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Complex Regulatory Pathways Connect Vfm Quorum-sensing and Cyclic-di-GMP Signaling to Bacterial Virulence in the Phytopathogen Dickeya Dadantii 3937

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COMPLEX REGULATORY PATHWAYS CONNECT VFM QUORUM-SENSING AND CYCLIC-DI-GMP SIGNALING TO BACTERIAL VIRULENCE IN THE PHYTOPATHOGEN *DICKEYA DADANTII* 3937

by

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ABSTRACT

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The bacterial second messenger Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) regulates multiple cellular behaviors in most bacteria. Bacterial c-di-GMP signaling involves enzymes that synthesize and degrade c-di-GMP, c-di-GMP binding effectors, and targets that are acted upon by the effectors. So far, the c-di-GMP signaling pathways have been understudied. In this work, we explore the c-di-GMP signaling network further in the phytopathogen *Dickeya dadantii* 3937.

In Chapter 2, we identified VfmE as a c-di-GMP binding transcriptional activator that represses pectate lyase production under a high c-di-GMP condition. VfmE was found to bind c-di-GMP *in vitro* via the RxxxR motif, similar to PilZ domain containing proteins. Distinct differences were observed in pectate lyase (Pel) production when a c-di-GMP insensitive mutant of VfmE was expressed in the cell under a high c-di-GMP condition. We also found that VfmE involves SlyA in the regulation of Pel, thus uncovering a complex regulatory network involving Vfm quorum-sensing, c-di-GMP signaling, and pectate lyase production.

In chapter 3, we studied the regulatory mechanism of type III secretion system (T3SS) gene expression by VfmE and the incorporation of c-di-GMP in the regulatory pathway. We demonstrated that VfmE positively regulates the expression of T3SS needle component *hrpA* at the transcriptional level via HrpL; the master regulator of T3SS. A c-di-GMP insensitive mutant of VfmE lacked the c-di-GMP related phenotype in *hrpA* transcription. Our study showed the multitiered regulation of *hrp* genes by VfmE and a high c-di-GMP level caused by deletion of *ecpC.*

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LIST OF ABBREVIATIONS

Ap	Ampicillin
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
DGC	Diguanylate cyclase
Ecp	EAL-domain containing proteins
Gcp	GGDEF-domain containing proteins
Gm	Gentamicin
GFP	Green fluorescent protein
Hrp	Hypersensitive response and pathogenicity
IPTG	Isopropyl-β-D-galactopyranoside
Km	Kanamycin
LB	Luria-Bertani broth
MM	Minimal medium
OD	Optical density
PCR	Polymerase chain reaction
PCWDE	Plant cell-wall-degrading enzyme
PDE	Phosphodiesterase
Pel	Pectate lyase
QS	Quorum-sensing
Sp	Streptomycin
T2SS	Type II secretion system
T3SS	Type III secretion system
TCS	Two-component system
Vfm	Virulence factor modulating cluster

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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, formerly known as *Erwinia chrysenthemi* 3937, is a Gram-negative plant pathogenic bacterium that causes soft-rot disease in a wide range of agricultural plants, such as tomato, potato, and chicory (Reverchon & Nasser, 2013). *D. dadantii* exhibits rod-shaped cells, as observed under the microscope. Dimensions of the bacterial cells are 1.8 μm in length and 0.6 μm in diameter. It shows motility with the help of its flagella and does not form spores (Yuan, 2016). The bacterium was initially classified as *Erwinia chrysanthemi* (Burkholder et al., 1953). After taxonomic studies and 16S DNA sequence analysis, it was reclassified into a new genus *Dickeya* (named after American phytopathologist Robert S. Dickey) (Samson et al., 2005). Based on further characterization and serological study, *Erwinia chrysanthemi* 3937 as the new species *D. dadantii* 3937 (Samson et al., 2005).

D. dadantii causes wilt and soft rot diseases on a wide range of host plants, including several economically important vegetables such as potato, tomato, cabbage, and carrot (Czajkowski et al., 2011). Besides the above-mentioned plants, *Dickeya* sp. can also infect *Arabidopsis thaliana* and *Saintpaulia ionantha,* making it a suitable microbe for host-pathogen interaction study (Reverchon & Nasser, 2013). *Dickeya* sp. has been reported to thrive well in both soil and water habitats (Cother & Gilbert, 1990; Reverchon & Nasser, 2013; Robert-Baudouy et al., 2000). Once the bacterium confronts a susceptible host and the temperature is above 30ºC with high humidity, it can instigate infection via transition to pathogenic mode. *D. dadantii*, being a phytopathogen, can infect different parts of the plant. In the pulpy and tender areas,

such as tubers and leaves, *D. dadantii* causes localized maceration of plant tissues, termed “soft rot” (Yuan, 2016).

The complete genome sequence of *D. dadantii* 3937 is available (https://asap.genetics.wisc.edu/asap/home.php), making it suitable for usage as a model organism to study host-microbe interactions (Glasner et al., 2011). This bacterium shares similarities with other Gram-negative bacteria including *Escherichia coli* and animal pathogens belonging to the genera *Yersinia* and *Salmonella*.

1.1.2 Virulence mechanism of *D. dadantii* 3937

D. dadantii initially infects the apoplast of plant tissues. During the early stages of infection, *D. dadantii* employs the type III secretion system (T3SS) to suppress the host immune response (Z. Cui et al., 2018), and its virulence was compromised in *D. dadantii* T3SS knockout strains (Yang et al., 2002). T3SS delivers effector proteins into the host cells to induce cell death and is important for *D. dadantii* to maintain its population *in planta* (Yang et al., 2002). T3SS assembly is initiated by the T3SS basal body formation, followed by the transport of needle subunits to the outer membrane space, thus forming a functional translocation system (Deng et al., 2017). During later stages of infection, *D. dadantii* secretes cell-wall degrading enzymes (CWDEs) through the type II secretion system (T2SS) (Robert-Baudouy et al., 2000). Plant leaves and soft tissues show substantial maceration and necrosis caused by the CWDEs, such as pectate lyases (Pel), proteases, cellulases, and polygalacturonases (Collmer & Keen, 1986; Roy et al., 1999).

1.1.2.1 Bacterial secretion systems

The cytoplasmic membrane of Gram-negative bacteria separates the cytoplasm from the periplasm (Kuhn, 2019). This membrane is selectively permeable and repulsive towards polar and ionic compounds. For transportation of water-soluble polar molecules and ions, there are several transmembrane proteins that secrete a number of effector proteins in response to environmental cues, allowing the organism to adapt and survive in different conditions, adhere to surfaces, or display pathogenicity in hosts (Costa et al., 2015).

The type II secretion system is common in pathogenic and non-pathogenic Gram-negative bacteria. It secretes folded proteins from the periplasm into the extracellular space. Secreted substrates comprise hydrolyzing enzymes that are essential for bacterial survival and growth in a host, and toxins (Nivaskumar & Francetic, 2014).

The type III secretion system is a double membrane-embedded needle like structure that is found in various plant and animal pathogenic Gram-negative bacteria. T3SS transfers bacterial effector proteins into the cytoplasm or the plasma membrane of target eukaryotic host cells. The effector molecules attune or suppress host cell functions and immune response and aid in bacterial invasion and colonization (Büttner, 2012; Cornelis, 2006).

1.1.2.2 The type III secretion system and its regulation in *D. dadantii* 3937

In *D. dadantii,* 39 gene clusters are named *hrp/hrc/dsp.* These geners are involved in T3SS formation and function (Peng, 2009). The *hrp* stands for hypersensitive response and pathogenicity. The expression of T3SS is tightly regulated in *D. dadantii* and can be induced by minimal medium (MM) and in the plant hosts but is repressed

by rich media (Yuan et al., 2020). The T3SS genes of *D. dadantii* are controlled at multiple steps by different regulators. HrpX/HrpY is the two-component system that activates T3SS via upregulating the transcription of *hrpS*. HrpS is a σ^{54} enhancer binding protein that promotes the binding of RpoN (σ^{54}) to RNA polymerase, resulting in the initiation of *hrpL* transcription (Chatterjee, Cui and Chatterjee 2002; Yap et al. 2005). HrpL, an alternative σ factor, has been termed the master regulator of T3SS in *D. dadantii*. Transcription of the *hrp* regulon genes including *hrpA*, *hrpN*, and *dspE,* are activated by HrpL (Tang et al. 2006). *hrpA* encodes the protein that forms the T3SS needle subunits, *hrpN* encodes a T3SS harpin protein, and *dspE* encodes an effector protein that is injected into the host cells (Chatterjee *et al*. 2002; Wei and Beer, 1995). HrpL, the alternative sigma factor, binds to the *hrp* box promoter sequence of the *hrp* regulon genes to activate their expression. (Tang et al., 2006; S. Yang et al., 2010). HrpX/HrpY-HrpS-HrpL pathway regulates the expression of the *hrpL* gene (Yap et al. 2005). Also, the second messenger Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) has been previously reported to suppress T3SS expression via RpoN (Yi et al., 2010). Additionally, the *hrpL* mRNA is regulated by the regulator of a secondary metabolism (Rsm) system through the GacS-GacA-*rsmB*-RsmA pathway (Yang et al., 2007). The Rsm system in *D. dadantii* includes RsmA and *rsmB*. RsmA is a small RNA-binding protein that binds *hrpL* mRNA and promotes its degradation. On the other hand, *rsmB* is an untranslated small RNA that binds to RsmA, forming an inactive ribonucleo-protein complex, thereby neutralizing its negative regulatory effect (Yang et al., 2007; Zou et al., 2012). GacS/A is a second two component system (TCS) that regulates T3SS genes of *D. dadantii 3937* (Yang et al., 2007). When T3SS is induced, GacA upregulates the transcription of *hrpA*, *hrpN*, and *dspE* through the Rsm pathway at the post-transcriptional level by increasing the

expression of *rsmB*. The increased level of *rsmB* inhibits the degradation effect of RsmA on *hrpL* mRNA, thereby upregulating T3SS expression (Yang et al., 2007; Zou et al., 2012) (Fig. 1.1).

1.1.2.3 The regulation of type II secretion system and pectate lyases in *D. dadantii* 3937

D. dadantii secretes pectate lyases and cellulases into the host via an ATP-dependent T2SS, the Out pathway. The Sec system exports the unfolded pectinases to the periplasmic space, where they are folded by chaperones. Then the T2SS (the Out system) translocates Out proteins and pectinolytic enzymes into the extracellular space, where they may interact with host plant tissues (Andro et al., 1984; Condemine et al., 1992; Lindeberg & Collmer, 1992; Robert-Baudouy et al., 2000). The genome of *D. dadantii* encodes several Pel proteins: PelA, PelB, PelC, PelD, and PelZ. Among these pectate lyases, PelD is regarded as one of the major endo-pectate lyase enzymes that shows activity on low methoxylated homogalacturonan and catalyzes β-elimination at random galactosidic bonds (Antúnez-Lamas et al., 2009; Roy et al., 1999). The Pel enzymes degrade pectin, which undergoes catabolism in the extracellular space and the resulting oligomers are utilized by the bacteria as growth substrates. One of the intermediate products of this catabolic pathway is polygalacturonate (PGA) (Robert-Baudouy et al., 2000). The production of pectate lyases can be induced in *D. dadantii* by adding 0.5% PGA to the respective culture medium (Yuan et al., 2020). Pectate lyase production is controlled by a number of regulators in the cell. The Vfm quorum-sensing system and SlyA are two of the major factors that upregulate Pel production in *D. dadantii* and *D. zeae* (Haque et al., 2009; Lv et al., 2019; Nasser et al., 2013; Reverchon et al., 2010; Zou et al., 2012). The c-

di-GMP represses virulence by lowering Pel production via its effector proteins (Reverchon et al., 2016; Yi et al., 2010b; Yuan et al., 2018; Yuan, Zeng, et al., 2020) (Fig.1.1-1.2).

1.1.3 The bacterial second messenger c-di-GMP

c-di-GMP is a ubiquitous bacterial second messenger that has been reported to regulate multiple cellular behaviors that include biofilm formation, swimming motility, and the expression of T3SS (Hengge, 2009). The intracellular c-di-GMP concentration is controlled by two different groups of enzymes namely, diguanylate cyclase (DGC) and phosphodiesterase (PDE). DGCs are GGDEF domain-containing proteins that are responsible for the synthesis of c-di-GMP from two GTP molecules (Hengge, 2009; Römling & Simm, 2009). On the other hand, PDEs are EAL or HD-GYP domain-containing proteins that conduct the hydrolysis of c-di-GMP into 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG) or GMP (Hengge, 2009; Römling & Simm, 2009). C-di-GMP has been known to bind to its effector proteins thereby controlling various cellular phenotypes (Fig. 1.2). The reported c-di-GMP effectors are diverse in structures. The different types of effectors include PilZ-domain containing proteins, GGDEF domain containing proteins with an I-site, degenerate GGDEF or EAL domain containing proteins, and RNA riboswitches (Romling et al., 2013). Our previous studies indicated that two DGCs, GcpA and GcpL, two PDEs, EcpC and EGcpB, and two effectors, YcgR and BcsA, are directly involved in the c-di-GMP mediated regulation of various cellular functions and virulence in *D. dadantii* (Yi et al. 2010; Yuan et al. 2015, 2018, 2019) High concentration of intracellular c-di-GMP has been previously reported to repress virulence factors, Pel and T3SS, by binding to its effector proteins (Yi et al.,

2010; Yuan et al., 2015, 2018; Yuan et al., 2020). However, there is a large number of c-di-GMP effector proteins that are uncharacterized.

1.1.4 Bacterial quorum-sensing system

Quorum-sensing (QS) is the bacterial cell to cell communication system that involve small signaling molecules that can either pass through the membrane or recognized by trans-membrane receptor proteins (Von Bodman, Bauer and Coplin 2003). QS is required for the regulation of cell-density-dependent cellular behavior, pathogenicity, and host colonization in various pathogenic bacteria (Baltenneck et al., 2021; Miller & Bassler, 2001; von Bodman et al., 2003). In Gram-negative bacteria, the most common signaling molecule for quorum-sensing is N-acyl-homoserine lactone (AHL). Gram-positive bacteria use autoinducing peptides as quorum-sensing signals (Leoni & Rampioni, 2018; Miller & Bassler, 2001). Quorum-sensing was observed to regulate virulence and biofilm formation in animal pathogens (Ball et al., 2017; C. Cui et al., 2018; Miller & Bassler, 2001; Mukherjee et al., 2017; Srivastava et al., 2011; Zhang et al., 2018) and plant pathogens (Baltenneck et al., 2021; Liu et al., 2022; Miller & Bassler, 2001; Potrykus et al., 2018; Sibanda et al., 2018; von Bodman et al., 2003). Previous reports demonstrated QS as a regulator of secretion systems and virulence factor production, including CWDEs in phytopathogenic bacteria (Lv et al., 2019; Nasser et al., 2013; Pena et al., 2019). The regulatory pathways of QS are complex. The QS can be regulated by two-component systems (Cui et al., 2018). Multiple reports suggest that the second messenger c-di-GMP plays an important role in regulating cellular processes during the transition from low cell density to high cell density during bacterial growth by controlling quorum-sensing (Y. Deng et al., 2013; Kim et al., 2018; Liang et al., 2020; Lin Chua et al., 2017; Schmid et al., 2017;

Srivastava et al., 2011; Zhang et al., 2018). In *D. dadantii* and *D. zeae*, QS controls virulence, cellular behaviors, and motility (Liu et al., 2022; Lv et al., 2019; Nasser et al., 2013; Potrykus et al., 2018).

1.1.4.1 The Vfm quorum-sensing system

In *D. dadantii*, two QS systems have been reported. The classic Exp (AHL-QS) system uses AHL autoinducer (Potrykus et al., 2018). The other quorum-sensing system is known as the Vfm QS system (virulence factor modulating cluster), which is conserved in the *Dickeya* genus (Hugouvieux-Cotte-Pattat et al., 2022; Nasser et al., 2013; Potrykus et al., 2018). The *vfm* genes direct the synthesis of an extracellular signal (likely short signaling peptides) and constitute a novel QS system that is different from the usual AHL-QS (Hugouvieux-Cotte-Pattat et al., 2022; Lv et al., 2019; Nasser et al., 2013). The signal produced by the signal synthesis genes is exported to the extracellular space by VfmF-G exporter. The signal is then detected by the sensor kinase VfmI, and it phosphorylates VfmH, the response regulator. VfmI-H forms a two-component system that regulates cellular behaviors and pathogenicity. VfmH activates transcription of an AraC type transcriptional regulator *vfmE,* and other operons including *vfmIH* and *vfmAZBCD*. VfmE activates both the transcription of the CWDE genes and the transcription of the entire *vfm* operon (Lv et al., 2019; Nasser et al., 2013). VfmE was previously reported to directly upregulate genes encoding cellulases, proteases, and pectate lyases in *D. dadantii,* thereby controlling its virulence (Nasser et al., 2013) (Fig. 1.3).

Figures

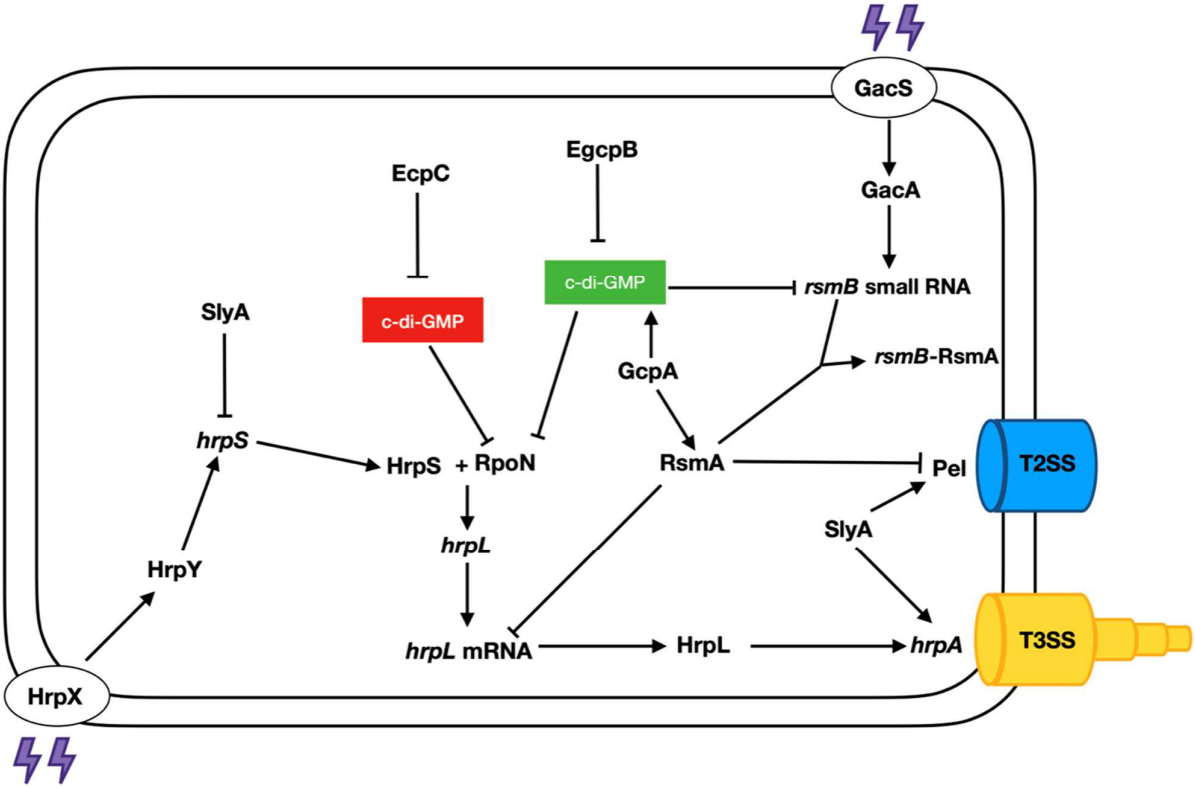

Fig. 1.1. Regulatory mechanism of pectinase (Pel) and type III secretion system (T3SS) in *Dickeya dadantii* 3937.

Figure modified from (Haque et al., 2009; Yuan et al., 2015, 2018, 2019). HrpX/Y two-component system and HrpS, along with σ^{54} RpoN, upregulate HrpL at the transcriptional level resulting in high HrpA production. GacS/A-RsmB-RsmA pathway regulates T3SS at the post-transcriptional level. EcpC, EGcpB, and GcpA control both Pel and T3SS genes via c-di-GMP. SlyA upregulates Pel production and *hrpA* transcription but represses *hrpS*. Positive and negative regulations are designated by arrows and bars, respectively.

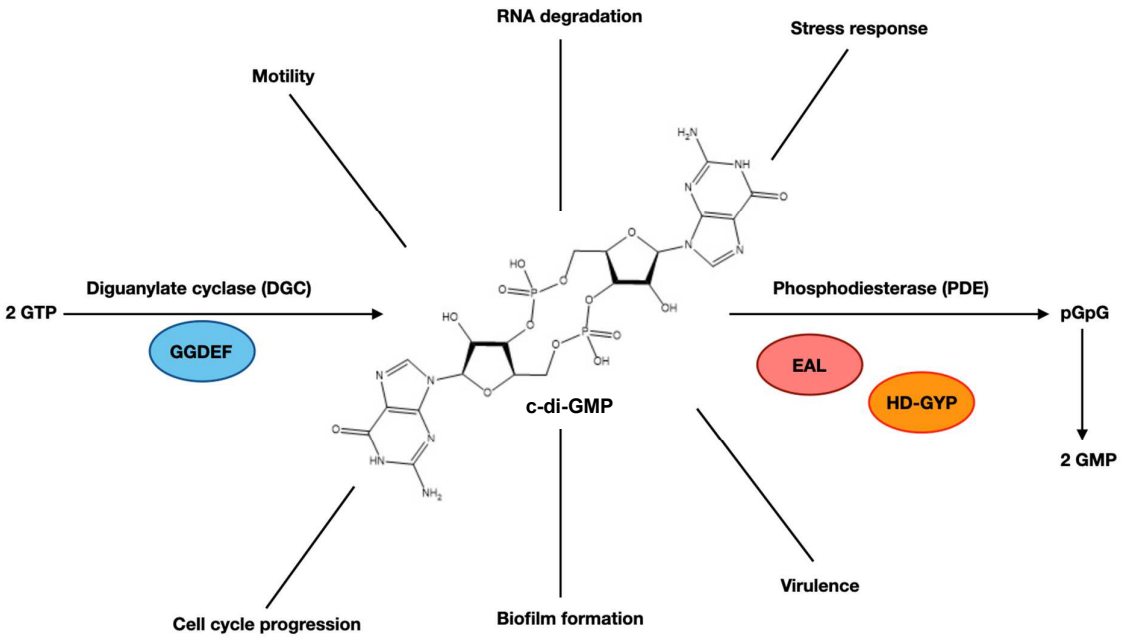

Fig. 1.2. Regulation of intracellular c-di-GMP and its effects on bacterial cellular behaviors.

Figure modified from (Hengge, 2009). The c-di-GMP is synthesized from two GTP molecules by diguanylate cyclases (DGCs) containing GGDEF domain. Phosphodiesterases (PDEs) contain either an EAL or HD-GYP domain and degrade c-di-GMP into linear pGpG, which is further converted to 2 GMP molecules. c-di-GMP regulates multiple cellular behaviors, like motility, biofilm formation, and virulence.

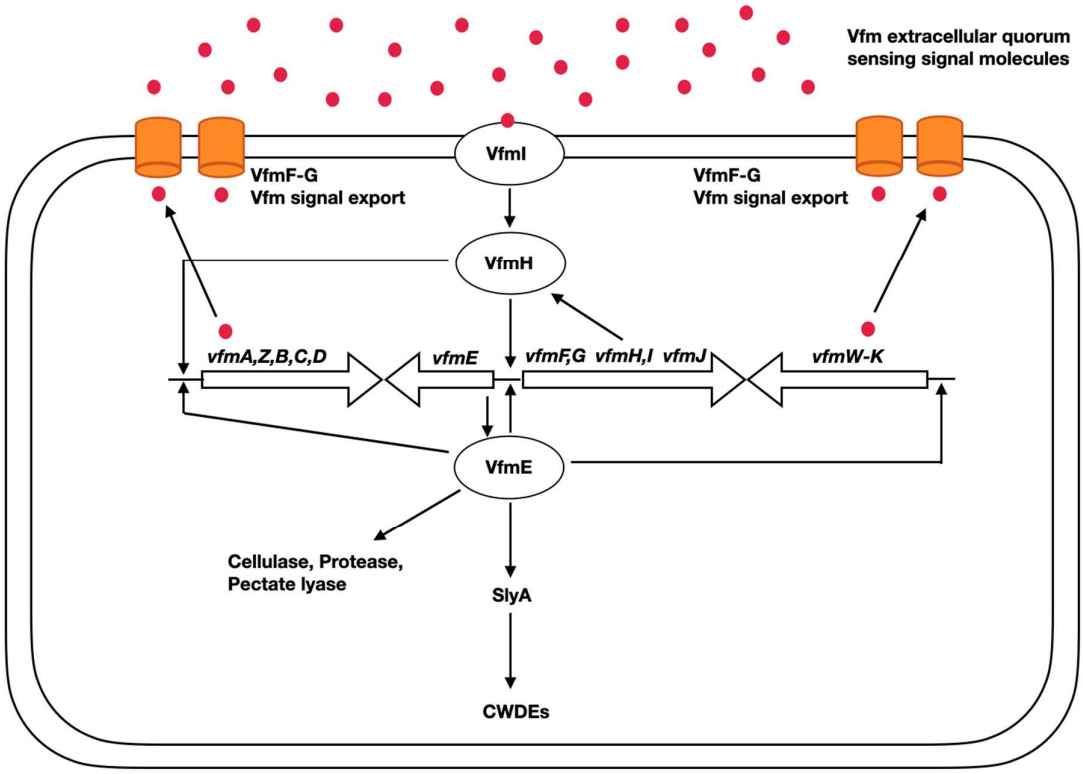

Fig. 1.3. Regulatory mechanism of Vfm quorum-sensing system in *D. dadantii* 3937 and *D. zeae* EC1.

Figure modified from (Lv et al., 2019; Nasser et al., 2013). The extracellular Vfm QS signal is sensed by VfmI, a sensory kinase that activates VfmH, the response regulator. VfmH activates the transcription of *vfmAZBCD* and *vfmE.* VfmE upregulates itself, *vfmAZBCD, vfmFGIHJ,* and *vfmKLMNOPQRSTUVW* operons and these operons are responsible for signal production and transport. VfmE positively regulates SlyA and CWDEs. Positive regulation is designated by arrows.

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Chapter 2

The Vfm quorum-sensing master regulator VfmE binds to c-di-GMP and alters pectate lyase production in the phytopathogen *Dickeya dadantii*

Abstract

Dickeya dadantii is a phytopathogenic bacterium that causes diseases on a wide range of host plants. The pathogen secretes pectate lyases (Pel) through the type II secretion system (T2SS). These Pels degrade the cell wall in host plants. The virulence of *D. dadantii* is controlled by the second messenger cyclic diguanylate monophosphate (c-di-GMP), and the homeostasis of c-di-GMP is maintained by a number of diguanylate cyclases and phosphodiesterases. Deletion of a phosphodiesterase *ecpC* repressed *pelD* transcription, and such repression can be suppressed by an additional deletion in *vfmE*. VfmE is an AraC type transcriptional regulator in the Vfm quorum-sensing system. Our results suggest that VfmE is a c-di-GMP effector that functions as an activator of *pel* at low c-di-GMP concentrations and a repressor of *pel* at high c-di-GMP concentrations through regulation of the transcriptional activator SlyA. Multiple sequence alignment with known c-di-GMP effectors identified a “RWIWR” motif in VfmE that we demonstrate is required for c-di-GMP binding. Mutation of Arg93 to Asp in the RxxxR motif eliminates the c-di-GMP-related phenotypes in Pel activity. Our results show that VfmE is not only a quorum-sensing regulator but also a c-di-GMP effector, suggesting that *D. dadantii* integrates the c-di-GMP signaling network with the Vfm quorum-sensing pathway during environmental adaptation.

Introduction

Dickeya dadantii, a bacterium of the *Enterobacteriaceae* family, is a pectinolytic plant pathogen causing soft rot, wilt, and blight diseases on a wide range of important crop species, such as tomato and potato (Reverchon & Nasser, 2013). *D. dadantii* causes

infection in the apoplast of plants tissue. Large maceration areas on plant leaves and tissues appear due to their degradation by the plant-cell-wall degrading enzymes (CWDEs), such as pectate lyases (Pel), proteases, cellulases, and polygalacturonases (Collmer & Keen, 1986; Roy et al., 1999). *D. dadantii* secretes Pels into the host via the type II secretion system (T2SS), and it encodes several Pel genes: *pelA*, *pelB*, *pelC*, *pelD*, and *pelZ*. Among these pectate lyases, PelD is regarded as the major endo-pectate lyase enzyme that acts on low methoxylated homogalacturonan and catalyzes β-elimination at random galactosidic bonds (Antúnez-Lamas et al., 2009; Roy et al., 1999). Despite the identification of the virulence factors, how *D. dadantii* senses environmental and host signals and modulates the expression of such virulence factors remains unclear.

Bis-(3'-5') -cyclic dimeric guanosine monophosphate (c-di-GMP) is a ubiquitous bacterial second-messenger molecule that regulates many cellular processes such as CWDE production (Hengge, 2009). Low intracellular c-di-GMP levels are associated with a planktonic lifestyle, while high c-di-GMP levels promote a sessile life cycle, such as biofilm formation. The turnover of intracellular c-di-GMP levels is subject to the regulation of two types of enzymes, diguanylate cyclase (DGC) and phosphodiesterase (PDE). DGCs are GGDEF domain-containing proteins that synthesize c-di-GMP from two GTP molecules. In contrast, PDEs are EAL or HD-GYP domain containing proteins that hydrolyze c-di-GMP into 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG) or two molecules of guanosine monophosphates (GMP). The c-di-GMP controls a wide variety of cellular functions through the binding of different effectors. The reported c-di-GMP effectors are structurally diverse, including PilZ-domain proteins, GGDEF domain proteins with an I-site, degenerate GGDEF or EAL domain proteins, and RNA riboswitches (Hsieh et al., 2020; Römling et al., 2013). Our

previous studies of *D. dadantii* reported that two PDEs, EcpC and EGcpB (or EcpB), and one DGC, GcpA, are involved in the regulation of pectate lyase production (Yi et al., 2010; Yuan et al., 2018, 2019, 2020). However, the effectors involved in the regulation of the CWDEs in *D. dadantii* remain unresolved.

Quorum-sensing (QS) is the bacterial intercellular communication system that depends on small signaling molecules that are perceived by the cells either via transmembrane receptor proteins or they are membrane-permeable. QS is required for pathogenicity and host colonization in many plant-pathogenic bacteria (von Bodman et al., 2003). Two QS systems were reported previously in *D. dadantii*: The Exp (AHL-QS) system uses an N- acyl-homoserine lactone (AHL) autoinducer, which is ubiquitous amongst Gram-negative bacteria. The other quorum-sensing system is known as the Vfm QS system (Virulence Factors Modulating cluster), which is conserved in *Dickeya* species (Potrykus et al., 2018). The *vfm* genes synthesize an extracellular signal and constitute a novel quorum-sensing system in *Dickeya*. The signal is perceived by the two-component system VfmI–VfmH, which activates the expression of an AraC regulatory protein VfmE. VfmE activates both the transcription of the CWDE genes and the expression of the *vfm* operon. The *vfmP* is one of the QS signal-producing genes in the *vfm* operon that encodes amino acid activating enzymes and displays similarities to the adenylation (A) domains of the non-ribosomal peptide gramicidin synthetase (NRPS) (Nasser et al., 2013).

In this study, we show that transposon insertion in *vfmE* suppresses the reduced *pelD* expression observed in the PDE deletion mutant Δ*ecpC*. We then show that VfmE regulates *pelD* through the transcriptional regulator SlyA, and the regulation is dependent on cellular c-di-GMP levels. Furthermore, we demonstrate that VfmE binds to c-di-GMP through a PilZ-like mechanism and is involved in the regulation of CWDE

production. This study identified a previously characterized quorum-sensing regulator as a novel c-di-GMP effector and unveiled interacting pathways of quorum-sensing and secondary messenger c-di-GMP that together regulate virulence in *D*. *dadantii*.

Materials and methods

Bacterial strains, plasmids, primers, and media

The details of bacterial strains and plasmids used are listed in Table 1. The media used for growing *D. dadantii* strains are 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 minimal medium (MM). The strains were cultured at 28°C (S. Yang et al., 2008; S. Yang, Peng, et al., 2007). *Escherichia coli* strains were cultured in LB medium at 37°C. Antibiotic supplements to the growth medium were added in the following concentrations: ampicillin (100 µg/ml), kanamycin (50 µg/ml), gentamicin (10 µg/ml), and streptomycin (100 µg/ml). The *D. dadantii* 3937 genomic sequence data is available in the annotation package for community analysis of genomes (ASAP) (https://asap.ahabs.wisc.edu/asap/home.php). PCR Primers used in this study are reported in Table 2.

Mutant construction and complementation

The *vfmE* and *vfmP* genes were deleted from the genomic DNA by marker exchange mutagenesis as previously described (Yang et al., 2002). In brief, the upstream and downstream regions of the target gene were PCR amplified with specific set of primers (Table 2). The kanamycin (Km) cassette was PCR amplified from pKD4 plasmid (Datsenko & Wanner, 2000) and fused in between the two flanking regions by overlap

PCR. The PCR construct was cloned into the suicide plasmid pWM91. The final constructed plasmid was transformed into *D. dadantii* 3937 by conjugation with *E. coli* strain S17-1 λ-pir. For selection of strains with chromosomal deletions, recombinant strains were grown on a kanamycin medium and plated on a 5% sucrose plate. Cells resistant to sucrose due to SacB-mediated sucrose toxicity were inoculated on ampicillin plate, and the cells that were ampicillin-sensitive were PCR confirmed using outside primers. The DNA fragment containing two flanking regions and a kanamycin cassette, was confirmed by sequencing. Marker-less mutants were constructed by excision of the Km cassette from the marker exchange mutants using pFLP2 plasmid encoding the FLP (flippase) recombinase enzyme in *E. coli* S17-1 λ-*pir*. After conjugation, the strains sensitive to kanamycin and resistant to sucrose were selected and analyzed by sequencing using outside primers. For constructing the *slyA* mutants, the pWM91:*slyA* plasmid from previous research was used (Zou et al., 2012). Complemented strains were generated by the following method: the promoter and open reading frame region of the specific genes were PCR amplified followed by cloning into low copy number plasmid pCL1920 (Table 1). The resulting plasmids were then PCR confirmed followed by electroporation into mutant cells.

Green fluorescent protein (GFP) reporter plasmid construction and flow cytometry assay

The reporter plasmid, pAT-*vfmE*, was generated by cloning the promoter region of *vfmE* into the promoter probe vector pPROBE-AT with the ribosomal binding site upstream of the *gfp* reporter gene (Leveau & Lindow, 2001; Miller et al., 2000). The reporter plasmids pAT-*pelD,* pAT-*slyA,* pAT-*gcpA,* pAT-*egcpB,* and pAT-*ecpC* were constructed previously complying with the same method (Peng et al., 2006; S. Yang,

Zhang, et al., 2007; Yi et al., 2010; Zou et al., 2012). Promoter activities of the respective genes were determined by quantifying GFP intensity by flow cytometry (BD Biosciences, San Jose, CA) as previously described (Peng et al., 2006). Overnight LB cultures of bacterial cells harboring reporter plasmids were inoculated 1:100 into MM media. Promoter activity of the strains was measured at 24 h by detecting GFP intensity using flow cytometry. For measuring the *pelD* promoter activity, bacterial cultures were grown for 24 h in MM medium supplemented with 0.1% polygalacturonic acid (PGA).

Pectate lyase activity assay

Extracellular Pel activity was quantified using a spectrometer as described previously (Matsumoto et al., 2003). In brief, bacterial cells were cultured in LB medium with 0.1% polygalacturonic acid as a supplement and grown at 28°C for 16 h. Centrifugation at 15000 r.p.m. for 2 min of 1 ml bacterial cultures were done. After collecting the supernatant, 10 µl of the supernatant was mixed with 990 µl of the Pel reaction buffer (0.05% PGA, 0.1 M Tris-HCl [pH 8.5] and 0.1 mM $CaCl_2$, pre-warmed to 30°C). Pel activity was measured at absorbance 230 (A_{230}) for 3 min. The calculation was based on one unit of Pel activity equals to an increase of 1×10^{-3} optical density 230 (OD_{230}) in 1 min. The cell density OD_{600} value of the liquid cell cultures of each sample was measured. The final value of OD_{230} was normalized by the OD_{600} value of each of the sample cultures (OD_{230} / OD_{600}).

Virulence assay

The local maceration assay of soft tissue was done using russet potato (*S. tuberosum*) as described (Yi et al., 2010). For each potato, 50 µl of a bacterial suspension at 10^6

CFU ml^{-1} were injected with a syringe in the middle of each sliced half of 1 cm thickness. Phosphate buffer saline (PBS, 50 mM, pH 7.4) was used to resuspend the bacterial cells. A potato in triplicates was used for each bacterial strain in this experiment. Potato slices inoculated with the bacterial strains were kept in a growth chamber at 28°C with 100% relative humidity. The necrosis of potato tissue was assessed by the following method: the soft necrotic tissue was scooped out, and the weight of the necrotic tissue was measured for each sample. The fluid from the necrotic tissue was collected (1 ml), serial dilution was done, and the CFU count of each of the strains was obtained to determine the survival ability of the strains in the host system.

Construction of R93D point mutation of VfmE

The open reading frame of *vfmE,* along with its natural promoter, was cloned into the low copy number plasmid pCL1920 by primers vfmE-F-HindIII and vfmE-R-XbaI (Table 2). To generate amino acid substitution in the RxxxR motif of VfmE protein, single nucleotide substitution was performed by PCR amplification of two fragments containing the desired mutation with specific primers, on both DNA strands and then combining them into one by fusion PCR. Briefly, two primers, *vfmE*-R93D-1 and *vfmE*-R93D-2 (Table 2), were utilized to create *vfmE*^{R93D}, which changed the RxxxR motif to RxxxD. The nucleotide substitution was confirmed by DNA sequencing. The pCL1920:*vfmE*^{R93D} plasmid construct was used to check the complementation of the *pelD* promoter activity and total Pel activity in Δ*vfmE* and Δ*vfmE* Δ*ecpC* mutant backgrounds. The pMAL-c6T:*vfmE*^{R93D} plasmid was used to overexpress the mutated protein for the c-di-GMP binding ELISA experiment.

Protein expression and purification

The full length of *vfmE* and *vfmE*^{R93D} were cloned into the expression vector pMAL-c6T (New England Biolabs) by primers *vfmE*-F-BamHI and *vfmE*-R-HindIII (Table 2) under *tac* promoter. The sequences of the constructs were verified by DNA sequencing. The constructs carrying *vfmE* and *vfmE*^{R93D} were transformed into NEB Express® *E. coli* competent cells strain for protein expression and purification. NEB Express® *E. coli* cells containing empty pMAL-c6T, which expresses only 6X-His-MBP, was used as control. Briefly, expression of fusion proteins was induced by the addition of isopropyl-thio-galactopyranoside (IPTG) at a final concentration of 0.1 mM, and the bacterial cultures were then incubated at 30°C for 4 h. Then bacterial cells were collected by centrifugation, followed by suspension in MBP column binding buffer and sonication. The crude cell extracts were centrifuged at 15,000 r.p.m. for 15 min to remove cell debris. The supernatant containing the soluble proteins was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot using Anti-MBP monoclonal primary antibody (New England Biolabs) and Anti-Mouse IgG-HRP conjugated secondary antibody (Southern Biotech). The presence of full-length 6X-His-MBP-VfmE and 6X-His-MBP-VfmE^{R93D} in the cell lysate was further confirmed by Mass spectrometry (University of Wisconsin-Madison). A his-tagged variant of YcgR from *D.dadantii* was purified by the method used previously (Yuan et al., 2015). The cell lysate containing the recombinant protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot using HRP-conjugated Anti-His monoclonal antibody (Invitrogen). The supernatant of the cell lysates containing the soluble proteins was used for the c-di-GMP binding assay.

c-di-GMP binding assay

The assay was modified from the c-di-AMP binding protein detection assay (Underwood et al., 2014). The protein concentrations in the cell lysates containing 6XHis-MBP-VfmE, 6XHis-MBP-VfmE^{R93D}, 6XHis-MBP, and 6X-His-YcgR were measured by standard Bradford assay and then diluted to 50 µg/ml in coating buffer containing 3.03g Na_2CO_3, 6.0g $NaHCO_3$ in 1L water, pH 9.6. Immulon 96-well plates (ThermoFisher Scientific) were coated with the diluted proteins (100 µl/well) overnight (16 h) at 4℃. The wells were blocked with 100 µl 1% Bovine Serum Albumin in phosphate buffer saline (pH7.4) for 1 h at room temperature. Then 100 µl of 25nM Biotinylated c-di-GMP (Biolog) was added to each well and incubated at room temperature for 2 h. 100 µl of 1:15,000 HRP-conjugated streptavidin was added to the wells and incubated at room temperature for 1 h. The wells were washed three times with 200 µl phosphate buffer saline (pH7.4) containing Tween 20 (PBST) in between every step. Then, 100 µl of Pierce TMB substrate (ThermoFisher Scientific) was added to each well and incubated for 30 minutes at room temperature. The colorimetric reaction was stopped by adding 100 µl 2M H_2SO_4. The absorbance was measured at 450nm using a plate reader. For the competition assay, 10 µl of 250nM unlabeled c-di-GMP (Biolog) was added to each well. For control of the competition assay, 10 µl sterile water was added to each of the samples.

Measurement of intracellular c-di-GMP levels

Intracellular c-di-GMP concentrations were determined by using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS), as previously described (Edmunds et al., 2013). Inoculation of overnight bacterial cultures were done 1:100 into 30 ml LB medium in a flask. Bacterial cells were harvested in 50 ml centrifuge tubes when OD_{600} of bacterial culture reached about 0.8

(mid- to late-exponential growth stage), for 30 min at 4000 r.p.m. After discarding the supernatant, the cell pellet was resuspended in 1.5 ml extraction buffer (40% acetonitrile–40% methanol in 0.1 N formic acid). The cells were resuspended in extraction buffer to release the c-di-GMP at −20°C overnight and then centrifuged at 13,000 r.p.m for 1 min. The supernatant was analyzed by HPLC-MS/MS at Michigan State University.

Statistical analysis

Statistical comparisons were made using one-way ANOVA using IBM SPSS 25 software (IBM, Armonk, NY). When ANOVA yielded significance ($P < 0{\cdot}05$); further analysis was performed using the Tukey's multiple comparisons test. Data are presented as means ± standard error of mean.

Results

Identification of a transposon mutant that alleviates the repression of Pel under high c-di-GMP condition

Previous studies demonstrated that EcpC positively regulates the pectate lyase and transcription of *pelD* through c-di-GMP, yet the effector(s) that perceive the c-di-GMP signal and execute the transcriptional regulation remains unknown (Yi et al., 2010; Yuan et al., 2020). To identify the potential c-di-GMP effector involved in this regulation, the promoter of *pelD* was cloned to a *gfp* (green fluorescent protein) reporter plasmid and subsequently transferred into a Δ*ecpC* mutant. As EcpC is a phosphodiesterase that degrades c-di-GMP, the expression of *pelD* was significantly reduced in the *ecpC* mutant compared to the wild type (Yi et al., 2010) (Fig 2.1A). Next, we constructed a transposon library in the Δ*ecpC* mutant carrying the *pelD*

promoter-*gfp* fusion (P_{pelD}::*gfp*) and screened for mutants with *pelD* transcription restored to the wild type level by monitoring the GFP intensity. Out of 1650 transposon mutants screened, 10 mutants restored *pelD* expression in Δ*ecpC* at a similar level as in the wild type (higher expression relative to Δ*ecpC*) (Table 3). Two of the 10 mutants contained transposon insertions in the open reading frames of *vfmE* (ABF-0016073) and *vfmP* (ABF-0019406). As these genes are quorum-sensing regulators, we further analyzed the functions of these genes in c-di-GMP sensing and virulence regulation.

To confirm our transposon mutagenesis data, we generated mutants by deleting *vfmE* or *vfmP* in the wild type and Δ*ecpC* backgrounds and then tested the expression of *pelD* using a promoter fluorescence reporter. We observed reduced *pelD* expression in *vfmE* and *vfmP* single deletion mutants (Fig.2.1A). This suggests that VfmE and VfmP positively regulate *pelD* gene expression, which is consistent with previous research on *vfm* regulons (Nasser et al., 2013). Similar to the Δ*ecpC vfmE* transposon mutant, an increased expression of *pelD* was also observed in the Δ*vfmE* Δ*ecpC,* and Δ*vfmP* Δ*ecpC* double deletion mutants (Fig.2.1A). The altered *pelD* expression could be complemented in Δ*vfmE* and Δ*vfmE* Δ*ecpC* mutants by introducing the *vfmE* gene with its native promoter cloned in pCL1920, a low copy number plasmid (Fig 2.1B). A similar trend was observed with extracellular Pel activity. The total extracellular Pel activity was reduced in Δ*ecpC* and Δ*vfmE* mutants, but Δ*vfmE* Δ*ecpC* showed a restored Pel activity similar to the WT level (Fig 2.3B). Thus, although VfmE behaves as a positive regulator of PelD in WT *D. dadantii*, VfmE negatively regulates *pelD* expression in Δ*ecpC* background, which reveals a complex role of VfmE in pectate lyase regulation. Since deletion of *vfmE* and double deletion of *vfmE* and *ecpC* showed opposite effects on *pelD* expression, we carried out virulence assays on potatoes. Consistent with the *pelD* expression results, Δ*ecpC*

showed reduced necrotic tissue formation compared to the wild type in potato (Figs 2.2A-B). Reduced necrosis of potato tissues in the Δ*vfmE* single deletion mutant was observed, but more necrosis was observed in the Δ*vfmE* Δ*ecpC* double mutant that was on par with the WT strain. The virulence phenotypes observed in Δ*vfmE* and Δ*vfmE* Δ*ecpC* mutants could be complemented by the expression of *vfmE* gene in-trans (Figs 2.2A-B). The above data conclude that Δ*vfmE* and Δ*ecpC* reduce the Pel activity, but Δ*vfmE* Δ*ecpC* double mutant restores the Pel production to the WT level. In addition, in-trans expression of *vfmE* can complement the phenotypes under low c-di-GMP background but fails to complement the phenotypes under high c-di-GMP background.

VfmE controls pectate lyase activity through the transcriptional regulator SlyA

SlyA, a regulator of the SlyA/MarR family, was reported to be an important regulator of virulence genes, and it upregulates pectate lyase production in *D. dadantii* (Haque et al., 2009; Reverchon et al., 2010). In *Dickeya zeae*, VfmE positively regulates *slyA* (Lv et al., 2019). In order to elucidate whether *slyA* is involved in the pathway of *pelD* regulation by VfmE, we checked the promoter activity of *slyA* in Δ*vfmE* mutants. We found that Δ*vfmE* and Δ*vfmP* single deletion mutants showed a reduced *slyA* promoter activity (Fig 2.3). The *ecpC* single mutant also showed reduced *slyA* activity. Surprisingly, double deletion of *vfmE/vfmP* and *ecpC* did not show a further reduction in *slyA* promoter activity but rather restored the *slyA* expression back to the WT level (Fig 2.3A). This suggests that even though VfmE/VfmP positively regulates *slyA* transcriptionally in the wild type background, such positive regulation was reversed to become a negative regulation when *ecpC* was mutated, which is analogous to the *pelD* expression observed in these strains (Fig 2.1A).

Since the expression of *pelD* and *slyA* both showed inverse phenotypes in Δ*vfmE* and Δ*vfmE* Δ*ecpC* mutant backgrounds, we investigated whether the restored *slyA* transcription in Δ*vfmE* Δ*ecpC* was the reason for the restored extracellular Pel activity. Chromosomal deletion mutants of *slyA* were constructed in WT, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* backgrounds, and the total extracellular *pel* activity was measured. Compared to the WT, reduced *pel* activities were detected in Δ*ecpC,* Δ*vfmE*, Δ*slyA,* Δ*slyA* Δ*ecpC,* and Δ*vfmE* Δ*slyA* mutants. The reduced *pel* activity in single deletion mutants of *vfmE* and *ecpC* was restored to the WT level in the Δ*vfmE* Δ*ecpC* double mutant. However, such restoration was abolished by an additional mutation of *slyA* in the triple deletion mutant Δ*vfmE* Δ*slyA* Δ*ecpC*, which showed further reduction in Pel activity compared to Δ*ecpC,* Δ*vfmE*, Δ*slyA,* Δ*slyA* Δ*ecpC,* Δ*vfmE* Δ*slyA* mutants (Fig 2.3B). These data suggests that the upregulation of *pelD* promoter activity or total Pel activity in the Δ*vfmE* Δ*ecpC* double mutant could be attributed to the increased transcription of *slyA.*

VfmE is regulated by cellular c-di-GMP regulatory system

As EcpC is a phosphodiesterase that degrades intracellular c-di-GMP, the intracellular c-di-GMP level in Δ*ecpC* is 2 times higher than the WT as previously determined (Yuan et al., 2015). Under such a condition, the *vfmE* promoter activity was significantly reduced in the Δ*ecpC* mutant than in the WT. The promoter activity of *vfmE* was validated in Δ*vfmP* and in Δ*vfmE*; in both strains, the *vfmE* promoter activity showed a drastic reduction as compared to the WT. This is expected because VfmP is the synthase of the quorum-sensing signaling molecule, and VfmE, in the presence of the quorum-sensing signaling molecules, activates its own transcription (Nasser et al., 2013). The Δ*vfmE* Δ*ecpC* mutant showed a similar level of *vfmE* transcription as

of the Δ*ecpC* mutant (Fig 2.5). These data indicate that the transcription of *vfmE* was regulated by EcpC and VfmE via the same pathway. We observed a lower *vfmE* transcription in Δ*vfmP* mutant compared to Δ*vfmE* because Δ*vfmP* mutant shuts off the entire Vfm quorum-sensing system and production of QS molecules. However, under Δ*vfmE* mutant, a smaller amount of VfmH is produced (Nasser et al., 2013), which can transcribe *vfmE* promoter at a lower level.

Multiple sequence alignment revealed a potential c-di-GMP binding RxxxR motif in VfmE

We hypothesized that VfmE is a c-di-GMP binding protein, the function of which is highly dependent on the intracellular c-di-GMP concentrations. Also, as the *vfmE* promoter is regulated by VfmE itself, the repression of *vfmE* transcription by high c-di-GMP (Δ*ecpC*) can be potentially through inactivation of the activator VfmE. Many proteins, such as YcgR in *Escherichia coli* and CuxR in *Sinorhizobium meliloti*, bind to c-di-GMP through the "RxxxR" motif (Boyd & O'Toole, 2012; Hengge, 2009; Nieto et al., 2019; Römling et al., 2013; Schäper et al., 2017). To determine if such "RxxxR" signature motif also exist in VfmE, multiple sequence alignment of VfmE (WP_013320004.1) with other known "RxxxR" motif containing c-di-GMP binding proteins Alg44 (WP_134306546.1), CuxR (WP_010975317.1), DgrA (WP_003230551.1), BcsA (NP_417990.4), and YcgR (NP_415712.1) was performed. Although an RxxxR sequence was identified in VfmE, the amino acid sequences flanking the motif are highly variable (Fig 2.6A). We used the AraC domain of VfmE to search against the NCBI protein database and have identified multiple homologues in various species, with only a minority of them containing the RWIWR motif (Fig 2.6B). This suggests that the RWIWR motif may have evolved in the AraC family regulators

to adapt these proteins to bind c-di-GMP. Using a protein structure predicting tool Phyre2, the RWIWR motif was identified at the N-terminal end of the protein. A helix-turn-helix domain, with a putative DNA binding function, was identified at the C-terminal end (Fig 2.6C).

c-di-GMP binds to WT VfmE but not VfmE^{R93D}

To determine if VfmE indeed contains c-di-GMP binding ability, we performed a modified Enzyme-Linked Immunosorbent Assay (ELISA) (Underwood et al., 2014). In brief, wells of an ELISA plate were first coated with VfmE protein, followed by biotinylated c-di-GMP. The binding of the biotin-labeled c-di-GMP was detected by HRP-conjugated streptavidin with TMB coloring substrate. HRP produced a measurable color change of TMB, and the color intensity was detected by a plate reader at 450 nm. For this experiment, a known c-di-GMP effector YcgR from *D. dadantii* was used as a positive control (Yuan et al., 2015), and Maltose Binding Protein (MBP) was used as a negative control (Roelofs et al., 2011). Wells containing only sterile water were used for the reference of background binding. We observed a high level of 450nm absorbance in VfmE and YcgR-treated wells and a low level of 450 nm absorbance in the MBP and water controls (Fig 2.7A). To confirm the specificity of the c-di-GMP binding, we performed a competition assay where we added an equal concentration of unlabeled c-di-GMP to the wells. We observed a reduced absorbance in both VfmE and YcgR-treated wells, whereas no reduction was observed in MBP and water-treated wells. This result reveals that VfmE binds c-di-GMP with specificity comparable to a known c-di-GMP effector YcgR.

To determine if the identified RWIWR motif is essential for the c-di-GMP binding of VfmE, we constructed a single amino acid substitution at the 93rd position of VfmE,

which changed the RWIWR motif to RWIWD. The purified VfmE^{R93D} protein was tested in the c-di-GMP binding assay. Compared to a high 450 nm absorbance observed in YcgR and VfmE treated wells, a reduced 450 nm absorbance was observed in VfmE^{R93D} treated wells at a similar level of the MBP and water controls (Fig 2.7B). This confirms that the Arg^{93} motif in VfmE is indeed essential for c-di-GMP binding.

VfmE^{R93D} recovers the Pel production regardless of high c-di-GMP condition

To investigate the regulatory mechanism of VfmE on *pel* expression under a high c-di-GMP condition, we tested the *pelD* promoter activities of wild type, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* mutants and complementing them with *vfmE*^{WT} or *vfmE*^{R93D} cloned into plasmid pCL1920 under the native promoter of *vfmE*. Consistent with previous results, Δ*ecpC* and Δ*vfmE* showed reduced *pelD* expression compared to WT, and Δ*vfmE* Δ*ecpC* showed expression on par with WT. The plasmid containing *vfmE*^{WT} gene complemented the *pelD* expression in Δ*vfmE* to WT level and Δ*vfmE* Δ*ecpC* to Δ*ecpC* level. However, *pelD* expression was on par with WT level when the plasmid containing *vfmE*^{R93D} was transformed into Δ*vfmE* Δ*ecpC,* suggesting VfmE^{R93D} recovered the *pelD* expression regardless of the high c-di-GMP condition in Δ*ecpC* (Fig 2.8A).

We further confirm the finding by performing the extracellular Pel activity. The total extracellular Pel activity was measured by spectrophotometry using 16 h cultures (Matsumoto et al., 2003). We observed reduced Pel activities for Δ*ecpC* and Δ*vfmE* mutants. The Pel activity in Δ*vfmE* Δ*ecpC* was higher than either Δ*ecpC* or Δ*vfmE* mutant and on par with WT level. The Pel activity was complemented in Δ*vfmE* (to WT level) and Δ*vfmE* Δ*ecpC* (to Δ*ecpC* level) with the plasmid containing *vfmE*^{WT} gene. Similar to the *pelD* expression result, *vfmE*^{R93D} recovered the Pel activity in Δ*vfmE*

and Δ*vfmE* Δ*ecpC* to WT level regardless of high c-di-GMP condition (Fig 2.8B). The above results demonstrated that VfmE is a positive regulator of Pel activity in *D. dadantii,* and the c-di-GMP binding motif mediates binding to c-di-GMP to convert VfmE to a repressor at higher c-di-GMP concentrations.

Since in-trans expression of $vfmE^{R93D}$ recovered the Pel activity to WT levels regardless of the high c-di-GMP condition (Δ*ecpC*), we checked the effect on virulence by infection assay in potato. Similar to the result in Pel activity, $vfmE^{R93D}$ recovered the virulence in *ΔvfmE* Δ*ecpC* to WT level regardless of high c-di-GMP background (Fig 2.2). This further confirms that the Arg^{93} in VfmE is essential to repress virulence under high c-di-GMP conditions. To check the survival of the mutants in the host, the fluid from the necrotic tissue was collected, and the colony forming units (CFU) of each of the strains was measured. We observed that Δ*ecpC* and Δ*vfmE* mutant had 10-fold lower CFU in the host. Similar to the finding of Pel activity, Δ*vfmE* Δ*ecpC* had CFU similar to the WT. Complementation with *vfmE* in the Δ*vfmE* Δ*ecpC* mutant resulted in CFU similar to Δ*ecpC,* while complementation with $vfmE^{R93D}$ in this strain resulted in CFU similar to WT (Fig 2.2C). These results show that c-di-GMP binding is important for VfmE function *in vivo*. VfmE takes part in virulence and survival in the host, and the c-di-GMP binding motif in VfmE is a key determinant of VfmE function in response to c-di-GMP.

Discussion

Although in a previous study, a single knockout of *vfmE* decreased the expression of *pel* genes (Nasser et al., 2013), transposon insertion into *vfmE* in Δ*ecpC* background unexpectedly recovered the reduced *pelD* promoter activity to WT level (Figs 2.1A-B). Previously, a MarR family transcriptional regulator SlyA was reported to positively

regulate Pel production and virulence in *D. dadantii* (Haque et al., 2009; Reverchon et al., 2010) and was regulated by VfmE positively in *D. zeae* (Lv et al., 2019). Consistent with the previous finding, *vfmE* positively regulates *slyA* transcription. However, similar to the finding of *pelD* expression in Δ*vfmE* Δ*ecpC* mutant, the *slyA* expression was also recovered to WT level in Δ*vfmE* Δ*ecpC* compared to Δ*vfmE* or Δ*ecpC* single mutants (Fig 2.3A). We suggest that the recovery of Pel activity in Δ*vfmE* Δ*ecpC* double mutant was due to upregulated *slyA* transcription. The Δ*vfmE,* Δ*slyA,* and Δ*vfmE* Δ*slyA* mutants showed a similar level of Pel activities, indicating that VfmE and SlyA are in the same regulatory pathway. Further, deletion of *slyA* in Δ*ecpC* background did not recover the Pel activities as seen in the Δ*vfmE* Δ*ecpC* mutant (Fig 2.3B), and deletion of *slyA* in Δ*vfmE* Δ*ecpC* mutant suppressed the recovery phenotype of Δ*vfmE* Δ*ecpC* mutant in the Pel activity (Fig 2.3B). These results suggest that VfmE positively regulates Pel activity in low c-di-GMP conditions through activation of SlyA, and this function is inhibited under high c-di-GMP conditions (i.e., Δ*ecpC*) (Fig. 2.9). We speculate the presence of an additional unidentified transcriptional activator of *slyA* which is repressed by VfmE under high c-di-GMP condition but can activate *slyA* when *vfmE* is deleted under Δ*ecpC* mutant.

A reduced pectate lyase activity was observed in the triple mutant, Δ*vfmE* Δ*slyA* Δ*ecpC*, compared to the double mutant Δ*vfmE* Δ*slyA*. It is possible that the decreased pectate lyase synthesis is caused by high c-di-GMP levels due to the *ecpC* mutation in the triple mutant. Furthermore, the differences in Pel activities in Δ*vfmE* and Δ*slyA* mutants suggest that VfmE regulates pel transcription not only via *slyA* but also via an additional regulator, although these differences are not statistically significant. SlyA plays an important role in this regulatory pathway because losing *slyA* suppresses Pel activity and cannot be recovered in *vfmE* and *ecpC* double deletion. We speculate

that *slyA* expression is increased if *vfmE* is deleted in high c-di-GMP condition (Δ*ecpC*); however, further investigation is required to resolve this linkage. The smaller maceration areas in the *vfmE* and *ecpC* mutants were observed because of lower pectate lyase production, although we cannot rule out the possibility that the slower growth of the *vfmE* mutant in the potato may also be a partial cause.

As VfmE is an activator of its own transcription (Nasser et al., 2013) and Δ*vfmE* and Δ*vfmE* Δ*ecpC* mutants showed similar *vfmE* transcription, we hypothesize that under high-c-di-GMP conditions, c-di-GMP binding inhibits VfmE activity. Previous research indicated that AraC family transcriptional regulators can bind c-di-GMP via RxxxR motif (Schäper et al., 2017), and a conserved RWIWR motif was also found in VfmE by multiple sequence alignment (Fig 2.6A). The c-di-GMP binding ELISA experiment shows that VfmE binds c-di-GMP (Fig 2.7A). Further, swapping the Arg^{93} residue to an aspartate residue in the $RWIWR^{93}$ motif ($RWIWD^{93}$) abolishes the binding of VfmE to c-di-GMP (Fig 2.7B). Our in vitro ELISA binding experiments and in vivo phenotypic assay are consistent with VfmE binding c-di-GMP via the RWIWR motif, similar to the PilZ-like mechanism.

VfmE was previously reported to regulate several DGCs and PDEs in *D. zeae* (Lv et al., 2019). Our results show that VfmE positively regulates a DGC *gcpA* (Fig 2.4A) and a PDE *egcpB* at the transcriptional level (Fig 2.4B) but does not directly regulate PDE *ecpC* transcription (Fig 2.4C). Although VfmE was found to control some c-di-GMP regulatory genes, the intracellular c-di-GMP level remains unaffected in Δ*vfmE* mutant (Fig 2.4D). This implies that VfmE does not have a dominant role in regulating overall cellular c-di-GMP levels in the conditions tested.

High c-di-GMP levels have been previously reported to antagonize quorum-sensing in *Burkholderia cenocepacia* H111 and *Pseudomonas sp.* (Liang et al., 2020;

Lin Chua et al., 2017; Schmid et al., 2017). In this study, we observed that the high c-di-GMP concentration caused by deletion of *ecpC* represses *vfmE* transcription (Fig 2.5). Since VfmE is a self-activator, the above observation indicates that c-di-GMP inhibits the transcription of *vfmE* via VfmE. Reduction in *vfmE* transcription indicates reduction of the transcription levels of the *vfm* gene cluster (Nasser et al., 2013). This trend is consistent with our observations of c-di-GMP and VfmE on *pel* transcription (Fig. 2.9). Since the transcription of *vfmE* is dependent on the vfm quorum-sensing, low transcription of *vfmE* is an indication of reduced vfm quorum-sensing under *ecpC* deletion mutant (high c-di-GMP condition). This hypothesis is verified by the mutant $VfmE^{R93D}$ that is able to recover the Δ*vfmE* phenotypes in normal c-di-GMP levels and in a high c-di-GMP environment of Δ*ecpC* background (Figs 2.8A-C). $VfmE^{R93D}$ displays the same molecular function as $VfmE^{WT}$ but is insensitive to c-di-GMP, which does not allow the cells to appropriately respond to the high c-di-GMP environment. This also suggests that the C-terminal helix-turn-helix domain of VfmE can work independently to activate its regulons while the role of c-di-GMP binding motif RWIWR is for suppression of the activity.

We propose that Vfm signal induces the transcription of *vfmE* to regulate Pel production, and high c-di-GMP levels suppress VfmE activity through the binding of RWIWR. Thus, VfmE is one of the activators of Pel but acts as a repressor when the c-di-GMP concentration is high. c-di-GMP is a ubiquitous bacterial second messenger that regulates multiple cellular functions, such as virulence. Given that VfmE is a key member in Vfm quorum-sensing, this study has provided a strong link between quorum-sensing and c-di-GMP signaling to mediate various responses to environmental changes.

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Table 1 List of strains and plasmids

Strains and plasmids	Relevant characteristics[a]	Reference or source
Dickeya dadantii		
3937	Wild type	(Hugouvieux-Cotte-Pattat, N.)
Δ*ecpC*	Δ*ecpC*, ABF-0020364 deletion mutant	(Yi et al., 2010)
Δ*vfmE*	Δ*vfmE*::*Km;* Km^r, ABF-0016073 deletion mutant	This study
Δ*vfmP*	Δ*vfmP*::*Km;* Km^r, ABF-0019406 deletion mutant	This study
Δ*vfmE* Δ*ecpC*	Δ*vfmE* Δ*ecpC*::*Km;* Km^r, ABF-0016073 and ABF-0020364 double deletion mutant	This study
Δ*vfmP*Δ*ecpC*	Δ*vfmP*Δ*ecpC*::*Km;* Km^r, ABF-0019406 and ABF-0020364 double deletion mutant	This study
Δ*slyA*	Δ*slyA*::*Km;* Km^r, ABF-0015312 deletion mutant	This study
Δ*vfmE*Δ*slyA*	Δ*vfmE*Δ*slyA*::*Km;* Km^r, ABF-0016073 and ABF-0015312 double deletion mutant	This study
Δ*ecpC*Δ*slyA*	Δ*ecpC*Δ*slyA*::*Km;* Km^r, ABF-0020364 deletion and ABF-0015312 double deletion mutant	This study
Δ*vfmE*Δ*ecpC*Δ*slyA*	Δ*vfmE*Δ*ecpC*Δ*slyA*::*Km;* Km^r, ABF-0016073, ABF-0020364 and ABF-0015312 triple deletion mutant	This study
Escherichia coli		
DH5α	*supE*44 Δ*lac*U169 (*ϕ*80*lacZ*ΔM15) *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
NEB Express®	*fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10*--TetS*)2 [dcm] R(zgb-210::Tn10*--TetS*) endA1 Δ(mcrC-mrr)114::IS10*	New England Biolabs
Plasmids		
pKD4	Template plasmid for kanamycin cassette, Km^r	(Datsenko & Wanner, 2000)
pWM91	Sucrose-based counter-selectable plasmid, Ap^r	(Metcalf et al., 1996)
pWM91: *vfmE*	pWM91 harboring flanking regions of *vfmE* with kanamycin cassette in between, Km^r, Ap^r	This study
pWM91: *vfmP*	pWM91 harboring flanking regions of *vfmP* with kanamycin cassette in between, Km^r, Ap^r	This study
pWM91: *slyA*	pWM91 harboring flanking regions of *slyA* with kanamycin cassette in between, Km^r, Ap^r	(Zou et al., 2012)
pET21b	Overexpression and purification vector, Ap^r	Novagen

pET21b: *ycgR*$_{3937}$	Overexpression of *ycgR*$_{3937}$ in expression vector	(Yuan et al., 2015)
pMAL-c6t	Overexpression and purification vector, Ap^r	New England Biolabs
pMAL-c6t: *vfmE*	Overexpression of *vfmE* in expression vector	This study
pMAL-c6t: *vfmE*^{R93D}	Overexpression of *vfmE*^{R93D} in expression vector	This study
pCL1920	Low copy number plasmid, lac promoter, Sp^r	(Lerner & Inouye, 1990)
pCL-*vfmE*	*vfmE* with natural promoter cloned in pCL1920, Sp^r	This study
pCL- *vfmE*^{R93D}	*vfmE*^{R93D} with natural promoter cloned in pCL1920, Sp^r	This study
pPROBE-AT	Promoter-probe vector, promoter-less *gfp*, Ap^r	(Miller et al., 2000)
pAT-*pelD*	pPROBE-AT containing *pelD* promoter-*gfp* transcriptional fusion, Ap^r	(Peng et al., 2006)
pAT-*gcpA*	pPROBE-AT containing *gcpA* promoter-*gfp* transcriptional fusion, Ap^r	Lab stock
pAT-*egcpB*	pPROBE-AT containing *egcpB* promoter-*gfp* transcriptional fusion, Ap^r	(Yi et al., 2010)
pAT-*ecpC*	pPROBE-AT containing *ecpC* promoter-*gfp* transcriptional fusion, Ap^r	(Yi et al., 2010)
pAT-*slyA*	pPROBE-AT containing *slyA* promoter-*gfp* transcriptional fusion, Ap^r	(Zou et al., 2012)
pFLP2	Plasmid containing the *flp* (flippase) gene, Amp^r	Lab stock

[a]Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sp^r, streptomycin resistance

Table 2 List of primers

Primers	Sequences (5'-3')	Amplicon
vfmE-A-XhoI	AATACTCGAGTCGTTTCCTGTTCATCTGC	*vfmE* deletion
vfmE-B	GAAGCAGCTCCAGCCTACACCGGTGTCATCC	
vfmE-C	CTAAGGAGGATATTCATATGTCTGTAATTAA	
vfmE-D-NotI	AATATTATGCGGCCGCTCGGCAAAATGATCGAC	
vfmP-A- XhoI	AATACTCGAGGCTGGGTTATGTGCGC	*vfmP* deletion
vfmP-B	GAAGCAGCTCCAGCCTACACCTTCTACCCGACGTCCTAGC	
vfmP-C	CTAAGGAGGATATTCATATGACCGGCCCGTTTCC	
vfmP-D- NotI	AATATTATGCGGCCGCTTCAAAGCAGATGTGGTA	
vfmE-R93D-1	TGCGCTGGATTTGGGACGTACGCCTGACGC	*vfmE* site-directed mutant
vfmE-R93D-2	GCGTCAGGCGTACGTCCCAAATCCAGCGCA	
vfmE-for-BamHI	TATCGTCGACGGATCCATGAGCTTGCAGAACACCTACG	*vfmE* overexpression
vfmE-rev-HindIII	GTTTTATTTGAAGCTTATTACAGAGGTTCTGGATATTATCCAGCAG	
vfmE-for-XbaI	AATATCTAGATGACTGGCCCTTCCGCTGA	*vfmE* complementation
vfmE-rev-HindIII	TTATAAGCTTATACCGCCACTTATTCAGT	
vfmE-p1-SalI	AATAGTCGACCTTTTTCCAGCGCCTGAATGACCCGCGC	*vfmE* promoter
vfmE-p2-EcoRI	TTATGAATTCCGTATGGGCGTAGGTGTTCTGCAAGCTC	
P1	GCGATTGTGTAGGCTGGAGCTGCTTC	Kanamycin cassette amplification from pKD4 plasmid
P2	GCTGACATGGGAATTAGCCATGGTCC	

Table 3 Transposon insertion mutant list

Mutant code	Identified genes	Alternate ID	ASAP ABF number
E1-28	D-alanine—poly(phosphoribitol) ligase subunit 1	*vfmP*	ABF-0019406
E5-24	DNA phosphorothioation-specific restriction system protein	*dptH*	ABF-0017224
E6-8	AraC family transcription regulator	*vfmE*	ABF-0016073
E10-16	Hypothetical protein	-	ABF-0046736
E12-26	Cold shock protein associated with 30S ribosomal subunit	*raiA*	ABF-0016995
E15-20	Permease component of an ABC superfamily amino acid transporter	-	ABF-0014997
E16-2	Short-chain dehydrogenase/reductase family oxidoreductase	-	ABF-0019913
E16-20	Short-chain dehydrogenase/reductase family oxidoreductase	-	ABF-0019913
E27-16	Permease/ATP-binding component of an ABC superfamily cysteine transporter	*cydD*	ABF-0020036
E29-29	Starvation sensing protein	*rspB*	ABF-0017131

Figures

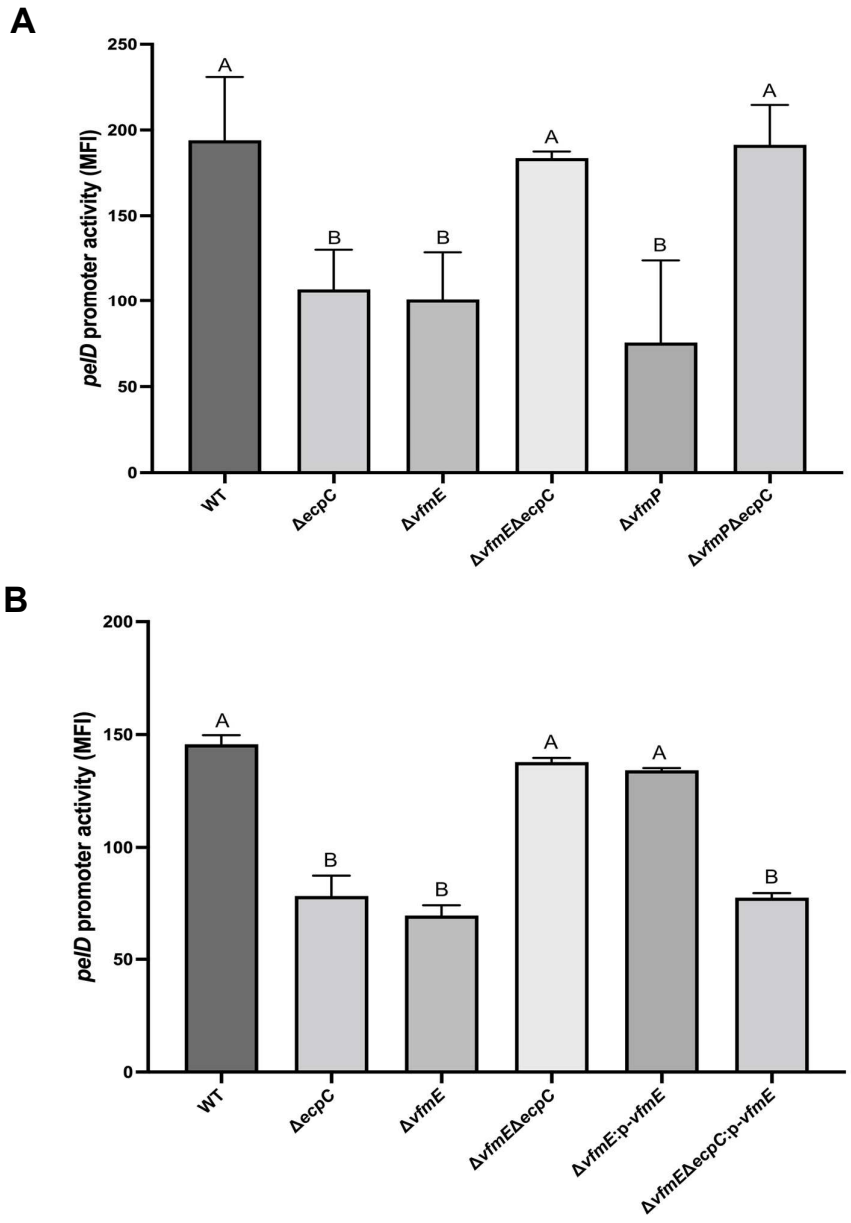

Fig. 2.1. Deletion of *vfmE* and *vfmP* in wild type (WT) decreases *pelD* promoter activity; on the other hand, deletion of both *vfmE* and *vfmP* under high c-di-GMP background (*ΔecpC*) restores the *pelD* promoter activity to WT level.

The pPROBE-AT plasmid harboring *pelD*-GFP promoter region was measured by flow cytometry. (A) *pelD* promoter activity in wild type, Δ*ecpC,* Δ*vfmE,* Δ*vfmE* Δ*ecpC,*

Δ*vfmP,* and Δ*vfmP* Δ*ecpC* was measured. (B) Complementation of *pelD* promoter activity in wild type, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* harboring low copy number empty pCL1920 plasmid (controls) and Δ*vfmE* and Δ*vfmE* Δ*ecpC* strains harboring pCL1920:*vfmE.* Mean Fluorescence Intensity (MFI); average GFP fluorescence intensity of total bacterial cells examined. The MFI is the log fluorescence intensity given by the Cell Quest software (BD Biosciences, San Jose, CA). Values are representative of three experiments, and three replicates were used for each experiment. The cultures were grown in MM medium for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

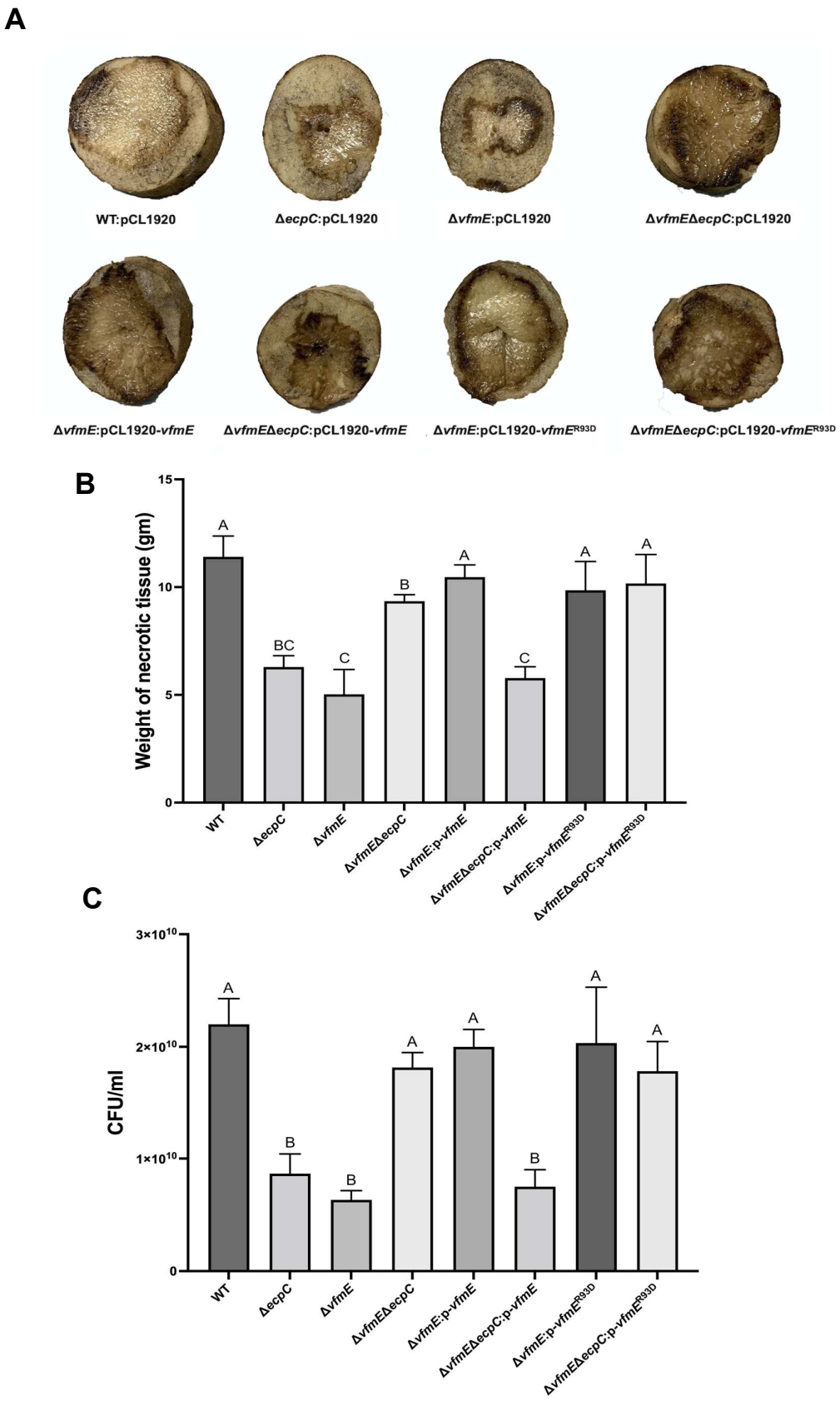
A
WT:pCL1920
ΔecpC:pCL1920
ΔvfmE:pCL1920
ΔvfmEΔecpC:pCL1920
ΔvfmE:pCL1920-vfmE
ΔvfmEΔecpC:pCL1920-vfmE
ΔvfmE:pCL1920-vfmER93D
ΔvfmEΔecpC:pCL1920-vfmER93D
B
Weight of necrotic tissue (gm)
15
10
5
0
A
BC
C
B
A
C
A
A
WT
ΔecpC
ΔvfmE
ΔvfmEΔecpC
ΔvfmE:p-vfmE
ΔvfmEΔecpC:p-vfmE
ΔvfmE:p-vfmER93D
ΔvfmEΔecpC:p-vfmER93D
C
CFU/ml
3×10^10
2×10^10
1×10^10
0
A
B
B
A
A
B
A
A
WT
ΔecpC
ΔvfmE
ΔvfmEΔecpC
ΔvfmE:p-vfmE
ΔvfmEΔecpC:p-vfmE
ΔvfmE:p-vfmER93D
ΔvfmEΔecpC:p-vfmER93D

Fig. 2.2. Deletion of *vfmE* in wild type (WT) reduces the maceration area in potato; on the other hand, deletion of *vfmE* under high c-di-GMP background (*ΔecpC*) restores the maceration area to WT level.

The maceration areas caused by *D. dadantii* strains (A) Observed in potato host by wild type, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* and the corresponding complementation with pCL1920 plasmid harboring *vfmE* and *vfmE*^{R93D} with its natural promoter. (B) The weights of the necrotic tissues measured in WT, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* mutants and the complementation strains. Values are representative of two experiments, and three replicates were used for each experiment. (C) Bacterial survival rate in host**.** The CFU of bacterial cells was measured from the necrotic tissue fluid. Values are representative of two independent experiments, and three replicates were used for each experiment. The potato slices with inoculated strains were incubated for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

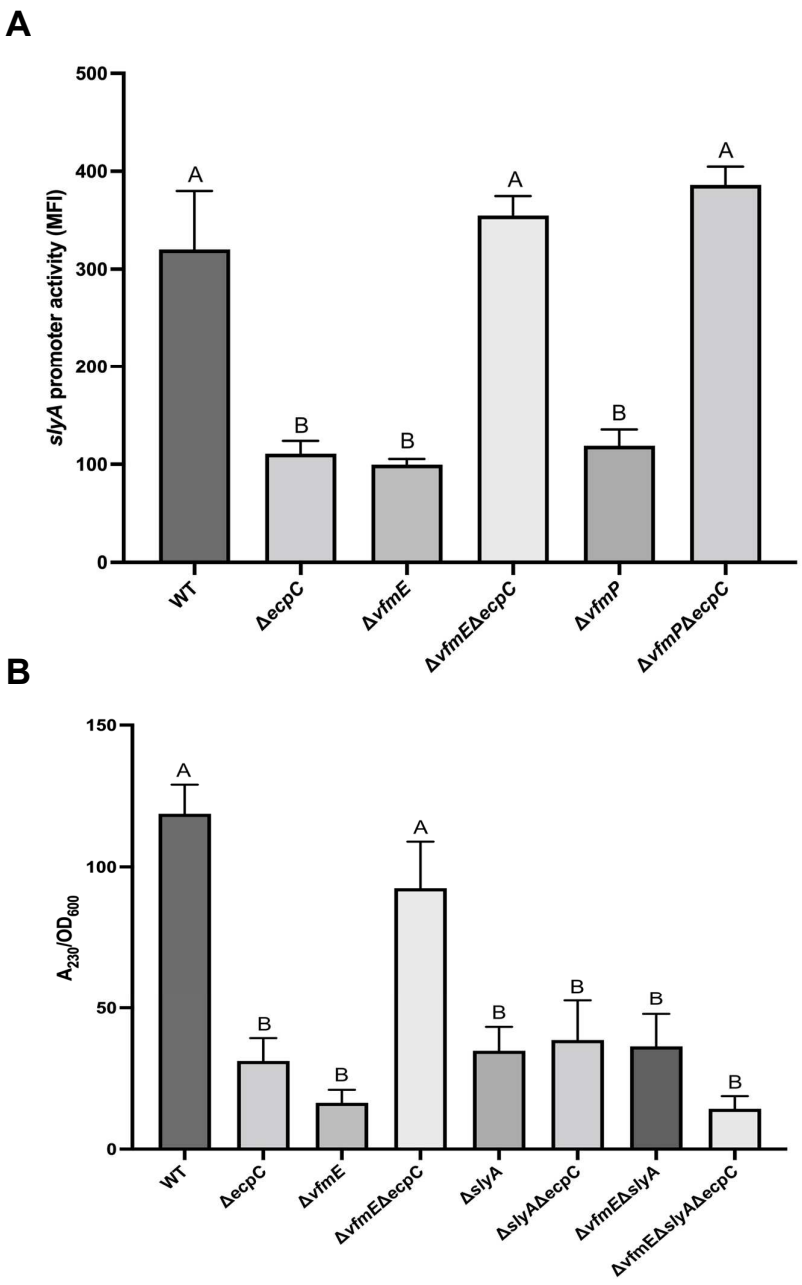

Fig 2.3. Regulation of Pel by VfmE involves *slyA.*

(A) The pPROBE-AT plasmid harboring *slyA*-GFP promoter region was measured by flow cytometry. *slyA* promoter activity in wild type, Δ*ecpC,* Δ*vfmE,* Δ*vfmE* Δ*ecpC,* Δ*vfmP,* and Δ*vfmP* Δ*ecpC* was measured. Mean Fluorescence Intensity (MFI); average GFP fluorescence intensity of total bacterial cells examined. The experiment

was performed with three replicates. The cultures were grown in MM medium for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

(B) The total Pectate Lyase activity was measured by spectrophotometry. The total Pel activity in wild type, Δ*ecpC,* Δ*vfmE,* Δ*vfmE* Δ*ecpC,* Δ*slyA,* Δ*slyA* Δ*ecpC,* Δ*vfmE* Δ*slyA* and Δ*vfmE* Δ*slyA* Δ*ecpC* was measured. The OD_{230} value of Pel activity was normalized by OD_{600} value of the cell culture. Values are representative of two experiments, and three replicates were used for each experiment. The cultures were grown in LB medium for 16 h.Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

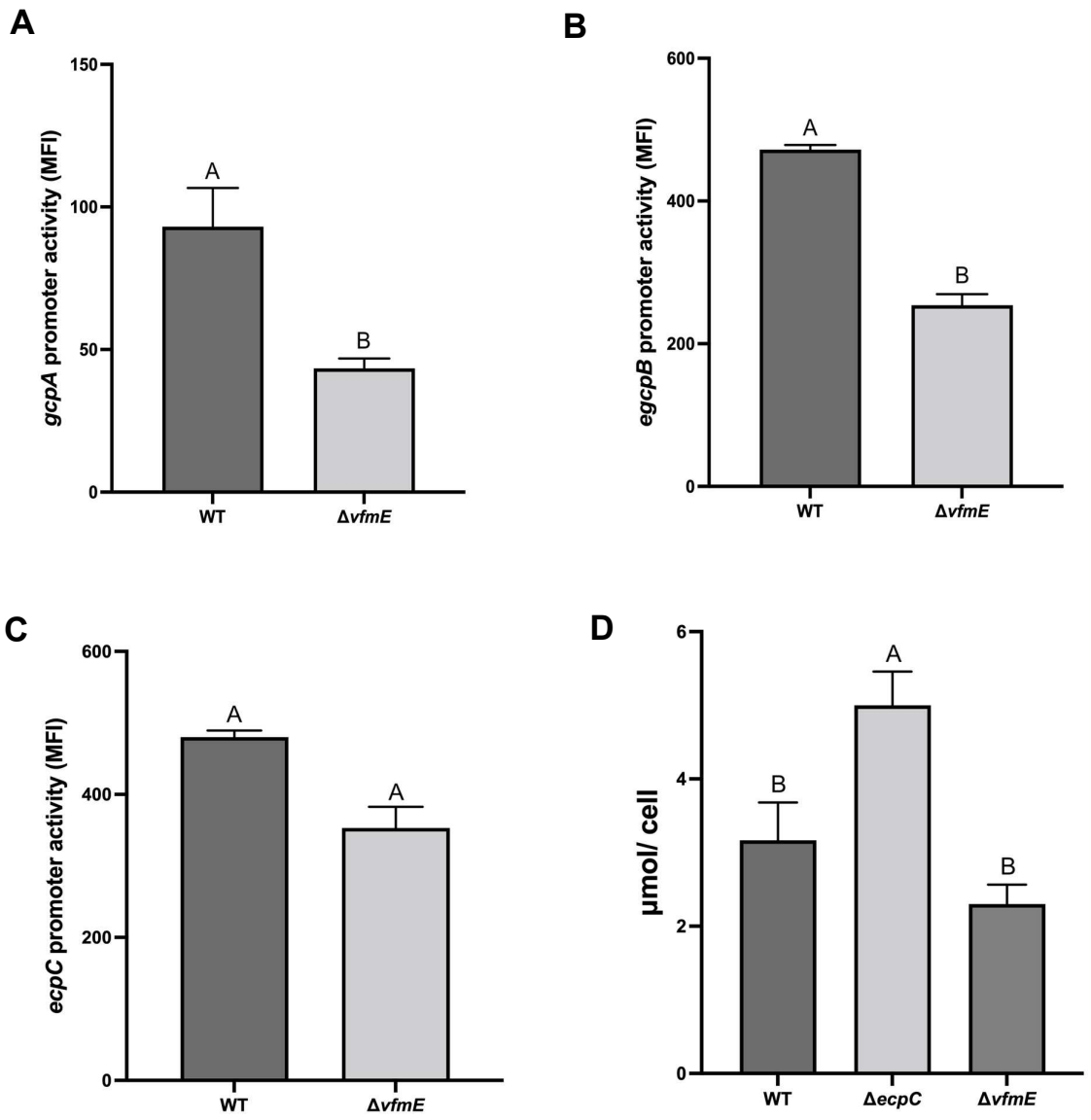

Fig. 2.4. VfmE affects the transcription of c-di-GMP modulating proteins

The promoter activities of pPROBE-AT plasmids harboring *gcpA*-GFP, *egcpB*-GFP, and *ecpC*-GFP promoter regions were measured by flow cytometry. (A) *gcpA* promoter activity in wild type and Δ*vfmE* mutant. (B) *egcpB* promoter activity in wild type and Δ*vfmE* mutant. (C) *ecpC* promoter activity in wild type and Δ*vfmE* was measured. (D) The intracellular c-di-GMP was measured by LC-MS method in wild type, Δ*ecpC,* and Δ*vfmE* mutants. Values are representative of four experiments, and three replicates were used for each experiment. Values of mean fluorescence intensity

(MFI) are an average green fluorescent protein fluorescence intensity of ~10,000 bacterial cells measured by flow cytometry. The cultures were grown in MM medium for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

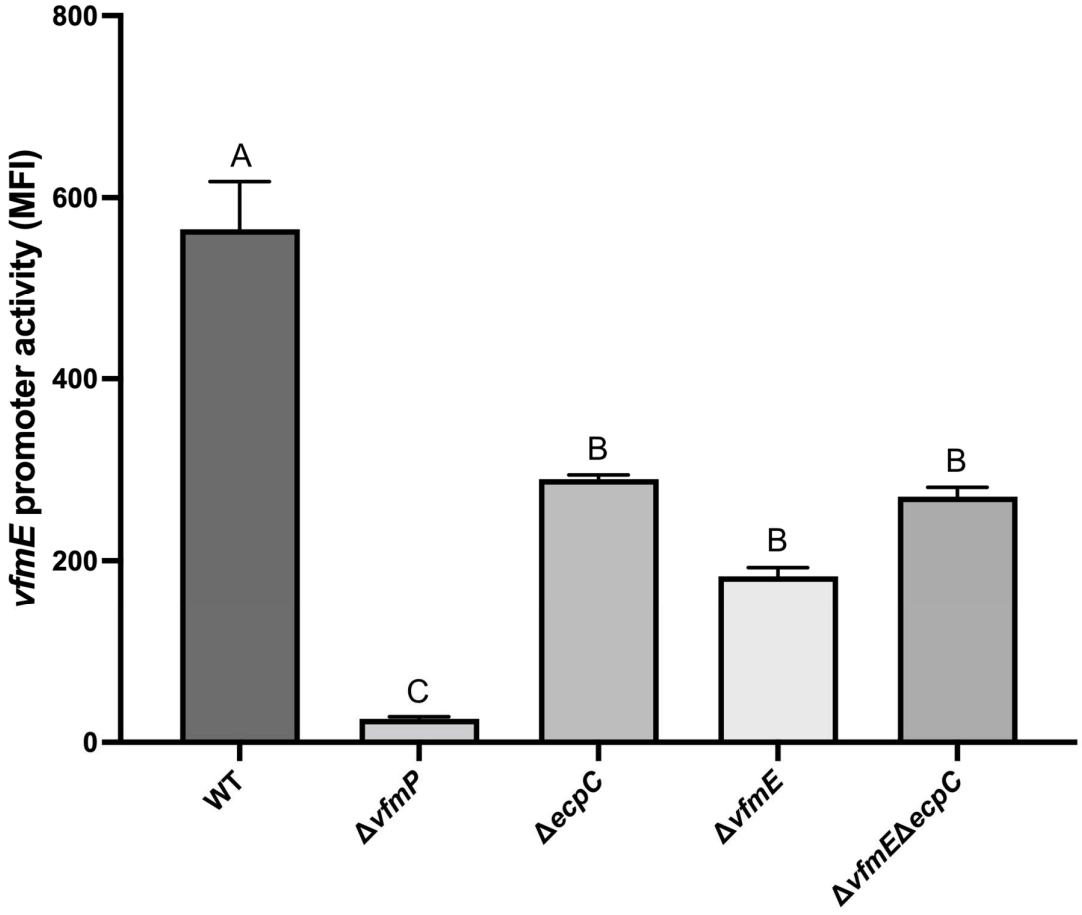

Fig. 2.5. EcpC regulates *vfmE* at the transcriptional level.

The promoter activity of pPROBE-AT plasmid harboring *vfmE*-GFP promoter region was measured by flow cytometry. The *vfmE* promoter activities were measured in wild type, Δ*vfmP,* Δ*vfmE,* Δ*ecpC,* and Δ*vfmE* Δ*ecpC* mutants. Mean Fluorescence Intensity (MFI); average GFP fluorescence intensity of total bacterial cells examined. The experiment was performed with three replicates. The cultures were grown in MM medium for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters for by one-way ANOVA with Tukey's posthoc test.

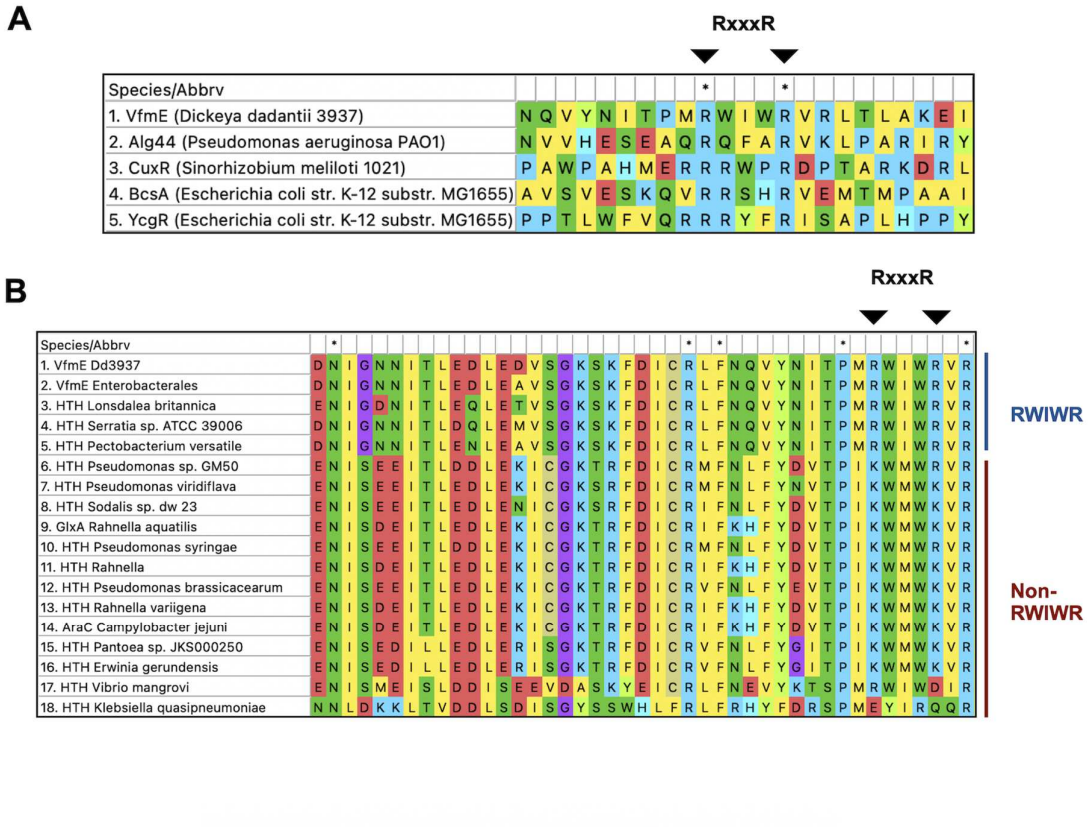

Fig. 2.6. The conserved RxxxR c-di-GMP binding motif of VfmE and other c-di-GMP binding proteins with RxxxR motifs.

(A) Denotes the multiple sequence alignment of VfmE (WP_013320004.1) with other known RxxxR motif containing c-di-GMP binding proteins Alg44 (WP_134306546.1), CuxR (WP_010975317.1), DgrA (WP_003230551.1), BcsA (NP_417990.4) and YcgR (NP_415712.1) showing aligned RxxxR motif. Asterisk marks indicate the conserved amino acid residues.

(B) Denotes the multiple sequence alignment of VfmE and other AraC family proteins across various bacteria. The ones containing RWIWR motif is labeled separately from the ones without RWIWR motif. Asterisk marks indicate the conserved amino acid residues.

(C) A homology modeling of VfmE was done (Kelley et al., 2015). The predicted c-di-GMP binding “RWIWR” motif is colored. Arg89 is colored Blue, Trp90 in Red, Ile91in Yellow, Trp92 in Green, and Arg93 in Magenta. The C-terminal helix-turn-helix DNA-binding domain is colored teal, orange, and teal respectively.

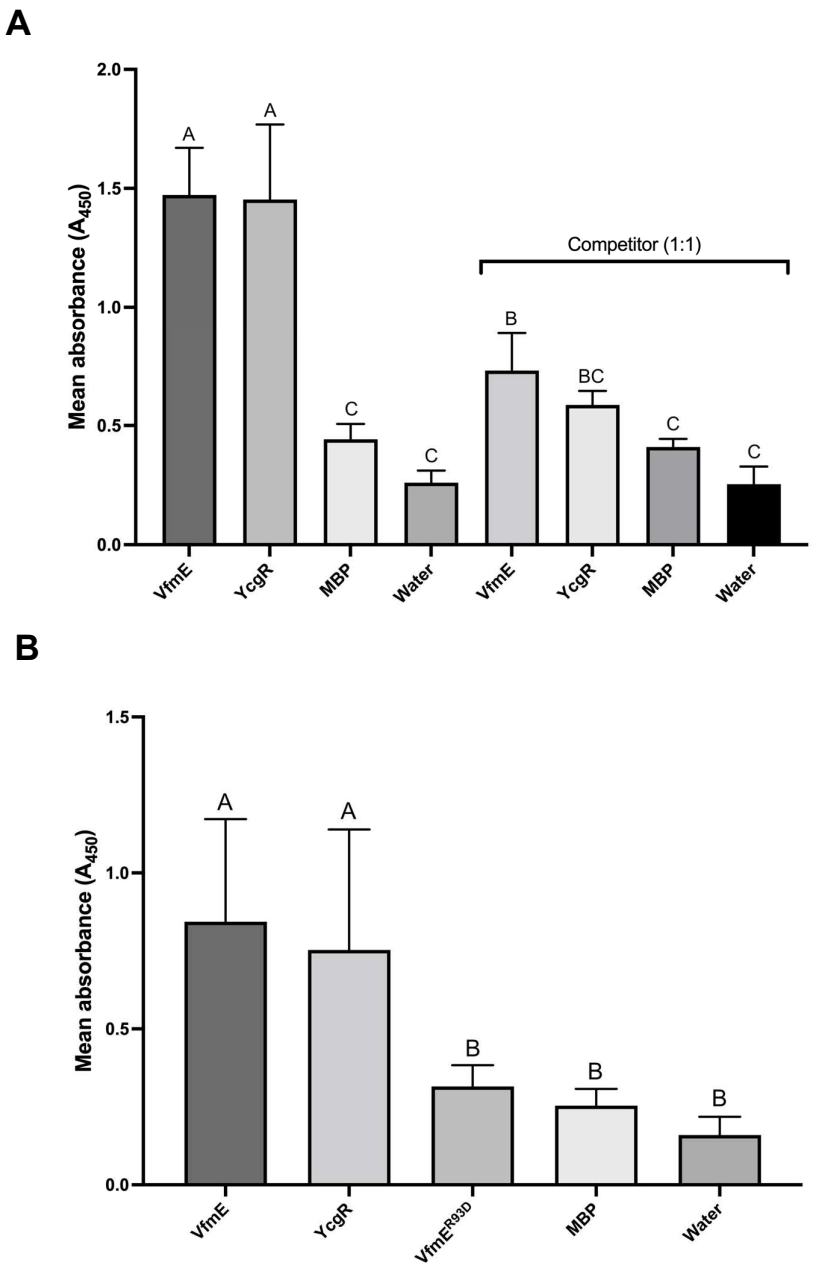

Fig. 2.7. VfmE is a c-di-GMP binding protein.

The c-di-GMP binding ability of VfmE was determined by ELISA (A) Absorbance at 450 nm observed in VfmE, using YcgR, MBP, and sterile water as controls. A similar experiment was done by adding non-labeled c-di-GMP as a specific competitor. (B) Absorbance at 450 nm observed in VfmE and VfmE^{R93D}, keeping YcgR, MBP, and sterile water as controls. Values are representative of two independent experiments,

and three replicates were used for each experiment. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

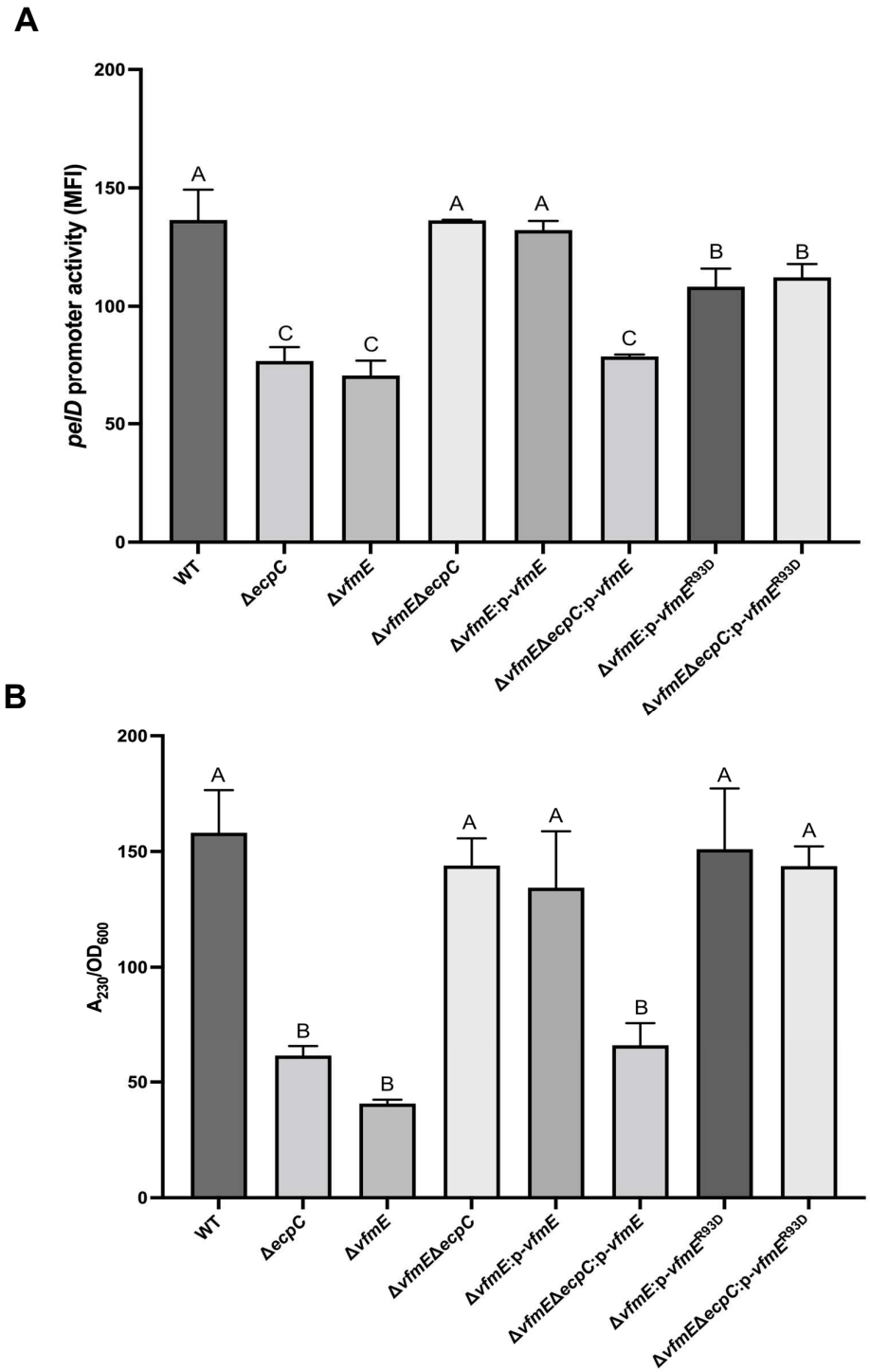

Fig 2.8. VfmE protein with an R93D substitution differentially affects pectate lyase activity under Δ*ecpC* mutant.

(A) The promoter activity of pPROBE-AT plasmid harboring *pelD*-GFP promoter region was measured by flow cytometry. *pelD* promoter activity in wild type, Δ*ecpC,*

Δ*vfmE,* and Δ*vfmE* Δ*ecpC* was measured. Complementation of *pelD* promoter activity in wild type, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* harboring low copy number empty pCL1920 plasmid (controls) and Δ*vfmE* and Δ*vfmE* Δ*ecpC* strains harboring pCL1920:*vfmE* and pCL1920:*vfmE*^{R93D}. Values are representative of three experiments, and three replicates were used for each experiment. Mean Fluorescence Intensity (MFI); average GFP fluorescence intensity of total bacterial cells examined. The cultures were grown in MM medium for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

(B) The total pectate lyase activity was measured by spectrophotometry. Complementation of total Pel activity in wild type, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* harboring low copy number empty pCL1920 plasmid (controls) and Δ*vfmE,* and Δ*vfmE* Δ*ecpC* strains harboring pCL1920:*vfmE,* and pCL1920:*vfmE*^{R93D}. The total Pel activity in wild type, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* strains was measured. The OD_{230} value of Pel activity was normalized by OD_{600} value of the cell culture. Values are representative of three experiments, and three replicates were used for each experiment. The cultures were grown in LB medium for 16 h.Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

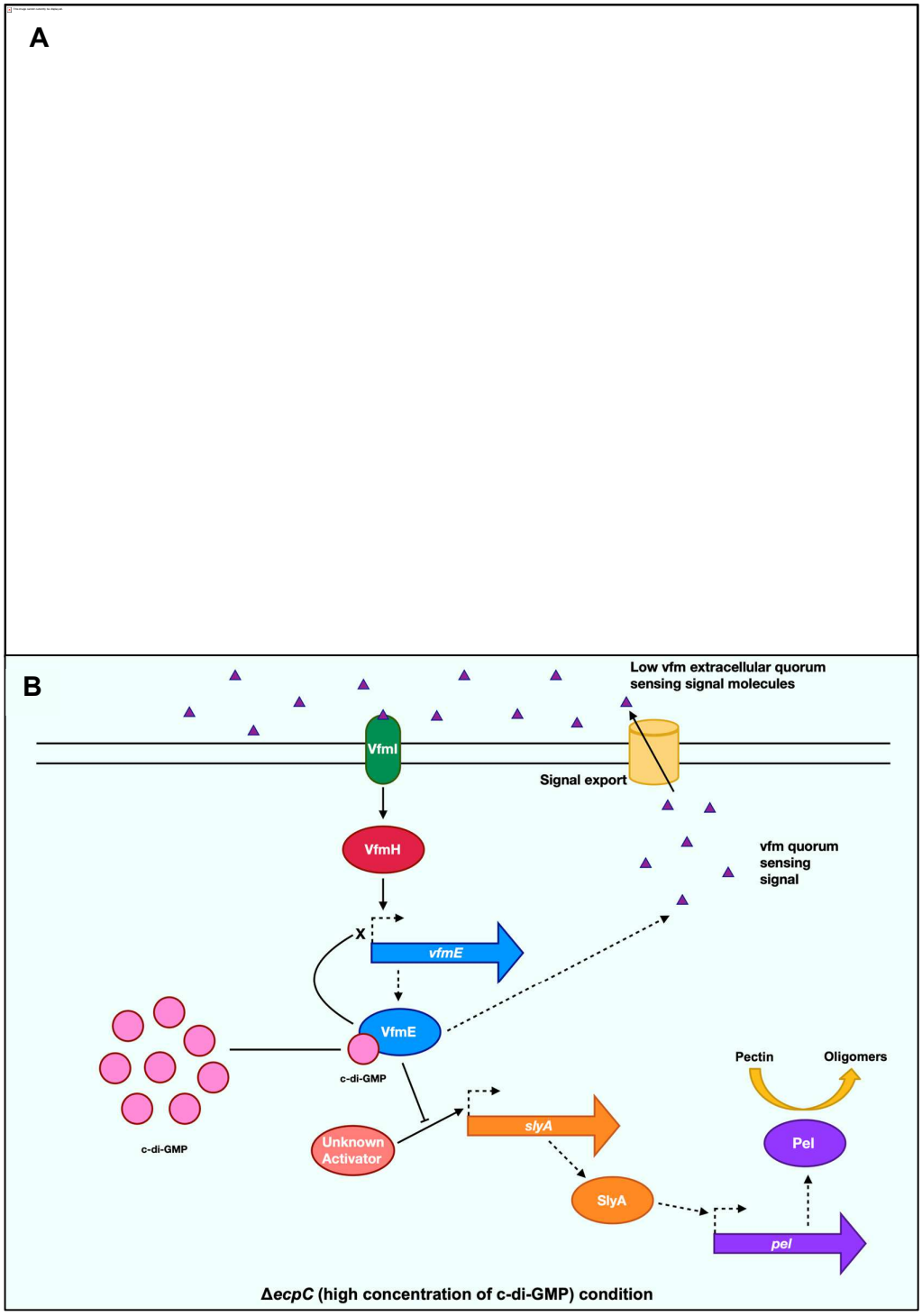

Fig 2.9. Regulatory mechanism of VfmE.

The regulatory mechanisms of VfmE on Pel production under low and high c-di-GMP conditions. (A) VfmE upregulates *pel* production through *slyA* transcription, resulting in a high Pel activity when the c-di-GMP level is WT (low) level. In this condition, VfmE

also upregulates its own transcription resulting in a high vfm quorum-sensing molecule production which corresponds to high *vfmE* transcription. (B) Under a high c-di-GMP condition (i.e., Δ*ecpC*), c-di-GMP bound VfmE represses the unidentified activator of *slyA* resulting in low *slyA* transcription and Pel activity. High c-di-GMP caused by deletion of *ecpC* also reduces *vfmE* transcription, resulting in a low vfm quorum-sensing molecule production which corresponds to low *vfmE* transcription. Solid arrows indicate higher production of SlyA, Pel, *vfmE,* and vfm quorum-sensing molecules, dotted arrows indicate lower production of SlyA, Pel, *vfmE,* and vfm quorum-sensing molecules.

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Chapter 3

The *Dickeya dadantii* AraC family transcriptional regulator VfmE uses cyclic di-GMP to regulate the type III secretion system

Abstract

Dickeya dadantii is a phytopathogenic bacterium that utilizes the type III secretion system (T3SS) to secrete effector proteins to disarm the host immune response. Cyclic diguanylate monophosphate (c-di-GMP), a bacterial second messenger, negatively regulates the expression of T3SS genes in *D. dadantii* and the concentration of c-di-GMP is maintained by multiple diguanylate cyclase and phosphodiesterase enzymes. Deletion of a phosphodiesterase-encoding gene *ecpC* suppressed the expression of the T3SS needle subunit-encoding gene *hrpA*, and this regulatory mechanism includes VfmE. VfmE, an AraC family transcription factor in the Vfm quorum-sensing system, was recently reported to bind c-di-GMP. We observed that deletion of *vfmE* reduced the expression of T3SS regulon genes and further demonstrated that VfmE regulated *hrpA* independently and also via the T3SS master regulator HrpL. Lastly, since VfmE binds c-di-GMP via its RxxxR motif, we found that R93D mutation of VfmE abolished the c-di-GMP-mediated suppression of *hrpA* transcription. In summary, we revealed a novel pathway by which the Vfm quorum-sensing system regulates the *hrp* genes of T3SS.

Introduction

Dickeya dadantii is a Gram-negative plant pathogen. This bacterium causes soft-rot disease in a wide range of economically important crops and vegetables, such as cabbage, chicory, tomato, and potato (Reverchon & Nasser, 2013). This bacterium infects the apoplast of plant tissue. During the initial stage of infection, *D. dadantii* utilizes the type III secretion system (T3SS) to suppress the host immune response (Cui et al., 2018), and full virulence was compromised in *D. dadantii* T3SS mutants (C.-H. Yang et al., 2002). T3SS delivers effector proteins into the host cells to cause

infection and is important for *D. dadantii* to maintain its population in planta (C.-H. Yang et al., 2002). The expression of T3SS is well regulated in *D. dadantii.* It is induced by growth in minimal medium (MM) and in plant hosts but is not expressed during growth in rich media (Yuan et al., 2020). T3SS assembly is initiated by the recruitment of T3SS subunits to the cell envelope. Once the envelope-localized T3SS basal body is formed, the rod and the needle is assembled (Deng et al., 2017).

The T3SS genes of *D. dadantii* are regulated by multiple cascades of regulators. The HrpX/HrpY two-component system upregulates the T3SS by activating transcription of *hrpS*, which encodes a σ^{54} enhancer binding protein. HrpS aids in the binding of RpoN (σ^{54}) to RNA polymerase, as a result initiating *hrpL* transcription (Chatterjee, Cui and Chatterjee 2002; Yap et al. 2005). *D. dadantii* HrpL, the master regulator of T3SS, is an alternative σ-factor. HrpL activates the transcription of *hrp* regulon genes, including *hrpA*, *hrpN*, and *dspE* (Tang et al. 2006). *hrpA* encodes the T3SS needle subunits, *hrpN* encodes a T3SS harpin protein, and *dspE* encodes an effector protein (Chatterjee *et al*. 2002; Wei and Beer, 1995). HrpL activates the transcription of *hrp* genes by binding to the *hrp* box in their promoter regions (Tang et al., 2006; S. Yang et al., 2010). *hrpL* transcription is regulated by the HrpX/HrpY-HrpS pathway in addition to RpoN (Yap et al. 2005).

Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger that regulates multiple cellular mechanisms such as biofilm formation, swimming motility, virulence, and expression the of T3SS (Hengge, 2009). The metabolism of c-di-GMP is controlled by two classes of enzymes, including diguanylate cyclase (DGC) and phosphodiesterase (PDE). DGCs produce c-di-GMP and PDEs degrade c-di-GMP (Hengge, 2009; Römling & Simm, 2009). C-di-GMP controls a wide variety of cellular functions through the allosteric bindings between c-

di-GMP and different effectors. The reported c-di-GMP effectors include PilZ-domain proteins, proteins with GGDEF domain and an I-site, proteins with degenerate GGDEF or EAL domain, and RNA riboswitches (Romling et al., 2013). Our previous studies reported that two PDEs, EcpC and EGcpB, two DGCs, GcpA and GcpL, and three effectors, including YcgR, BcsA, and VfmE, are actively involved in the regulation of several cellular behaviors in *D. dadantii* (Yi et al. 2010; Yuan et al. 2015, 2018, 2019; Banerjee et al. 2022). In our previous study, we deciphered how VfmE responds to c-di-GMP signaling and regulates Pel, but the regulation of T3SS by VfmE still needs to be uncovered.

Quorum-sensing (QS) is the bacterial intercellular communication system that depends on small signaling molecules (Von Bodman, Bauer and Coplin 2003). QS is required for pathogenicity and bacterial colonization in the host system by many plant pathogenic bacteria (Von Bodman, Bauer and Coplin 2003). *D. dadantii* has two quorum-sensing systems: the classic Exp, a N-acyl-homoserine lactone (AHL)-QS system and the Vfm (Virulence Factors Modulating cluster) QS system. The Vfm QS is conserved among *Dickeya* species (Nasser et al., 2013). The *vfm* genes produce an extracellular signal that is perceived by the two-component system VfmI/VfmH. VfmH activates the expression of an AraC type transcriptional regulator VfmE. VfmE, in turn, activates expression of the *vfm* operons and of genes encoding cell-wall degrading enzymes (CWDEs) (Lv et al., 2019; Nasser et al., 2013).

Our recent study showed that *D. dadantii* VfmE is a c-di-GMP binding effector that acts as a repressor of CWDEs under a high c-di-GMP conditiom (Δ*ecpC*) (Banerjee *et al.* 2022). The Vfm QS system was predicted to have a regulatory connection with the T3SS *hrp* genes (Reverchon et al., 2016); however, no detailed research was done to elucidate the regulatory pathway of T3SS expression by the

vfm genes. In this work, we unveiled that VfmE regulates expression of *hrpA* and *hrpL*, and this regulation is dependent on c-di-GMP signaling. Furthermore, we demonstrated that VfmE responds to high intracellular c-di-GMP levels caused by the deletion of *ecpC* and suppresses *hrpA* transcription in the *ecpC* mutant background. This study identified a novel T3SS regulatory pathway involving the Vfm QS master regulator VfmE and c-di-GMP.

Materials and methods

Bacterial strains, plasmids, primers, and growth conditions

Table 4 contains information about the bacterial strains and plasmids used in this study. The media used for growing *D. dadantii* strains are 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% $MgSO_4$) and low nutrient T3SS inducing minimal medium (MM) at 28°C (S. Yang et al., 2008; S. Yang, Peng, et al., 2007). The growth conditions for *Escherichia coli* strains were LB at 37°C. Antibiotic supplements to the growth medium were added as listed: ampicillin (100 μg/ml), kanamycin (50 μg/ml), gentamicin (10 μg/ml), and streptomycin (100 μg/ml). The *D. dadantii* 3937 genomic data is available in the annotation package for community analysis of genomes (ASAP) (https://asap.ahabs.wisc.edu/asap/home.php). Table 5 contains the primers used for PCR in this report.

Mutant construction and complementation

The *vfmE* deletion and complementation strains were generated previously (Banerjee et al., 2022). Overexpression of *rpoN* and *hrpL* was done by cloning *rpoN* and *hrpL*

coding sequences under their natural promoters in a high copy number plasmid, pML123, and in a medium copy number plasmid pBBR1MCS-5 (Table 4). The resulting plasmid constructs were confirmed by PCR and electroporated into the different mutant strain cells for further assay.

Construction of green fluorescent protein (GFP) reporter plasmid and flow cytometry assay

The reporter plasmids, pAT-*hrpL* and pAT-*hrpA*, were previously constructed by cloning the promoter regions of *hrpL* and *hrpA* into the promoter probe vector pPROBE-AT, which contains *gfp* gene as a reporter (Leveau & Lindow, 2001; Miller et al., 2000; S. Yang, Peng, et al., 2007). The transcriptional activity of the genes was observed by measuring GFP intensity using flow cytometry (BD Biosciences, San Jose, CA) following the previously described protocol (Peng et al., 2006). Overnight LB cultures of bacterial cells with reporter plasmid were inoculated 1:100 into MM media. Promoter activity was checked at 24 h by quantifying GFP intensity using flow cytometry.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

The mRNA level of *hrpL* was measured by qRT-PCR. Briefly, 24 h MM medium cultures of bacterial cells were collected. Total RNA extraction was done using a PureLink RNA Mini Kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. DNase treatment (Invitrogen, Carlsbad, CA, USA) was carried out. Synthesis of cDNA was done using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time PCR was used to quantify the cDNA levels of different

samples using PowerUp SYBR Green Master Mix (Life Technologies, Carlsbad, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression (Livak & Schmittgen, 2001), with *rplU* gene as the internal control for transcription (Mah et al., 2003). The experiment was done with triplicates.

Statistical analysis

One-way ANOVA was used in the statistical analysis using IBM SPSS 25 software (IBM, Armonk, NY). Significance was checked at a 95% confidence interval ($P < 0{\cdot}05$); multiple comparisons were done by Tukey's test. Data were shown as means ± standard error of the mean.

Results

Deletion of *vfmE* downregulates *hrpA* transcription and cannot be complemented under a high c-di-GMP (Δ*ecpC*) background

We previously demonstrated that VfmE is a c-di-GMP binding protein that inversely affects CWDE pectate lyases production under a high c-di-GMP (Δ*ecpC*) background (Banerjee *et al.* 2022). To elucidate the regulation of VfmE on T3SS genes, we checked the transcription of P_{hrpA}*::gfp* fusion (*hrpA* promoter-*gfp*) in wild type (WT), Δ*ecpC*, Δ*vfmE*, and Δ*vfmE* Δ*ecpC* mutants. We observed that *hrpA* transcription was significantly reduced in Δ*ecpC*, suggesting that high c-di-GMP represses T3SS genes as per previous findings (Yi et al. 2010). *hrpA* transcription in the Δ*vfmE* mutant was reduced compared to WT, and was further reduced in Δ*vfmE* Δ*ecpC* double mutant compared to Δ*vfmE* or Δ*ecpC* single mutants (Fig.3.1A). The altered *hrpA* transcription could be restored to the WT levels in Δ*vfmE* but not in Δ*vfmE* Δ*ecpC* by introducing the *vfmE* gene with its native promoter cloned in pCL1920, a low copy

number plasmid (Fig. 3.1B). Together, these data suggest that VfmE is required for the expression of *hrpA* in *D. dadantii*. *hrpA* transcription could be restored to WT levels by in-trans expression of *vfmE* under low c-di-GMP background but not under high c-di-GMP background.

VfmE upregulates *hrpA* transcription via *hrpL*

Since HrpL is the master regulator that controls *hrpA* transcription, we tested whether VfmE affects the transcription of *hrpL* by comparing the GFP intensity of P_{hrpL}*::gfp* in WT, Δ*ecpC*, Δ*vfmE*, and Δ*vfmE* Δ*ecpC*, respectively. We observed that Δ*ecpC* showed reduced *hrpL* transcription as per previous report (Yi et al. 2010). Δ*vfmE* also showed significantly reduced *hrpL* transcription compared to WT. The Δ*vfmE* Δ*ecpC* double mutant showed reduced *hrpL* transcription similar to the single mutants (Fig. 3.2A). A reduced *hrpL* mRNA level was observed, as Δ*ecpC*, Δ*vfmE*, and Δ*vfmE* Δ*ecpC* showed an approximately 4-fold reduction in the mRNA level of *hrpL* relative to the WT bacteria (Fig. 3.2B).

Next, we investigated whether overexpression of *hrpL* in Δ*vfmE* or Δ*vfmE* Δ*ecpC* recovers *hrpA* transcription. We cloned the *hrpL* gene under its natural promoter in a medium copy number plasmid pBBR1-MCS-5 (pBBR1MCS-5-*hrpL*) to complement the reduced *hrpL* in the mutant strains. We observed that *hrpA* transcription increased to near WT levels when pBBR1MCS-5-*hrpL* was introduced into Δ*ecpC,* Δ*vfmE*, and Δ*vfmE* Δ*ecpC* (Fig. 3.2C), suggesting that VfmE upregulates *hrpA* expression via *hrpL.*

VfmE upregulates *hrpL* transcription by a mechanism that does not involve *rpoN*

A previous study has shown that high c-di-GMP levels caused by the deletion of *ecpC* represses *hrpL* transcription by reducing *rpoN* (σ^{54}) mRNA levels (Yi et al. 2010). To elucidate whether VfmE reduces *hrpL* transcription via RpoN, we measured the *hrpL* promoter activity by overexpression of *rpoN* in a high copy number plasmid pML123. We found that *hrpL* transcription was increased in Δ*ecpC*, approaching WT levels, but was unaffected in Δ*vfmE* or Δ*vfmE* Δ*ecpC* (Fig. 3.3). This result indicates that EcpC upregulates *hrpL* via RpoN whereas VfmE upregulates *hrpL* via an uncharacterized mechanism independent of RpoN.

VfmE^{R93D} upregulates *hrpA* but not *hrpL* transcription regardless of c-di-GMP levels

Recently, we found that VfmE is a c-di-GMP effector and the arginine residue at position 93 is essential for c-di-GMP binding (Banerjee *et al.* 2022). To further understand VfmE-mediated T3SS regulation, we tested the *hrpA* promoter activities in WT, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* in the presence of *vfmE*^{WT} or *vfmE*^{R93D}. Consistent with previous findings, Δ*ecpC* and Δ*vfmE* showed reduced *hrpA* expression compared to WT, and Δ*vfmE* Δ*ecpC* showed further reduced *hrpA* transcription. More important, *in trans* expression of *vfmE*^{WT} gene complemented the *hrpA* expression in Δ*vfmE* to WT levels but not in Δ*vfmE* Δ*ecpC*. However, *hrpA* expression was on par with WT levels when the plasmid containing *vfmE*^{R93D} was transformed into both Δ*vfmE* and Δ*vfmE* Δ*ecpC,* suggesting that VfmE^{R93D} recovers the *hrpA* expression regardless of the high c-di-GMP condition in Δ*ecpC* (Fig 3.4A).

We also determined the complementation phenotypes of *hrpL* transcription with *vfmE*^{WT} and *vfmE*^{R93D}, respectively. In line with previous findings, Δ*ecpC* and Δ*vfmE* showed reduced *hrpL* expression compared to WT, and Δ*vfmE* Δ*ecpC* showed further

reduced *hrpL* transcription. The plasmid containing *vfmE*^{WT} gene complemented the *hrpL* expression in Δ*vfmE* to WT levels and Δ*vfmE* Δ*ecpC* to Δ*ecpC* levels. The *hrpL* expression was on par with WT levels when the plasmid containing *vfmE*^{R93D} was transformed into Δ*vfmE* but not in Δ*vfmE* Δ*ecpC,* suggesting that VfmE^{R93D} could recover the *hrpL* expression under the WT c-di-GMP conditions but not under high c-di-GMP conditions caused by *ecpC* deletion (Fig 3.4B). Our observation suggested that VfmE^{R93D} was insensitive to c-di-GMP and recovered *hrpA* but not *hrpL* transcription under high c-di-GMP conditions implies that VfmE could regulate *hrpA* in a HrpL-independent manner and this regulation involves elevated c-di-GMP levels.

Discussion

D. dadantii utilizes the T3SS to invade plant cells, and the expression of the T3SS gene cluster is negatively regulated by c-di-GMP (Yi et al. 2010). In this study, we propose a novel regulatory pathway that connects c-di-GMP and the Vfm QS system to regulate the expression of T3SS genes in *D. dadantii*. This is the first report that established a connection between Vfm QS, c-di-GMP signaling, and the expression of T3SS genes in *D. dadantii.*

Previous studies have shown that genes belonging to the Vfm QS system are regulated by *hrp* gene regulators in *D. dadantii* (Reverchon et al., 2016). Interestingly, in this study, we demonstrated that deletion of *vfmE* which encodes the master regulator of the Vfm QS system,reduces the expression of *hrpA* and *hrpL*, (Fig. 3.1A). Since VfmE was recently reported to bind c-di-GMP (Banerjee *et al.* 2022), we observed a further reduced *hrpA* transcription caused by *vfmE* deletion under high c-di-GMP background, suggesting an additive effect on *hrpA* transcription (Fig. 3.1A).

This phenotype was not complemented under a high c-di-GMP condition indicating that high c-di-GMP levels inhibit VfmE activation of *hrpA* promoter (Fig. 3.5). Research conducted in *Escherichia coli* and *Aeromonas hydrophila* demonstrated that QS positively regulates the expression of T3SS genes (Pena et al., 2019), which is in line with our findings that the Vfm QS system upregulates *hrpL* and *hrpA* in *D. dadantii*. Our data suggest a multitiered regulation of the *hrp* gene cluster by VfmE. Unlike EcpC, which involves *rpoN* for upregulation of *hrpL* (Yi et al. 2010), VfmE regulates *hrpL* via a novel pathway that requires further elucidation. We also observed that both VfmE and EcpC control *hrpA* expression in a *hrpL* dependent manner.

Our study showed that complementation with *vfmE*^{R93D} (c-di-GMP insensitive mutant of VfmE) restored the *hrpA* phenotype to WT level in Δ*vfmE* mutant under both low and high c-di-GMP backgrounds (Fig. 3.4A). However, the transcription of *hrpL* gene was not complemented under the high c-di-GMP condition (Fig. 3.4B). This corresponds to the repression of *hrpL* via an additional component, *rpoN,* by c-di-GMP in the Δ*ecpC* mutant (Yi et al. 2010). Moreover, the upregulation of *hrpA* transcription but not *hrpL* transcription by VfmE^{R93D} under high c-di-GMP condition indicates that although VfmE regulates *hrpA* via *hrpL*, it can also regulate *hrpA* by a mechanism that bypass *hrpL*. Further studies are needed to understand the regulation of VfmE on *hrpA* as well as other T3SS regulon genes such as *hrpN* and *dspE*.

In conclusion, our data revealed a model in which the vfm signal induces the transcription of *vfmE,* which influences the T3SS *hrp* genes. High c-di-GMP levels suppress VfmE activity through the binding of RxxxR motif, resulting in low expression of the T3SS components. VfmE is a novel activator of *hrpA* but acts as a repressor when the c-di-GMP level is high. This study provides insight into the regulatory pathways of T3SS genes by VfmE, the master regulator of the Vfm QS system. It also

demonstrates the complexity of c-di-GMP signaling and the QS system and unveils various strategies of *D. dadantii* during different stages of infection.

Table 4 List of strains and plasmids

Strains and plasmids	Relevant characteristics[a]	Reference or source
Dickeya dadantii		
3937	Wild type	(Hugouvieux-Cotte-Pattat, N.)
Δ*ecpC*	Δ*ecpC*, ABF-0020364 deletion mutant	(Yi et al. 2010)
Δ*vfmE*	Δ*vfmE*::*Km;* Km^r, ABF-0016073 deletion mutant	(Banerjee et al., 2022)
Δ*vfmE* Δ*ecpC*	Δ*vfmE* Δ*ecpC*::*Km;* Km^r, ABF-0016073 and ABF-0020364 double deletion mutant	(Banerjee et al., 2022)
Plasmids		
pCL1920	Low copy number plasmid, lac promoter, Sp^r	(Lerner & Inouye, 1990)
pCL-*vfmE*	*vfmE* with natural promoter cloned in pCL1920, Sp^r	(Banerjee et al., 2022)
pCL- *vfmE*^{R93D}	*vfmE*^{R93D} with natural promoter cloned in pCL1920, Sp^r	(Banerjee et al., 2022)
pPROBE-AT	Promoter-probe vector, promoter-less *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, Ap^r	(Yang et al. 2007)
pBBR1MCS-5	Medium copy number plasmid, Gm^r	(Kovach et al., 1995)
pBBR1MCS-5-*hrpL*	*hrpL* with natural promoter cloned in pBBR1MCS-5, Gm^r	This study
pML123	High copy number plasmid, Gm^r	Lab stock
pML123-*rpoN*	*rpoN* gene cloned in pML123, Gm^r	Lab stock

[a]Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sp^r, streptomycin resistance, Gm^r gentamicin resistance

Table 5 List of primers

Primers	Sequences (5'-3')	Amplicon
hrpL-for-EcoRI *hrpL*-rev-SalI	AATAGAATTCTTGTGCATCGAAGCGTTCCAGTAAGGCATC TTATGTCGACTTGACAGGTTCCTTCACCTCCCCTGATTAA	*hrpL* overexpression
hrpL-F *hrpL*-R	CCCCTGCCAATACCGTGGACTGGGAGC GCGATAGCGCGCCTGTTTGAAGTGA	*hrpL* mRNA amplification for qRT-PCR

Figures

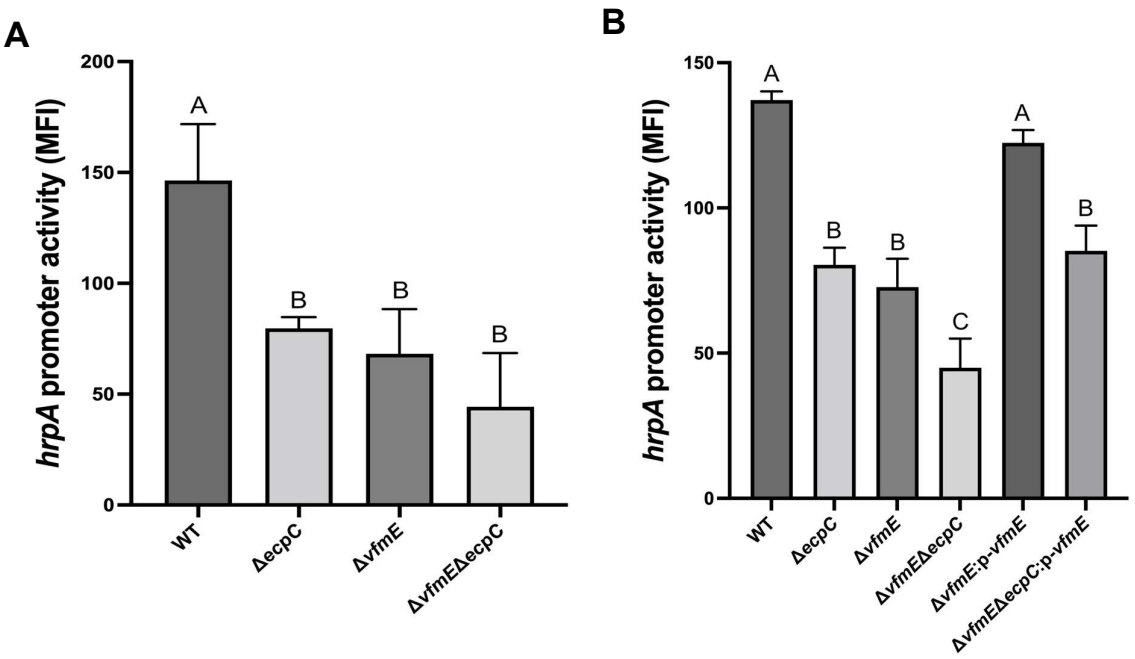

Fig. 3.1. Deletion of *vfmE* downregulates *hrpA* transcription, was not complemented under a high c-di-GMP (Δ*ecpC*) background.

The promoter activity of *hrpA* in pPROBE-AT reporter plasmid harboring *hrpA-gfp* was measured by flow cytometry. *hrpA* promoter activity in WT, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* was measured. Complementation of *hrpA* promoter activity in WT, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* harboring low copy number empty pCL1920 plasmid (controls) and Δ*vfmE* and Δ*vfmE* Δ*ecpC* strains harboring pCL1920:*vfmE*. Values of mean fluorescence intensity (MFI) are an average green fluorescent protein fluorescence intensity of bacterial cells measured by flow cytometry. Values are representative of three experiments, and three replicates were used for each experiment. The cultures were grown in MM medium for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

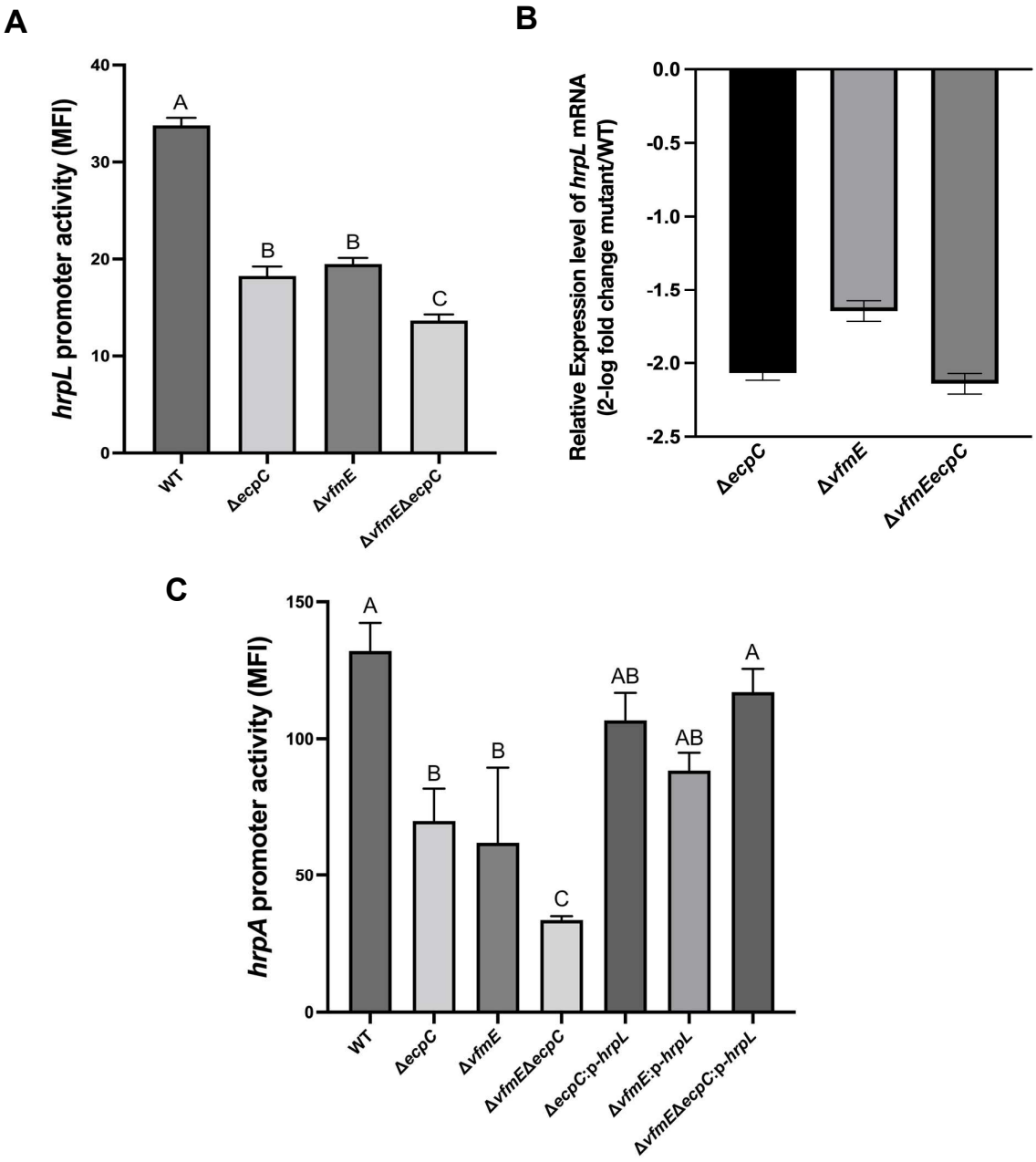

Fig. 3.2. VfmE upregulates *hrpA* transcription via *hrpL.*

(A) The promoter activity of *hrpL* in pPROBE-AT reporter plasmid harboring *hrpA-gfp* was measured by flow cytometry. *hrpL* promoter activity in WT, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* was measured. (B) *hrpL* mRNA levels were examined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The mutant/WT ratio for *hrpL* gene expression was calculated as described in Materials

and Methods. The results are displayed as a 2-log fold-change of the *hrpL* mRNA levels of the mutant vs. WT. (C) The promoter activity of *hrpA* in pPROBE-AT reporter plasmid harboring *hrpA-gfp* was measured by flow cytometry. *hrpA* promoter activity in WT, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* was measured with over-expression of *hrpL* in pBBR1-MCS-5 plasmid. Values of mean fluorescence intensity (MFI) are an average green fluorescent protein fluorescence intensity of bacterial cells measured by flow cytometry. Values are representative of three experiments, and three replicates were used for each experiment. The cultures were grown in MM medium for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

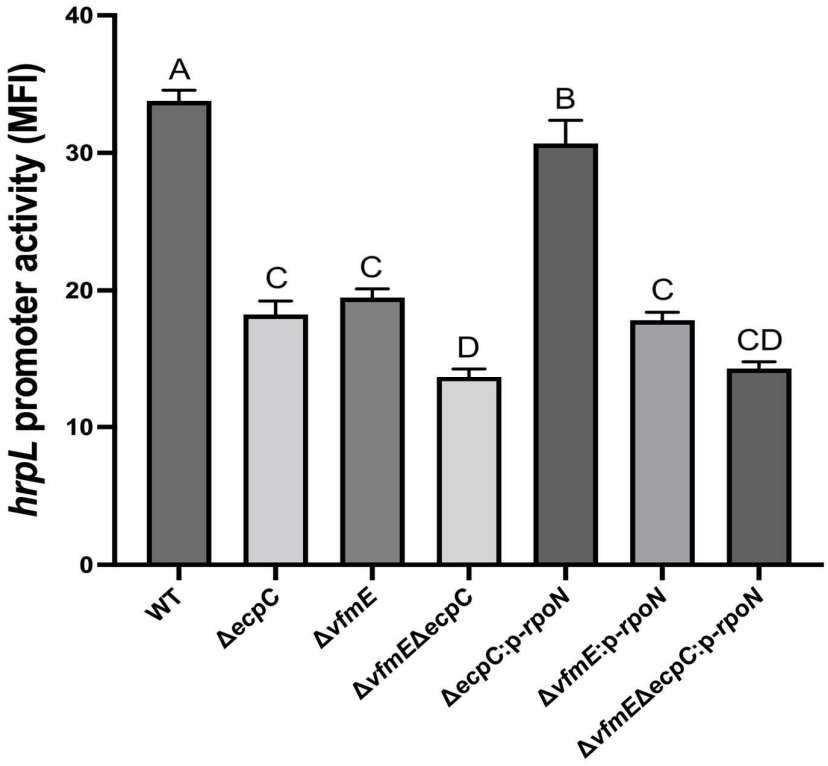

Fig. 3.3. VfmE upregulates *hrpL* transcription by a mechanism that does not involve *rpoN.*

The promoter activity of pPROBE-AT plasmid harboring *hrpL*-GFP promoter region was measured by flow cytometry. *hrpL* promoter activity in WT, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* was measured with over-expression of *rpoN* in pML123 plasmid. Values of mean fluorescence intensity (MFI) are an average green fluorescent protein fluorescence intensity of bacterial cells measured by flow cytometry. Values are representative of three experiments, and three replicates were used for each experiment. The cultures were grown in MM medium for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

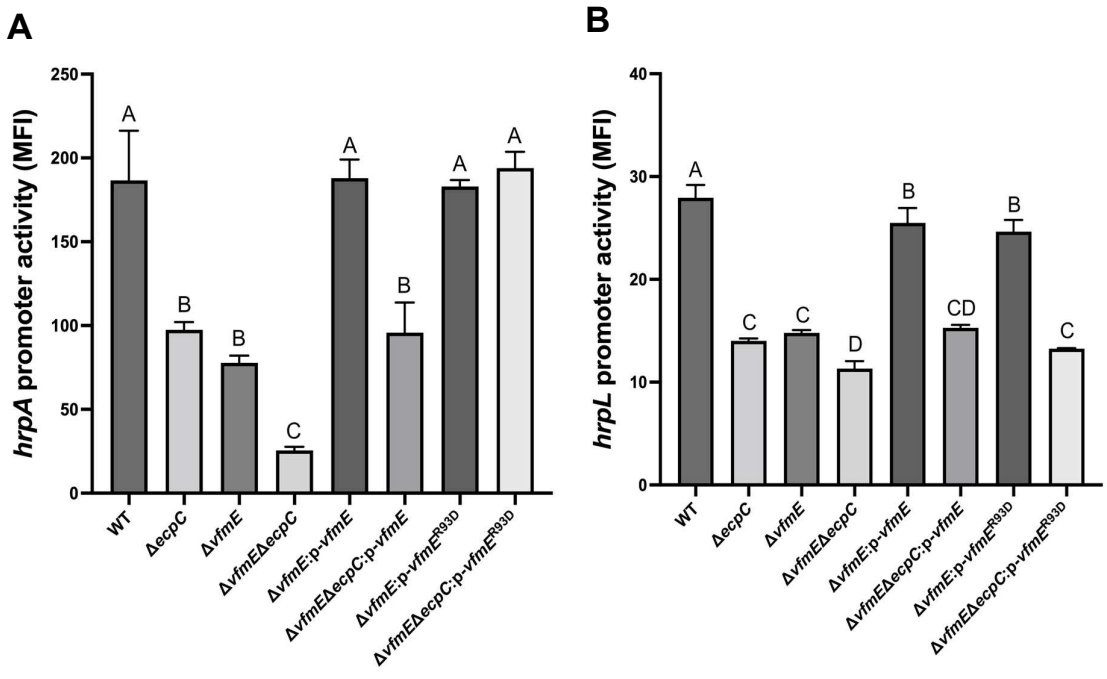

Fig. 3.4. VfmE^{R93D} upregulates *hrpA* transcription regardless of c-di-GMP condition but not *hrpL*

(A) The promoter activity of pPROBE-AT plasmid harboring *hrpA*-GFP promoter region was measured by flow cytometry. Complementation of *hrpA* promoter activity in wild type, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* harboring low copy number empty pCL1920 plasmid (controls) and Δ*vfmE* and Δ*vfmE* Δ*ecpC* strains harboring pCL1920:*vfmE* or pCL1920:*vfmE*^{R93D}. Values of mean fluorescence intensity (MFI) are an average green fluorescent protein fluorescence intensity of bacterial cells measured by flow cytometry. (B) The promoter activity of pPROBE-AT plasmid harboring *hrpL*-GFP promoter region was measured by flow cytometry. Complementation of *hrpL* promoter activity in WT, Δ*ecpC,* Δ*vfmE,* and Δ*vfmE* Δ*ecpC* harboring low copy number empty pCL1920 plasmid (controls) and Δ*vfmE* and Δ*vfmE* Δ*ecpC* strains harboring pCL1920:*vfmE* or pCL1920:*vfmE*^{R93D}. Values of mean fluorescence intensity (MFI) are an average green fluorescent protein fluorescence

intensity of bacterial cells measured by flow cytometry. Values are representative of three experiments, and three replicates were used for each experiment. The cultures were grown in MM medium for 24 h. Different upper-case letters represent treatment groups with significant statistical difference ($P < 0.05$), whereas treatments with no significant differences were assigned the same letters by one-way ANOVA with Tukey's posthoc test.

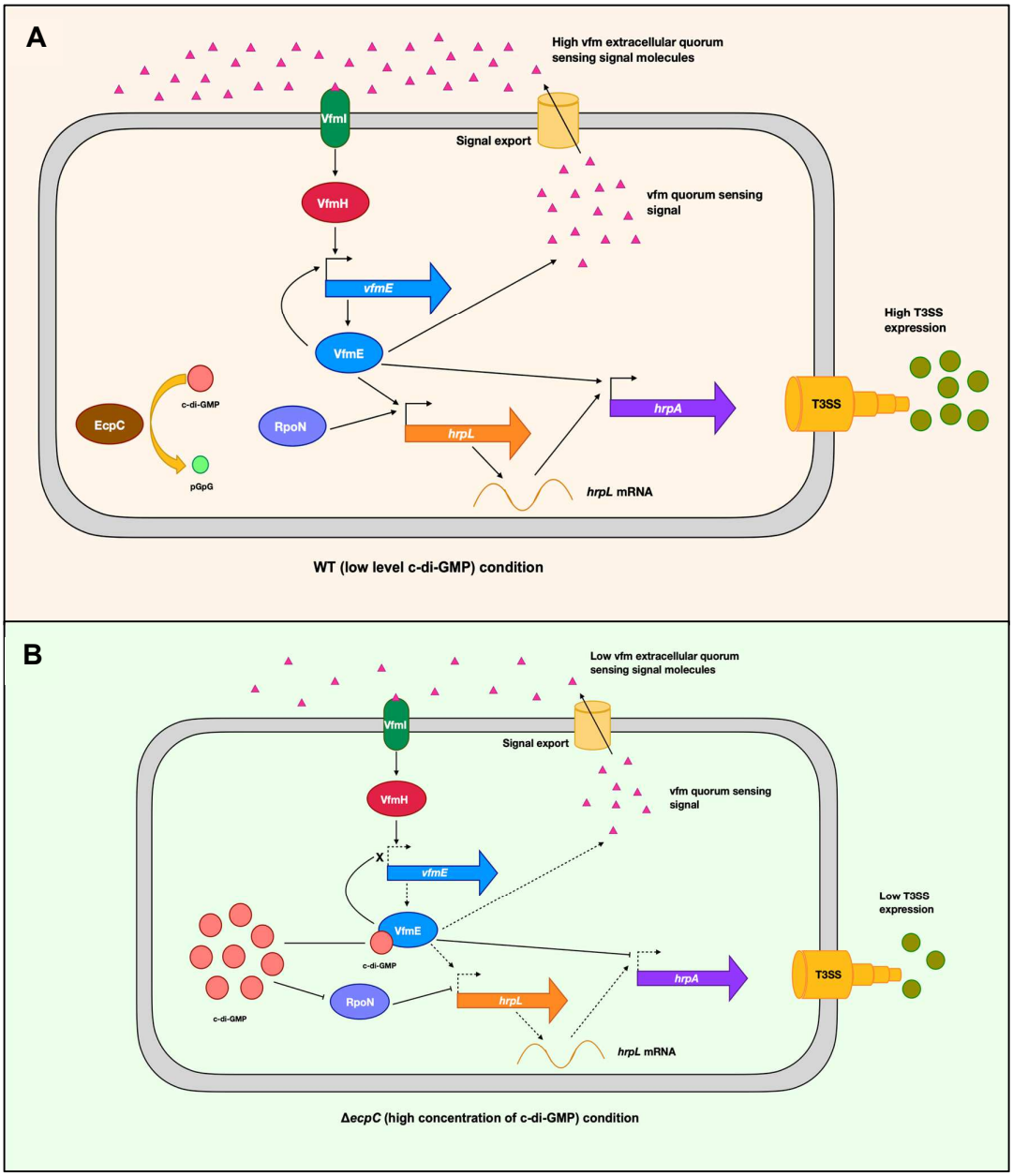

Fig 3.5. Regulatory mechanism of VfmE on *hrp* genes.

The regulatory mechanisms of VfmE on T3SS expression under WT and high c-di-GMP conditions. (A) VfmE upregulates *hrpL* and *hrpA* transcription resulting in a high T3SS gene expression when the c-di-GMP level is WT (low) level. In this condition, VfmE also upregulates its own transcription resulting in a high vfm QS molecule production corresponding to high *vfmE* transcription. (B) Under a high c-di-GMP

condition (i.e., Δ*ecpC*), c-di-GMP bound VfmE fails to activate *hrpL* or represses the *hrpA* transcription. High c-di-GMP caused by deletion of *ecpC* also reduces *vfmE* transcription, resulting in a low vfm QS molecule production which corresponds to low *vfmE* transcription. In addition, the *hrpL* transcription is suppressed by high c-di-GMP concentration caused by deletion of *ecpC* via RpoN. This causes a lower expression of T3SS components. Solid arrows indicate higher production of *vfmE*, *hrpL*, *hrpA*, and vfm QS molecules, dotted arrows indicate lower production of *vfmE*, *hrpL*, *hrpA*, and vfm QS molecules.

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Summary

In the above studies, we have demonstrated that VfmE, an AraC family transcription factor, is also a c-di-GMP binding effector that controls not only the Vfm quorum-sensing but also virulence in response to environmental changes. We also show that VfmE upregulates *pelD* transcription through SlyA, a MarR family transcriptional regulator, and this activity is repressed when intracellular c-di-GMP levels are high (Δ*ecpC*). We have observed that VfmE interacts with c-di-GMP via a PilZ-like mechanism and alters the CWDE production in response to c-di-GMP signaling. Furthermore, we have identified the RWIWR motif of VfmE to be the important motif for c-di-GMP binding and substitution of the conserved 93rd arginine abolished c-di-GMP binding and its related phenotypes.

Previous reports had predicted a connection between the Vfm QS system and the T3SS (Reverchon et al., 2016). In this study, we have established the linkage between the two systems. VfmE regulates *hrpA* and *hrpL* genes of T3SS, and the c-di-GMP signaling plays an important role in this regulatory pathway. It is important to note that although VfmE is an activator of T3SS gene expression, high intracellular c-di-GMP caused by deletion of *ecpC* suppresses its activity in T3SS regulation.

Overall, this study identified a novel regulatory mechanism of a previously characterized quorum-sensing master regulator VfmE that is also a c-di-GMP effector. This study also demonstrates a complex regulatory network between quorum-sensing and secondary messenger c-di-GMP signaling pathways *D. dadantii*. Thus our research established a strong connection between two different signaling pathways that together control different virulence factors and cellular responses to environmental changes.

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EXPERIENCE

- **Graduate Teaching Assistant, University of Wisconsin-Milwaukee, USA — 2017-2022**
  Laboratory course instructor of undergraduate Biology course.

- **Research Technician, T3 Bioscience LLC, Milwaukee, USA — 2018-2022**
  Research work based on natural compound discovery against multi-drug resistant Pseudomonas aeruginosa and fungal pant pathogens.

- **Finance Secretary, Graduate Organization of Biological Sciences, UW-Milwaukee, USA — 2019-2021**
  Student officer at graduate student organization of the university.

- **Research Assistant, Indian Statistical Institute, Kolkata, India — 2016-2017**
  Project based on Face Recognition and Social Cognition.

- **Summer Intern, Indian Statistical Institute, Kolkata, India — 2015**
  Project based on Neuro-ethics.

EDUCATION

- **PhD dissertator — University of Wisconsin-Milwaukee, USA, 2017-2022 (expected)**
  Microbiology (primary area) and Biochemistry (secondary area), CGPA: 3.726/4

- **M.Sc — St. Xavier's College, University of Calcutta, Kolkata, India, 2016**
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- **B.Sc — St. Xavier's College, University of Calcutta, Kolkata, India, 2014**
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PUBLICATIONS

JOURNAL

- **Banerjee, B.**, Das, S., Ghosh, S., Banerjee, J., Bhowmick, S., Basu, T., Mukherjee, R., Roy, P., Das, S., Mukherjee, K., Mitra, A.K. (2016). Bioremediation by Salt-tolerant Brevibacillus sp. as a cost-effective solution for the detergent polluted marine and estuarine zone, Avishkaar: A Xaverian Journal of Research; 21-30, ISSN: 2277-8411 (print), 2278-1048 (online).
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- **Banerjee, B**., Das, S., Ghosh, S., Banerjee, J., Bhowmick, S., Basu, T., Mukherjee, R., Roy, P., Das, S., Mukherjee, K., Roy, L. (2016). Biodegradation of sodium

dodecyl sulphate (SDS) and other commercial detergents by salt tolerant Brevibacillus sp. and other bacterial isolates from the Bay of Bengal coastal beach, Indian Science Congress Mysuru, Karnataka, Environmental Science, p. 76-77.

- **Banerjee, B.**, Yang, C-H. (2020). Regulation of c-di-GMP, Type II and Type III Secretion Systems in Dickeya dadantii by the Vfm Quorum-sensing System, ASM Microbe, Chicago, IL (conference cancelled).
- **Banerjee, B.**, Yu, M., Yang, C-H. (2021). AraC family transcription factor VfmE differentially regulates pectate lyase production by binding to c-di- GMP in a plant pathogen Dickeya dadantii, IS-MPMI Plant-microbe interactions in the environment – Navigating a complex world 2021.
- **Banerjee, B.**, Yang, C-H. (2022). The Vfm quorum-sensing master regulator VfmE is a c-di-GMP binding effector that regulates pectate lyase and type III secretion system in phytopathogen Dickeya dadantii, ASM Microbe, Washington DC.

PRESENTATIONS

POSTER

- 100th Indian Science Congress, Kolkata, 2012 International Conference on Molecular Biology and its Application, Jadavpur University, Kolkata, 2014
- Biological Science Research Symposium, UW-Milwaukee, 2019
- Biological Science Research Symposium, UW-Milwaukee, 2020 (online)
- American Society of Microbiology Microbe 2020, Chicago, IL (conference cancelled, held online)
- IS-MPMI Plant-microbe interactions in the environment – Navigating a complex world 2021 conference (online)
- American Society of Microbiology Microbe 2022, Washington DC

ORAL

- National Symposium, Indian Phytopathological Society, 2015
- National Seminar organized by Institute of Management Study, Kolkata
- and Institute of Cost Accountants of India, 2015
- Departmental seminar, St. Xavier's college, Kolkata, 2015
- 103rd Indian Science Congress, Mysore, 2016

- Biological Science Research Symposium, UW-Milwaukee, 2022 (online)

INVITED TALKS

- Department of Microbiology, St. Xavier's College, Kolkata, 2018

HONORS AND AWARDS

- All India 83rd rank in 4th National Cyber Olympiad, 2004
- Achieved distinction in International Assessment for Indian Schools, (University of New South Wales), 2005
- Distinctive rank in 9th National Science Olympiad, 2006
  2nd prize at inter-college Acharya Satyendranath Basu Memorial Science
- and Technology Fair, Kolkata, 2012
- DST INSPIRE Scholarship (Govt. of India), 2011-2016
- Indian Academy of Science Summer Fellowship, 2015
- 2nd prize for oral presentation at KSHITIJ, IIT Kharagpur, India, 2016
- Chancellor's Graduate Student Award, University of Wisconsin Milwaukee, 2017-2020
- Raymond E. Hatcher Scholarship, Dept. of Biological Sciences, UW- Milwaukee, 2020
- Ruth I. Walker Scholarship, Dept. of Biological Sciences, UW-Milwaukee, 2021
- 1st prize for oral presentation at Biological Science Research Symposium, UW-Milwaukee, 2022 (online)