A Substrate-Activated Efflux Pump, DesABC, Confers Zeamine Resistance to Dickeya zeae

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ABSTRACT Zeamines are a family of polyamino phytotoxins produced by Dickeya zeae EC1. These phytotoxins are also potent antibiotics against a range of microorganisms. To understand how D. zeae EC1 can protect itself from the antimicrobial activity of zeamines, we tested whether the ABC transporter genes within the zms (zeamine synthesis) gene cluster were related to zeamine resistance. Our results ruled out the possible involvement of these ABC transporters in zeamine resistance and instead unveiled an RND (resistance-nodulation-cell division) efflux pump, DesABC, which plays an important role in zeamine resistance in D. zeae EC1. The desAB genes are located next to the zms gene cluster, but desC is at a distant location in the bacterial genome. Null mutation of the desABC genes in a zeamine-minus derivative of strain EC1 led to about an 8- to 32-fold decrease in zeamine tolerance level. This efflux pump was zeamine specific and appeared to be conserved only in Dickeya species, which may explain the high potency of zeamines against a wide range of bacterial pathogens. Significantly, expression of the desAB genes was abolished by deletion of zmsA, which encodes zeamine biosynthesis but could be induced by exogenous addition of zeamines. The results suggest that sophisticated and coordinated regulatory mechanisms have evolved to govern zeamine production and tolerance. Taken together, these findings documented a novel signaling role of zeamines and the first resistance mechanism against zeamines, which is a family of potent and promising antibiotics against both Gram-positive and Gram-negative bacterial pathogens.

IMPORTANCE Zeamines are a family of newly identified phytotoxins and potent antibiotics produced by D. zeae EC1. Unlike most bacterial organisms, which are highly sensitive, D. zeae EC1 is tolerant to zeamines, but the mechanisms involved are unknown. Our study showed, for the first time, that a new RND efflux pump, DesABC, is indispensable for D. zeae EC1 against zeamines. We found that the DesABC efflux pump was zeamine specific and appeared to be conserved only in the Dickeya species, which may explain the high potency of zeamines against a wide range of bacterial pathogens. We also showed that expression of DesABC efflux system genes was induced by zeamines. These findings not only provide an answer to why D. zeae EC1 is much more tolerant to zeamines than other bacterial pathogens but also document a signaling role of zeamines in modulation of gene expression.

KEYWORDS RND efflux pump, antimicrobial resistance, phytotoxin, rice stem rot, zeamines

The phytopathogen Dickeya zeae can cause severe infections on both dicotyledonous and monocotyledonous plants (1). Similar to other species in the Dickeya genus, the virulence of D. zeae is linked to cell motility, biofilm formation, and production of cell wall-degrading enzymes (1–3), except that D. zeae also produces a family of

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phytotoxins, known as zeamines (4, 5). Zeamines are a family of structurally related polyamino compounds that play important roles in the pathogenicity of D. zeae EC1. Inactivation of zmsA, the key gene responsible for the biosynthesis of all zeamine compounds, abrogated the infectivity of D. zeae EC1 on rice, potato, and Chinese cabbage (4).

Zeamines are produced by Dickeya species and Serratia plymuthica strains with the zms gene cluster, including D. zeae EC1 and S. plymuthica RVH1 (4–9). Among them, zeamine, zeamine I, and prezeamine are the derivatives of a polyamino chain zeamine II, with polyketide moiety conjugating at the terminal amino group of zeamine II (4–7, 9). Apart from their important role in the virulence of D. zeae EC1, zeamines are also potent antibiotics with broad-spectrum activity against various organisms, including multidrug-resistant bacteria, fungi, oomycetes, and nematodes (8–10). Evidence shows that zeamines target the outer membrane of Gram-negative bacteria in a way reminiscent of the cationic antimicrobial peptide polymyxin B (11). Organization of the zms gene clusters is genetically well conserved in D. zeae EC1 and S. plymuthica RVH1, with genes encoding polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), and fatty acid synthases (FASs). In addition, five genes within the zms gene cluster were predicted to encode transporter proteins, including four encoding putative ATPases and permeases associated with the ABC transporter system and one encoding a potential HlyD superfamily protein (6, 8, 12). One of the predicted ABC transport systems encoded by zmn20 and zmn21 was proposed to be a zeamine transporter and associated with zeamine resistance in S. plymuthica RVH1 (6), but this speculation has not yet been validated experimentally.

Multidrug resistance (MDR) efflux pumps are membrane-associated proteins that can export a wide range of antibiotics and confer intrinsic antibiotic-resistant ability to bacteria. The efflux pumps can be classified into five superfamilies: MFS (major facilitator superfamily), ABC (ATP-binding cassette), SMR (small multidrug resistance), MATE (multidrug and toxic compound extrusion), and RND (resistance-nodulation-cell division) (13). In Gram-negative bacteria, RND efflux pumps play important roles in MDR due to their broad-spectrum substrate profile (14). The RND efflux pump is a tripartite complex system comprised of an outer membrane channel, an adaptor, and an inner membrane protein, all of which are required for the full function of antibiotic transportation (15). The genes responsible for encoding RND efflux pumps are commonly presented as a single operon in bacteria, like the MexAB-OprM efflux pump in Pseudomonas aeruginosa (16), but there are also exceptional cases with the gene encoding outer membrane channel protein placed in another location in the genome (17). In RND efflux pumps, antibiotic specificity is determined by the inner membrane protein. Antibiotics belonging to different families can enter into the inner membrane proteins through three putative entrance channels opening to the central cavity of inner membrane protein, the inner membrane, and periplasmic space of bacterial cells (18). The multiple active binding sites in the porter region of inner membrane proteins make it possible for the RND efflux pumps to transport a variety of structurally unrelated antibiotics produced by bacteria themselves (19) or from the environment (20, 21).

How D. zeae EC1 protects itself from the antimicrobial activity of zeamines remains unknown. While the MICs of zeamines for most bacterial pathogens are low, in the range of 0.3 to 10 μg/ml (9), our preliminary assay results showed that the zeamine producer D. zeae EC1 could tolerate up to 1,800 μg/ml of zeamines, suggesting a high-level resistance mechanism(s) is encoded by the D. zeae EC1 genome. In this study, we tested whether the transporter genes within the zms gene cluster, which were speculated to play roles in zeamine resistance (6), and the adjacent genes encoding RND efflux pump are associated with zeamine resistance in D. zeae EC1. Our results rule out the possible involvement of the ABC transporter genes within the zms gene cluster in zeamine resistance but lead to identification of a RND efflux pump, DesABC, that confers a high level of zeamine tolerance in D. zeae EC1. Substrate specificity assay against a range of antibiotics showed that DesABC appeared to only confer resistance against zeamines. In addition, the DesABC efflux system was found to be functionally
conserved in *Dickeya* species. Interestingly, consistent with the zeamine-specific pattern of DesABC, we found that the transcriptional expression of its coding genes, *desAB*, was stimulated by the presence of zeamines, suggesting that DesABC co-evolved with the genes encoding zeamine biosynthesis to ensure high-level production of the antibiotics.

### RESULTS

The ABC transporter systems encoded by the *zms* gene cluster are not required for zeamine resistance in *D. zeae* EC1. As the transporter systems present within the antibiotic biosynthesis gene clusters often confer resistance to the encoded antibiotics, we conducted bioinformatics analysis of the five transporter genes, i.e., *zmsP*, *zmsQ*, *zmsR*, *zmsL*, and *zmsM* (NCBI accession no. WP_016943528.1, WP_016943529.1, WP_016943530.1, WP_016943542.1, and WP_016943543.1, respectively) within the *zms* gene cluster of *D. zeae* EC1 (Fig. 1) (12). These genes share similar genetic organization (Fig. 1) and high levels of identity and similarity in amino acids (above 69% and 82%, respectively) (Table S2) to their homologs found in *S. plymuthica* and other *Dickeya* species and strains with the *zms* gene clusters (8). Among them, sequence analysis showed that *zmsP* encodes a potential HlyD family protein, *zmsR* and *zmsM* encode potential ABC transporter permeases, and *zmsQ* and *zmsL* encode potential ABC transporter ATPases. The homologs of *zmsM* and *zmsL* were hypothesized to encode a zeamine transporter, conferring self-resistance against zeamines in *S. plymuthica* RVH1 (6). As a previous study indicated that the HlyD family protein could form a functional transport system with ABC transporter proteins (22), ZmsP was considered a part of the ABC transporter systems encoded by the *zms* gene cluster. To elucidate the potential roles of these genes in zeamine resistance, in-frame deletion was performed to generate the deletion mutants of *zmsR* and *zmsM*, respectively, which represent the two putative transport systems encoded by the *zms* gene cluster of *D. zeae* EC1. Zeamines were purified from the cell culture of *D. zeae* EC1 and confirmed by liquid chromatography-mass spectrometry (LC-MS) (see Fig. S1 in the supplemental material) and used for determination of MIC against different bacterial strains in this study. The results showed that inactivation of *zmsR* or *zmsM* could not cause any change in the MIC of zeamines compared with those of the wild-type strain EC1 (data

**FIG 1** Organization of transporter genes within and adjacent to the *zms* gene cluster in *Dickeya* species and *Serratia plymuthica* strains. The organization of genes was drawn using Illustrator for Biological Sequences (34). Data were derived from NCBI and updated to 24 July 2018.
not shown). These findings preclude the possible association of these ABC transport systems with zeamine resistance, and their roles remain to be further investigated.

**DesABC efflux system is required for zeamine resistance.** In addition to the five potential transporter genes within the *zms* gene cluster, further bioinformatics analysis unveiled two genes encoding RND efflux pump proteins located adjacent to the *zms* gene cluster of *D. zeae* EC1 and another three *Dickeya* species and strains (Fig. 1). One of the gene clusters encodes a putative AcrA-like adaptor protein, and the other encodes a potential AcrB-like inner membrane protein. The AcrAB-ToIC RND efflux system has been well characterized as being associated with multiple antibiotic resistance in *Escherichia coli*, including β-lactams, tetracycline, chloramphenicol, and rifampin, with *acrAB* located together in the genome and *tolC* at a distant location (15). We proposed to name these two genes *desA* and *desB*, for *Dickeya* efflux system proteins *Aa* and *B* (Fig. 1). Interestingly, the *desAB* genes are not present within the vicinity of the *zms* gene cluster in *S. plymuthica* strains (Fig. 1).

Topological analysis of DesB revealed typical features of inner membrane transporter protein in an RND efflux system with 12 transmembrane helix domains (TM) and 2 large periplasmic loops spanning from TM1 to TM2 and TM7 to TM8 (Fig. S2) (23). In RND efflux systems, a tripartite complex is required for the full function of substrate transportation. To identify the outer channel protein for DesAB, a BLAST search was conducted to identify the homolog of *E. coli* outer membrane protein TolC. The result showed that only one *tolC* homolog (74% identity and 86% similarity at the amino acid level) is present in the genome of *D. zeae* EC1, which was designated *desC* accordingly.

To elucidate the role of the DesABC system in zeamine resistance, three *des* genes were deleted in-frame separately at the background of the strain defective in zeamine production, i.e., *zmsA* in-frame deletion mutant. Inactivation of *desA* and *desB* led to about an 8-fold decrease in the MIC of zeamines, while deletion of *desC* led to about a 32-fold decrease in MIC (Table 1). Consistent with the above-described results, in trans expression of *desB* and *desC* in the corresponding mutants could increase the zeamine resistance level of the mutants (Table 1). In addition, we found that heterologous expression of *desABC* under the control of the *lac* and *tetO* promoter in *E. coli* DH5α increased the MIC of zeamines by 2-fold (Table 1). These results demonstrate the role of the RND system DesABC in self-protection of *D. zeae* EC1 against the antimicrobial activity of zeamines.

**DesABC efflux system is zeamine specific and functionally conserved in Dickeya species.** DesABC belongs to the RND efflux systems, in which the inner membrane proteins associated with recognition and binding have been well characterized to aid in understanding their substrate profiles (14). For example, MexY from *P. aeruginosa* is required for streptomycin resistance (24), MexB and AcrB from *P. aeruginosa* and *E. coli* are associated with chloramphenicol and tetracycline resistance (25, 26), CmeB from *Campylobacter jejuni* plays a role in resistance against ampicillin, chloramphenicol, gentamicin, and tetracycline (27), and AdeB in *Acinetobacter baumannii* BM4454 is involved in tetracycline, chloramphenicol, gentamicin, and kanamycin resistance (28). To understand the potential substrate profile of the DesABC efflux pump, a phylogenetic

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**TABLE 1 Zeamine susceptibility of Dickeya zeae and Escherichia coli derivatives**

| Strain                              | ZEA MIC (µg/ml) |
|-------------------------------------|-----------------|
| Δ*zmsA                              | 1,800           |
| Δ*zmsAΔdesB                         | 225             |
| Δ*zmsAΔdesA                         | 225             |
| Δ*zmsAΔdesC                         | 56.25           |
| Δ*zmsAΔdesB(pBB-desB)               | 1,800           |
| Δ*zmsAΔdesB(pBB-desB<sub>3937</sub>) | 1,800           |
| Δ*zmsAΔdesC(pBB-desC)               | 900             |
| DH5αΔ*                              | 3.52            |
| DH5α(pBB, pAmob)                    | 3.52            |
| DH5α(pBB-desAB, pAmob-desC)         | 7.03            |

<sup>a</sup>ZEA, zeamines.
The phylogenetic tree was constructed with DesB of *D. zeae* EC1 and its homologs (sequence similarity above 93%) found by blastp search in *Dickeya* species, other proteobacterial species, including the homologs (sequence similarity above 80%) from *S. plymuthica* strains containing the *zms* gene cluster, as well as the above-mentioned inner membrane proteins with known functions (Table S3). The DesB phylogeny was largely consistent with known evolutionary relationships among the bacterial genomes. All of the *Dickeya* DesB proteins formed a monophyletic clade in the tree, whereas the homologs from other genera were more distantly related (Fig. S3). Notably, the inner membrane proteins with known substrate profiles were clustered together on the tree and showed considerable divergence from the DesB homologs from *Dickeya* species. The result suggests that DesB and its homologs from *Dickeya* species have a different substrate profile than their counterparts from other bacterial species.

The substrate profile of DesABC was then examined experimentally by MIC assay. The results showed that in trans expression of *desB* from *D. zeae* EC1 or *desB3937* from *D. dadantii* 3937, which lacks the *zms* gene cluster, in the *zmsA-desB* double deletion mutant of *D. zeae* EC1 could fully restore the zeamine resistance (Table 1), suggesting that the *desB* orthologs from other *Dickeya* species (Table S3) have a similar function in zeamine resistance. MIC assay was also performed using antibiotics which belong to different classes and have different targets (Table S4). The results showed that neither inactivation of *desB* nor overexpression of *desAB* genes in *D. zeae* EC1 could affect the MICs of ampicillin, tetracycline, kanamycin, gentamicin, streptomycin, and chloramphenicol (Table S5). The above data are consistent with the phylogenetic analysis results (Fig. S3), indicating that the DesABC system has a distinct substrate specificity.

**DesABC efflux system is essential for *D. zeae* survival against zeamines.** As the DesABC system was found to be required for zeamine resistance, a survival assay was conducted against zeamines using the *zmsA* mutant, the *zmsA-desB* double deletion mutant, and the double mutant complemented with *desB*. Bacterial cells were added to LS5 salts, without carbon source, supplemented with zeamines at a final concentration of 2-fold the MIC of the *zmsA-desB* double deletion mutant, and bacterial cell numbers were measured at different time points upon treatment with zeamines to evaluate the role of DesABC in *D. zeae* EC1 survival. The results showed that inactivation of *desB* resulted in a sharp decline in survival rate, whereas its parental and complemented strains could maintain upon to a three-log larger amount of survivors than the *desB* mutant at 30 min after treatment (Fig. 2). These results indicate that the DesABC efflux pump plays an indispensable role in the survival of strain EC1 when the bacterial cells were treated with zeamines.

**DesABC efflux system confers bacterial self-resistance against zeamines at the late stage of bacterial growth.** To investigate the protective spectrum of DesABC during cell growth, an in-frame deletion mutant of *desB* was constructed using *D. zeae*
The cell growth curves and zeamine production of wild-type strain EC1 and its desB mutant were compared in LS5 medium, which was optimized for zeamine production (29). At the early stage after inoculation (12 to 24 h), the growth rate and zeamine production were comparable between strain EC1 and its desB mutant (Fig. 3A). However, the growth of the desB mutant was arrested in the subsequent stages after 24 h (Fig. 3A), and similarly, accumulation of zeamines in bacterial supernatant was also flattened after 24 h (Fig. 3B). These results suggest that D. zeae cells could tolerate a certain level of zeamines in the absence of the DesABC efflux system, but along with bacterial growth and zeamine accumulation, the DesABC efflux pump...
becomes indispensable for protecting bacterial cells against the detrimental effect of zeamines.

To determine the relationship between expression of DesABC genes and zeamine resistance, the *gfp* coding region was placed under the promoter of *desAB*, and the transcriptional fusion construct pDesABgfp was prepared. The expression of *desAB* was evaluated by monitoring the fluorescence of wild-type strain EC1 containing the pDesABgfp construct grown in LS5 medium by using a CytoFLEX flow cytometer system. The results showed that expression of *desAB* was bacterial population density dependent, showing a basal-level expression at the early growth stage (12 to 20 h) and rapidly increased expression at 20 h onward after inoculation (Fig. 3C). As the DesABC system was critical to the bacterial growth at the late growth stage (Fig. 3A), the above results indicate that zeamine resistance mediated by the DesABC efflux system is positively related to the expression level of *desAB* genes.

Expression of *desAB* is induced by zeamines. Expression of *desAB* genes was consistent with zeamine production during cell growth (Fig. 3), suggesting that *desAB* expression is influenced by zeamines. To test this possibility, the pDesABgfp construct was introduced into the *D. zeae* ΔzmsA mutant. Our previous results showed that deletion of *zmsA* abolished production of all the zeamines (4). The *gfp* expression level driven by the promoter of *desAB* in wild-type strain EC1 and the ΔzmsA mutant was monitored at different time points during bacterial growth in LS5 medium. The results showed that the growth patterns of both strains were similar (Fig. 4A), but the

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**FIG 4** Expression of *desAB* is positively related to the exposure of zeamines. (A) Growth curves of EC1(pDesABgfp) and ΔzmsA(pDesABgfp) strains were measured in the flasks with LS5 medium at 12 h, 16 h, 20 h, and 24 h after inoculation. (B) The *desAB* expression patterns in the wild-type strain EC1 and ΔzmsA zeamine-minus mutant. The relative fluorescence was expressed as the fluorescence monitored at specific time points normalized to the fluorescence of strain EC1(pDesAB_gfp) at 12 h after inoculation. (C) qPCR analysis of *desB* gene expression in strain EC1 and the ΔzmsA mutant. (D) Relative fluorescence of ΔzmsA(pDesAB_gfp) and 3937(pDesAB3937_gfp) strains with exogenous addition of polymyxin B (PMB) or zeamines (ZEA). The same amounts of water (WAT) and methanol (MET) were added as controls. The ΔzmsA(pDesAB_gfp) or 3937(pDesAB3937_gfp) strain was grown in LS5 medium and adjusted to an OD<sub>600</sub> of about 0.5 (±0.05). Bacterial cells were then treated with different concentrations of polymyxin B or zeamines for 8 h. The relative fluorescence of cells with different treatments was normalized to the fluorescence of cells in water or methanol control, which was set as 1. Data in the graph show the means from three repeats and standard deviations (A, B, and D) or standard errors (C).
expression levels of desAB in these strains were varied substantially (Fig. 4B). The expression of desAB in strain EC1 was increased along with bacterial growth but remained flat in the toxin-minus ΔzmsA mutant (Fig. 4B).

For validation of the findings described above, the transcript levels of the DesABC efflux system genes in D. zeae strain EC1 and the zmsA mutant were determined at an optical density at 600 nm (OD600) of about 1.5 (approximately 20 h after inoculation). The quantitative PCR (qPCR) results showed that although the transcript level of desC was comparable between strain EC1 and the ΔzmsA mutant (fold change of less than 2; data not shown), the desB transcript level in strain EC1 was significantly higher than that in the ΔzmsA mutant, which was hardly detectable (Fig. 4C). The basal level of expression of desAB noticed in the zmsA mutant coupled with their induced expression in wild-type strain EC1 suggest that expression of the desAB genes rely on the product of zmsA, i.e., zeamines.

To further confirm this assumption, expression of desAB was monitored in the ΔzmsA(pDesABgfp) strain treated with zeamines. The result showed that a supplement of zeamines at a final concentration of 100 μg/ml did not decrease the cell growth of the ΔzmsA(pDesABgfp) strain but led to about a 30-fold higher expression of desAB genes compared with that of the control without addition of zeamines (Fig. 4D). Notably, a low concentration of zeamines, 5 μg/ml, could significantly induce the expression of desAB genes in D. zeae EC1 and their homologs in D. dadantii 3937. As a control, we also tested whether expression of desAB could be induced by polymyxin B by incubation of the ΔzmsA(pDesABgfp) strain with polymyxin B at the same concentration as zeamines. The results showed that unlike zeamines, polymyxin B could not trigger the expression of desAB genes (Fig. 4D). Cumulatively, these results unveil a novel and specific signaling role of zeamines in triggering the expression of the desAB genes in Dickeya species and strains.

**DISCUSSION**

Unlike most bacterial pathogens, which are highly sensitive to zeamines (9, 10), the zeamine producer D. zeae EC1 can withstand a high level of zeamines. In this study, we identified a novel RND efflux pump, DesABC, that plays a role in resistance against zeamines, especially at the late stage of bacterial growth, when zeamines were accumulated at a high level. Null mutation of the efflux pump could lead to up to about a 32-fold decrease in zeamine resistance. In contrast, the DesABC efflux pump was not functional against a range of conventional antibiotics, including ampicillin, tetracycline, kanamycin, gentamicin, streptomycin, and chloramphenicol. Furthermore, we also showed that desAB expression was growth and zeamine dependent and documented a novel signaling role of zeamines in regulation of desAB transcription. Moreover, we found that deletion of the desB gene could substantially reduce the accumulation of zeamines, suggesting that zeamine biosynthesis and resistance are modulated by coordinated and sophisticated regulatory mechanisms.

The zeamine biosynthesis genes and desAB were clustered in the genomes of D. zeae EC1 and another three Dickeya species and strains according to bioinformatics analysis (Fig. 1). However, despite their functional relevance (Fig. 4), the results from this study suggest that desAB and zeamine biosynthesis genes are not tightly linked during evolution. This is evident as the genomes of multiple Dickeya species and strains, such as D. zeae Ech586 and D. dadantii 3937, contain desAB but not the zeamine biosynthesis genes. It is possible that the common ancestor of Dickeya contains both desAB and zeamine biosynthesis genes, but some Dickeya species and strains subsequently lost the ability to produce zeamines while maintaining DesABC as a defense mechanism. How the genes responsible for zeamine biosynthesis and resistance have originated during evolution remains to be further investigated. Interestingly, the desAB genes were not found in the vicinity of the zms gene cluster in S. plymuthica strains (Fig. 1), which is also known for production of zeamines (6, 7), suggesting that D. zeae and S. plymuthica have different evolutionary origins of the genes encoding zeamine biosynthesis and resistance.
The RND family proteins associated with antibiotic resistance can commonly transport a broad spectrum of substrates, which is determined by the corresponding inner membrane proteins of the efflux pumps (14). Most inner membrane proteins in the RND family found in human bacterial pathogens, including *E. coli* (15), *P. aeruginosa* (16), *Burkholderia* (30), and *Acinetobacter* (31), are related to transportation of multiple structurally dissimilar antibiotics. In contrast, we found that the DesABC efflux system of *D. zeae* EC1 was zeamine specific (Table 1; see also Table S5 in the supplemental material). Interestingly, two RND efflux pump systems found in *Erwinia amylovora*, a plant bacterial pathogen causing fire blight disease, also displayed narrow substrate specificity. Overexpression of these two RND efflux systems, MdtABC and MdtUVW, only resulted in the increment of MIC of several phytochemicals but had no effect on various conventional antibiotics (21), including some antibiotics used in our study (Table S5). Given that both *D. zeae* and *E. amylovora* are plant pathogens with little exposure to conventional antibiotics, these findings agree with the previous findings about the linkage between intrinsic resistance and the habitat of environmental bacteria (32). The narrow substrate specificity of MdtABC and MdtUVW in *E. amylovora* and DesABC in *D. zeae* EC1 may reflect the ancient role of the corresponding RND efflux pumps for bacteria to survive in hazardous environments generated by bacteria themselves or by other organisms.

Intriguingly, our results showed that inactivation of the *desABC* genes could cause different levels of decrement in the MIC of zeamines. Mutation of *desA* or *desB* led to only about an 8-fold decrease in MIC compared with that of the control strain (Table 1), while inactivation of *desC* could result in about a 32-fold decrease. A plausible explanation is that similar to its homolog tolC in *E. coli* (15), other proteins in strain EC1 are able to replace DesAB and form a functional transportation system with DesC to efflux zeamines. In addition, heterologous expression of *desABC* in *E. coli* resulted in only about a 2-fold increment in the MIC of zeamines, which was not comparable to the MIC changes when *desABC* were deleted in strain EC1. We first checked the possibility of whether the differences in GC content and codon usage of these two bacterial species affect the expression efficiency of *desABC* genes in *E. coli*. Our previous study showed that the GC content of the *D. zeae* EC1 genome is 53.43% (12), which is not identical but not substantially different from the GC content (50.8%) of *E. coli* strain K-12 (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/Escherichia%20coli%20K-12). Strain K-12 is the parental strain of *E. coli* strain DH5α used in this study. At the codon usage level, both bacteria have more or less similar codon usage patterns, except that the rarely used codon CUA in *E. coli* is a frequently used codon in the coding sequence of *desB* in *D. zeae* EC1 (Table S6). We then examined the potential toxic effect of overexpressed DesABC in *E. coli*, as other overexpressed membrane proteins commonly exhibit detrimental effects on bacterial growth (33). We found that *E. coli* growth was markedly retarded when DesABC were overexpressed (Fig. S4). Taken together, it is most likely that overexpressed DesABC membrane proteins affect the bacterial normal physiological functions and, hence, compromise the ability to withstand zeamines.

Our data indicate that zeamine biosynthesis and DesABC-mediated resistance mechanisms are well coordinated by a sophisticated mechanism(s). The level of *desAB* gene expression was increased along with accumulation of zeamines (Fig. 3B and C and 4B), and deletion of *zmsA*, the gene essential for zeamine biosynthesis (4), caused an arrest of the transcriptional expression of *desAB* (Fig. 4B and C). Significantly, exogenous addition of zeamines to the *zmsA* mutant could boost *desAB* expression by more than 30-fold compared with that for water or solvent control (Fig. 4D). The findings thus demonstrated that in addition to their roles as phytotoxins and antibiotics (4, 5, 8–10), zeamines can also act as signals in modulation of gene expression (Fig. 4D). The signal role of zeamines in induction of *desAB* expression was further confirmed in *D. dadantii* strain 3937 (Fig. 4D), which does not contain a *zms* gene cluster. Considering the wild distribution of *zms* gene clusters and *desAB* homologs in *Dickeya* species and strains (Fig. 1 and Table S3) (8), we hypothesize that *Dickeya* species and strains have evolved
a dedicated pathway to sense extracellular zeamines in self-protection against the detrimental effect of these antibiotics. The key regulators in this pathway might at least include a sensor or receptor protein that detects and responds to zeamines and a transcriptional regulator that modulates the expression of desAB. A gene that encodes a proposed two-component system sensor (NCBI accession number WP_029456608.1) was found near the desAB locus. However, inactivation of this gene did not affect the MIC of zeamines, which seems to preclude its potential link with the regulation of desAB (data not shown). In addition, given that the cellular levels of zeamines are important in induction of the zeamine resistance genes (Fig. 4D), several regulators known to be associated with the regulation of zeamine production and virulence, such as the acylhomoserine lactone (AHL) synthase ExpI (1), transcriptional regulator SlyA (3), and global regulator Fis (46), might also influence the transcriptional expression of desAB through modulating the production of zeamines or even more direct regulatory mechanisms, which demands further investigation.

In summary, this study documented a first resistance mechanism against zeamines, which are a new family of potent antibiotics with a broad spectrum of antimicrobial activities. This resistance mechanism is mediated by a novel and substrate-specific RND efflux pump, DesABC. Interestingly, this study also unveiled a signaling role of zeamines in modulation of desAB expression at the transcriptional level, which further expands our understanding about zeamines. In addition, the findings from this study suggest that D. zeae EC1 contains other mechanisms implicated in zeamine resistance besides the DesABC efflux system. This is evident as the MIC of zeamines for D. zeae EC1 was more than 500-fold higher than that for the zeamine-sensitive E. coli DH5α, whereas inactivation of desC in D. zeae EC1 led to only about a 32-fold decrease in the MIC of zeamines (Table 1). A thorough understanding of the zeamine resistance mechanisms and the cognate regulatory networks might pave the way for practical application of these potent antibiotics and also could provide new insight on the control and prevention of this important bacterial pathogen.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The strains and plasmids used in this study are listed in Table 2. D. zeae EC1 and derivatives were routinely grown at 28°C in Luria-Bertani (LB) medium, minimal medium (MM) [10.5 g K₂HPO₄, 4.5 g KH₂PO₄, 2.0 g (NH₄)₂SO₄, 2.0 g mannitol, 2.0 g glycerol, 0.2 g MgSO₄·7H₂O, 0.01 g CaCl₂, 0.005 g FeSO₄·7H₂O, and 0.002 g MnCl₂·4H₂O per liter, pH 7.0], or L55 medium (5.25 g K₂HPO₄, 2.25 g KH₂PO₄, 10.0 g sucrose, 3.6 g NH₄NO₃, 1.0 g KCl, and 0.25 g MgSO₄·7H₂O per liter, pH 7.0) as indicated (29). E. coli strains were routinely grown at 37°C. The following antibiotics were supplemented when necessary: streptomycin, 50 μg/ml; kanamycin, 50 μg/ml; ampicillin, 50 μg/ml; chloramphenicol, 15 μg/ml.

**Construction of deletion and complementation strains.** Oligonucleotide primers used in this study are listed in Table S1 in the supplemental material. DNA manipulation was conducted by following methods described previously (5). Briefly, for gene in-frame deletion, fusion fragments containing the downstream and upstream regions of target genes were cloned into pKNG101 and transformed into E. coli CC118 for construction of gene in-frame deletion constructs. Triparental mating was performed by using wild-type strain EC1 or a zmsA in-frame deletion mutant as a recipient strain. Mutants were screened on an MM agar plate supplemented with 5% (wt/vol) sucrose, and desired deletions were confirmed by PCR and DNA sequencing. For complementation, the open reading frames (ORFs) of target genes were cloned into pBBR1-MCS4 and genes were expressed under the control of the lac promoter. The desired expression constructs were confirmed by PCR and DNA sequencing and introduced into corresponding mutants by triparental mating. The complementation strains were screened on MM agar plates containing ampicillin and verified by PCR. For construction of the strain expressing desABC heterologously, desC was cloned and expressed under the control of the tetO promoter in pAmob, while desAB were cloned into pBBR1-MCS4 and expressed under the control of the lac promoter. The resultant constructs, pBB-desAB and pAmob-desC, were cotransformed into E. coli DH5α for heterologous expression of the desABC efflux pump genes.

**Preparation of zeamines.** Overnight starter culture of wild-type strain EC1 grown in LB medium was inoculated (0.1%, vol/vol) into L55 medium and grown at 28°C with rotation at 100 rpm for 48 h. The cells were then removed by centrifugation at 10,000 rpm at 4°C for 10 min. Approximately 10-liter supernatants were then passed slowly through the column containing 500 g of absorbent resin XAD7 (Sigma) at a flow rate of 1 ml/min by following the method described previously (4). The column was consecutively eluted with 2 liters of double-distilled H₂O and 1 liter of methanol prior to elution with 2 liters of acetone to obtain the elutes containing zeamines. The acetone in the elutes was evaporated, and the residues were dissolved in methanol to obtain crude zeamine antibiotics. For confirmation, liquid chromatography-mass spectrometry (LC-MS) was performed using an Agilent 1260 infinity system.
**TABLE 2** Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Source or reference |
|-------------------|---------------------------|---------------------|
| **Strains**       |                           |                     |
| *Dickeya zeae*    |                           |                     |
| EC1               | Wild-type strain of *D. zeae* | 1                   |
| EC1(pDesAB<sub>gfp</sub>) | EC1 carry pDesAB<sub>gfp</sub> vector, Kan<sup>r</sup> | This study          |
| ΔzmsA            | In-frame deletion of zmsA in EC1 | Laboratory collection |
| ΔzmsA(pDesAB<sub>gfp</sub>) | ΔzmsA carrying pDesAB<sub>gfp</sub> vector, Kan<sup>r</sup> | This study          |
| ΔzmsAΔdesAB      | ΔzmsA carrying the in-frame deletion of desA | This study          |
| ΔzmsAΔdesB       | ΔzmsA carrying the in-frame deletion of desB | This study          |
| ΔzmsAΔdesB<sub>pBB</sub> | ΔzmsAΔdesB carrying pBBR1-MCS4 vector, Amp<sup>r</sup> | This study          |
| ΔzmsAΔdesB<sub>pBB-desB</sub> | ΔzmsAΔdesB carrying pBB-desB vector, Amp<sup>r</sup> | This study          |
| ΔzmsAΔdesB<sub>pBB-desB<sub>3937</sub></sub> | ΔzmsAΔdesB carrying pBB-desB<sub>3937</sub> vector, Amp<sup>r</sup> | This study          |
| ΔzmsAΔdesC       | ΔzmsA carrying the in-frame deletion of desC | This study          |
| ΔzmsAΔdesC<sub>pBB-desC</sub> | ΔzmsAΔdesC carrying pBB-desC vector, Amp<sup>r</sup> | This study          |
| ΔdesB            | In-frame deletion of desB in EC1 | This study          |
| ΔdesR            | In-frame deletion of desR in EC1 | Laboratory collection |
| ΔzmsM            | In-frame deletion of zmsM in EC1 | Laboratory collection |
| **Escherichia coli** |                           |                     |
| DHS<sub>sa</sub>  | F<sup>−</sup> pBla<sub>sa</sub>ΔZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17<sub>K</sub> m<sub>K</sub> <sup>−</sup> m<sub>K</sub> <sup>−</sup> | TransGen Biotech, China |
| DHS<sub>sa</sub>(pBB, pAmob) | DHS<sub>sa</sub> harboring both pBBR1-MCS4 (Amp<sup>r</sup>) and pAmob (Tetr, Chlr) | This study          |
| DHS<sub>sa</sub>(pBB-desAB, pAmob-desC) | DHS<sub>sa</sub> harboring both pBB-desAB (Amp<sup>r</sup>) and pAmob-desC (Chlr) | This study          |
| CC118            | Host strain for replication of pKNG101 and derivate plasmids | Laboratory collection |
| HB101(pRK2103)   | thr leu thi recA hasdR hasdM pro, Kan<sup>r</sup> | Laboratory collection |
| *Dickeya dadantii* |                          |                     |
| 3937(pDesAB<sub>3937</sub><sub>gfp</sub>) | 3937 carrying pDesAB<sub>3937</sub><sub>gfp</sub>, Kan<sup>r</sup> | This study          |
| **Plasmids**     |                           |                     |
| pKNG101          | Suicide vector for gene in-frame deletion, Str<sup>r</sup> | Laboratory collection |
| pKNG-desA        | pKNG101 harboring flanking region of desA | This study          |
| pKNG-desB        | pKNG101 harboring flanking region of desB | This study          |
| pKNG-desC        | pKNG101 harboring flanking region of desC | This study          |
| pBB              | Low-copy-number vector pBBR1-MCS4 with lac promoter, Amp<sup>r</sup> | Lab collection     |
| pBB-desB         | pBBR1-MCS4 harboring ORF of desB gene from EC1, Amp<sup>r</sup> | This study          |
| pBB-desB<sub>3937</sub> | pBBR1-MCS4 harboring ORF of desB gene homolog from *Dickeya dadantii* 3937, Amp<sup>r</sup> | This study          |
| pBB-desAB        | pBBR1-MCS4 harboring ORF of desAB genes from EC1, Amp<sup>r</sup> | This study          |
| pBB-desC         | pBBR1-MCS4 harboring ORF of desC gene from EC1, Amp<sup>r</sup> | This study          |
| pAmob            | pACYC184 with mob region cloned from pBBR1-MCS4 inserting in BstZ17I restriction site, Tett, Chlr<sup>r</sup> | This study          |
| pAmob-desC       | pAmob harboring desC gene from EC1 under the control of tetracycline resistance gene promoter tetO, Chlr<sup>r</sup> | This study          |
| pPROBE<sub>NT</sub> | Promoterless gfp transcriptional reporter plasmid, Kan<sup>r</sup> | 43                  |
| pDesAB<sub>gfp</sub> | gfp transcriptional fusion with upstream region of desAB in EC1 | This study          |
| pDesAB<sub>3937</sub><sub>gfp</sub> | gfp transcriptional fusion with upstream region of desAB<sub>3937</sub> found in 3937 | This study          |

<sup>a</sup>Abbreviations: Amp<sup>r</sup>, ampicillin resistance; Tett, tetracycline resistance; Chlr<sup>r</sup>, chloramphenicol resistance; Kan<sup>r</sup>, kanamycin resistance; Str<sup>r</sup>, streptomycin resistance.

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trimAl v1.4 (37) with the “-gappyout” option. The filtered MSA was analyzed by IQ-TREE v1.6.5 (38) to first perform a model selection with the “-m” option (39), and then we carried out a maximum-likelihood tree inference under the best-fit model ("LG+R5") with 1,000 ultrafast bootstrap support (40).

**Survival assay.** The survival assay was conducted by following the kill curve method described previously, with minor modifications (41). Briefly, fresh bacterial cultures in LB medium at exponential phase were collected and adjusted to an OD_{600} of about 1.0 (±0.05). Cells from 1 ml culture were harvested (4,000 rpm, 4°C, 5 min) and washed twice with LSS salts (LSS medium without sucrose). Bacterial cells were then resuspended in LSS salts and added to 96-well plates with LSS salts containing zeamines. The final concentration of zeamines in the assay was at 2-fold the MIC of the zmsA-desB mutant. The plates were incubated at 28°C with agitation at 200 rpm, and the survivors were determined at specific time points by plating appropriate bacterial dilutions on LB plates.

**Growth kinetics assay measured in the flasks with LSS medium.** Bacterial growth curves in LSS medium were measured by following the procedures described for zeamine preparation, with minor modifications. Briefly, overnight cell cultures were adjusted to an OD_{600} of about 0.5 (±0.05) before inoculation at a ratio of 0.1%, vol/vol, and flasks were kept at 28°C with shaking at 150 rpm. The optical density at 600 nm was measured at different time points, as indicated, by the NanoDrop 2000c (Thermo Fisher Scientific, MA, USA) with proper dilutions when necessary.

**Zeamine production assay.** The assay of zeamine production was conducted by following a method described previously, with minor modifications (4). Briefly, 25 ml LB agar was poured in 10- by 10-cm square plates and overlaid with 7.5 ml 1% (wt/vol) agarose containing about 1.5 × 10^8 fresh E. coli DH5α cells. The wells, at 4-mm diameter, were punched in the plate, and 30 μl of cell-free supernatants (filter sterilized with a 0.22-μm pore filter) were added in each well. The plates were incubated at 37°C for 24 h, and the inhibition zone around the wells was measured. For semiquantification, one unit of zeamines was defined as the amount that could generate a 2-mm-diameter inhibitory zone around the well. The number of zeamine units per milliliter was calculated from the bioassay by the fold change of sample volume used in the test (30 μl) to the total volume (1 ml).

**Construction of transcriptional fusion construct pDesAB_{gfp} and pDesAB_{3937gfp} and flow cytometry analysis.** As there is only a 20-bp interval region located between the ORFs of desA and desB, the desAB genes were considered to be located in the same operon. The promoter region of desAB genes was predicted by using the online tool provided by BPROM (42) (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). The 204-bp DNA fragment upstream of the ORF of desAB was amplified using the primer pair P-desAB-F and P-desAB-R (Table S1) and ligated into the pPROBE-NT (Tiangen Biotech, Co., Ltd., Beijing, China) by following the manufacturer’s protocol. Specific primers for gfp promoterless were separately mobilized into wild-type EC1 and the ΔzmsA mutant by triparental mating with the helper strain HB101(pRK2013) to construct EC1(pDesAB-gfp) and ΔzmsA(pDesAB-gfp). 3937(pDesAB_{3937gfp}) was constructed by a similar method. Expression of desAB and desAB_{3937} was analyzed by monitoring the average fluorescence of 50,000 cells when bacteria were grown in flasks with LSS medium at different time points by a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) by following the method previously described (44).

**RNA extraction and real-time qPCR analysis.** Bacterial cells were cultured and harvested at an OD_{600} of about 1.5 (±0.05). RNA extraction was performed using the RiboPure RNA purification kit, bacteria (Thermo Fisher Scientific, MA, USA), by following the manufacturer's instructions. The purity of RNA was determined by gel electrophoresis, and the A_{260}/A_{280} and A_{260}/A_{230} ratios were determined using a NanoDrop 2000c (Thermo Fisher Scientific, MA, USA). In qPCR analysis, an aliquot of 300 ng RNA sample was used for genomic DNA elimination and cDNA synthesis by a FastKing RT kit (with gDNase) (Tiangen Biotech, Co., Ltd., Beijing, China) by following the manufacturer’s protocol. Specific primers for the desC, desB, and 16S rRNA genes (Table S1) were designed by AlleleID 6.0 (PRIMER Biosoft). The 204-bp DNA fragment upstream of the ORF of desAB was amplified using the primer pair P-desAB-F and P-desAB-R (Table S1) and ligated into the pPROBE-NT (Tiangen Biotech, Co., Ltd., Beijing, China) by following the manufacturer’s protocol. Specific primers for desAB genes were considered to be located in the same operon. The promoter region of desAB genes was predicted by using the online tool provided by BPROM (42) (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). The 204-bp DNA fragment upstream of the ORF of desAB was amplified using the primer pair P-desAB-F and P-desAB-R (Table S1) and ligated into the pPROBE-NT (Tiangen Biotech, Co., Ltd., Beijing, China) by following the manufacturer’s protocol. Specific primers for the desC, desB, and 16S rRNA genes (Table S1) were designed by AlleleID 6.0 (PRIMER Biosoft). The housekeeping gene 16S rRNA was used as a reference. The PCR efficiency of each gene was determined using five DNA standards at different concentrations (10, 1, 0.1, 0.01, and 0.001 μg/ml). The qPCR analysis was conducted on a Quantstudio 6 Flex system using PowerUp SYBR green master mix (Thermo Fisher Scientific) with the following cycle profile: 1 cycle at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 57°C for 15 s, and 72°C for 30 s. The experiment was repeated three times, each time with triplicates. Data were analyzed using the 2^{-ΔΔCT} method as previously described (45).

**Statistical analysis.** Experiments were individually performed at least twice with three replicates each time. Data shown are the means from three replicates, and error bars indicated the standard deviations or standard errors. Statistical comparison was performed by using Student’s t test in GraphPad Prism 5.0 software (GraphPad, La Jolla, CA). A P value of less than 0.05 was considered significant.

**Data availability.** The genome sequence of D. zeae EC1 is accessible in NCBI under accession number NZ_CP006929.1. The amino acid sequence of TolC in E. coli K-12 AG100 is accessible in NCBI under accession number WP_000735278.1.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00713-19.

- **FIG S1**, PDF file, 0.3 MB.
- **FIG S2**, PDF file, 0.3 MB.
- **FIG S3**, DOCX file, 1.8 MB.
- **FIG S4**, PDF file, 0.3 MB.
TABLE S1, DOCX file, 0.03 MB.

TABLE S2, DOCX file, 0.03 MB.

TABLE S3, DOCX file, 0.03 MB.

TABLE S4, DOCX file, 0.02 MB.

TABLE S5, DOCX file, 0.02 MB.

TABLE S6, DOCX file, 0.03 MB.

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Z.L., L.H., F.H., X.Z., Z.S., J.Z., Y.C., M.L., and Y.C. performed experiments. Z.L. and L.-H.Z. designed experiments, analyzed the data, and wrote the paper.

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

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