A Novel Multidomain Polyketide Synthase Is Essential for Zeamine Production and the Virulence of *Dickeya zea*

Jianuan Zhou,¹ Haibao Zhang,² Jien Wu,² Qiongguang Liu,¹ Pinggen Xi,¹ Jasmine Lee,² Jinling Liao,¹ Zide Jiang,¹ and Lian-Hui Zhang²

¹Department of Plant pathology, South China Agricultural University, Guangzhou 510642, People's Republic of China;

²Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673

Submitted 6 April 2011. Accepted 26 May 2011.

Dickeya zea is the causal agent of the rice foot rot disease, but its mechanism of infection remains largely unknown. In this study, we identified and characterized a novel gene designated as *zmsA*. The gene encodes a large protein of 2,346 amino acids in length, which consists of multidomains arranged in the order of N-terminus, β -ketoacyl synthase, acyl transferase, acyl carrier protein, β -ketoacyl reductase, dehydratase. This multidomain structure and sequence alignment analysis suggest that *ZmsA* is a member of the polyketide synthase family. Mutation of *zmsA* abolished antimicrobial activity and attenuated the virulence of *D. zea*. To determine the relationship between antimicrobial activity and virulence, active compounds were purified from *D. zea* EC1 and were structurally characterized. This led to identification of two polyamino compounds, i.e., zeamine and zeamine II, that were phytotoxins and potent antibiotics. These results have established the essential role of *ZmsA* in zeamine biosynthesis and presented a new insight on the molecular mechanisms of *D. zea* pathogenicity.

Dickeya zea is the causal agent of devastating rice foot rot disease, which is a major concern of the agricultural industry in rice planting countries and regions. The pathogen used to be named *Erwinia chrysanthemi* pv. *zea*, as one of the six pathovars of the *Erwinia chrysanthemi* complex. Based on phylogenetic analysis, the members of *Erwinia chrysanthemi* complex were reclassified into a novel genus, *Dickeya* gen. nov., and the six pathovars were renamed *Dickeya chrysanthemi*, *Dickeya dadantii*, *Dickeya dianthicola*, *Dickeya dieffenbachiae*, *Dickeya paradisiaca*, and *Dickeya zea* (Samson et al. 2005). While the other members of genus *Dickeya* only infect dicotyledons, *D. zea* has the ability to infect both monocotyledons and dicotyledons (Goto 1979; Hussain et al. 2008; Sinha and Prasad 1977), suggesting that this pathovar may have acquired one or more additional virulence factors in the process of evolution that enables expanding its host range. Extensive biochemical and genetic studies on the pathovars that infect dicotyledon plants have unveiled a range of virulence factors. For example, in *D. dadantii* (*E. chrysanthemi* 3937), pectinases, cellulases, protease isozymes, xylanase, and phospholipase were found to play variously important roles in plant

tissue maceration (Collmer and Bauer 1994; Collmer et al. 1985; Keen et al. 1984; Kotoujansky et al. 1985; Reverchon et al. 1985; Van Gijsegem et al. 1985). In addition, iron metabolism (Expert 1999), siderophores (Franza et al. 2005), indigoidine pigment (Reverchon et al. 2002), extracellular polysaccharide (Condemine et al. 1999), and the type III secretion system (López-Solanilla et al. 2001; Yang et al. 2002, 2004; Yap et al. 2005) are also known to play roles in bacterial systemic infections and disease symptom development. Collectively, these virulence factors are responsible for soft-rot symptom development and eventual tissue collapse. However, little is known about the pathogenic mechanisms of *D. zea*.

Our previous study showed that the acyl-homoserine lactone (AHL) quorum-sensing (QS) system is implicated in the regulation of certain virulence traits in *D. zea* (Hussain et al. 2008). Null mutation of the *expI* gene encoding AHL synthase in the *D. zea* EC1 abolishes AHL signal production, increases bacterial swimming and swarming motility, disables formation of cell aggregates, and attenuates the bacterial virulence on potato tubers. It was noticed that the mutation only marginally reduces the inhibitory activity of *D. zea* on rice seed germination (Hussain et al. 2008), suggesting that the pathogen may likely produce other virulence factors independent of AHL-type QS regulation.

Recently, we found that *D. zea* DZ1 produces a novel polyamino-amide antibiotic designated as zeamine (Wu et al. 2010), which displayed potent inhibitory activities against a range of gram-positive and gram-negative bacterial pathogens. Our preliminary study showed that zeamine could inhibit germination of rice seeds but did not seem to have an obvious effect on rice roots. To determine the role of zeamine in bacterial pathogenesis, zeamine-negative mutants were identified by transposon mutagenesis and bioassay. This study reports the identification and characterization of the *zmsA* gene, which encodes a novel multimodular polyketide synthase with 2,346 amino acids (aa). Disruption of this gene drastically reduced the pathogenicity of strain DZ1. In addition, chemical analysis was performed, and the results showed that *zmsA* is essential for production of zeamine and its derivative zeamine II, which are phytotoxins and potent antibiotics.

RESULTS

Screening of zeamine-negative mutants.

Our preliminary results showed that the purified antibiotic zeamine from *D. zea* DZ1 (Wu et al. 2010) was able to inhibit germination of rice seeds, suggesting that zeamine could be one of the key virulence factors produced by *D. zea*. To identify the genes involved in zeamine production, we used

Corresponding author: Lian-Hui Zhang; E-mail: lianhui@imcb.a-star.edu.sg; Telephone: +65-6586 9686; Fax: +65-6779 1117.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and one supplementary table are published online.

Escherichia coli DH5 α , which is sensitive to zeamine (Wu et al. 2010), as an indicator strain in bioassays. A mutant library of *D. zeae* EC1, which is a pathogenic isolate shown previously to cause rice infections (Hussain et al. 2008), was gener-

Fig. 1. Analysis of antibiotics production by wild-type EC1 and selected transposon mutants. Bioassay plates were prepared by pouring about 20 ml of Luria Bertani agar medium, which was overlaid with 5 ml of 1% agarose containing 1.0×10^8 cells of fresh *Escherichia coli* DH5 α after solidification. **A**, EC1 and its derivatives were spotted onto the bioassay plate by adding 1 μ l of the corresponding bacterial culture at optical density at 600 nm = 1.5. **B**, Filter-sterilized bacterial supernatants of overnight bacterial culture in optimized minimal medium (20 μ l from each sample) were added to a well in bioassay plates. The bioassay plates were incubated in 37°C for 24 h before photography.

ated by using a Tn5 transposon mutagenesis approach. Tn5 mutants were spotted on *E. coli* DH5 α -overlaid plates to screen for the altered phenotypes in antibiotic activity. Zeamine-defective mutants were purified by subculture and were further confirmed by bioassay. From about 16,000 transposon mutants, 21 zeamine-defective mutants were identified. Among them, a few mutants lacking antibiotic activity, including Em9, Em11, Em54, Em67, Em76, and Em107, were used in this study. Bioassay using either bacterial cells or culture supernatants showed that the mutants lost the ability to produce antibiotics to inhibit the growth of *E. coli* (Fig. 1A and B; Supplementary Table S1), indicating that transposon insertions disrupted one or more genes essential for zeamine production.

The inhibition zone of strain EC1 supernatants was clearly larger than that produced by spotting the EC1 cells onto bioassay plates (Fig. 1), suggesting that optimized minimal (OM) medium is better than Luria Bertani (LB) medium (both mediums are defined below) in supporting the production of antibiotics by *D. zeae*. This was confirmed by semiquantitative analysis. The results showed that, while the growth of EC1 in OM medium was initially slower than that in LB, its relative antibiotic activity in OM medium was at least fourfold higher than that in LB medium at stationary phase (Supplementary Fig. S1).

Sequence characterization of the zeamine-negative mutants.

By using thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR), the sequences flanking the Tn5 insertion were amplified and cloned. To obtain the full gene sequences, TAIL-PCR was conducted consecutively for several rounds by using primers designed based on the DNA sequence obtained from the previous round of TAIL-PCR products. A total of 8,189 bp of DNA sequences were obtained by assembling the TAIL-PCR fragments. Sequence analysis revealed an open reading frame (ORF) of 7,041 bp, and Tn5 was found inserted in various positions within this ORF in all the mutants used in this study. (Table 1; Fig. 2A). The ORF commences at 557 bp in the obtained sequence with an ATG translation initiation codon, 11 bp downstream from a ribosome binding site (RBS) (GAGG). The closest match (TTTCCT-14 $\times$ -TATAAT) to the consensus -35 (TTGACA) and -10 (TATAAT) sequences for *E. coli* σ^{70} promoters occurs at 62 bp upstream of the translation initiation codon (Fig. 2B). The promoter analysis data suggest that this ORF promoter is likely under the control of housekeeping sigma factor σ^{70} , which directs gene transcrip-

Table 1. The strains and plasmids used in this study

Strain or plasmid	Relevant phenotypes and characteristics ^a	Source or reference
<i>Dickeya zeae</i>		
EC1	Wild-type rice foot rot pathogen	Hussain et al. 2008
Em54	EC1 defective in zeamine production with Tn5 inserted at bp 4,410 of the <i>zmsA</i> ORF	This study
Em54(zmsA)	EM54 containing expression construct pLAFR-zmsA	This study
Em9	EC1 defective in zeamine production with Tn5 inserted at bp 867 of the <i>zmsA</i> ORF	This study
Em11	EC1 defective in zeamine production with Tn5 inserted at bp 5,238 of the <i>zmsA</i> ORF	This study
Em67	EC1 defective in zeamine production with Tn5 inserted at bp 4,410 of the <i>zmsA</i> ORF	This study
Em76	EC1 defective in zeamine production with Tn5 inserted at bp 5,549 of the <i>zmsA</i> ORF	This study
Em107	EC1 defective in zeamine production with Tn5 inserted at bp 2,935 of the <i>zmsA</i> ORF	This study
Δ zmsA	A <i>zmsA</i> knockout mutant derived from strain EC1 containing Gm ^r gene	This study
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i> λ <i>pir</i>	Laboratory collection
HB101(pRK2013)	<i>Thr leu thi recA hsdR hsdM pro</i> , Km ^r	Laboratory collection
Plasmid		
pMD18-T	Ligation vector. Ap ^r	TaKaRa, Shiga, Japan
pTGN	pBSL202 carrying a 2-kb <i>XbaI</i> - <i>SmaI</i> fragment containing a mini Tn5 with <i>gfp-nptII</i> fusion gene	Tang et al. 1999
pLAFR3	Tc ^r , Mob ⁺ , Cos, low-copy cloning vector	Laboratory collection
pDSK519	Broad host range IncQ cloning vector, Km ^r	Laboratory collection
pLAFR-zmsA	pLAFR3 containing <i>zmsA</i> coding region at the downstream of <i>lac</i> promoter	This study

^a ORF = open reading frame. Ap^r, Gm^r, Km^r, or Tc^r = ampicillin, gentamycin, kanamycin, or tetracycline resistant.

tion in growing cells. This notion is consistent with the time-course analysis that antibiotic production started at early exponential phase and plateaued upon entering stationary phase. Surprisingly, a BLAST search using the coding sequence of this ORF against the published genome of *Dickeya zeae* Ech1591 in the National Center for Biotechnology Information (NCBI) database did not reveal any significant homologs.

Protein in silico analysis.

The ORF (designated *zmsA*) encodes a protein of 2,346 aa. A BLAST search against the NCBI protein database showed that its best homolog is the putative polyketide synthase XBJ1_2953 (2,274 aa) from *Xenorhabdus bovienii* SS-2004, sharing 55% identity with a query coverage of 98%. *X. bovienii* is a gram-negative symbiotic bacterium associated with the nematode *Sterinernema glaseri* (Dunphy et al. 1985). For understanding enzymatic characteristics, we specifically compared the protein sequence of ZmsA with those of characterized homologs, including PfaA, P3-A6-PKS, and EryAI. Among them, PfaA is an omega-3 polyunsaturated fatty acid

synthase from the deep-sea bacterium *Photobacterium profundum* (Allen and Bartlett 2002). ZmsA and PfaA share about 33% aa identity. The polyketide synthase P3-A6-PKS from *Chromobacterium violaceum* is involved in synthesis of the anticancer agent FK228 (Cheng et al. 2007). The enzyme is homologous to the N-terminal region of ZmsA and shares about 64% identity. Interestingly, the polyketide synthase EryAI from *Saccharopolyspora erythraea*, essential for production of antibiotic erythromycin (Weber et al. 1990), contains multiple regions sharing about 24 to 30% identical amino acids to the N- and C-terminal regions of ZmsA.

Domain structure analysis showed that ZmsA contains three conserved domains, i.e., a β -ketoacyl (KS)-acyl carrier protein (ACP) synthase domain, a β -ketoacyl reductase (KR) domain, and an acyltransferase (AT) domain (Fig. 2C). Its domain structure is most similar to XBJ1 and PfaA, except the latter contains five phosphopantetheine binding (P) domains. In contrast, P3-A6-PKS contains a KS and an AT domain only, whereas EryAI contains two sets of KS-AT-KR domains and an extra AT domain at the N-terminal (Fig. 2C). The duplicate domain struc-

Fig. 2. Physical map and sequence analysis of *zmsA* gene. **A**, Physical map of the DNA fragment containing the *zmsA* gene. The relative transposon insertion sites in corresponding mutants are indicated by vertical arrows. **B**, *zmsA* promoter analysis. The predicted ribosomal binding site and putative promoter elements are underlined. The direction of transcription is indicated by a horizontal arrow. **C**, ZmsA domain structure analysis. The amino sequences of XBJ1, PfaA, P3-A6-PKS, and EryAI were obtained from the National Center for Biotechnology Information. KS = β -ketoacyl-cyl carrier protein synthase domain, AT = acyltransferase domain, KR = a ketoreductase domain, and P = phosphopantetheine binding domain. Domain analysis was performed using the SMART program.

ture of EryAI explains its multiregional similarity to ZmsA. Taken together, the above sequence and domain analysis indicate that ZmsA belongs to the widely conserved polyketide synthase family and contains distinct variations in amino-acid sequence and in domain architecture with its homologs.

The *zmsA* mutants show decreased virulence on rice, potato, and Chinese cabbage.

Given that *D. zeae* infects both monocotyledon and dicotyledon plants (Hussain et al. 2008), rice seeds, potato tubers, and Chinese cabbage were inoculated with EC1 and *zmsA* transposon mutant Em54, respectively. The results showed that rice seed germination was abolished when inoculated with EC1 (Fig. 1A), whereas all the rice seeds treated with mutant Em54 germinated and grew well (Fig. 1B), similar to the control seeds treated with LB medium (Fig. 1C). Quantitative analysis of randomly selected 30 seedlings showed that the average length of the main root and shoot of rice seeds treated with Em54 reached over 15 mm 1 week after inoculation, a sharp contrast to the zero growth of seeds treated with wild-type strain EC1 (Fig. 1E). Similarly, potato tubers and Chinese cabbage inoculated with Em54 showed substantially reduced rotting zones as compared with those treated with the wild-type EC1, at 24 h after inoculation (Fig. 3F).

The complemented strain Em54(pLAFR-*zmsA*) expressing wild-type *zmsA* under the control of *lac* promoter in vectors

pLAFR3 failed to restore the virulence phenotype (data not shown). As no error was found in the DNA sequence of the cloned *zmsA* and the *zmsA* transcripts were detectable by reverse transcription (RT)-PCR, a likely possibility is the polar effect caused by Tn5 insertion that affects transcriptional expression of downstream genes involved in zeamine biosynthesis. This is likely, as antibiotic production is commonly encoded by multiple genes within one or more operons (Bangera and Thomashow 1999; Ikeda et al. 1999; Peirú et al. 2005; Schewecke and Leadlay 1995). The role of *zmsA* in bacterial virulence was confirmed by the finding that the pathogenicity of the other five independent *zmsA* mutants, including Em9, Em11, Em67, Em76, and Em107, were also significantly reduced on potato and rice, as compared with the wild-type control. To provide further validation, we generated the deletion mutant $\Delta zmsA$ by replacement of an internal fragment of *zmsA* with a gentamycin-resistant gene, using wild-type strain EC1 as the parental strain. Inoculation results showed that the deletion mutant had similar phenotypes in both virulence and antibiotic production as the transposon mutants (Fig. 3D, E, and F).

Mutation of *zmsA* abolishes production of one or more phytotoxins inhibiting rice seed root germination.

Given that mutation of *zmsA* abolished the bacterial antibiotic activity and virulence on rice, we tested whether one or more products of ZmsA could inhibit germination of rice seeds.

Fig. 3. Mutation of *zmsA* attenuates the virulence of *Dickeya zeae*. Rice seeds were treated with **A**, EC1, **B**, mutant Em54, **C**, Luria Bertani medium, and **D**, $\Delta zmsA$. Photos were taken 7 days after incubation at 28°C. **E**, Quantitative analysis of the effect of EC1 and its mutants on rice root and shoot growth. The data are averages of 30 randomly selected seeds, and error bars indicate standard deviation. **F**, Quantitative analysis of the virulence of EC1 and its mutants on potato and Chinese cabbage. The data are means of three repeats, and error bars indicate standard deviation.

We compared the biological activity of supernatants of wild-type EC1 and the *zmsA* mutant Em54 on *E. coli* growth and rice seed germination. In addition, the crude extracts were prepared equivalent to a 500× concentration of culture supernatants, using XAD-7 partition chromatography. EC1 supernatant treatments of rice seeds only caused inhibition on root germination but did not affect shoot germination (Fig. 4, middle), but both root and shoot germination were blocked when treated with the crude extracts of EC1 (Fig. 4, right). The results indicate that rice root germination is more sensitive to the products of *ZmsA* than shoot germination. As expected, rice root and shoot grew well when treated with the Em54 culture supernatants (Fig. 4, left).

D. zeae EC1 produces two antibiotic-like phytotoxins.

The above data indicate that *ZmsA* is not only essential for production of antibiotics, but also for phytotoxic activity. To determine the relationship between antibiotic and phytotoxin activity, we purified the toxic substances from strain EC1, using *E. coli* and rice seeds as bioassay indicator. Crude toxin extracts eluted from XAD-7 partition resins were subjected to Sephadex LH-20 gel filtration chromatography. Both antibiotic and phytotoxin activity were found in the same fractions. The active fractions were combined and were concentrated for reverse-phase high-performance liquid chromatography (HPLC) separation. The major antibiotic activity was found in two fractions with retention times at 29 and 31 min, respectively (Fig. 5A and B). These two fractions also provided about 78 and 100% relative inhibitory activity on rice seed root germination (Fig. 5C).

The fraction at 31 min was a colorless solid, accounting for around 60% of the total toxin, based on its dry mass relative to the fraction at 29 min. Accurate electrospray ionization-mass spectrometry (ESI-MS) revealed a molecular formula of $C_{49}H_{105}O_4N_6$, $[M+H]^+/e$, 841.8218, (calculated 841.8192), identical to the antibiotic zeamine purified from *D. zeae* DZ1 (Wu et al. 2010). Further nuclear magnetic resonance (NMR) analysis confirmed that the active compound in fraction 31 is zeamine (Fig. 6).

The fraction at 29 min, which accounted for around 40% of the total toxin, was collected and vacuum-dried as a colorless solid. Accurate ESI-MS revealed a molecular formula of $C_{40}H_{88}ON_5$, $[M+H]^+/e$, 654.6993 (calculated 654.6983). MS data supported a saturated structure without any rings or double

bonds. In normal ESI analysis, the following peaks were noticed: 655, 638, 621, 604, 587, and 569, which represent $M+H$, $M+H-NH_3$, $M+H-2NH_3$, $M+H-3NH_3$, $M+H-4NH_3$, and $M+H-4NH_3-H_2O$. These fragmentation patterns suggest that there are four secondary amino groups and one secondary hydroxyl group in the skeleton. ^1H and ^{13}C spectra revealed the existence of a primary amino group. Due to signal overlap, it was difficult to identify the positions of those secondary amino groups. Therefore, C-C bond cleavage in ESI analysis was employed to identify the positions of those amino groups. In ESI analysis, there were peaks at 638, 592, 566, 464, 438, 337, 331, 227, and 100, which agree well with the forty-carbon polyamino compound designated as zeamine II (Fig. 6). The structural similarity of zeamine and zeamine II is also clear when their ESI spectra are compared (Supplementary Figs. S2 and S3), which showed a range of identical molecular ions, including 655, 638, 621, 604, 587, 569, 557, 328, 311, 302, and 293.

Zeamines are phytotoxins that inhibit rice seed germination.

The phytotoxin activities of zeamines were tested using a rice seed germination bioassay. The results showed that rice shoot and root germination was not affected when treated with zeamine or zeamine II at a final concentration of 60 μM , albeit reduced growth was observed. Root germination was inhibited by 240 μM of toxin, and shoot generation was affected with 480 μM of toxin (Fig. 7). Rice shoot germination was almost completely blocked when the concentration of toxins reached about 720 μM (data not shown). Consistent with the results of bacterial supernatant treatments, rice root germination was more sensitive than shoot germination to the purified zeamines (Fig. 7).

DISCUSSION

D. zeae is a bacterial pathogen that infects both monocot and dicot plants (Hussain et al. 2008), but its virulence determinants have not yet been characterized. In this study, we identified and sequenced a gene designated *zmsA*, encoding a novel multidomain polyketide synthase. Mutation of *zmsA* abolished the antimicrobial activity of *D. zeae* EC1. The *zmsA* mutants were avirulent when challenged against rice seeds and showed substantially reduced disease symptoms on potato tubers and Chinese cabbage. We presented evidence that *D. zeae* EC1 produced two polyamino-type antibiotics and phytotoxins, zeamine and zeamine II, through chromatographic purification and structural characterization. Treatment of rice seeds with zeamines inhibited germination and growth. Taken together, these data have established that the *ZmsA* is a key enzyme essential for production of zeamines, which are phytotoxins and play a key role in bacterial virulence.

Among the characterized homologs, the modular domain structure of *ZmsA* is most similar to PfaA, P3-A6-PKS, and EryAI (Fig. 2C), which are involved in synthesis of omega-3 fatty acid, FK228, and 2,6-deoxyerythronolide (Allen and Bartlett 2002; Cheng et al. 2007; Weber et al. 1990), respectively. Structural comparison shows that the common structure shared by zeamines and the products of *ZmsA* homologs is the fatty acid chain (Fig. 6). Bioinformatics analysis indicated that *ZmsA* belongs to the widely conserved polyketide synthase family, with three conserved domains including KS-ACP synthase, KR, and acyltransferase (AT) domains (Fig. 2C). KS-ACP synthase is an enzyme involved in fatty acid synthesis, and it results in formation of acetoacetyl ACP. KR belongs to a family of enzymes that catalyzes the transfer of electrons from one molecule to another, usually utilizing NADP or NAD as cofactors. AT is a type of transferase enzyme that catalyzes transfer of an acyl group from one molecule to another. These enzymatic prop-

Fig. 4. Mutation of *zmsA* abolishes the inhibitory activity of *Dickeya zeae* on rice seed germination. Rice seeds were treated with Em54 supernatants (left), EC1 supernatants (middle), and EC1 crude extracts (right) for 5 h, before germination on filter papers moisturized with corresponding solutions in a 28°C incubator. The experiments were repeated twice with 20 replicates for each treatment. Photos were taken 1 week postinoculation.

erties together with its similarities to the polyketide synthases involved in synthesis of omega-3 fatty acid, FK228, and 2,6-deoxyerythronolide (Fig. 6), suggest that ZmsA is involved in synthesis of the fatty acid backbone of zeamines.

BLAST searches with DNA and protein sequences of ZmsA did not identify any significant homologs in the published genome sequence of *D. zea* Ech1591. Similarly, ZmsA homologs were not found in the genome sequences of closely related bacterial species, including *Dickeya dadantii*, *Erwinia amylovora*, *Pectrobacterium atrosepticum*, and *Pectrobacterium carotovorum*. It is noted that Ech1591 is the causal agent of onion soft rot, whereas strain EC1, used in this study, is the pathogen causing rice foot rot (Hussain et al. 2008). It is possi-

ble that strain EC1 may acquire *zmsA* and other genes involved in zeamine biosynthesis through horizontal gene transfer. The notion is supported by the finding that the GC content of strain Ech1591 is about 53%, whereas the 8,189-bp sequence obtained from this study contain only about 47% of GC. Genome-sequencing analysis of strain EC1 and other isolates would be essential for understanding the diversity and genome evolution among *D. zea* isolates.

Null mutation of ZmsA substantially attenuated the virulence of strain EC1 on dicot plants and abolished the bacterial inhibitory activity against germination of rice seeds, suggesting that ZmsA products play a key role in bacterial pathogenesis. Consistent with the role of ZmsA in antibiotic production and in

Fig. 5. High-performance liquid chromatography (HPLC) separation and bioassay of the antibacterial compounds produced by *Dickeya zea* EC1. **A**, The UV absorbance ($\lambda = 210$ nm) profile of the HPLC fractions of the active components after Sephadex LH-20 gel filtration. **B**, Semiquantitative comparison of the effect of HPLC fractions on *Escherichia coli* growth and **C**, on rice seed germination, by measuring the relative activities of each fraction.

virulence, zeamine and zeamine II were shown to be active phytotoxins that inhibit rice seed germination. Identification of zeamines as key virulence determinants may provide useful clues for design and development of new treatments for prevention and control of the disease caused by *D. zeae*. For example, enzymatic inactivation of the phytotoxin albicidin through biocontrol agents or transgenic plants was shown to be effective in control of sugarcane leaf scald disease caused by *Xanthomonas albilineans* (Zhang and Birch 1997a and b; Zhang et al. 1999). It is also interesting to note that, while a high concentration of zeamine was needed for inhibition of rice seed germination (Fig. 7), the minimal inhibitory concentration values of zeamine against gram-positive and gram-negative bacteria were mostly in the low range between 0.3 and 10 μ M (Wu et al. 2010). The differential toxicities of zeamine on prokaryotes and eukaryotes may suggest promising pharmaceutical potentials, which await further exploration.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions.

The bacterial strains and plasmids used in the present study are listed in Table 1. *Escherichia coli* was routinely grown at 37°C in LB medium (10 g/l Bacto tryptone, 5 g/l yeast extract, and 10 g/l NaCl, pH7.0). *D. zeae* EC1 and its derivatives were either grown at 28°C in YEB medium (10 g of Bacto tryptone per liter, 5 g of yeast extract per liter, and 5 g of NaCl per liter, 5 g of sucrose per liter, and 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.0) or were grown in minimal medium as indicated (Hussain et al. 2008). For large-scale purification of antibiotics from bacterial cultures, bacteria were grown in the optimized minimal (OM) medium (10.5 g of K_2HPO_4 per liter, 4.5 g of KH_2PO_4 per liter, 2 g of $(\text{NH}_4)_2\text{SO}_4$ per liter, 2 g of mannitol per liter, 2 g of glycerol per liter, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter, 5 mg of FeSO_4 per liter, 10 mg of CaCl_2 per liter, and 2 mg of MnCl_2 per liter, pH 7.0). Antibiotics were added at the following final concentrations when required: ampicillin (Ap), 100 μ g/ml; kanamycin (Km), 100 μ g/ml; gentamycin (Gm), 20 μ g/ml; and tetracycline (Tc), 15 μ g/ml. The optical density (OD_{600}) of the bacterial culture was measured by using a UNIC 7200 spectrophotometer at 600 nm.

Transposon mutagenesis.

Transposon Tn5, carried by pTGN in *E. coli* S17-1, was transferred into *D. zeae* DZ1 by conjugation (Larsen et al. 2002;

Tang et al. 1999). Briefly, conjugal mating was performed by mixing overnight cultures of donor and recipient strains in about a 2:1 ratio onto LB agar plates and incubating them at 28°C for 6 h. Tn5 mutants were selected on minimal medium agar plates containing Km, and the mutants were then screened for altered phenotypes in toxin production, using the toxin bioassay method described in the following section.

Sequence analysis of toxin-deficient mutants.

To identify the mutated gene, TAIL-PCR was performed (Liu and Whittier 1995). The genomic DNAs were extracted using MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, WI, U.S.A.). Two sets of nested primers were used in this study. For sequencing the forward DNA strand, the primers Gt-447 (5'-GTGCAAGCAGATTACGGTGACGAT), Gt-464 (5'-TGACGATCCCGCAGTGGCTCTC), and Gt-487 (5'-ATACAAAGTTGGGCATACG) were designed; for reverse DNA strand, SP1 (5'-TGTTGCATCACCTTCACCCTCTCC), SP2 (5'-GTTCTTCTCCTTTACTCATT) and SP3 (5'-TTATGAGTCTCCAGTTTGTTCAGTT) were designed, based on the DNA sequence of Tn5 used in this study. These nested primers were paired with an arbitrary primer AD6 (5'-NGTASASWGTNAWCAA) for PCR amplification, and the amplified fragments were ligated with pMD18-T. The resulting plasmid

Fig. 7. The dosage effect of zeamines on rice seed germination. The rice seeds in the top and bottom panel were treated with zeamine II and zeamine, respectively. The photos were taken 7 days after treatment. The experiments were repeated at least three times with similar results.

Fig. 6. Chemical structures of zeamines and the products of ZmsA homologs.

was transformed into the competent cells of *E. coli* DH5 α , and the transformants were selected on LB agar supplemented with 100 μ g of Ap per milliliter, which were then sent to Shanghai Invitrogen Biotechnology Co. (Shanghai, China) for DNA sequencing. Further, TAIL-PCR experiments were performed using AD6 and the primers designed based on the identified sequences to obtain the full gene sequences.

Complementation and deletion analysis.

Primers Em54-1 (5'-CGGGATCCCGATAATACCAAGGAGTAAG) and Em54-2 (5'-CCCAAGCTTGGGTAATTGGCCTTATCTC) were designed based on sequence data of *zmsA* and were used to amplify its coding region. The PCR product was then subjected to *Bam*HI and *Hind*III digestion, as was the expression plasmid vector pLAFR3. The digested PCR product and vector were then purified and ligated in such a way that the coding region of *zmsA* was placed under the control of the *lac* promoter carried by the vector. The ligated mixture was transfected into *E. coli* DH5 α competent cells, and transformants were selected on LB agar supplemented with 15 μ g of Tc per milliliter and were confirmed by DNA sequencing. The corresponding complemented strain of Em54 were generated by conjugal triparental mating, and the transformants were selected on minimal medium agar plates supplemented with 100 μ g of Km per milliliter and 15 μ g of Tc per milliliter. The resultant strain Em54(*zmsA*) was confirmed by PCR analysis. For generation of the *zmsA* knockout mutant, two pairs of primers were designed to amplify the 5' and 3' end fragments of *zmsA*, which were fused with the Gm-resistance gene *aacC1* fragment amplified from the plasmid mini-Tn7T-Gm. This resultant 5'-fragment-*aacC1*-3'-fragment was cloned in the suicide vector pK18. The ligation mixture was used to transform *E. coli* DH5 α (λ pir), and the transformants were selected on LB plate containing Km. After sequence verification, the plasmid pK18-*zmaacC1sA* was introduced into strain EC1 by electroporation. The EC1 transformant colonies were grown on LB plate containing Km and Gm. The selected colonies were cultured in liquid LB supplemented with the same antibiotics.

Pathogenicity assay against potato tubers and Chinese cabbage.

Potato tubers were obtained from local stores. After being washed with tap water and dried on a paper towel, potato tubers were surface-sterilized with 70% ethanol and were then sliced evenly about 5 mm in thickness. Each slice was then placed in a petri dish lined with Whatman no. 3 filter paper moistened with sterilized water. Bacterial cells (2 μ l at an OD₆₀₀ = 1.2) were added to the center of the sliced potato tuber after piercing it with a pipette tip. The potato tubers were then incubated at 28°C. The potato tubers were observed regularly for symptom development. Chinese cabbage was similarly sterilized and cut to 2-cm² pieces for inoculation. The same volume of LB medium was inoculated as a negative control. Each assay was repeated three times with triplicates.

Rice seed germination assay and determination of bacterial population associated with rice seeds.

Fifty rice seeds were immersed in 20 ml of bacterial culture of OD₆₀₀ = 1.2, equivalent to about 1.15×10^9 bacterial CFU per ml, at room temperature for 5 h. The rice seeds were then washed three times with sterilized water and were 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 conditions, and sterilized water was added when necessary. LB medium was used as a negative control. The rate of seed germination was determined one week after treatment. The experiments were repeated at least twice.

For determination of bacterial cell numbers, rice seeds were incubated with bacteria as described above. The rice seeds were then washed three times with sterilized water and were transferred onto two moistened Whatman no. 3 filter papers in a petri dish and were incubated at 28°C for 24 h. For measurement of total bacterial cell numbers tightly associated with rice seeds, five randomly selected rice seeds were washed three times with sterilized water before being ground in a sterilized mortar. For measurement of bacterial population within rice seeds, rice seeds were surface-sterilized by immersing in 75% alcohol for 5 s before being washed three times with sterilized water. The ground seed residues were suspended in 1 ml of LB medium, and bacterial CFU were determined by preparing a serial dilution and spreading 100 μ l of the dilution on LB agar plates. Colonies grown on the plates were counted after 24 h, and wild-type and mutant strains were confirmed by their ability to produce zeamine antibiotics and resistance to Km, respectively. Each treatment was repeated three times.

Purification of bactericidal compounds.

The start cultures of *D. zeae* strains were prepared in OM medium by incubation at 28°C, with shaking at 200 rpm, overnight, and were then added to the same medium (0.2% vol/vol) and grown at the same temperature, 120 rpm, for 24 h. Bacterial cells were removed by centrifugation at $11,000 \times g$, 15°C, for 15 min. The supernatants were adjusted to pH 10, with 5 M NaOH, and were concentrated by about tenfold using a rotary evaporator. The concentrates were extracted by three volumes of organic solvents (n-butanol/ethyl acetate = 2:1) twice. The organic solvents were removed by rotary evaporation. The active compounds in the residues, dissolved in methanol, were subjected to gel filtration chromatography on Sephadex LH-20, with methanol as the eluting solvent. The fractions with antibiotic activity were combined and were concentrated by using a rotary evaporator. Further purification was done by using a Waters 2690 HPLC separation module equipped with an automatic injector (Milford, MA, U.S.A.) and a reverse-phase column (Phenomenex Luna 5 μ C₁₈ 250 $\times$ 4.60 mm), which was connected to a UV detector to monitor the elutes at λ = 210. A gradient solvent program (CH₃CN in H₂O) (5% [0 to 5 min], 5 to 75% [5 to 50 min], 75 to 95% [50 to 51 min], 95% [51 to 55 min], 95 to 5% [55 to 56 min], 5% [56 to 60 min]) was used for HPLC separation at a speed of 1 ml/min. Fractions (1 ml) were collected, vacuum dried, and dissolved in the same volume of 5% methanol in water for bioassay of antibiotic activity. HPLC separation was repeated several times to obtain sufficient samples for chemical structure analysis.

NMR and MS analyses.

Purified sample (8 mg) was dissolved in CD₃OD. The ¹H NMR spectra were obtained at 400 MHz, and the ¹³C NMR spectra were obtained at 100 MHz (Bruker DRX400). MS analysis was done with a Micromass VG 7035 mass spectrometer (electron ionization) and a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer (ESI).

Bioassay of antibiotics.

The bactericidal activity was assayed using *E. coli* DH5 α following the method described previously (Zhang et al. 1998). Briefly, the bioassay plate was prepared by pouring about 20 ml of LB agar medium, which, after solidification, was overlaid with 5 ml of 1% agarose, at about 50°C, containing 1.0×10^8 cells of fresh *E. coli* DH5 α . DZ1 and its transposon mutants were spotted onto the plates using toothpicks. For liquid assay, wells of 4 mm in diameter were punched in the plates. Supernatants of overnight bacterial cultures grown in minimal medium to OD₆₀₀ = 1.5 were filter-sterilized and 20 μ l

was added to the wells. Plates were incubated overnight at 37°C, and the mutants defective in production of an inhibition zone were selected for further study.

For semiquantification, the sample was diluted in a twofold series and was assayed as described above. One unit of antibiotic activity was arbitrarily defined as the amount that generates a 2-mm diameter inhibition zone around the well. The number of antibiotic units per milliliter of sample was calculated by multiplying the final dilution that produces a 2-mm inhibition zone by the total volume of sample (1,000 µl) and then dividing by the volume used in the bioassay (20 µl).

LITERATURE CITED

- Allen, E. E., and Barlett, D. H. 2002. Structure and regulation of the omega-3 polyunsaturated fatty acid synthase genes from the deep-sea bacterium *Photobacterium profundum* strain SS9. *Microbiol.* 148:1903-1913.
- Bangera, M. G., and Thomashow, L. S. 1999. Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. *J. Bacteriol.* 181:3155-3163.
- Cheng, Y. Q., Yang, M., and Matter, A. M. 2007. Characterization of a gene cluster responsible for the biosynthesis of anticancer agent FK228 in *Chromobacterium violaceum* no. 968. *Appl. Environ. Microbiol.* 73:3460-3469.
- Collmer, A., and Bauer, D. W. 1994. *Erwinia chrysanthemi* and *Pseudomonas syringae*: Plant pathogens trafficking in extracellular virulence proteins. *Curr. Top. Microbiol. Immunol.* 192:43-78.
- Collmer, A., Schoedel, C., Roeder, D. L., Ried, J. L., and Rissler, J. F. 1985. Molecular cloning in *Escherichia coli* of *Erwinia chrysanthemi* genes encoding multiple forms of pectate lyase. *J. Bacteriol.* 161:913-920.
- Condemine, G., Castillo, A., Passeri, F., and Enard, C. 1999. The PecT repressor coregulates synthesis of exopolysaccharides and virulence factors in *Erwinia chrysanthemi*. *Mol. Plant Microbe Interact.* 12:45-52.
- Dunphy, G. B., Rutherford, T. A., and Webster, J. M. 1985. Growth and virulence of *Steinernema glaseri* influenced by different subspecies of *Xenorhabdus nematophilus*. *J. Nematol.* 17:476-482.
- Expert, D. 1999. Withholding and exchanging iron: Interactions between *Erwinia* spp. and their plant hosts. *Annu. Rev. Phytopathol.* 37:307-334.
- Franza, T., Mahé, B., and Expert, D. 2005. *Erwinia chrysanthemi* requires a second iron transport route dependent of the siderophore achromobactin for extracellular growth and plant infection. *Mol. Microbiol.* 55:261-275.
- Goto, M. 1979. Bacterial foot rot of rice caused by a strain of *Erwinia chrysanthemi*. *Phytopathology* 69:213-216.
- Hussain, M. B. B. M., Zhang, H. B., Xu, J. L., Liu, Q., Jiang, Z., and Zhang, L. H. 2008. The acyl-homoserine lactone-type quorum-sensing system modulates cell motility and virulence of *Erwinia chrysanthemi* pv. *zeae*. *J. Bacteriol.* 190:1045-1053.
- Ikeda, H., Nonamiya, T., Usami, M., Ohta, T., and Omura, S. 1999. Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. *Proc. Natl. Acad. Sci. U.S.A.* 96:9509-9514.
- Keen, N. T., Dahlbeck, D., Staskawicz, B., and Belser, W. 1984. Molecular cloning of pectate lyase genes from *Erwinia chrysanthemi* and their expression in *Escherichia coli*. *J. Bacteriol.* 59:825-831.
- Kotoujansky, A., Diolez, A., Boccara, M., Bertheau, Y., Andro, T., and Coleno, A. 1985. Molecular cloning of *Erwinia chrysanthemi* pectinase and cellulase structural genes. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:781-785.
- Larsen, R. A., M. M. Wilson, A. M. Guss, and W. W. Metcalf. 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Pv2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Arch. Microbiol.* 178:193-201.
- Liu, Y. G., and Whittier, R. F. 1995. Thermal asymmetric interlaced PCR: Automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics* 5:674-681.
- López-Solanilla, E., Llama-Palacios, A., Collmer, A., García-Olmedo, F., and Rodríguez-Palenzuela, P. 2001. Relative effects on virulence of mutations in the *sap*, *pel*, and *hrp* loci of *Erwinia chrysanthemi*. *Mol. Plant Microbe Interact.* 14:386-393.
- Peirú, S., Menzella, H. G., Rodríguez, E., Carney, J., and Gramajo, H. 2005. Production of the potent antibacterial polyketide erythromycin C in *Escherichia coli*. *Appl. Environ. Microbiol.* 71:2539-2547.
- Reverchon, S., Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy, J. 1985. Cloning of genes encoding pectolytic enzymes from a genomic library of the phytopathogenic bacterium, *Erwinia chrysanthemi*. *Gene* 35:121-130.
- Reverchon, S., Rouanet, C., Expert, D., and Nasser, W. 2002. Characterization of indigoidine biosynthetic genes in *Erwinia chrysanthemi*. *Mol. Microbiol.* 29:1407-1418.
- Samson R., Legendre, J. B., Christen R, Fisher-Le Saux M., Achouak W., and Gardan, L. 2005. Transfer of *Pectobacterium chrysanthemi* (Burkholder et al., 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. *Int. J. Syst. Evol. Microbiol.* 55:1415-1427.
- Schewecke, T., and Leadlay, P. F. 1995. The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. *Proc. Natl. Acad. Sci. U.S.A.* 92:7839-7843.
- Sinha, S. K., and Prasad, M. 1977. Bacterial stalk rot of maize, its symptoms and host-range. *Zentbl. Bakteriell. Parasitenkd. Infektkrankh. Hyg.* 132:81-88.
- Tang, X., Lu, B. F., and Pan, S. Q. 1999. A bifunctional transposon mini-Tn5gfp-km which can be used to select for promoter fusions and report gene expression levels in *Agrobacterium tumefaciens*. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 179:37-42.
- Van Gijsegem, F., Toussaint, A., and Schoonejans, E. 1985. *In vivo* cloning of pectate lyase and cellulase genes of *Erwinia chrysanthemi*. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:787-792.
- Weber, J. M., Leung, J. O., Maine, G. T., Potenz, R. H. B., Paulus, T. J., and DeWitt, J. P. 1990. Organization of a cluster of erythromycin genes in *Saccharopolyspora erythraea*. *J. bacterial.* 172:2372-2383.
- Wu, J., Zhang, H. B., Xu, J. L., Cox, R. J., Simpson, T. J., and Zhang, L. H. 2010. ¹³C labeling reveals multiple amination reactions in the biosynthesis of a novel polyketide polyamine antibiotic zeamine from *Dickeya zeae*. *Chem. Comm.* 46:333-335.
- Yang, C.-H., Gavilanes-Ruiz, M., Okinaka, Y., Vedel, R., Berthuy, I., Boccara, M., Chen, J. W.-T., Perna, N. T., and Keen, N. T. 2002. *hrp* genes of *Erwinia chrysanthemi* 3937 are important virulence factors. *Mol. Plant Microbe Interact.* 15:472-480.
- Yang, S., Perna, N. T., Cooksey, D. A., Okinaka, Y., Lindow, S. E., Ibekwe, A. M., Keen, N. T., and Yang, C. H. 2004. Genome-wide identification of plant-upregulated genes of *Erwinia chrysanthemi* 3937 using a GFP-based IVET leaf array. *Mol. Plant Microbe Interact.* 17:999-1008.
- Yap, M. N., C. H. Yang, C. H., Barak, J. D., Jahn, C. E., and Charkowski, A. O. 2005. The *Erwinia chrysanthemi* type III secretion system is required for multicellular behavior. *J. Bacteriol.* 187:639-648.
- Zhang, L. H., and Birch, R. G. 1997a. The gene for albicidin detoxification from *Pantoea dispersa* encodes an esterase which attenuates pathogenicity of *Xanthomonas albilineans* to sugarcane. *Proc. Natl. Acad. Sci. U.S.A.* 94:9984-9989.
- Zhang, L. H., and Birch, R. G. 1997b. Mechanisms of biocontrol of sugar cane leaf scald disease by *Pantoea dispersa*. *J. Appl. Microbiol.* 82:448-454.
- Zhang, L. H., Xu, J. L., and Birch, R. G. 1998. Factors affecting biosynthesis by *Xanthomonas albilineans* of albicidin antibiotics and phytotoxins. *J. Appl. Microbiol.* 85:1023-1028.
- Zhang, L. H., Xu J. L., and Birch, R. G. 1999. Engineered detoxification confers resistance against a pathogenic bacterium. *Nature Biotech.* 17:1021-1024.

AUTHOR-RECOMMENDED INTERNET RESOURCES

NCBI BioProject *Dickeya zeae* Ech1591 webpage:
www.ncbi.nlm.nih.gov/genomeprj/59297
 SMART database: smart.embl-heidelberg.de