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The Acyl-Homoserine Lactone-Type Quorum-Sensing System Modulates Cell Motility and Virulence of *Erwinia chrysanthemi* pv. zeae▽

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***Erwinia chrysanthemi* pv. zeae is one of the *Erwinia chrysanthemi* pathovars that infects on both dicotyledons and monocotyledons. However, little is known about the molecular basis and regulatory mechanisms of its virulence. By using a transposon mutagenesis approach, we cloned the genes coding for an *E. chrysanthemi* pv. zeae synthase of acyl-homoserine lactone (AHL) quorum-sensing signals ($expI_{Ecz}$) and a cognate response regulator ($expR_{Ecz}$). Chromatography analysis showed that $expI_{Ecz}$ encoded production of the AHL signal *N*-(3-oxo-hexanoyl)-homoserine lactone (OHHL). Null mutation of $expI_{Ecz}$ in the *E. chrysanthemi* pv. zeae strain EC1 abolished AHL production, increased bacterial swimming and swarming motility, disabled formation of multicell aggregates, and attenuated virulence of the pathogen on potato tubers. The mutation also marginally reduced the inhibitory activity of *E. chrysanthemi* pv. zeae on rice seed germination. The mutant phenotypes were rescued by either exogenous addition of AHL signal or in *trans* expression of $expI_{Ecz}$. These data demonstrate that the AHL-type QS signal plays an essential role in modulation of *E. chrysanthemi* pv. zeae cell motility and the ability to form multicell aggregates and is involved in regulation of bacterial virulence.**

Erwinia chrysanthemi pv. *zeae* is the major pathogen responsible for bacterial stalk rot of maize around the world (20, 29, 31). The pathogen was also found to cause rice foot rot in Asian countries, including Japan, China, India, Indonesia, and South Korea (11, 17). *E. chrysanthemi* pv. zeae is a member of the pathovars of the gram-negative bacterium *Erwinia chrysanthemi*, which has been divided, on the basis of its pathogenicity on host plants and certain biochemical and physiological differences, into six pathovars: pv. chrysanthemi, pv. dianthicola, pv. dieffenbachiae, pv. paradisiaca, pv. parthenii, and pv. zeae (7). The variations among these pathovars were subsequently verified by molecular genetic analysis (20, 21). While the rationality of this pathovar classification is still under debate and a new scheme of classification has been proposed (29), in which *E. chrysanthemi* pv. zeae was delineated as a novel species, *Dickeya zeae*, the distinct status of *E. chrysanthemi* pv. zeae in different taxonomy schemes may reflect its unshakeable differences from other pathovars, which will hereby be collectively referred to as *E. chrysanthemi* strains for convenience and to be consistent with numerous previous studies. Recent constant outbreaks of rice foot rot disease caused by *E. chrysanthemi* pv. zeae have stirred serious concern (17); however, little is known about the molecular bases of its host specificity and the pathogenic mechanisms of this important pathovar of *E. chrysanthemi*.

Among the closely related bacterial pathogens, a few *E. crysanthemi* strains that infect dicotyledonous plants, such as strains EC3937 and EC16, have been characterized extensively at biochemical and genetic levels. They are known to cause the soft rot disease that is characterized by foul-smelling rot and eventual collapse of plant tissues. The pathogens produce a range of pectinases as key virulence factors which degrade various components of pectins (5, 15, 28), as well as other degradative enzymes such as cellulase isozymes, protease isozymes, xylanase, and phospholipase (5, 15, 28). In addition, the pigment indigoidine and the siderophores chrysobactin and achromobactin have been implicated in the bacterial systemic infections (8, 9, 27). Production of the pectate lyases is regulated by the transcriptional repressor KdgR, whose repression is released by the presence of pectin degradation products such as 2-keto-3-deoxygluconate (28). In addition, the acyl-homoserine lactone (AHL)-type quorum-sensing (QS) signals may be implicated in the regulation of virulence. The QS system of *E. chrysanthemi* includes the AHL-dependent transcription factor ExpR and an enzyme, ExpI, which is responsible for the synthesis of *N*-(3-oxo-hexanoyl)-homoserine lactone (OHHL) and *N*-hexanoyl-homoserine lactone (22). Of these two AHLs, OHHL is the most abundant and was thus postulated to be the most physiologically important QS signal in *E. chrysanthemi* (22). Mutation of the AHL synthase gene, *expI*, results in a decrease of some pectinase gene expression but does not seem to significantly change the total pectinase activity of the pathogen (22).

In our preliminary work, we found that *E. chrysanthemi* pv. zeae strain EC1, which was isolated from a rice plant showing typical foot rot symptoms, was able to cause infections on both dicotyledonous and monocotyledonous plants. In contrast, *E. chrysanthemi* strain EC3937, which infects dicot plants, did not cause any visible symptoms or adverse effect on rice plants. It is curious how well the AHL QS system is conserved and whether it plays a similar role in different pathovars of *E.*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant phenotypes and characteristics[a]	Source or reference
Strains		
E. chrysanthemi. pv. zeae		
EC1	Wild-type rice foot rot pathogen, isolated from Guangdong Province, China	This study
WM3	EC1 defective in AHL production with Tn*5* inserted at bp 172 of the $expI_{Ecz}$ ORF	This study
WM6	EC1 defective in AHL production with Tn*5* inserted at bp 265 bp of $expI_{Ecz}$ ORF	This study
WM8	EC1 defective in AHL production with Tn*5* inserted at bp 265 of $expI_{Ecz}$ ORF	This study
WM3expI	Complementary strain of WM3 carrying plasmid construct pDSK-expI	This study
WM6expI	Complementary strain of WM3 carrying plasmid construct pDSK-expI	This study
WM8expI	Complementary strain of WM3 carrying plasmid construct pDSK-expI	This study
Other bacteria		
EC3937	Wild-type *E. crysanthemi* isolated from *Saintpaulia*	A. Charkowski
NT1(*traR*, *tra*::*lacZ749*)	*A. tumefaciens* strain harboring pJM749 and pSVB33, AHL biosensor strain	24
DH5α	*E. coli* strain; *supE44* Δ*lacU169*(ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* λ*pir*	Laboratory collection
S17-1	*E. coli* strain; pro res$^-$ mod$^+$ integrated copy of RP4; mob$^+$	Laboratory collection
BW20767	*E. coli* strain; RP4-2-Tc::Mu-1 Kan::Tn*7* integrant *leu-63*::IS*10 recA1 zbf-5 creB510 hsdR17 endA1 thi uidA* (Δ*MluI*)::*pir*$^+$	16
Plasmids		
pRL27	Harboring Tn*5* for mutagenesis	16
pDSK519	Broad-host-range IncQ cloning vector; Km^r	A. Kerr
pDSK-GenR	Gentamicin resistance gene cloned from plasmid pTGN and inserted at XhoI site of pDSK519; Gen^r	This study
pDSK-expI	*expI* coding region cloned at EcoRI and BamHI sites and under control of *lac* promoter of pDSK-GenR	This study

[a] Abbreviations: Tra^c, constitutive in AHL production and Ti plasmid conjugative transfer; Tra^i, AHL production and Ti plasmid conjugative transfer are inducible by opine inducer; Amp^r, ampicillin resistant; Kan^r, kanamycin resistant; Tet^r, tetracycline resistant; Rif^r, rifampin resistant; Ery^r, erythromycin resistant; Chl^r, chloramphenicol resistant; Suc^r, sucrose resistant; Gen^r, gentamicin resistant.

chrysanthemi. In this study, we identified the *expR-expI* homologues in *E. chrysanthemi* pv. zeae strain EC1 through transposon mutagenesis. We showed that disruption of the gene encoding AHL signal production results in significant changes in bacterial cell motility and in formation of cell aggregates and resulted in partially decreased bacterial virulence.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was routinely maintained at 37°C in LB medium, which contains (per liter) 10 g Bacto tryptone, 5 g yeast extract, and 10 g NaCl (pH 7.0). *E. chrysanthemi* strains were either grown at 28°C in yeast extract broth (YEB), which contains (per liter) 10 g Bacto tryptone, 5 g yeast extract, 5 g sucrose, 5 g NaCl, and 1 mM $MgSO_4 \cdot 7H_2O$ (pH 7.0) or grown in minimal medium or in SOBG medium as indicated (33, 34). Antibiotics were added at the following concentrations when required: gentamicin, 25 μg/ml; and kanamycin, 100 μg/ml.

Transposon mutagenesis. Transposon Tn*5*, carried by the suicide plasmid pRL27 in *E. coli* strain BW20767, was transferred into *E. chrysanthemi* pv. zeae strain EC1 by conjugation as previously described with minor revisions (16). 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. Tn*5* mutants were then selected on minimal medium agar plates containing kanamycin. These mutants were then screened for the defective phenotype in QS signal production using the AHL bioassay method described in the following section.

AHL bioassay. *Agrobacterium tumefaciens* NT1 containing a *tra-lacZ* fusion gene was used an AHL biosensor (24). Briefly, plates containing 20 ml of minimal agar medium supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml) were used for the bioassay. The solidified medium was cut into separate slices (1 cm in width). An *Erwinia* colony was streaked to one end of an agar slice, and then the fresh cultures of the AHL biosensor strain at an optical density at 600 nm (OD_{600}) of ≈0.1 were spotted at progressively further distances from the *Erwinia* bacteria. The plates were incubated at 28°C for 24 h. The blue spots of the AHL biosensor indicated the AHL activity (6). The AHL signals from liquid culture were extracted and concentrated as described previously (14), and chromatography analyses were performed following the method described previously (30). The standard AHL molecules used in this study were synthesized and purified as described previously (35).

DNA cloning and sequencing. The DNA fragment containing a Tn*5* insert was cloned by using the plasmid rescue approach described previously (16). The genomic DNAs of the mutants were extracted with the MasterPure DNA purification kit (EPICENTRE Biotechnologies), digested with BamHI, and ligated using T4 DNA ligase. The ligation mixture was transformed into *E. coli* DH5α/λ*pir*, and the transformants containing Tn*5* plasmids were selected on LB agar supplemented with 100 μg/ml kanamycin. The flanking regions of the Tn*5* insert were sequenced using three primers, with two designed based on the Tn*5* sequence (16)—i.e., tpnRL17-1 (5′-AACAAGCCAGGGATGTAACG) and tpnRL13-2 (5′-CAGCAACACCTTCTTCACGA)—and one primer, ExpR1 (5′-CCCATACTTGCCCAGTAGAG), designed based on the sequence information.

Complementation of the AHL-deficient mutants of strain EC1. Primers ExpIB1 (5′-CGGGATCCTCACCAGGTGAGCTATTGCG) and ExpIB2 (5′-CGGAATTCGCTTGGGGTTGAAATGAACC) were designed based on sequence data of $expI_{Ecz}$ and used to amplify its coding region. The PCR product was then subjected to BamHI and EcoRI digestion, as was the plasmid expression vector pDSK-Gen^r. The digested PCR product and vector were then purified and ligated in such a way that the coding region of *expI* was placed under the control of the *lac* promoter carried by the vector. The ligation mixture was transferred to *E. coli*, and transformations were selected on LB agar supplemented with 25 μg/ml gentamicin and confirmed by DNA sequencing. The corresponding complemented strains of WM3, WM6, and WM8 were generated by conjugal triparental mating, and the transformants were selected on minimal medium agar plates supplemented with 100 μg/ml kanamycin and 25 μg/ml gentamicin. The resultant strains, WM3expI, WM6expI, and WM8expI, were confirmed by PCR and phenotype analysis.

Swimming, swarming, and cell aggregation assay. For determination of swimming motility, the plate containing about 20 ml of semisolid Bacto tryptone agar medium (per liter contains 10 g Bacto tryptone, 5 g NaCl, 3 g agar) supplemented with X-Gal (40 μg/ml) was either spotted with bacteria using a toothpick or

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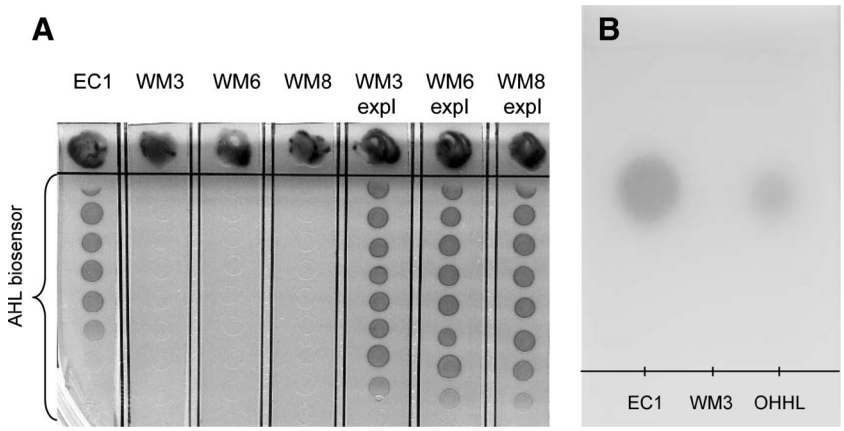

FIG. 1. Mutation of the $expI_{Ecz}$ gene of *E. chrysanthemi* pv. zeae strain EC1 abolished AHL signal production. (A) Diffusion plate assay of AHL QS signal production by strain EC1 and its derivatives. Strain EC1 and its derivatives were streaked separately on the top of the agar bar. The AHL biosensor strain *A. tumefaciens* NT1(*traR tra*::*lacZ749*) was spotted on the rest of the agar bar. The biosensor spots turned blue in the presence of AHL signals. (B) Characterization of the AHL signals produced by strain EC1 using thin-layer chromatography. Synthetic OHHL was spotted as a standard control.

inoculated with 1 μl of an overnight bacterial culture. The plates were incubated at 28°C for 6 h, and the diameter of the bacterial zone was measured. The swarming motility was assayed under the same conditions, except for the medium, which contains (per liter) 5 g peptone, 3 g yeast extract, and 4 g agar. The experiment was repeated three times in triplicates.

For observation of cell aggregation, overnight start cultures of strain EC1 and its derivatives were diluted to an OD_{600} of 0.5 and 50 μl of each bacterial dilution was added to a 50-ml Falcon tube containing 10 ml of fresh LB medium. The tubes were incubated at 28°C with shaking at 250 rpm for 6 h before microscopy examination.

Pathogenicity assay. Potato (*Solanum tuberosum* L. var. Bintje) tubers were obtained from local stores. After being washed with tap water and dried on a paper towel, potato tubers were surfaced sterilized with 70% ethanol and 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 μl at an OD_{600} of 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 for 24 h. The potato tubers were observed regularly for symptom development. Each assay was repeated three times with triplicate determinations each time.

Rice seed germination assay. Overnight bacterial cultures were diluted in 10-fold series, and the CFU (defined as the number of viable cells per ml) of each dilution was determined using heterotrophic plate counting assay (14). Fifty seeds of rice variety Texian 13 were added to 20 ml of a bacterial dilution and incubated at room temperature for 5 h. The rice seeds were then washed three times with sterilized water and transferred onto two moistened Whatman paper no. 3 filter papers in a petri dish. The seeds were then incubated at 28°C under 16-h-light–8-h-dark conditions, and sterilized water was added when necessary. Rice seeds were incubated with same amount of sterilized water as a blank control. The rate of seed germination was determined 1 week after treatment. The experiment was repeated four times with triplicates. The presented data were normalized based on the corresponding bacterial CFU.

Assay for exoenzymes. The cellulose enzyme activity was determined by plate assay. Briefly, 5 μl of fresh bacterial culture at an OD_{600} around 1.5 was spotted onto a LB agar plate containing 0.5% carboxymethylcellulose as a substrate. After 24 h of incubation at 30°C, the plate was flooded with 0.1% aqueous Congo red (Sigma) for 15 min and then washed with 1 M NaCl three times. The Congo red-stained carboxymethylcellulose became red, and the clearing zone around the bacterial colony indicated the cellulase activity. For assay of protease and pectinase, the bacterial supernatants were obtained by centrifugation and were filter sterilized. The pectate lyase activity and proteolytic enzyme activity were determined using polygalacturonic acid and azocasein as substrates, respectively, following the methods described previously (2, 4).

Nucleotide sequence accession number. The nucleotide sequence obtained in this study has been deposited in the GenBank database under accession no. EU142019.

RESULTS

Screening of the *E. crysanthemi* pv. zeae mutants defective in AHL production. Similar to the well-characterized *E. chrysanthemi* strain EC3937 (22), the rice pathogen *E. chrysanthemi* pv. zeae strain EC1 produced detectable AHL signals as indicated by the AHL-biosensor strain *Agrobacterium tumefaciens* NT1 (*traR tra*::*lacZ749*) (Fig. 1), in which the *lacZ* gene encoding a β-galactosidase is under the control of an AHL-dependent promoter (24). Tn*5* transposon mutagensis was then used to identify the genes involved in AHL production. Mutants showing altered AHL production were selected. Out of the 15,000 mutants screened, three transposon mutants, designated as WM3, WM6, and WM8, showed an absence of AHL production (Fig. 1).

For identification of the AHL signals produced by *E. chrysanthemi* pv. zeae, the overnight cultures of strain EC1 and its mutant WM3 were extracted by ethyl acetate as described previously (14). The organic solvent in each extract was evaporated, and the signal was dissolved in methanol as a 200× concentrate. Thin-layer chromatography analysis showed that strain EC1 produced one AHL signal in a position corresponding to OHHL (Fig. 1). In agreement with the results of the plate assay, no OHHL signal was detected from the extracts from the mutant WM3 (Fig. 1).

Cloning and sequence analysis of the *luxI* and *luxR* homologues from *E. chrysanthemi* pv. zeae strain EC1. The Tn*5* insertion flanking regions from three mutants were cloned and sequenced. Sequence analysis showed that Tn*5* was inserted respectively in bp 172 (WM3) and 265 (WM6 and WM8) of an open reading frame (ORF). Comparison of the DNA sequence of this ORF using BLAST revealed about 86% identity with the *expI* of *E. chrysanthemi* strain 3937 (NCBI accession no. X96440), which codes for QS signal OHHL biosynthesis (22). This ORF was thus designated as $expI_{Ecz}$. At the peptide level, $ExpI_{Ecz}$ shares about 92% identity with the ExpI of *E. chrysanthemi* strain 3937.

Further sequencing the downstream of *expI* revealed an-

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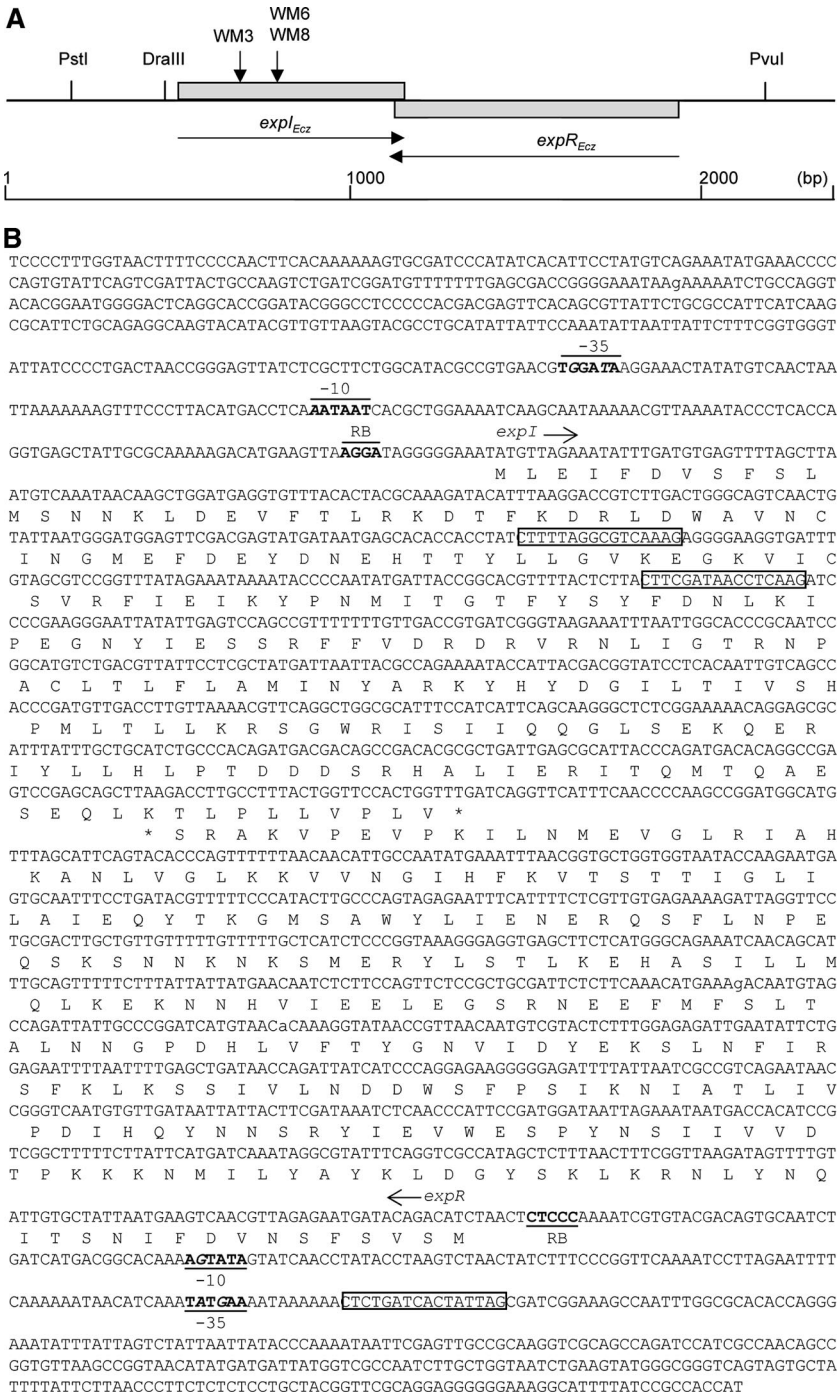

FIG. 2. Physical map and sequence analysis of the DNA fragment containing the genes involved in AHL QS signal biosynthesis and regulation. (A) Physical map of the 2.3-kb fragment containing the $expI_{Ecz}$ and $expR_{Ecz}$ genes. The transposon insertion sites in the AHL-deficient mutants WM3, WM6, and WM8 are marked by vertical arrows. The direction of transcription is indicated by horizontal arrows. (B) Nucleotide sequences of $expI_{Ecz}$ and $expR_{Ecz}$ and the predicted peptide products. The predicted ribosomal binding sites (RB) and putative promoter elements are indicated by boldface type and underlines. The predicted *lux* box consensus sequences were boxed.

other ORF (Fig. 2A). A BLAST search found that this ORF, designated $expR_{Ecz}$, shares 85.9% identity with the *expR* gene of *E. chrysanthemi* strain 3937 (22). In addition to high sequence similarity, the $expRI_{ecz}$ gene of *E. chrysanthemi* pv. zeae strain EC1 also shares the same genome organization with the *expRI* of *E. chrysanthemi* (22). The $expI_{Ecz}$ and $expR_{Ecz}$ genes are oppositely oriented with 33 bp overlapped at the 3′ end of the genes (Fig. 2A and B). The promoter elements, including the −10 and −35 regions, and the ribosome binding site were identified in the corresponding promoter regions of the two genes. A putative *lux* box, which is the binding site of LuxR-type transcription factors (24, 32), was found at the region

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close to the −35 element in the promoter of $expR_{Ecz}$. No *lux* box was found in the promoter of $expI_{Ecz}$. Instead, two putative *lux* box sequences were found in the 5′ region of the $expI_{Ecz}$ coding sequence (Fig. 2B).

Overexpression of $expI_{Ecz}$ in the AHL⁻ mutants restored the QS signal production. The wild-type $expI_{Ecz}$ was then cloned from the *E. chrysanthemi* pv. zeae strain EC1 by PCR amplification. For the complementation test, the gene was placed under the control of the *lac* promoter in expression vector pDSK-Gen^r. The resultant construct was introduced into the mutants WM3, WM6, and WM8 separately. Bioassay results showed that AHL produced in the three mutants was fully restored (Fig. 1), demonstrating that $expI_{Ecz}$ is the gene responsible for AHL production in strain EC1.

Mutation of $expI_{Ecz}$ drastically enhanced bacterial swimming and swarming motility. *E. chrysanthemi* pv. zeae strain EC1 appeared blue on the medium containing X-Gal (Fig. 1), presumably due to production of β-galactosidase. This property was used to improve the visibility of EC1 cells on the swimming assay plate. The results showed that the three $expI_{Ecz}$ mutants had a significantly increased swimming motility compared to wild-type EC1 (Fig. 3A). The swimming motility was restored to the wild-type level in the corresponding complemented strains WM3expI, WM6expI, and WM8expI, which expressed the $expI_{Ecz}$ gene in *trans*.

To determine if exogenous OHHL was able to modulate the bacterial swimming motility, the swimming assay was performed in the presence or absence of the OHHL signal. The results showed that addition of 2 μM OHHL was able to decrease the swimming motility of the mutants to the level of wild-type strain EC1 (Fig. 3B).

Similarly, mutation of *expI* resulted in drastically increased swarming motility. The mutant WM3 phenotype was restored to the wild-type level by either expression of $expI_{Ecz}$ in *trans* or exogenous addition of OHHL (Fig. 3C).

AHL signal-induced formation of cell aggregates in *E. chrysanthemi* pv. zeae liquid culture. Bacteria swim and swarm by rotating their flagella. Electron microscopy analysis, however, did not reveal obvious differences in flagellar location and number when the wild-type strain, EC1, was compared with its AHL-deficient mutants. Interestingly, however, close examination by the naked eye found that strain EC1 grown in LB medium produced many cell clumps in liquid culture, even under vigorous shaking conditions, whereas no visible cell clumps were noticed in the culture of its AHL-deficient mutants throughout bacterial growth. Microscopy analysis confirmed the formation of multicell aggregates by strain EC1 and the planktonic living status of the AHL-deficient mutant WM3 bacterial cells (Fig. 4). For quantitative comparison, microscope counting was used to determine the total numbers of cell aggregates in 10 randomly selected fields of vision. While none was found in WM3 culture, strain EC1 on average had about seven multicell aggregates per field of vision, with each aggregate containing at least a few hundred cells 50 to 100 μm in diameter. The findings suggest that AHL QS signal might induce the formation of *E. chrysanthemi* pv. zeae cell aggregates. The speculation was confirmed by either addition of 2 μM exogenous OHHL to WM3 culture (data not shown) or in *trans* expression of the OHHL synthase gene $expI_{Ecz}$ in the mutant WM3 (Fig. 4). Microscope observation also found that

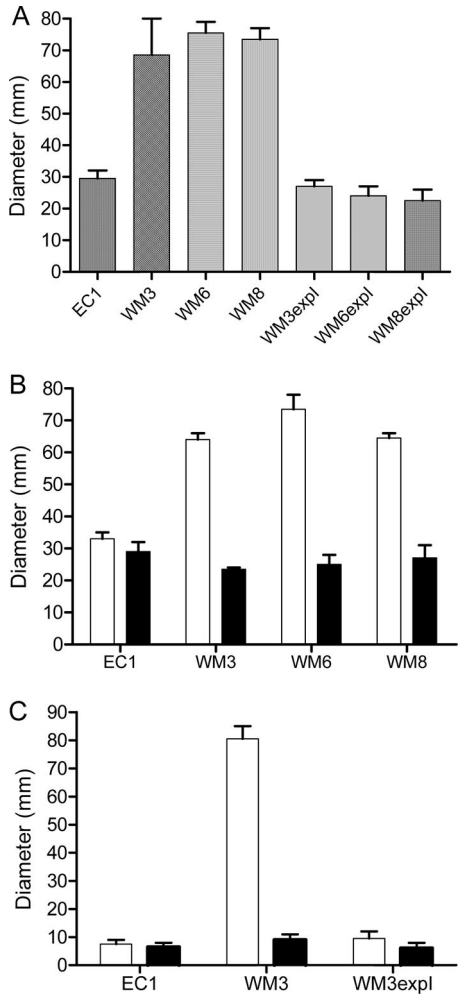

FIG. 3. Mutation of the gene encoding AHL biosynthesis enhanced EC1 swimming motility. (A) Swimming distance of strain EC1 and its derivatives. (B) Swimming motility of EC1 and its AHL-deficient mutants in the absence (open bars) and presence (filled bars) of 2 μM OHHL. (C) Swarming motility of EC1 and its derivatives in the absence (open bars) and presence (filled bars) of 2 μM OHHL.

the planktonic cells were swimming rapidly and constantly, whereas cell aggregates were virtually standing still.

We then tested the formation of cell aggregates in SOBG medium, which supports the type III secretion system-dependent pellicle formation of *E. chrysanthemi* in stationary culture (33). Under the same stationary conditions, both EC1 and its QS-defective mutant form similar pellicles, except that the mutant pellicles appeared slightly thinner than those of the wild type (data not shown). The difference between the wild type and its mutant became obvious when bacteria were grown under shaking conditions. Although unlike in LB medium, mutation of $expI_{Ecz}$ did not completely abolish cell aggregate formation, EC1 and the complemented strain WM3expI formed significantly bigger cell aggregates (~200 to 300 μm in diameter) than the mutant WM3 (~50 to 80 μm in diameter) (Fig. 4). These data, together with the size difference of the cell aggregates formed in LB and SOBG media (50 to 100 μm versus 200 to 300 μm) and the previous findings that a func-

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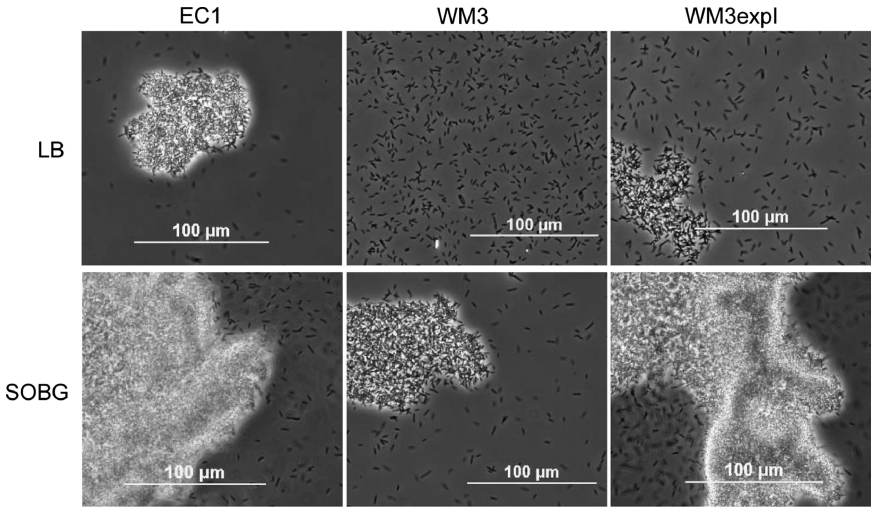

FIG. 4. AHL signal induced the formation of cell aggregates by *E. chrysanthemi* pv. zeae. Bacterial strains were grown at 28°C with shaking in LB (top) or SOBG (bottom) medium for 6 h. Photos were taken at ×400 under an Olympus BX50 microscope.

tional type III secretion system is required for the pellicle but not for biofilm formation by *E. chrysanthemi* (33), suggest that several pathways may be involved in the formation of the *E. chrysanthemi* pv. zeae cell aggregates in SOBG medium, including the one regulated by OHHL signals.

AHL-deficient mutants showed partially decreased virulence against potato tubers. The ability of strain EC1 and the AHL-deficient mutants to cause maceration in potato tubers was investigated. Similar to *E. chrysanthemi* strain EC3937 (Fig. 5B), which is known to cause infections in dicot plants, *E. chrysanthemi* pv. *zeae* strain EC1 could also cause soft rot symptoms on potato tubers (Fig. 5A). When inoculated on potato tubers, the AHL-deficient mutants WM3 (Fig. 5C) and WM6 (Fig. 5E) caused smaller maceration zones than the wild-type strain EC1 and their corresponding complemented strains (Fig. 5D and F). In addition, the mutant-infected plant tissues generated less tissue fluid in the vicinity of maceration than those infected by the wild type and the complemented strains.

Mutation of $expI_{Ecz}$ marginally decreased the bacterial ability to inhibit rice seed germination. Given that the major impact of pathovar *E. chrysanthemi* pv. zeae on rice is the inhibition of rice seed germination (17), we compared the pathogenic effects of *E. chrysanthemi* strain EC3937 and *E. chrysanthemi* pv. zeae strain EC1 on rice seeds. In each treatment, 50 rice seeds were placed in a 20-ml bacterial solution (10^6 CFU bacterial cells per ml) for 5 h at room temperature before washing and letting seeds germinate. Treatment of rice seeds with EC1 resulted in total inhibition of rice seed germination, whereas under the same conditions the *E. chrysanthemi* strain EC3937 did not show any detectable inhibitory effect (Fig. 6A). The QS mutant WM3, however, also completely inhibited the rice seeds' germination when the same inoculum concentration was used (Fig. 6B).

To analyze potential quantitative differences, we prepared a series of dilutions of bacterial cultures with water and determined the abilities of different bacterial strains to inhibit rice seed germination. While *E. chrysanthemi* strain EC3937 had no

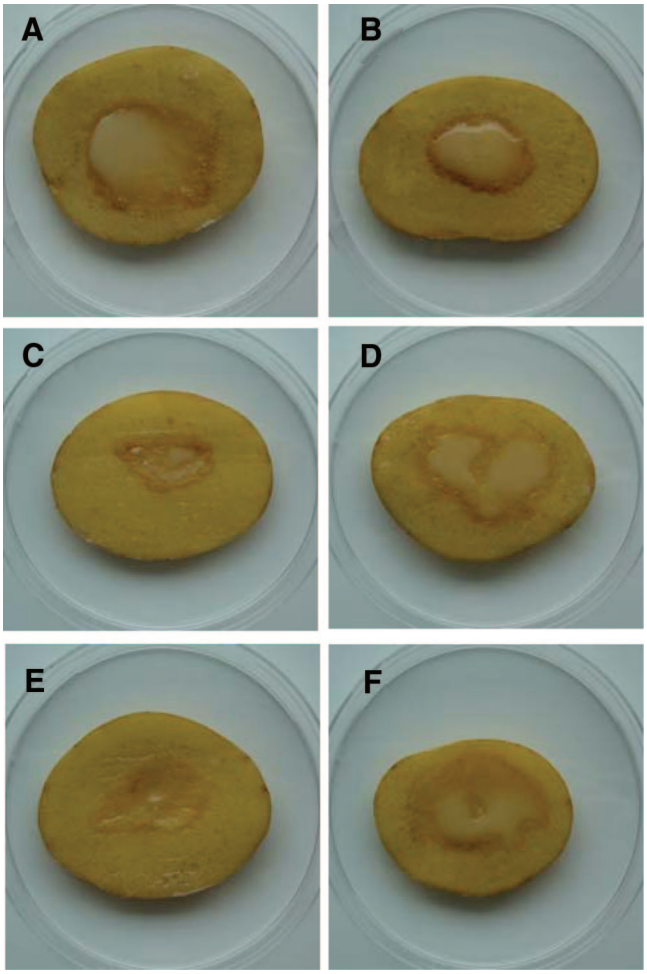

FIG. 5. AHL-deficient mutants showed attenuated soft rot syptoms on potato tubers. Each cut tuber was inoculated with 2 μl of fresh bacterial cells at an OD_{600} of 1.2. The bacterial strains inoculated were EC1 (A), EC3937 (B), WM3 (C), WM3expI (D), WM6 (E), and WM6expI (F). Photographs were taken 24 h after incubation at 28°C. The experiment was repeated three times with similar results. The photographs show a set of representative samples.

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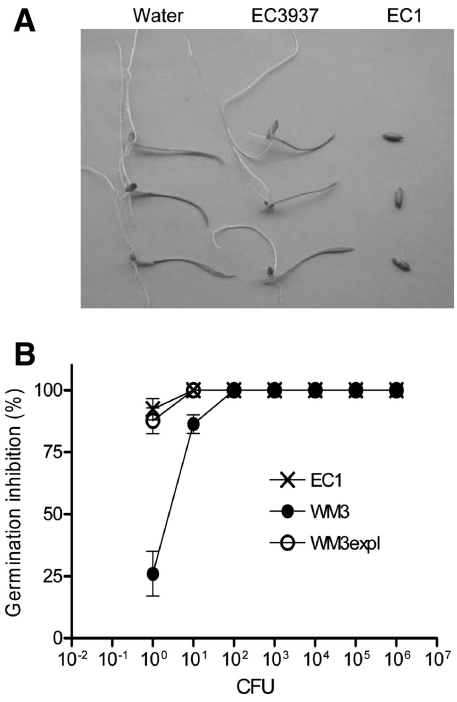

FIG. 6. *E. chrysanthemi* pv. *zeae* inhibited rice seed germination. (A) Rice seedling symptoms after treatment with strains EC1 and EC3937 (10^6 CFU of bacterial cells per ml), respectively. Water was used as a control. The photo was taken 1 week after treatment. (B) Quantitative comparison of the rice seed germination inhibitory activities of EC1 and its derivatives. In each treatment, 50 rice seeds were added to a tube containing 20 ml of bacterial suspension containing a certain number of bacterial cells as indicated. The mixtures were incubated at room temperature for 5 h before washing. The washed seeds were then transferred to a clean plate with moisturized filter papers and incubated at 28°C under 16-h-light–8-h-dark conditions. The rate of germination was determined 1 week later.

effect on rice seed germination regardless of inoculated bacterial cell numbers (data not shown), surprisingly, *E. chrysanthemi* pv. zeae strain EC1 showed an unusual strong inhibitory ability even at an inoculum concentration as low as 1 viable cell per ml (Fig. 6). This was somewhat unexpected because under this condition, the ratio of seeds to viable bacterial cells was 5:2. Plausible explanations are that the pathogen could proliferate during incubation by utilizing the nutrients from rice seeds and that more bacterial cells survived on rice seeds than on the LB agar plates used for counting the bacterial CFU. Mutation of the $expI_{Ecz}$ gene in *E. chrysanthemi* pv. zeae strain EC1 only had a marginal effect on its inhibitory activity against rice seed germination. Unlike *E. chrysanthemi* strain EC3937, the mutant WM3 at a low concentration of 100 CFU still provided full inhibition of rice seed germination. The difference between WM3 and its wild type, EC1, in germination inhibition became obvious only when the bacterial cell numbers were diluted to less than 10 CFU (Fig. 6). In contrast, the inhibitory effect of the complemented strain WM3expI was basically indistinguishable from that of wild-type strain EC1 regardless of inoculum concentration (Fig. 6).

Mutation of $expI_{Ecz}$ did not significantly reduce the production of exoenzymes. As exoenzymes are common virulence factors of many plant bacterial pathogens, we tested whether disruption of *E. chrysanthemi* pv. zeae QS signaling affected the production of exoenzymes. The mutant WM3 showed comparable activities of protease (7.7 ± 1.4 U/ml) and pectate lyase (0.036 ± 0.005 U/ml) to those of EC1 (7.8 ± 1.1 U/ml and 0.040 ± 0.004 U/ml, respectively). Similarly, WM3 and EC1 generated indistinguishable cellulose activities based on the almost identical sizes of clear zones in the plate assay (data not shown).

DISCUSSION

Our research aims were to identify the QS genes of *E. chrysanthemi* pv. zeae and to determine their potential roles in regulation of the bacterial physiology and pathogenesis. In this study, we cloned and sequenced the $expI_{Ecz}$ and $expR_{Ecz}$ genes, which encode an AHL synthase and the corresponding AHL-dependent transcription factor (Fig. 2), respectively. We showed that mutation of $expI_{Ecz}$ in *E. chrysanthemi* pv. zeae strain EC1 abolished the production of AHL QS signal OHHL (Fig. 1), markedly increased swimming and swarming motility (Fig. 3), eliminated formation of cell aggregates in LB medium (Fig. 4), and partially attenuated bacterial virulence (Fig. 5 and 6). The AHL QS system has been characterized in several *E. chrysanthemi* strains (3), but its role in regulation of bacterial behaviors has not been clearly established. Inactivation of the *expI* gene in *E. chrysanthemi* strain EC3937 results in decreased expression of some pectinase genes, but the role of the QS system in bacterial physiology remains elusive (22, 26). Similarly, mutation of the same gene in *E. chrysanthemi* strain EC16 has no apparent effect on its virulence in witloof chicory leaves (13). To our knowledge, the data from this study show for the first time that the AHL QS system of *E. chrysanthemi* pv. zeae controls several obvious phenotypes, illustrating its role in modulation of bacterial physiology and virulence.

E. chrysanthemi pv. zeae appears to be different from other gram-negative bacterial strains in the mode of QS regulation of bacterial motility. In other bacterial organisms, null mutation of AHL biosynthesis or enzymatic degradation of AHL signal in bacteria either has no effect on swimming motility, as in the case of *Pseudomonas aeruginosa* (25), or results in decreased swimming and swarming motility, such as in *Yersinia enterocolitica* (1). In contrast, inactivation of AHL-synthase gene $expI_{Ecz}$ in the bacterial pathogen *E. chrysanthemi* pv. zeae resulted in a hypermotile phenotype, which was restored to the wild-type level by exogenous addition of AHL signals (Fig. 3). Interestingly, mutation of $expI_{Ecz}$ also drastically changed the growth pattern of *E. chrysanthemi* pv. zeae by shifting from the multicell aggregate form of the wild-type strain to the planktonic form of the mutants (Fig. 4). This changed growth pattern of the AHL-deficient mutants could largely, if not entirely, account for their hypermotile phenotype, as flagellar rotation in cell aggregates could be severely restricted.

Our data show that the decreased virulence of the AHL-deficient mutants is unlikely due to altered exoenzyme production as EC1 and its mutants displayed similar activities of proteases, pectinases, and cellulases. However, the increased motility and reduced ability to form multicell aggregates of the AHL-deficient mutants of *E. chrysanthemi* pv. zeae seem to be consistent with their decreased virulence phenotype (Fig. 3 to 6). Previous studies suggest that both motility and the ability to

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form multicell aggregates might contribute to bacterial colonization or virulence (1, 18, 19, 23). The hypermotile mutants of *Vibrio fischeri* are significantly delayed in colonization of host organism (18). The ability to form multicell aggregates enhances the bacterial survival on host plants under various environmental conditions (19). It was also noted that conditions that promote virulence gene expression may become unfavorable to the expression of motility genes (23). Culture of *Salmonella enterica* on motility agar plates significantly induces the expression of the virulence gene but at the same time down-regulates the expression of flagellar genes. This regulation is mediated by SirA, a two-component response regulator of the FixJ family (23). Further investigation of AHL-regulated bacterial motility and aggregation at genetic and genomic levels would be important to delineate the molecular mechanisms of their association with *E. chrysanthemi* pv. zeae virulence.

The QS system of *E. chrysanthemi* pv. zeae seems to share a similar evolutionary origin to that of its closely related *E. chrysanthemi* pathovars (12). The notion is supported by several lines of evidence. First, the $expI_{Ecz}$-$expR_{Ecz}$ gene region is highly homologous (86%) at the nucleotide level to the *expI-expR* DNA fragment of *E. chrysanthemi* strain EC3937 (22). Second, while the orientation of the two genes in the strain EC1 genome differs from that of the *luxI-luxR* operon of marine bacterium *Vibro fischeri* (NCBI accession no. Y00509), it is similar to those of *E. chrysanthemi* strain EC3937 (NCBI accession no. X96440) as well as other *Erwinia* species (10). Third, similar to strain EC3937, in which null mutation of *expI* only causes a slight decrease in transcriptional expression of some pectinase genes but does not change the overall pectinase activity (22), inactivation of $expI_{Ecz}$ in strain EC1 also did not significantly affect its total pectate lyase activity.

It is interesting to note the sharp contrast in virulence of *E. chrysanthemi* pv. zeae and the reference strain *E. chrysanthemi* EC3937 on dicots and monocots. *E. chrysanthemi* pv. zeae strain EC1 produced similar symptoms to *E. chrysanthemi* strain EC3937 on dicoteledonous plants (Fig. 5), but it differed from the *E. chrysanthemi* strain in its strong virulence on rice seeds (Fig. 6). At an inoculum concentration as low as 1 CFU per ml, strain EC1 still caused about 90% inhibition of rice seed germination. In sharp contrast, under the conditions used in this study *E. chrysanthemi* strain EC3937 did not show any inhibitory effect on rice seeds, which germinated normally even in the presence of 10^6 CFU of the pathogen per ml (Fig. 6). Intriguingly, the AHL-defective mutants of *E. chrysanthemi* pv. zeae showed only a marginal decrease in inhibitory activity against rice seed germination. Albeit the mechanism remains unknown, these data taken together suggest *E. chrysanthemi* pv. zeae may differ from the other *E. chrysanthemi* pathovars by producing a unique virulence factor(s) responsible for the strong inhibitory activity against rice seed germination and that production of this virulence factor(s) may not or only partially depend on the AHL-type QS system identified in this study.

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