THE STUDY OF THE PHYTOPATHOGEN *DICKEYA DADANTII* 3937 CPX SIGNALING ON THE REGULATION OF VIRULENCE AND ANTIMICROBIAL RESISTANCE

by

Daqing Jiang

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ABSTRACT

THE STUDY OF THE PHYTOPATHOGEN *DICKEYA DADANTII* 3937 CPX SIGNALING ON THE REGULATION OF VIRULENCE AND ANTIMICROBIAL RESISTANCE

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Bacteria respond to environmental cues through a variety of mechanisms. Two-component systems (TCSs) are a conserved mechanism used by bacteria to accurately respond and sense environmental changes. The monitoring of envelope perturbance is linked to TCS CpxA/CpxR in animal-infecting pathogens. The study of the TCS response regulator (RR) CpxR is largely unexplored in phytopathogens. This work focuses on the genetic linkage between *cpxR* and T3SS expression regulation. We identified the multiple roles of CpxR on several T3SS regulators and its participation in the bacterial second messenger (c-di-GMP) signaling cascade. Moreover, a compound library screening revealed a novel Cpx inducer CHIR-090, an inhibitor of lipid A biosynthesis. Although the linkage between Cpx response and lipid A biosynthesis remains unknown, we propose a potential role of CpxQ (a 3′-UTR sRNA from CpxR regulon gene *cpxP* mRNA) for relieving cells from the stress caused by lipopolysaccharide perturbance.

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LIST OF ABBREVIATIONS

Ap	Ampicillin
c-di-GMP, cdG	Cyclic diguanosine monophosphate
Cpx	Conjugative pilus expression
DGC	Diguanylate cyclase
Ecp	EAL-domain containing protein
EPS	Exopolysaccharides
ETI	Effector-triggered immunity
Gcp	GGDEF-domain containing protein
GFP	Green florescent protein
Hrp	Hypersensitive response and pathogenicity
Km	Kanamycin
LB	Lysogeny media
LPS	lipopolysaccharide
MM	Minimal medium
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PTI	Pattern-triggered immunity
PCR	Polymerase chain reaction
PCWDE	Plant cell-wall-degrading enzymes
PDE	Phosphodiesterase
Pel	Pectate lyase
RR	Response regulator

SK	Sensor kinase
Sp	Spectinomycin
TCS	Two-component system
T3SS	Type III secretion system
T3SEs	T3SS effectors

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Chapter 1

Introduction

1.1 *Dickeya dadantii* 3937

1.1.1 Background and Significance of *Dickeya dadantii* 3937

Erwinia chrysanthemi 3937 was first recorded as the causal agent of a wilt symptom on an ornamental plant Chrysanthemums (*Chrysanthemum morifolium*) in New York, United States in the 1950s (Burkholder *et al*., 1953). Historically, the pathogen was first referred to in the nomenclature as *Pectobacterium chrysanthemi* 3937, and then *Erwinia chrysanthemi* 3937. In 2005, it was proposed to *Dickeya dadantii* 3937. The reclassification into a novel genus, *Dickeya*, is due to better phenotypical, phylogenetical, and serological analysis, which allowed all previously characterized isolates to be assigned into a single species *D. dadantii* (Gardan, 2005).

This Gram-negative, opportunistic phytopathogenic bacterium causes soft rot, wilt, and blight diseases on a wide range of important crop species, including potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), and cabbage (*Brassica rapa subsp. pekinensis*) (Czajkowski *et al*., 2011, Perombelon & Kelman, 1980). It can also infect the model plant *Arabidopsis*. In addition, Africa violet (*Saintpaulia ionantha*) is a model host plant for its virulence assays (Reverchon & Nasser, 2013).

Since the pervasiveness of *Dickeya* spp. in Europe causes crop failure of its susceptible hosts, such as potatoes, and has a detrimental effect on the global agricultural industry, the study of the pathogenesis and the regulation of extracellular enzyme synthesis of *D. dadantii* is urgent (Czajkowski et al., 2011).

The genome of *D. dadantii* (https://asap.genetics.wisc.edu/asap/home.php) has been available for a decade, which serves as an ideal phytopathogenic genetic platform for researchers to study its

pathogenesis on the molecular level (Glasner *et al*., 2011). Genetically, *D. dadantii* 3937 belongs to the *Enterobacteriaceae* family. A study of its orthologs reveals its most closely related genus is *Pectobacterium atrosepticum*, which is one of the causal agents of potato blackleg disease. (Charkowski, 2006, Gardan, 2005).

1.1.2 The virulence determinants of *D. dadantii* 3937

D. dadantii is a necrotrophic phytopathogen that feeds on the nutrients released by decayed plant tissues. Prior to the maceration of plant tissues, *D. dadantii* expresses cellulose fibrils, exopolysaccharides (EPS), and the CdiA/HecA type V secretion system to facilitate attachment to the plant surface (Rojas *et al*., 2002, Reverchon & Nasser, 2013). An exo-biosurfactant is reported to promote surface wettability to further enhance this attachment (Hommais *et al*., 2008). To enter the plant apoplast, *D. dadantii* utilizes flagella and chemotaxis (*motAB-cheAWDRBYZ*), two virulence determinants, to swim/swarm towards environmental cues, such as jasmonic acid, a signal released from decayed plant tissue (Antunez-Lamas *et al*., 2009). Once inside of the host apoplast, *D. dadantii* launches host-pathogen interactions by producing a variety of plant cell-wall-degrading enzymes (PCWDE) to macerate plant tissues, including a plethora of pectinases, exo-proteases, rhamnogalacturonate lyases, exopolygalacturonases, and exo-cellulases (Letoffe *et al*., 1990, Py *et al*., 1991, Hugouvieux-Cotte-Pattat, 2016). The secretion of pectin-degrading proteins is via an ATP-dependent membrane-bound protein complex known as type II secretion system (T2SS) (Pineau *et al*., 2014). The T2SS, also known as the Out secretion system, is important for the extracellular delivery of pectinases and cellulase, whereas the PrtDEF type I secretion system is responsible for the secretion of proteases (Delepelaire, 1998).

It is important to note that evolutionary and ecological pressures drive the plant immune system to recognize alien cells/molecules and generate programmed cell death at the site of infection. This strategy of cellular triage meant to eradicate phytopathogens is known as the hypersensitive response (HR) (Reverchon *et al*., 2016). As plant pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin and lipopolysaccharides (LPSs), pattern-triggered immunity (PTI) is activated to treat invading pathogens. The co-evolution of plant hosts with its pathogens has driven bacteria to develop mechanisms that counter PTI (McCann & Guttman, 2008). An effective weapon *D. dadantii* utilizes in this interminable arms race between pathogen and host is the type III secretion system (T3SS), a virulence determinant that represses the plant immune response (Yang *et al*., 2002). *D. dadantii* utilizes the T3SS to translocate a set of T3SEs (T3SS effector proteins) to suppress or subvert host defense signaling. The delivery of effector proteins directly into the host cells enables bacteria to manipulate the host's common cellular processes, including plant defense response. T3SS also plays a critical role in maintaining the bacterial population in the fight against plant hosts (Yang et al., 2002).

1.1.3 Bacterial secretion systems

The basic morphological design of the cell membrane is comprised of layers of plasma membranes that separate the cytoplasm from its environment. For example, Gram-negative bacteria have a double phospholipid membrane (Kuhn, 2019). In many cases, this barrier is impermeable to the majority of inorganic ions and polar compounds. Apart from the blockage of certain ions and molecules, this pliable membrane is also studded with a number of protein complexes, such as protein secretion systems, which have the capacity to alter their surroundings

by secreting a plethora of effector proteins to respond to a variety of different environments. Secreted effectors can participate and interact with molecules in the surrounding environment, allowing organisms to establish their niche.

Despite their common secretion properties, different effector proteins have to be recognized by their corresponding secretion systems in a temporal- and spatial- dependent manner. A number of protein secretion apparatuses are identified in bacteria so far, which are numbered Type I through Type IX, many of which contribute to virulence in a variety of bacterial pathogens (Abby *et al*., 2016).

In addition to the secretion systems, another type of protein complexes located in the bacterial membrane are two-component systems (TCSs), which will be addressed in section 1.2.

1.1.3.1 Type III secretion system

Type III secretion systems (T3SSs) are present in a large number of Gram-negative pathogenic bacteria, including *D. dadantii*. T3SS apparatuses resemble needle-like structures spanning from the inner to the outer membrane (Galán & Collmer, 1999). They translocate a series of proteins known as Type III Effector proteins (T3SEs) from the bacterial cytoplasm directly into host cells to respond to host immune systems.

In addition to the injection of T3SEs into host cells, T3SSs are required for bacterial pathogens to further spread around the infection site. In the plant defense model, plant basal level defense involves monitoring the presence of PAMPs or microbial-associated molecular patterns (MAMPs), such as bacterial flagellum (Zipfel & Felix, 2005). The presence of PAMPs triggers a first layer defense known as pattern-triggered immunity (McCann & Guttman, 2008). To counteract this, the T3SS of bacteria secretes T3SEs in a spatial- and temporal-dependent manner

to silence plant basal level defense, and disrupt or modify plant membrane-bound receptors (McCann & Guttman, 2008). Studies have revealed a wide variety of T3SEs that have different roles in combating host defense signaling. The failure of the timely perception of PAMPs by plant hosts can result in susceptibility to the infection agent and eventual plant death.

Another strategy plants have evolved to eradicate infection from pathogens is effector-triggered immunity (ETI). Plant NB-LRR proteins play a major role in the detection of T3Es related proteins and ultimately trigger another layer of defense response. In response, natural selection has driven bacterial pathogens to use other sets of T3SEs to avoid or suppress the NB-LRR protein-mediated defense response (Jones & Dangl, 2006). The co-evolution between pathogen and host cells indicates the underlying sophisticated molecular mechanisms of the time-sensitive assemblage and expression of T3SS, and this work provides more insight into how bacteria regulate T3SS expression via multiple signaling cascades.

1.1.3.2 The regulation of T3SS expression in *D. dadantii*

In *D. dadantii*, there are 39 gene clusters termed with *hrp*/*hrc*/*dsp* related to T3SS (Peng, 2009). The *hrp* stands for hypersensitive response and pathogenicity, while *hrc* is known as hypersensitive response conserved. A highly regulated hierarchy of the appropriate expression of T3SS is determined by the transcriptional and post-transcriptional regulation of an extracytoplasmic factor (ECF) sigma factor, HrpL (Zwiesler-Vollick *et al*., 2002). Transcriptional regulation of HrpL expression occurs through the HrpX/HrpY two-component system, which increases the transcription of *hrpS* via an unknown mechanism. *hrpS* encodes a σ^{54} enhancer binding protein and facilitates the binding of RpoN (σ^{54}) to an RNA polymerase (RNAP) (Fig. 1). The alternative sigma factor σ^{54} binds to the RNAP, forming RNAP

holoenzymes. RpoN conserved binding motif TGGCACG-N_4-TTGCT is located within -61 to -45 of *hrpL* promoter region (Lloyd *et al*., 2017). The RNAP holoenzymes recognize the σ^{54} binding site at the *hrpL* promoter region and initiate the transcription of *hrpL*. HrpL activates the transcription of *hrp* gene clusters such as *hrpA*, *hrpN*, and *dspE* (Chatterjee *et al*., 2002). *hrpA* encodes the T3SS needle subunit, *hrpN* encodes a T3SS harpin protein, whereas *dspE* encodes a virulence factor (Peng, 2009).

The genetic regulation of the presence of HrpL is also subject to post-transcriptional regulation by the two-component system GacS/GacA (Fig. 1). The GacS/A system regulation works through RsmA/*rsmB* pairs. In the presence of RsmA protein, *hrpL* mRNA is degraded by RsmA binding (Lebeau *et al*., 2008). In T3SS inducing conditions, the GacS/A two-component system stimulates the transcription of *rsmB*, a small regulatory RNA encoding gene. RsmB specifically binds to RsmA, and the negative impact of RsmA on *hrpL* mRNA is neutralized.

A T3SS negative regulator, PecT, was reported recently (Yuan *et al*., 2019). It is a LysR-type transcriptional regulator that represses *rsmB* expression to further downregulate T3SS expression through the negative impact of RsmA on *hrpL* mRNA. Additionally, sRNA ArcZ and Hfq might interact with each other to regulate the expression of *pecT* (Fig. 2) (Yuan et al., 2019).

The structural and genetic similarities between T3SS and flagella indicate a close link between these two protein complexes. It is no surprise that the flagellar master regulator FlhDC showed a positive role on T3SS (Fig. 2) (Yuan *et al*., 2015). Regulation of flagellar gene expression follows a three-tier hierarchy in enterobacteria such as *Escherichia coli* and *Salmonella* (Wang *et al*., 2006). The class I regulation occurs on the flagellar master regulator FlhDC, class II FliA is an alternative sigma factor that regulates class III genes, such as flagellum components (Wang et al., 2006). In *D. dadantii*, mutation of *flhDC* showed a basal level expression of T3SS genes,

whereas *fliA* deletion mutant showed no change of *hrpL* or *hrpA*/*N* expression. Two pathways of FlhDC-mediated T3SS expression have been reported (Fig. 2); they are the FlhDC-EcpC-RpoN-HrpL pathway and the FlhDC-*rsmB*-RsmA-HrpL pathway (Yuan et al., 2015).

1.1.4 c-di-GMP and regulation of T3SS expression

Accumulated evidence shows that *bis*-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a global regulatory molecule that regulates many cellular processes such as biofilm formation, motility, and T3SS (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. GGDEF domain-containing proteins (diguanylate cyclase, DGC) and EAL or HD-GYP domain proteins (phosphodiesterase, PDE). DGCs synthesize c-di-GMP from two molecules of GTP while PDEs hydrolyze c-di-GMP into 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG) or two molecules of guanosine monophosphates. In *D. dadantii*, a total of 18 such proteins were identified from genomic analysis of GGDEF and EAL domains (Yi *et al*., 2010). The nomenclature refers to phosphodiesterases as Ecp (EAL domain containing proteins) while diguanylate cyclases as Gcp (GGDEF domain containing proteins) in *D. dadantii* 3937. Two PDEs, EcpC and EGcpB, participate in the regulation of biofilm formation, swimming, pectate lyase production, and T3SS gene expression (Fig. 2) (Yi et al., 2010). A lower amount of RpoN transcripts detected in Δ*ecpC* and Δ*egcpB* indicates c-di-GMP signaling participates in T3SS regulation at least on the *hrpL* transcriptional level (Fig. 2).

A known DGC, GcpA, showed a dominant role in several virulence factor regulations. Its presence is necessary for cell survival since attempts to delete *gcpA* in *D. dadantii* were

unsuccessful (Yuan *et al*., 2018). The regulation of GcpA on T3SS expression might depend on its negative impact on *rsmB* RNA (Fig. 2) (Yuan et al., 2018).

The binding of c-di-GMP to different effectors is necessary for cells to control a wide variety of functions. 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 (Chou & Galperin, 2016).

Two PilZ-domain proteins, $YcgR_{3937}$ and $BcsA_{3937}$, were identified as c-di-GMP effectors. The binding affinity of $YcgR_{3937}$ to c-di-GMP was confirmed by isothermal titration colorimetry (ITC) assay (Yuan et al., 2015). $YcgR_{3937}$ and $BcsA_{3937}$ differentially participate in T3SS regulation in response to intracellular c-di-GMP levels. However, the molecular mechanism of how c-di-GMP regulates T3SS awaits further elucidation (Fig.2) (Yuan et al., 2015).

1.2 Bacterial two-component systems

To sense various extracellular signals, the bacterial cell membrane is equipped with signal transduction systems to accurately respond and adapt to its environment (Kuhn, 2019). A basic stimulus-response from a classical two-component system involves a membrane-associated sensor kinase (SK) that catalyzes three functions, autophosphorylation, phosphorylation, and dephosphorylation. Depending on different TCSs, activated SK phosphorylates or dephosphorylates its corresponding response regulator (RR) receiver domain (REC) (Mizuno, 1998). RRs can mediate the cellular response, mostly through alteration of the bacterial transcriptional profile (Zschiedrich *et al*., 2016).

On average, the Gram-negative bacterial genome consists of 30 TCSs (Schaller *et al*., 2011). Though there are exceptions; for example, *Myxococcus xanthus* has over 200 TCSs (Shi *et al*.,

2008). An examination of genome of *D. dadantii* genome showed 30 TCSs (Yap *et al*., 2008). Several TCSs were studied in *D. dadantii*. Two TCSs, HrpX/HrpY and GacA/GacS, were to be involved in T3SS regulation (Fig.2) (Chatterjee et al., 2002, Tang *et al*., 2006). TCS PhoP-PhoQ, which in turn, is regulated by GacA, plays a role in resisting harsh environments (Manjurul Haque *et al*., 2012, Yang *et al*., 2008b). TCS CpxA/CpxR is essential for plant infection in *D. dadantii*, but the mechanism behind the regulation of CpxR on the virulence target is not well understood (Bontemps-Gallo *et al*., 2015).

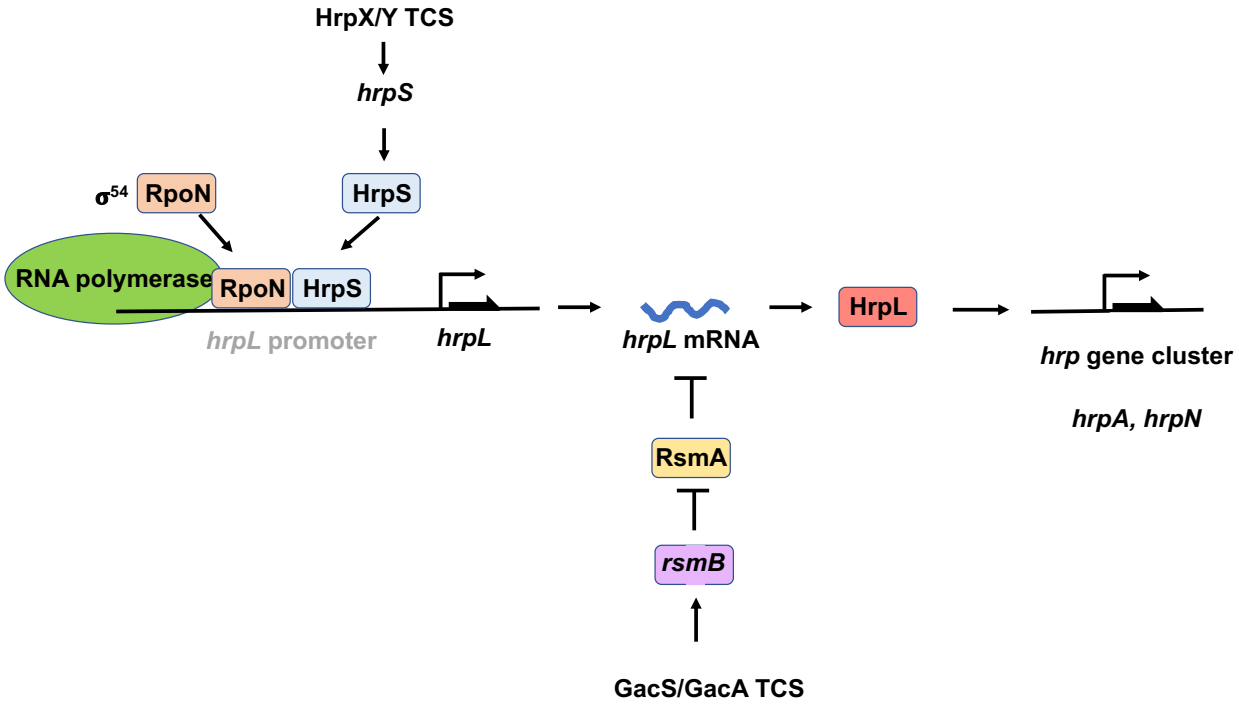
1.2.1 CpxA/CpxR two-component system

In Gram-negative bacteria, the cell envelope is bracketed by the outer membrane and inner membrane (Kuhn, 2019). It is a unique structure that contributes to essential cellular behaviors (Rogers & Perkins, 1968). TCS CpxA/CpxR was identified in the F plasmid conjugation defective phenotype in *E. coli*. Thus, Cpx (conjugative pilus expression) was initially known to be related to the envelope structural protein complex (McEwen & Silverman, 1980). Around a decade later, Silhavy's lab linked Cpx mutants to envelope located protein misfolding toxicity. They showed that the Cpx mutation conferred resistance to envelope stress (Cosma *et al*., 1995). The resistant phenotype observed from the Cpx mutation was later shown to be related to the gain-of-function of *cpxA* mutants (*cpxA**). Genomic alterations of the *cpxA* encoding region have the potential of activating its phosphorylation activity and muting its phosphatase activity, and thus constitutively activate CpxR (Raivio & Silhavy, 1997). Later, accumulated evidence showed TCS CpxA/CpxR was not only involved in the adaptation to envelope perturbation but also played an important role in animal bacterial pathogenesis (Raivio, 2014).

CpxA/CpxR is a typical TCS with a histidine kinase CpxA that senses envelope signals and alters the phosphorylation state of its cognate RR, CpxR (Fig. 3) (Weber & Silverman, 1988).

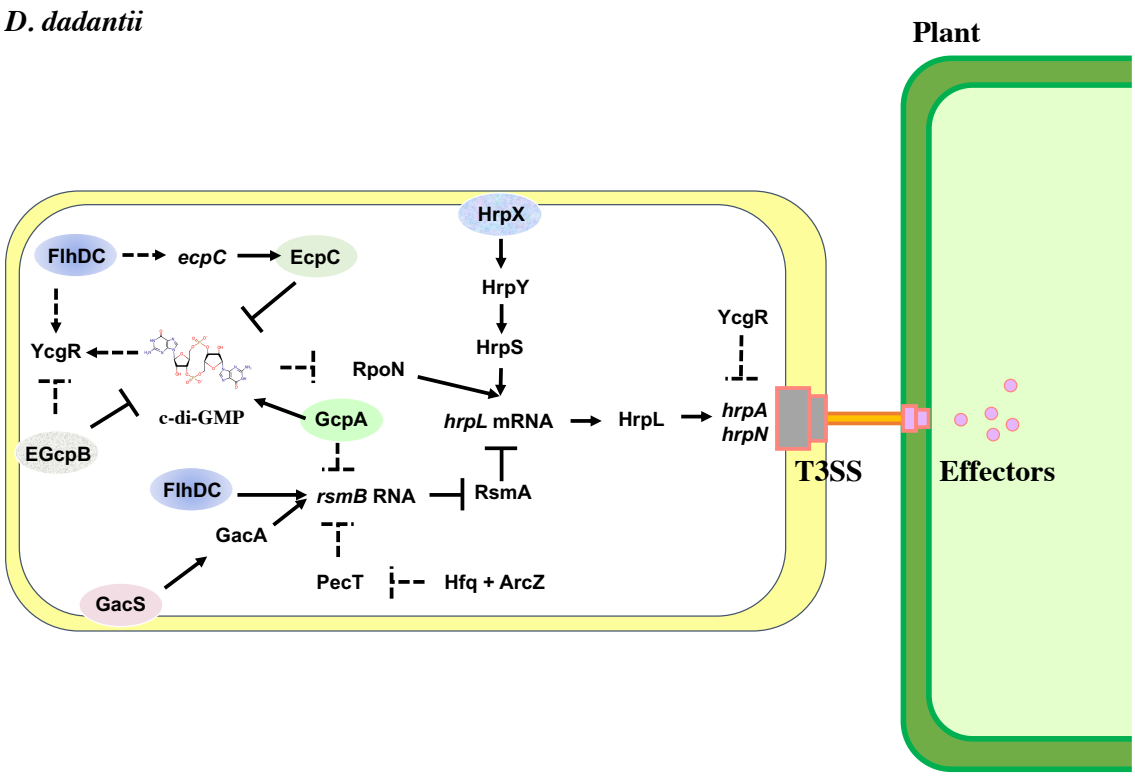
Two auxiliary proteins, NlpE and CpxP, were linked to the Cpx response. OM lipoprotein NlpE activates Cpx signaling either through overexpression of the *nlpE* locus or artificial mislocalization of OM NlpE in the inner membrane by genetically modified *nlpE* locus (DiGiuseppe & Silhavy, 2003). *cpxP* transcription is one of the most activated target genes under Cpx inducing conditions (Raivio, 2014). CpxP is a periplasmic chaperone protein that binds to misfolded proteins in the cell envelope and brings them to the periplasmic protease DegP for degradation (DiGiuseppe & Silhavy, 2003). CpxQ, a small RNA, was only recently discovered and linked to Cpx response (Chao *et al*., 2012, Chao & Vogel, 2016). It is a 3′-UTR sRNA from *cpxP* transcripts and is highly conserved in *E. coli*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and *Klebsiella pneumoniae* (Chao & Vogel, 2016).

The study of TCS CpxA/CpxR in animal pathogens raises the question of whether CpxA/CpxR plays a similar role in phytopathogens such as in *D. dadantii*. In this work, we addressed the potential role of CpxR on virulence regulation, aiming to enhance our understanding of the complex T3SS regulatory network in *D. dadantii*.



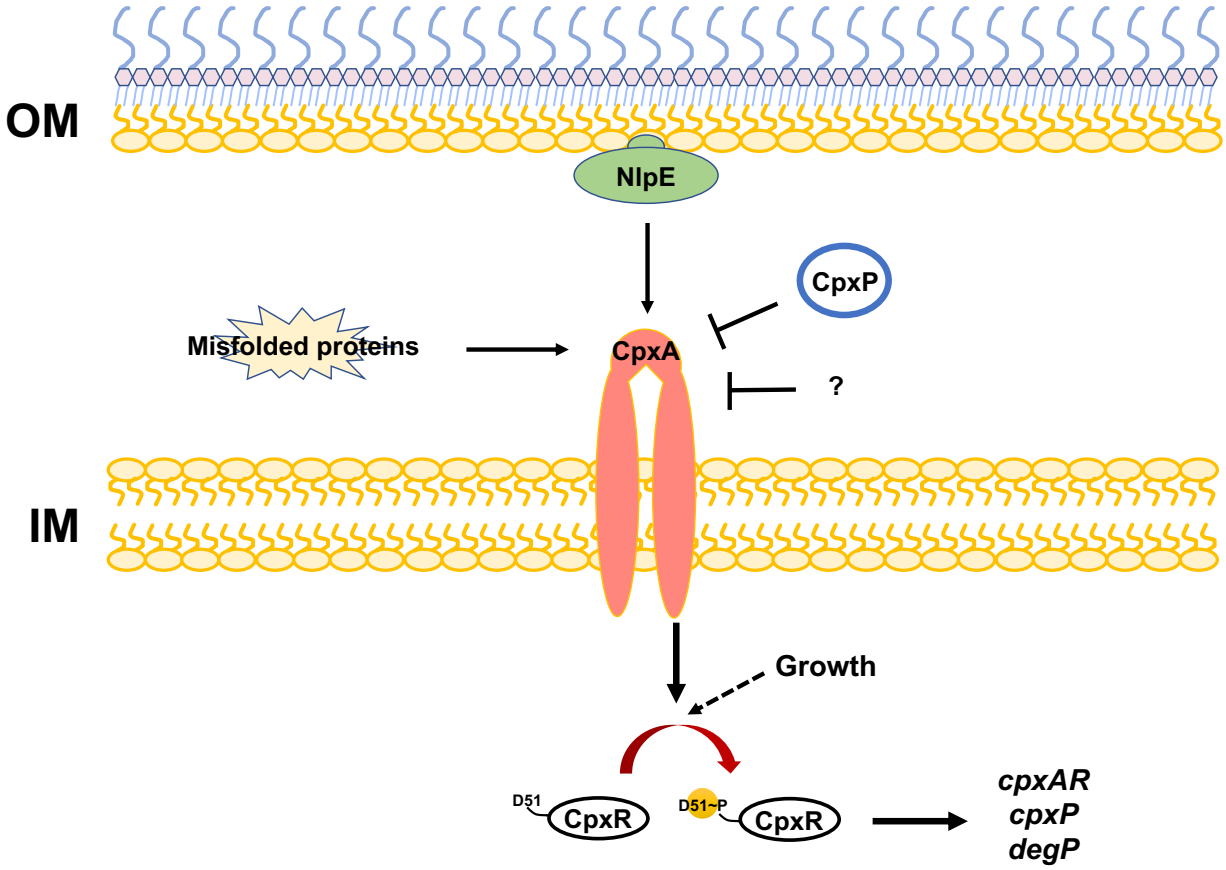
Source: modified from (Li *et al*., 2010)

Figure 1. The regulatory cascade of T3SS expression in *Dickeya dadantii*. A detailed description of the regulatory cascade is described in the text. HrpX/HrpY two-component system (TCS) activates the transcription of *hrpS*, which encodes a σ^{54} enhancer binding protein. HrpS recrutes the binding of RpoN (σ^{54}) to the promoter region of *hrpL* and increases the transcription of an alternative sigma factor HrpL. *hrpL* mRNA also subject to the post-transcriptional regulation by the inhibitor protein RsmA. The negative effect of RsmA is neutralized by *rsmB* sRNA via GacS/GacA TCS.



Source: modified from (Yuan et al., 2015, Yi et al., 2010, Yuan et al., 2019)

Figure 2. c-di-GMP signaling cascade on T3SS regulation in *Dickeya dadantii*. The master regulator FlhDC sits on the top of the hierarchy of the flagellar regulation, and it also shows an impact on the regulation of the T3SS expression of *D. dadantii* through at least three regulators: EcpC, FliA (not shown in the figure), and YcgR. EcpC is a phosphodiesterase that degrades bacteria second messenger c-di-GMP (cdG), and FlhDC indirectly influences the transcription of *ecpC*. Another phosphodiesterase EGcpB participates in T3SS regulation by degrading intracellular c-di-GMP, and the local EGcpB-generated-cdG pool influences RpoN RNA stability. EGcpB also showed an impact on YcgR regulation, which is a c-di-GMP effector that participates in T3SS regulation. FlhDC and PecT influence the expression of *hrpL* mRNA through RsmA-*rsmB* pair at post-transcriptional level.



Source: modified from (Mitchell & Silhavy, 2019)

Figure 3. **The components of Cpx response system.** CpxA/CpxR two-component system is located in the inner membrane (IM) and cytoplasm, respectively. NlpE is an outer membrane (OM) lipoprotein that could induce Cpx response via CpxA. Periplasmic chaperone CpxP and other unknown factors inhibit the phosphorylation of CpxA. Inducing signals such as envelope misfolded proteins and NlpE activate CpxA autophosphorylation and phosphorylation activity, the activated CpxA is able to transfer the phosphor group to the 51st aspartic acid of its cognate response regulator CpxR. Growth factors also influence the phosphorylation state of CpxR. The CpxR-P up-regulates or down-regulates a wide range of target genes. OM refers to outer membrane, and IM refers to inner membrane.

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Chapter 2

The phytopathogen *Dickeya dadantii* 3937 *cpxR* locus gene participates in the regulation of virulence and global c-di-GMP network

Abstract

Bacteria utilize signal transduction systems to sense and respond to their external environment. The two-component system (TCS) CpxA/CpxR senses envelope protein misfolded stress and responds by up-regulating envelope protein factors and down-regulating several virulence factors in several animal pathogens. *Dickeya dadantii* is a phytopathogen equipped with a type III secretion system (T3SS) for manipulating host immune response. We found that deletion of *cpxR* enhanced the expression of a T3SS marker gene *hrpA* in a designated T3SS inducing minimal medium (MM). Multiple T3SS and c-di-GMP regulators were also up-regulated in the Δ*cpxR* mutant. Subsequent analysis revealed that deletion of the phosphodiesterase gene *egcpB* in Δ*cpxR* abolished the enhanced T3SS expression. This suggested that CpxR suppresses EGcpB levels, causing low T3SS expression in MM. Furthermore, we found that the Δ*cpxR* mutant displays low c-di-GMP phenotypes in biofilm formation and swimming. Increased production of cellular c-di-GMP by *in trans* expression of a diguanylate cyclase gene *gcpA* was negated in the Δ*cpxR* mutant. Here, we propose that the TCS CpxA/CpxR regulates T3SS expression by manipulating the c-di-GMP network, in turn modifying multiple physiological activities involved in the response to environmental stresses in *D. dadantii*.

Introduction

The phytopathogen *Dickeya dadantii* 3937 (previously named *Erwinia chrysanthemum* 3937), a Gram-negative bacteria, was initially isolated and characterized from a lesion spot of a wilted African violet (*Saintpaulia ionantha*) in 1953 (Burkholder et al., 1953). Later research revealed *D. dadantii* has a wide range of hosts and is capable of infecting many economically important plants, including potatoes, tomatoes, and cabbages (Czajkowski et al., 2011). The typical symptom caused by *D. dadantii* is "soft rot", which occurs at the site of infection. The macerated plant tissue releases a substantial amount of oligosaccharides that provide nutrients for the bacteria to multiply. The bacteria also have an epiphytic lifestyle when they reside on the surface of leaves or underground in water. Because of their ability to adapt to various nutrient conditions, they have become an emerging threat to crop production, and prevention strategies are required to address *D. dadantii* infection.

Bacterial pathogens rely primarily on signal transduction systems to adapt to different environments and tackle host immune responses. One-component systems and two-component systems (TCSs) are known to sense and respond to a myriad of environmental signals and correspondingly change the bacteria transcriptional profile. The mode of molecular communication of TCSs is based on the phosphotransfer from a histidine-phosphate group to the conserved aspartate site of its cognate response regulator (Mizuno, 1998). The sensing mechanisms also assist pathogenesis in motility and chemotaxis (Prüß, 2017) for driving the cell into plant tissues through natural opening or wounds and further towards the apoplast, where they can digest polysaccharides from plant cell walls through a battery of plant cell wall degrading enzymes (PCWDEs). The *D. dadantii* type III secretion system (T3SS), an envelope

spanning complex, is regulated by at least two TCSs, HrpX/HrpY and GacA/GacS. TCS HrpX/HrpY transcriptionally activates T3SS expression via increasing the transcription of *hrpL*, encoding the T3SS master regulator HrpL (Wei & Beer, 1995). The transcription initiation of *hrpL* requires the binding of RpoN (a sigma factor σ^{54}) to an RNA polymerase and an RpoN-enhancer-binding protein HrpS. The transcription of HrpS is activated by TCS HrpX/HrpY (Tang et al., 2006). The *hrpL* transcripts are also subjected to posttranscriptional regulation by RsmA, which binds to *hrpL* mRNA and promotes its degradation. On the other hand, TCS GacA/GacS activates the transcription of RsmB regulatory RNA that binds to the RsmA protein and neutralizes negative effect of RsmA on *hrpL* mRNA. In turn, the alternative sigma factor HrpL binds to the *hrp* box region (GGAACC-$N_{15/16}$-CCACNNA) and activates the transcription of *hrpA* and *hrpN* (Yang *et al*., 2010). *hrpA* encodes the T3SS needle protein and *hrpN* encodes a harpin. The *D. dadantii* T3SS also contributes to multicellular behaviors, including pellicle formation, a cell aggregation behavior observed within the laboratory culture surface-liquid-air interface in Enterobacteria (Mee-Ngan *et al*., 2005).

All membrane-associated TCSs and secretion systems are located in the cell envelope. In Gram-negative bacteria, the envelope consists of three parts with a periplasmic space in between two lipid bilayers; the outer membrane (OM) and the inner membrane (IM). The cell envelope is the central hub for exchanging materials and signals between extracellular and intracellular environments. TCS CpxA/CpxR monitors envelope perturbance and is essential to maintain envelope health. CpxA/CpxR is a classical TCS with an inner membrane located CpxA histidine kinase and a cytoplasmic response regulator CpxR. The conserved 51st aspartate residue of CpxR receives a phosphor group from CpxA and alters the transcription of a diverse range of Cpx

regulon genes. The *cpxP* locus is one of the most induced transcriptional loci under Cpx inducing conditions and is frequently used as a Cpx response indicator (MacRitchie *et al*., 2012). The role of CpxP in *Escherichia coli* is both an inhibitor of CpxA autophosphorylation and a P-pilus binding protein that sends misfolded P-pilus proteins for degradation by periplasmic protease DegP, whose transcription is also Cpx dependent (Raivio *et al*., 2000, Danese *et al*., 1995, Danese & Silhavy, 1998).

A previous study demonstrated that TCS CpxA/CpxR is involved in the early stages of the infection process and motility in *D. dadantii* (Bontemps-Gallo et al., 2015), yet the details of its downstream regulation have not been explained. In *D. dadantii*, the bacterial second messenger, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) serves as a global signaling molecule that regulates T3SS expression via multiple c-di-GMP related components (Yi et al., 2010, Yuan et al., 2015, Yuan et al., 2019, Yuan et al., 2018). Cellular c-di-GMP levels are regulated by two types of enzymes; diguanylate cyclases (GGDEF domain-containing DGCs) and phosphodiesterases (EAL or HD-GYP domain-containing PDEs), which synthesize and degrade the second messenger, respectively. c-di-GMP acts as a versatile ligand that binds to PilZ domain proteins, degenerate GGDEF or EAL domain proteins, or RNA riboswitches to modulate a specific output (Hengge, 2009). Hence, the dynamic intracellular c-di-GMP concentrations facilitate the ability of the cell to adequately respond to its environment. Analysis of *D. dadantii* genome revealed 12 DGCs, 4 PDEs, and 2 dual-domain proteins (GGDEF-EAL domain). A panel of deletion mutants of DGC and PDE displayed various phenotypes, suggesting some DGCs/PDEs have specific roles in cellular behaviors. For example, *D. dadantii* GcpA (a DGC) controls pectate lyase (Pel) production via GcpA mediated repression of H-NS,

which is in the same pathway that up-regulates RsmB and downregulates RsmA (Yuan et al., 2018). However, GcpA mediated T3SS expression occurs via a different route that bypasses the H-NS and Rsm system (Yuan et al., 2018).

In this study, we unveil the link between the Cpx signaling cascade and c-di-GMP in the regulation of the T3SS. In contrast to the LB medium used in a CpxR-related T3SS study in an animal pathogen *Yersinia pseudotuberculosis* (Liu *et al*., 2012), the plant apoplast mimicking minimal medium (MM) is used to induce and study T3SS expression. We demonstrate the negative role of CpxR on T3SS expression and the involvement of CpxR in the transcription of several T3SS regulators such as HrpS and RpoN. This study proposes that the virulence, biofilm, and motility phenotypes linked to *cpxR* deletion are mediated through downstream c-di-GMP regulators. The involvement of this TCS in the already sophisticated T3SS regulatory mechanism, bringing us closer to elucidating how the T3SS functions under various stresses in phytopathogens.

Experimental procedures

Bacterial strains, plasmids, primers, and culture

The bacterial strains and media used in this work are listed in Table S1. All the primer information is listed in Table S2 in the supplemental material. *Escherichia coli* strains were grown in lysogeny broth (LB) medium (1% tryptone, 1% NaCl and 0.5% yeast extract) at 37°C. *D. dadantii* strains were cultured in LB broth, 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) at 28°C (Yang *et al*., 2008). Antibiotics were added to the

culture at the listed concentrations: ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), spectinomycin (100 μg ml^{-1}). Genomic data of *Dickeya* was retrieved from a systematic annotation package for community analysis of genomes (ASAP) (https://asap.ahabs.wisc.edu/asap/home.php).

Mutant construction and complementation

Marker exchange mutagenesis was used to construct Δ*cpxR* or other mutants (Yang et al., 2002). In brief, the downstream and upstream of the target gene locus (e.g., *cpxR* locus) were each amplified by PCR using specific primers (Table 2), the achieved DNA fragments were ligated with the kanamycin (Km) cassette fragments (~1.5 kb) from the pKD4 plasmid (Datsenko & Wanner, 2000) using three-way cross-over PCR. The right sized DNA fragments were then digested and ligated to pwm91, the suicide plasmid. After conjugation using *E. coli* S17-1 λ-pir strain, pwm91 plasmid was transformed into *D. dadantii*. Following a selection of recombinants grown in 10% sucrose MG agar medium, colonies showing sucrose resistance due to the loss of SacB-mediated toxicity were then plated onto an LB ampicillin plate, and the ampicillin-sensitive cells were picked and stored in -80° C for future analysis. Constructed mutants were further confirmed by sequence analysis via the target gene outside primers.

To construct double mutants, removal of the Km cassette from maker exchange mutants was first performed. To remove the Km cassette, the pFLP2 plasmid encoding the FLP (flippase) recombinase enzyme in *E. coli* S17-1 λ-pir was conjugated with mutant harboring Km cassette in LB agar media. After excision of Km cassette through pFLP2 plasmid, strains were selected on Km sensitive and sucrose resistant petri plates for sequence analysis using outside primers. The double mutant was then constructed using maker exchange mutagenesis as mentioned above. To generate complemented strains, a low-copy-number plasmid pCL1920 was used to express the open reading frame (ORF) regions of target genes. The target region was cloned to pCL1920 and

downstream of a leaky *lac* operon. Constructed vector was then transformed into *Dickeya* strains. All constructed plasmids were confirmed by sequencing and phenotype.

Transcriptional analysis from the GFP reporter plasmid

To construct GFP reporter plasmid pAT-*cpxP*, pAT-*cpxR*, and pAT-*degP*, the promoter regions and small portion of open reading frames of target genes were amplified by PCR and ligated to the probe vector pPROBE-AT(Leveau & Lindow, 2001). The mean fluorescence intensity (MFI) was measured by flow cytometry (BD Biosciences, San Jose, CA, USA) after culture in LB or MM medium, and bacterial cell suspensions were collected in different time periods. Samples were diluted 100x and detected by flow cytometry (Peng *et al*., 2006). To study T3SS expression, two T3SS subunits (HrpA, HrpN) and the T3SS master regulator expression HrpL were chosen. pAT-*hrpA*, pAT-*hrpN,* and pAT-*hrpL* were constructed previously and their promoter activities were measured as described above (Yi et al., 2010).

Pel activity assay and potato virulence assay

Dickeya Pel activity was measured by spectrometry as described in (Matsumoto *et al*., 2003). In brief, overnight cultures of *Dickeya* strains in LB broth were transferred 1:100 to MM media supplemented with 0.1% PGA at 28˚C for 16 h; two mL of bacteria culture was collected and centrifuged at 13 000 g for 2 min, the supernatant was then transferred to a new Eppendorf tube for further analysis. To measure the Pel activity, 10 μL of the supernatant was mixed with 990 μL of reaction buffer [0.05% polygalacturonic acid (PGA), 0.1 M Tris-HCl (pH 8.5) and 0.1 mM $CaCl_2$, prewarmed to 30 ˚C]. Spectrometry was set to absorbance 230 nm to measure the Pel activity for 3 min. The calculation is based on the basis that one unit of Pel activity is equal to an increase of 1×10^{-3} OD_{230} in 1 min. The potato maceration assay was performed using potatoes purchased in a local supermarket. One hundred microliter overnight bacterial cultures ($OD_{590} = 1.0$)

were injected into surface sterilized potatoes. The images were taken after 3 days of incubation at 28˚C.

Determination of the intracellular c-di-GMP concentration

We adopted a c-di-GMP responsive riboswitch method where *Vibrio cholerae* Vc2 riboswitch is cloned into pRS414 (Sudarsan *et al*., 2008). Plasmid pRS414-Vc2 was electrotransformed into *D. dadantii* 3937 and its derivatives, and the c-di-GMP levels were represented by the β-galactosidase activity (Sudarsan et al., 2008) as described below.

GUS reporter assay on c-di-GMP concentration

Five hundred μL overnight cell cultures grown in MM were harvested by centrifugation at 10 000 g for 3 min. The cell pellet was resuspended in 1 × PBS buffer. After the addition of 50 μL 0.1 % SDS (sodium dodecyl sulfate), 50 μL of chloroform was added. After each addition of agent the sample was vigorously vortexed. Samples were then centrifuged for 1 min under 10 000 g. One hundred μL of the upper fraction was collected and mixed with 890 μL 1 × PBS buffer. Following the addition of 10 μL of 10 mM MUG (Sigma), 100 μL of the mixture was measured at the excitation of 365nm and emission at 455 nm at 0, 5, 10, 15, and 20 minutes. One hundred μL of upper fraction from GUS assay was used in the Bradford assay (Bio-Rad, Hercules, CA, USA) to standardize the GUS value. Briefly, 700 μL H_2O and 200 μL Bradford agent were mixed with 100 μL of upper fraction from GUS assay and determine the absorbance at 595 nm.

Biofilm formation assay and swimming assay

Biofilm formation assay was tested as described before (Yi et al., 2010). In brief, *Dickeya* and its derivatives were streaked on LB agar plate, and single colonies of different strains were cultured in LB broth at 28˚C overnight. Bacteria broth was transferred 1:100 into MM media in 1.5 mL Eppendorf tubes, and 200 μL MM was then transferred into 96-well Microplate and incubated for

48 h. 1% crystal violet was used to stain the sessile cells for 15 min. The planktonic cells were removed by several gentle washes with H_2O. After 5 h air dry process, the stained cells were dissolved in 90% ethanol and examined under optical density (OD) 590. Swimming assay was tested as described before (Yuan et al., 2015). Cells were first grown in LB broth overnight at 28˚C. The sample was then adjusted to OD_{590} = 1.0, and 10 μL overnight bacterial sample was inoculated in a swimming assay plate (MG plates containing 0.2% agar) at 28˚C. Swimming results were represented as the diameter of the radial growth after 16 h.

Statistical analysis

Calculation of means and standard deviations of experimental values was performed using Excel. Statistical analysis was calculated using one-way ANOVA with post-hoc Tukey HSD (Honestly Significant Difference) test or Student's t-test (Microsoft, Redmond, WA).

Results

CpxR is a negative regulator of T3SS expression

To study T3SS regulation, genes encoding two subunits of the T3SS protein complex, the needle subunit HrpA and the harpin protein HrpN, were selected for expression analysis. Previously, our lab identified a diguanylate cyclase (GcpD), mutant of *gcpD* locus showed enhanced *hrpA* expression (data not shown). We conducted a transposon screening for identification of any Δ*gcpD*-mediated T3SS regulator(s) based on a *hrpA::GFP* reporter system. One transposon mutant that demonstrated a significant increase in *hrpA* expression was selected for further analysis. The insertion site was found to be within the *cpxR* gene, which encodes a response regulator from a typical TCS CpxA/CpxR. To further verify the relationship between CpxR and *hrp* gene expression, we constructed a *cpxR* deletion mutant (Δ*cpxR*), and its impact on the T3SS expression was examined. A significant increase of *hrpA* and *hrpN* expression was observed in Δ*cpxR*, and the enhanced T3SS expression was restored to wild-type level by complementation of a *cpxR* gene in a low copy number plasmid (pCL1920-*cpxR*) (Fig.1A-1B). Since CpxR regulates T3SS in the wild-type background, we further investigated the regulatory effect of CpxR in wild-type *D. dadantii*.

The expression of T3SS is tightly regulated and involves multiple regulatory cascades. The T3SS master regulator HrpL is an alternative sigma factor that activates *hrp* gene transcription by recruiting RNA polymerase to a *hrp* box region (GGAACC-$N_{15/16}$-CCACNNA) (Tampakaki *et al*., 2010). Deletion of the *cpxR* locus resulted in an increase in *hrpL* transcription (Fig. 1C). The expression of *cpxR* in trans restored the *hrpL* promoter activity back to wild-type levels (Fig.

1C), indicating that CpxR participates in T3SS repression through regulation of *hrpL* transcription in *D. dadantii*.

Transcription of key T3SS regulators was increased in the Δ*cpxR* mutant

To further identify the potential CpxR-regulated T3SS regulators, several upstream T3SS regulators were selected and their transcriptions were analyzed upon deletion of *cpxR*. Studies showed that at least two regulatory cascades regulate *hrpL* at the transcriptional and posttranscriptional levels. The transcriptional regulation of *hrpL* is through HrpS which facilitates the binding of RpoN to the promoter region of HrpL (Tang et al., 2006). We examined *hrpS* and *rpoN* promoter activity under the Δ*cpxR* mutant. At 24 h, transcription of *hrpS* and *rpoN* was both significantly increased in the Δ*cpxR* mutant (Fig. 2 A, B). This observation indicates that CpxR controls T3SS expression by regulating transcriptional levels of HrpS and RpoN.

TCS GacA/GacS regulates T3SS by up-regulating the transcription of the regulatory small RNA RsmB. RsmB binds to the RsmA and neutralizes the activity of RsmA on *hrpL* mRNA degradation. (Liu *et al*., 1998, Chatterjee et al., 2002). The promoter activities of *rsmB* and *rsmA* were therefore examined. Although RsmA and RsmB are expected to work counteractively in T3SS expression, both *rsmA* and *rsmB* transcriptions were increased in Δ*cpxR* (Fig. 2 C, D). This posed a puzzle in identifying the role of CpxR on T3SS regulation. Since the increased *rsmB* might sequester the negative effect of RsmA, we tried to solve this discrepancy by examining pectate lyase (Pel) activity that is also subject to the regulation by the RsmA/RsmB system(Yuan et al., 2015). The HrpS-RpoN-HrpL pathway does not regulate Pel production in *D. dadantii*. RsmA promotes the degradation of Pel mRNA while the RsmB sRNA binds to RsmA to neutralize its negative effect

on Pel mRNA (Liu *et al*., 1997). If RsmA/RsmB plays a major role in T3SS regulation through CpxR, a higher Pel activity is expected in the Δ*cpxR*. The total Pel activity was measured spectrophotometrically to determine the net effect of the Rsm system in T3SS inducing minimal medium. In Δ*cpxR*, a slight but significant reduction of Pel activity was observed (Fig. 2 E). This result is supported by the reduced Pel activity in Δ*cpxR* through virulence assays we conducted on potato tubers. The production of Pel is essential for the phytopathogen to degrade plant cell walls (Hugouvieux-Cotte-Pattat, 2016). The reduction of virulence on potato tubers in the Δ*cpxR* pCL1920 strain was recovered in Δ*cpxR* pCL-*cpxR,* which showed similar maceration to the wild-type strain (Fig. 2 F). Since inverse phenotypes were observed in T3SS and Pel in Δ*cpxR*, the above results demonstrate RsmA/RsmB does not play a major role in the T3SS regulation through CpxR. This also indicates CpxR regulates Pel through other regulator(s), not the Rsm system.

CpxR regulates T3SS expression through CpxP in minimal medium

Our next step was to link any Cpx response to T3SS. Apart from minimal medium, we also included the nutrient rich LB broth for comparison (Bontemps-Gallo et al., 2015). Nutrient-rich media resulted in basal T3SS expression in phytobacteria. As reported in other studies, *cpxP* and *degP* are two Cpx regulon genes (Price & Raivio, 2009, Bontemps-Gallo et al., 2015). The promoter regions of *cpxP* and *degP* were amplified by PCR and ligated to the pPROBE-AT vector containing green fluorescent protein. The promoter activity was measured by determining fluorescence intensity through flow cytometry similar to previous promoter activity measurements. Our Δ*cpxR* construct showed no expression of *cpxP* in both LB and MM (Fig 3 A, B). CpxP is one of the most highly induced Cpx components (Price & Raivio, 2009), and a further enhancement of *cpxP* transcription was observed in LB by *in trans* expression of *nlpE*, which encodes a known

Cpx pathway activator (Snyder *et al*., 1995) (Fig. 3B). However, the NlpE-induced Cpx response was insignificant in MM (Fig. 3A). A complementation strain harboring pCL1920-*cpxR* restored the *cpxP* transcription to WT level, while pCL1920-*cpxR*^{D51A}, a point mutation on the phosphorylate site on CpxR, was incapable of restoring *cpxP* transcription (Fig. 3 A, B). *degP* transcription was suppressed in Δ*cpxR* in LB only (Fig. 3C, D) and slightly induced by NlpE in LB (Fig. 3D). Deletion of *cpxR* did not fully abolish the expression of *degP*, suggesting *degP* expression is not solely dependent on CpxR. Since there was no observable change of *degP* transcription in Δ*cpxR* in MM, the T3SS inducing medium for phytobacteria, the suppressive effect of CpxR on T3SS expression may not go through DegP in *D. dadantii*. On the other hand, the necessity of the presence of CpxR on *cpxP* transcription suggests that the *cpxP* locus may be involved in T3SS regulation. Thus, a follow-up experiment including Δ*cpxR* with over-expressed *cpxP* was conducted. *In trans* expression of *cpxP* in Δ*cpxR* suppressed *hrpA* expression at levels similar to *in trans* expression of *cpxR* in Δ*cpxR* at 24 h (Fig. 3E). This further demonstrates that *cpxP* is involved in T3SS expression in *D. dadantii*.

CpxR controls T3SS expression through manipulation of genes affecting c-di-GMP turnover

Our previous investigation of T3SS regulation showed a strong link between several T3SS regulators and bacterial second messenger c-di-GMP signaling. Several c-di-GMP components were shown to participate in T3SS expression (Yuan et al., 2018, Yi et al., 2010). GcpA, a diguanylate cyclase, was able to increase intracellular c-di-GMP concentrations to downregulate T3SS expression (Yuan et al., 2018). It was also discovered that EGcpB and EcpC, two phosphodiesterases that catalyze hydrolysis of c-di-GMP, upregulated T3SS expression (Yi et

al., 2010). We were interested in determining if the regulation of T3SS by CpxR is through c-di-GMP. We first examined the effect of *cpxR* deletion on the transcription of several reported DGC and PDEs. Interestingly, the results showed that *gcpA*, *egcpB*, and *ecpC* expression were all increased in Δ*cpxR* (Fig. 4 A-C). DGCs and PDEs have contradictive roles in cellular c-di-GMP levels, which raised the question of what could be causing the net increase in T3SS expression in Δ*cpxR*. We then analyzed intracellular c-di-GMP levels through a c-di-GMP reporter that harbors a transcriptional fusion of β-glucuronidase to a 110-nucleotide Vc2 RNA riboswitch, a high affinity c-di-GMP aptamer (Sudarsan et al., 2008). The result showed that there was no significant difference in c-di-GMP levels between WT and Δ*cpx*R in MM (Fig. 4D). To verify the potential strength of Δ*cpxR* mediated T3SS expression and c-di-GMP signaling, we sought to confirm two c-di-GMP associated phenotypes in nutrient limiting media: biofilm formation and motility. The induced motility and the repressed biofilm formation observed in Δ*cpxR* (Fig. 5 A, B) led us to speculate whether artificial manipulation of intracellular c-di-GMP would alter the c-di-GMP related phenotypes in Δ*cpxR*. First, we compared *hrpA* promoter activity in *egcpB* and *ecpC* mutants. The single mutants of Δ*ecpC* and Δ*egcpB* repressed *hrpA* promoter activity (Fig. 5C), which as reported before is due to the increased global c-di-GMP levels (Yi et al., 2010). The double mutants Δ*cpxR*Δ*egcpB* and Δ*cpxR*Δ*ecpC* had significantly different levels of *hrpA* promoter activity (Fig. 5C). Deletion of *cpxR* in Δ*ecpC* showed increased *hrpA* activity, especially at 24 h, while no significant difference was observed between Δ*egcpB* and Δ*cpxR*Δ*egcpB* (Fig. 5C). Deletion of *cpxR* did not increase *hrpA* expression in Δ*egcpB*, indicating that *hrpA* expression in Δ*cpxR* is dependent on EGcpB. To further bolster our comprehension of the Δ*cpxR*-regulated c-di-GMP cascade, we increased the level of c-di-GMP by *in trans* expressed *gcpA* (pCL-*gcpA*) in WT and Δ*cpxR*. While the WT strain harboring pCL-

gcpA showed increased c-di-GMP levels, there was no difference in c-di-GMP levels between WT and Δ*cpxR* harboring pCL-*gcpA* (Fig. 5D). This observation suggests that the increased c-di-GMP levels by *gcpA* is negated by enhanced EGcpB expression in Δ*cpxR*. The Δ*cpxR* mutant harboring empty vector showed a noticeable but insignificant reduction of c-di-GMP levels (Fig 4D, 5D). This could be because the basal level of c-di-GMP is too low to be detected by this method.

Discussion

Signal transduction systems provide remarkable flexibility for bacteria to respond to a variety of external and cellular signals. The CpxA/CpxR two-component system (TCS) is one of the most highly conserved signal transduction systems in Enterobacteriaceae and has been extensively studied in animal pathogens, but the regulatory role of Cpx on virulence factors in phytopathogens is rarely reported. Transposon mutagenesis and gene deletion of *cpxR* significantly increased *hrpA* and *hrpN* expression in minimal medium, the T3SS-inducing medium of phytobacteria. Expression of two T3SS regulators, *rpoN* and *hrpS*, were both increased in Δ*cpxR*, indicating CpxR might regulate T3SS expression via these regulators. Among the two known CpxR-regulated genes, *cpxP* and *degP* (Bontemps-Gallo et al., 2015, Price & Raivio, 2009), only *cpxP* expression was significantly reduced in Δ*cpxR* mutant in MM, suggesting that CpxP plays a more important role in *cpxR*-mediated T3SS expression in MM.

Our understanding of the Cpx response comes largely from model organisms such as *E. coli*, *Y. pseudotuberculosis*, and *Shigella flexneri* (Fei *et al*., 2021, Acosta *et al*., 2015, Vogt *et al*., 2010). The initial characterization of the Cpx response showed a direct relationship to envelope stress

factors such as periplasmic proteins DegP, DsbA, and PpiA (Danese et al., 1995). Later research of TCS CpxA/CpxR showed that the physiological role of the Cpx response was even more diverse than originally thought. For instance, a broad-scale analysis of *E. coli* MC4100 Cpx response revealed uncharacterized targets that belong to a wide range of cellular processes (Price & Raivio, 2009). Moreover, various Cpx signal inducers were identified. These included external stresses, such as alkaline pH, misfolded P-pilus, and attachment to abiotic surfaces through NlpE (Danese & Silhavy, 1998, Jones *et al*., 1997, Snyder et al., 1995). Intracellular signals have also been shown to invoke Cpx response, such as altered LPS biosynthesis (Delhaye *et al*., 2016), or the small phosphor donor acetyl-phosphate, that is generated through the AckA-Pta pathway (Wolfe, 2010), and can directly enter into the Cpx pathway through direct CpxR phosphorylation. The broad range of Cpx targets and Cpx inducers raise the question of the specific role of Cpx response on cellular behaviors. We have also identified some stress-inducing compounds that significantly increase CpxP transcription (data not shown), suggesting CpxR in *D. dadantii* may also have a similar role in stress response as in other bacteria. On top of the above findings, the T3SS-related transposon screening in this study has unveiled a novel role of CpxR in T3SS regulation in *D. dadantii*.

Although the mechanism of T3SS regulation has been reported previously, the participation of multiple layers of regulation pathways in T3SS expression is not surprising, considering the deep complexities of host-pathogen molecular interactions. In this study, we addressed the crosstalk between T3SS expression and various signaling cascades. Our key model is based on the induced T3SS expression in plant apoplast mimicking minimal media (MM). There is a distinct difference between the study of animal pathogens and phytopathogens since most animal pathogens can express the T3SS in nutrient-rich media. For example, the study of *Y. pseudotuberculosis* requires

brain heart Infusion (BHI) broth, a nutrient-rich medium, to induce T3SS expression (Liu et al., 2012). The majority of Cpx studies in *Enterobacteriaceae* family used the nutrient-rich medium lysogeny broth that, in contrast, suppresses T3SS expression in plant pathogens (Yang *et al*., 2008a). In this study, NlpE induced Cpx response was not significant in MM when compared to LB (Fig. 3A, C), indicating Cpx-mediated T3SS regulation in *D. dadantii* in MM could be different from the reported organisms.

Several studies indicate that CpxR participates in the regulation of secretion systems in several animal pathogens. CpxR might directly suppress *Y. pseudotuberculosis* Ysc-Yop T3SS through binding to the promoter regions of *lcrF* and *yop* (Liu et al., 2012) where *lcrF* encodes an AraC-like transcriptional activator responsible for Ysc-Yop T3SS transcription (Cornelis *et al*., 1989). The direct role of CpxR binding to the Dot/Icm translocation system proteins (a type IV_B secretion system) was also observed in the study of *Legionella pneumophila* through gel mobility shift assays (Altman & Segal, 2008). Conversely, the study of enteropathogenic *E. coli* (EPEC) Δ*cpxR* showed minimal effect on T3SS, but a CpxR-regulated protease DegP plays an essential role in EPEC T3SS assembly at the posttranscriptional level (MacRitchie et al., 2012). The variability of Cpx responses observed in various pathogens with diverse conditions are worth noting and await further clarification.

We hypothesized that CpxR of *D. dadantii* might affect some global responses that alter multiple T3SS regulators such as RpoN, HrpS, or HrpL, as reported in this study. The bacterial second messenger c-di-GMP is a global regulator that regulates multiple phenotypes in *D. dadantii* (Yuan et al., 2018). The study of this single ribonucleotide also raises the question of its specificity.

Global and local c-di-GMP pools have been proposed to explain the seemingly contradictory cellular behaviors (Hengge, 2021). Although the specific c-di-GMP effector(s) of the T3SS remain unknown in *D. dadantii*, surface plasmon resonance (SPR) assays suggested the T3SS ATPase HrcN is a potential c-di-GMP binding target (Trampari *et al*., 2015). There is a possibility that CpxR binds to the promoter regions of the DGC GcpA or PDEs EGcpB and EcpC, but this theory needs further confirmation. Through c-di-GMP binding riboswitch experiments, we were able to measure the relative c-di-GMP levels in MM. No significant differences in global c-di-GMP levels were observed between the wild-type and Δ*cpxR* (Fig. 5D), and we speculate that deletion of *cpxR* might affect the T3SS through altering local c-di-GMP pools. Interestingly, *in trans* expression of *gcpA* in Δ*cpxR* did not show increased c-di-GMP concentration compared to the WT. There are two possibilities to explain this phenomenon: 1. The increased c-di-GMP might be accordingly degraded by the increased expression of PDEs; or 2. The function of GcpA is suppressed. Since EGcpB was a strong c-di-GMP remover (Fig. 4D) and the variation of *gcpA* expression was minor (Fig. 4A) in MM, we prefer the hypothesis that c-di-GMP was cleared by EGcpB in Δ*cpxR*. Although the difference of c-di-GMP levels between WT and Δ*cpxR* may be too subtle to be detected, the low c-di-GMP phenotypes such as induced swimming and repressed biofilm formation were found in Δ*cpxR* (Fig. 5. A, B).

In this study, the negative role of CpxR on *D. dadantii* T3SS expression in MM was identified, and a model for the crosstalk between CpxR and c-di-GMP in T3SS regulation is proposed (Fig. 6). CpxR suppresses T3SS expression, which might be through the suppression of PDEs such as EGcpB. Biologically, the suppression may inform the bacteria not to convert to a virulent lifestyle when potential stress is present. Conversely, the loss of CpxR (i.e., Δ*cpxR*) could give an artificial

signal to the cell that the surrounding environment will be accommodating, leading it to explore and become virulent. The diverse role of CpxR in complex bacterial signal transduction systems presents a key foundation for potential drug targets that could facilitate bacterial management and disease control in the future.

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*cpxR*::Km	Δ*cpxR*::*Km*; Km^r, ABF-0019170 deletion mutant	This study
Δ*cpxR*	Δ*cpxR* clean mutant	This study
gcpA^{D418A}	*gcpA*^{D418A}::Km; Kmr, ABF-0020368 site-directed mutant	(Yuan et al., 2018)
Δ*egcpB*	Δ*egcpB::Km*; Km^r, ABF-0020123 deletion mutant	(Yi et al., 2010)
Δ*ecpC*	Δ*ecpC::Km*; Km^r, ABF-0020364 deletion mutant	(Yi et al., 2010)
egcpB-HA	Chromosomal *egcpB*-HA; ABF-0020123	(Yuan et al., 2019)
Δ*cpxR egcpB*-HA	Chromosomal *egcpB*-HA in Δ*cpxR* clean mutant;	This study
Escherichia coli		
DH5α	F$^-$ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1	Lab stock
S17-1 λpir	λ(pir) *hsdR* pro *thi*; chromosomally integrated RP4-2 Tc::Mu Km::Tn7	Lab stock
Plasmid		
pKD4	The template used to PCR kanamycin cassette, Km^r	(Datsenko & Wanner, 2000)
pWM91	A sucrose-based counter-selectable plasmid used for marker exchange mutation, Ap^r	(Metcalf *et al*., 1996)
pCL1920	Low copy number plasmid, *lac* promoter Sp^r	(Lerner & Inouye, 1990)
pFLP2	Flippase FRT excision plasmid for clean mutant construction, Ap^r	(Hoang *et al*., 1998)
pCL1920-*gcpA*	Low copy number plasmid expressing *gcpA* locus, Sp^r	(Yuan et al., 2018)
pCL1920-*cpxR*	Low copy number plasmid expressing *cpxR* locus, Sp^r	This study

pCL1920-*cpxR* ^{D51A}	*cpxR* site-directed mutated locus cloned in pCL1920	This study
pCL1920-*nlpE*	*nlpE* cloned in pCL1920, Sp^r	This study
pCL1920-*cpxP*	*cpxP* cloned in pCL1920, Sp^r	This study
pRS414-Vc2	c-di-GMP reporter vector, Ap^r	(Sudarsan et al., 2008)
pPROBE-AT	Promoter-probe vector using *gfp*, Ap^r	(Miller *et al*., 2000)
pAT-*hrpA*	pPROBE-AT vector containing *hrpA* promoter region with *gfp* transcriptional fusion, Ap^r	(Yang *et al*., 2007)
pAT-*hrpN*	pPROBE-AT vector containing *hrpN* promoter region with *gfp* transcriptional fusion, Ap^r	(Yang et al., 2007)
pAT-*hrpL*	pPROBE-AT vector containing *hrpL* promoter region with *gfp* transcriptional fusion, Ap^r	(Yang et al., 2007)
pAT-*rpoN*	pPROBE-AT vector containing *rpoN* promoter region with *gfp* transcriptional fusion, Ap^r	(Yi et al., 2010)
pAT-*hrpS*	pPROBE-AT vector containing *hrpS* promoter region with *gfp* transcriptional fusion, Ap^r	(Yi et al., 2010)
pAT-*rsmA*	pPROBE-AT vector containing *rsmA* promoter region with *gfp* transcriptional fusion, Ap^r	(Yi et al., 2010)
pAT-*rsmB*	pPROBE-AT vector containing *rsmB* promoter region with *gfp* transcriptional fusion, Ap^r	(Li *et al*., 2015)
pAT-*cpxR*	pPROBE-AT vector containing *cpxR* promoter region with *gfp* transcriptional fusion, Ap^r	This study
pAT-*cpxP*	pPROBE-AT vector containing *cpxP* promoter region with *gfp* transcriptional fusion, Ap^r	This study
pAT-*gcpA*	pPROBE-AT vector containing *gcpA* promoter region with *gfp* transcriptional fusion, Ap^r	(Yuan et al., 2018)
pAT-*egcpB*	pPROBE-AT vector containing *egcpB* promoter region with *gfp* transcriptional fusion, Ap^r	(Yuan et al., 2015)
pAT-*ecpC*	pPROBE-AT vector containing *ecpC* promoter region with *gfp* transcriptional fusion, Ap^r	(Yuan et al., 2015)

pAT-*degP*	pPROBE-AT vector containing *degP* promoter region with *gfp* transcriptional fusion, Ap^r	This study

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

Table 2. List of primers

Primers	Sequences (5'–3')	Use
cpxR-A-XhoI	AATA**CTCGAG**TCAGGGCAGGATAGTCAC	Δ*cpxR*::Km deletion
cpxR-B	GAAGCAGCTCCAGCCTACACCCATGATTTACCTACCTCCACGGGCA	
cpxR-C	CTAAGGAGGATATTCATATGAGCGTCTGGGTCAGGTGGT	
cpxR-D-NotI	AATATTAT**GCGGCCGC**ATGCGGTTCAGTTCGTTGC	
cpxR-For-Hind III	AAAT**AAGCTT**TGCCCGTGGAGGTAGGTAAATCA	*cpxR* expression
cpxR-Rev-XbaI	AAAA**TCTAGA**TCATGCGGTGGAAACCATCAGATAG	
cpxR-D51A-1	GTTGACCTGTTATTGCTGGCTATCATGATGCCGAAGAAA	site-directed *cpxR* expression
cpxR-D51A-2	TTTCTTCGGCATCATGATAGCCAGCAATAACAGGTCAAC	
cpxP-For-Hind III	AAAT**AAGCTT**CAAGGCAATTAGACTAAGTCCATCG	*cpxP* expression
cpxP-Rev-XbaI	AAAA**TCTAGA**AAACACGCATTACTCGGTACTATCA	
nlpE-For-Hind III	AAT**AAGCTT**TTACGGCTGCTGGCGTGGAT	*nlpE* expression
nlpE-Rev-XbaI	AAA**TCTAGA**ATAACGCCACCGAACAGAG	
*cpxP*_AT_SalI	AATA**GTCGAC**GCTTCGCCTGCCTGTTGTTG	*cpxP* promoter
*cpxP*_AT_EcoRI	TTAT**GAATTC**GCATCAGGTCACGCATTT	
*degP*_AT_SalI	TTT**GTCGAC**GATTTTTGCCAGTTGGTGAATGACG	*degP* promoter
*degP*_AT_EcoRI	TTT**GAATTC**CATCGTCGCCTTCACCCT	
P1	TGTGTAGGCTGGAGCTGCTTCG	Kanamycin cassette amplification
P2	CATATGAATATCCTCCTTAGTTCCTATTCC	

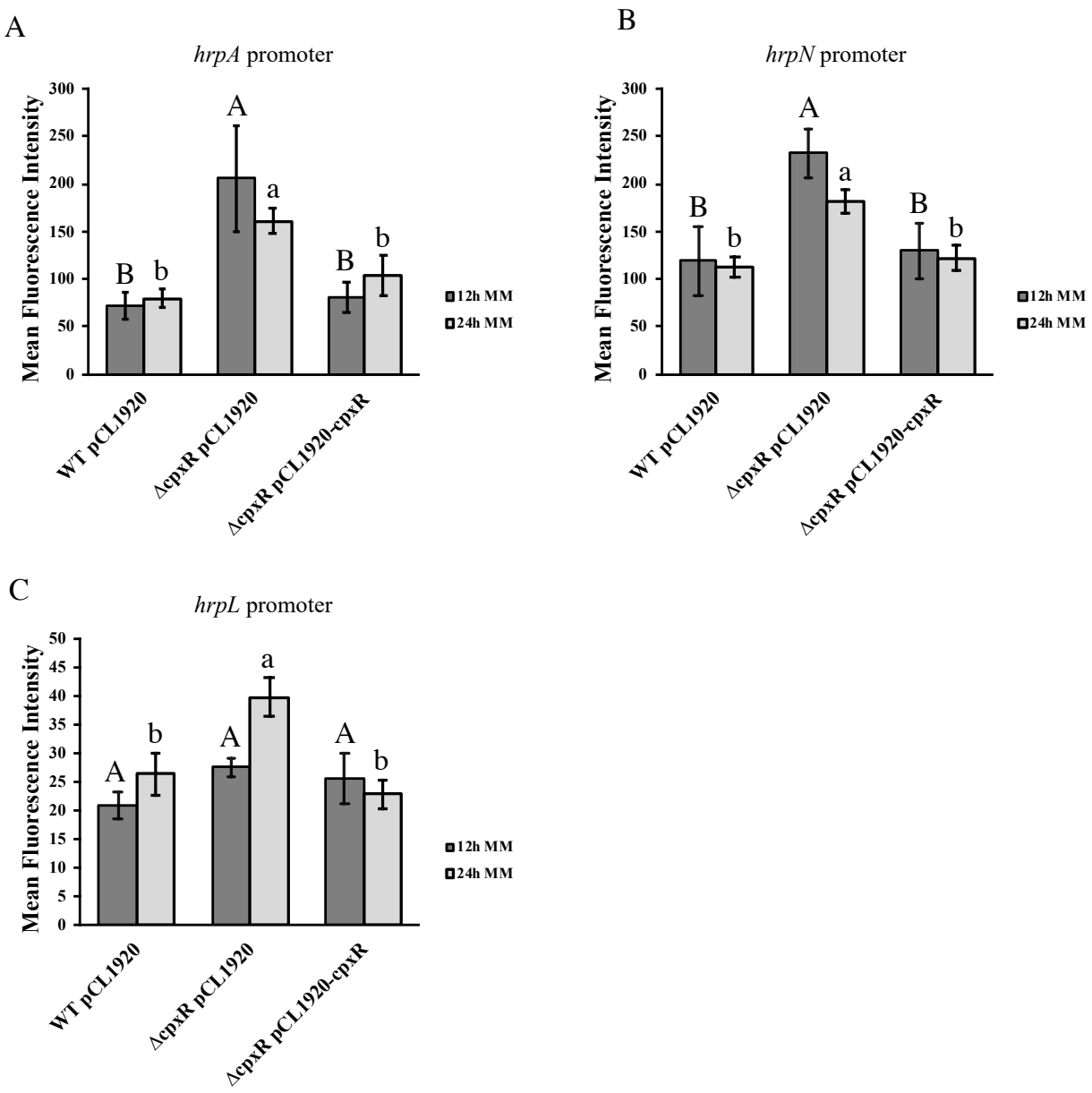

Figure 1. Transcriptional study of the role of CpxR on T3SS expression in *D. dadantii*. Cells grown in MM for 12 and 24 h were collected and their promoter activity was measured using flow cytometry. (A, B) The transcription of two representative T3SS components, HrpA and HrpN, was examined. Wild-type strains harboring empty vector pCL1920 were compared with Δ*cpxR*-pCL1920 and Δ*cpxR* pCL1920-*cpxR*. (C) The T3SS regulator HrpL expression was examined as described above. The experiments were repeated at least three times, and the

representative figures were chosen to represent T3SS regulation. Different upper- or lower-case letters represent a significant difference from a one-way ANOVA test.

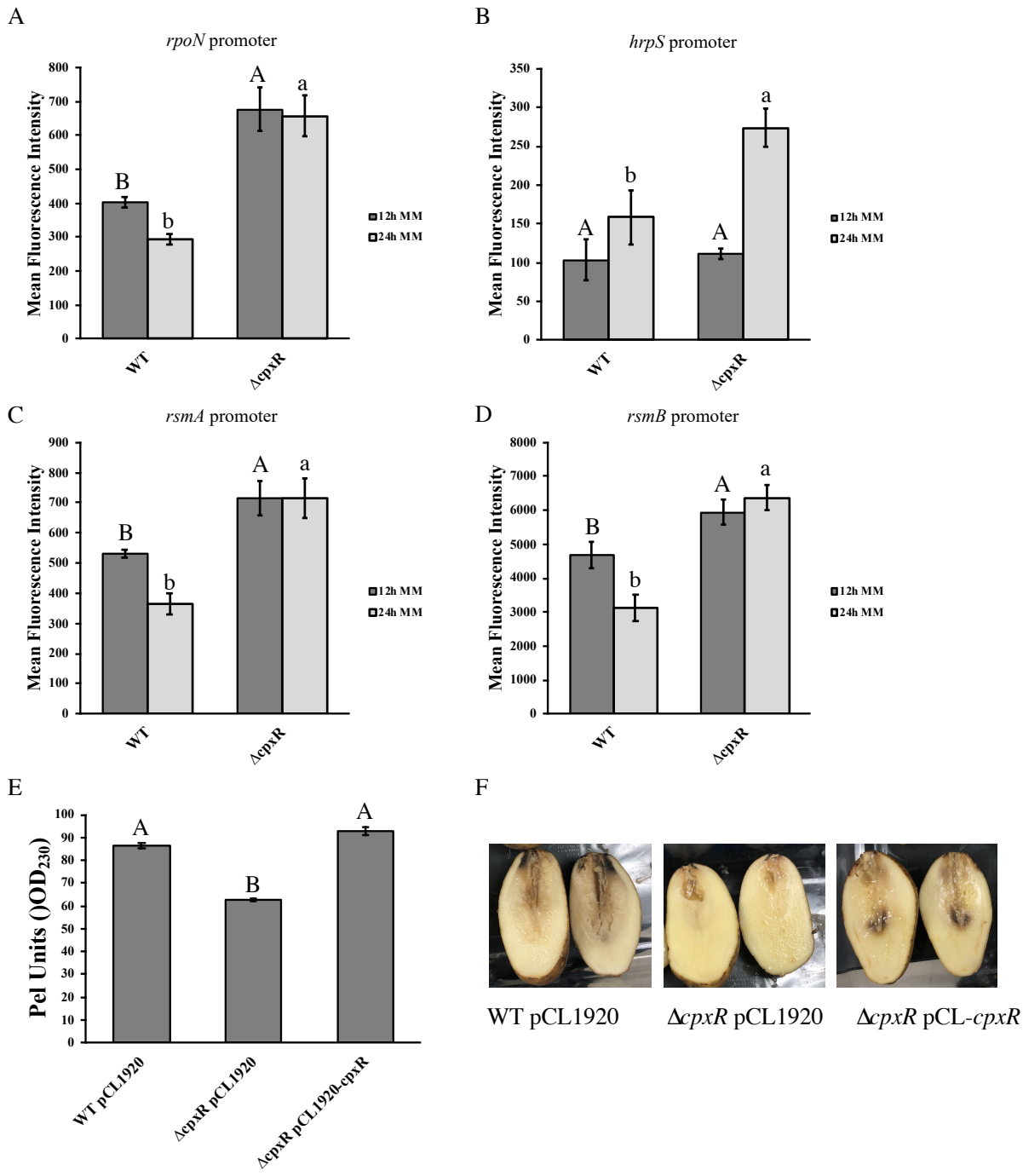

Figure 2. The effect of Δ*cpxR* on T3SS regulator was examined. (A, B) The promoter activities of *rpoN* and *hrpS* were examined in *D. dadantii* and Δ*cpxR* Strains were cultured in LB and transferred to MM for T3SS induction. (C, D) The transcription of *rsmA* and *rsmB* under wild-type and *cpxR* deletion background. (E) Pectate lyase assay. Wild-type strains were

compared to Δ*cpxR* pCL1920 and Δ*cpxR* pCL1920-*cpxR*. The cells were transferred from an overnight culture grown in LB to MM plus 0.1% PGA to induce Pel production. (F) Virulence assay performed on potato tubers with three strains; The wild-type and its derivatives Δ*cpxR* and Δ*cpxR* complementation were examined. The experiments were repeated at least three times and the representative figures were chosen to represent T3SS regulation. Different upper- or lower-case letters represent a significant difference from a one-way ANOVA test.

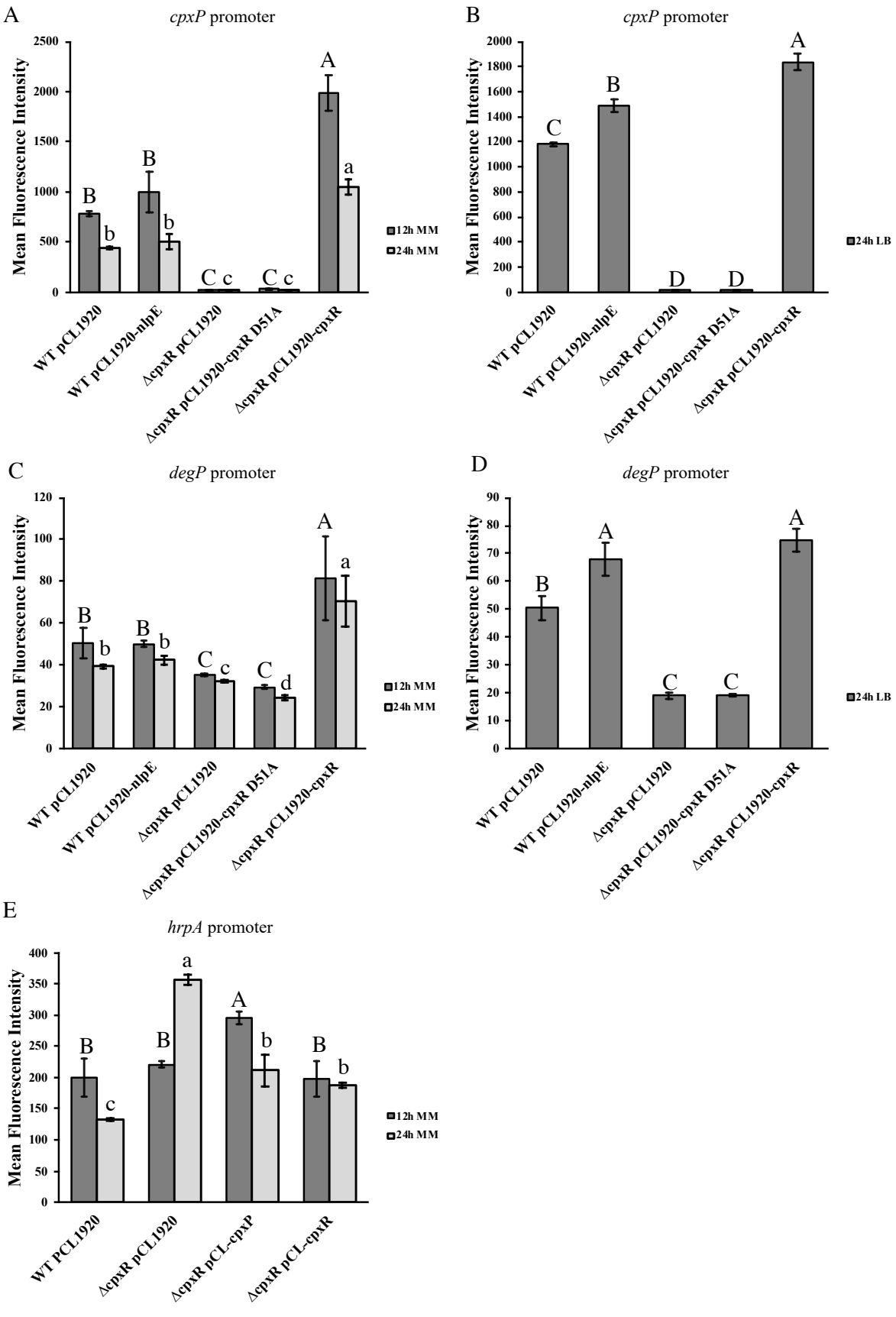
A
cpxP promoter
Mean Fluorescence Intensity
12h MM
24h MM
WT pCL1920
WT pCL1920-nlpE
ΔcpxR pCL1920
ΔcpxR pCL1920-cpxR D51A
ΔcpxR pCL1920-cpxR
B
cpxP promoter
Mean Fluorescence Intensity
24h LB
C
degP promoter
Mean Fluorescence Intensity
12h MM
24h MM
D
degP promoter
Mean Fluorescence Intensity
24h LB
E
hrpA promoter
Mean Fluorescence Intensity
12h MM
24h MM
WT PCL1920
ΔcpxR pCL1920
ΔcpxR pCL-cpxP
ΔcpxR pCL-cpxR

Figure 3. The *Dickeya* Cpx response on known CpxR targets was examined both in LB and in MM. (A – D) *D. dadantii cpxP* and *degP* loci transcription were measured in wild-type strain, Δ*cpxR* harboring empty vector, and Δ*cpxR* complementation with pCL1920-*cpxR* and pCL1920-*cpxR* ^{D51A} in LB and MM. (E) Comparison of the *hrpA* expression with *in trans* expression of *cpxP* in Δ*cpxR*. Different upper- or lower-case letters represent a significant difference from a one-way ANOVA test. Experiments were repeated three times and one representative result was chosen.

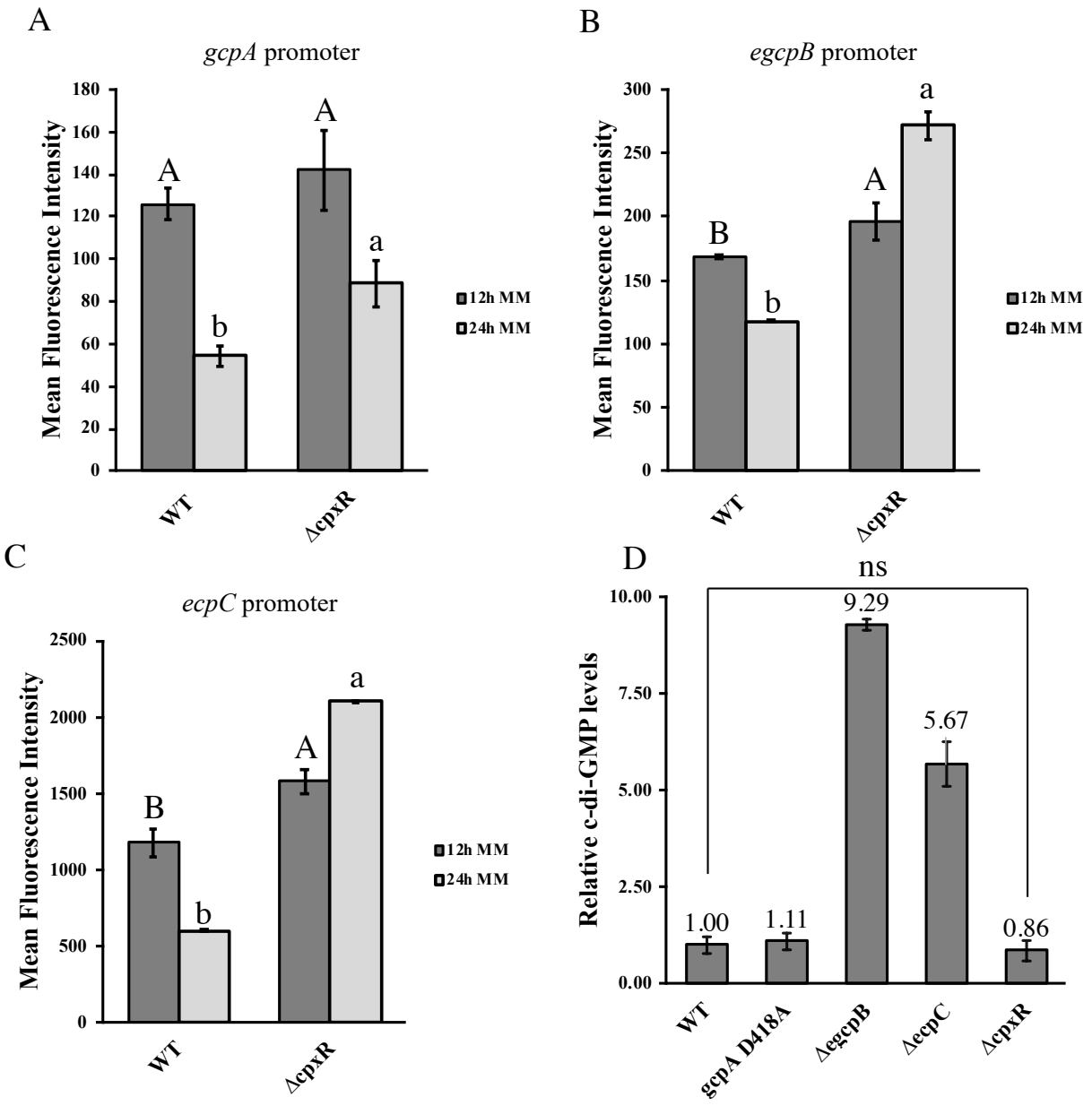

Figure 4. Effects of CpxR on GcpA, EGcpB, and EcpC transcription. (A – C) Deletion of *cpxR* on the transcription of diguanylate cyclase GcpA and two phosphodiesterases EGcpB and EcpC. Promoter activities were examined in MM media for 12 and 24 h. (D) Relative c-di-GMP levels were examined in WT *D. dadantii* and its derivatives *gcpA* ^{D418A}, Δ*egcpB*, Δ*ecpC*, and Δ*cpxR*. The data represents GUS values of each gene relative to the WT. Three technical replicates were

performed, and one test was shown in the figure. Different upper- or lower-case letters represent a significant difference from a one-way ANOVA test.

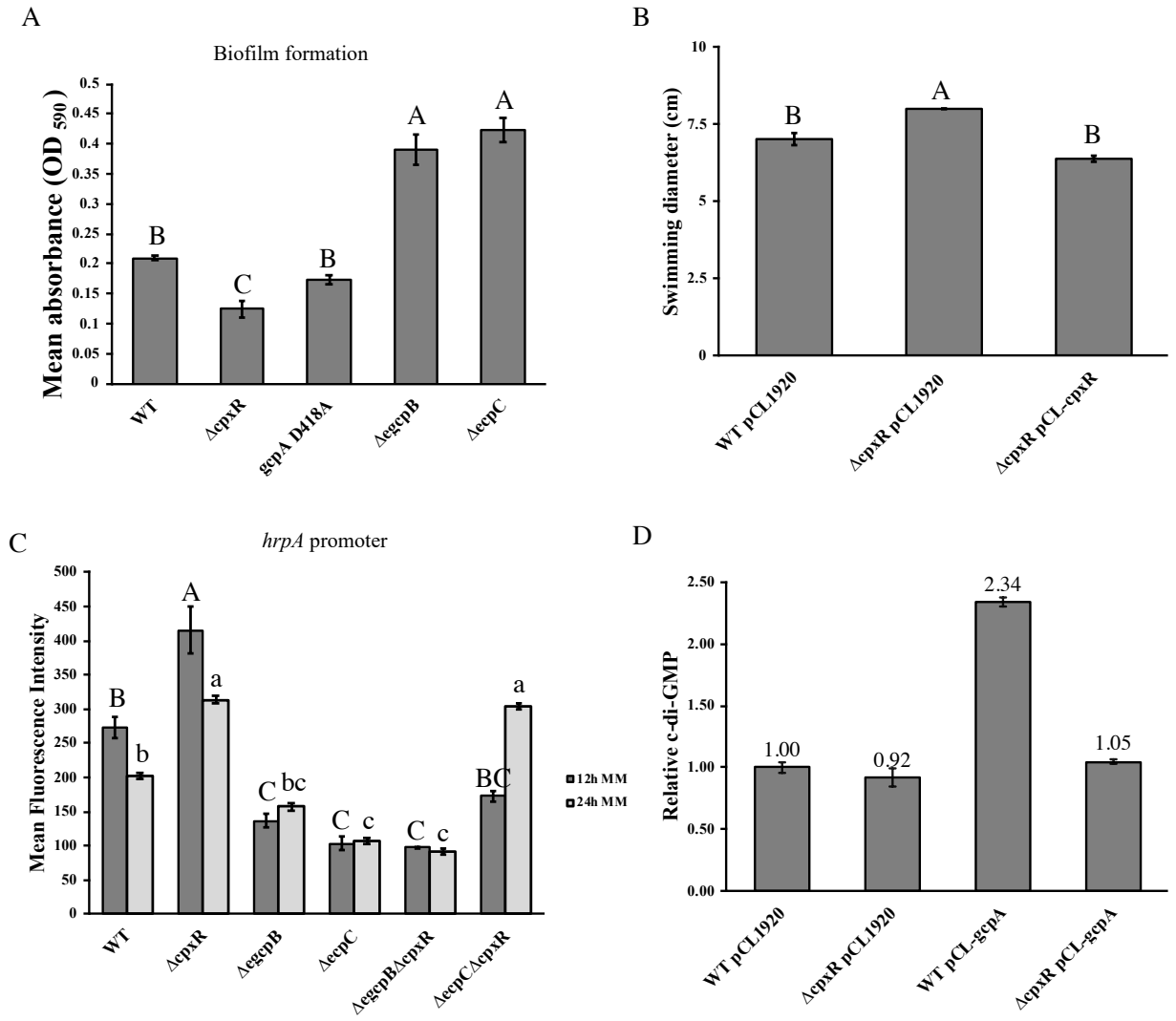

Figure 5. Comparison of the global regulator c-di-GMP and Cpx response on regulation of the T3SS. (A) Biofilm formation of *Dickeya* wild-type and *gcpA*^{D418A}, phosphodiesterase mutants Δ*egcpB* and Δ*ecpC*. (B) Motility assay of *Dickeya* wild-type and its derivatives Δ*cpxR* (pCL1920 or pCL1920-*cpxR*). (C) T3SS expression analysis from *hrpA* transcription analysis in wild-type, Δ*egcpB*, Δ*ecpC*, Δ*cpxR*, Δ*ecpC*Δ*cpxR* and Δ*egcpB*Δ*cpxR* mutant. (D) Relative c-di-GMP concentrations of different *D. dadantii* cells in MM. Calculation was made by measuring the β-galactosidase activity using a c-di-GMP riboswitch fusion into the pRS414 vector. Different upper- or lower-case letters represent a significant difference from a one-way ANOVA test. Experiments were repeated three times and one representative value was chosen.

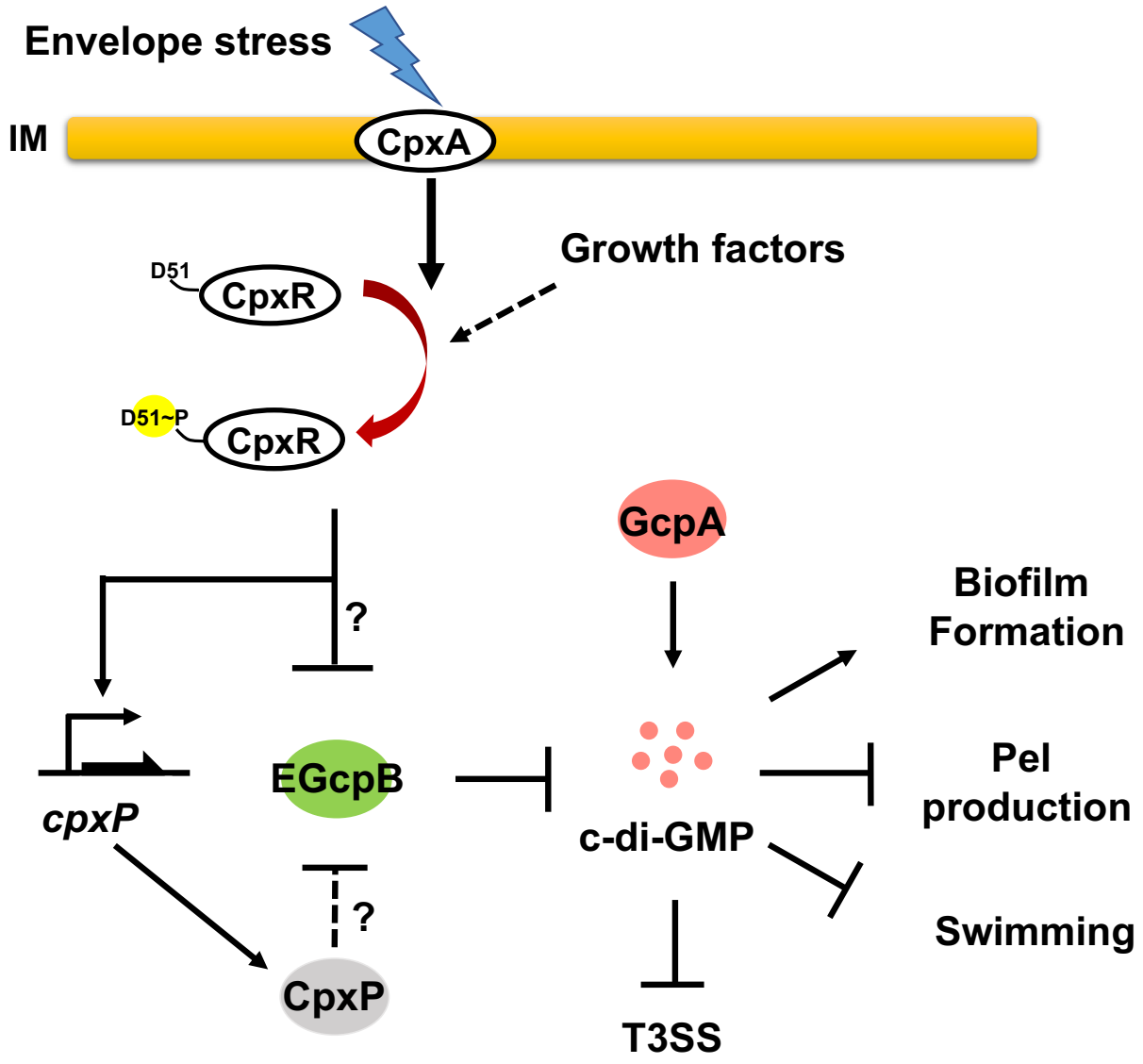

Figure 6. Proposed mechanism of how CpxR regulates T3SS in *D. dadantii*. Envelope stress or growth factors activate CpxR by phosphorylation at residue D51. Activated CpxR suppresses EGcpB, a phosphodiesterase (a c-di-GMP remover), probably through CpxP or an unknown mechanism (dash lines). High c-di-GMP concentration inhibits T3SS expression, swimming, and Pel production, and promotes biofilm formation. Deletion of *cpxR* removes the suppression of EGcpB and hence increases T3SS expression.

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Chapter 3

Identification of a novel Cpx inducer CHIR-090 and the related mechanism on stress resistance

Abstract

The sRNA regulation represents a valuable, underexplored field to study bacterial transcriptional and post-transcriptional regulation. One under-studied piece of the CpxA/CpxR two-component system (TCS) is the newly identified sRNA, CpxQ. In combination with the highly expressed *cpxP* transcripts under Cpx inducing conditions, CpxQ is co-transcribed from the 3' UTR of *cpxP* mRNA. Using a *cpxP*-GFP transcriptional reporter, we screened a library of potential antimicrobial compounds. Out of the 199 compounds, we identified a compound TS168 (CHIR-090) that induced the highest *cpxP* expression in *Dickeya dadantii*. CHIR-090 was found previously as an antibiotic that inhibits LPS biosynthesis. Notably, the induced Cpx response plays an essential role in cell survival under CHIR-090 toxicity. We observed that the absence of the Cpx response by mutation of *cpxR* impaired cell tolerance to CHIR-090. In contrast, complementation of *cpxR* or *cpxQ* in Δ*cpxR* rescued cells from CHIR-090 stress, indicating the potential role of CpxQ in stress/antimicrobial resistance. In this work, we linked the Cpx response, including the conserved sRNA CpxQ, to the restoration of the lipopolysaccharide (LPS) biosynthesis disturbance caused by CHIR-090. Moreover, CHIR-090 showed a differential regulatory role on the T3SS *hrpA* transcription in the Δ*cpxR* mutant under different doses.

Introduction

The cell membrane organization of Gram-negative bacteria is unique in several aspects. Gram-negative bacteria possess a so-called "diderm" cell membrane, which consists of two phospholipid bilayers (Sutcliffe, 2010). The cytoplasmic membrane, also known as the inner membrane (IM), is a continuous phospholipid bilayer. The outer membrane (OM) is an asymmetric bilayer. The periplasmic leaflet of the OM is composed of phospholipids, whereas the outer leaflet is composed almost entirely of lipopolysaccharide (LPS) (Sperandeo *et al*., 2019). The periplasm is an aqueous space that contains the inelastic peptidoglycan layer, which bridges IM and OM, enabling the resistance to turgor forces from the cytoplasm (Rogers & Perkins, 1968). Various protein complexes integrate with the cell membrane and give cells a wide range of essential functionalities to develop precise cell responses (Kuhn, 2019). For example, the IM contains essential machinery such as respiratory complexes, IM-spanning transporters, and phosphotransferase systems (PTS). The incorporation of the lipopolysaccharide (LPS) anchored at the OM of the cell wall allows the bacteria to form a barrier for small external molecules. Moreover, LPS is also involved in pathogenicity. As a group of bacterial glycolipids, LPS interacts with the host and modulates its innate immune response (Kagan, 2017). The chemical structure of LPS in several *Enterobacteria* organisms has been fully elucidated, such as in the model organism *Escherichia coli* (Kuhn, 2019). The structure of LPS consists of three domains: the highly conserved lipid A linked to a core oligosaccharide, which is itself connected to the structurally diverse O-antigen (Raetz, 1990, Raetz & Whitfield, 2002). In contrast to the lipid A, the O-antigen is highly variable, made from repeating oligosaccharide units. The contrasting features from the hydrophobic lipid A moiety and the hydrophilic O-antigen also

pose a challenge for the cell when transporting LPS, and maintaining OM asymmetry (Sperandeo *et al*., 2019).

The biosynthesis of Kdo_2-lipid A subunit and the attachment of O-antigen repeats to lipid A subunit occur in the cytoplasm and the cytoplasmic side of the IM, respectively (Raetz & Whitfield, 2002). LPS formed on the cytoplasmic side of the IM has to be flipped to the periplasmic side of the IM, and this is done by an ABC (ATP binding cassette) transporter, MsbA (Doerrler *et al*., 2004). It extracts LPS from the cytoplasmic side of the IM, and when modified, the mature LPS is escorted across the periplasm and anchored at the OM (Sperandeo *et al*., 2019). Since this process is energetically unfavorable, the protein machinery known as LptABCDEFG complex (abbreviated from lipopolysaccharide transport) plays an essential role in facilitating the transmission of LPS from IM to the external surface of the OM (Sperandeo *et al*., 2007).

Besides the biosynthesis of LPS, more than 100 genes are involved in regulating LPS assembly onto the OM in *E. coli* (Klein & Raina, 2019). The biosynthesis of LPS starts from *N*-acetylglucosamine linked to a UDP (UDP-Glc*N*Ac), the precursor of Kdo_2-lipid A. Cytoplasmic enzyme LpxA first adds an acyl chain (R-3-hydroxymyristate) to UDP-Glc*N*Ac, forming UDP-3-*O*-(acyl)-Glc*N*Ac (Raetz & Whitfield, 2002). The second step is deacetylation of UDP-3-*O*-(acyl)-Glc*N*Ac by LpxC; this reaction is irreversible, and thus it is one of the central regulatory points of LPS biosynthesis (Young *et al*., 1995). Next, a second acyl chain is added by LpxD, making UDP-2,3-diacyl-GlcN (Kelly *et al*., 1993). The other six enzymes (termed LpxH, LpxB, LpxK, WaaA, LpxL, and LpxM) subsequently make the core of lipid A, a hexaacylated diglucosamine, and the complete lipid A-core moiety is then attached by O-antigen oligosaccharide (Sperandeo *et al*., 2019).

Currently, LpxC has garnered attention for developing novel antibiotics, as its orthologues are highly conserved among Enterobacteriaceae, thus making it a promising target. The most potent LpxC inhibitor known so far is *N*-aroyl-$_{L}$-threonine hydroxamic acid (CHIR-090) (Barb *et al*., 2007, McClerren *et al*., 2005).

Two-component system CpxA/CpxR is a well-studied signal transduction system known to monitor and maintain a healthy cell envelope (Price & Raivio, 2009). The Cpx system senses signals either directly through IM localized CpxA or indirectly through the cytoplasmic response regulator CpxR via small phosphor donors (Wolfe, 2010, Raivio & Silhavy, 1997). CpxP demonstrated several roles in maintaining envelope homeostasis. It can act as a periplasmic chaperone protein that inhibits CpxA phosphorylation activity through a feedback mechanism (Raivio *et al*., 1999). It also shows binding affinities to periplasmic proteins such as misfolded P-pilus proteins and sends misfolded protein to protease DegP for degradation (Raivio et al., 1999, Danese & Silhavy, 1998). Importantly, CpxP transcription is highly responsive to Cpx-inducing conditions. Thus, the modulation of the Cpx response can be detected by the examination of CpxP transcription. In line with the Cpx response study, a recent study on Hfq associated sRNA revealed a post-transcriptional regulator, CpxQ, in the Cpx signaling pathway (Chao et al., 2012). It is generated through RNaseE degradation from the *cpxP* mRNA, and Hfq plays a role in protecting CpxQ from degradation by other RNases (Chao & Vogel, 2016). However, the role of CpxQ awaits further characterization in phytopathogens.

In Chapter 2, we analyzed the role of Cpx response on virulence regulation in *D. dadantii* 3937. The *cpxR* mutant of *D. dadantii* showed increased transcription of multiple type III secretion system (T3SS) regulators. To expand our understanding of Cpx response on pathogenicity, we sought to identify any Cpx inducers from a compound library. Several of the compounds were

identified on the repression of T3SS in *D. dadantii* previously (Yuan *et al*., 2020). We introduced a transcriptional reporter pAT-*cpxP* into *D. dadantii* wild-type cells and tested various compounds' abilities on CpxP transcription alteration.

In this work, a screening of Cpx response modulators revealed a bactericidal molecule, CHIR-090, that increased CpxP transcription at least 8-fold. Since CHIR-090 is a novel antibiotic, further investigation of CHIR-090 toxicity on *D. dadantii* was also performed and analyzed.

Experimental procedures

Bacterial strains, plasmids, primers, and media

Dickeya dadantii strains and plasmids used in this work are listed in Table 1 & 2. Wild-type *D. dadantii* was grown in lysogeny broth (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) overnight and then transferred into MM at 28°C for experimental analysis (Yang et al., 2008b). Primer design is based on the *D. dadantii* genome sequence (https://asap.ahabs.wisc.edu/asap/home.php), and primers used in this work are listed in Table 2.

Mutant construction and complementation

Δ*cpxR* mutant was constructed by marker exchange mutagenesis (Yang et al., 2002). In brief, primers flanking the upstream and downstream *cpxR* locus were amplified and ligated to kanamycin cassette. The PCR product was then ligated to pWM91 plasmid for conjugation recombination in *E. coli* S17-1 λ-*pir*. Successful mutants were achieved from selection of kanamycin resistant, ampicillin, and sucrose sensitive strains on mannitol-glutamic acid (MG) medium (1% mannitol, 0.2% glutamic acid, 0.05% potassium phosphate monobasic, 0.02% NaCl, and 0.02% MgSO4) plus kanamycin (50 μg/ml) and sucrose (0.1 g/mL) agar plate.

The *in trans* expression of *cpxR, cpxP,* and *cpxQ* was achieved by incorporating each target gene coding region into the low copy number plasmid pCL1920 and then transferred into Δ*cpxR*.

Screening and detection of Cpx inducer

GFP reporter pPROBE-AT plasmid was used to examine *cpxP* transcription under various compounds. The *cpxP* promoter region was amplified by the flanking primers and ligated to the pPROBE-AT vector (Miller et al., 2000) to produce pAT-*cpxP*, and the plasmid was then transferred to WT for further screening. A hundred μM in a final concentration of each compound was added into MM media, and promoter activity was examined after 6 h incubation in 28 ˚C. Measurement of average GFP intensity through flow cytometry (BD Biosciences, San Jose, CA) was performed as described in Chapter 2.

Promoter detection

As described in the screening method, the *hrpA* and *cpxR* promoter activities were tested under the same growth conditions except for different timing. Cells were grown in LB broth before transferring into minimal medium (MM) at the ratio of 1:100. Bacterial samples were then collected and diluted 100x in PBS buffer for the assays in flow cytometry (BD Biosciences, San Jose, CA, USA) detection.

Inhibition assay of CHIR-090 on *Dickeya* strains

Cells were first grown in LB overnight. Overnight cultures were then transferred into MM media with or without 5 μM CHIR-090. After 24 h, cell cultures were serial diluted, and 10 μL of the bacterial suspension was spotted on LB plate for comparison.

Statistical analysis

Excel (Microsoft, Redmond, WA) was used to calculate means and standard deviations. Screened inducer was confirmed at least three repeats. The statistical analysis was performed using a two-tailed Student's t-test.

Results

Identification of novel Cpx inducers from a compound library

A total of 199 compounds were screened for their effect on CpxP transcription in the minimal medium (MM). One compound, assigned as TS168, significantly induced *cpxP* promoter activity (Fig 1). TS168 denotes the compound CHIR-090 (Table 3), which was reported as an inhibitor of the deacetylase LpxC in lipid A biosynthesis in *E. coli* and *Pseudomonas aeruginosa* (Clements *et al*., 2002). Lipid A is the hydrophobic portion of the lipopolysaccharide (LPS), a major component of the Gram-negative bacterial OM (Sperandeo *et al*., 2019). UDP-3-*O*-acyl-Glc*N*Ac deacetylase LpxC is the primary regulatory point of LPS synthesis, and its inhibition renders cells sensitive to external stresses (Sperandeo *et al*., 2019).

Induction of *cpxP* promoter by CHIR-090 is dosage-dependent

Further analysis of the CHIR-090 found that the dosage of the compound concentration at 100 μM during the screening process led to poor growth of wild-type cells. To minimize the effect of growth inhibition, we optimized the CHIR-090 concentration to 5 μM so that the CHIR-090 affected Cpx response without affecting growth of the wild-type cells (Fig. 2).

CpxR and CpxQ restored CHIR-090 induced stress in Δ*cpxR* mutant

We compared the effects of 5 μM CHIR-090 on the WT, Δ*cpxR,* and Δ*cpxR* with the complementation vector of *cpxP* or *cpxQ* vector. Previously, we showed the abolishment of *cpxP* transcription in Δ*cpxR*. Cells that grew with or without 5 μM CHIR-090 after 24 h were serially diluted, and 10 μL was spotted on LB plate for comparison. Deletion of *cpxR* significantly impacted cell recovery under the effect of CHIR-090 compared to the Δ*cpxR* with DMSO (Fig. 3). The sharp reduction of cell concentration was restored by complementation of pCL1920-*cpxR*, whose *cpxR* expression was driven by a leaky *lac* promoter. When we compared the samples with *in trans* expression of *cpxP* and *cpxQ*, Δ*cpxR*(pCL1920-*cpxP*) slightly increased the cell concentration, while Δ*cpxR*(pCL1920-*cpxQ*) restored cell concentration almost to that of the WT. The above results suggest that the Cpx response plays an essential role in resisting CHIR-090 toxicity, and the two Cpx elements, CpxR and CpxQ, could complement the loss of Δ*cpxR* in this situation.

CHIR-090 showed dose and time-dependent regulation on T3SS expression

Since our initial goal was to link T3SS expression to potential compounds, we sought to investigate the T3SS expression under 5 μM of CHIR-090, the concentration that does not affect normal cell growth (Fig. 3). Both Cpx regulon genes *cpxP* and *cpxR* were used in this study. As expected, the addition of CHIR-090 induced *cpxP* and *cpxR* expression in the WT (Fig. 4). However, the expression of *hrpA*, one of the T3SS subunit genes, was not affected under 5 μM of CHIR-090 (Fig. 4). Based on the negative role of CpxR on T3SS from Chapter 2, we included a lowered concentration of CHIR-090 with the Δ*cpxR* mutant in a follow-up experiment. Although the effect of CHIR-090 on *hrpA* expression in WT was not significantly altered at 12 h, T3SS expression was significantly lowered in both 2.5 μM and 1.25 μM CHIR-090 in Δ*cpxR*

mutant at the same time point (12 h) (Fig. 5). Interestingly, *hrpA* expression was significantly reduced in 2.5 μM of CHIR-090 but not in 1.25 μM treatment group at 24 h (Fig. 5). The restoration of Δ*cpxR hrpA* expression in the late time point, specifically at 1.25 μM of CHIR-090 exhibits a time and dosage dependent regulation of CHIR-090 in the absence of Cpx response.

Discussion

In this work, we screened 199 compounds and identified a novel Cpx inducer CHIR-090. CHIR-090 is an antibiotic that binds to LpxC (Tomaras *et al*., 2014). LpxC is the second enzyme involved in the synthesis of the lipid A moiety of LPS. In this study, we confirmed that wild-type cells were more resistant to the toxicity of CHIR-090 than were cells of the Δ*cpxR* mutant. Our analysis of the Cpx response also suggests that the sRNA CpxQ is related to CHIR-090 induced stress. The refined mechanisms of how CpxQ regulated stress resistance on CHIR-090 remain unknown.

Studies of TCS CpxA/CpxR on antibiotic resistance have been reported for decades, and the effect of Cpx response on cell resistance to toxicity argues a multifactorial role for Cpx in influencing this resistance (Thorbjarnardóttir *et al*., 1978, Rainwater & Silverman, 1990, Cosma et al., 1995, Mileykovskaya & Dowhan, 1997, Raivio, 2014). For example, the impact of Cpx signaling on β-lactam resistance has been investigated in *Klebsiella pneumoniae*; this study indicates that the presence of Cpx response is necessary for the survival of *K. pneumoniae* under β-lactam toxcity (Srinivasan *et al*., 2012). Conversely, the impact of Cpx signaling activation in *Haemophilus ducreyi* leads cells to be more sensitive to antimicrobial peptides (Rinker *et al*., 2011). Thus, one should take into account that the mechanism of Cpx response varies between different organisms.

CpxQ, the non-intergenic class of sRNA, is an under-studied Cpx component in the stress response. To date, two studies have investigated the regulatory role of CpxQ under IM stress. The first studied the *Salmonella* CpxQ through RNA-seq, where researchers identified a role of CpxQ on the regulation of IM-associated genes transcription, involving the NhaB sodium-proton antiporter and periplasmic chaperone Skp. They suggested a multi-target role of CpxQ in the IM stress response (Chao & Vogel, 2016). The other investigation of CpxQ sRNA was conducted in *E. coli*, where the role of CpxQ in the repression of Skp and CpxP production was confirmed by immunoblotting. Furthermore, the jamming toxicity caused by *lamB*(A23D) mutation could be relieved by CpxQ-mediated repression of Skp (Grabowicz *et al*., 2016), indicating the *cpxP*-CpxQ pair might differentially regulate the different cellular compartments.

Multiple signaling pathways work in tandem to maintain cell envelope homeostasis. The adverse conditions caused by the tight binding of CHIR-090 to the zinc-dependent amidase LpxC lead to the perturbance of lipid A biosynthesis (Tomaras et al., 2014). We hypothesize that *D. dadantii* Cpx response might participate in the alleviation of LPS biosynthesis defects caused by the bactericidal effect of CHIR-090. Lipid A is the hydrophobic portion of the LPS, and it serves as an anchor point for the LPS molecule to localize onto the OM (Raetz, 1990, Raetz & Whitfield, 2002). Lack of lipid A might result in the enrichment of LPS in the IM and OM symmetry (Sperandeo *et al*., 2019).

In this study, the Cpx response confers resistance to a low dose of bactericidal compound CHIR-090 (Fig. 3). Reports of three proteins, the IM protease FtsH (Führer *et al*., 2006, Ogura *et al*., 1999), YciM/LapB heat-shock proteins (Klein *et al*., 2014, Mahalakshmi *et al*., 2014), and an unknown protein (Emiola *et al*., 2016) have shown a regulatory role on the deacetylase LpxC turnover. Notably, FtsH also controls the WaaA degradation, the third to last enzyme that

catalyzes lipid A biosynthesis (Katz & Ron, 2008). Furthermore, previous research has proven that the loss of FtsH activity triggers Cpx response in *E. coli* (Shimohata *et al*., 2002). Stress studies of the FtsH protease suggests FtsH can release SecYEG apparatus jamming stress by degradation of SecY (Van Stelten *et al*., 2009). This process is regulated by an IM protein YccA, which inhibits FtsH protease activity (Kihara *et al*., 1998). Interestingly, *yccA* is a Cpx regulon gene (Yamamoto & Ishihama, 2006). It was initially identified in an aminoglycoside resistance study (Kashyap *et al*., 2011). Since several lines of evidence suggested a potential link of FtsH with Cpx response under CHIR-090 stress, we hypothesize a scenario in which the LpxC turnover is regulated by FtsH protease. Together with CpxR or CpxQ playing a role in the repression of YccA, Cpx activation might lead to the induction of FtsH protease activity and release CHIR-090 toxicity. Further study is required to examine any of the above hypotheses.

Conclusion

Bacterial Cpx response was initially characterized in *Escherichia coli* MC4100 strain in 1980 (McEwen & Silverman, 1980). Thirty years later, genetic studies on Cpx response were expanded to various animal infecting pathogens (Price & Raivio, 2009). However, the observation of the contrasting role of CpxR to specific targets under similar Cpx-regulated conditions indicates that Cpx response is both strain- and culture-dependent. Under this concept, Nancy L. Price *et al*. summarized Cpx pathway regulation in the *E. coli* MC 4100, the strain initially characterized Cpx response (Price & Raivio, 2009). They concluded that the role of Cpx response towards various Cpx-regulated genes might be due to the different strengths of Cpx regulation, which might link to the position of the CpxR consensus binding site near the promoter region of the target genes. They also confirmed that the well-documented *cpxP* serves as the most responsive Cpx regulon gene (Price & Raivio, 2009).

In *Dickeya. dadantii*, CpxP was also shown to be the strongest Cpx-regulated target under our tested conditions [both in LB and in minimal media (MM)]. When referring to the second CpxR-regulated gene, *degP*, we observed the Cpx-signaling modulator NlpE functions as an activator in LB broth but not in MM. Since MM is a distinct medium used for plant bacterial pathogens to induce the expression of T3SS, the observation that Cpx response is media-dependent lets us cautiously refer to other work when interpreting Δ*cpxR*-regulated T3SS in *D. dadantii*. We examined the transcriptional data of several T3SS essential regulators through flow cytometry. The *cpxR* null mutant showed an induced T3SS expression compared to the wild-type strain in MM.

CpxR exhibited a negative role in the transcription of several T3SS regulators. The *rsmA* and *rsmB* sRNA transcriptions were both induced in Δ*cpxR*. Since *rsmB* has an antagonistic effect on

the inhibitor protein, RsmA, the repression of both activator and repressor through the same pathway (*rsmB*/RsmA pair) is further confirmed through Pel production; this phenotype is also regulated by the inhibitor RsmA and *rsmB* sRNA. The reduced Pel production in Δ*cpxR* rules out the possibility of *rsmB* induced T3SS expression since enhanced *rsmB* sRNA positively affects T3SS expression and Pel production.

Current genetic research focuses on the comprehensive understanding of the cross-talk among multiple global signaling pathways. The impact of CpxR on the biosynthesis of the ubiquitous signaling molecule c-di-GMP is examined in this study. It is not surprising to observe a link between Cpx response and c-di-GMP signaling since a report had shown *E. coli* YdeH, a GGDEF-containing protein, is a Cpx-regulated gene (Yamamoto & Ishihama, 2006). CpxR might repress T3SS through its negative effect on RpoN and HrpS transcription. Yi proposed two phosphodiesterases, EGcpB and EcpC, might regulate T3SS through the regulation of RpoN mRNA stability (Yi et al., 2010). Increased expression of DGC GcpA, and two PDEs EGcpB and EcpC was observed in Δ*cpxR.* After manipulating the intracellular c-di-GMP levels through DGC/PDEs, our results indicate that EGcpB plays a major role in the Δ*cpxR*-induced T3SS.

We further asked whether alteration of Cpx response through any Cpx response inducer(s) could impact the T3SS expression. To this end, we screened a compound library and identified a novel Cpx inducer, CHIR-090. This antimicrobial compound was reported to have a high binding affinity to the LpxC protein, a conserved enzyme involved in the lipid A biosynthesis (Raetz *et al.*, 2006). Lipid A forms the lipid portion of lipopolysaccharide (LPS) and enables the anchoring of LPS to the outer membrane of Gram-negative bacteria (Sperandeo *et al*., 2019).

This work linked Cpx response to CHIR-090 sensitivity in the *cpxR* null mutant. We showed CHIR-090 is recognized by the Cpx response in *D. dadantii*. While it remains unclear how the

CHIR-090 caused stress in *D. dadantii*, it is possible that the envelope hemostasis of the bacterial cells is sharply altered after being treated with CHIR-090, as observed from the 9-fold increase of the *cpxP* transcription. This phenotype is possibly through CpxA sensing the perturbance of the outer membrane composition, but a further test is needed to verify this hypothesis. We showed a restoration of CHIR-090 sensitivity in Δ*cpxR* using the complementation plasmid pCL1920-*cpxR* or pCL1910-*cpxQ*. These two Cpx components suggest a possible stress signaling pathway involving a small RNA. In short, although CpxR-mediated adaptation to stress conditions is not rare, the link of this stress signaling pathway to the T3SS regulation is intriguing. The connection between the Cpx stress response and the cellular decision of virulence factor expression in phytopathogen awaits further investigation.

Table 1. List of strains and plasmids

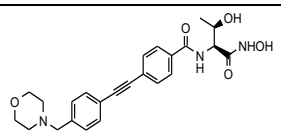
Strains and plasmids	Relevant characteristics[a]	Reference or source
Dickeya dadantii		
3937	Wild type	Hugouvieux-Cotte-Pattat, N.
Δ*cpxR*::Km	Δ*cpxR*::*Km*; Km^r, ABF-0019170 deletion mutant	This study
Escherichia coli		
DH5a	F^- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Lab stock
S17-1 lpir	λ(pir) *hsdR* pro *thi*; chromosomally integrated RP4-2 Tc::Mu Km::Tn7	Lab stock
Plasmid		
pKD4	The template used to PCR kanamycin cassette, Km^r	(Datsenko & Wanner, 2000)
pWM91	A sucrose-based counter-selectable plasmid used for marker exchange mutation, Ap^r	(Metcalf et al., 1996)
pCL1920	Low copy number plasmid, *lac* promoter Sp^r	(Lerner & Inouye, 1990)
pCL1920-*cpxR*	Low copy number plasmid expressing *cpxR* locus, Sp^r	This study
pCL1920-*cpxP*	*cpxP* cloned in pCL1920, Sp^r	This study
pCL1920-*cpxQ*	*cpxQ* cloned in pCL1920, Sp^r	This study
pPROBE-AT	Promoter-probe vector using *gfp*, Ap^r	(Miller et al., 2000)
pAT-*hrpA*	pPROBE-AT vector containing *hrpA* promoter region with *gfp* transcriptional fusion, Ap^r	(Yang et al., 2007)
pAT-*cpxR*	pPROBE-AT vector containing *cpxR* promoter region with *gfp* transcriptional fusion, Ap^r	This study
pAT-*cpxP*	pPROBE-AT vector containing *cpxP* promoter region with *gfp* transcriptional fusion, Ap^r	This study

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

Table 2. List of primers

Primers	**Sequences (5'–3')**	**Use**
cpxR-A-XhoI	AATA**CTCGAG**TCAGGGCAGGATAGTCAC	Δ*cpxR*::Km deletion
cpxR-B	GAAGCAGCTCCAGCCTACACCCATGATTTACCTACCTCCACGGGCA	
cpxR-C	CTAAGGAGGATATTCATATGAGCGTCTGGGTCAGGTGGT	
cpxR-D-NotI	AATATTAT**GCGGCCGC**ATGCGGTTCAGTTCGTTGC	
cpxP-For-Hind III	AAAT**AAGCTT**CAAGGCAATTAGACTAAGTCCATCG	*cpxP* expression
cpxP-Rev-XbaI	AAAA**TCTAGA**AAACACGCATTACTCGGTACTATCA	
*cpxP*_AT_SalI	AATA**GTCGAC**GCTTCGCCTGCCTGTTGTTG	*cpxP* promoter
*cpxP*_AT_EcoRI	TTAT**GAATTC**GCATCAGGTCACGCATTT	
*cpxR*_AT_SalI	AATA**GTCGAC**GCATCAGGTCACGCATTT	*cpxR* promoter
*cpxR*_AT_EcoRI	TTAT**GAATTC**GCTTCGCCTGCCTGTTGTTG	
cpxQ-For-Hind III	AAAT**AAGCTT**TGATAGTACCGAGTAATGCGTGTTT	*cpxQ* expression
cpxQ-Rev-XbaI	AAAA**TCTAGA**AAACGGATGGCTTACGATCAGGGCA	
cpxR-For-Hind III	AAAT**AAGCTT**TGCCCGTGGAGGTAGGTAAATCA	*cpxR* expression
cpxR-Rev-XbaI	AAAA**TCTAGA**TCATGCGGTGGAAACCATCAGATAG	
P1	TGTGTAGGCTGGAGCTGCTTCG	Kanamycin cassette amplification
P2	CATATGAATATCCTCCTTAGTTCCTATTCC	

Table 3 CHIR-090 chemical name and structure

ID	Chemical Name	Structure
TS168	(2S,3R)-N-(2-Hydroxy-1-hydroxycarbamoylpropyl)-4-(4-morpholin-4-ylmethylphenylethynyl)benzamide (**CHIR-090**) MW 437.49	

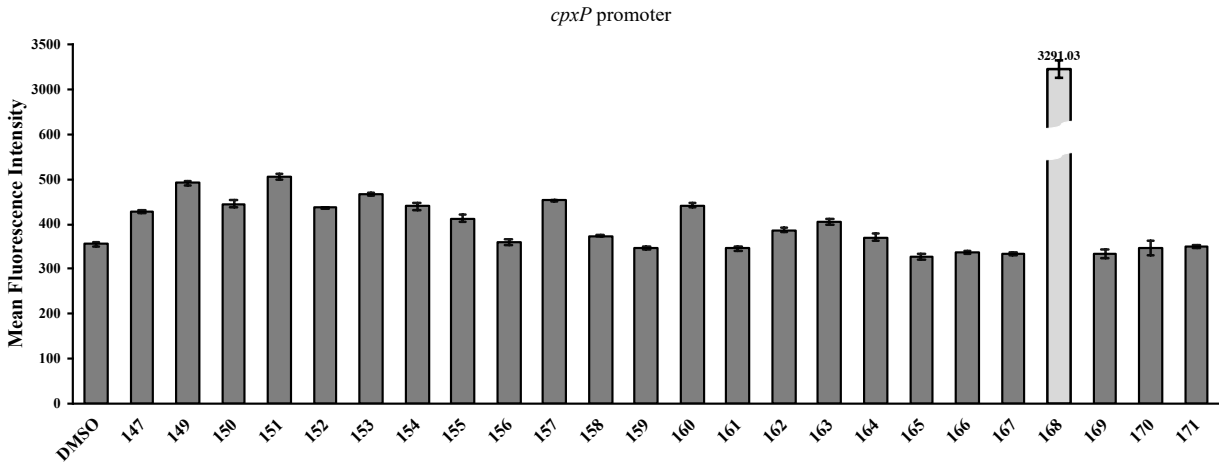


Figure 1. A representative figure of the compound library screening on Cpx response. Mean fluorescent intensity of *cpxP-gfp* was used as a Cpx response indicator, and cells were grown in MM with DMSO or 100 μM of the corresponding compounds were added. Shown in the figure is a representative figure of CpxP induction value from compound 147 – 171.

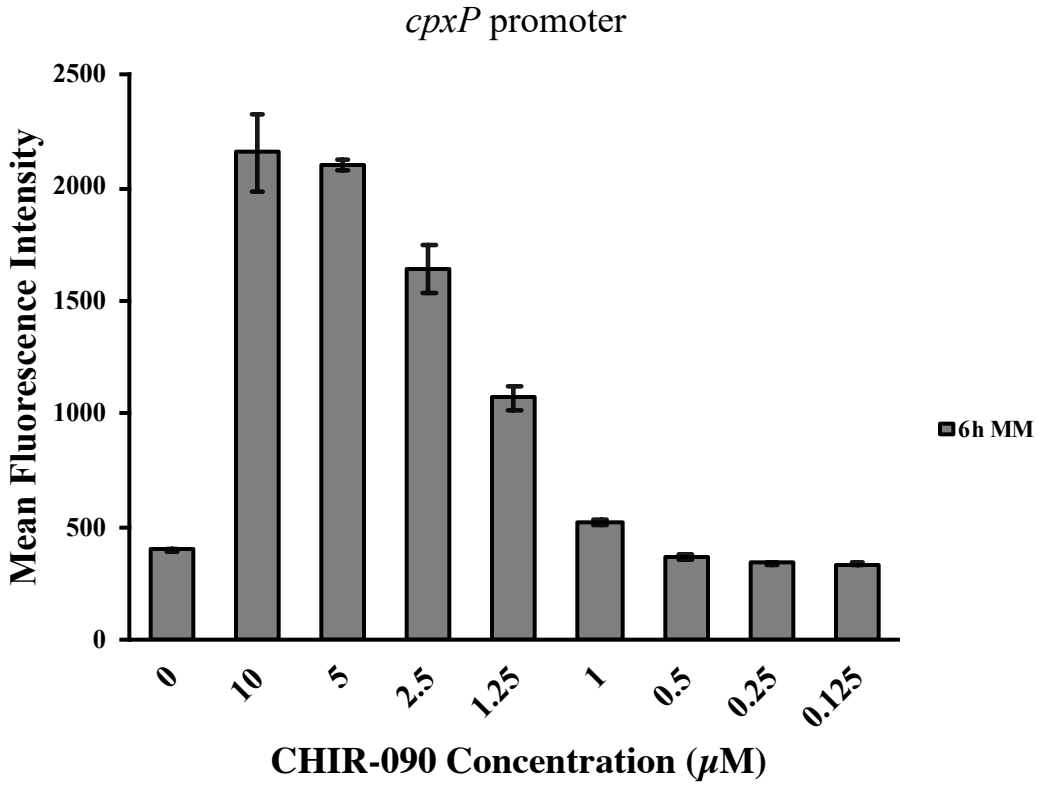


Figure 2. Concentration-dependent induction of *cpxP* promoter activity. Various CHIR-090 concentrations were each tested for *cpxP* transcriptional induction in MM. Mean fluorescent intensity was tested by flow cytometry.

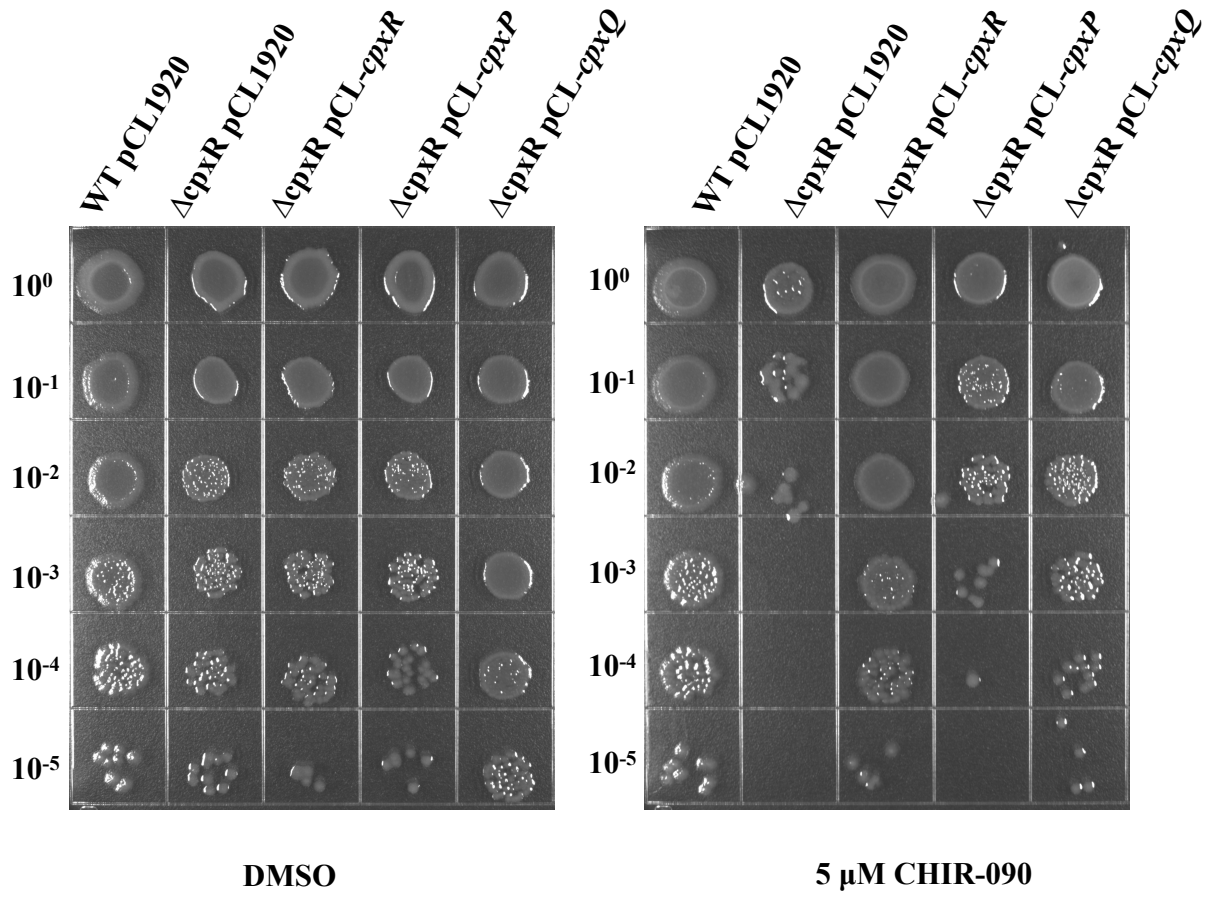


Figure 3. The effect of Cpx inducer CHIR-090 on the survival rate of the tested strains in 5 μM concentrations. DMSO was used as a negative control. Cells grown with or without 5 μM CHIR-090 were serially diluted after 24 h, and 10 μL of the bacterial suspension was spotted on LB plate for comparison.

Tested strains: WT strains, Δ*cpxR* with pCL1920, or *in trans* expression of *cpxR*, *cpxP*, and *cpxQ* in Δ*cpxR* strain.

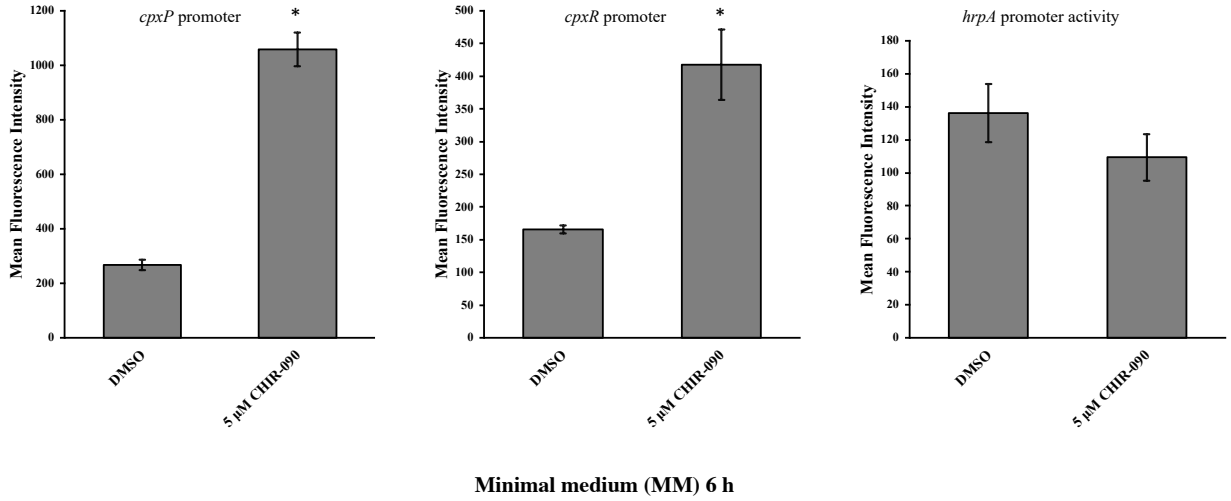


Figure 4. The measurement of the expression of two Cpx regulon genes (*cpxP* and *cpxR*) and one CpxR regulated-gene (*hrpA*) in the presence of 5 μM of CHIR-090 in WT cells. The data values represent means with standard deviations. * represents statistically significant difference ($P<0.05$).

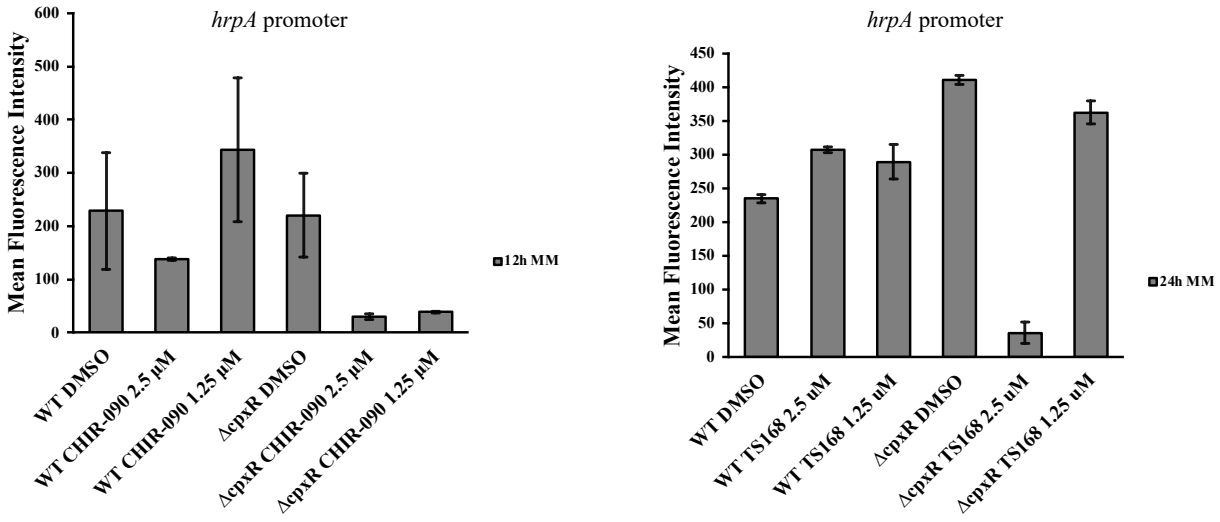


Figure 5. T3SS transcriptional expression examination using a *hrpA*-GFP reporter plasmid (pAT-*hrpA*). 2.5 μM and 1.25 μM of CHIR-090 were tested on their participation in the T3SS regulation in the WT and Δ*cpxR* strains.

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CURRICULUM VITAE

DAQING JIANG

EDUATION

Ph.D. in Biological Sciences in University of Wisconsin-Milwaukee

Dissertation: The study of the phytopathogen *Dickeya dadantii* 3937 Cpx signaling on the regulation of virulence and antimicrobial resistance — Dec. 2021

Dissertation Advisor: Dr. Ching-Hong Yang, Ph.D.

AWARDS AND PRESENTATION

Chancellor's Graduate Student Fellowship, University of Wisconsin — 2016 - 2020

Biological Sciences Symposium, University of Wisconsin, Milwaukee — 2019

RESEARCH EXPERIENCE

Doctoral study in microbiology genetics — Aug. 2016 – Now
Department of Biological Sciences, University of Wisconsin-Milwaukee
Advisor: Ching-Hong Yang

Research project: Utilization and development of edible and medicinal fungi, — Jun. 2016 - Sept. 2014
Zhejiang A&F University
Advisor: Haiping Lin

Undergraduate research on the anti-fungal activities of Alfalfa extracts, — Jun. 2014 – Sept. 2013
Department of Forestry, Southwest Forestry University
Advisor: Qian Liang

TEACHING EXPERIENCE: UNIVERSITY OF WISCONSIN- MILWAUKEE

Teaching assistant - General Survey of Microbiology (4 credits) 2017-2021
The surveys of bacteria, fungi and microbial disease applications lab lecture

MANUSCRIPTS IN PREPARATION

The phytopathogen *Dickeya dadantii* 3937 *cpxR* locus gene participates in the regulation of virulence and global c-di-GMP network

CONFERENCE ABSTRACTS/POSTERS

Jiang, D., Yuan, X., Yang, C. (2020) Investigation of The Regulatory Mechanism of CpxA-CpxR Two-component System on The Type III Secretion System of *Dickeya dadantii* 3937

Jiang, D., Yuan, X., Yang, C. (2019) Investigation of the regulatory mechanism of the type III secretion system by a putative diguanylate cyclase GcpD in *Dickeya dadantii* 3937