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Deciphering the Multi-tiered Regulatory Network That Links Cyclic-di-GMP Signaling to Virulence and Bacterial Behaviors

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DECIPHERING THE MULTI-TIERED REGULATORY NETWORK THAT LINKS CYCLIC-DI-GMP SIGNALING TO VIRULENCE AND BACTERIAL BEHAVIORS

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ABSTRACT

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Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger that regulates multiple cellular behaviors in most major bacterial phyla. C-di-GMP signaling in bacterial often includes enzymes that are responsible for the synthesis and degradation of c-di-GMP, effector proteins or molecules that bind c-di-GMP, and targets that interact with effectors. However, little is known about the specificity of c-di-GMP signaling in controlling virulence and bacterial behaviors. In this work, we have investigated the c-di-GMP signaling network using the model plant pathogen *Dickeya dadantii* 3937.

In Chapter 2, we characterized two PilZ domain proteins that regulate biofilm formation, swimming motility, Type III secretion system (T3SS) gene expression, and pectate lyase production in high c-di-GMP level conditions. $YcgR_{3937}$ binds c-di-GMP both *in vivo* and in *vitro*. Next, we revealed a sophisticated regulatory network that connects the sRNA, c-di-GMP signaling, and flagellar master regulator FlhDC. We proposed FlhDC regulates T3SS through three distinct pathways, including the FlhDC-FliA-$YcgR_{3937}$ pathway; the FlhDC-EcpC-RpoN-HrpL pathway; and the FlhDC-*rsmB*-RsmA-HrpL pathway. Genetic analysis showed that EcpC is the most dominant factor for FlhDC to positively regulate T3SS expression.

In chapter 3, we constructed a panel of single-deletion mutants, in which each GGDEF

and/or EAL domain protein coding gene was individually either deleted or inactivated. Various cellular outputs were investigated using these mutants. We showed that GGDEF domain protein GcpA negatively regulates swimming motility, pectate lyase production, and T3SS gene expression. GcpD and GcpL only negatively regulate the expression of T3SS and swimming motility but not the pectate lyase production.

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Ap	Ampicillin
C-di-GMP	Cyclic diguanosine monophosphate
CFP	Cyan fluorescent protein
Cm	Chloramphenicol
DGC	Diguanylate cyclase
Ecp	EAL-domain containing protein
FRET	Förster resonance energy transfer
Gcp	GGDEF-domain containing protein
GFP	Green florescent protein
Gm	Gentamycin
Hrp	Hypersensitive response and pathogenicity
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITC	Isothermal tritration colorimetry
Km	Kanamycin
LB	Luria-Bertani media
MM	Minimal medium
OD	Optical density
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
Pel	Pectate lyase
Real-time	RT-PCR Real-time reverse transcription polymerase chain reaction
Sp	Spectinomycine
T2SS	Type II secretion system
T3SS	Type III secretion system
YFP	Yellow florescent protein

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Chapter 1

Introduction

1.1 *Dickeya dadantii* 3937

1.1.1 Background and Significance of *Dickeya dadantii* 3937

Dickeya dadantii 3937 (former name *Erwinia chrysanthemi* 3937) was first observed to cause disease on greenhouse stocks of *Chrysanthemums morifolium* in New York in the 1950s. Studies of cell morphology, culture conditions and biochemical characteristics determined that this isolated organism was a new species of *Erwinia*, and was thus named as *Erwinia chrysanthemi* (Burkholder et al. 1953). Thereafter, taxonomical analysis using 16S rDNA sequencing and DNA-DNA hybridization techniques reclassified *E. chrysanthemi* 3937 into a new genus *Dickeya* (named for American phytopathologist Robert S. Dickey), which is different from other *Erwinia* sp. (Gardan 2005; Ma et al. 2007). Thus, a new name was given to *Erwinia chrysanthemi* 3937 as *Dickeya dadantii* 3937.

D. dadantii is known to cause diseases on a wide range of host plants throughout the world, including tropical, subtropical, and temperate regions. Based on literature provided by the European and Mediterranean Plant Protection Organization (EPPO), this pathogen infects a wide range of ornamental and horticultural host plants, including many economically important vegetables such as potato, tomato and carrot (Czajkowski et al. 2011). *D. dadantii* cells can live in soils and water-logged environments as either epiphytes or saprophytes (Cother and Gilbert 1990; Robert-Baudouy et al. 2000; Reverchon and Nasser 2013). Several studies reported that *D. dadantii* is able to survive for weeks in cattle fecal material, months in sterile distilled water, or on other non-host plants without infection (Cother and Gilbert 1990; Lohuis 1990; Nelson 2009). However, once *D. dadantii* encounters a susceptible host under favorable conditions such as high temperature (above 30°C) and high humidity, it can quickly

shift to its pathogenic state and initiate infection. As a plant pathogen, *D. dadantii* causes a variety of disease symptoms within several parts of the plant. In the fleshy and succulent areas, such as tubers and leaves, *D. dadantii* causes severe maceration or decaying of these plant tissues, which is a localized symptom often referred to as "soft rot." Additionally, *D. dadantii* is able to infect xylem vessels, resulting in a systemic infection that causes wilting. Grenier and colleagues reported that *D. dadantii* is capable of infecting pea aphid, which may serve as an insect vector for *Dickeya* disease transmission (Grenier et al. 2006).

The complete genome of *D. dadantii* 3937 has been sequenced, and thus this organism is widely used as a model system (Glasner et al. 2011). Genetically, it is a close relative of *Escherichia coli*, and animal pathogens *Yersinia* and *Salmonella*. Physically, *D. dadantii* is a Gram-negative, rod-shaped bacterium, whose cells measure 1.8 μm in length and 0.6 μm in diameter. It is also motile, due to its peritrichous flagella, and does not form spores.

1.1.2 Virulence mechanisms of *D. dadantii* 3937

The initial attachment of *D. dadantii* to the plant surface is crucial for its pathogenicity. In order to increase this attachment, *D. dadantii* secretes the CdiA/HecA type V secreted protein, resulting in enhanced adherence of bacteria to the plant surface (Rojas et al. 2002). It also produces a biosurfactant and cellulose fibrils that contribute to the bacterial colonization and aggregation on leaves, respectively (Hommais et al. 2008; Jahn et al. 2011; Prigent-Combaret et al. 2012). As a phytopathogen, *D. dadantii* has a strong response to jasmonic acid, which is an environmental stimulus produced by wounded plant tissue. Chemotaxis and motility then enable this pathogen to move to these wounded sites and facilitate the invasion of *D. dadantii*

to the apoplast (Antúnez-Lamas et al. 2009a; Antúnez-Lamas et al. 2009b). In the apoplast, plants can sense molecules originating from pathogens, which triggers of a battery of immune responses. Pathogen-associated molecular patterns (PAMP)/pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) are two well-defined modes of plant immunity against pathogens (Dubery et al. 2012; Gassmann and Bhattacharjee 2012; Spoel and Dong 2012). PTI is activated via recognition of pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), such as the bacterial flagellin and lipopolysaccharide (LPS). However, bacterial pathogens utilize the Type III secretion system (T3SS) to successfully evade the PTI and attenuate the host defense mechanisms. The T3SS allows the bacteria to translocate several effector proteins directly into the host cell cytoplasm. As a result, ETI will be provoked by specific recognition of these effector proteins. The plant then synthesizes proteins to neutralize the injected bacterial effectors, which is associated with apoptosis, or programmed cell death, called the hypersensitive response (HR) in the non-host plant such as tobacco (Bauer et al. 1995; Pieterse et al. 2009). *D. dadantii* can degrade the plant cell wall, which is correlated with its ability to express and secrete plant cell wall degrading enzymes (PCWDE) that include pectinases, proteases, cellulases, and polygalacturonases (Collmer and Keen 1986; Roy et al. 1999; Herron et al. 2000; Kazemi-Pour et al. 2004). Pectinases and cellulases are secreted by the Out pathway, which is the Type II secretion system (T2SS) (Andro et al. 1984; Condemine et al. 1992b; Lindeberg and Collmer 1992). Proteases are secreted by the PrtDEF Type I secretion system (Shevchik et al. 1998).

1.1.2.1 Type II secretion system

D. dadantii 3937 secretes a plethora of PCWDEs to cause severe maceration and wilting of host tissue. These enzymes are released via an ATP-dependent secretion system notated as the T2SS. The T2SS, also known as the Out system, translocates Out proteins and pectinolytic enzymes into the extracellular space, which interact with host plant tissues (Condemine et al. 1992b; Lindeberg and Collmer 1992; Sandkvist 2001). As a result, these enzymes break down the long-chain carbohydrate pectin into smaller sugars, which can then be used in bacterial metabolism.

The regulation of pectate lyase production is complex, including modifications of DNA topology, quorum-sensing and other regulatory systems that are associated with bacterial physiological and metabolic status (Römling et al. 2013) (Fig. 1). During the early and intermediate stages of infection, bacterial chromosomal DNA is relaxed in response to the oxidative and acidic stresses and provokes a strong negative effect on *pel* gene transcription (Ouafa et al. 2012). Major pectate lyase repressors, such as Fis, H-NS, PecT, PecS, and KdgR, directly bind to the relaxed *pel* gene promoters and regulate their expression (Condemine et al. 1992a; Castillo et al. 1998; Rodionov et al. 2004; Lautier and Nasser 2007; Hommais et al. 2008; Ouafa et al. 2012). A recent study showed that PecT preferentially binds relaxed *pel* promoters (Hérault et al. 2014). Negative regulation of pectate lyase production is also provided by a two-component regulatory system PhoP/PhoQ, and the ferric uptake receptor Fur, both of which are activated under acidic conditions (Franza et al. 2002; Venkatesh et al. 2006; Wu et al. 2014). RsmA, a global post-transcriptional regulator, degrades *pel* mRNA and promotes its time-dependent degradation (Charkowski et al. 2012). During the advanced stages of infection, alkalinization of plant tissue increases the pH value, resulting in

inactivation of PhoP/PhoQ and Fur, and activation of *pel* operon activator MfbR (Reverchon et al. 2010). GacS/GacA, another two-component system, is activated, which in turn represses the expression of *rsmA* and *pecT* (Yang et al. 2008b; Charkowski et al. 2012). Degradation of pectin to 2-keto-3-deoxygluconate (KDG) relieves the *pel* promoter from its cognate repressor KdgR, which becomes inactive when complexed to KDG (Rodionov et al. 2004). The cAMP receptor protein CRP, which is activated during differential carbon utilization, and a newly defined quorum-sensing system Vfm, also assist in positive regulation of pectate lyase production (Hugouvieux-Cotte-Pattat et al. 1996; Nasser et al. 2013; Reverchon and Nasser 2013). In addition, a DNA supercoiling state decreases the Fis and H-NS repression of the *pel* genes (Reverchon and Nasser 2013).

1.1.2.2 Type III secretion system and its regulatory mechanism

The T3SS is required for complete pathogenicity of *D. dadantii* 3937 (Yang et al. 2002; Yang et al. 2004). It is encoded by two divergent operons, which include approximately 32 ORFs located in the *hrp*/*hrc*/*dsp* gene clusters (Yang et al. 2002; Yang et al. 2010; Glasner et al. 2011). Evolutionarily, the T3SS and bacterial flagellum share a common ancestor, and the basal structure of the T3SS shows many similarities with bacterial flagellum (Young et al. 1999; Lee and Galán 2004; Pallen et al. 2005; Erhardt et al. 2010). In addition, FlhDC, the master regulator of flagellar transcription, positively regulates the expression of T3SS in *Pectobacterium carotovorum* and *D. dadantii* 3937 (Cui et al. 2008; Yuan et al. 2015).

The expression of T3SS genes is modulated by two main regulatory pathways at both the transcriptional and post-transcriptional levels (Yap et al. 2005; Tang et al. 2006; Yang et al.

2008a; Yang et al. 2008b) (Fig. 2). In the HrpX/HrpY-HrpS-HrpL pathway, HrpX/HrpY is a two-component system, which activates the expression of *hrpS*. HrpS is an NtrC-family transcriptional enhancer protein, which interacts with the sigma factor RpoN (σ^{54}), and then activates the transcription of *hrpL* (Yap et al. 2005). HrpL is a member of the extracytoplasmic factor (ECF) family alternative sigma factors that activates the expression of T3SS structural and effector genes, such as *hrpA*, *hrpN* and *dspE*, which encode the T3SS pilus protein, a harpin protein and a virulence effector, respectively (Wei and Beer 1995; Chatterjee et al. 2002; Tang et al. 2006). The T3SS gene expression is also controlled by the GacS/A-RsmB-RsmA-HrpL pathway at the post-transcriptional level (Chatterjee et al. 2002; Yang et al. 2008b). The two component system GacS/GacA positively controls the expression of a regulatory small RNA (sRNA) RsmB (Yang et al. 2008b). RsmB binds to its target protein RsmA with high affinity and neutralizes its activity against *hrpL* mRNA (Liu et al. 1998; Chatterjee et al. 2002). In *D. dadantii* 3937, RsmA is a small RNA-binding protein that binds to the 5' untranslated region of *hrpL* mRNA, and facilitates its time-dependent degradation (Chatterjee et al. 1995). Besides the above mentioned regulators, the expression of T3SS genes is also modulated by the polynucleotide phosphorylase (PNPase), a regulator of the SlyA/MarR family (SlyA), a global bacterial second messenger bis-(3'-5')-cyclic di-GMP (c-di-GMP), and some natural phenolic compounds in the plant (Yang et al. 2008a; Yi et al. 2010; Zeng et al. 2010; Zou et al. 2012).

1.1.2.3 Role and regulatory mechanism of chemotaxis and motility

Chemotaxis and motility are crucial for *D. dadantii* 3937 to reach the interior of the plant

in order to cause disease (Antúnez-Lamas et al. 2009a). Bacterial cells use chemotactic signaling to move away from hostile surroundings and towards favorable conditions (Lux and Shi 2004). In the chemotaxis signaling system, methyl-accepting chemotaxis proteins (MCPs) function as transmembrane receptors, which sense the environmental cues and transmit the chemotactic signal to the cytoplasm (Parkinson et al. 2005). Several cellular proteins such as CheA and CheY, a histidine kinase and a response regulator respectively, then transfer the chemical signal to the flagellar switch (Wadhams and Armitage 2004). In many bacterial species, motility is determined by the rotation of flagella. In general, bacterial cells move forward when the rotation of flagella is counter-clockwise; they tumble in place to change direction when the flagellar rotation is clockwise (Eisenbach 1996).

Intensive studies have established a hierarchical regulation of the flagellar assembly system in *E. coli* and other enteric bacteria (Chilcott and Hughes 2000; Aldridge and Hughes 2002) (Fig. 3). The expression of a functional chemotaxis and flagellar system require more than 50 genes, which are divided among at least 17 operons in the flagellar regulon (Chilcott and Hughes 2000). These genes are referred to as class I, class II, and class III, depending upon the expression of these genes at early, middle, or late stages. In addition, the expression of the previous transcriptional class is required for the expression of the next class (Kutsukake et al. 1990). The class I genes include two genes that are transcribed from the *flhDC* operon. FlhD and FlhC form a hetero-oligomeric complex ($FlhD_4FlhC_2$), which functions as a transcriptional activator for the class II genes (Liu and Matsumura 1994; Wang et al. 2006). Proteins encoded by the class II genes include those necessary for the basal body and hook of the flagellum known as the hook-basal body intermediate structure, and the transcriptional

regulators FlgM and FliA (Chilcott and Hughes 2000). FliA is an alternative sigma factor (σ^{28}) that determines the transcription of σ^{28} RNA polymerase-specific class III genes (Ide et al. 1999; Schaubach and Dombroski 1999). The protein products of class III operons are required to form a complete flagellum, such as the outer subunits of the flagellum, chemotaxis and the flagellar motor (Chilcott and Hughes 2000; Aldridge et al. 2006). FlgM binds to FliA, resulting in an inhibition of FliA-dependent transcription. This negative regulation on FliA will be relieved upon completion of the hook-basal body intermediate structure (Ohnishi et al. 1992).

1.1.3 A bacterial second messenger c-di-GMP

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a common bacterial second messenger found in most major bacterial phyla (Römling et al. 2013). It was first discovered in 1987 as an allosteric activator for cellulose synthase in *Gluconacetobacter xylinus* (Ross et al. 1987; Tal et al. 1998). It is now established that c-di-GMP is involved in the regulation of many cellular activities, including biofilm formation, motility, cell cycle, antibiotic production, virulence, and other processes (Dow et al. 2006; Cotter and Stibitz 2007; Fineran et al. 2007; Ryan et al. 2007; Tamayo et al. 2007; Wolfe and Visick 2008; Duerig et al. 2009; Hengge 2009; Yi et al. 2010). The synthesis and breakdown of c-di-GMP are dependent on two groups of enzymes, the diguanylate cyclase (DGC) enzymes and the c-di-GMP-specific phosphodiesterase (PDE) enzymes, respectively (Fig. 4). DGC activity is associated with the GGDEF domain, which converts two molecules of guanosine-5'-triphosphate (GTP) to c-di-GMP (Paul et al. 2004; Solano et al. 2009). PDE

activity is associated with either the EAL or the HD-GYP domains, which degrade c-di-GMP to 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) or two molecules of guanosine monophosphate (GMP), respectively (Schmidt et al. 2005; Tamayo et al. 2005; Ryan et al. 2006a). Studies of the DGCs and PDEs revealed that most GGDEF, EAL and HD-GYP domains are linked to various N-terminal sensory input domains, such as PAS, GAF, CHASE, and REC domain (Galperin 2004; Ryan et al. 2006b; Hengge 2009). Signals received by the above mentioned sensor domains include numerous environmental cues, such as light, oxygen, and redox conditions, as well as other cellular signals including antibiotics, polyamines or intercellular signaling molecules (Galperin et al. 2001; Galperin 2004; Jenal and Malone 2006). Recently, Townsley and colleagues reported that temperature also serves as an environmental signal to regulate c-di-GMP-dependent biofilm formation in *Vibrio cholerae* (Townsley and Yildiz 2015), however, many primary signals in the c-di-GMP signaling network are yet to be identified. C-di-GMP binds to diverse classes of receptors in order to regulate bacterial activities. These include PliZ domain receptors, inactive GGDEF, EAL and HD-GYP domain receptors, and two types of RNA riboswitches (Sudarsan et al. 2008; Krasteva et al. 2012; Ryan et al. 2012; Römling et al. 2013).

The genomes of many bacteria contain a large number of enzymes involved in the synthesis and breakdown of c-di-GMP. For example, *V. cholerae* encodes more than 50 GGDEF or EAL domain proteins, *E. coli* has 29 GGDEF or EAL domain proteins, whereas *Caulobacter crescentus* has 14 (Hengge 2009). In *D. dadantii*, there are 12 GGDEF, 4 EAL, and 2 GGDEF and EAL dual domain proteins. The multiplicity of these proteins raises questions of the specificity of c-di-GMP signaling. To determine whether they regulate

diverse cellular outputs redundantly or in a temporal and spatial manner, the functions of each GGDEF and/or EAL domain proteins need to be investigated in *D. dadantii* 3937.

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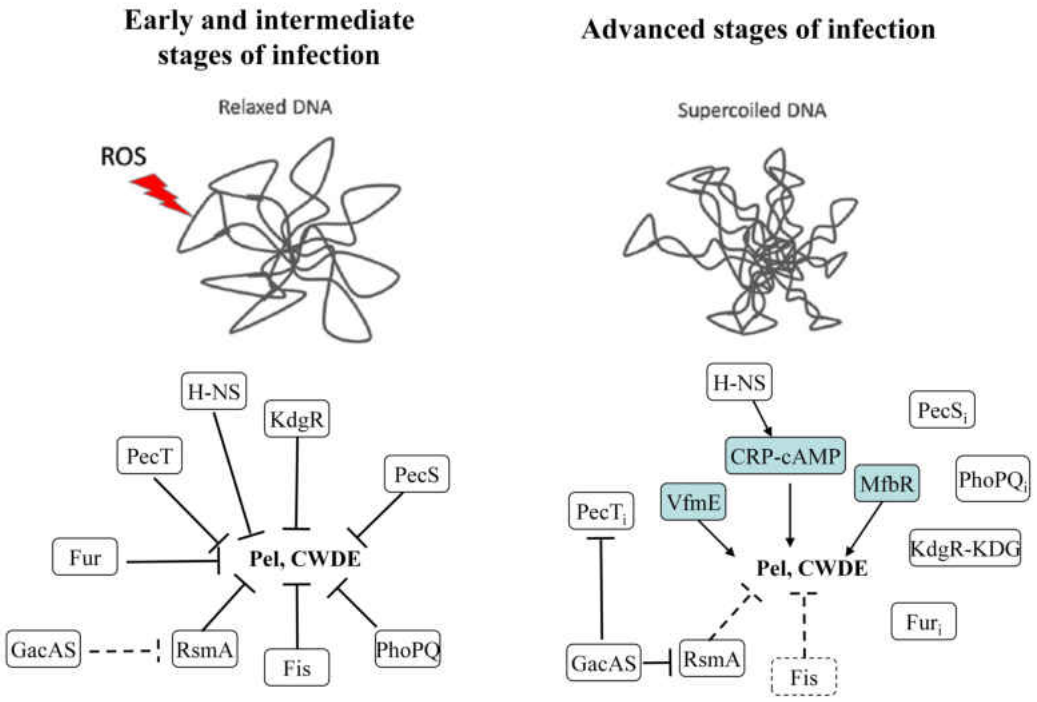


Source: modified from (Reverchon and Nasser 2013)

FIG 1 Regulation of pectate lyase production during *D. dadantii* 3937 pathogenesis. During early and intermediate stages of infection, generation of reactive oxygen species (ROS) and low pH conditions in the plant cause bacterial DNA relaxation, and activation of several pectate lyase repressors including PhoP-PhoQ and Fur. The relaxed DNA facilitates major regulators such as Fis and H-NS to bind and repress the *pel* gene expression. During advanced stages of infection, the in plant condition is altered resulting in higher pH value, increased acetate, 2-keto-3-deoxygluconate (KDG) concentration, and quorum-sensing signals. These changes inactivate the repressors mentioned above, while activate the activators for petate lyase production. Fis production is growth phase regulated, and is mainly produced during the early exponential growth phase while decreasing in concentration during the stationary growth phase. Arrows and bars indicate activation and repression of gene expression, respectively.

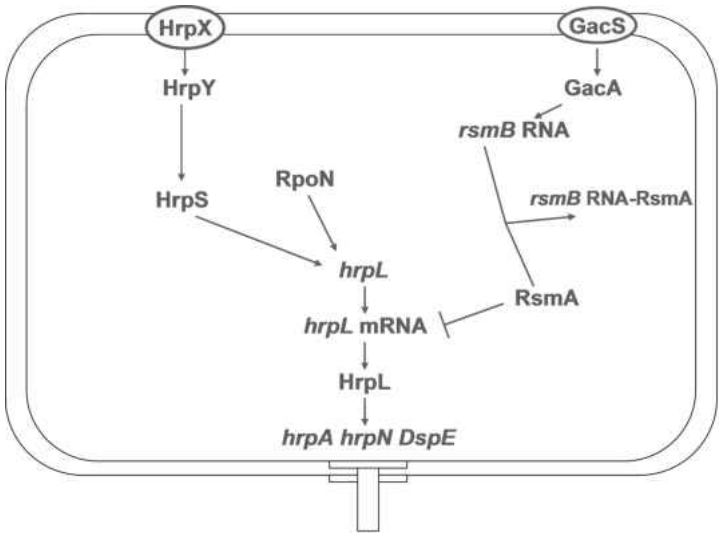
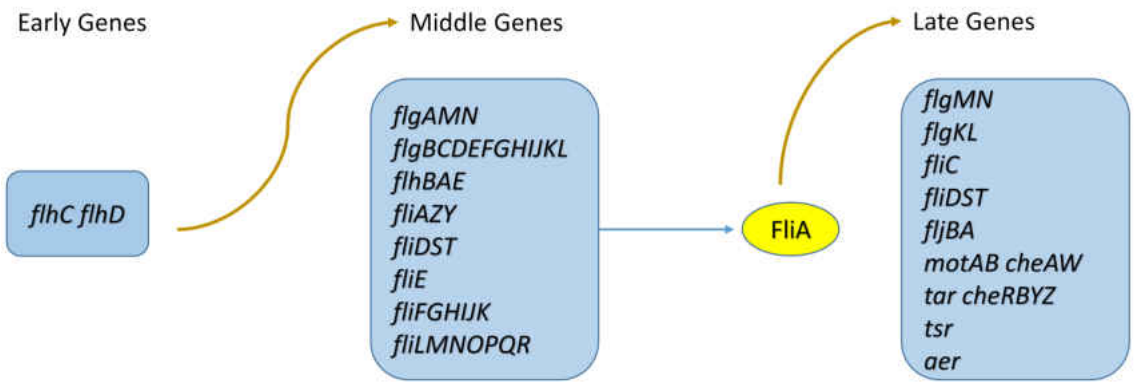


FIG 2 Regulatory mechanism of T3SS in *D. dadantii* 3937. HrpX/Y-HrpS pathway positively regulates HrpL at the transcriptional level. GacS/A-RsmB-RsmA pathway controls T3SS at the post transcriptional level. Arrows and bars indicate positive and negative regulation, respectively.



Source: modified from (Chilcott and Hughes 2000)

FIG 3 Flagellar transcriptional hierarchy in bacteria. The flagellar master regulator FlhDC, which is encoded from two early (class I) genes, is required for the transcription of middle (class II) genes. Proteins encoded by middle genes include those necessary for the flagellar hook-basal body structure, and two regulatory proteins, FlgM and FliA. FliA is an alternative sigma factor that activates the transcription of late (class III) genes to accomplish the flagellar assembly.

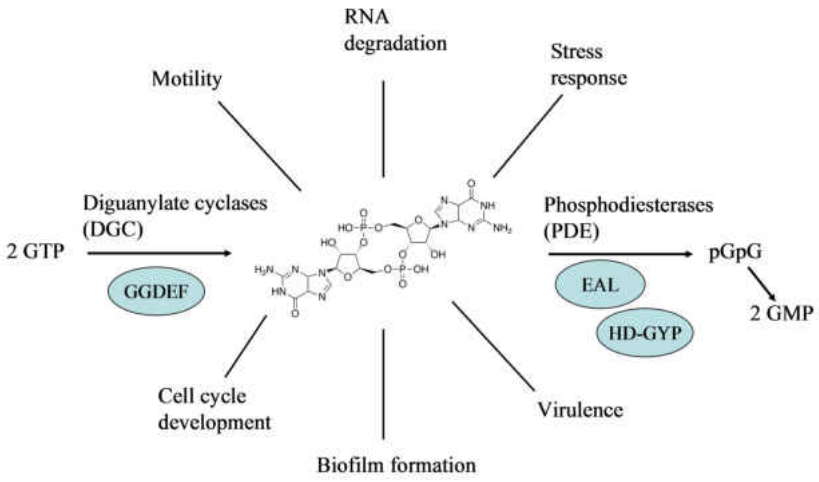


FIG 4 Modulation of c-di-GMP and its regulatory effects on diverse cellular behaviors. Diguanylate cyclases (DGCs) contain GGDEF domain, which synthesize c-di-GMP from two molecules of GTP. Phosphodiesterases (PDEs) with either an EAL or HD-GYP domain, break down c-di-GMP.

Chapter 2

Cross-talk between a regulatory small RNA, cyclic-di-GMP signaling, and flagellar regulator FlhDC for virulence and bacterial behaviors

ABSTRACT

Dickeya dadantii is a globally dispersed phytopathogen which causes diseases on a wide range of host plants. This pathogen utilizes the type III secretion system (T3SS) to suppress host defense responses, and secretes pectate lyase (Pel) to degrade the plant cell wall. Although the regulatory small RNA (sRNA) RsmB, cyclic diguanylate monophosphate (c-di-GMP), and flagellar regulators have been reported to affect the regulation of these two virulence factors and multiple cell behaviors such as motility and biofilm formation, the linkage between these regulatory components that coordinate the cell behaviors remain unclear. Here we reveal a sophisticated regulatory network that connects the sRNA, c-di-GMP signaling, and flagellar master regulator FlhDC. We propose multi-tiered regulatory mechanisms that link the FlhDC to the T3SS through three distinct pathways including the FlhDC-FliA-$YcgR_{3937}$ pathway; the FlhDC-EcpC-RpoN-HrpL pathway; and the FlhDC-*rsmB*-RsmA-HrpL pathway. Among these, EcpC is the most dominant factor for FlhDC to positively regulate T3SS expression.

INTRODUCTION

Dickeya dadantii 3937, belonging to the *Enterobacteriaceae* family, is a Gram-negative plant pathogen that causes soft rot, wilt, and blight diseases on a wide range of plant species, including many economically important vegetables such as potato, tomato and chicory (Czajkowski et al. 2011). There are many virulence factors that contribute to the pathogenesis of *D. dadantii* at different stages of infection. For example, during the primary stage of infection, *D. dadantii* produces several factors that enhance its adhesion to the plant surface, such as cellulose fibrils, CdiA-type V secreted proteins and a biosurfactant (Rojas et al. 2002;

Hommais et al. 2008; Jahn et al. 2011; Prigent-Combaret et al. 2012). Chemotaxis and motility are essential when *D. dadantii* needs a favorable site to enter into the plant apoplast (Antúnez-Lamas et al. 2009). In the apoplast, *D. dadantii* uses a Type III secretion system (T3SS) to further invade the plant host (Bauer et al. 1994; Yang et al. 2002) by translocating virulence effector proteins into the host cytoplasm, thereby causing disease symptoms (Hueck 1998; He et al. 2004; Mota et al. 2005). At later stages of infection, large areas of maceration on plant leaves and tissues occur due to the production and secretion of plant cell wall degrading enzymes, such as pectate lyases, proteases, cellulases and polygalacturonases (Collmer and Keen 1986; Roy et al. 1999; Herron et al. 2000; Kazemi-Pour et al. 2004).

The T3SS of *D. dadantii* is encoded by a group I *hrp* gene cluster, in which the alternative sigma factor HrpL is required to activate most *hrp* operons (Alfano and Collmer 1997). Two regulatory pathways to control the expression of *hrpL* have been discovered in *D. dadantii* (Yap et al. 2005; Tang et al. 2006; Yang et al. 2008a; Yang et al. 2008b). The first pathway is through the two-component signal transduction system (TCS) HrpX/HrpY, which directly activates *hrpS* transcription. HrpS is a σ^{54} (RpoN)-enhancer binding protein, that binds a σ^{54} -containing RNA polymerase holoenzyme and initiates the transcription of *hrpL* (Chatterjee et al. 2002; Yap et al. 2005; Tang et al. 2006). Hence, HrpL is able to activate most genes downstream in the T3SS regulatory cascade, such as *hrpA*, *hrpN*, and *dspE*, which encode the T3SS pilus protein, a harpin protein, and a virulence effector, respectively (Wei and Beer 1995; Chatterjee et al. 2002; Tang et al. 2006). *hrpL* is also post-transcriptionally regulated by the RsmA/*rsmB* RNA-mediated pathway (Chatterjee et al. 2002; Yang et al. 2008b). RsmA is a small RNA-binding protein that binds to the 5' untranslated region of *hrpL*

mRNA, and facilitates its degradation (Chatterjee et al. 1995). RsmB is an untranslated regulatory RNA that binds to RsmA and sequesters its negative effect on *hrpL* mRNA (Liu et al. 1998; Chatterjee et al. 2002). The global two-component system GacS/A upregulates RsmB RNA production, which alternatively increases downstream T3SS gene expression (Yang et al. 2008b). How these regulatory pathways are coordinated to regulate T3SS gene expression remains unclear.

Recent work from our laboratory demonstrated that a bacterial second messenger bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a global regulatory signal in *D. dadantii* controlling the expression of T3SS-encoding genes, the production of pectate lyase, swimming and swarming motility, and biofilm formation (Yi et al. 2010). This is in agreement with the function of c-di-GMP in many other bacterial species showing that c-di-GMP regulates diverse cellular activities (Cotter and Stibitz 2007; Hengge 2009; Schirmer and Jenal 2009; Römling 2012). The synthesis and degradation of c-di-GMP are controlled by two types of enzymes performing opposing functions. They are the GGDEF domain-containing diguanylate cyclases (DGC), which convert two molecules of GTP to c-di-GMP (Paul et al. 2004; Solano et al. 2009), and the EAL or the HD-GYP domain-containing phosphodiesterases (PDE), which break down c-di-GMP into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) or 2 guanosine monophosphates, respectively (Schmidt et al. 2005; Tamayo et al. 2005; Ryan et al. 2006). In order for c-di-GMP to exert such diverse influences in the cell, a range of cellular c-di-GMP effectors have been identified including PilZ domain proteins, transcription factors, enzymatically inactive GGDEF and/or EAL domain proteins and RNA riboswitches. These effectors are able to directly interact with

c-di-GMP which either activates or represses their activity (Hengge 2009; Breaker 2011; Ryan et al. 2012).

It has long been established that flagellar gene expression and assembly is a highly regulated process and occurs in a hierarchical manner. In *Escherichia coli* and other enteric bacteria, FlhDC is the master regulator, also defined as class I operon in flagellar assembly genes (Wang et al. 2006). FlhDC activates the expression of class II operons which encode the basal body and hook of the flagellum and an alternative σ factor (σ^{28}) FliA. FliA is required for the activation of class III operons which encode proteins for the outer subunits of the flagellum, chemotaxis and the flagellar motor (Chilcott and Hughes 2000; Aldridge et al. 2006). Recently, it has been reported that FlhDC regulates the expression of genes encoding GGDEF domains in *E. coli* (Pesavento et al. 2008). In addition, FlhDC positively regulates T3SS gene expression and extracellular enzyme production in *Pectobacterium carotovorum* by activating the expression of *rsmB* regulatory RNA (Cui et al. 2008). The homolog of FlhDC was also found in the genome of *D. dadantii* 3937, but its regulatory function has not yet been fully characterized.

C-di-GMP control of flagellar motility has been well studied in some bacterial species (Ryjenkov et al. 2006; Hengge 2009). For example, the PilZ-domain protein YcgR slows down flagellar rotation by directly binding to switch complex proteins under elevated c-di-GMP conditions (Fang and Gomelsky 2010; Paul et al. 2010). C-di-GMP also directly controls motility by transcriptional regulation of flagellar synthesis in *Vibrio cholerae* (Srivastava et al. 2013) and indirectly through induction of extracellular polysaccharides, which inhibit motility via undescribed mechanisms in *V. cholerae* and *Salmonella* (Srivastava

et al. 2013; Zorraquino et al. 2013).

In this study, we further investigated the impact of the PDEs EGcpB and EcpC on c-di-GMP-regulated behaviors in *D. dadantii* 3937. We identified two PilZ domain proteins $YcgR_{3937}$ and $BcsA_{3937}$, and determined their roles and functional relationship with EGcpB and EcpC. Then we systematically investigated the multi-tiered regulatory pathways linking the flagellar master regulator FlhDC to c-di-GMP signaling and T3SS gene expression. We found that EcpC is the major contributor that controls the T3SS through FlhDC.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, primers, and media

The bacterial strains and plasmids used in this study are listed in Table 2. *D. dadantii* 3937 and mutant strains were stored at –80°C in 20% glycerol. *D. dadantii* strains were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl), mannitol-glutamic acid (MG) medium (1% mannitol, 0.2% glutamic acid, 0.05% potassium phosphate monobasic, 0.02% NaCl, and 0.02% $MgSO_4$) or low-nutrient T3SS inducing MM at 28°C (Yang et al. 2007; Yang et al. 2008b). *E. coli* strains were grown in LB at 37°C. Antibiotics were added to the media at the following concentrations: ampicillin (100 μg/ml), kanamycin (50 μg/ml), gentamicin (10 μg/ml), chloramphenicol (20 μg/ml), tetracycline (12 μg/ml) and spectinomycin (100 μg/ml). The *D. dadantii* 3937 genome sequence can be retrieved from ASAP (https://asap.ahabs.wisc.edu/asap/home.php). Primers used for PCR in this report are listed in Table 3.

Mutant construction and complementation

The *flhDC*, *fliA*, $bcsA_{3937}$ and $ycgR_{3937}$ genes were deleted from the genome by marker exchange mutagenesis (Yang et al. 2002). Briefly, two fragments flanking each target gene were amplified by PCR with specific primers (Table 3). The kanamycin cassette was amplified from pKD4 (Datsenko and Wanner 2000), and was cloned between two flanking regions using three-way cross-over PCR. The PCR construct was inserted into the suicide plasmid pWM91, and the resulting plasmid was transformed into *D. dadantii* 3937 by conjugation using *E. coli* strain S17-1 λ-pir. To select strains with chromosomal deletions, recombinants, grown on kanamycin medium, were plated on 5% sucrose plate. Cells that were resistant to sucrose due to SacB-mediated toxicity were then plated on ampicillin plate, and the ampicillin sensitive cells were confirmed by polymerase chain reaction (PCR) using outside primers. Finally, the DNA fragment which contains two flanking regions and kanamycin cassette was sequencing confirmed.

To generate complemented strains, the promoter and ORF region of target genes were amplified and cloned into low-copy-number plasmid pCL1920 (Table 2). The resulting plasmids were then confirmed by PCR and electroporated into mutant cells.

Biofilm formation assay

Biofilm formation was determined by using a method that was previously described (Yi et al. 2010). In brief, bacterial cells grown overnight in LB media were inoculated 1:100 in MM media in 1.5 ml polypropylene tubes. After incubation at 28°C for 48 h, cells were stained with 1% crystal violet (CV) for 15 min. The planktonic cells were removed by several rinses

with H_2O. The CV-stained bound cells were air dried for 1 h, then dissolved in 90% ethanol, and the OD_{590} of the solution was measured to quantify the biofilm formation.

Swimming motility assay

Swimming motility was tested by inoculating 10 µl of overnight bacterial cultures (OD_{600}=1.0) onto the center of MG plates containing 0.2% agar. The inoculated plates were incubated at 28°C for 20 h, and the diameter of the radial growth was measured (Antúnez-Lamas et al. 2009).

Pectate lyase activity assay

Extracellular Pel activity was measured by spectrometry as previously described (Matsumoto et al. 2003). Briefly, bacterial cells were grown in MM media supplemented with 20% glycerol and 1% polygalacturonic acid at 28°C for 20 h. For extracellular pel activity, 1 ml bacterial cultures were centrifuged at 15,000 rpm for 2 min, supernatant was then collected and 10 µl of the supernatant was added to 990 µl of the reaction buffer (0.05% PGA, 0.1 M Tris-HCl [pH 8.5], and 0.1 mM $CaCl_2$, prewarmed to 30°C). Pel activity was monitored at A_{230} for 3 min and calculated based on one unit of Pel activity equals to an increase of 1×10^{-3} OD_{230} in 1 min.

GFP reporter plasmid construction and flow cytometry assay

To generate the reporter plasmids pAT-*ycgR*$_{3937}$ and pAT-*ecpC*, the promoter regions of *ycgR*$_{3937}$ and *ecpC* were PCR amplified and cloned into the promoter probe vector

pPROBE-AT, which contains the ribosomal binding site upstream of the *gfp* gene, respectively (Miller et al. 2000; Leveau and Lindow 2001). The reporter plasmids pAT-*hrpA*, pAT-*hrpN*, pAT-*hrpL* and pAT-*rsmB* were constructed previously following the same procedure (Yang et al. 2007; Li et al. 2014). Promoter activity was monitored by measuring GFP intensity through flow cytometry (BD Biosciences, San Jose, CA) as previously described (Peng et al. 2006). Briefly, bacterial cells with reporter plasmid were grown in LB media overnight and inoculated 1:100 into MM media. Samples were collected at 12 h and 24 h, respectively, and promoter activity was analyzed by detecting GFP intensity using flow cytometry.

Determination of intracellular c-di-GMP concentration

Intracellular c-di-GMP concentrations were determined by using ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS-MS), that has been described previously (Edmunds et al. 2013). Overnight bacterial cultures were inoculated 1:100 into 30 ml LB media in a flask. After the OD_{600} of bacterial culture reached about 0.8, corresponding to mid- to late-exponential growth, cells were centrifuged in 50-ml polystyrene centrifuge tubes for 30 min at 4,000 rpm. The supernatant was then removed, and the pellet was resuspended in 1.5 ml extraction buffer (40% acetonitrile–40% methanol in 0.1 N formic acid). To lyse the cell and release intracellular c-di-GMP, cells resuspended in extraction buffer were left at -20°C for 30 min, and then centrifuged at 13,000 rpm for 1 min. The supernatant was collected and analyzed by UPLC-MS-MS.

Protein expression and purification

The full length *ycgR*$_{3937}$ was cloned into the expression vector pET21b after PVR with primers *ycgR*$_{3937}$-for-NdeI and *ycgR*$_{3937}$-rev-EcoRI (Table 3). To construct the site-specific point mutation in the RxxxR motif of YcgR$_{3937}$ PilZ domain, single nucleotide substitution was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Briefly, a primer set, *ycgR*$_{3937}$-R124D-1 and *ycgR*$_{3937}$-R124D-2 (Table 3), was used to generate *ycgR*$_{3937}^{R124D}$, which changed the RxxxR motif to RxxxD. Substitution was confirmed by DNA sequencing. The constructs carrying *ycgR*$_{3937}$ and *ycgR*$_{3937}^{R124D}$ were transformed into *E. coli* BL21 stains for protein expression and purification. Briefly, expression of fusion proteins was induced by addition of isopropyl-thio-galactopyranoside at a final concentration of 0.5 mM and the bacterial cultures were then incubated at 16°C for 12 h. Then bacterial cells were collected by centrifugation, followed by suspension in phosphate buffered saline and sonication. The crude cell extracts were centrifuged at 12,000 rpm for 25 min to remove cell debris. The supernatant containing the soluble proteins was collected and mixed with preequilibrated Ni^{2+} resin (GE Healthcare, Piscataway, NJ, U.S.A.) for 3 h at 4°C, then placed into a column and extensively washed with buffer containing 30 mM Tris-HCl (pH 8.0), 350 mM NaCl, 0.5 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, and 30 mM imidazole. The proteins were subsequently eluted with buffer containing 300 mM imidazole. The purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

ITC assay

The binding of $YcgR_{3937}$ and $YcgR_{3937}^{R124D}$ to c-di-GMP was detected on ITC200 (MicroCal, Northampton, MA) following the manufacturer's protocol. In brief, 2 μl of c-di-GMP solution (500 μM) was injected at 2 min intervals via a 60 μl syringe into the sample cell containing $YcgR_{3937}$ or $YcgR_{3937}^{R124D}$ proteins (50 μM) with constant stirring at 20°C, and the heat change accompanying these additions was recorded. The titration experiment was repeated three times, and the data were calibrated with a buffer control and fitted with the single-site model to determine the binding constant (K_d) using the MicroCal ORIGIN version 7.0 software.

Förster resonance energy transfer (FRET) analysis

To construct the c-di-GMP sensor *in vivo*, encoded by plasmid pMMB67EHGent-*ycgR*$_{3937}$ (YFP-$YcgR_{3937}$-CFP), the *ycgR*$_{3937}$ fragment was amplified using specific primers (Table 3) and cloned into pMMB67EHGent vector. The resulting plasmid was transferred into *D. dadantii* 3937 by electroporation. Bacterial strains containing the pMMB67EHGent vector or derivative plasmids were incubated in LB medium at 28°C with a range from 0 to 100 μM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 10 μg/ml gentamycin for 12 or 24 hr to express various amounts of $YcgR_{3937}$-based c-di-GMP sensors. After incubation, the cells placed on a glass-bottom dish were ready for FRET imaging. Accurate determination of apparent FRET efficiency for cells expressing the $YcgR_{3937}$-based c-di-GMP sensor was performed by spectrally resolved FRET imaging (Raicu et al. 2009) using an optical micro-spectroscope (OptiMiS TruLine, Aurora Spectral Technologies, Milwaukee, WI). The imaging system was equipped with a Ti-Sapphire laser (Tsunami, Spectra-Physics) with a

tuning range of 690–1040 nm and delivering pulses with a width of < 100 fs at a repetition rate of 80 MHz. In this system, the excitation beam is shaped into a line by employing a curved mirror placed at the back focal plane of the scanning lens (Biener et al. 2013). This set-up features a reduced acquisition time and increased overall sensitivity. The incident light is focused through an infinity-corrected oil-immersion objective (100× magnification, NA 1.4, Nikon Instruments, Melville, NY) to a line with diffraction-limited thickness on the sample. The emitted light is passed through a transmission grating and projected onto a cooled electron-multiplying CCD camera (EMCCD; Andor, iXon 897).

Dishes containing cells expressing the c-di-GMP sensor were placed on the microscope sample stage and irradiated at 800 nm with femtosecond light pulses to obtain emission spectra consisting of signals from donors and acceptors for every pixel in an image. Emission spectra also were separately acquired for cells expressing donors or acceptors alone, which were excited at 800 nm and 960 nm, respectively; the measured fluorescence intensities were normalized to the maximum value to obtain elementary spectra for donors and acceptors. The elementary spectra where then used to unmix the donor and acceptor signals for the cells expressing the c-di-GMP sensor following a procedure described elsewhere (Raicu and Singh 2013). The signals corresponding to the donor in the presence of acceptor (k^{DA}) and acceptor in the presence of donor (k^{AD}), respectively, were used to compute the FRET efficiency at each pixel in an image, using the same method as described before (Raicu et al. 2009).

For data analysis, an automatic computer algorithm, based on thresholding, masking, and segmentation, was performed. First, an image was generated (labeled as F^D) by correcting for FRET the digital image of the donor in the presence of acceptor, k^{DA}, and multiplying by the

donor spectral integral, as described elsewhere (Patowary et al. 2013). Then, a threshold for the donor emission, based on Otsu's algorithm, was chosen (Otsu 1975). Next, a mask of the F^D image was formed using this threshold. The mask was segmented using a MATLAB function "boundaries" (Gonzalez et al. 2004). The segments' boundaries were plotted to assist the user in removing segments containing multiple bacteria. Once the segments were approved by the user, the mask was used to select all the pixels corresponding to individual cells. Fluorescence images contained an average of 50 cells per image. Between 5 and 11 images were acquired for each sample type. Average FRET efficiency values were computed over all cells in an image and then mean values and standard errors of the mean (i.e., standard deviation divided by the square root of the number of images) were computed for each sample type.

Northern blotting analysis

To measure the RNA levels of *rsmB* in wild type, Δ*flhDC*, Δ*fliA* and complemented strains, bacterial cells grown in MM for 12 h were harvested and total RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO). The residual DNA was removed with a Turbo DNA-free DNase kit (Ambion, Austin, TX). Northern blotting analysis was performed using biotin-labelled probe and a biotin detection system (BrightStar Psoralen-Biotin and Bright Star BioDetect, Ambion). 16S rRNA was used as an internal control.

qRT-PCR analysis

The mRNA levels of *rpoN* and *hrpL* were measured by qRT-PCR. Briefly, bacterial cells

cultured in MM for 12 h were harvested and total RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Extracted RNA was treated with Turbo DNase I (Ambion, Austin, TX), and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The cDNA level of target genes was quantified by qRT-PCR using a Real Master Mix (Eppendorf, Westbury, NY, USA), as described previously (Peng et al. 2006). Data were analyzed using a Relative Expression Software Tool (Pfaffl et al. 2002). The expression level of *rplU* was used as an endogenous control for data analysis (Mah et al. 2003).

Virulence assay

The local leaf maceration assay was performed using the leaves of Chinese cabbage (*B. campestris*) and African violet (*S. ionantha*) as described (Yi et al. 2010). For African violet, 50 µl of bacterial suspension at 10^6 CFU/ml were syringe infiltrated in the middle of each symmetric side of the same leaf. Phosphate buffer (50 mM, pH 7.4) was used to suspend the bacterial cells. Five replicate plants were used for each bacterial strain, and four leaves were inoculated in each plant. For Chinese cabbage, 10 µl of bacterial suspension at 10^7 CFU/ml were inoculated into the wounds punched with a sterile pipette on the leaves. Five leaves were used for each strain. Inoculated African violet plants or Chinese cabbage leaves were kept in growth chamber at 28°C with 100% relative humidity. To evaluate disease symptoms, APS Assess 1.0 software (Image Analysis Software for Plant Disease Quantification) was used to determine the leaf maceration area.

Statistical analysis

Means and standard deviations of experimental results were calculated using Excel (Microsoft, Redmond, WA) and the statistical analysis was performed using a two-tailed student's t-test.

RESULTS

Elevated c-di-GMP levels were detected in *D. dadantii* Δ*egcpB*, Δ*ecpC*, and Δ*egcpB*Δ*ecpC*

Previously, we identified two PDE-encoding genes *egcpB* (former name was *ecpB*), and *ecpC* in *D. dadantii* (Yi et al. 2010). Deletion of these PDE-encoding genes resulted in increased biofilm formation and reduced swimming motility, pectate lyase production, T3SS gene expression, and overall virulence, suggesting that the c-di-GMP level in these mutants is increased compared to the wild-type strain (Yi et al. 2010). To determine if these phenotypes observed in the above PDE mutants were indeed associated with elevated c-di-GMP levels, we performed liquid chromatography-mass spectrometry to measure the intracellular c-di-GMP concentration in the wild type and the PDE mutants (The levels of c-di-GMP in Δ*egcpB,* Δ*ecpC*, and Δ*egcpB*Δ*ecpC* were measured by Devanshi Khokhani through collaboration with Christopher Waters). As expected, our results showed an increased c-di-GMP concentration in Δ*egcpB*, Δ*ecpC*, and Δ*egcpB*Δ*ecpC* in comparison with the wild-type strain (Fig. 1), suggesting that the two PDEs EGcpB and EcpC indeed reduce c-di-GMP concentration in *D. dadantii* 3937. The fact that the double-deletion mutant had the highest level of c-di-GMP indicated that the effect of EGcpB and EcpC was not completely redundant, which is consistent with the previous report that Δ*egcpB*Δ*ecpC* showed more drastic changes phenotypically than either Δ*egcpB* or Δ*ecpC* (Yi et al. 2010).

PilZ domain proteins regulated biofilm formation, swimming motility, and pectate lyase production in *D. dadantii* under elevated c-di-GMP conditions

C-di-GMP effectors are responsible for directly sensing intracellular changes in c-di-GMP levels and regulating cellular activity. PilZ domain proteins are the most widely distributed c-di-GMP effectors in bacteria (Hengge 2009). After searching the genome of *D. dadantii* 3937 genome using the Pfam program, we found two genes, $ycgR_{3937}$ (ABF-0014564) and $bcsA_{3937}$ (ABF-0017612), encoding PilZ domains (Fig. 2). Domain structure analysis using the simplified modular architecture research tool (SMART) revealed that $YcgR_{3937}$, similar to the *E.coli* YcgR protein, has an N-terminal YcgR domain and a C-terminal PilZ domain, and $BcsA_{3937}$ is an *E. coli* BcsA-like protein, which has an N-terminal cellulose synthesis domain and a C-terminal PilZ domain (Fig. 2A). Amino acid sequence alignments of the reported PilZ domains from *E. coli* and those identified in *D. dadantii* 3937, suggested that the c-di-GMP binding motif (RxxxR) is conserved in the PilZ domain of both $YcgR_{3937}$ and $BcsA_{3937}$ proteins (Fig. 2B).

To investigate whether the regulatory pathway of EGcpB and EcpC is mediated by the two PilZ domain proteins, we constructed $ycgR_{3937}$ and $bcsA_{3937}$ gene deletion mutants in the wild type, Δ*egcpB* and Δ*ecpC* backgrounds, and examined biofilm formation, swimming motility, and pectate lyase production in these mutants. As shown in Figure 3, compared with the wild type, there was no detectable impact on biofilm formation, swimming motility, or pectate lyase production when $bcsA_{3937}$ and $ycgR_{3937}$ were deleted in the wild-type background (Fig. 3). This is in agreement with earlier results demonstrating that increased

c-di-GMP level is required for triggering the activity of PilZ-domain proteins (Paul et al. 2010). Compared with Δ*egcpB* and Δ*ecpC*, no further changes in swimming motility were detected when *bcsA*$_{3937}$ was deleted in these backgrounds (Fig. 3A). However, both Δ*bcsA*$_{3937}$Δ*egcpB* and Δ*bcsA*$_{3937}$Δ*ecpC* were fully restored to wild-type levels in biofilm formation (Fig. 3B). A full restoration of pectate lyase production was also observed when *bcsA*$_{3937}$ was deleted in either the Δ*egcpB* and Δ*ecpC* backgrounds (Fig. 3C). Moreover, deletion of *ycgR*$_{3937}$ in the Δ*egcpB* and Δ*ecpC* backgrounds led to partial restoration of swimming motility and biofilm formation, and full restoration of pectate lyase production (Fig. 3D, 3E, 3F).

To conclude, we propose that PilZ domain proteins BcsA$_{3937}$ and YcgR$_{3937}$ participate in the regulation of biofilm formation and pectate lyase production at elevated levels of c-di-GMP in *D. dadantii* 3937. In addition, YcgR$_{3937}$, but not BcsA$_{3937}$, regulates swimming motility when the intracellular levels of c-di-GMP are elevated.

YcgR$_{3937}$ and BcsA$_{3937}$ differentially regulate T3SS gene expression under elevated c-di-GMP conditions

Next, we wanted to determine whether YcgR$_{3937}$ and BcsA$_{3937}$ mediate regulation of T3SS gene expression, since EGcpB and EcpC affected T3SS gene expression in *D. dadantii* 3937 (Yi et al. 2010). The promoter activity of the *hrpA* gene, which encodes the T3SS pilus protein, was measured in wild-type and mutant strains. As expected, deleting the *ycgR*$_{3937}$ and *bcsA*$_{3937}$ gene in the wild-type background did not affect *hrpA* promoter activity. Interestingly, a further reduction of *hrpA* expression was observed in Δ*bcsA*$_{3937}$Δ*egcpB* and

Δ*bcsA*$_{3937}$Δ*ecpC* compared with the Δ*egcpB* and Δ*ecpC* backgrounds, respectively (Fig. 4A), suggesting that BcsA$_{3937}$ might regulate T3SS gene expression in parallel with EGcpB and EcpC. In contrast, the Δ*egcpB*Δ*ycgR*$_{3937}$ mutant partially restored *hrpA* promoter activity to the wild-type level compared with the *egcpB* single mutant (Fig. 4B). But there was no detectable impact on T3SS gene expression when *ycgR*$_{3937}$ was deleted in the Δ*ecpC* background (Fig. 4B). Thus, we concluded that EGcpB, but not EcpC, affected T3SS gene expression through YcgR$_{3937}$.

Binding of YcgR$_{3937}$ to c-di-GMP is required for regulating T3SS gene expression

Since the above results demonstrated that YcgR$_{3937}$ was in the signaling pathway of EGcpB to regulate the T3SS, we were interested in determining whether this regulation was related to its binding to c-di-GMP. First, we examined whether YcgR$_{3937}$ bound c-di-GMP *in vivo* and *in vitro*. The results from isothermal tritration colorimetry (ITC) assay revealed that the purified YcgR$_{3937}$ protein was capable of binding c-di-GMP at a 1:1 stoichiometric ratio with an estimated dissociation constant (K_d) of 413±64 nM (Fig. 5A). In contrast, the YcgR$_{3937}^{R124D}$ protein failed to bind c-di-GMP due to the mutation of the second arginine in the RxxxR motif in YcgR$_{3937}$, which is in agreement with the notion that these arginine residues are critical for the recognition of c-di-GMP by PilZ domains (Ryjenkov et al. 2006) (Fig. 5B). To probe the interaction between YcgR$_{3937}$ and c-di-GMP in living cells, we constructed a biosensor, in which YcgR$_{3937}$ was fused to yellow (YFP) and cyan (CFP) fluorescent proteins at the N and C termini, respectively. The CFP and YFP acted as a donor-acceptor pair in a process of Förster resonance energy transfer (FRET), which relies on

the distance-dependent transfer of energy from an excited donor fluorescent protein to an acceptor fluorescent protein (Raicu and Singh 2013). Previous studies using a biosensor derived from *Salmonella enterica* serovar Typhimurium protein YcgR (YFP-YcgR-CFP) in diverse Gram-negative bacterial species demonstrated that the YcgR-based c-di-GMP sensor undergoes a conformational change that pushes the donor and acceptor apart when c-di-GMP binds to the PilZ domain of YcgR; this leads to reduction in the overall FRET efficiency, which is inversely proportional to the concentration of c-di-GMP in the cell (Benach et al. 2007; Christen et al. 2007; Christen et al. 2010; Kulasekara et al. 2013). As shown in Table 1 (second and third columns), significant differences between the FRET efficiencies in the wild-type and Δ*egcpB*Δ*ecpC* strains were observed, which were consistent with the result from mass spectrometry assay showing higher concentrations of c-di-GMP for the Δ*egcpB*Δ*ecpC* strain than the wild type (Fig. 1). To conclude, these results strongly suggest that $YcgR_{3937}$ directly interacts with c-di-GMP in *D. dadantii* 3937.

Next, we performed a chromosomal replacement of $ycgR_{3937}$ with $ycgR_{3937}^{R124D}$ in the Δ*egcpB* background, and checked T3SS gene expression in this strain. As shown in Figure 4B, the promoter activity of the *hrpA* gene was recovered to a level similar to that in the Δ*egcpB*Δ$ycgR_{3937}$ double mutant. Based on these results, we propose that $YcgR_{3937}$ negatively regulates T3SS gene expression only under high c-di-GMP conditions in the Δ*egcpB* background, and that this activity is triggered by directly sensing the intracellular c-di-GMP concentration via the $YcgR_{3937}$ PilZ domain. Similar experiments were also carried out in the Δ*ecpC* background. No further change in *hrpA* gene expression was detected (Fig. 4B), which was consistent with the above data showing that $YcgR_{3937}$ does not mediate T3SS gene

expression regulation via EcpC.

In a study by Tuckerman et al., an *E. coli* protein complex, termed "degradosome" contained a DGC and PDE which mediate the c-di-GMP-dependent RNA processing (Tuckerman et al. 2011). We used a bacterial adenylate cyclase two-hybrid (BACTH) system to test whether there is a physical interaction between $YcgR_{3937}$ and EGcpB or EcpC in *D. dadantii*. No positive signal was detected using different protein combinations, suggesting that neither EGcpB nor EcpC directly interacts with $YcgR_{3937}$ (data not shown).

The flagellar master regulator FlhDC positively controls the expression of the T3SS regulon in *D. dadantii*

Studies comparing the flagellum and the T3SS in several bacterial species demonstrated a close link between these two nanomachines in terms of structure, function and expression regulation (Young et al. 1999; Lee and Galán 2004; Pallen et al. 2005; Erhardt et al. 2010). In enteric bacteria such as *E. coli* and *Salmonella*, the flagellar gene regulon has a three-tier hierarchy, which is controlled by the class I master regulator FlhDC, and class II alternative sigma factor FliA (Macnab 1996). FliA is required for the activation of all flagellar class III genes that encode the structural components of the flagellum (Liu and Matsumura 1994). Homologs of both FlhDC and FliA are present in *D. dadantii*. Deletion of *flhDC* or *fliA* led to significantly reduced motility, indicating that they are important regulators for motility in *D. dadantii* (Fig. 6). To determine whether there is a similar gene expression hierarchy in *D. dadantii*, we examined the promoter activity of *fliA* in wild-type and Δ*flhDC* strains. The results showed that the promoter activity of *fliA* was reduced dramatically in the Δ*flhDC*

mutant, and was restored to the wild-type level in the complemented strain (Fig. 7A), suggesting that FlhDC strictly controls the expression of *fliA*.

In *P. carotovorum*, Cui and colleagues discovered that the expression of T3SS *hrp* regulon is controlled by FlhDC (Cui et al. 2008). Therefore, we asked whether the homologs of FlhDC and FliA in *D. dadantii* regulate the T3SS. To test this, we first examined the promoter activity of *hrpL*, *hrpA* and *hrpN* in the wild-type, Δ*flhDC*, and Δ*fliA* strains. Deletion of *flhDC* significantly decreased the promoter activity of *hrpA* (3.9-fold), *hrpN* (6.6-fold) and *hrpL* (1.9-fold) under T3SS-inducing conditions (Fig. 7B). Complementation of Δ*flhDC* by expression of *flhDC in trans* restored the *hrpL*, *hrpA* and *hrpN* promoter activities to the wild-type level (Fig. 7B). In contrast, similar promoter activities for *hrpL*, *hrpA* and *hrpN* were observed between the wild-type and Δ*fliA* strains (Fig. 7C), suggesting that FliA does not impact T3SS gene expression. These results implied that FlhDC positively controls the expression of T3SS independently of FliA.

FlhDC controls expression of *ecpC*, *ycgR*$_{3937}$, but not *egcpB*

The data above illustrated that the c-di-GMP degrading enzymes EGcpB and EcpC positively regulate the expression of T3SS, while YcgR$_{3937}$ partially mediates the regulatory pathway downstream of EGcpB. In addition, the flagellar master regulator FlhDC also positively regulates T3SS gene expression. To understand the regulatory connections between these systems, we further examined the expression status of *ecpC*, *egcpB*, and *ycgR*$_{3937}$ in the Δ*flhDC* and Δ*fliA* mutants. As shown in Figure 8A, the promoter activity of *ecpC* dropped by 70% in the Δ*flhDC* mutant, but was not significantly affected in Δ*fliA*, suggesting that FlhDC

positively regulates the expression of *ecpC*, and the regulation was probably independent of FliA. In comparison, the promoter activity of *egcpB* was not affected by mutation of either *flhDC*, or *fliA* (Fig. 8B), while that of $ycgR_{3937}$ was reduced in both Δ*flhDC* and Δ*fliA* (Fig. 8C). These results indicated that expression of *egcpB* is not regulated by FlhDC or FliA, and that FlhDC positively regulates the expression of $ycgR_{3937}$ through FliA.

FlhDC positively controls the expression of the PDE gene *ecpC* (Fig. 8A) and the T3SS gene *hrpL* (Fig. 7B). As EcpC positively regulates an alternative sigma factor RpoN, which is required to activate the transcription of *hrpL* in *D. dadantii* 3937 (Yi et al. 2010), we hypothesized that FlhDC exerted its effects on T3SS gene expression via induction of *ecpC*. To test whether FlhDC regulates T3SS gene expression by activating the expression of *hrpL* through EcpC, a quantitative real time RT-PCR was performed to measure the levels of *rpoN* and *hrpL* transcripts in the wild type and Δ*flhDC* mutant. As shown in Figure 8D, a considerable decrease in the *rpoN* and *hrpL* transcript level was detected in Δ*flhDC* compared with the wild-type strain. Taken together, these results strongly suggest that FlhDC regulates T3SS gene expression through the FlhDC-EcpC-RpoN-HrpL pathway independently of FliA.

FlhDC positively controls *rsmB* expression at the post-transcriptional level

The GacS/A-*rsmB*-RsmA network has been well-studied as a major regulatory pathway controlling the T3SS of *D. dadantii* (Yang et al. 2008b). In *P. carotovorum*, FlhDC promotes the transcription of *gacA* via an unknown mechanism, which in turn positively controls the expression of *rsmB* (Cui et al. 2008). Therefore, to investigate whether and at which level FlhDC regulates RsmB, we first examined the promoter activity of *rsmB* in the wild-type,

Δ*flhDC* and Δ*fliA* strains under T3SS-inducing conditions. Interestingly, no difference in *rsmB* promoter activity was detected between the wild type and the mutants (Fig. 9A). We then determined the RNA levels of *rsmB* in the above mentioned strains by Northern blotting. The results showed that *rsmB* RNA level was reduced in Δ*flhDC*, but increased in Δ*fliA* when compared with the wild type (Fig. 9B). Complementation assays using low-copy number plasmid pCL1920 containing *flhDC* and *fliA* genes restored the Δ*flhDC* and Δ*fliA* phenotypes to the wild-type levels, respectively (Fig. 9B). RsmB positively regulates the production of pectate lyase by sequestering the effect of the post-transcriptional regulator RsmA (Yang et al. 2008b). To further investigate the impact of FlhDC and FliA on RsmB, we used a spectrophotomeric assay to monitor the pectate lyase production of wild-type, Δ*flhDC* and Δ*fliA* strains, and the complemented strains. The results showed that the pectate lyase production was reduced in Δ*flhDC* while increased in Δ*fliA* compared with the wild-type strain (Fig. 9C). To conclude, we propose that FlhDC and FliA divergently post-transcriptionally regulate the *rsmB* RNA level in *D. dadantii* 3937, and that these effects may contribute to the attenuated T3SS gene expression in Δ*flhDC*.

FlhDC regulates T3SS gene expression mainly through EcpC

The findings outlined above revealed three potential pathways through which FlhDC regulates T3SS gene expression. They are the FlhDC-FliA-$YcgR_{3937}$ pathway, the FlhDC-EcpC-RpoN-HrpL pathway, and the FlhDC-*rsmB*-RsmA-HrpL pathway. To determine which pathway is the most dominant one, we first excluded the FlhDC-FliA-$YcgR_{3937}$ pathway. This is because a negative impact on the T3SS through

YcgR$_{3937}$ was observed (Fig. 4B), which is in contrast to the phenotype in Δ*flhDC* where the T3SS gene expression levels were lower than in the wild type (Fig. 7B). Next, to compare the other two pathways, FlhDC-EcpC-RpoN-HrpL and FlhDC-*rsmB*-RsmA-HrpL, we engineered two constructs containing genes *ecpC* and *rsmB in trans* using low-copy number plasmid pCL1920, respectively. The resulting plasmids were transferred into wild-type and Δ*flhDC* strains harboring a *hrpA-gfp* reporter plasmid pAT-*hrpA*. The results for transcriptional assays showed that Δ*flhDC* strain with plasmid pCL1920 expressing *rsmB* was unable to restore the *hrpA* promoter activity to the wild-type level. In contrast, Δ*flhDC* strain with the plasmid pCL1920 expressing *ecpC* restored the *hrpA* promoter activity to the wild-type level (Fig. 10). Based on these results, we concluded that the positive effect of *D. dadantii* 3937 FlhDC on T3SS gene expression is mainly controlled through the FlhDC-EcpC-RpoN-HrpL pathway.

Motility regulators are required for the virulence of *D. dadantii*

Since FlhDC and FliA affected multiple phenotypes, such as swimming motility (Fig. 6), pectate lyase production, and T3SS, which are known to contribute to *D. dadantii* pathogenesis (Beaulieu and Van Gijsegem 1990; Yang et al. 2002; Antúnez-Lamas et al. 2009), virulence assays were performed to assess the effects of Δ*flhDC* and Δ*fliA* in the leaves of the host plant Chinese cabbage (*Brassica campestris*). Compared with the wild type, deletion mutants of *flhDC* and *fliA* were significantly reduced in maceration ability in planta (Fig. 11). Complementation assays restored the mutant phenotypes to the wild-type level. Similar results were also observed in African violet (*Saintpaulia ionantha*) when inoculated with these bacterial strains (Fig. 12). These data suggested that FlhDC and FliA are both

essential for the full pathogenesis of *D. dadantii* 3937.

Since the findings outlined above showed that FlhDC and FliA regulated swimming motility in the same direction, but not pectate lyase production or T3SS, we speculated that motility might play a determinate role in the FlhDC-regulated virulence. When *ecpC* was expressed *in trans* in Δ*flhDC*, it restored *hrpA* promoter activity and pectate lyase production (Fig. 10 and 13A). In contrast, expression of *rsmB* in Δ*flhDC* was able to restore pectate lyase production, but not T3SS gene expression (Fig. 10 and 13A). However, neither *ecpC* nor *rsmB* expression restored the swimming motility in Δ*flhDC* (Fig. 13B), which suggests that FlhDC, the flagellar master regulator, controls flagellar gene expression independently from EcpC or RsmB. As expected, neither *ecpC* nor *rsmB* expression in Δ*flhDC* strain restored its virulence in the leaves of Chinese cabbage (Fig. 11). These results supported the notion that motility is essential for the FlhDC-regulated virulence.

DISCUSSION

In this study, we identified two PilZ-domain proteins $YcgR_{3937}$ and $BcsA_{3937}$ in *D. dadantii* 3937 and demonstrated that these proteins regulate diverse cellular activities under elevated c-di-GMP conditions. $YcgR_{3937}$ specifically bound c-di-GMP as an effector both *in vivo* and *in vitro*, and this binding ability was required for mediating the regulation of T3SS gene expression by EGcpB. In addition, we demonstrated that the flagellar master regulator FlhDC regulates T3SS gene expression mainly through induction of the PDE *ecpC* under our experimental conditions.

We detected increased c-di-GMP concentrations in the PDE mutants including Δ*egcpB*,

Δ*ecpC*, and Δ*egcpB*Δ*ecpC* (Fig. 1), which supports the idea that EGcpB and EcpC regulates various cellular activities by modulating c-di-GMP levels. It has been proposed for many bacterial species that the regulation of c-di-GMP signaling is controlled in a temporal and spatial manner in the cell (Hengge 2009). EGcpB and EcpC probably control the degradation of c-di-GMP derived from different c-di-GMP pools, since deleting both of them had an additive effect on the increase of overall cellular c-di-GMP level. The changes in the c-di-GMP level are sensed at least partially by two PilZ domains proteins $YcgR_{3937}$ and $BcsA_{3937}$, since further deletion of them in the individual PDE mutants could restore some of the phenotypes to near wild-type level (Fig. 3B, 3C, 3D, 3E, 3F).

In *E. coli* and *Salmonella*, the regulatory role of YcgR was found to be strictly associated with motility (Ryjenkov et al. 2006; Fang and Gomelsky 2010; Paul et al. 2010). Here, we showed that $YcgR_{3937}$ not only regulates bacterial motility, but is mainly involved in the regulation of other activities including biofilm formation, pectate lyase production, and T3SS gene expression (Fig. 3 and Fig. 4). This is probably due to differences in the c-di-GMP signaling network between different bacterial species. In addition, $YcgR_{3937}$ positively regulates T3SS gene expression in the Δ*egcpB* background, but not the Δ*ecpC* background, suggesting that EGcpB and EcpC might have different mechanisms in affecting T3SS gene expression. Whether there are other c-di-GMP effectors mediating the downstream signaling pathway of EcpC needs further investigation.

BcsA in *E. coli* and *Salmonella* strains was shown to play a role in synthesizing cellulose, a major component of the extracellular matrix (Zogaj et al. 2001; Hengge 2009; Zorraquino et al. 2013). Here, we showed $BcsA_{3937}$ regulates biofilm formation and pectate lyase production

under the elevated levels of c-di-GMP (Fig. 3B, 3C), which is similar to $YcgR_{3937}$. $BcsA_{3937}$ of *D. dadantii* positively regulates biofilm formation, which might be due to the ability of $BcsA_{3937}$ to produce cellulose when c-di-GMP is elevated (Jahn et al. 2011). Dissimilar to $YcgR_{3937}$, $BcsA_{3937}$ was not found to affect the regulation of motility (Fig. 3A). A recent study in *Salmonella* demonstrated that BcsA and YcgR coordinately regulate swimming motility, in which BcsA produces cellulose to block the rotation of the flagellar (Zorraquino et al. 2013). It is possible that, similar to *Salmonella,* $BcsA_{3937}$ regulates swimming motility in the Δ*ycgR* background under high-c-di-GMP-level condition. Moreover, $BcsA_{3937}$ and $YcgR_{3937}$ regulate T3SS gene expression in opposite directions in the Δ*egcpB* and Δ*ecpC* backgrounds (Fig. 4). These data suggest that the regulation of T3SS by c-di-GMP signaling system in *D. dadantii* involves multiple components and is very complex.

It has been shown that YcgR interacts with the flagellar switch complex proteins FliG and FliM to regulate swimming motility (Fang and Gomelsky 2010; Paul et al. 2010). The point mutation R118D in the RxxxR motif of YcgR abolished its binding ability to c-di-GMP, and also weakened its binding to FliM or FliG, suggesting that the c-di-GMP binding ability of YcgR is required for its strong interaction with flagellar switch complex in responding to intracellular c-di-GMP changes (Fang and Gomelsky 2010; Paul et al. 2010). Here, our *in vitro* ITC and *in vivo* FRET assays confirmed that $YcgR_{3937}$ is a c-di-GMP binding protein, and the RxxxR motif in the PilZ domain is required for the binding activity (Table 1 and Fig. 5). In addition, by chromosomally replacing the wild-type $YcgR_{3937}$ with $YcgR_{3937}^{R124D}$, we showed that the binding to c-di-GMP is essential for its regulatory role in T3SS gene expression (Fig. 4B). Recently, Morgan and colleagues presented crystal structures of the

c-di-GMP-activated BcsA complex, which confirmed that the biological activity of BcsA is promoted through the allosteric effect of c-di-GMP (Morgan et al. 2014). We tried to examine whether the binding ability of the PilZ domain of $BcsA_{3937}$ to c-di-GMP is responsible for the phenotypes of biofilm formation, pectate lyase production and T3SS expression. However, several attempts of integrating the *$bcsA_{3937}$* gene with amino acid replacements in the PilZ motif into chromosome of *D. dadantii* were unsuccessful. In addition, the ITC and FRET assays were not performed in $BscA_{3937}$ because over-expression of $BcsA_{3937}$ in *E. coli* led to poor growth and cell death.

We demonstrated that the flagellar master regulator FlhDC plays a role in regulating T3SS gene expression. Three unique pathways were uncovered, including the FlhDC-FliA-$YcgR_{3937}$ pathway, the FlhDC-EcpC-RpoN-HrpL pathway, and the FlhDC-*rsmB*-RsmA-HrpL pathway, and a model of FlhDC regulation of T3SS genes was developed (Fig. 14). In the first regulatory pathway, the FlhDC-controlled sigma factor FliA activates the expression of *$ycgR_{3937}$* at the transcriptional level. Under high-c-di-GMP-level conditions caused by Δ*egcpB*, $YcgR_{3937}$ binds c-di-GMP and negatively regulates the expression of the T3SS regulon gene *hrpA*. Although the regulatory effect of YcgR on T3SS was not reported previously, the FlhDC-FliA-YcgR pathway was identified in *S.* Typhimurium (Frye et al. 2006). In the FlhDC-EcpC-RpoN-HrpL pathway, FlhDC controls the expression of *ecpC*, a phosphodiesterase-encoding gene at the transcriptional level. EcpC lowers the intracellular c-di-GMP concentration by degrading c-di-GMP, which positively affects the transcription of *hrpL* through the sigma factor RpoN at the post-transcriptional level (Fig. 8A, 8D) (Yi et al. 2010). It is important to note that this regulation is different in *E.*

coli, where the expression of *yhjH* (*ecpC* homolog) is activated by FliA (Pesavento et al. 2008), whereas the expression of *ecpC* of *D. dadantii* is regulated by FlhDC but independent of FliA (Fig. 8A). In addition, computational and DNase footprinting analyses in *E. coli* and *S.* Typhimurium of the FlhDC-regulon gene promoter regions have identified a consensus FlhDC binding sequence, in which two repeats of FlhDC-binding boxes AA(C/T)G(C/G)N_{2-3}AAATA(A/G)CG, are separated by a nonconserved sequence of 10-12 nucleotides (Claret and Hughes 2002; Stafford et al. 2005). In our work, we did not find this binding sequence within the 500bp from the 5' *ecpC* start codon, suggesting that FlhDC might not directly activate *ecpC* by binding to its promoter region. Finally, in the FlhDC-*rsmB*-RsmA-HrpL pathway, we discovered that FlhDC positively regulates the production of RsmB RNA at the post-transcriptional level, while FliA negatively regulates it (Fig. 9B). RsmB binds to RsmA, which neutralizes RsmA's negative impact on *hrpL* mRNA (Liu et al. 1998; Chatterjee et al. 2002). In *P. carotovorum*, FlhDC was reported to positively regulate *rsmB* through the *rsmB* transcriptional activator GacA at the transcriptional level (Cui et al. 2008). The promoter activity of *rsmB* is controlled by GacA in *D. dadantii* (Yang et al. 2008b). However, we did not detect any significant impact on the promoter activity of *rsmB* from the deletion of either *flhDC* or *fliA* (Fig. 9A), suggesting that despite the overall impact of FlhDC on *rsmB* being the same between *P. carotovorum* and *D. dadantii*, the underlying regulatory mechanisms are different. Furthermore, since RsmB has also been reported to positively regulate the production of pectate lyase in *D. dadantii* (Yang et al. 2008b), our observation that the pectate lyase production increased in Δ*fliA* compared with the wild-type strain is in agreement with the earlier statement. Owing to the fact that FlhDC

also regulates the expression of the phosphodiesterase gene *ecpC* (Fig. 8A), the reduced pectate lyase production observed in Δ*flhDC* may be due to a coordinated regulation of FlhDC on both the *rsmB*-RsmA system and the c-di-GMP signaling system (Yi et al. 2010). Expressing *ecpC* or *rsmB* using plasmid pCL1920 in Δ*flhDC* strain restored the pectate lyase production to near wild-type levels (Fig. 13A), which supports the above hypothesis. Finally, since we observed that FlhDC hierarchically regulates the expression of T3SS encoding genes, we further determined which of the three components, $YcgR_{3937}$, EcpC or *rsmB*, contributes to the FlhDC's positive effect on the T3SS. We first excluded $YcgR_{3937}$ due to its negative impact on the T3SS. Our results showed that expression of *ecpC* using low-copy-number plasmid pCL1920 in the Δ*flhDC* background is able to restore the promoter activity of T3SS encoding gene *hrpA* to the wild-type level (Fig. 10). No significant difference was detected when *rsmB* was expressed under the same condition (Fig. 10). To conclude, these data suggest that FlhDC regulates the T3SS mainly through the FlhDC-EcpC-RpoN-HrpL pathway.

Previous studies in *D. dadantii* 3937 demonstrated that swimming motility, pectate lyase production, and the T3SS are essential virulence factors that contribute to the pathogenicity of *D. dadantii* in host plant (Bauer et al. 1994; Yang et al. 2002; Yang et al. 2008b; Antúnez-Lamas et al. 2009). Here we uncovered a master regulator FlhDC, which positively regulates swimming motility, pectate lyase production, and T3SS expression (Fig. 6, 7B and 9C). The sigma factor FliA was found to positively regulate swimming motility but negatively regulates pectate lyase production and has no impact on T3SS expression (Fig. 6, 7C and 9C). Interestingly, our results showed significant reductions in maceration ability in planta for both

Δ*flhDC* and Δ*fliA* strains (Fig. 11 and Fig. 12). We also observed that expression of *ecpC in trans* in Δ*flhDC* restored *hrpA* promoter activity and pectate lyase production (Fig. 10 and 13A), but not swimming motility or overall virulence in Chinese cabbage (Fig. 11 and 13B). In addition, expression of *rsmB in trans* in Δ*flhDC* restored only pectate lyase production, but not *hrpA* promoter activity, swimming motility or virulence in Chinese cabbage (Fig. 10, 11, 13A and 13B). Thus, these results together with the previous report that several motility-deficient mutants were severely impaired in virulence (Antúnez-Lamas et al. 2009), imply that the reduced virulence of Δ*flhDC* and Δ*fliA* strains might be due to their defective swimming motility, which cannot be restored by pectate lyase production or T3SS gene expression. Therefore, we conclude that swimming motility, pectate lyase production and T3SS gene expression are essential in determining the full virulence of *D. dadantii* 3937 in host plants.

Many studies have demonstrated that the bacterial T3SS and the flagellum are evolutionarily related, since they share similarities in structure, function, and sequences of the main components (Young et al. 1999; Lee and Galán 2004; Pallen et al. 2005; Erhardt et al. 2010). In *Salmonella*, the type III effector SptP missing its chaperone-binding domain was secreted through the flagellar system instead of the T3SS, implying that these effectors carry ancient signals that could be recognized by the flagellar system (Lee and Galán 2004). Recently, it was demonstrated that the flagellin protein FliC in *Pseudomonas syringae* could be translocated into plant cells by the T3SS and induce immune responses (Wei et al. 2013). Here, our work provides novel insights that further support a connection between flagella and T3SS by showing that the flagellar master regulator FlhDC of *D. dadantii* 3937 also regulates

the transcription of the T3SS genes in a c-di-GMP-dependent manner.

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Sample	12 hrs/0 μM	12 hrs/50 μM	12 hrs/100 μM	24 hrs/50 μM
Wild type	0.253 ± 0.002 (n=10)	0.310 ± 0.002 (n=11)	0.363 ± 0.001 (n=5)	0.402 ± 0.003 (n=10)
Δ*egcpB*Δ*ecpC* mutant	0.257 ± 0.003 (n=10)	0.291 ± 0.002 (n=10)	0.339 ± 0.004 (n=5)	0.405 ± 0.002 (n=10)

Table 1 Mean ± SEM (standard errors of the mean) of apparent FRET efficiency for wild-type and Δ*egcpB*Δ*ecpC* cells expressing the c-di-GMP sensor YFP-YcgR$_{3937}$-CFP. Various induction levels were tested (listed as "time/μM" IPTG in the table) to establish the dynamic range of the sensor. The sensor was sensitive to changes in the concentrations of c-di-GMP when it was incubated for approximately 12 hr with 50 to 100 μM IPTG. An order-of-magnitude estimate of the sensor concentration based on the intensity of donor emission corrected for FRET, F^D (Patowary et al. 2013), as described in the Materials and Methods section, suggested that the sensor expression level varied between roughly 10 molecules per cell, for 12 hr incubation with 0 μM IPTG (first column), and 1,000 sensor molecules per cell, for 24 hr incubation with 50 μM IPTG (fourth column). The sensor concentration around which the sensor responded to changes in c-di-GMP concentrations, shown in the second and third columns in the Table, were on the order of 100 sensor molecules per cell. Within that concentration range, significant differences between the FRET efficiencies of the wild-type and Δ*egcpB*Δ*ecpC* mutant were observed. n = number of FRET images. FRET images contained an average of 50 cells per image.

Table 2 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics[a]	Reference or source
Dickeya dadantii		
3937	Wild type	Hugouvieux-Cotte-Pattat, N.
Δ*flhDC*	Δ*flhDC*::*Km*; Km^r, ABF-0018763 and ABF-0018762 deletion mutant	This study
Δ*fliA*	Δ*fliA*::*Km;* Km^r, ABF-0019722 deletion mutant	This study
Δ$ycgR_{3937}$	Δ$ycgR_{3937}$::*Km;* Km^r, ABF-0014564 deletion mutant	This study
$ycgR_{3937}^{R124D}$	$ycgR_{3937}^{R124D}$::*Km;* Km^r, ABF-0014564 site-directed mutant	This study
Δ*egcpB*Δ*ecpC*	Δ*egcpB*Δ*ecpC*, ABF-0020123 and ABF-0020364 double deletion mutant	(Yi et al. 2010)
Δ*egcpB*Δ $ycgR_{3937}$	Δ*egcpB*Δ$ycgR_{3937}$::*Km;* Km^r, ABF-0020123 and ABF-0014564 double deletion mutant	This study
Δ*ecpC*Δ $ycgR_{3937}$	Δ*ecpC*Δ$ycgR_{3937}$::*Km;* Km^r, ABF-0020364 and ABF-0014564 double deletion mutant	This study
Δ*egcpB*Δ $ycgR_{3937}^{R124D}$	Δ*egcpB*Δ$ycgR_{3937}^{R124D}$::*Km;* Km^r, ABF-0020123 deletion and ABF-0014564 site-directed mutant	This study
Δ*ecpC*Δ $ycgR_{3937}^{R124D}$	Δ*ecpC*Δ$ycgR_{3937}^{R124D}$::*Km;* Km^r, ABF-0020364 deletion and ABF-0014564 site-directed mutant	This study
Δ $bcsA_{3937}$	Δ$bcsA_{3937}$::*Km;* Km^r, ABF-0017612 deletion mutant	This study
Δ*egcpB*Δ $bcsA_{3937}$	Δ*egcpB*Δ$bcsA_{3937}$::*Km;* Km^r, ABF-0020123 and ABF-0017612 double deletion mutant	This study
Δ*ecpC*Δ$bcsA_{3937}$	Δ*ecpC*Δ$bcsA_{3937}$::*Km;* Km^r, ABF-0020364 and ABF-0017612 double deletion mutant	This study
Escherichia coli		
DH5α	*supE*44 □*lac*U169 (*ϕ*80*lacZ*□M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Lab stock
S17-1 λpir	λ(pir) *hsdR* pro *thi*; chromosomally integrated RP4-2 Tc::Mu Km::Tn7	Lab stock
Plasmids		
pKD4	Template plasmid for kanamycin cassette, Km^r	(Datsenko and Wanner 2000)
pKD3	Template plasmid for chloramphenicol cassette, Cm^r	(Datsenko and Wanner 2000)
pWM91	Sucrose-based counter-selectable plasmid, Ap^r	(Metcalf et al. 1996)
pET21b	Overexpression and purification vector, Ap^r	Novagen
pET21b: $ycgR_{3937}$	Overexpression of $ycgR_{3937}$ in expression vector	This study
pET21b: $ycgR_{3937}^{R124D}$	Overexpression of $ycgR_{3937}^{R124D}$ in expression vector	This study
pCL1920	Low copy number plasmid, lac promoter, Sp^r	(Lerner and Inouye 1990)
pCL-*flhDC*	*flhDC* cloned in pCL1920, Sp^r	This study

pCL-*fliA*	*fliA* cloned in pCL1920, Sp^r	This study
pCL-*ecpC*	*ecpC* cloned in pCL1920, Sp^r	This study
pCL-*rsmB*	*rsmB* cloned in pCL1920, Sp^r	This study
pPROBE-AT	Promoter-probe vector, promoterless *gfp*, Ap^r	(Miller et al. 2000)
pAT-*hrpA*	pPROBE-AT containing *hrpA* promoter-*gfp* transcriptional fusion, Ap^r	(Yang et al. 2007)
pAT-*hrpN*	pPROBE-AT containing *hrpN* promoter-*gfp* transcriptional fusion, Ap^r	(Yang et al. 2007)
pAT-*hrpL*	pPROBE-AT containing *hrpL* promoter-*gfp* transcriptional fusion, Ap^r	(Yang et al. 2007)
pAT-*rsmB*	pPROBE-AT containing *rsmB* promoter-*gfp* transcriptional fusion, Ap^r	(Li et al. 2014)
pAT- $ycgR_{3937}$	pPROBE-AT containing $ycgR_{3937}$ promoter-*gfp* transcriptional fusion, Ap^r	This study
pAT-*ecpC*	pPROBE-AT containing *ecpC* promoter-*gfp* transcriptional fusion, Ap^r	This study
pMMB67EHGent	pMMB67EHGent, Gm^r	(Kulesekara et al. 2006; Christen et al. 2010)
pMMB67EHGent-CFP	pMMB67EHGent::*mCYPet*, Gm^r	(Christen et al. 2010)
pMMB67EHGent-YFP	pMMB67EHGent::*mYPet*, Gm^r	(Christen et al. 2010)
pMMB67EHGent-Spy	pMMB67EHGent::*mYPet_synthSpy_mCYPet,* Gm^r	(Christen et al. 2010)
pMMB67EHGent-$ycgR_{3937}$	pMMB67EHGent::*mYPet_* $ycgR_{3937}$*_mCYPet,* Gm^r	This study

[a]Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Sp^r, spectinomycin resistance.

Table 3 Primers used in this study

Primers	Sequences (5'-3')	Amplicon
flhDC-A-BamHI	GGTGGATCCTCGAAGCAGGTATAATG	*flhDC* deletion
flhDC-B	GGCAAGCTTTTTCGTCATTTATTAATCG	
flhDC-C	GACAAGCTTCAGCCACCCTCCAGAGCAG	
flhDC-D-XhoI	CAACTCGAGGCAAGCCATCCCCCATCAG	
fliA-A-BamHI	GGGGGATCCTCAAAAAAGTTGCTTTGT	*fliA* deletion
fliA-B	ACAGAATTCGCACCAGGGGAACATAGC	
fliA-C	GGGGAATTCGGAAGAACTGAACCTAAAGG	
fliA-D-XhoI	TTTCTCGAGTCGGCATCGGCTTTGAG	
ycgR$_{3937}$-A-XhoI	AATACTCGAGACCCATAAAGGCGGCATTTT	*ycgR*$_{3937}$ deletion
ycgR$_{3937}$-B	GAAGCAGCTCCAGCCTACACCATTTTATTACGCCTGGCGT	
ycgR$_{3937}$-C	CTAAGGAGGATATTCATATGGGTGATCACCGACGTGGAAT	
ycgR$_{3937}$-D-NotI	AATATTATGCGGCCGCTGGCTTTCTGGGCATAAGTA	
ycgR$_{3937}$-R124D-1	GCAGGCGAGTTGATATCGAAAAAGTTACGGCGCTGG	*ycgR*$_{3937}$
ycgR$_{3937}$-R124D-2	CCAGCGCCGTAACTTTTTCGATATCAACTCGCCTGC	site-directed
		mutant
bcsA$_{3937}$-A-XhoI	ATAATACTCGAGGACGGATAACCGCCGTGCAA	*bcsA*$_{3937}$ deletion
bcsA$_{3937}$-B	GAAGCAGCTCCAGCCTACACATGCAGGGTTTCCGTGCCC	
bcsA$_{3937}$-C	CTAAGGAGGATATTCATATGCGTCGATATTCCGCTGGCCC	
bcsA$_{3937}$-D-NotI	AATATTATGCGGCCGCCAGCCAGACGCTGCTGGACA	
ycgR$_{3937}$-for-NdeI	ATATACATATGATGACGGTGGGGATGGAT	*ycgR*$_{3937}$
ycgR$_{3937}$-rev-EcoRI	AATTAGAATTCATGCGCAGCCGTTTGCGCTTTT	overexpression
		and purification
ycgR$_{3937}$-for-SpeI	GCGCACTAGTATGGATGTAGTGGATAACAATATGAAAGAGC	*ycgR*$_{3937}$ clone into
ycgR$_{3937}$-rev-KpnI	AGTAC	pMMB67EHGent
	ATTAGGTACCGCGCAGCCGTTTGCGCTTTT	
flhDC-for-XbaI	GGCGTCTAGATAAGCAGCTGTGGTGTTTTT	*flhDC*
flhDC-rev-HindIII	ATTTAAGCTTTTGATCGCTTTGCCGTTGTT	complementation
fliA-for-XbaI	GGAATCTAGAAAATTGGCTGAGCAACAGGA	*fliA*
fliA-rev-HindIII	CCGTAAGCTTGATATCGAAATAATTGGCGT	complementation
ecpC-for-XbaI	ATACTCTAGAAAGCATATCCTTCAATGGCG	*ecpC* expression
ecpC-rev-HindIII	ATACTAAGCTTCAACAAAGCAGGCATAGCAG	
rsmB-for-XbaI	AGGGTCTAGATTGACGATCTGGAATGCACG	*rsmB* expression
rsmB-rev-HindIII	TTTTAAGCTTAGGCTGCCATAACGGGCTCG	
ycgR$_{3937}$-p1-BamHI	TTTGGATCCCTTTGCTACCGTGCGTC	*ycgR*$_{3937}$ promoter
ycgR$_{3937}$-p2-EcoRI	GCTGAATTCCTCAAGGATCTTGCTGA	
ecpC-p1-BamHI	TTATAGGATCCTTAGAATTGGGCGGCACCGG	*ecpC* promoter
ecpC-p2-EcoRI	AATAGAATTCGTGCCTCCCCGGTGATGGAG	
rplU-for-qRT	GCGGCAAAATCAAGGCTGAAGTCG	qRT-PCR analysis
rplU-rev-qRT	CGGTGGCCAGCCTGCTTACGGTAG	
rpoN-for-qRT	ACTGGCGCTGGAAAGCAACC	qRT-PCR analysis
rpoN-rev-qRT	GGCAGCTCGTCGGGCATATC	

hrpL-for-qRT	GATGATGCTGCTGGATGCCGATGT	qRT-PCR analysis
hrpL-rev-qRT	TGCATCAACAGCCTGGCGGAGATA	
rsmB-for-Northern	CGCGATTTTTGTACGGCTAT	Northern blotting
rsmB-rev-Northern	CGATTTCTCGGTTCCCTCTT	analysis

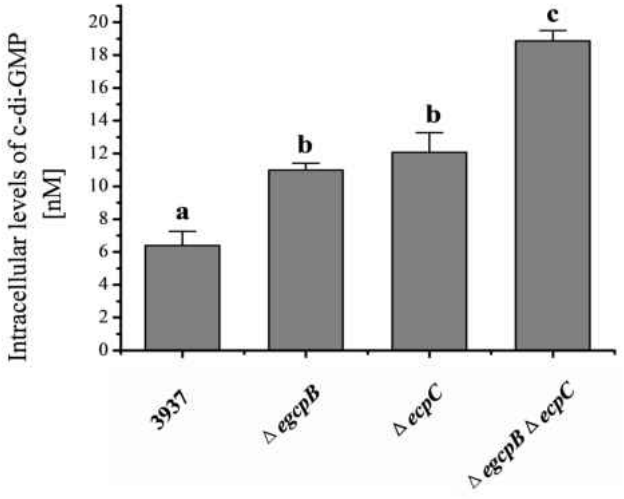

FIG 1 Measurement of intracellular levels of c-di-GMP in wild-type *Dickeya dadantii*, Δ*egcpB*, Δ*ecpC*, and Δ*egcpB*Δ*ecpC*. Assays were performed as described in Materials and Methods. Error bars indicate standard errors of the means. Different lowercase letters above the bar indicate statistically significant differences between treatments ($P<0.05$ by Student's *t* test).

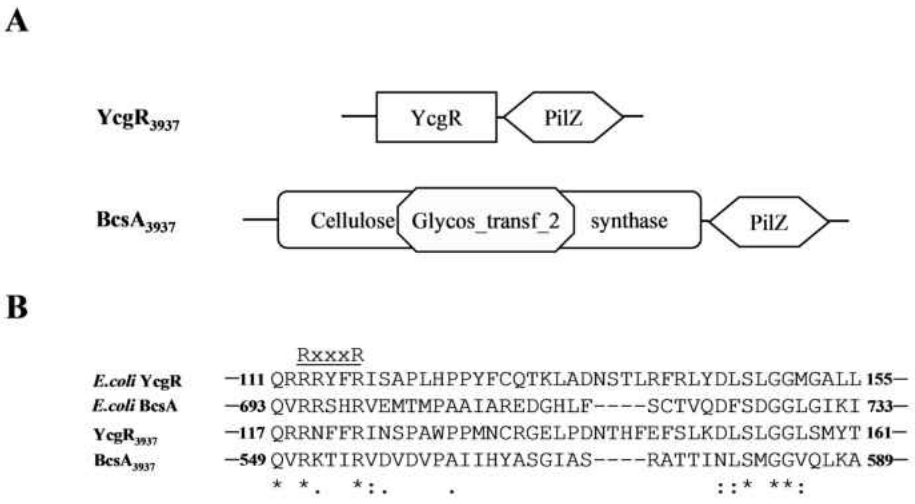

FIG 2 Analysis of PilZ-domain proteins. (A) PilZ-domain proteins $YcgR_{3937}$ and $BcsA_{3937}$ in *Dickeya dadantii* 3937. Protein domains were predicted by the simplified modular architecture research tool (SMART). (**B**) Amino acid sequence alignment for the PilZ domains in *E. coli* and *D. dadantii*. c-di-GMP binding motif RxxxR is marked. "*" means that the residues are identical in all sequences in the alignment, ":" means that conserved substitutions have been observed, "." Means that semi-conserved substitutions are observed.

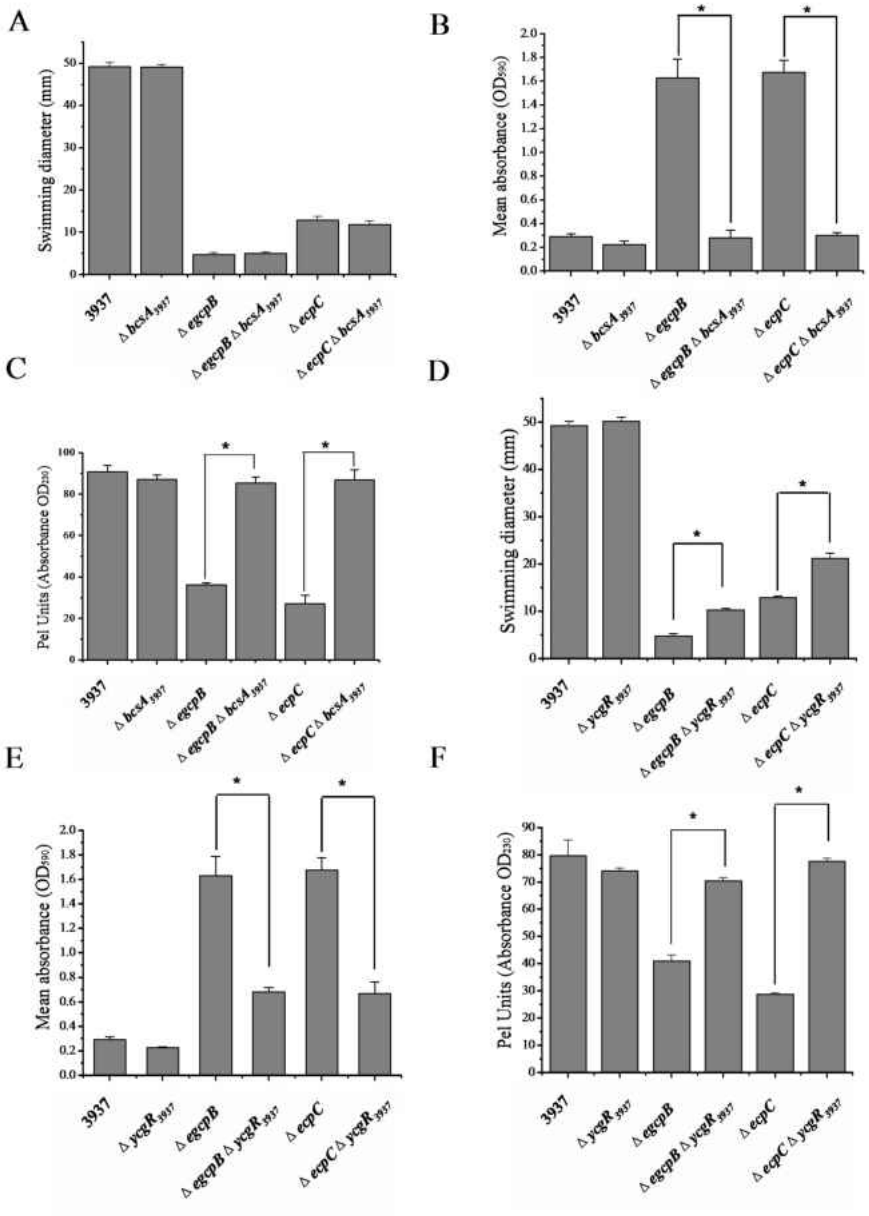

FIG 3 The impact of mutation of *bcsA*$_{3937}$ and *ycgR*$_{3937}$ on various virulence phenotypes were examined. Bacterial swimming motility (A), biofilm formation (B) and pectate lyase production (C) were measured in the parental strain *D. dadantii* 3937, Δ*bcsA*$_{3937}$, Δ*egcpB*, Δ*egcpB*Δ*bcsA*$_{3937}$, Δ*ecpC*, and Δ*ecpC*Δ*bcsA*$_{3937}$, respectively. The same assays were also tested in the parental strain 3937, Δ*ycgR*$_{3937}$, Δ*egcpB*, Δ*egcpB*Δ*ycgR*$_{3937}$, Δ*ecpC*, and Δ*ecpC*Δ*ycgR*$_{3937}$ (D-F). Assays were performed as described in Materials and Methods. The experiments were repeated three independent times with similar results. The figure represents results from one experiment which includes three to five technical replicates. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (P<0.05 by Student's t test).

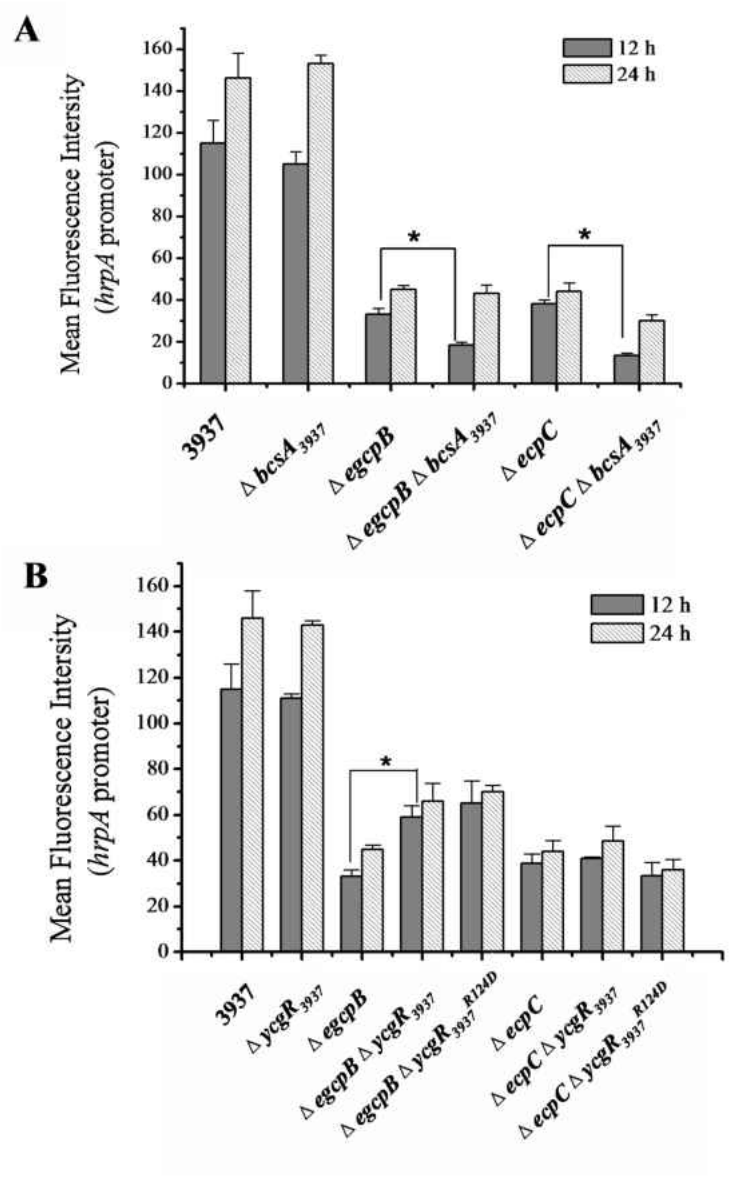

FIG 4 The impact of mutation of $bcsA_{3937}$ and $ycgR_{3937}$ on *hrpA* promoter activity was examined. (A) The *hrpA* promoter activity was measured in the parental strain *D. dadantii* 3937, Δ$bcsA_{3937}$, Δ*egcpB*, Δ*egcpB*Δ$bcsA_{3937}$, Δ*ecpC*, and Δ*ecpC*Δ$bcsA_{3937}$, respectively. Cells cultured under T3SS-inducing condition were used to measure the mean fluorescence intensity (MFI) by flow cytometry. The same assays were performed in the parental strain 3937, Δ$ycgR_{3937}$, Δ*egcpB*, Δ*egcpB*Δ$ycgR_{3937}$, Δ*egcpB* $ycgR_{3937}^{R124D}$, Δ*ecpC*, Δ*ecpC*Δ$ycgR_{3937}$, and Δ*ecpC* $ycgR_{3937}^{R124D}$ (B). The experiments were repeated three independent times with similar results. The figure represents results from one experiment which includes three technical replicates. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (P<0.05 by Student's t test).

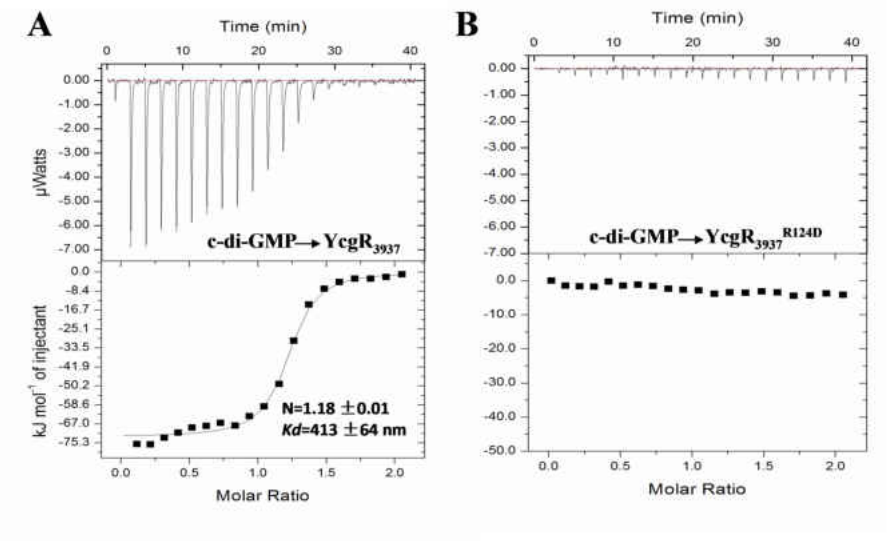

FIG 5 Isothermal titration calorimetric analysis of c-di-GMP binding to wild-type $YcgR_{3937}$ (A) or the point mutation version $YcgR_{3937}^{R124D}$ (B). Calorimetric titration for c-di-GMP (500 μM) titrated into test proteins (50 μM) is shown. Derived values for K_d and stoichiometry (N) are shown.

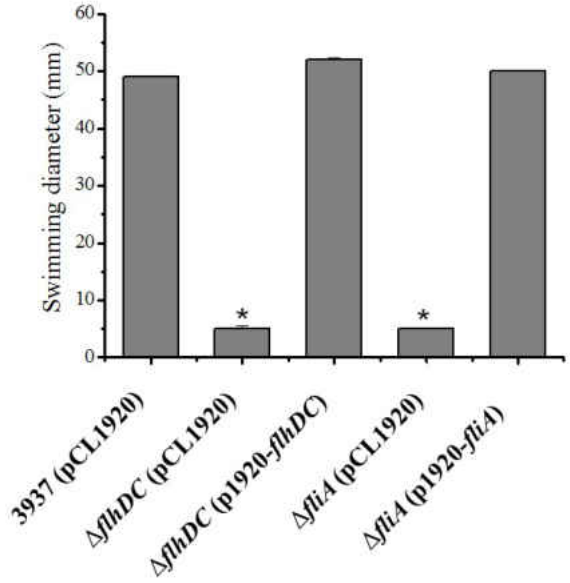

FIG 6 Swimming motility was measured in *D. dadantii*. All results are shown from one representative experiment, three independent experiments were performed and three replicates were used for each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ($P<0.05$ by Student's *t* test).

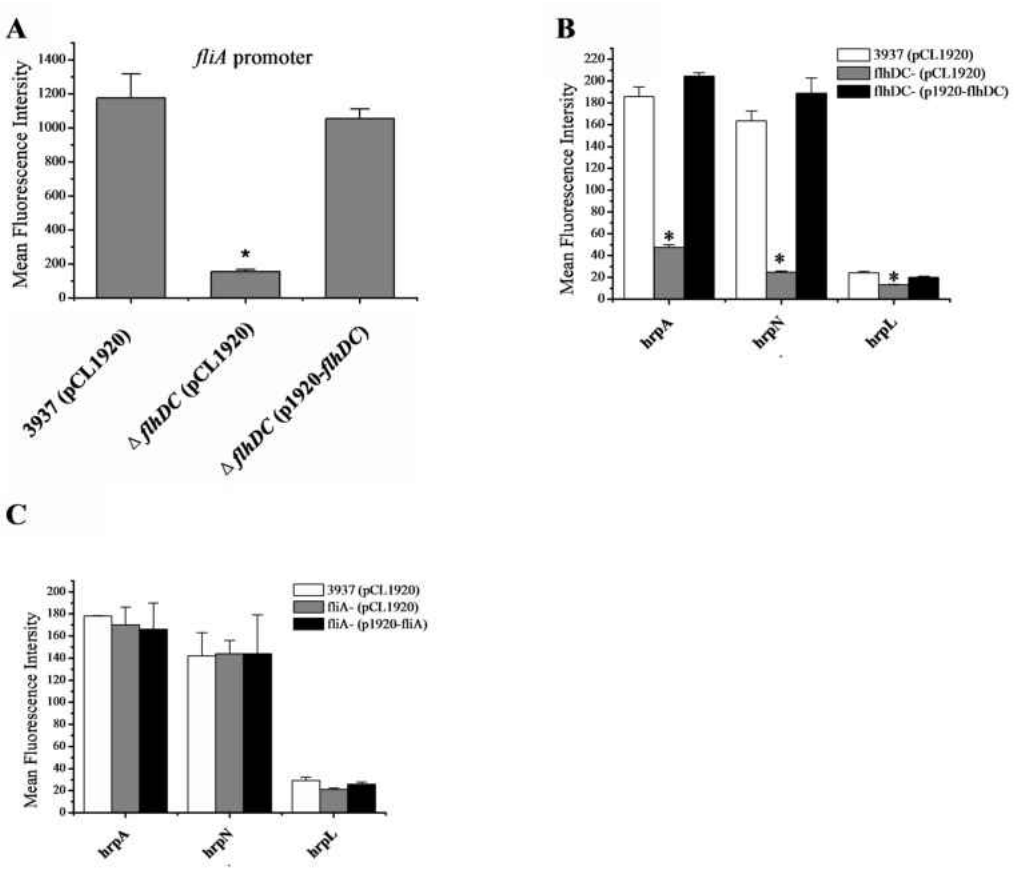

FIG 7 The impact of mutation of *flhDC* and *fliA* on the T3SS gene expression in *D. dadantii* 3937 was examined. (**A**) Promoter activity of *fliA* was measured using plasmid pAT-*fliA* in wild-type strain *D. dadantii* harboring empty vector pCL1920, the Δ*flhDC* harboring empty vector pCL1920, and Δ*flhDC* harboring pCL-*flhDC*. (**B** and **C**) Promoter activity of T3SS regulon genes *hrpA*, *hrpN*, and *hrpL* was measured in the *D. dadantii* 3937 harboring empty vector pCL1920, the Δ*flhDC* harboring empty vector pCL1920, the Δ*fliA* harboring empty vector pCL1920, and their complemented strains using reporter plasmids pAT-*hrpA*, pAT-*hrpN* and pAT-*hrpL*, respectively. Three independent experiments were performed and three replicates were used in each experiment. Values are a representative of three experiments. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (P<0.05 by Student's t test).

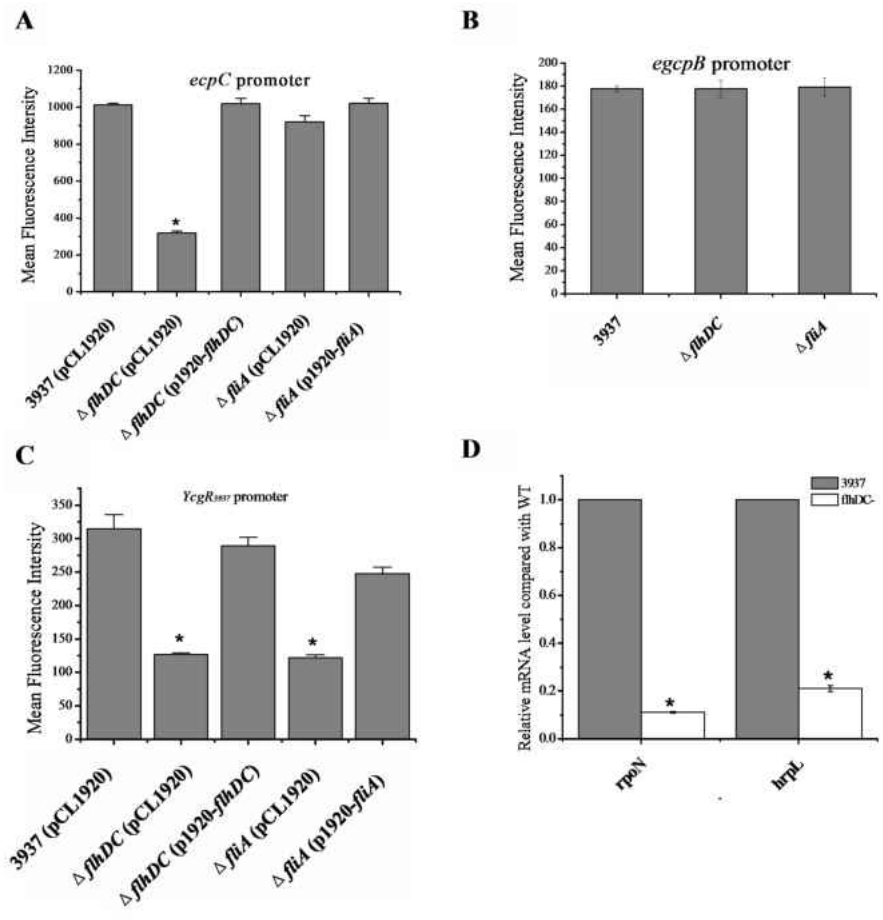

FIG 8 FlhDC, independently of FliA, regulates the T3SS master regulator HrpL at transcriptional level through EcpC-RpoN-HrpL pathway in *D. dadantii* 3937. But FlhDC positively regulates transcription of $ycgR_{3937}$ through FliA. (**A**) Promoter activity of *ecpC* was measured in the wild-type *D. dadantii*, the *flhDC* and *fliA* mutant strains, and the *flhDC* and *fliA* complemented strains, respectively. (**B**) Promoter activity of *egcpB* was measured in wild-type *D. dadantii*, Δ*flhDC*, and Δ*fliA* strains. (**C**) Promoter activity of $ycgR_{3937}$ was measured in wild-type *D. dadantii* harboring empty vector pCL1920, the Δ*flhDC* harboring empty vector pCL1920, the Δ*fliA* harboring empty vector pCL1920, and their complemented strains. (**D**) Relative mRNA levels of *hrpL* and *rpoN* were examined using quantitative real time RT-PCR in the wild-type *D. dadantii* and the Δ*flhDC*. Values are a representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ($P<0.05$ by Student's *t* test).

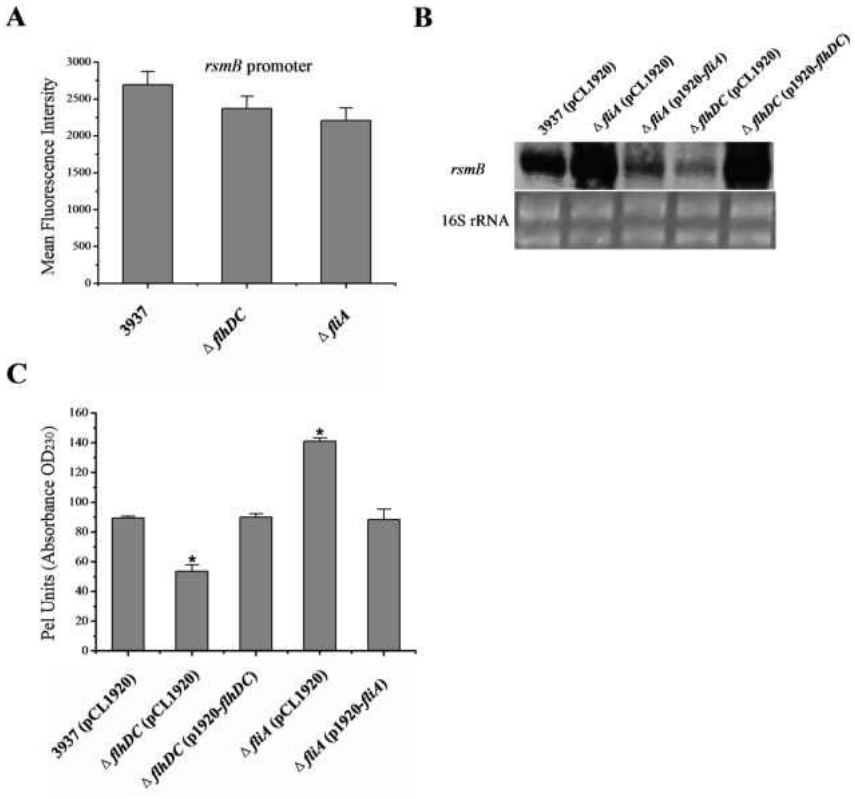

FIG 9 FlhDC and FliA inversely regulate RsmB at a post-transcriptional level. (A) Promoter activity of *rsmB* was measured in the wild-type *D. dadantii*, the *flhDC* mutant and the *fliA* mutant strains. (**B**) Northern blot analysis of *rsmB* mRNA in the wild-type *D. dadantii* harboring empty vector pCL1920, Δ*fliA* harboring empty vector pCL1920, Δ*fliA* harboring plasmid pCL1920-*fliA*, Δ*flhDC* harboring empty pCL1920, and Δ*flhDC* harboring pCL1920-*flhDC*. 16S rRNA was used as RNA loading control. (**C**) Pectate lyase production assay was performed in in the wild-type *D. dadantii* harboring empty vector pCL1920, Δ*fliA* harboring empty vector pCL1920, Δ*fliA* harboring plasmid pCL1920-*fliA*, Δ*flhDC* harboring empty pCL1920, and Δ*flhDC* harboring pCL1920-*flhDC*. Values are a representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ($P<0.05$ by Student's *t* test).

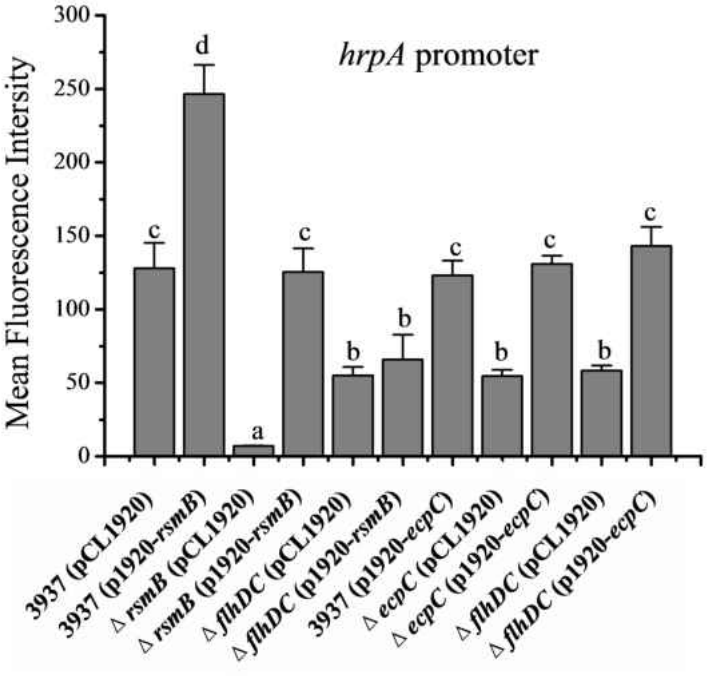

FIG 10 Promoter activity of *hrpA* in different *D. dadantii* 3937 strains was examined. Values are a representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. Different lowercase letters above the bar indicate statistically significant differences between treatments ($P<0.05$ by Student's *t* test).

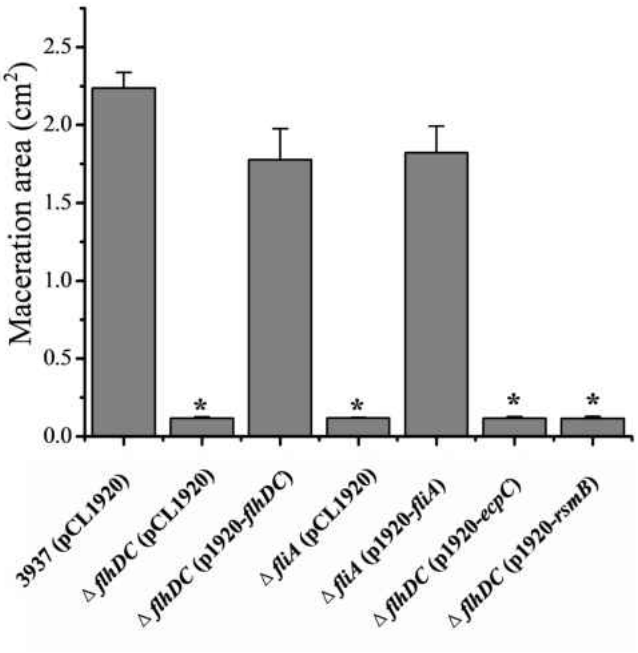

FIG 11 FlhDC and FliA positively regulate the virulence of *D. dadantii* 3937 on Chinese cabbage (*Brassica campestris*). Bacterial cells of the wild-type *D. dadantii* harboring empty vector pCL1920, Δ*fliA* harboring empty vector pCL1920, Δ*fliA* harboring plasmid pCL1920-*fliA*, Δ*flhDC* harboring empty pCL1920, Δ*flhDC* harboring pCL1920-*flhDC*, Δ*flhDC* harboring pCL1920-*ecpC*, and Δ*flhDC* harboring pCL1920-*rsmB* strains were inoculated in the leaves of Chinese cabbage. The maceration symptom was measured 24 hours post-inoculation. Maceration assays were performed as described in Materials and Methods. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (P<0.05 by Student's t test).

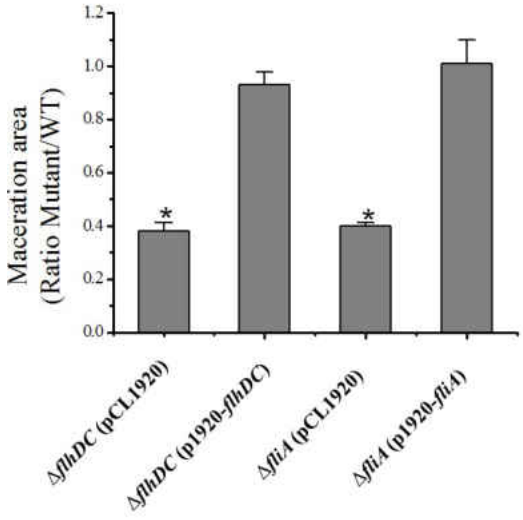

FIG 12 Measurement of *D. dadantii* virulence to African violet (*Saintpaulia ionantha*). Bacterial cells of the wild-type *D. dadantii*, *flhDC* and *fliA* mutant strains, and complemented strains were inoculated in the leaves of African violet. The maceration symptom was measured 2 days post-inoculation. Maceration assays were performed as described in Materials and Methods. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ($P<0.05$ by Student's *t* test).

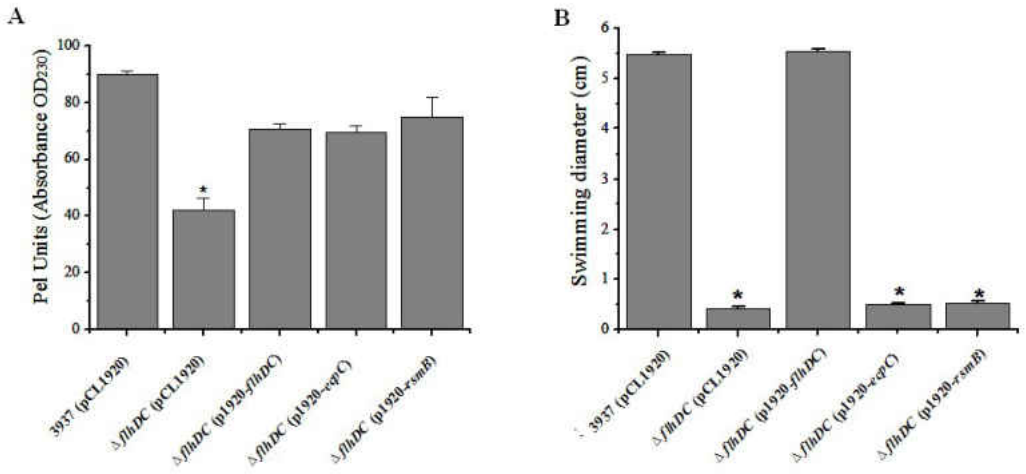

FIG 13 Pectate lyase production and swimming motility were measured in the parental strain *D. dadantii* 3937 harboring empty vector pCL1920, Δ*flhDC* harboring empty pCL1920, Δ*flhDC* harboring pCL1920-*flhDC*, Δ*flhDC* harboring pCL1920-*ecpC*, and Δ*flhDC* harboring pCL1920-*rsmB*, respectively Assays were performed as described in Materials and Methods. The experiments were repeated three independent times with similar results. The figure represents data from one experiment which includes three to five technical replicates. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ($P<0.05$ by Student's *t* test).

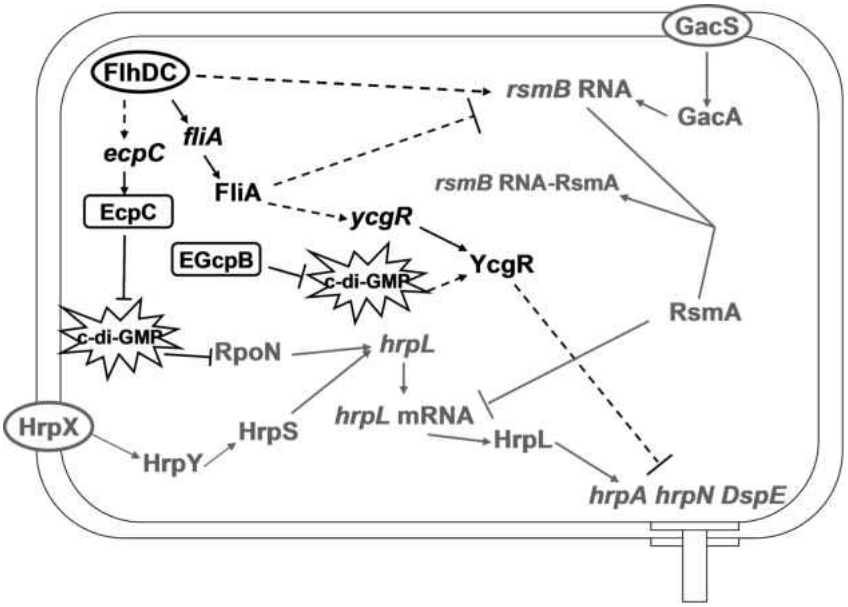

FIG 14 Model for the type III secretion system (T3SS) regulatory network in *D. dadantii* 3937. The *D. dadantii* 3937 T3SS is regulated by the HrpX/HrpY-HrpS-HrpL and the GacS/GacA-*rsmB*-RsmA-HrpL pathways. In this study, the flagellar master regulator FlhDC was observed to hierarchically regulate the expression of T3SS encoding genes. (1) FlhDC positively regulates the PilZ domain protein encoding gene $ycgR_{3937}$ at transcriptional level through a sigma factor FliA. Under high c-di-GMP levels (Δ*egcpB*), $YcgR_{3937}$ binds c-di-GMP, which negatively regulates the T3SS. (2) FlhDC controls the expression of phosphodiesterase encoding gene *ecpC*. EcpC degrades intracellular c-di-GMP, which counteracts the negative impact of c-di-GMP on the RpoN, which is required for the transcription of *hrpL*. (3) FlhDC and FliA divergently regulate the regulatory small RNA RsmB at the post-transcriptional level. ⊥represents negative control; → represents positive control. The dotted lines indicate regulatory mechanisms identified in this study.

Chapter 3

Study of GGDEF and EAL domain proteins

in c-di-GMP signaling in *D. dadantii* 3937

ABSTRACT

In *Dickeya dadantii* 3937, a bacterial second messenger cyclic diguanylate monophosphate (c-di-GMP) regulates several cellular behaviors, including biofilm formation, swimming and swarming motility, Type III secretion system (T3SS) gene expression, and pectate lyase production. The modulation of c-di-GMP is achieved by two types of enzymes, which include the GGDEF-domain-containing diguanylate cyclases (DGCs) and the EAL- or the HD-GYP-domain-containing phosphodiesterases (PDEs). In *D. dadantii* 3937, there are 12 GGDEF-domain-containing proteins (Gcp), 4 EAL-domain-containing proteins (Ecp), and 2 EAL-GGDEF-dual-domain-containing proteins (EGcp). However, the redundancy of these proteins and their individual functions in the c-di-GMP signaling remain unknown. In this study, we investigated the phenotypes of various cellular behaviors using eighteen single-deletion mutants, in which each GGDEF and/or EAL domain protein coding gene was individually either deleted or inactivated. Our results showed that GcpA negatively regulates swimming motility, pectate lyase production, and T3SS gene expression. Interestingly, GcpD and GcpL only negatively regulate the expression of T3SS and swimming motility but not the pectate lyase production. These results suggest that different GGDEF and EAL domain proteins can regulate different cellular processes.

INTRODUCTION

Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) was first reported in *Gluconacetobacter xylinus* as an allosteric regulator of cellulose synthesis (Ross et al. 1987). This molecule is now recognized as a bacterial second messenger that regulates diverse

cellular functions involving motility, biofilm formation, virulence against animal and plant targets, and cell cycle progression (Cotter and Stibitz 2007; Hengge 2009; Schirmer and Jenal 2009; Römling 2012). The synthesis of c-di-GMP is modulated by diguanylate cyclases (DGCs) that have a GGDEF domain, which converts two GTP molecules to c-di-GMP (Paul et al. 2004; Solano et al. 2009). Phosphodiesterases (PDEs) contain either an EAL or HD-GYP domain that break down c-di-GMP into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) or GMP, respectively (Schmidt et al. 2005; Tamayo et al. 2005; Ryan et al. 2006). Studies in many Gram-negative bacteria revealed that genes encoding GGDEF and/or EAL domains are widely distributed in their genomes. For example, the *Escherichia coli* K-12 strain contains 29 GGDEF and/or EAL domain encoding genes, whereas *Vibrio cholerae* has 53 (Waters et al. 2008; Povolotsky and Hengge 2012). Although the reason for this gene redundancy remains unknown, individual DGC or PDE may sense different environmental signals to modulate the intracellular c-di-GMP level, since many of them contain N-terminal signaling domains (PAS, GAF, CHASE and REC) that are associated with their GGDEF, EAL, or HD-GYP domains (Hengge 2009). The sophisticated c-di-GMP-mediated signaling network includes transcriptional, post-transcriptional, and posttranslational regulation. In order to exert such diverse influences, c-di-GMP has a range of cellular effectors, such as PilZ domain proteins, transcription factors, enzymatically inactive GGDEF, EAL or HD-GYP domain proteins and RNA riboswitches (Hengge 2009; Breaker 2011; Ryan et al. 2012). In *Dickeya dadantii* 3937, two PilZ domain proteins, $YcgR_{3937}$ and $BcsA_{3937}$, were reported to regulate several cellular behaviors and virulence (Yuan et al. 2015).

EcpC is one of the EAL-domain-containing-proteins that exhibit PDE activity in *D.*

dadantii 3937. It is involved in the regulation of biofilm formation, swimming and swarming motility, pectate lyase production, and T3SS gene expression (Yi et al. 2010). EGcpA (former name CsrD) and EGcpB (former name EcpB) are two EAL-and-GGDEF-dual-domain-containing proteins. Deletion of *egcpB* increased intracellular c-di-GMP level and displayed similar phenotypes as the Δ*ecpC*, suggesting that EGcpB displays PDE activity (Yi et al. 2010; Yuan et al. 2015). Although EGcpA exhibits PDE activity, its regulatory effects on *D. dadantii* are mainly via the global post-translational regulator RsmA (Wu et al. 2014). EGcpA positively controls sRNA RsmB expression at the transcriptional level, whereas it negatively regulates RsmB at the post-transcriptional level through the osmoregulated periplasmic glucan synthesis proteins OpgGH (Wu et al. 2014). RsmB binds to RsmA that sequesters RsmA activity (Liu et al. 1998).

In the present study, we constructed a panel of strains, each with a deletion or inactivation of one of the eighteen genes that encode GGDEF and/or EAL domains in *D. dadantii* (Δ*egcpA*, Δ*egcpB*, Δ*gcpB*, Δ*gcpC*, Δ*gcpD*, Δ*gcpF*, Δ*ecpA*, and Δ*ecpC* were previously constructed by Xuan Yi). The effects of these mutations on diverse cellular behaviors were investigated. GcpD and GcpL influenced T3SS and swimming motility, respectively. GcpA regulated swimming motility, pectate lyase production, and T3SS gene expression.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, primers, and media

The bacterial strains and plasmids used in this study are listed in Table 1. *D. dadantii* strains

were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl), mannitol-glutamic acid (MG) medium (1% mannitol, 0.2% glutamic acid, 0.05% potassium phosphate monobasic, 0.02% NaCl, and 0.02% MgSO4) or low-nutrient T3SS inducing MM at 28°C (Yang et al. 2007; Yang et al. 2008). *Escherichia coli* strains were grown in LB at 37°C. Antibiotics were added to the media at the following concentrations: ampicillin (100 μg/ml), kanamycin (50 μg/ml), gentamicin (10 μg/ml), chloramphenicol (20 μg/ml), tetracycline (12 μg/ml) and spectinomycin (100 μg/ml). The *D. dadantii* 3937 genome sequence can be retrieved from ASAP (https://asap.ahabs.wisc.edu/asap/home.php). Primers used for PCR in this study are listed in Table 1.

Mutant construction and complementation

The GGDEF and/or EAL domain encoding genes were deleted from the genome by marker exchange mutagenesis (Yang et al. 2002). Briefly, two fragments flanking each target gene were amplified by PCR with specific primers (Table 1). The kanamycin cassette was amplified from pKD4 (Datsenko and Wanner 2000) and was cloned between two flanking regions using three-way cross-over PCR. The PCR construct was inserted into the suicide plasmid pWM91, and the resulting plasmid was transformed into *D. dadantii* 3937 by conjugation using *E. coli* strain S17-1 λ-pir. To select strains with chromosomal deletions, recombinants, grown on kanamycin medium, were plated on 5% sucrose plate. Cells that were resistant to sucrose due to the loss of SacB-mediated toxicity were then plated on ampicillin plate, and the ampicillin sensitive cells were confirmed by polymerase chain reaction (PCR) using outside primers. Finally, the DNA fragment which contains two flanking regions and the

kanamycin cassette was confirmed by sequencing. To construct the site-specific point mutation in the GGDEF motif of the GcpA GGDEF domain, single nucleotide substitution was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Briefly, a primer set, *gcpA*-D418A-1 and *gcpA*-D418A-2 (Table 1), was used to generate $gcpA^{D418A}$, which changed the SGDEF motif to SGAEF. Substitution was confirmed by DNA sequencing.

To generate complemented strains, the promoter and ORF regions of target genes were amplified and cloned into low-copy-number plasmid pCL1920 or high-copy-number plasmid pML123 (Table 1). The resulting plasmids were then confirmed by PCR and electroporated into mutant cells.

Swimming motility assay

Swimming motility was tested by inoculating 10 μl of overnight bacterial cultures (OD_{600}=1.0) onto the center of MG plates containing 0.2% agar. The inoculated plates were incubated at 28°C for 20 h, and the diameter of the radial growth was measured (Antúnez-Lamas et al. 2009).

Detection of bacterial flagella

A droplet of overnight bacterial culture was placed onto carbon-stabilized Formvar supports on 200-mesh copper transmission electron microscopy (TEM) grids. The samples were then preserved by adding formaldehyde (final concentration, 2%). Specimens were imaged using a Hitachi H-9000NAR TEM operating at 80 to 100 kV.

Biofilm formation assay

Biofilm formation was determined by using a method that was previously described (Yi et al. 2010). In brief, bacterial cells grown overnight in LB media were inoculated 1:100 in MM media in 1.5 ml polypropylene tubes. After incubation at 28°C for 48 h, cells were stained with 1% crystal violet (CV) for 15 min. The planktonic cells were removed by several rinses with H_2O. The CV-stained bound cells were air dried for 1 h, then dissolved in 90% ethanol, and the OD_{590} of the solution was measured to quantify the biofilm formation.

GFP reporter plasmid construction and flow cytometry assay

To generate the reporter plasmids pAT-*ycgR*$_{3937}$ and pAT-*ecpC*, the promoter regions of *ycgR*$_{3937}$ and *ecpC* were PCR amplified and cloned into the promoter probe vector pPROBE-AT, which contains a ribosomal binding site upstream of the *gfp* gene (Miller et al. 2000; Leveau and Lindow 2001). The reporter plasmids pAT-*hrpA*, pAT-*hrpN*, pAT-*hrpL* and pAT-*rsmB* were constructed previously following the same procedure (Yang et al. 2007; Li et al. 2014). Promoter activity was monitored by measuring GFP intensity through flow cytometry (BD Biosciences, San Jose, CA) as previously described (Peng et al. 2006). Briefly, bacterial cells with reporter plasmid were grown in LB media overnight and inoculated 1:100 into MM media. Samples were collected at 12 h and 24 h, respectively, and promoter activity was analyzed by detecting GFP intensity using flow cytometry.

Pectate lyase activity assay

Extracellular Pel activity was measured by spectrometry as previously described (Matsumoto et al. 2003). Briefly, bacterial cells were grown in MM media supplemented with 20% glycerol and 1% polygalacturonic acid (PGA) at 28°C for 20 h. For extracellular pel activity, 1 ml bacterial cultures were centrifuged at 15000 rpm for 2 min, supernatant was then collected and 10 µl of the supernatant was added to 990 µl of the reaction buffer (0.05% PGA, 0.1 M Tris-HCl [pH 8.5], and 0.1 mM $CaCl_2$, prewarmed to 30°C). Pel activity was monitored at A_{230} for 3 min and calculated based on one unit of Pel activity being equal to an increase of 1×10^{-3} OD_{230} in 1 min.

Statistical analysis

Means and standard deviations of experimental results were calculated using Excel (Microsoft, Redmond, WA) and the statistical analysis was performed using a two-tailed student's t-test.

RESULTS

Identification of GGDEF and EAL domain containing proteins in *D. dadantii* 3937

In agreement with many other bacterial species showing that GGDEF and EAL domains coding genes are abundant in their genomes (Hengge 2009), we found, in *D. dadantii* 3937 genome, 12 GGDEF-domain-encoding genes, 4 EAL-domain-encoding genes, and 2 EAL-GGDEF-dual-domain-encoding genes by using the Pfam program. New names were then given to these genes as *gcp* (GGDEF-domain-containing protein encoding gene), *ecp* (EAL-domain-containing protein encoding gene) and *egcp* (EAL-GGDEF-domains-containing protein encoding gene) (Fig. 1). Further studies of the

encoded protein domain structure revealed that the N-terminal regions of certain proteins such as GcpA, EGcpB, and EcpD, contain either sensor domains or transmembrane domains that are associated with their GGDEF and/or EAL domains (Fig. 1). This suggests a temporal and/or spatial c-di-GMP specificity in *D. dadantii* 3937 (Hengge 2009). The GGDEF active-site (A-site) motif and EAL motif are crucial for their domain activities. To assess the significance of the identified domains, we performed amino acid sequence alignments between the reported GGDEF and EAL domains from *Caulobacter crescentus*, *Vibrio cholerae*, *Pseudomonas aeruginosa* and *D. dadantii* 3937 (Fig. 2). The results suggest that the majority of the GGDEF A-site motifs are enzymatically active, except for a degenerate YHSDF motif in the GGDEF domain of EGcpA. In addition, the essential glutamate residue in the EAL motifs is conserved in all of the *D. dadantii* EAL domains, suggesting potential phosphodiesterase activity among the EAL-domain-containing proteins.

Construction of GGDEF and EAL single deletion mutants

C-di-GMP signaling is involved in the regulation of diverse cellular behaviors and virulence in *D. dadantii* 3937 (Yi et al. 2010; Wu et al. 2014; Yuan et al. 2015). Previous study showed that two PDEs, EGcpB and EcpC, degrade c-di-GMP, resulting in positive effects on swimming motility, pectate lyase production and T3SS gene expression, while negative effects on biofilm formation (Yi et al. 2010). EGcpA, a homologue of *E. coli* CsrD, exhibits PDE activity but mainly regulates the sRNA RsmB level in order to control pectate lyase production and T3SS gene expression (Wu et al. 2014). Therefore, to further elucidate the c-di-GMP signaling network in *D. dadantii* 3937, we used allelic exchange mutagenesis to

construct the remaining *gcp* and *ecp* mutants. As a result, 14 new single-deletion mutants were constructed. Several attempts to delete *gcpA* were not successful, suggesting that *gcpA* may be essential for viability of *D. dadantii* 3937. We then constructed a $gcpA^{D418A}$ site-directed mutant, in which the essential aspartic acid in the GGDEF A-site motif was replaced by alanine, and is has no defectiveness in bacterial growth when compared with the wild-type strain (data not shown).

Biofilm formation in *D. dadantii* GGDEF and/or EAL single deletion mutants

Biofilm formation is crucial for the virulence of many pathogens, and the second messenger c-di-GMP is known to promote biofilm formation (Cotter and Stibitz 2007; Kuchma et al. 2007). To test which Gcp and Ecp are involved in regulating this cellular behavior, we measured the biofilm forming ability in all GGDEF and/or EAL single-deletion mutants. As shown in Figure 3A, both Δ*egcpB* and Δ*ecpC* strains displayed an over 2-fold increase in biofilm production compared with the wild type, which is consistent with their PDE activities (Yi et al. 2010). Δ*egcpA* showed an even higher biofilm production than the PDE mutants, which may be due to its additive effects on c-di-GMP and sRNA RsmB. By contrast, no other single-deletion mutants showed a detectable difference in biofilm formation compared with the wild type (Fig. 3A). Thus, we concluded that EGcpA, EGcpB and EcpC inhibit biofilm formation in *D. dadantii* 3937.

GcpA, GcpL, and EGcpA regulate swimming motility in *D. dadantii*

Next, we measured the bacterial swimming motility in single GGDEF and/or EAL

deletion mutants. In agreement with our previous data (Yi et al. 2010), we observed a dramatic reduction of swimming motility in both Δ*egcpB* and Δ*ecpC* (Fig. 3B). $gcpA^{D418A}$ A-site mutant and Δ*gcpL* showed increased swimming motilities when compared with the wild-type strain (Fig. 3B). Complementation assays were performed by expressing *gcpA* and *gcpL in trans* using high copy number plasmid pML123, which drastically reduced the swimming motility (Fig. 3C). Next, we used transmission electron microscopy (TEM) to observe the physical differences between the wild-type, $gcpA^{D418A}$ A-site mutant, and Δ*ecpC* strains. Interestingly, our results showed that $gcpA^{D418A}$ A-site mutant produced much more flagella than the wild type (Fig. 4). In addition, reduced flagellar numbers were observed in Δ*ecpC* compared to the wild-type strain. Thus, these results indicated that GcpA and EcpC may play a role in regulation of flagellar number and this may impact swimming motility in *D. dadantii*.

Deletion of *egcpA* in *D. dadantii* increased swimming motility (Fig. 3B), suggesting that the PDE activity of EGcpA is not a major player in this regulation (Wu et al. 2014). In addition, our previous work demonstrated that EGcpA represses RsmB at post-transcriptional level (Wu et al. 2014). Therefore, we hypothesized that EGcpA repressed swimming motility through its negative effects on sRNA RsmB. To confirm this, we compared swimming motility in wild type, Δ*rsmB*, and Δ*egcpA*. Our results showed that deletion of *rsmB* reduced swimming motility (Fig. 5), suggesting EGcpA represses RsmB at the post-transcriptional level to regulate swimming motility in *D. dadantii* 3937.

GcpA inhibits pectate lyase production in *D. dadantii* 3937

The ability to produce a variety of polysaccharidases and polysaccharide lyases enables *D. dadantii* 3937 to degrade the plant cell wall (Collmer and Keen 1986). To investigate which DGC and PDE are potentially involved in regulating pectate lyase production, we measured the activity of pectate lyase in each GGDEF and/or EAL deletion mutants. *egcpB* and *ecpC* deletion mutants showed reduced pectate lyase activity, and Δ*egcpA* showed increased pectate lyase activity, which is consistent with previous results (Yi et al. 2010; Wu et al. 2014) (Fig. 6A). In addition, $gcpA^{D418A}$ A-site mutant showed increased pectate lyase activity compared with the wild-type strain (Fig. 6A). Expression of *gcpA* from the plasmid pCL1920 in the $gcpA^{D418A}$ A-site mutant was able to restore the wild-type phenotype (Fig. 6B). Next, to elucidate at which level GcpA regulated pectate lyase production, we checked the promoter activity of *pelD*, one of the major pectate lyase production genes, in wild type and $gcpA^{D418A}$ A-site mutant strains. Our results showed that the promoter activity of *pelD* was 10-fold higher in $gcpA^{D418A}$ A-site mutant than in the wild type at 12 h (Fig. 6C), suggesting that GcpA transcriptionally regulated the expression of *pel* genes in order to control the pectate lyase production. Further studies were performed on the effects of GcpA on several pectate lyase regulators, revealing that only the promoter activity of *fur* and the protein levels of RsmA were reduced in $gcpA^{D418A}$ A-site mutant compared with the wild type (Fig. 6D and 6E). This indicated that the regulatory effect of GcpA on pectate lyase production may be through two *pel* repressors, Fur and RsmA. In summary, GGDEF and EAL dual domain proteins EGcpA and EGcpB, EAL domain protein EcpC, and GGDEF domain protein GcpA are involved in pectate lyase production in *D. dadantii*.

GcpA and GcpD regulate T3SS gene expression in *D. dadantii* 3937

D. dadantii utilizes the T3SS to invade plants by directly translocating virulence factors into the plant cell (Bauer et al. 1994; Yang et al. 2002). C-di-GMP inhibits the expression of T3SS genes in *D. dadantii* 3937 (Yi et al. 2010). To investigate which DGC and PDE affect T3SS gene expression, we examined the promoter activities of *hrpA*, a T3SS regulon gene, in wild-type and in each GGDEF and/or EAL deletion mutant strain. In agreement with our previous results, deletion of *egcpB* and *ecpC* reduced *hrpA* promoter activity, while deletion of *egcpA* increased it (Fig. 7A) (Yi et al. 2010; Wu et al. 2014). Additionally, the $gcpA^{D418A}$ A-site mutant and Δ*gcpD* showed increased *hrpA* promoter activities compared with the wild type (Fig. 7A). Plasmid complementation of *gcpA* and *gcpD* restored the wild-type phenotype (Fig. 7B). Since HrpL is the master regulator for T3SS and c-di-GMP was reported to transcriptionally regulate *hrpL* via RpoN, we compared the promoter activity of *hrpL* between wild type, $gcpA^{D418A}$ A-site mutant and Δ*gcpD*. As shown in Fig. 7C, the promoter activity of *hrpL* in Δ*gcpD* was higher than in the wild type, and the promoter activity in $gcpA^{D418A}$ A-site mutant was similar to that in the wild type. Therefore, this result suggested that although GcpA and GcpD regulate T3SS gene expression, their regulatory mechanisms are different. GcpD, but not the GcpA, may synthesize c-di-GMP to negatively regulate *hrpL* transcription via RpoN.

DISCUSSION

The bacterial second messenger c-di-GMP has a pleiotropic role in regulating multiple cellular behaviors; however, how this small molecule triggers one cellular output but not

another is still unclear. To understand the c-di-GMP signaling specificity in *D. dadantii* 3937, we investigated the function of each GGDEF and/or EAL domain protein in the regulation of four cellular outputs including biofilm formation, swimming motility, pectate lyase production, and T3SS gene expression. In agreement with previous results (Yi et al. 2010), deletion of two PDE-encoding genes, *egcpB* or *ecpC*, increased biofilm formation but repressed swimming motility, pectate lyase production, and T3SS gene *hrpA* promoter activity (Fig. 3A, 3B, 6A and 7A). Although EGcpA was reported to exhibit PDE activity, it controls pectate lyase production and T3SS gene expression mainly through its regulation on the small regulatory RNA RsmB (Wu et al. 2014). In this study, we observed enhanced biofilm and swimming motility when *egcpA* was deleted (Fig. 3A and 3B), suggesting that this protein is more crucial in regulating diverse *D. dadantii* behaviors than it has been reported previously (Wu et al. 2014). Further studies showed that EGcpA may control swimming motility through its negative regulation on RsmB, since deletion of *rsmB* resulted in attenuated swimming motility in *D. dadantii* (Fig. 5). The homolog of *D. dadantii* Rsm system has also been shown to be involved in swimming motility in *E. coli*, *Pectobacterium wasabiae* and *Serratia* sp. (Kõiv et al. 2013; Wilf et al. 2013; Yakhnin et al. 2013).

In contrast to the above mentioned GGDEF and/or EAL domain proteins that were involved in multiple cellular behaviors, GcpL and GcpD were observed to regulate swimming motility and T3SS gene expression, respectively. Our results showed that GcpL negatively regulated swimming motility, which is consistent with its predicted DGC activity (Fig. 3B). However, deletion of *gcpL* had no impact on biofilm formation, pectate lyase production, and T3SS gene expression (Fig. 3A, 6A and 7A). GcpD repressed T3SS gene *hrpA* promoter

activity, but was not involved in other cellular behaviors (Fig. 7A). Since c-di-GMP was known to control the T3SS master regulator HrpL via RpoN, and RpoN activates *hrpL* expression (Yi et al. 2010), we tested *hrpL* promoter activity in wild-type and Δ*gcpD* strains. As shown in Fig. 7C, the *hrpL* promoter activity was increased in Δ*gcpD* when compared with the wild type, suggesting that GcpD may regulate T3SS through RpoN.

The GGDEF A-site motif of GcpA (SGDEF) was predicted to be essential for its DGC activity (Fig. 2). Site-directed mutagenesis of the aspartic acid to alanine in GcpA A-site ($GcpA^{D418A}$) increased swimming motility, pectate lyase production, and T3SS gene *hrpA* promoter activity when compared with the wild-type strain (Fig. 3B, 6A and 7A), suggesting that GcpA is an active DGC and its A-site motif is involved in c-di-GMP formation in *D. dadantii* 3937. We performed several attempts to delete *gcpA* in the wild-type background without success, which suggests multiple function of GcpA in *D. dadantii*, independent to its DGC activity. C-di-GMP represses swimming motility through a PilZ domain protein YcgR which affects the activity of flagellar rotation subunit FliG (Ryjenkov et al. 2006). A recent study in *Salmonella* demonstrated that cellulose negatively regulates swimming motility under high c-di-GMP conditions, and this regulation is coordinated with YcgR (Zorraquino et al. 2013). Here, we observed increased number of flagella in $gcpA^{D418A}$ and reduced flagella number in Δ*ecpC* when compared with the wild type. To our knowledge, this is the first report that connected c-di-GMP signaling to regulation of flagellar number in bacteria, suggesting that c-di-GMP in *D. dadantii* may modulate the production of flagella, in collaboration with flagellar rotation, for complete motility regulation. GcpA repressed pectate lyase production at the transcriptional level, and this regulation may partially depend on Fur and RsmA.

Intriguingly, different from GcpD, GcpA may repress T3SS gene expression via an unidentified pathway that is independent of RpoN, since deletion of *gcpA* had no influence on *hrpL* promoter activity (Fig. 7C).

In conclusion, we uncovered several GGDEF and/or EAL domain proteins that specifically or generally regulate diverse cellular behaviors in *D. dadantii*. To fully appreciate the c-di-GMP signaling in this bacterium, future experiments will be performed to elucidate the potential correlation and interaction between these c-di-GMP specific proteins for different cellular processes, and to investigate the molecular mechanisms that control these regulatory interactions.

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Table 1

Strains and plasmids	Relevant characteristics[a]	Reference or source
Dickeya dadantii		
3937	Wild type	Hugouvieux-Cotte-Pattat, N.
gcpA^{D418A}	*gcpA*^{D418A}*::Km;* Km^r, ABF-0020368 site-directed mutant	This study
Δ*gcpB*	Δ*gcpB::Km;* Km^r, ABF-0016029 deletion mutant	Lab stock
Δ*gcpC*	Δ*gcpC::Km;* Km^r, ABF-0019499 deletion mutant	Lab stock
Δ*gcpD*	Δ*gcpD::Km;* Km^r, ABF-0014719 deletion mutant	Lab stock
Δ*gcpE*	Δ*gcpE::Km;* Km^r, ABF-0019019 deletion mutant	This study
Δ*gcpF*	Δ*gcpF::Km;* Km^r, ABF-0016283 deletion mutant	Lab stock
Δ*gcpG*	Δ*gcpG::Km;* Km^r, ABF-0019796 deletion mutant	This study
Δ*gcpH*	Δ*gcpH::Km;* Km^r, ABF-0015146 deletion mutant	This study
Δ*gcpI*	Δ*gcpI::Km;* Km^r, ABF-0017509 deletion mutant	This study
Δ*gcpJ*	Δ*gcpJ::Km;* Km^r, ABF-0019128 deletion mutant	This study
Δ*gcpK*	Δ*gcpK::Km;* Km^r, ABF-0019798 deletion mutant	This study
Δ*gcpL*	Δ*gcpL::Km;* Km^r, ABF-0015843 deletion mutant	This study
Δ*ecpA*	Δ*ecpA::Km;* Km^r, ABF-0015066 deletion mutant	Lab stock
Δ*ecpC*	Δ*ecpC::Km;* Km^r, ABF-0020364 deletion mutant	Lab stock
Δ*ecpD*	Δ*ecpD::Km;* Km^r, ABF-0020048 deletion mutant	This study
Δ*ecpE*	Δ*ecpE::Km;* Km^r, ABF-0020067 deletion mutant	This study
Δ*egcpA*	Δ*egcpA::Km;* Km^r, ABF-0015649 deletion mutant	This study
Δ*egcpB*	Δ*egcpB::Km;* Km^r, ABF-0020123 deletion mutant	Lab stock
Escherichia coli		
DH5α	*supE*44 □*lac*U169 (*ϕ*80*lacZ*□M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Lab stock
S17-1 λpir	λ(pir) *hsdR* pro *thi*; chromosomally integrated RP4-2 Tc::Mu Km::Tn7	Lab stock
Plasmids		
pKD4	Template plasmid for kanamycin cassette, Km^r	(Datsenko and Wanner 2000)
pKD3	Template plasmid for chloramphenicol cassette, Cm^r	(Datsenko and Wanner 2000)
pWM91	Sucrose-based counter-selectable plasmid, Ap^r	(Metcalf et al. 1996)
pCL1920	Low copy number plasmid, lac promoter, Sp^r	(Lerner and Inouye 1990)
pCL-*gcpD*	*gcpD* cloned in pCL1920, Sp^r	This study
pPROBE-AT	Promoter-probe vector, promoterless *gfp*, Ap^r	(Miller et al. 2000)
pAT-*hrpA*	pPROBE-AT containing *hrpA* promoter-*gfp* transcriptional fusion, Ap^r	(Yang et al. 2007)
pAT-*hrpL*	pPROBE-AT containing *hrpL* promoter-*gfp* transcriptional fusion,	(Yang et al. 2007)

	Ap^r	
pAT-*hrpS*	pPROBE-AT containing *hrpS* promoter-*gfp* transcriptional fusion, Ap^r	(Yi et al. 2010)
pAT-*rsmA*	pPROBE-AT containing *rsmA* promoter-*gfp* transcriptional fusion, Ap^r	(Yi et al. 2010)
pAT-*rsmB*	pPROBE-AT containing *rsmB* promoter-*gfp* transcriptional fusion, Ap^r	(Li et al. 2014)
pML123	RSF1010-derived expression and *lac*-fusion broad –host-range vector, Gm^r	(Labes et al. 1990)
pML-*gcpA*	*gcpA* cloned in pML123, Gm^r	This study
pML-*gcpL*	*gcpL* cloned in pML123, Gm^r	This study
pRK415	Broad-host-range cloning vector, Tc^r	(Keen et al. 1988)
Primers	**Sequences (5'-3')**	**Amplicon**
gcpA-D418A-1	ATCGCCGTAGCGGCGCCGAATTCATCATCTTG	*gcpA* site-directed
gcpA-D418A-2	CAAGATGATGAATTCGGCGCCGCTACGGCGAT	mutant
gcpA-A-SacI	GGCGAGCTCGAGAAAGAACTGGTTG	*gcpA* site-directed
gcpA-B	GAAGCAGCTCCAGCCTACACATTACTGGTGGG	mutant
gcpA-C	GGAATAGGAACTAAGGAGGATATTCATATGACGCCGATTCG	
gcpA-D-KpnI	AAAGGTACCCGGGCGAACTCAGCGACAT	
gcpE-A-XhoI	AATACTCGAGGGGCGTCGACAGTATGAATG	*gcpE* deletion
gcpE-B	GAAGCAGCTCCAGCCTACACTAACCAGCTCTTGCCACTCA	
gcpE-C	CTAAGGAGGATATTCATATGTCAAAAAGGCCGATAACGCG	
gcpE-D-NotI	AATATTATGCGGCCGCTCAATGTTAATCGCGACCCG	
gcpG-A-SacI	CCGGACGCGGGATAGTCTTCACCAGTCG	*gcpG* deletion
gcpG-B	GAAGCAGCTCCAGCCTACACATTAGTTATATTTACCG	
gcpG-C	CTAAGGAGGATATTCATATGCCGCGCCCGACCTCTGC	
gcpG-D-KpnI	ATCTGATTTTGCCGGGAAATA	
gcpH-A-SacI	GCAGAGCCGGAATGCCTTCAT	*gcpH* deletion
gcpH-B	GAAGCAGCTCCAGCCTACACATCACTTTTCCTGCATT	
gcpH-C	CTAAGGAGGATATTCATATGCCGTTTTTCGCACCGAAACT	
gcpH-D-KpnI	CAAATATTACAAACAGCACGG	
gcpI-A-BamHI	AAAGGATCCCTGCCCTACTTCAACAGCTC	*gcpI* deletion
gcpI-B	GAAGCAGCTCCAGCCTACACACCAGCATAATCA	
gcpI-C	GGAATAGGAACTAAGGAGGATATTCATATGCAGTCTGAATG	
gcpI-D-KpnI	AAAGGTACCCGATTGCAAGATCGACGG	
gcpJ-A-SacI	TACCCCGCCATCCCCTCCTCT	*gcpJ* deletion
gcpJ-B	GAAGCAGCTCCAGCCTACACAATTCAGATGCATGCCGTCA	
gcpJ-C	CTAAGGAGGATATTCATATGCGACCACACAAGAAATGGTG	
gcpJ-D-KpnI	GCGATCAATCGAAAATCGTGC	
gcpK-A-SacI	GTGATCCGCAAACTGAGCCAG	*gcpK* deletion
*gcp*K-B	GAAGCAGCTCCAGCCTACACAAAATAAATAAGATAAACAA	
gcpK-C	CTAAGGAGGATATTCATATGTAACGGAAAATTACTCG	
gcpK-D-KpnI	GGATCTGTGTGAGGTCAGGGT	
gcpL-A-BamHI	AAAGGATCCGGACAGCGGGAGACGG	*gcpL* deletion

gcpL-B	GAAGCAGCTCCAGCCTACACACAATCGGCACAA	
gcpL-C	GGAATAGGAACTAAGGAGGATATTCATATGCTCGTTATGGC	
gcpL-D-KpnI	AAAGGTACCGGGTAATTACGTGGCTGGG	
ecpD-A-BamHI	AAAGGATCCGAGGCCTATGGAGGGGC	*ecpD* deletion
ecpD-B	GAAGCAGCTCCAGCCTACACACATATTCAGTTG	
ecpD-C	GGAATAGGAACTAAGGAGGATATTCATATGCTGCGTTATCGA	
ecpD-D-KpnI	AAAGGTACCGCGAGCAGTTGGCCGTAC	
ecpE-A-BamHI	AAAGGATCCCGCGTGAAAAGAATTGGGG	*ecpE* deletion
ecpE-B	GAAGCAGCTCCAGCCTACACAGATCATCTCTAG	
ecpE-C	GGAATAGGAACTAAGGAGGATATTCATATGGCAAGTCGTGG	
ecpE-D-KpnI	AAAGGTACCCGGAAGTTGCTGGTGATA	
egcpA-A-BamHI	GCCAGCGCATCTTTATAGTTG	*egcpA* deletion
egcpA-B	GAAGCAGCTCCAGCCTACACACCCTGCGCTTAACTCCGTA	
egcpA-C	CTAAGGAGGATATTCATATGTGTCAAAATCCGAACAA	
egcpA-D-KpnI	GATGAAGTGCTGCAGCATTTT	
gcpD-for-XbaI	TTTATCTAGAATTTCGTGGTGCTGGACTGG	*gcpD*
gcpD-rev-HindIII	TTCCAAGCTTCGATAAATGAATAATGACGT	complementation
gcpA-for-SacI	GGCGAGCTCGAGAAAGAACTGGTTG	*gcpA*
gcpA-rev-HindIII	GCCAAGCTTCGACGCCAGTGAAAAC	complementation
gcpL-for-SacI	GGCGAGCTCTAGTCTGTCCCCAATG	*gcpL*
gcpL-rev-HindIII	GCCAAGCTTTGTCGCACATTCGTGA	complementation

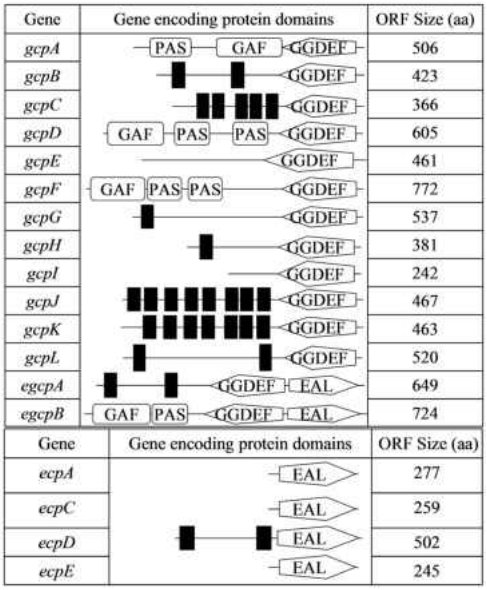

FIG 1 GGDEF and/or EAL domain proteins in *Dickeya dadantii* 3937. Proteins were shown with the encoded gene names and protein length. Protein domains were predicted by the simplified modular architecture research tool (SMART).

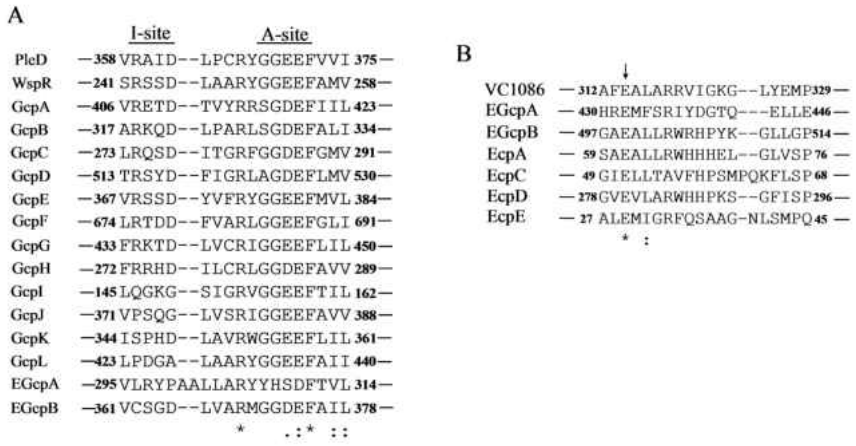

FIG 2 (A) Amino acid sequence alignment for the GGDEF domains in *D. dadantii*. PleD and WspR are two active DGCs from *Caulobacter crescentus* and *Pseudomonas aeruginosa*, respectively. Inhibition site RxxD motif (I-site) and enzymatic activity site GGDEF motif (A-site) are marked. (B) Amino acid sequence alignment for the EAL domains in *D. dadantii*. VC1086 is an active PDE from *Vibrio cholerae*. The arrow indicates glutamate residue in the EAL motif. "*" means that the residues are identical in all sequences in the alignment, ":" means that conserved substitutions have been observed, "." Means that semi-conserved substitutions are observed.

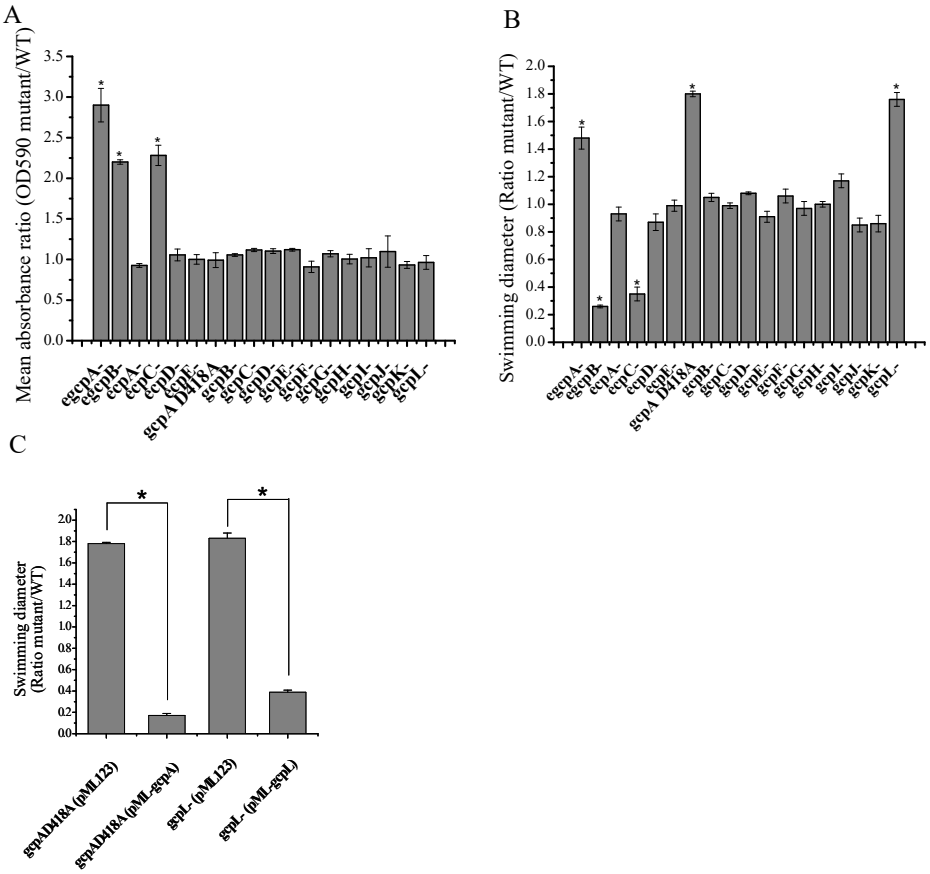

FIG 3 Biofilm formation and swimming motility in wild-type *D. dadantii*, GGDEF and/or EAL single deletion mutants and complementation strains. (A) Biofilm formation of wild type and mutant strains cultured at 28°C in MM media for 48 hours. Ratio of mutant/WT was performed for data analysis. (B) Swimming diameter of the wild-type *D. dadantii* and mutant strains culture at 28°C in 0.2% MG agar plates for 16 h. Ratio of diameter were calculated following mutant/WT. (C) Swimming activity in wild type, $gcpA^{D418A}$, Δ*gcpL* mutants and their complementation strains. All results are from one representative experiment, three independent experiments were performed and three replicates (five replicates for biofilm formation assay) were used for each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ($P<0.05$ by Student's *t* test).

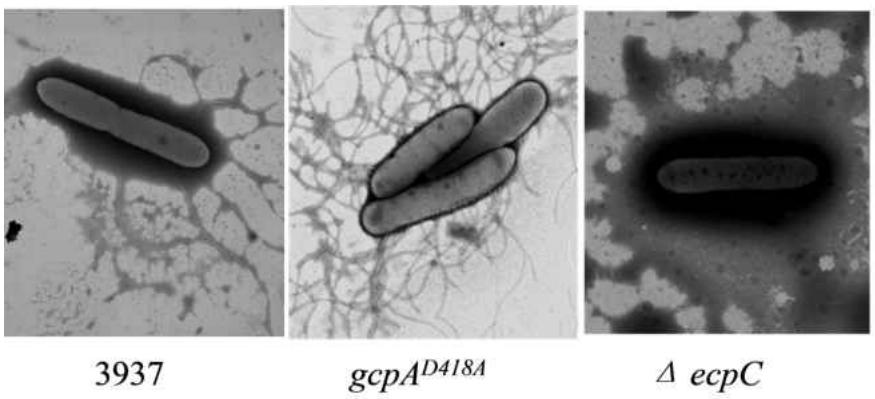

FIG 4 TEM pictures of the wild-type *D. dadantii* and mutant strains.

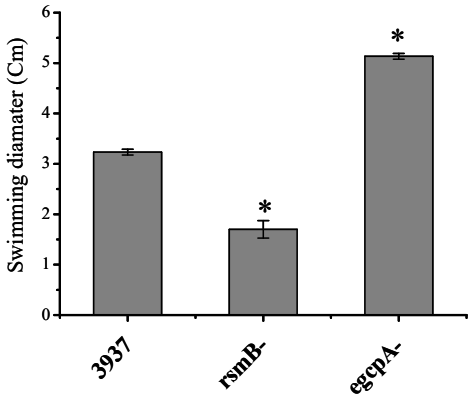

FIG 5 Swimming motility in *D. dadantii* 3937. Swimming diameter of the wild-type *D. dadantii* and mutant strains was measured at 16 h incubation at 28°C. All results are from one representative experiment, three independent experiments were performed and three replicates were used for each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (P<0.05 by Student's t test).

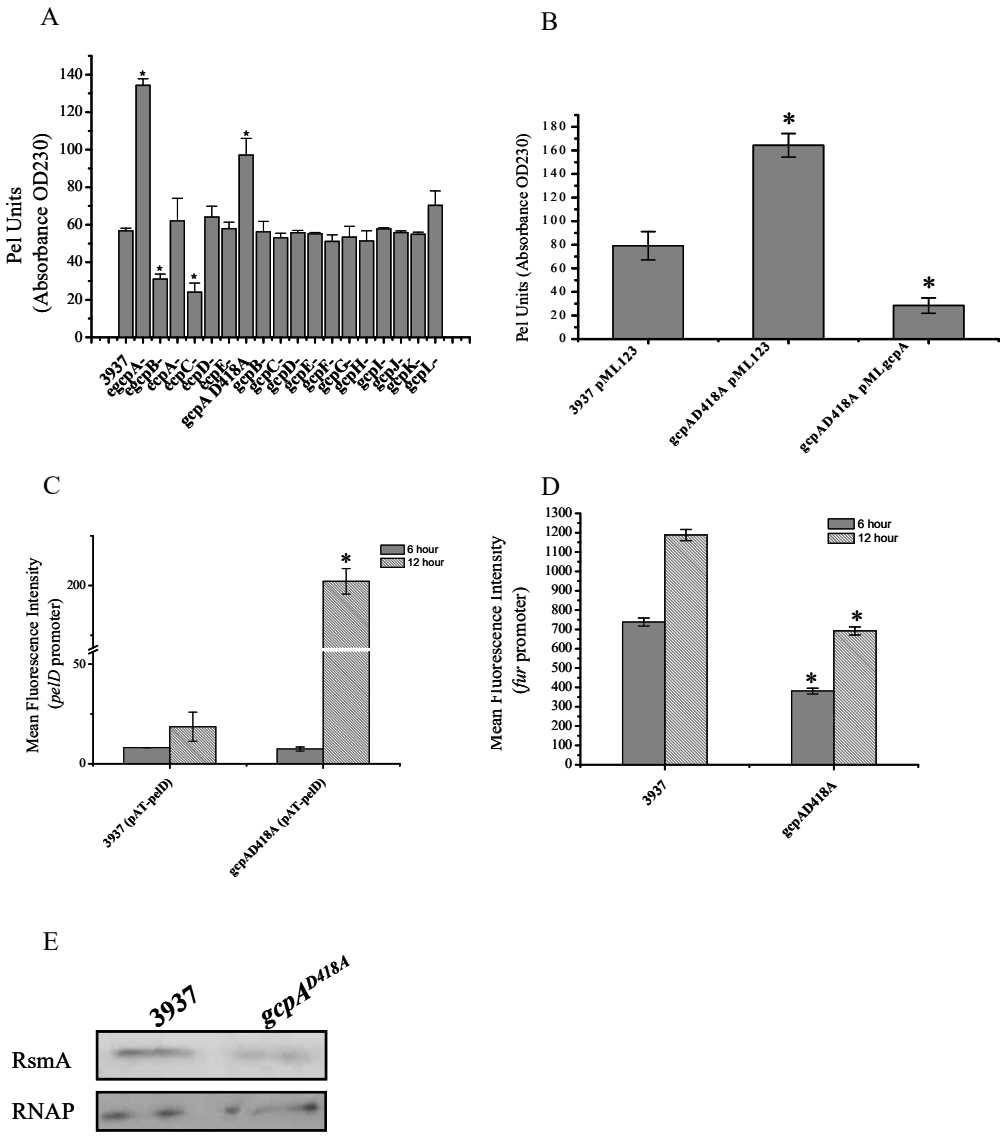

FIG 6 Pectate lyase production, gene promoter activities and RsmA protein levels in *D. dadantii* 3937. (A) Pectate lyase production of wild type and mutant strains cultured in MM+0.1% PGA for 12 h at 28°C. (B) Pectate lyase activity in wild type, $gcpA^{D418A}$ mutant and complementation strain. (C) *pelD* promoter activity in wild type and $gcpA^{D418A}$ A-site mutant strains. (D) *fur* promoter activity in wild type and $gcpA^{D418A}$ A-site mutant strains. (E) RsmA protein levels in wild type and $gcpA^{D418A}$ A-site mutant strains. All results are from one representative experiment, three independent experiments were performed and three replicates were used for each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (P<0.05 by Student's t test).

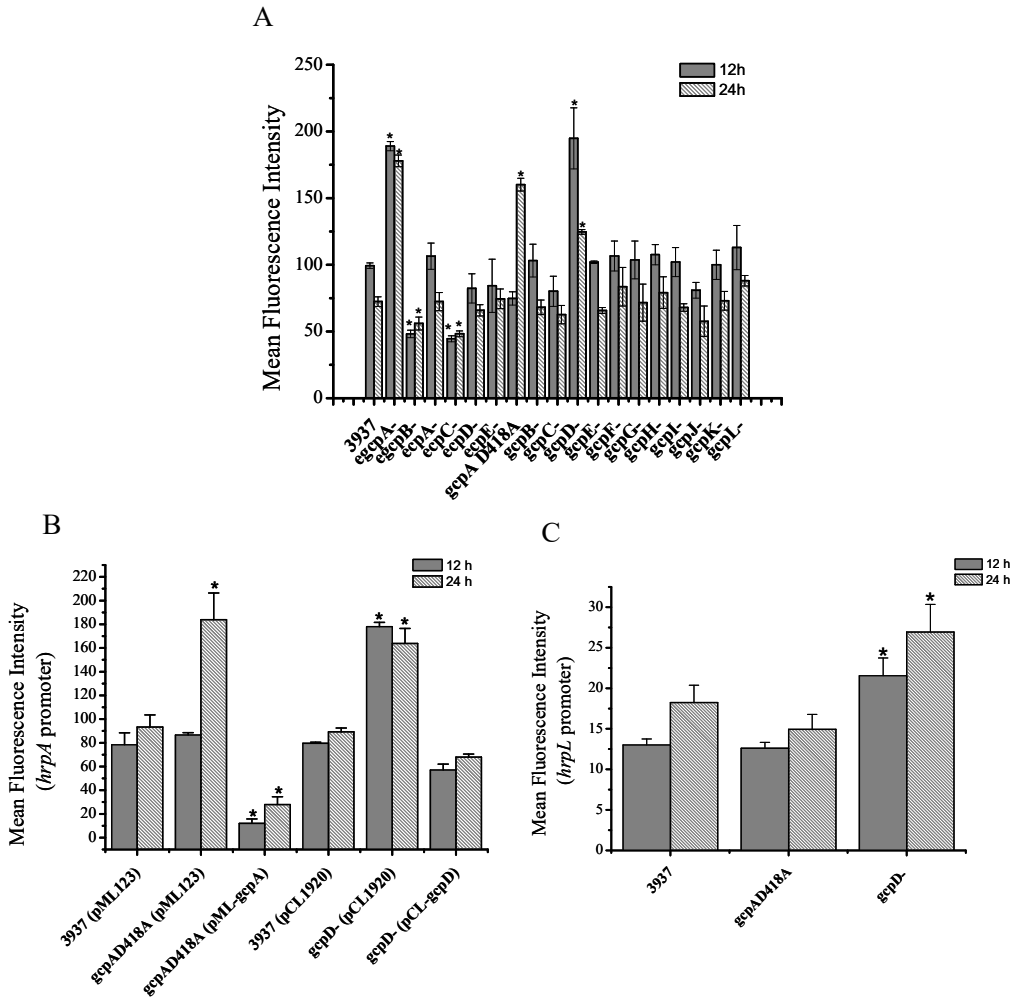

FIG 7 T3SS gene *hrpA* (A and B) and *hrpL* (C) promoter activities in wild-type *D. dadantii*, GGDEF and/or EAL single deletion mutants and their complementation strains. Cells cultured in minimal medium were used to measure the mean fluorescence intensity (MFI) of *hrpA-gfp* by flow cytometry at 12 and 24 h, respectively. One representative experiment was chosen, and three independent experiments were performed. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (P<0.05 by Student's t test).

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Awards and Presentation

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Biological Sciences Symposium, University of Wisconsin, Milwaukee, WI, 2013, 2015, and 2016

Outstanding Graduate Poster Award, University of Wisconsin, Milwaukee, WI, 2016

Invited seminar "Deciphering the multi-tier regulatory network that links the flagellar master regulator FlhDC to c-di-GMP signaling and the type III secretion system, an important virulence factor of pathogenic bacteria**"** Milwaukee Microbiology Society, Milwaukee, WI, March 11th 2015

Teaching Experience

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Elements of Biology, (**Teaching Evaluation (TE): 4.3/5.0**)

Developed laboratory instruction syllabus and assisted with overall class structure, including weekly lab practicum, and administered all grading for assigned laboratory sections.

Teaching Assistant, University of Wisconsin, Milwaukee, WI, 2013, 2015, and 2016

Experimental Microbiology, (**TE: 4.8/5.0**)

Assisted in the development the course structure, assessment of student performance, and grading of laboratory work. Designed special projects assigned by the course professor. Implementation of enhanced techniques for improving student experimental outcomes.

Publications and Papers

Xiaochen Yuan, Devanshi Khokhani, Xiaogang Wu, Fenghuan Yang, Gabriel Biener, Benjamin J. Koestler, Valerica Raicu, Chenyang He, Christopher M. Waters, George W. Sundin, Fang Tian and Ching-Hong Yang. Cross-talk between a regulatory small RNA, cyclic-di-GMP signalling and flagellar regulator FlhDC for virulence and bacterial behaviours. Environmental Microbiology, 2015, 17-11: 4745-4763.

Susu Fan, Fang Tian, Jianyu Li, William Hutchins, Huamin Chen, Fenghuan Yang, **Xiaochen Yuan**, Zining Cui, Ching-Hong Yang and Chenyang He. Identification of phenolic compounds that suppress the virulence of *Xanthomonas oryzae* on rice via the type III secretion system. Molecular Plant Pathology, 2016, accepted.

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