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Small RNA, Cyclic-di-GMP and Phenolic Compounds Regulate the Type III Secretion System in Bacterial Phytopathogens

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SMALL RNA, CYCLIC-DI-GMP AND PHENOLIC COMPOUNDS REGULATE THE TYPE III SECRETION SYSTEM IN BACTERIAL PHYTOPATHOGENS

by

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ABSTRACT

SMALL RNA, CYCLIC-DI-GMP AND PHENOLIC COMPOUNDS REGULATE THE TYPE III SECRETION SYSTEM IN BACTERIAL PHYTOPATHOGENS

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Type III Secretion System (T3SS) is an essential virulence factor in many Gram-negative bacterial pathogens. Expression of T3SS consumes large amount of energy. Hence it is tightly regulated by bacteria through several mechanisms. In this work we screened a library of phenolic compounds and found several compounds that dramatically downregulate T3SS in *Erwinia amylovora* 273. Additionally, the role of small RNA (sRNA) chaperone, Hfq, and a secondary messenger, cyclic-di-GMP in T3SS regulation in *Dickeya dadantii* 3937 was also examined.

Chapter 1 provides a brief overview of the history and virulence mechanisms of two phytopathogens – *Erwinia amylovora* 273 and *Dickeya dadantii* 3937. In chapter 2, a chemical library of phenolic compounds was screened. Several compounds inhibited expression of T3SS in *E. amylovora* 273. *trans*-4-methoxy cinnamic acid (TMCA) and Benzoic acid (BA) inhibited T3SSexpression through HrpS-HrpL pathway. Additionally, TMCA altered T3SS expression through the $rsmB_{Ea}$-$RsmA_{Ea}$ system. Additionally, *trans*-2-(4-hydroxyphenyl)-ethenylsulfonate (EHPES) induced T3SS expression. Finally, TMCA and BA inhibited the hypersensitive response (HR) by inhibiting expression of T3SS.

In chapter 3, we investigated the role of a second messenger, cyclic-di-GMP in the regulation of T3SS expression in *D. dadantii* 3937. A PilZ domain protein, YcgR regulated expression of T3SS in a c-di-GMP-dependent manner. A point mutation was created by replacing the crucial arginine residue in the RRxxxR motif of PilZ domain by aspartic acid. This mutation in YcgR altered its ability to regulate T3SS expression. BcsA, another PilZ domain protein positively regulated T3SS.

In chapter 4, we examined the role of Hfq in regulation of expression of T3SS. *hfq* mutant dramatically reduced the expression of T3SS genes such as, *hrpA*, *hrpN* and *dspE*. Hfq controlled T3SS expression by regulating expression of a response regulator, GacA which in turn regulates expression of *rsmB*. RsmB is an untranslated sRNA that positively regulates expression of the master regulator of the T3SS. *hfq* mutant also altered the expression of another sRNA, ArcZ that also regulated T3SS. Additionally, Hfq modulated the c-di-GMP levels in *D. dadantii*. Overall, the study suggested that Hfq regulated T3SS through Rsm system. The mechanism of Hfq regulating the c-di-GMP levels remains to be determined.

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LIST OF ABBREVIATIONS

AP	Alkaline phosphatase
Ap	Ampicillin sodium
ATP	Guanosine tri phosphate
BA	Benzoic acid
CDI	Contact dependent Inhibition
c-di-GMP	bis-(3′-5′)-cyclic di-Guanosine Mono Phosphate
Cm	Chloramphenicol
CV	Crystal Violet
DFO	Desferrioxamine
DGC	Diguanylate cyclases
DIPM	DspE-interacting protein from *Malus*
DMSO	Dimethyl Sulfoxide
ECF	Extracytoplasmic factor
Ecp	EAL-containing protein
EHEC	Enterohaemorrhagic *Escherichia coli*
EHPES	*trans*-2-(4-hydroxyphenyl)-ethenylsulfonate
EPPO	European and Mediterranean Plant Protection Organization
EPS	Exopolysaccharides
Gcp	GGDEF-containing protein
GFP	Green Fluorescent Protein
Gm	Gentamicin
GTP	Adenosine tri phosphate
HEE	*hrp* effectors and elicitors
HIM	Hrp Inducing minimal
HIPM	HrpN-interacting protein from *Malus*
HR	Hypersensitive Response
HrBP1	HrpN binding protein
Hrc	HR and conserved
Hrp	Hypersensitive responsive and pathogenicity
IC_{50}	Minimum inhibition concentration
IPTG	Isopropyl-thio-galactopyranoside
ITC	Isothermal Titration Calorimetric
KDG	2-keto-3-deoxygluconate
Km	Kanamycin sulfate
LB	Luria-Bertani medium
LRR	Leucine-rich repeat
MCHA	*trans*-2-Methylcinnamohydroxamic acid
MES	Morpholinoethanesulfonic acid
MG	Mannitol Glutamic acid medium
MM	Minimal salts Medium
mRNA	Messenger RNA
MS	Mass Spectrometry
nt	Nucleotide
OCA	*o*-coumaric acid
OD	optical density
OPG	osmoregulated periplasmic glucans
PCA	*p*-coumaric acid
PCR	Polymerase Chain Reaction

PCWDE	Plant cell wall degrading enzymes
PDE	Phosphodiesterases
Pel	Pectate lyase gene products
PGA	Polygalacturonate
PNPase	Polynucleotide phosphorylase
PR	Pathogenesis related
qPCR	Quantitative PCR
RBS	Ribsosome Binding Site
REST	Relative Expression Software Tool
Rif	Rifampin
RpoN	RNA polymerase subunit, nitrogen-limitation sigma factor
RpoS	RNA polymerase subunit, stationary phase sigma factor
RT	Room temperature
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sp	Spectinomycin
sRNA	Small Ribose Nucleic Acid
T1SS	Type I Secretion System
T2SS	Type II Secretion System
T3SS	Type III Secretion System
T5SS	Type V Secretion System
TAHA	*trans*-3-(2-Thienyl) acrylhydroxamic acid
TCA	*t*-cinnamic acid
TCS	Two-component system
TM	Transmembrane domain
TMCA	Trans-4-methoxy-cinnamic acid
UTR	Untranslated region
WT	Wild-type

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“You look at science (or at least talk of it) as some sort of demoralizing invention of man, something apart from real life, and which must be cautiously guarded and kept separate from everyday existence. But science and everyday life cannot and should not be separated. Science, for me, gives a partial explanation for life. In so far as it goes, it is based on fact, experience and experiment.”

-Rosalind Franklin, Biophysicist, Physical Chemist (1920-1958)

Chapter 1
Introduction

Introduction

The genus *Erwinia*, named after the phytobacteriologist Erwin F. Smith, was established in 1920 to unite all Gram negative, fermentative, non-sporulating, peritrichous flagellated plant pathogenic bacteria (Winslow et al., 1920). The phytopathogens belonging to this genus cause diseases of trees, flowers, tubers, fruits and vegetables that lead to quantitative and/or qualitative crop losses. *Pectobacterium* spp. Waldee, 1945 (formerly *Erwinia carotovora*) and *Dickeya* spp. (formerly *Erwinia chrysanthemi*, Burkholder et al. 1953) species are related soft rot enterobacterial pathogens with broad host ranges. These species formerly were known as the soft rot *Erwinia* spp., but several studies have shown that the soft rot enterobacteria and *Erwinia amylovora*, the type strain of the *Erwinia* genus, are too divergent to be included in one clade. Therefore, the soft rot *Erwinia* spp. were moved to two new genera as *Pectobacterium* and *Dickeya* (Gardan et al., 2003; Hauben et al., 1998; Samson et al., 2005 and Waldee, 1945). This chapter summarizes the biology of *Erwinia amylovora* and *Dickeya dadantii*, including their virulence mechanisms and type III secretion system as one of the major pathogenicity determinants.

1.1 *Erwinia amylovora* 273

1.1.1 Background and significance of *Erwinia amylovora* 273

E. amylovora is the first proven bacterial plant pathogen, and was shown to be the causative agent of the necrotic disease called fire blight (Burrill, 1883; Baker, 1971). Symptoms such as water soaking of infected tissue, wilting and necrosis give tissues a scorched blackened appearance, giving rise to the name "Fireblight"**.** It causes fire blight mainly in plants in the family Rosaceae, including apple and pear in the subfamily Pomoideae, and in a few other rosaceous plants such as *Rubus* spp

(Eden-Green & Billing, 1974). Historically, fire blight was initially restricted to the northeast part of North America, with its occurrence first reported more than 200 years ago. Thereafter, it spread to New Zealand, Europe, the Middle East, Korea, Japan, and, Australia (Vanneste, 1995; Bonn and van der Zwet, 2000). The disease cycle of fire blight comprises of the entry of the pathogen through blossoms, the primary route of infection, along with wounding sites on plants. In blossom infection, *E. amylovora* is transmitted to the stigma by pollinating agents such as insects or rainfall. Bacteria associated with the stigmas can be washed away into nectaries, where readily available nutrients facilitate their growth (Wilson, 1989). Since nectathodes are not cutinized, *E. amylovora* has direct access to the underlying tissues, where the infection is established. Also, bacteria present in the intercellular spaces alter the host membrane leading to ion and nutrient leakage (Youle and Cooper, 1987). Eventually, the continued presence of *E. amylovora* leads to host cell collapse and decompartmentalization, accompanied by disease symptoms such as water soaking and tissue necrosis. In susceptible hosts, bacteria present in the intercellular spaces of the cortical parenchyma can enter older tissues resulting in systemic disease. The host defense response limits spreading of pathogen by sealing off the diseased tissues through deposition of cork layers in the cortex. This results in the formation of cankers wherein *E. amylovora* can overwinter. In the following spring season, when plant growth is resumed, these dormant bacterial populations are supplied with increased amount of nutrients which leads to resurgence of disease. In addition, bacterial ooze exuded from the cankers provide an inoculum that can be transmitted to other parts of the plant which eventually can result in the destruction of entire orchards of trees. Strain Ea273 (ATCC 49946) of *E. amvlovora* was isolated from an infected apple tree growing in a western New York orchard in the 1970s.

The genome of *E. amylovora* consists of a circular chromosome of 3.8 Mb, and, two plasmids, AMYP1 (28 Kb) and AMYP2 (71 Kb). The size of the genome is shorter than other plant pathogens that have genomes of 4.5 Mb to 5.5 Mb. Analysis of the *E. amylovora* sequence and its comparison with sequenced genomes of closely related enterobacteria revealed signs of pathoadaptation to rosaceous hosts (Sebaihia et al., 2010). *E. amylovora* is closely related to the well studied non phytopathogens *Escherichia coli* and *Pantoea agglomerans* (*Erwinia herbicola*). Therefore, it provides a good model for studying the evolution of plant pathogenicity.

1.1.2 Virulence Mechanisms of *E. amylovora* 273

Type III secretion systems (T3SS) are essential determinants of the interaction of many Gram-negative bacteria with their hosts. It is a highly complex and specialized nanomachine that injects bacterial proteins into the host cell cytoplasm that translocate bacterial proteins into eukaryotic host cells. The sequence similarities between components of T3SSs and those of the flagellar assembly machineries suggest an evolutionary relation in prokaryotes (Galan et al., 1992; Blocker et al., 2003). The T3SS is induced by its contact with the host. It is an energy expensive task to secrete proteins and translocate them into the host cells. Therefore, the expression of genes encoding proteins secreted downstream in the pathway need to be regulated for which there are dedicated cytoplasmic chaperones. It differs from other secretion pathways in Gram-negative bacteria by the absence of primary sequence that is conserved in regions of secreted proteins involved in targeting except within some species (Miao and Miller, 2000). Also, there is lack of a cleaved signal sequence in secreted polypeptides and a periplasmic secretion intermediate (Miao and Miller, 2000). T3SSs and flagella share all the above mentioned characteristics except for the ability to translocate proteins into eukaryotic cells.

In the mid-1980s, a transposon mutagenesis approach resulted in a number of mutants of *E. amylovora* that lost the pathogenicity in apple (host) and the ability to elicit the hypersensitive response (HR) in tobacco (non-host). The HR is the manifestation of rapid and localized cell death followed by the infiltration of a certain number of bacteria into the leaf tissue. Based on these phenotypes, mutated genes were referred as "*hrp*" genes for *h*ypersensitive *r*esponse and *p*athogenicity. Some of the mutants resulted in loss of pathogenicity in the host, but not the HR elicitation in tobacco. These mutated genes were called "*dsp*" for *d*isease *s*pecific genes. The *hrp* and *dsp* genes exist as the *hrp*/*dsp* gene clusters in the genomic DNA. Several other virulence factors are also expressed once *E. amylovora* enters the host plant. Secreted exopolysaccharides (EPS) such as amylovoran plug the xylem channels that prevent the transport of water within the plant and eventually lead to wilting of shoots (Sjulin and Beer, 1978). Bacteria deficient in amylovoran synthesis are non-pathogenic (Bellemann and Geider, 1992). Amylovoran synthesis requires the expression of 12 *ams* genes which in turn are controlled by RcsA and RcsB, identified as regulators of capsule synthesis (Rcs) in many enterobacterial species. Two transcriptional regulators, RcsA and RcsB, bind as a heterodimer to the promoter of *amsG*, the first reading frame in the operon for amylovoran biosynthesis (Bernhard et al., 1990; Chatterjee et al., 1990; Wehland et al., 1999). Another EPS, levan, is synthesized by levansucrase encoded by the *lsc* gene. Levan is also considered to be a virulence factor (Gross et al., 1992) whose expression is positively regulated by RlsA (Zhang and Geider, 1999). The *rlsA* gene is located next to *dspF/B* in the pathogenicity island region. Amylovoran and levan have been reported to contribute to biofilm formation in *E. amylovora* (Koczan et al., 2009). Other than EPS, role of type I fimbriae, flagella, type IV pili, and curli of *E. amylovora* in biofilm formation have

also been examined (Koczan et al., 2011). This study indicated that the cell surface attachment structures are equally important in different stages of biofilm formation. Moreover, defects in many of the genes encoding these appendages result in decreased virulence *in planta* (Koczan et al., 2011). In contrast to many species of *Erwinia*, *E. amylovora* lacks the ability to degrade cell wall components by the action of carbohydrate-degrading enzymes (Seemuller and Beer, 1976). However, *E. amylovora* is reported to produce and secrete PrtA, a metalloprotease, in minimal medium (Zhang et al., 1999). PrtA is secreted by the type I secretion system (T1SS), which is comprised of three structural proteins, PrtD, PrtE, and PrtF. *E. amylovora* mutated in the *prtD* gene was unable to secrete PrtA, which resulted in reduced colonization of apple leaves by *E. amylovora* (Zhang et al., 1999). Under iron limiting conditions, *E. amylovora* also expresses siderophores that bind to Fe^{+++} in order to acquire iron. These siderophores are then taken up by the receptors on the outermembrane and delivered to the cell. *E. amylovora* produces and secretes siderophores such as cyclic desferrioxamine (DFO) which is encoded by *dfoA* gene. These iron bound siderophores are then taken up by a siderophore receptor called FoxR, encoded by *foxR*. Both the *dfoA* and *foxR* mutants result in reduced necrotic symptoms and reduced bacterial growth compared to the wild-type strain (Dellagi et al., 1998), indicating that the iron acquisition system is essential for virulence and survival of *E. amylovora.* Desferrioxamine also protects *E. amylovora* against oxidative stress (Venisse et al., 2003). Lastly, *E. amylovora* is also known to harbor the *srl* operon which encodes proteins that metabolize sorbitol. However, the ability to use sorbitol is important for causing disease only in apple shoots (Aldridge et al., 1997).

1.1.2.1 Type III secretion system

The *hrp/dsp* cluster consists of the *hrp*/*hrc* region that contains 25 genes including the four regulatory genes such as *hrpXY*, *hrpS* and *hrpL* that regulate other *hrp* genes, and nine "*hrc*" (for HR and conserved) genes. The *hrp* genes form a protein secretion/translocation pathway, called the ''Hrp T3SS'', to secrete and deliver proteins from bacteria to plant apoplasts or cytoplasm. The nine *hrc* genes encode proteins that constitute the core structural components of the Hrp T3SS. Based on homology of the Hrc proteins to those of *Pseudomonas syringae*, *hrcC* encodes for the outer membrane protein while the other *hrp* genes encode for the inner membrane proteins and form the basal structure of the Hrp T3SS. *hrpA* encodes a pilin protein for the Hrp T3SS pilus that extends outside the bacterial cell and reaches the host cell membrane. The HEE (*hrp* effectors and elicitors) region consists of seven genes; two (*hrpN* and *hrpW*) encode harpins, two are *dsp* genes (*dspA*/*E* and *dspB/F*), one is a *yopJ* homolog (*eopB*), and two encode putative chaperones (*orfA* and *orfC*) (Oh et al., 2005). Harpins are glycine rich and heat stable proteins. HrpN and HrpW encode for the harpin proteins in *E. amylovora*. Based on mutagenesis experiments, HrpN is involved in disease development and is also considered as the first cell free elicitor of HR in tobacco (Wei et al., 1992). HrpW has a putative pectate lyase domain in its C-terminus, but it does not have virulence function (Kim and Beer, 1998; Gaudrialt et al., 1998). Based on electron microscopic studies, unlike effector proteins that are translocated to the plant cytoplasm, harpins are targeted to the intercellular spaces of the plant tissues (Perino et al., 1999). Other research groups have shown that HrpN affects the ion channels in the plasma membrane of the plant and also inhibits the ATP synthesis in tobacco cell culture leading to disruption of mitochondrial function (Wei et al., 1992; El-Mahroof et al., 2001; Xie and Chen, 2000). A HrpN-interacting protein from *Malus* (HIPM) was

found in apple (Oh C. S. 2005) and a HrpN binding protein (HrBP1) was found in Arabidopsis. DspA/E is an effector protein secreted through the HrpT3SS and its secretion is dependent on DspB/F. It is a pathogenicity factor in *E. amylovora* because *dspA/E* mutants are not pathogenic to apple shoots; immature pear fruit slices (Bogdanove et al., 1998) or pear seedlings. On the other hand, *dspB*/*F* mutant exhibit reduced virulence which indicates that in absence of the DspB/F, small amount of DspE/A may be secreted. DspE-interacting protein from *Malus* (DIPM) apple has been found. This protein contains a leucine-rich repeat (LRR), a transmembrane domain (TM), and a kinase domain. However, the *in planta* interactions of this protein with DspE/A remain to be determined. EopB (*Erwinia* outer protein B) is one of the first characterized secreted proteins of *E. amylovora* and its translocation to plant cells has been demonstrated (Oh. C. S. 2005). However, EopB does not play a role in virulence or pathogenicity of *E. amylovora* in immature pear fruit and in HR elicitation in tobacco because when mutated the responses did not differ from that of the wild-type strain in pear fruit and *N. tabacum* cv. *Xanthi*, respectively (Kim J.F. 1997).

1.1.2.1.1 Regulation of the Type III secretion system

Expression of *E. amylovora hrp* genes is repressed in rich media, while it is induced *in planta* or in minimal media that simulate the conditions of plant apoplasts. The environmental signals that affect gene expression include carbon and nitrogen sources, pH, temperature, and osmolarity (Wei et al., 1992). HrpL, a member of a subfamily of eubacterial σ factors that regulate extracytoplasmic functions, is the master regulator of the *hrp* genes of *E. amylovora* (Wei and Beer, 1995). HrpL recognizes a conserved sequence motif located at the promoter regions of the HrpL-dependent secretory *hrp* operons, harpin genes, and *dsp* genes (Xiao and Hutcheson,

1994). Expression of *hrpL* is also reported to be partially dependent on the σ54/HrpS. A σ54 consensus sequence has been found in the promoter region of *hrpL* (Wei et al., 2000), and expression of *hrpL* is partially controlled by HrpS which belongs to the NtrC family of σ54 enhancer binding proteins (Wei and Beer, 1995). The two component system HrpX/Y is required for the expression of *hrpL* (Fig. 1) (Wei et al., 2000). They are members of the two-component system (TCS) regulatory protein family that is widely used for prokaryotic gene expression. HrpX is a sensor kinase that likely perceives environmental signals, and HrpY is the corresponding response regulator that transmits the signal from HrpX to *hrpL*. *hrpY* is absolutely required for the T3SS expression and *hrpL* expression, whereas *hrpX* is only partially involved in *hrpL* activation. Expression of *hrpS* and *hrpXY* is induced by conditions that mimic the environment of the apoplast of the plant (Wei et al., 1992; Wei et al., 2000). *hrpXY* shows high basal-level expression in nutrient rich medium and is increased by three fold in the Hrp Inducing minimal (HIM) medium. Studies have also indicated that the expression of HrpXY is also autoregulated. On the other hand, the expression of *hrpS* which is also induced in HIM medium has been shown to be independent of HrpXY, and is also not autoregulated (Wei et al., 2000). Finally, the HrpL σ factor which recognizes a conserved promoter motif, GGAACC-N_{15}-CCACTAAT directs transcription of the remaining *hrp* and *dsp* genes. This results in the formation of the secretion machinery and virulence proteins that interact with plant cells.

Additionally, an orthologue of the PhoPQ TCS has been genetically characterized in *E. amylovora* (Nakka et al., 2010). Three different cues have been shown to activate the PhoPQ system including a mild acidic pH, antimicrobial peptides, and low Mg^{++} (Bader et al., 2005; Brodsky and Gunn 2005; Garcia Vescovi et al., 1996; Prost et al., 2007). The PhoPQ system negatively regulates gene

expression of two novel T3SS in *E. amylovora* (Nakka et al., 2010). In several enterobacteria it has been demonstrated that RsmA, which is a global small RNA binding regulatory protein, acts by reducing the half-life of target mRNA (Liu et al., 1998; Romeo 1998). *rsmB* is a noncoding regulatory small RNA (Liu et al., 1998) that sequesters multiple units of RsmA and neutralizes its degradation effect on various mRNA species. A functional homolog of *rsmB* has been determined in *E. amylovora,* and it has been shown that $rsmB_{Ea}$ is able to neutralize the negative effect of $RsmA_{Ea}$ (Fig. 1) (Ma et al., 2001). In several enterobacteria it has been shown that a TCS, GacSA, positively regulates the expression of such small RNAs. Similar to HrpXY, expression of GacSA is also induced by low pH. A study on TCS systems in *E. amylovora* indicates the possibility of GacSA regulating HrpXY (Zhao, 2009). However, the role of GacSA in regulating the T3SS remains to be determined.

1.2 Antibiotic resistance

Antibiotics are either naturally occurring or human-made compounds. They have been successfully used to prevent and treat infections caused by pathogenic bacteria. Other than the antimicrobial activity, antibiotics such as oxytetracycline have also been reported to have growth promoting effects in animals (Chopra and Roberts, 2001). However, there are concerns regarding antibiotics being used as animal feed additives, since there are reports showing acquisition of resistant enteric flora by the animals that did not receive the antibiotics. Moreover, antimicrobial resistance in plant pathogenic target bacteria began to appear in the 1960s, a few years after introduction of the use of streptomycin (Jones, 1982; Burr and Norellii, 1990). Additionally, resistance has also been found to be linked with copper resistance (Pohronezny, 1994; Scheck, 1996). Genetically, resistance genes may be chromosomal or carried on plasmids or transposons; all genetic forms are found in

environmental, human, and plant pathogenic strains of bacteria (Sundin and Bender, 1996). Interestingly, tetracycline resistance has not been reported in target bacterial plant pathogens, but has been found in other plant surface–associated (phylloplane) bacteria (Schanbel and Jones, 1999).

Streptomycin and oxytetracycline treatments have been used as prophylactic treatment to control fire blight damage during blossom time, when fire blight damage is the most devastating (Johnson and Stockwell, 1998). Blossom time may extend 6 weeks or more and differs among species and varieties. At the time of harvest, streptomycin activity was still detectable in leaves of (Shaffer and Goodman, 1969). The 1992 Environmental protection agency fact sheet on streptomycin [7, p. 5] indicates that "all ecological effects data requirements are satisfied" and that streptomycin is nontoxic to birds, freshwater invertebrates, and honeybees and is slightly toxic to fish (both cold-water and warm-water species). Interestingly, streptomycin is reported to be "toxic to algae." After two decades, streptomycin resistant strains were isolated from pear orchards in California, Oregon and Washington (McManus and Jones, 1994). Oxytetracycline is currently being used as a replacement for streptomycin. The use of oxytetracycline may lead to the selection of *E. amylovora* strains that have acquired tetracycline resistance genes from other orchard bacteria (Schnabel and Jones, 1999). Kasugamycin is currently seen as an alternative to previously used antibiotics (McGhee and Sundin, 2011).

An alternative approach to the development of new antimicrobials is to target bacterial virulence. This approach can be used to attenuate pathogens in the host. Specific mechanisms such as adhesion, invasion, and subversion of host defense, toxin secretion, and, chemical signaling required for promoting infection and hence persisting in a pathogenic cascade to cause disease symptoms are attractive targets for

the development of antimicrobials (Cegelski, 2008). Microorganisms lacking their virulence properties may undergo a reduced selection pressure for drug-resistant mutation without threatening their existence. Such virulence-specific therapeutics may also avoid the undesirable dramatic alterations to microbial populations of the host that are associated with current antibiotics. Additionally, biocontrol agents (Lindow et al., 1996; Stockwell et al., 1996), transgenic plants or novel chemical compounds are also being investigated in order to avoid the use of antibiotics.

1.3 Plant phenolic compounds

Phenolic compounds are secondary metabolites found throughout the plant kingdom. Along with their key roles as pigments, antioxidants, metal chelators, as signaling agents, and as UV light screens, they are known to be involved in resistance against pathogen attacks and hence help plants to survive. Plants harbor preformed phenolic compounds that play an important role in resistance, especially in non-host plants. Salicylic acid (SA) is one of the major signaling molecules that activates plant defence responses against invading pathogens. It is synthesized from phenylalanine (phenylpropanoid metabolism) and other alternative biosynthetic pathways. It is produced by plants locally, at the site of infection. However, it is also found in the phloem sap and in uninfected tissues. Analysis of transgenic plants expressing the bacterial *nahG* gene, which encodes for the enzyme salicylate hydroxylase that inactivates SA by converting it to catechol, indicated the importance of SA in defense response. Transgenic NahG plants are unable to accumulate SA, and are also incapable of developing systemic acquired resistance (SAR), indicating that salicylic acid accumulation is required for the expression of SAR (Gaffney et al., 1994; Pieterse and Van Loon, 1999). SA acts as a secondary signal molecule, the level of which increases during the induced defence process and it is likely that this increase

results from an increased expression of SA biosynthetic enzymes. Increased expression of such enzymes might not be induced by SA but by another earlier signal. This endogenous regulator, in turn, coordinates the induction of SAR related genes, along with the well-characterized pathogenesis related (PR) genes, in plants infected by pathogens. A mode of action for SA has been proposed by virtue of its ability to form a phenolic free radical upon inhibition of catalase and ascorbate peroxidase. It has been suggested that such phenolic radical is involved in lipid peroxidation, the product of which might activate defence gene expression.

1.4 *Dickeya dadantii* 3937

1.4.1 Background and Significance

This pathogen was originally isolated from diseased plants in the greenhouse stocks of *Chrysanthemum morifolium* in New York in 1950. The morphological, cultural, and, physiological characters of the new species of *Erwinia* were determined and were named as *Erwinia chrysanthemi* (Burkholder et al., 1953). The taxonomy of the soft rot enterobacteria has been complicated. It was not clear from the reports as to whether *Pectobacterium, Dickeya*, or perhaps another soft rot enterobacterial genus was being described. Therefore, several names were proposed for the species. Finally, after several revisions of the nomenclature of the complex taxonomy of the *Dickeya* pathogens (Samson et al., 2005; Ma et al., 2007) it was clear that the *Dickeya* pathogens are different from the other potato soft rot erwiniae, *Erwinia carotovora* subsp. *atroseptica* (now renamed *Pectobacterium atrosepticum*) and *Erwinia carotovora* subsp. *carotovora* (renamed *Pectobacterium carotovorum* subsp. *carotovorum*). Further analysis of *Pectobacterium chrysanthemi* using 16S rDNA, DNA–DNA hybridization and biochemical characterization revealed that it forms a distinct clade from the pectobacteria, and a new genus, *Dickeya*, was proposed

(named after the microbiologist Robert S. Dickey). This led to the renaming of *Erwinia chrysanthemi* 3937 to *Dickeya dadantii* 3937.

Dickeya spp. primarily infects the fleshy and succulent parts of the plants and causes localized symptoms. They can also infect the vascular system such as the xylem channels that transport water from the soil to the entire plant. This can lead to wilting and systemic infection of the host plants. While in soil, *Dickeya* can be easily carried to plants with the help of natural vectors such as insects and can cause soft rot in vegetables. Additionally, these bacteria can overwinter in insect pupae, rotten vegetables such as tubers and occasionally in soil. Under conditions of high temperature (80° F and upwards) and humidity, these bacteria actively start growing again and are often spread in the plants with the help of insects that lay eggs on the same plants. Plants are known to form a cork layer to restrict the spreading of the infecting larvae. However, this cork layer is quickly eaten away by the larvae. During this process, bacteria get the opportunity to spread from the parent tubers to other tubers or young stem and roots of the plant. This is called stolon-end infection. The red to red-brown lesions produced on the stems and occasionally on the roots expand rapidly in succulent tissues and cause extensive maceration of the affected tissues so that the diseased plants finally collapse. According to the data sheets of European and Mediterranean Plant Protection Organization (EPPO), *Dickeya* infects a wide range of plants including horticultural and ornamental host plants and therefore is known to cause disease in several parts of the world including tropical, subtropical and temperate regions. A small number of species of insects such as pea aphid are the alternate hosts of *D. dadantii* (Costechareyre et al., 2012; Grenier et al., 2006).

The genome of *Dickeya dadantii* 3937 has been sequenced as it is a widely used model system to study molecular biology and pathogenicity of this group of

bacteria. The complete circular chromosome of *D. dadantii* strain 3937 is approximately 5 Mb (Glasner et al., 2011). Orthologs of the *D. dadantii* proteins in other sequenced enterobacteria were identified and the highest number of putative orthologs was found in *Pectobacterium atrosepticum*, which is both phylogenetically and phenotypically very similar to *D. dadantii*.

1.4.2 Virulence Mechanisms of *D. dadantii* 3937

The plant cell wall is mainly composed of cellulose, hemicellulose and pectin. The virulence of *D. dadantii* 3937 is correlated with its ability to express and secrete plant cell wall degrading enzymes (PCWDE) that include a complete set of pectinases (Robert-Baudouy et al., 2000), extracellular proteases and a cellulase (Letoffe et al., 1990; Py et al., 1991). Pectinases and cellulase are secreted by the type 2 secretion system (T2SS) which is also known as the Out system (Andro et al., 1984; Condemine et al., 1992; Lindeberg and Collmer, 1992). This system involves the export of proteins through the inner membrane and the specific Out system for transfer through the outer membrane. Several extracellular proteases secreted through the type 1 secretion system (T1SS) are known to cleave the N-terminal residues of the pectate lyase, PelI after its secretion (Shevchik et al., 1998). These processed enzymes are translocated by the Sec or Tat translocons (Johnson et al., 2006) and accumulated in the periplasmic space. Moreover, the processed protein form acts as a defense elicitor in plants (Shevchik et al., 1998). The PCWDEs have different properties (acidic or basic pI, high and low optimum pH, periplasmic or extracellular and exo- or endo-mode of action) (Collmer and Keen, 1986). The ability to produce large quantities of a wide range of enzymes enables *D. dadantii* to invade and cause disease (Lapwood, 1957; Zucker & Hankin, 1970; Liao, 1989) in a wide range of hosts. More recently, (p)ppGpp, which is produced at high levels when cells are

starved, also affects PCWDE production (Wang et al., 2007). Also, the production of the osmoregulated periplasmic glucans (OPGs) that sense the environment plays an important role in pectate lyase production and secretion (Page et al., 2001). The PCWDEs randomly cleave, by β-elimination, the internal glycosidic linkages in pectic polymers, preferentially polygalacturonate (PGA), de-esterified pectin, or low esterified pectin and generate a series of unsaturated oligogalacturonates. The end products of pectin degradation by the extracellular endo-Pels enter the periplasm through porins and are subsequently internalized into the cytoplasm via the transporter systems (Blot et al., 2002; Condemine and Ghazi, 2007; Hugouvieux-Cotte-Pattat and Reverchon, 2001). In this cellular compartment, small oligomers are further degraded to two types of monomers, galacturonate and 5-keto-4-deoxyuronate, which are both converted to 2-keto-3-deoxygluconate (KDG). The transcriptional repressor KdgR is inactivated by KDG which, eventually leads to the derepression of the *pel* genes (Nasser et al., 1991; 1992; 1994). Moreover, KdgR has been reported to control the expression of genes encoding key gluconeogenic steps (Rodionov et al., 2004). Thus, KdgR may participate in the coordination of central carbon metabolism by modulating the direction of carbon flow. Another important protein secretion system required for complete pathogenicity of *D. dadantii* is the type III secretion system (T3SS). As mentioned in section 1.1.2.1, *D. dadantii* 3937 utilizes the T3SS in order to translocate several effector proteins to the host cell cytoplasm and attenuate the host defense mechanisms. Bauer and associates (1994) demonstrated the presence of *hrp* genes in *E. chrysanthemi.* $HrpN_{Ech}$ was shown to elicit an HR in nonhost tobacco and to be involved in soft rot pathogenesis in witloof chicory (Bauer et al., 1995). Deletion of the T3SS in *D.dadantii* resulted in reduced virulence in all disease-tolerant African violet varieties tested. The *hrp* translocation gene mutants

were severely reduced in virulence relative to the wild type. Moreover, studies on the susceptible varieties indicated that the T3SS was also required for high bacterial virulence (Yang et al., 2002). Other factors such as production of cellulose fibrils, allow *D. dadantii* to develop aggregates on a plant surface (Jahn et al., 2011; Prigent-Combaret et al., 2012). These aggregates are found embedded within an extracellular polymeric substance that maintains a hydrated surface around the bacteria and thus helps them to survive desiccation stress (Condemine et al., 1999). The Type V secretion systems (T5SS) is a type of simple secretion systems, also called two-partner secretion systems. They consists of an outer membrane TpsB protein that facilitates secretion of a larger TpsA protein comprising an N-terminal transport domain and a large hemagglutinin repeat region that forms a fiber-like structure. Tps systems are encoded in *D.dadantii* T3SS gene clusters and have been shown to function in contact-dependent growth inhibition (CDI) (Aoki et al., 2010). CDI is a phenomenon in which the TpsA protein, designated CdiA, binds to target bacterial cells and inhibits their growth by delivery of a C-terminal toxin domain, the CdiA-CT. Two CDI systems are present in *D. dadantii* 3937, each expressing a different CdiA-CT toxin. The CdiA3937-1 toxin is a tRNase, and the CdiA3937-2 toxin has DNase activity (Aoki et al., 2010). *D. dadantii* mutants lacking the CDI3937-1 system were outcompeted by CDI+ wild-type bacteria on chicory, whereas deletion of the CDI3937-2 system did not affect competition. These results indicate that CDI plays a role in adhesion of bacteria to leaves (Rojas et al., 2002). In addition, *D. dadantii* secretes a biosurfactant that increases wettability, probably resulting in increased plant surface colonization (Hommais et al., 2008). Motility and chemotaxis are essential for *D. dadantii* when searching for potential sites to enter into the plant apoplast. In particular, *D. dadantii* has a strong chemotactic response to jasmonic

acid produced by wounded tissue and may enable the bacterial cells to move towards plant wounds and facilitate plant systemic invasion (Antunez-Lamas et al., 2009). Once inside the apoplast, *D. dadantii* produces siderophores which allow the bacterium to cope with the restricted iron bioavailability in the plant. Iron acquisition is critical for *D. dadantii* virulence (Franza and Expert, 2013). *D. dadantii* produces several iron storage proteins, FntA, Bfr and Dps, that contribute to its virulence, illustrating the importance of a tight control of iron homeostasis during infection (Boughammoura et al., 2008). *D. dadantii* also produces antioxidant enzymes, such as superoxide dismutases, catalases and alkylhydroperoxide reductase (Santos et al., 2001; Glasner et al., 2011), a peptide methionine sulfoxide reductase that repairs oxidized proteins (El Hassouni et al., 1999), and the blue pigment indigoidine that can scavenge reactive oxygen species (ROS) (Reverchon et al., 2002). SapABCDF transport system, responsible for the import of antimicrobial peptides and their proteolytic degradation in the cytoplasm, is required for *D. dadantii* virulence (Lopez-Solanilla et al., 1998).

1.4.2.1 Type III secretion system

In addition to pectinolytic ability, the T3SS is also required for the complete pathogenicity of the bacterium (Yang et al., 2002; Yang et al., 2004). T3SS structural genes, many of which are homologous to flagellar export apparatus, are highly conserved across genera (Galan and Collmer, 1999). The T3SS of *D. dadantii* 3937 is encoded by two divergent operons, which contain the *hrp*/*hrc*/*dsp* gene clusters. A common feature of T3SSs is the extracellular pilus structure, encoded by hrpA, through which the secreted proteins travel across the plant cell wall (Peng et al., 2006). *hrpN* encodes a harpin protein that is translocated by the T3SS, and *dspE*

encodes an effector protein in *D. dadantii* 3937 (Yap et al., 2006). T3SS genes are activated by a transcriptional activator, HrpL, a member of the extracytoplasmic factor (ECF) family alternative sigma factor (Tang et al., 2006). HrpL forms a complex with core RNA polymerase (RNAP) and binds to the *hrp* box sequence in the promoter regions of T3SS genes. The T3SS genes regulated by HrpL are classified into two major categories; 1) T3SS substrate genes *dspA*/*E*, *hrpA*, *hrpK*, *hrpN*, and *hrpW*; and 2) T3SS apparatus genes such as *hrpP*, *hrcQ*, *hrpG*, and *hrpF* (Yang et al., 2010).

1.4.2.1.1 Regulation of type III secretion system

The T3SS in *D. dadantii* plays a role in pathogenicity during the initial stages of infection (Yang et al., 2004). The expression of T3SS genes is modulated by the constantly changing environment that includes abiotic and biotic factors, metabolic signals such as different carbon sources or those released by the plants, and divalent cations. For example, the T3SS genes are expressed at a very low level in nutrient-rich media, but are induced in infected plant tissues or in artificial *hrp*-inducing minimal medium (Alfano et al., 1997). In addition, some natural phenolic compounds in the plant either induce or inhibit the expression of T3SS genes in *D. dadantii* 3937 (Li et al., 2009; Yang et al., 2008). Intensive studies on the regulation of the T3SS in recent years have revealed a number of transcriptional and post-transcriptional regulatory elements (Büttner, 2012; Li et al., 2010; Tang et al., 2006; Yang et al., 2007). In *D. dadantii* 3937, two main regulatory pathways are involved in controlling the expression of the T3SS (Fig. 2). The TCS HrpX/HrpY activates the expression of *hrpS*, which encodes an NtrC-family transcriptional enhancer protein. HrpS interacts with the sigma factor RpoN (σ^{54}), and then activates the transcription of *hrpL* (Yap et al., 2005; Peng et al., 2005). HrpL then activates the expression of T3SS structural

and effector genes. The TCS GacS/GacA positively controls the T3SS via a regulatory cascade involving a regulatory small RNA (sRNA), RsmB. This sRNA binds to its target protein with high affinity and modulates its activity (Cui et al., 2001; Yang et al., 2008). In *D. dadantii* 3937, the RNA-binding protein RsmA binds to and promotes the degradation of *hrpL* mRNA. The degradation effect of RsmA on *hrpL* mRNA is neutralized by the action of the regulatory sRNA RsmB, whose expression is positively regulated by the TCS GacS/GacA (Cui et al., 2001; Yang et al., 2008). Additionally, polynucleotide phosphorylase (PNPase) encoded by *pnp*, downregulates *hrpL* transcription by reducing *rpoN* mRNA stability (Zeng et al., 2010). PNPase also reduces *hrpL* mRNA stability. Besides the HrpX/HrpY and GacS/GacA signaling pathways, expression of the T3SS is regulated by additional factors. For instance, a mutation of *pecT* or *pecS* has been shown to significantly influence the expression of *hrpN* (Nasser et al., 2005). PecS was reported to bind the *hrpN* regulatory region and inhibit *hrpN* transcript elongation in *D. dadantii* 3937. In addition, a novel regulator of the SlyA/MarR family, SlyA, was found to regulate *hrp* genes of the HrpL regulon in parallel with HrpL in *D. dadantii* (Zou et al., 2012). It negatively regulates the expression of *hrpL* by downregulating *hrpS* and upregulating *rsmA*. Interestingly, the authors showed that SlyA positively regulates the expression of *hrpA* and *hrpN*. The intracellular secondary messenger molecules bis-(3′-5′)-cyclic di-GMP (c-di-GMP), a global second messenger which controls the lifestyles of many bacteria, was reported to regulate the T3SS of *D. dadantii* 3937 (Jenal and Malone, 2006; Yi et al., 2010).

1.4.2.2 c-di-GMP – a secondary messenger

Bacterial cells use intracellular signal molecules to sense and respond to their physiological conditions. These signals may be small molecules, such as cyclic

diguanylate (c-di-GMP), whose levels are modified by diguanylate cyclases (DGCs) that contain a GGDEF domain and phosphodiesterases (PDEs) that contain either an EAL or HD-GYP domain, depending on the environmental conditions (Jenal and Malone, 2006). Moreover, these molecules may be tied directly to metabolic pathways, such as levels of glucose inside cells or glucans in the periplasm. Their effects may be both at the level of transcription, posttranscriptional, and, postrtranslational (Hengge 2009; Romling et al., 2005; Wolfe and Visick 2008). Cyclic-di-GMP acts as an intracellular secondary messenger molecule. It is often involved in switching cells from one lifestyle, such as motile, to another, such as a sessile biofilm cell (Jenal and Malone, 2006). This aids in the establishment of multicellular biofilm communities and in the transition from the virulent state in acute infections to the less virulent but more resilient state characteristic of chronic infectious diseases. Apart from these behaviors, cyclic di-GMP has also been shown to regulate virulence, the cell cycle, differentiation, and other processes (Fig. 3).

D.dadantii 3937 encodes numerous putative DGCs and PDEs that may act on c-di-GMP. Seven of the 18 proteins in these classes in *D. dadantii* were mutated (Yi et al., 2010) and two had significant effects on multiple phenotypes. Deletion of *ecpB* and *ecpC* enhanced biofilm formation and reduced virulence, motility, pectate lyase production, and the T3SS gene expression.

1.5 small RNA and Hfq

Gene expression is an essential and an energetically expensive task for all living cells. This makes it very important for the cell to decide the level of production of specific proteins and avoid the wastage of resources. Regulation of gene expression is guided by an intrinsic (physiological state of the cell) or extrinsic (environmental cue) factors. Several stages of regulation of gene expression include

transcriptional, post-transcriptional, translational, and, post-translational modifications.

The significance of post-transcriptional regulation by small non-coding RNAs has recently been recognized in both prokaryotes and eukaryotes. The discovery of MicF, involved in the down-regulation of the expression of *ompF*, a gene that encodes a major outer membrane porin of *Escherichia coli* (Mizuno et al., 1984) lead to unraveling of many other small regulatory RNAs (sRNAs) involved in gene regulation in bacteria. Among them, a subset of sRNAs act by imperfect base-pairing to target mRNAs and regulate their translation and stability, either positively or negatively. Unlike miRNA in eukaryotes that bind to the 3' untranslated region (UTR) of target mRNAs, bacterial sRNAs bind to the 5' UTR of the target mRNAs (Gottesman, 2005) that comprise the translation initiation region. The pairing therefore masks the ribosome binding site (RBS) and results in translational repression (Fig. 4A). These sRNAs fold into stem–loops and contain modular domains for sRNA biogenesis or function. The 5′ end or internal region of the sRNAs is highly conserved within homologous sRNAs. These regions serve as target recognition domains that select the regulated mRNAs by short pairing; such regions are referred as 'seed' regions. The common stem–loop structure at the 3′ end of sRNAs, followed by a short poly (U) stretch, is part of a Rho-independent transcription terminator and probably serves the additional function of preventing attack by 3′ exonucleases. In several cases, Hfq binds in an AU-rich single-stranded region upstream of the terminator and this binding might then expose the target-binding domain of sRNAs for the interrogation of potential mRNA partners. However, as most sRNAs are short and structured, the proximity of the stem–loop might be coincidental. In a few cases, the pairing occurs upstream of the RBS

(Waters and Storz, 2009). sRNAs can also activate expression of the target mRNAs by disrupting an inhibitory secondary structure that sequesters the RBS (Fig. 4B) (Waters and Storz, 2009; Gottesman and Storz, 2010). The potential base-pairing between sRNAs and target mRNAs ranges from 10 to 30 nucleotides (nt). However, more limited core-pairing appears to be sufficient for regulation (Waters and Storz, 2009). For example, a 14-nt sequence within the 31-nt potential pairing region of SgrS is sufficient to inhibit the translation of target *ptsG* (Maki et al., 2010).

Studies in *E. coli* have shown that these small RNAs find and anneal to their target mRNAs with the help of RNA binding protein, Hfq. The abundant protein Hfq was discovered forty years ago as a host factor required for in vitro replication of the RNA bacteriophage, Qβ (Fernandez et al., 1968). The role of Hfq in bacterial physiology began to be established through the broad phenotypes of *hfq* mutants of *E. coli* (Tsui et al., 1994). Hfq has been shown to be required for the fitness and virulence of an increasing number of bacterial pathogens. Although Hfq mutants are often sensitive to host defense mechanisms and highly attenuated in animal models, there is considerable variation in both severity and extent of phenotypes. Additionally, it was shown that Hfq is required for the synthesis of RpoS which is crucial for the virulence of *Salmonella typhimurium* (Brown and Eliott, 1996). The involvement of Hfq in sRNA mediated regulation was first recognized during the study on translational repression of *rpoS* mRNA (encoding the alternative sigma factor RpoS) by OxyS RNA that is induced in response to oxidative stress in *E. coli* (Zhang et al., 1998). Thereafter, Hfq was reported to play a major role in sRNA mediated regulation (Gottesman 2004; Valentin-Hansen et al., 2004). Hfq is a highly conserved protein encoded in a large number of bacteria (Sun et al., 2002). Structural analyses have revealed that it oligomerizes into homo-hexameric rings and is similar

to archaeal and eukaryotic Sm and Sm-like proteins integral to premRNA splicing and RNA degradation complexes (Schumacher et al., 2002). Moreover, like eukaryotic Sm and Sm-like proteins, studies have indicated that Hfq preferentially binds to A/U-rich sequences close to more structured region of RNA (Vytvytska et al., 2000; Zhang et al., 2002; Brescia et al., 2003). It is a heat stable protein and is known to exist as a pentamer or hexamer. Calculations have estimated the presence of 30,000 to 60,000 copies of Hfq protein per cell (Kajitani et al., 1994; Talukder et al., 1999). The majority of Hfq is in the cytoplasm in association with ribosomes, while a minor fraction associates with the nucleoid (Kajitani et al., 1994; Talukder et al., 2000). It has been demonstrated in *E.coli* that Hfq binds to polyriboadenylate RNA (Link et al., 2009). The study also suggests that Hfq acts as an RNA chaperone by binding sRNAs and mRNAs at two distinct binding faces, with mRNAs being recognized at (A-R-N) triplet repeats. The repetitive nature of each surface provides several contact sites with the same RNA and gives rise to cooperative effects, and facilitates the recruitment of the competitor RNA to favor exchange with the stably bound RNA. In vitro studies have shown that Hfq facilitates sRNA–mRNA base-pairing in several cases, such as OxyS-*fhlA* (Zhang et al., 2002), Spot42-*galK* (Moller et al., 2002), MicA-*ompA* (Rasmussen et al., 2005), and SgrS-*ptsG* (Kawamoto et al., 2006). Also, a recent study has demonstrated that, in vitro, Hfq is no longer required for translational inhibition once base-pairing is achieved (Maki et al., 2008). However, all Hfq-binding RNAs examined to date require Hfq for their regulatory functions in vivo. Interestingly, Hfq can also act alone to regulate gene expression by influencing polyadenylation or translation of mRNAs. Taken together, it is highly likely that the Hfq- mediated rapid association of sRNAs with target mRNAs is necessary to efficiently block ribosome loading to the RBS. Hfq promotes base-pairing by either

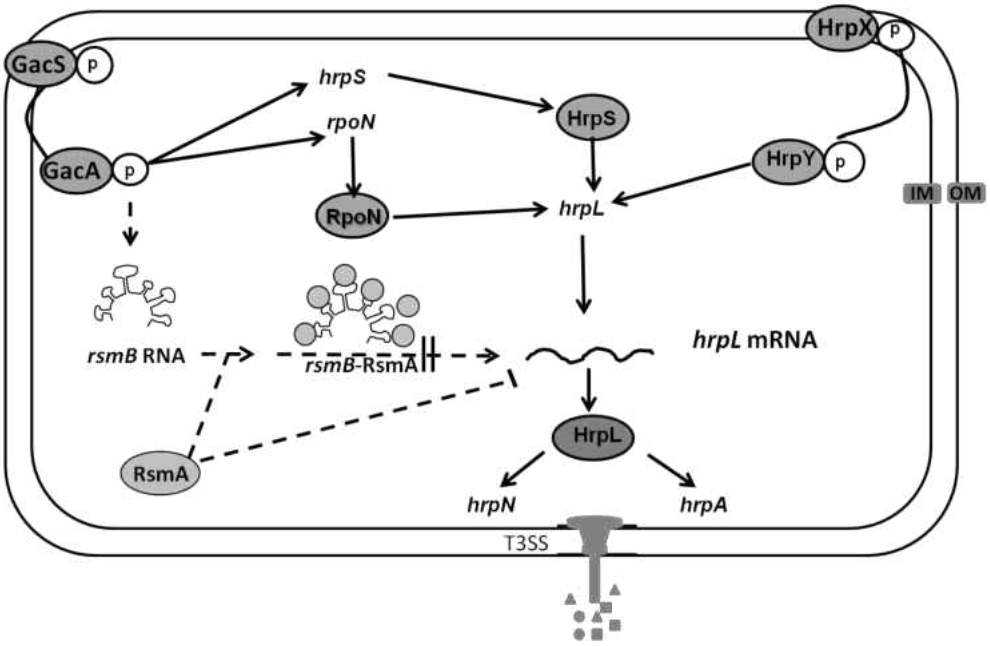
changing the RNA structures, acting as an RNA chaperone, thereby allowing accessibility of two complementary RNAs; or binding to a given sRNA and its target mRNAs at the same time. This leads to an increase in local concentrations of both the RNAs (Storz et al., 2004).

Apart from revealing regulated genes, regulon identification has indicated that sRNA can be a part of larger regulatory networks (Beisel and Storz, 2010). For example, *E. coli* Spot 42 RNA is part of a multi output feedforward loop, in which Crp both directly activates the target genes by binding to their promoters and indirectly activates the same genes by repressing the synthesis of the Spot 42 RNA, which acts as a repressor of the genes (Beisel and Storz, 2011).

Other prominent sRNA regulators act by modifying protein activity, for example, the very highly conserved *E. coli* CsrB and 6S RNAs. These sRNAs bind specific proteins rather than base pair to target RNAs (Babitzke and Romeo, 2007; Wassarman, 2007; Willkomm and Hartmann, 2005).

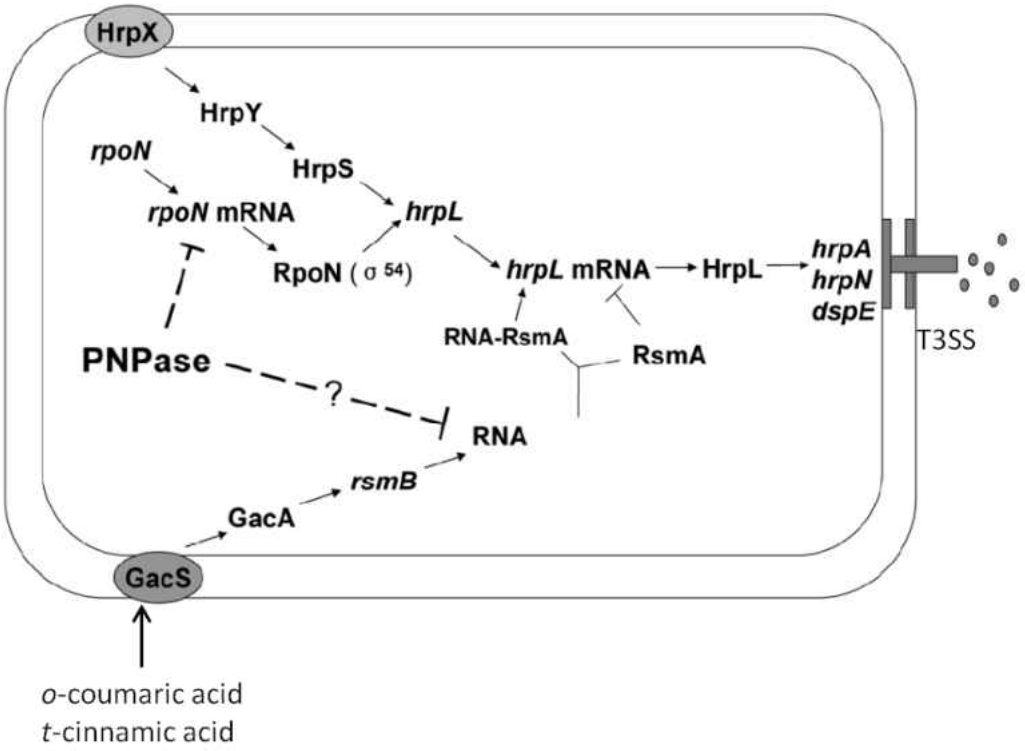
Mathematical modeling and simulations support the previous studies speculating the efficiency of sRNA based regulation (Shimoni et al., 2009). Transcriptional regulation involves recognition between amino acids and bases, and protein interaction is determined by recognition between amino acids. Regulation by sRNAs involves, in many of the studied cases, base pairing with the mRNA of the target gene. Therefore, it seems that evolutionary design of sRNAs that will regulate target genes by base pairing should be simpler than the evolution of the other regulatory molecules (Eddy, 2001). This evolutionary advantage of sRNAs along with their other properties, may suggest why these molecules are so widespread in all kingdoms of life.

1.6 Figures



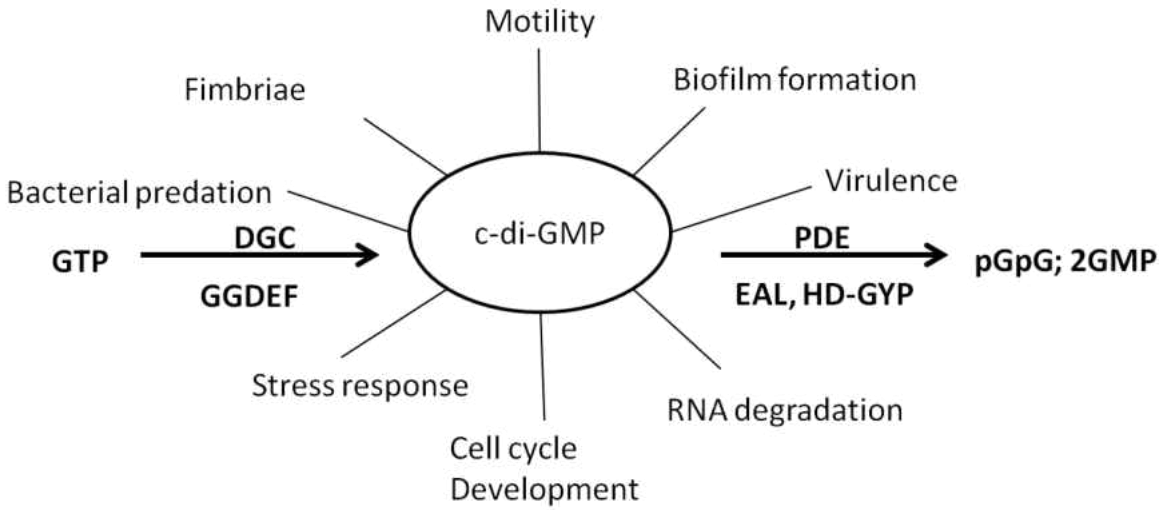
(Source: Modified from Wei et al., 2000)

Figure 1: Model of the *hrp* gene regulatory cascade in *E. amylovora*. Thick arrow lines: genes or operons. Dashed lines: hypothetical indirect regulation or hypothetical regulatory links based on evidence shown in *E. amylovora* or other plant-pathogenic bacteria. Ovals and circles: proteins. Arrowheads in thinner lines: directions of information flow. IM, inner membrane; OM, outer membrane; P, phosphate;



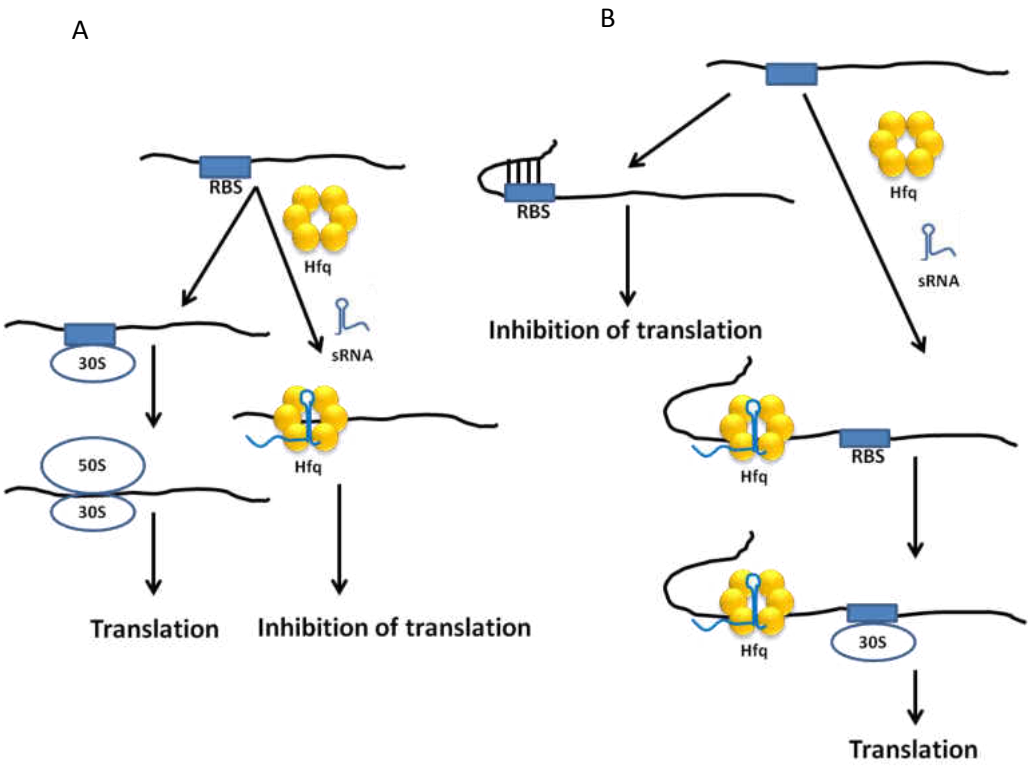
(Source: Zeng et al., 2010 and Yang et al., 2008)

Figure 2: **Model of T3SS and Gac-Rsm regulatory cascades of *D.dadantii* 3937**. Plant phenolic compounds *o*-coumaric acid (OCA) and *t*-cinnamic acid (TCA) induce expression of the type III secretion system (T3SS) genes. The T3SS and Gac-Rsm regulatory cascades were adopted as described in the text.



(Modified from Source: Waters, 2012)

Figure 3: **Various cellular behaviors controlled by Cyclic-di-GMP**. DGC enzymes, typically encoding a GGDEF domain, synthesize c-di-GMP from two GTP molecules. Phosphodiesterase enzymes (PDE), typically containing either an EAL or HD-GYP domain, degrade c-di-GMP molecules. These domains are named for the critical amino acids in the respective active sites of these two types of enzymes.



(Modified from Source: Vogel and Luisi, 2011)

Figure 4: **Common modes of Hfq activity**: **A**. Hfq in association with a small RNA (sRNA) may sequester the ribosome-binding site (RBS) of a target mRNA, and prevent binding of the 30S and 50S ribosomal subunits and thus repressing translation. **B**. In some mRNAs, a secondary structure in the 5′ untranslated region (UTR) form an inhibitory loop which can mask the RBS and inhibit translation. A complex formed by Hfq and a specific sRNA may activate the translation of such target mRNAs by exposing the translation initiation region for 30S binding.

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Chapter 2

Discovery of Plant Phenolic Compounds that act as Type III Secretion System Inhibitors or Inducers of the Fire Blight Pathogen, *Erwinia amylovora*

Abstract

Erwinia amylovora causes a devastating disease called fire blight in rosaceous plants. The type III secretion system (T3SS) is one of the important virulence factors utilized by *E. amylovora* in order to successfully infect its hosts. By using a green fluorescent protein (GFP) reporter construct combined with a high-throughput flow cytometry assay, a library of phenolic compounds and their derivatives was studied for their ability to alter the expression of the T3SS. Based on the effectiveness of the compounds on the expression of the T3SS pilus, the T3SS inhibitors 4-methoxy-cinnamic acid (TMCA) and benzoic acid (BA) and one T3SS inducer, *trans*-2-(4-hydroxyphenyl)-ethenylsulfonate (EHPES), were chosen for further study. Both the T3SS inhibitors (TMCA and BA) and the T3SS inducer (EHPES) were found to alter the expression of T3SS through the HrpS-HrpL pathway. Additionally, TMCA altered T3SS expression through the *rsmBEa*-RsmAEa system. Finally, we found that TMCA and BA weakened the hypersensitive response (HR) in tobacco by suppressing the T3SS of *E. amylovora*. In our study, we identified phenolic compounds that specifically targeted the T3SS. The T3SS inhibitor may offer an alternative approach to antimicrobial therapy by targeting virulence factors of bacterial pathogens.

Introduction

Erwinia amylovora is a Gram-negative bacterial plant pathogen that belongs to family *Enterobacteriaceae*. It is the causal agent of fire blight in rosaceous plant species such as apple, raspberry, cotoneaster, and pear. It can infect blossoms, leaves, succulent shoots, and immature fruits of these host plants (Norelli et al., 2003). The pathogen can enter the plant naturally through wounds created by wind, hail, or rain. Pollinating agents like insects and splashing rain help in spreading the infection. After establishing itself in the plant, bacterium moves through the vascular system and accumulate in the xylem. Symptoms observed in the infected plant parts, such as twigs and leaves include water soaking, discoloration, and wilt, followed by necrosis which gives the plant a fire-scorched appearance, hence the name fire blight (Koczan et al., 2009).

During infection, *E. amylovora* utilizes different virulence factors to cause disease in a susceptible host. These include the type III secretion system (T3SS); exopolysaccharides, such as amylovoran and levan (Metzger et al., 1994); metalloprotease PrtA (Zhang et al., 1999); iron-scavenging siderophore desferrioxamine (Dellagi et al., 1998); multidrug efflux pump AcrAB (Burse et al., 2004); and others (Aldridge et al., 1997, Bogs and Geider, 2000). The T3SS is encoded by hypersensitive response and pathogenicity *(hrp*) genes and is considered to be one of the major virulence determinants in *E. amylovora*. The T3SS forms a proteinaceous, syringe-like structure which secretes and translocates effector proteins from bacteria to the plant apoplast or cytoplasm (He et al., 2004; Mota and Cornelis, 2005). These effectors manipulate the host cellular activities in order to ensure survival of the bacteria and cause disease in the host (Nimchuk et al., 2001). In the case of resistant host plants and nonhost plants, such as tobacco, these effectors elicit

the hypersensitive response (HR) (Dangl and Jones, 2001). Since the T3SS is a conserved virulence factor in many Gram-negative bacteria, it is an attractive target for designing antimicrobial compounds.

The expression of *E. amylovora hrp* genes is environmentally regulated in response to nutritional conditions, including carbon and nitrogen sources, pH, and temperature (Wei et al., 1992). Under appropriate environmental conditions, HrpX/HrpY, a two-component signal transduction system (Wei et al., 2000) regulates the expression of *hrpL,* which encodes an alternative sigma factor that binds at the promoter region of the *hrp* genes encoding T3SS effectors, harpin proteins, chaperones, and the type III pilus (Xiao and Hutcheson, 1994; Kim et al., 1997) (Fig. 1). A σ^{54} consensus sequence has been found in the promoter region of *hrpL* (Wei et al.,2000), and expression of *hrpL* is partially controlled by HrpS (Wei and Beer, 1995), which belongs to the NtrC family of σ^{54} enhancer binding proteins. Unlike other plant pathogens such as *Pantoea stewartii* or *Dickeya dadantii*, HrpS is independent of the HrpXY signal in *E. amylovora* (Wei et al., 2000) (Fig. 1). In soft-rot pathogens such as *Pectobacterium carotovorum* and *D. dadantii,* regulation of the *hrp* genes is subject to transcriptional and post transcriptional regulation. RsmA, which is a global small RNA binding regulatory protein acts by reducing the half-life of *hrpL* mRNA (Liu et al., 1998; Romeo, 1998). *rsmB* is a noncoding regulatory small RNA (Liu et al., 1998) that sequesters multiple units of RsmA and neutralizes its degradation effect on various mRNA species. A functional homolog of *rsmB* has been determined in *E. amylovora,* and it has been shown that $rsmB_{Ea}$ is able to reverse the negative effect of $RsmA_{Ea}$ (Ma et al., 2001). In *P. carotovorum,* it has been shown that the GacS/GacA system upregulates *rsmB* production, which in turn positively regulates extracellular polysaccharide production, motility, and pathogenicity (Ma et

al., 2001; Cui et al., 2001). GacS/GacA responds to acidic pH conditions, and a previous study in *E. amylovora* (Zhao et al., 2009) along with other plant pathogens, such as *Pseudomonas syringae* (Chatterjee et al., 2003) indicated that it can positively regulate the T3SS which is also activated under condition such as acidic pH.

Phenolics are one of the secondary metabolites synthesized by plants. Apart from their role in pigmentation, growth and reproduction it also plays a key role in disease resistance (Aoki et al., 2001). Plants have developed a systemic acquired resistance (SAR) mechanism to protect themselves from pathogen invasion. SAR occurs at sites distant from the initial site of pathogen infection, and salicylic acid (SA) has been identified as a signaling molecule that acts during SAR development (Durrant and Dong, 2004; Yasuda et al., 2008). During this process, many natural compounds- for example, stilbenes, coumarins, and isoflavonoids -are produced in response to microbial attack and are effective against a broad-spectrum of pathogens (Matern, 1991; Hammerschmidt and Dann, 1999; Liu et al., 2006). Many of the above compounds induced in response to pathogen attack are derived from multiple branches of the phenylpropanoid biosynthesis pathway. Phenylpropanoids are a group of secondary metabolites exclusively produced by plants from L-phenylalanine (Delany et al., 1994; Glazebrook and Ausubel, 1994; Nawrath and Metraux, 1999; Achnine et al., 2004; Fagard et al., 2007; Ravirala et al., 2007). Although natural products occupy an important position in the area of plant disease management, researchers have encountered difficulty in isolating specific active components from plant extracts, which usually consists of a mixture of a large number of structurally related compounds with various degrees of bioactivity, or even opposing effects (growth inhibitors versus growth stimulants), and even some with cytotoxicity (Jaki et al., 2008).

Recently, we reported that plant phenolic compounds *p*-coumaric acid (PCA), *o*-coumaric acid (OCA) and *t*-cinnamic acid (TCA) were able to either inhibit or induce the expression of T3SS genes of a bacterial phytopathogen *D. dadantii* 3937 (Yang et al., 2008; Li et al., 2009). OCA and TCA induced the expression of the T3SS through the *rsmB*-RsmA pathway, and the inhibition of T3SS expression by PCA was found to be moderated through the HrpS-HrpL pathway. Given that the T3SS is conserved in many phytopathogenic bacteria, we hypothesized that some of the plant phenolic compounds found in plants and/or their derivatives may affect the T3SS of *E. amylovora*. In this report, an inventory of phenolic compounds, including newly synthesized derivatives was screened for their effect on T3SS expression of *E. amylovora*. Several of these compounds showed either induction or repression of the T3SS. Two T3SS inhibitors, *trans*-4-methoxy cinnamic acid (TMCA) and Benzoic acid (BA), and one inducer *trans*-2-(4-hydroxyphenyl)-ethenylsulfonate (EHPES), were chosen, and their effects on T3SS regulatory components were further investigated. In addition, the effectiveness of T3SS inhibitors TMCA and BA on suppression of HR of *E. amylovora* in tobacco was examined.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All bacteria strains were stored at –80°C in 20% glycerol. Wild-type *Erwinia amylovora* 273 (Ea273), *Escherichia coli* and *Agrobacterium* strains were routinely grown in Luria-Bertani (LB) medium at 28°C, 37°C, and 30°C respectively. For induction of T3SS genes, Ea273 was grown in HIM (*hrp* inducing minimal medium) supplemented with 10 mM mannitol as previously described (Huynh et al., 1989). When necessary, antibiotics were added to growth medium at the following concentrations: kanamycin

(50 μg/ml), ampicillin (100 μg/ml), chloramphenicol (40 μg/ml), gentamycin (15 μg/ml) and rifampin (50 μg/ml).

Recombinant DNA techniques

Preparation of genomic or plasmid DNA, PCR, restriction digestion, ligation, DNA electrophoresis, and transformations were performed as described by Sambrook and Russell (Sambrook and Russell, 2006). Primers used for PCR in this report are listed in Table 1.

Sources of the screened compounds

Compounds TS1-35, 108-113, 134-136 and 144-145 were purchased from commercial sources Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), and TCI (Tokyo, Japan). TS100-107 and 114-133 were synthesized via the routes described in our recent publication (Yamazaki et al., 2012). The remaining compounds were synthesized by the following methods:

(i) Synthesis of cinnamyl hydroxamates and amides from the corresponding esters

To an ice-cold solution of methyl cinnamate (5 mmol) dissolved in anhydrous MeOH (5 mL) and THF (5 mL) was added hydroxylamine hydrochloride or amine hydrochloride (15 mmol, 3 equiv) followed by 25% sodium methoxide in methanol solution (5.25 mL, 22.5 mmol, 4.5 equiv). The reaction mixture was stirred under argon at 0°C for 2 h, and then allowed to warm to an ambient temperature with continuous stirring overnight. The resulting yellow suspension was condensed to dryness with a rotary evaporator, and the residue was treated with 1N HCl aqueous solution (20 mL). The mixture was extracted with ethyl acetate (EtOAc) (3 times, 30 mL each time), and dried (anhydrous Na_2SO_4). Evaporation of the solvent afforded the crude products, which was purified by flash silica gel chromatography (elution

with 5 to 15% MeOH in Dichloromethane [DCM]) to yield the corresponding hydroxamic acid or amide.

TS137: white solid; ^{1}H NMR (300 MHz, CD_3OD): δ2.82 (s, 3H), 6.38 (d, *J*=15.9 Hz, 1H), 6.78 (d, *J*=8.4 Hz, 2H), 7.38 (d, *J*=8.4 Hz, 2H), 7.44 (d, *J*=15.9 Hz, 1H); MS (ESI): *m/z* 176 (M-1).

TS138: white sold; ^{1}H NMR (300 MHz, CD_3OD): δ3.41 (t, *J*=5.4 Hz, 2H), 3.65 (t, *J*=5.4 Hz, 2H), 6.43 (d, *J*=15.9 Hz, 1H), 6.77 (d, *J*=8.4 Hz, 2H), 7.38 (d, *J*=8.4 Hz, 2H), 7.45 (d, *J*=15.9 Hz, 1H); MS (ESI): *m/z* 206 (M-1).

TS139: light brown solid; ^{1}H NMR (300 MHz, DMSO-d_6): δ2.24 (t, *J*=8.1 Hz, 2H), 2.78 (t, *J*=8.1 Hz, 2H), 7.16-7.25 (m, 5H), 8.71 (s, 1H), 10.20 (s 1H); MS (ESI): *m/z* 164 (M-1).

TS 140: yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6): δ6.53 (d, *J*=15.9 Hz, 1H), 7.32-9.92 (m, 9H), 7.51 (d, *J*=15.9 Hz, 1H); MS (ESI): *m/z* 238 (M-1).

TS141: brown solid; ^{1}H NMR (300 MHz, DMSO-d_6): δ6.39 (d, *J*=15.9 Hz, 1H), 7.24 (m, 2H), 7.43 (d, *J*=15.9 Hz, 1H), 7.57 (m, 2H), 9.05 (brs, 1H), 10.74 (brs 1H); MS (ESI): *m/z* 180 (M-1).

TS142: light-brown solid; ^{1}H NMR (300 MHz, CD_3OD): δ2.40 (s, 3H), 6.22 (d, *J*=15.6 Hz, 1H), 6.87 (d, *J*=8.4 Hz, 2H), 7.48 (d, *J*=8.4 Hz, 2H), 7.52 (d, *J*=15.6 Hz, 1H); MS (ESI): *m/z* 176 (M-1).

TS143: white solid; ^{1}H NMR (300 MHz, DMSO-d_6): δ4.43 (s, 2H), 6.91-7.30 (m, 5H), 8.90 (brs, 1H), 10.77 (brs 1H); MS (ESI): *m/z* 166 (M-1).

(ii) Synthesis of cinnamyl hydroxamates from the corresponding carboxylic acids

To a stirred mixture of cinnamic acid (7 mmol) and diisopropylethylamine (DIEA) (2.45 mL, 17 mmol) in anhydrous DMF (20 mL) was added *O*-(benzotriazol-1-yl)-*N,N,N=,N=*-tetramethyluronium hexafluorophosphate HBTU (2.92g, 7.7 mmol)

at room temperature, followed by adding a solution of hydroxylamine hydrochloride (0.98g, 14 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (2.13g, 14 mmol) in anhydrous DMF (8 mL), and then the whole reaction mixture was stirred at room temperature for 1 h. After most of the DMF was removed with a rotary evaporator, the residue was dissolved in EtOAc (150 mL), then washed with H_2O (50 mL) and brine (50 mL), and dried (anhydrous Na_2SO_4). Evaporation of the solvent afforded the crude products, which was purified by flash silica gel chromatography (eluting with 5-15% MeOH in DCM) to afford the hydroxamic acid.

TS146: yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6): δ6.48 (d, *J*=15.6 Hz, 1H), 7.44 (d, *J*=15.6 Hz, 1H), 7.58 (d, *J*=7.2 Hz, 2H), 7.61 (d, *J*=7.2 Hz, 2H), 9.05 (brs, 1H), 10.76 (brs 1H), 11.29 (s, 1H); MS (ESI): *m/z* 189 (M-1).

TS147: yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6): δ4.49 (s, 2H), 6.42 (d, *J*=15.9 Hz, 1H), 7.32 (d, *J*= 7.2 Hz, 2H), 7.42 (d, *J*=15.9 Hz, 1H), 7.49 (d, *J*=7.2 Hz, 2H); MS (ESI): *m/z* 192 (M-1).

TS160: light-yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6): δ2.35 (s, 3H), 6.34 (d, *J*=15.6 Hz, 1H), 7.23 (m, 3H), 7.49 (d, *J*=7.5 Hz, 1H), 7.66 (d, *J*=15.6 Hz, 1H), 9.05 (brs, 1H), 10.78 (brs 1H); MS (ESI): *m/z* 176 (M-1).

TS161: light-brown solid; ^{1}H NMR (300 MHz, DMSO-d_6): δ6.24 (d, *J*=15.3 Hz, 1H), 7.06 (m, 1H), 7.34 (m, 1H), 7.60 (m, 1H), 7.66 (d, *J*=15.3 Hz, 1H), 9.10 (brs, 1H), 10.80 (brs 1H); MS (ESI): *m/z* 168 (M-1).

(iii) Synthetic method for TS165

A 100-mL round-bottom flask, equipped with a condenser and a stirring bar, was charged with benzyl 4-bromophenyl ketone (7.43g, 27 mmol), sodium acetate NaOAc (2.66g, 32.4 mmol, 1.2 eq), *tert*-butyl acrylate (4.70 mL, 32.4 mmol, 1.2 eq) and N-methylpyrrolidinone (NMP) (35 mL) at room temperature. In a separate 100

mL flask, palladium (II) acetate $Pd(OAc)_2$ (22.5 mg, 0.1 mmol) was dissolved in NMP (50 mL) with stirring, getting a 0.002M solution. At room temperature and under N_2, 6.75 mL (0.0135 mmol) of this solution was added in one portion to the reaction flask via a syringe. The reaction mixture was heated at 135°C with stirring in an oil bath for 40 min, resulting in a gray mixture. After being cooled to room temperature, the reaction mixture was quenched by adding water (200 mL), and the resulting suspension was extracted with EtOAc (3 times with100 mL each). The combined organic layers were washed with water (twice with 100 mL each) and brine (100 mL), and dried over anhydrous Na_2SO_4. The solution was filtered through a pad of celite to remove the catalyst, the celite was rinsed with EtOAc (twice with 40 mL each), and the solvents were evaporated with rotary evaporator to dryness, yielding *trans*-4-(benzylcarbonyl)cinnamic acid tert-butyl ester as oil (about 10g), which was used for next step reaction without further purification.

To a mixture of trimethylsulfoxonium iodide (15.9g, 72 mmol, 3.75 eq) and 60% NaH (2.54g, 63.5 mmol, 3.3 eq) was added anhydrous DMSO (85 mL) in one portion at rt, and the mixture was stirred under nitrogen for 1.5h. A solution of *trans*-4-(benzylcarbonyl)cinnamic acid tert-butyl ester obtained above (6.2g, 19.2 mmol) in anhydrous DMSO (40 mL) was added dropwise within 12 min to the resulting suspension at RT. After the whole mixture was stirred at RT for 3h, the reaction was quenched by brine (160 mL). The resulting mixture was extracted with ether (4 times with100 mL each), washed with brine (twice with 50 mL each), and dried. The solvent was evaporated with rotary evaporator to dryness, and the crude product was purified by flash column chromatography (eluting with 95:5 to 90:10 hexane/EtOAc) to afford *trans*-2-[(4'-benzylcarbonyl]phenylcyclo-propane-1-carboxylic acid tert-butyl ester (4.0g, 62% yield) as white solid.

To an ice-cold stirred solution of *trans*-2-[(4'-Benzylcarbonyl]phenylcyclopropane-1-carboxylic acid tert-butyl ester (1.1g, 3.2 mmol) dissolved in dichloromethane (12 mL) was added trifluoroacetic acid (TFA) (3.5 mL) in one portion, and the reaction mixture was stirred at 0°C for 1h, then allowed to warm to the ambient temperature, and the stirring was continued for additional 2h. The solvents were evaporated to dryness with rotary evaporator, and the solid residue was azeotroped with toluene (2×10 mL) to give *trans*-2-[(4'-benzylcarbonyl]phenyl-cyclopropane-1-carboxylic acid (TS165) (700 mg, 78% yield) as off-white solid. ^{1}H NMR (300 MHz, CD_3OD): δ1.35 (m, 1H), 1.50 (m, 1H), 1.80 (m, 1H), 2.58 (m, 1H), 3.60 (s, 2H), 6.86 (d, *J*=8.4 Hz, 2H), 7.08 (m, 5H), 7.14 (d, *J*=8.4 Hz, 2H); MS (ESI): m/z 279 (M-1).

GFP transcriptional reporter screening

To screen compounds that induce or inhibit the expression of *Erwinia amylovora* Ea273 T3SS, a 260-bp fragment containing the promoter region of *hrpA* was PCR amplified using the primer set p*hrpA*-F (5′-ATATGGATCCCGATAAAGAGCAGCGTAG) and p*hrpA*-R (5′-ATTAGAATTCTTAGACGCCTGAGCATTG). Underlined letters in the forward and reverse primers indicate the recognition sites for *Bam*HI and *Eco*RI, respectively. The amplified fragment and pPROBE-gfp[AAV], a broad-host-range vector carrying promoterless *gfp* (Miller et al., 2000), were digested with *Eco*RI and *Bam*HI, gel purified, and ligated to create promoter-probe construct called phrpA. This constructed plasmid was then transferred to Ea27*3* by electroporation. Expression of *hrpA* was analyzed using a FACS Calibur flow cytometer as previously described (BD Biosciences, San Jose, CA) (Peng et al., 2006). Wild-type Ea273 carrying the promoter-probe phrpA or pPROBE-gfp[AAV] (vector control) was grown in LB broth overnight and transferred to *hrp* inducing minimal medium (HIM) or HIM

supplemented with 100µM the compounds as described (Li et al., 2009). HIM was used in order to induce the *hrp* genes, which encode the proteins that form the T3SS structure, and also to express the virulence proteins secreted through it. Kanamycin was added to the respective media whenever promoter-probe construct was examined. The promoter activity of *hrpA* in Ea273 was monitored by measuring intensity of the green fluorescence protein (GFP) using flow cytometry. Two independent experiments were performed, and three replicates were used in each of the experiments. Minimum inhibition concentration (IC_{50}) was measured by diluting the selected compounds and measuring the fluorescence at 9 hrs of bacterial growth in HIM. Due to the low expression levels of *hrpS* and *hrpL*, promoter regions of *hrpS* and *hrpL* of Ea273 were cloned into pPROBE-AT at *Bam*HI and *Eco*RI sites. The pPROBE-AT contains a wild-type *gfp* reporter and has been routinely used in *D. dadantii* for measuring promoter activity of the bacterium (Yang et al., 2008).

RNA extraction and Quantitative PCR analysis

E. amylovora cells were cultured in LB medium overnight at 28°C and subcultured in HIM medium supplemented with DMSO or with 100µM TMCA, BA or EHPES for 6 hrs. Total bacterial RNA was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA) and treated with Turbo DNA-*free* DNase kit (Ambion, Austin, TX). cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) from 0.6 µg of total RNA. The Real Master Mix SYBR ROX (5 PRIME, Gaithersburg, MD) was used for quantitative PCR (qPCR) reactions to quantify the cDNA level of target genes in different samples. Data were collected by the Opticon 2 system (Bio-Rad, Hercules, CA) and analyzed using Relative Expression Software Tool as described by Pfaffl and co workers (Pfaffl et al., 2000). A housekeeping gene *rplU* was used as an

endogenous control for data analysis (Mah et al., 2003). The primer pairs used in this study are listed in Table 1.

Western blot analysis

HrpN protein level was determined by immunoblot using anti-HrpN antibody prepared by Proteintech company. Sample preparation was performed as previously described with modifications (Ham et al., 1998). Wild-type Ea273 was grown overnight in LB medium at 28°C. The culture was washed with *hrp* inducing medium (HIM) and resuspended in 40 ml of HIM to an optical density (OD) at 600 nm of 0.2. To determine the effect of inhibitors or inducers on the HrpN protein level, the compounds TMCA, BA or EHPES dissolved in DMSO were added to the culture at the final concentration of 100 μM and the cultures were grown with moderate shaking at 28 °C. After 24 h, the cells were collected by centrifugation at 3,500 × g for 10 min and resuspended in 20 ml phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 [pH 7.4]). After the cells were washed with PBS, cells were centrifuged and the pelleted cells were resuspended in 4 ml B-PER® Bacterial Protein Extraction Reagent (Pierce Biotechnology Inc., Rockford, IL). The resuspended cells were incubated in the shaker at 200 rpm for 15 mins at 28°C. Thereafter, the cells were centrifuged and the supernatant was collected as the soluble cell fraction protein. Optical density of the bacteria cells treated with different compounds was measured at 590nm and normalized before the protein extraction (Nikolaus et al., 2001). Protein concentration was further normalized for all the treatments with the help of the Bradford assay (Bio-Rad Protein Assay for microtiter plate) (DeLisa et al., 2003). Bovine Serum Albumin (BSA) was used as standard. 35μl of 1 × sample buffer (2% w/v sodium dodecyl sulfate, 2% v/v glycerol, 2 mM β-mercaptoethanol, 50 mM Tris-HCl (pH 6.8), and 0.01% w/v

bromophenol blue) was added to 100 µl of normalized sample protein and heat-treated at 95°C for 10 min using Dry Bath Incubator (Fisher Scientific, Pittsburgh, PA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), blotted to a polyvinylidene fluoride (PVDF) membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad laboratories, Hercules, CA), and subjected to immunodetection. The blotted membrane was then washed three times with PBS and incubated in blocking solution (5% w/v skim milk in PBS) overnight. After washing three times with the washing buffer (PBS containing 0.3% v/v Triton-X100), the membrane was incubated in washing buffer supplemented with an anti-hrpN polyclonal antibody (Proteintech company) for 1.5 h and then in the washing buffer supplemented with an anti-Rabbit IgG conjugated with alkaline phosphatase (AP) (Southern Biotech, Birmingham, AL) for another 1.5 h. Between the incubations membrane was washed three times with washing buffer. The membrane was then incubated in AP reaction buffer (100 mM Tris base, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$) for 5 min, and HrpN proteins were detected by the chromogenic detection method. The results were analyzed using ImageJ (http://imagej.nih.gov/ij/index.html) to obtain the relative intensity of the HrpN protein level.

RNA isolation and Northern blot analysis

E. amylovora cells grown in HIM supplemented with DMSO or 100 µM TMCA, BA, EHPES for 6 h were harvested and total RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, followed by DNase treatment with a Turbo DNA-free DNase kit (Ambion, Austin, TX). Each RNA sample was analyzed by Northern blot analysis using a NorthernMax kit (Ambion, Austin, TX) according to the manufacturer's instructions. Hybridization

probes used to detect the mRNA of target genes were PCR amplified and labeled with biotin using a BrightStar Psoralen-Biotin kit (Ambion, Austin, TX). Signals were developed using the Bright-Star BioDetect kit (Ambion, Austin, TX). 16S rRNA was visualized under UV transilluminator (Syngene, Frederick, MD) and used as an internal control for normalization of RNA.

Measurement of growth rate

E. amylovora cells were grown overnight in LB at 28°C. The cells were then resuspended in M9 medium supplemented with DMSO or 100 µM of the selected 56 compounds to have 0.05 OD_{590} and their growth rate were monitored till 24 hrs. The cultures were grown in 15 ml glass tubes and 200 µl of the cultures was transferred to the 96-well flat bottom plate (Corning, NY) in order to measure the absorbance at different time points by using Infinite M200 Pro™ plate reader (Tecan, Crailsheim, Germany). Two independent experiments were performed, and three replicate samples were used in each experiment.

Hypersensitive response assay

E. amylovora cells from overnight cultures grown in LB broth were resuspended in sterile dH_2O. Cell suspensions were mixed with compounds and incubated for 30 minutes before plant inoculation. *Nicotiana tabacum* cv. Xanthi plants were used for HR assays. Tobacco leaves were pressure infiltrated by using a needless syringe (Fu et al., 2006) with 8 x 10^5 cells /ml of *E. amylovora* and 100 µM TMCA or BA. Bacterial cell concentration was determined using serial dilution plating method. Plants were photographed and assessed for macroscopic tissue collapse indicative of HR 18 h post-inoculation. For transient gene expression studies, tobacco leaves were first infiltrated with *A. tumefaciens* strain GV3101 carrying pCPP5247. After that,

100µM TMCA and BA were infiltrated to the different sets of tobacco leaves at 0, 3, 6, 9, 12 and 24 h of post infiltration of *A. tumefaciens* strain.

Agrobacterium-mediated transient expression

A. tumefaciens strain GV3101 carrying pCPP5247 was grown at 30ºC in LB supplemented with appropriate antibiotics to stationary phase. Bacteria were sedimented by centrifugation at 3,500 x g for 10 min at room temperature and washed and resuspended in 10 mM morpholinoethanesulfonic acid (MES) (pH 5.5) and 100 µM acetosyringone to a final optical density at 590 nm (OD_{590}) of 0.5 (Badel et al., 2006). Cells were infiltrated into abaxial air spaces of *N. tabacum* cv. Xanthi using a 1-ml blunt syringe. Plants inoculated with bacteria were incubated at 24ºC with 12 h of daily illumination.

Statistical analysis

Means and standard deviations of experimental results were calculated using Excel (Microsoft, Redmond, WA) and the statistical analysis was performed using a two-tailed *t*-test.

Results

Various compounds either inhibited or induced the *hrpA* promoter activity.

In order to screen the compounds that would affect T3SS expression, a promoter-reporter fusion plasmid was constructed. The promoter of *hrpA,* which encodes the type III pilus (Kim et al., 1997; Jin et al., 2001) was cloned into the promoter-probe vector pPROBE-gfp[AAV] (Miller et al., 2000). This vector contains the promoterless green fluorescent protein (*gfp*) gene whose expression is driven by the promoter cloned immediately upstream. This construct was referred to as phrpA. *E. amylovora* 273 (Ea273) containing phrpA was cultured in a *hrp*-inducing medium (HIM) (Huynh et al., 1989) supplemented with dimethyl sulfoxide (DMSO; solvent of phenolic compounds), or 100 μM concentrations of each phenolic compound. The promoter activity of *hrpA* was measured at 6 and 9 hrs of induction by measuring GFP intensity using flow cytometry (Table 2) (Peng et al., 2006). Several of the compounds tested in the initial screen had a strong inhibitory effect on *hrpA* promoter activity, such as trans- cinnamic acid (TCA), benzoic acid (BA), salicylic acid (SA), and ortho-cinnamic acid (OCA). Many were found to induce *hrpA* promoter activity when added to HIM, such as diethyl *trans*-2-(4-hydroxyphenyl)-vinylphosphonate (TS114), 2, 4-Dihydroxycinnamic acid (TS2), *trans*-3-(4-hydroxyphenyl) acrylohydrazide (TS133), ethyl *trans*-2-(4-hydroxyphenyl)-ethenylsulfonate (EHPES) and methyl para-coumate (TS101). Mean GFP intensity (MFI) of the total bacterial population has been shown in table 2. This method of screening indicated that several compounds have an effect on the T3SS of *E. amylovora.* To eliminate the possibility that the decline in phrpA reporter fluorescence signal was due to the cell death or stasis of cells in HIM, the Ea273 cells carrying phrpA were grown in HIM supplemented with phenolic compounds, TMCA, BA, and EHPES for 9 hours.

Similar numbers of bacterial cells were observed between HIM supplemented with DMSO and EHPES, respectively (Fig. 2B). Interestingly, compared with DMSO, a slight increase in bacterial numbers was observed in HIM supplemented with TMCA and BA (Fig. 2B). Also, no plasmid loss was observed in Ea273 cells carrying phrpA grown in HIM supplemented with phenolic compounds (TMCA, BA, and EHPES) for 9 hours (data not shown).

Growth rate of *E. amylovora* was not affected by a majority of inhibitory or inducing compounds tested.

Out of 85 compounds that were screened, 56 of them exhibited greater than or equal to 50 % inhibition of *hrpA* promoter activity (Table 2). To exclude the possibility that the reduced GFP intensity was due to the effect on bacterial growth, these compounds were then tested for their effect on the growth rate of *E. amylovora*. Since HIM was not able to support the growth of *E. amylovora* beyond an optical density (OD) at 590nm of 0.15, M9 media was chosen to test the effect of the compounds on growth rate. Out of 56, 49 compounds did not cause a significant inhibition of the growth rate of the bacterium when the media was supplemented with 100μM of the compounds (Table 3). All the inducers found in the screening did not affect the growth rate.

Minimum T3SS-inhibitory concentrations of TMCA, SA, BA, MCHA, and TAHA

Out of 49 T3SS inhibitors, 4 compounds, TMCA, SA, BA, and TS161: *trans*-3-(2-Thienyl) acrylhydroxamic acid (TAHA) showed the highest inhibition of *hrpA* promoter activity and were chosen for further study. TS160, *trans*-2-Methylcinnamohydroxamic acid (MCHA) is a novel compound that has not been reported to be synthesized elsewhere. Therefore, although MCHA reduced the

growth rate of the *E. amylovora* at 100 µM, we were interested to test its effect on bacterial growth as well as the T3SS expression at lower concentrations. To compare the inhibitory effect of these compounds, we tested the half-maximal inhibitory concentration (IC_{50}) of these compounds on T3SS expression. Here, IC_{50} is defined as the concentration of compound that is required for the inhibition of 50% of the *hrpA* promoter activity compared to the DMSO control. No growth inhibition was observed in MCHA at concentrations of 12.5 and 25 µM (data not shown). At lower concentrations, such as 12.5µM, compounds MCHA and TAHA did not inhibit the *hrpA* promoter activity to the extent shown by TMCA, SA, or BA (Fig. 2A). TMCA and BA were further chosen for the experiments described below.

hrpA mRNA levels of _E. amylovora_ were affected by inhibitors and inducers

TMCA and BA showed an inhibitory effect, whereas EHPES induced *hrpA* promoter activity (Table 4). To confirm this finding, northern blot analyses were performed to examine the effect of the compounds on *hrpA* mRNA levels. Compared to the DMSO control, lower levels of *hrpA* mRNA were observed with the addition of 100 µM TMCA and BA, whereas higher levels of *hrpA* mRNA were observed with the addition of 100µM EHPES (Fig. 3). This result confirms the promoter activity result that the T3SS of Ea273 is affected by TMCA and BA (T3SS inhibitors) as well as EHPES (a T3SS inducer).

Inhibitors and inducers altered T3SS expression through HrpS-HrpL pathway

Based on the initial experiments, we found that T3SS inhibitors and inducers altered the *hrpA* expression levels. *hrpA* is a HrpL-regulon gene; in order to understand the effect of these inhibitors and inducers on the regulatory components of the T3SS, we measured both the promoter activity and mRNA levels of *hrpL*. Our

result showed that *hrpL* levels were also reduced by TMCA and BA, whereas they were induced by EHPES (Table 4 and Fig. 4).

Previously, it has been shown that HrpS and RpoN (σ54) are required for transcription of *hrp* genes in *P. syringae* pathovars (Xiao et al., 1994; Grimm et al., 1995) and *P. caratovora* (Chatterjee et al., 2002). In case of *E. amylovora*, it is known that *hrpL* transcription is positively regulated by HrpS (Wei and Beer, 1995). In order to find out whether the T3SS inhibitors and the inducer affect the expression of *hrpL* through *hrpS* and *rpoN*, expression of *hrpS* and *rpoN* were compared in the presence and absence of T3SS inhibitors and the inducer. We found that both the promoter activity and the mRNA levels of *hrpS* were decreased with inhibitor treatment and were elevated with the inducer treatment (Table 4 and Fig. 5). *rpoN* mRNA levels were not altered significantly by either inhibitors or the inducer (Fig. 5). This result suggests that the T3SS inhibitors (TMCA and BA) or inducer (EHPES) alter T3SS expression through HrpS-HrpL pathway.

TMCA alters the T3SS expression through $rsmB_{Ea}$-$RsmA_{Ea}$ system.

Previous work has identified *rsmB* in *E. amylovora***.** It has also been shown that $rsmB_{Ea}$ in *E. amylovora* positively regulates extracellular polysaccharide production, motility, and pathogenicity (Ma et al., 2001). Based on the sequence of *rsmB* in *E. amylovora* E9 (Ma et al., 2001), a *rsmB* sequence was located in the genome of Ea273 and the expression level of *rsmB* was determined in presence of the inhibitors or the inducer. Northern blot results showed that $rsmB_{Ea}$ levels were not affected by BA and EHPES (Fig. 4). A reduced level of $rsmB_{Ea}$ was found in T3SS inhibitor TMCA treatment. Similar $rsmA_{Ea}$ (*csrA* homolog) expression levels were observed among bacterial cells treated with TMCA, BA, EHPES, and DMSO control (Fig. 4). This result suggests that TMCA may also alter the T3SS expression through

the *rsmB*$_{Ea}$-RsmA$_{Ea}$ system. Similar levels of *rsmB* and *rsmA* RNAs were observed in bacterial cells treated with BA and EHPES.

Inhibitors and inducers do not alter the expression of *hrpX/hrpY*

In *E. amylovora*, *hrpX* and *hrpY* encode a functional two-component system where HrpX acts as a sensor kinase and HrpY acts as a response regulator. This two-component system is known to regulate the T3SS expression in response to appropriate environmental conditions (Wei et al., 2000). In order to determine whether the phenolic compounds affect the T3SS expression through the HrpX/Y system, mRNA levels of *hrpX* and *hrpY* were measured in presence of the T3SS inhibitors and the inducer. We observed that the inhibitors TMCA and BA or the inducer EHPES did not alter the *hrpX* and *hrpY* mRNA levels (Fig. 5). This result suggests that the phenolic compounds do not affect the expression of *hrpXY*.

HrpN levels were altered in presence of T3SS inhibitors and T3SS inducer

We found that the T3SS inhibitors and the inducer affected *hrpL* expression. *hrpN* of *E. amylovora* is a HrpL-regulon gene and its encoding protein is characterized as a major elicitor of the HR in plants. The *hrpN* was examined for its expression at both mRNA and protein levels. First, northern blot analysis of *E. amylovora* cells treated with TMCA, BA, or EHPES showed that the mRNA levels of *hrpN* were either significantly reduced or increased, respectively, compared to the DMSO control (Fig. 6A). In addition, Western blot analysis was performed using an anti-HrpN polyclonal antibody against the total cell fraction collected after 24 hrs of bacterial growth in HIM supplemented with the inhibitors TMCA, BA or the inducer EHPES. The amount of HrpN protein in the total cell fraction of *E. amylovora* treated with TMCA and BA was reduced, whereas the amount of HrpN protein in EHPES-treated cells was higher than the control treatment (DMSO) (Fig. 6B). These results

confirm the inhibitory and inducing effect of TMCA and BA, and EHPES on *hrpL,* respectively. Also, it indicates that the T3SS inhibitors and the inducer alter the HrpN protein levels. HrpN protein is secreted via the T3SS. To determine the effect of BA and TMCA on HrpN protein secretion, we performed Western Blot analysis on the supernatant fraction of the cell cultures treated with BA and TMCA. The results demonstrated that the secreted levels of HrpN are also reduced by the T3SS inhibitors BA and TMCA respectively (Fig. 6C).

TMCA and BA weakened the HR symptom in tobacco by targeting the T3SS of *E. amylovora*.

Based on the effect of inhibitors on the T3SS, TMCA and BA were evaluated for their effect on suppressing the HR in non-host tobacco by *E. amylovora*. Compared to the DMSO treatment, a reduced HR phenotype was observed in tobacco leaves when TMCA and BA were co-infiltrated with *E. amylovora* (Fig. 7A). To rule out the possibility that compounds TMCA and BA suppressed the plant defense response leading to a suppression of the HR, a tobacco plant transiently expressing the *hopQ1* effector gene of *P. syringae* DC3000 was used in this study. HopQ1 is an effector known to elicit the HR in tobacco (Schechter et al., 2004). An *Agrobacterium tumefaciens* GV3101 strain carrying the *hopQ1* effector gene of *P. syringae* DC3000 (pCPP5247) strain was infiltrated into tobacco leaves, which will allow the transient gene expression of *hopQ1* in tobacco plants (Badel et al., 2006). TMCA and BA were infiltrated at different time points after the leaves were infiltrated with *Agrobacterium* and the HR symptoms were monitored at 18 hrs post-infiltration. The results showed that the development of HR in the tobacco plants transiently expressing HopQ1, was not affected in the presence of TMCA or BA (Fig. 7B).

Discussion

The T3SS is a crucial virulence determinant among many Gram-negative bacteria. Since it is well conserved, this makes it a good candidate as a target for drug development. In animal pathogens such as *P. aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *Yersinia pestis*, large libraries of synthetic small molecules have been screened to identify potential candidates that can target the T3SS of the pathogens (Aiello et al., 2010; Keyser et al., 2008; Clatworthy et al., 2007; Muschiol et al., 2006). Phenolic compounds are one of the major classes of secondary metabolites found in the plant. About 10,000 structures of phenolics have been identified to date (Kennedy and Wightman, 2011). These compounds are usually involved in pigmentation, growth, resistance to pathogens, and reproductive functions of the plant. Our previous work showed that phenolic compounds such as TCA, OCA, and PCA specifically alter the T3SS expression of *D. dadantii* 3937 (Yang et al., 2008 ; Li et al., 2009). In addition, phenolic compound derivatives were found to either induce or inhibit the T3SS expression *of P. aeruginosa* (Yamazaki et al., 2012). In this study, a library consisting of 85 compounds, including several synthesized phenolic derivatives, was screened in an effort to identify compounds that alter the T3SS expression in *E. amylovora*.

Out of 85 compounds, several compounds showed a spectrum of inhibition or induction of the *hrpA* promoter activity (Table 2). Previous research has shown that low nutrients, pH, and temperature conditions can induce the T3SS (Wei et al., 1992). However, it is largely unknown whether there is any specific component that has the ability to trigger the T3SS of *E. amylovora*. It is interesting to identify several phenolic compounds that are able to induce T3SS expression. Among the tested compounds, EHPES has exhibited the strongest inducing activity. TMCA and BA

showed significant reduction of *hrpA* promoter activity, and did not have any impact on the growth rate of the bacteria (Table 2 and 3). Besides TMCA and BA, several other phenolic derivatives also show potent inhibitory effect against the T3SS of *E. amylovora*, such as SA (TS33), *trans*-4-mercaptocinnamic acid (TS26), and *trans*-4-dimethylaminocinnamic acid (TS112). There are electron-donating groups in TMCA (4-MeO group), TS26 (4-mercapto group) and TS112 (4-Me_2N group). When an electron-withdrawing group, such as nitro group, is presented in the 4-position, the corresponding *trans*-4-nitrocinnamic acid (TS28) affects the growth of the bacteria (Table 2). When the carboxylic acid group is replaced with N-hydroxyl carboamide, the resulting hydroxamic acids can maintain the inhibitory activity. For example, salicylhydroxamic acid (TS135) has demonstrated similar activity as TS33. However, some hydroxamic acids dramatically affect the bacterial growth, such as *trans*-4-bromocinnamohydroxamic acid (TS128) and *trans*-4-fluorocinnamohydroxamic acid (TS141).

It is known that SA can reduce the expression of virulence factors, such as flagella (required for motility) and fimbriae (capsule development required for biofilm formation) biosynthesis (Price et al., 2000). SA also plays a critical signaling role in the activation of plant defense responses against pathogen infection (Loake and Grant, 2007). It is worth to note that SA was found to be able to suppress the T3SS expression of *E. amylovora* in this study. It is also worth to mention that the SA suppression on T3SS expression of *E. amylovora* is specific; no effect of SA was observed on the T3SS expression in *D. dadantii* (Yang et al., 2008). Earlier studies on phenylpropanoid metabolism in cell suspension cultures has reported that TMCA (4-methoxy cinnamic acid) is one of the intermediates in the biosynthetic conversion of cinnamic acid to benzoic acid in the cells of *Vanilla planifolia* (Funk and

Brodelius, 1990). Also, the conversion of TCA into SA has been proposed to proceed via chain shortening to produce benzoic acid (BA), followed by hydroxylation at the C-2 position to derive SA (Yalpani and Raskin, 1993). It is interesting to uncover that the intermediates of the phenylpropanoid pathways such as TMCA and BA are capable of lowering the expression of the T3SS of *E. amylovora*.

In several plant pathogens mentioned above, HrpX phosphorylates HrpY, changing it into a transcriptional activator that initiates the expression of the T3SS. Measurement of mRNA levels of *hrpXY* suggested that both the T3SS inhibitors (TMCA and BA) and the T3SS inducer (EHPES) do not affect the expression of *hrpXY* (Fig. 5). Since HrpX/HrpY is activated by phosphorylation, the effect of these T3SS inhibitors and inducer on this two-component regulatory system is unclear at this stage. Consistent with the phenotype that we observed for *hrpA* expression, the T3SS inhibitors (TMCA and BA) and the T3SS inducer (EHPES) also exhibited an inhibitory or induction effect on the expression of both *hrpL,* as well as *hrpS,* respectively (Table 4 and Fig. 5). These results indicated that the compounds channel their inducing or inhibitory effect on the expression of HrpL via HrpS. Unlike other plant pathogens such as *D. dadantii* 3937, *Pantoea stewartii,* and *Erwinia herbicola* (Merighi et al., 2003; Nizan-Koren et al., 2003; Yap et al., 2005), *hrpS* expression is not regulated by the two component system HrpXY in *E. amylovora.* Validated from the sequence analysis of HrpS, it was observed that HrpS of *E. amylovora* possesses a helix–turn–helix (HTH) domain, but a very short N-terminal A domain and hence possibly lacks the phosphorylation receiver domain (Wei and Beer, 2000). In case of *P. syringae* pv. *tomato,* expression of *hrpS* is modulated by the GacA/S two component regulatory system (Chatterjee et al., 2003). Little is known about the regulatory effect of GacA/S on *hrpS* expression of *E. amylovora*. Together, our

results suggest that the inhibitors and the inducer affect the expression of *hrpA* through HrpS-HrpL regulatory pathway, possibly by targeting GacS/A (Fig. 1).

Studies in *P. carotovorum* and *P. syringae* have revealed that the regulatory role of GacS/A is mediated through the regulator of secondary metabolism (Rsm) system (Heeb and Haas, 2001). Further research in *P. carotovorum* and *D. dadantii* 3937 suggests that *hrpL* is also subject to post transcriptional regulation mediated by RsmA and *rsmB* RNA (Yang et al., 2008; Chatterjee et al., 2002). Also, it has been reported that phenolic compounds such as OCA and TCA induce the T3SS through the *rsmB*-RsmA pathway (Yang et al., 2008). In this study, since mRNA levels of *hrpL* were affected in presence of T3SS inhibitors or the inducer, we determined the $rsmB_{Ea}$ and $rsmA_{Ea}$ mRNA levels in *E. amylovora*. The $rsmB_{Ea}$ and $rsmA_{Ea}$ mRNA levels were not affected by the T3SS inhibitor BA and the T3SS inducer EHPES (Fig. 4). However, compared to the DMSO treatment (control), the mRNA levels of $rsmB_{Ea}$ was altered in Ea273 culture supplemented with TMCA. Although the regulatory mechanism of RsmA-*rsmB* on T3SS of *E. amylovora* is unclear, from other related bacterial pathogens reported, our result indicated that TMCA may exert an effect on the T3SS through $rsmB_{Ea}$-$RsmA_{Ea}$ pathway (Fig. 1). Finally, in *D. dadantii*, new T3SS regulatory components were found such as PecS, PecT (Nasser et al., 2005), SlyA (Zou et al., 2012), Polynucleotide phosphorylase (PNPase) (Zeng et al., 2010), and c-di-GMP phosphodiesterase (Yi et al., 2010). In addition, HexA (a PecT homolog) and KdgR of *P. carotovorum* subsp. *carotovorum* regulate harpin production through *rsmB* (Cui et al., 2008). Although the roles of these regulators on T3SS of *E. amylovora* have not been studied, it is possible that the phenolic compounds may exhibit an effect on the T3SS through some of these regulators.

It is known that HrpN is a harpin protein secreted by *E. amylovora* via its T3SS into the intercellular spaces of the plant (Perino et al., 1999) and it triggers the HR, i.e. programmed cell death, when inoculated in a non-host plant like tobacco (Wei et al., 1992 ; Barny, 1995). Therefore, we performed the HR assay in the non host plant, tobacco, by co-infiltrating *E. amylovora* with the inhibitors TMCA and BA. We found that both these inhibitors reduced the HR response (Fig. 7A). Further, plant assays showed that development of the HR in the tobacco plants that transiently expressed HopQ1 was not affected in the presence of BA as well as TMCA (Fig. 7B). These results demonstrate that the reduced HR in tobacco is due to suppression of the T3SS of *E. amylovora* by BA and TMCA, but not by suppression of the defense response of tobacco plants by these compounds. In consistency with the tobacco HR assay, Western blot analysis showed that T3SS inhibitors TMCA and BA suppressed the production and the secretion of the HrpN protein (Fig. 6B and C).

In summary, this study identified small molecule phenolic compounds that affect the T3SS of *E. amylovora*. Their effect on regulatory components of T3SS was elucidated. Based on the results, the structure-activity relationships of these compounds on T3SS expression were examined. T3SS inhibition effect exhibited by TMCA or BA and several other compounds indicate that phenolic compounds may provide an alternative strategy in bacterial disease control. In our future work, we are in the process of formulating T3SS inhibitors using the lead compounds identified in this study. Their effect on disease management will be further evaluated.

Table 1: Strains, plasmids and primers used in this study

Designation	Relevant characteristics or sequences (5'to 3')[a]	Reference or source
Strains		
Erwinia amylovora 273	Wild type strain of *Erwinia amylovora* 273 isolated from apple orchards of New York	Bogdanove et al., 1998
Agrobacterium tumefaciens GV3101	Wild type, Rif^r Gm^r	Badel et al., 2006
Plasmids		
pPROBE-AT	Long life GFP Promoter-probe vector, Ap^r	Miller et al., 2000
pPROBE-*gfp*[AAV]	Short life GFP Promoter-probe vector, Km^r	Miller et al., 2000
pCPP5247	pLN314 expressing HopQ1, Cm^r	Badel et al., 2006
p*hrpA*	pPROBE-gfp[AAV] derivative with PCR fragment containing *hrpA* promoter, Km^r	This study
p*hrpL*	pPROBE-AT derivative with PCR fragment containing *hrpL* promoter, Km^r	This study
p*hrpS*	pPROBE-AT derivative with PCR fragment containing *hrpS* promoter, Ap^r	This study
Primers		
Promoter cloning		
hrpA forward	ATATGGATCCCGATAAAGAGCAGCGTAG	This study
hrpA reverse	ATTAGAATTCTTAGACGCCTGAGCATTG	This study
hrpL forward	ATATGGATCCAATATGTTGCTGCGCTCGG	This study
hrpL reverse	ATTAGAA TTC AAATGATGCACGCGTCG	This study
hrpS forward	ATATGGATCCTTGTGGAGTGTAACCGC	This study
hrpS reverse	ATTAGAATTCAACCGCGACCAATTTTCC	This study
Northern blot probes		
hrpA forward	GCGGCATTATTACAGGTATGGC	This study
hrpA reverse	GAACTGAATAGCTTTAGCCGCG	This study
hrpL forward	GTCAACGATGGGCTACCGC	This study
hrpL reverse	CTGTTTCAGCGTGACGCG	This study
hrpN forward	AGTCTGAATACAAGTGGGCTGGG	This study
hrpN reverse	CGCCCAGCTTGCCAA	This study
$rsmB_{Ea}$ forward	TGCTCCCTGCTCATCCTTGA	This study
$rsmB_{Ea}$ reverse	CAGGAAGAGGTCAGGAACATCTCCAGG	This study

rsmA$_{Ea}$ forward	TCGTCGAGTTGGTGAAACCC	This study
rsmA$_{Ea}$ reverse	GTAACTCGTTTGCTGCGTCT	This study
qPCR		
rplU forward	ACAACACCGAGTAAGCGAAGGTCA	This study
rplU reverse	GCTTTAATCACGCCGCCTGAAACT	This study
hrpS forward	ATATGCGTGTCATTGTCGCAACGC	This study
hrpS reverse	CGCCCGTAAAGGTTGCAGTTGAAT	This study
rpoN forward	TGGTGAAAAAGTTAGTCTCGG	This study
rpoN reverse	CTGTTTACGCTGATTCGATG	This study
hrpX forward	GAGCCGCTCAAATTGCTCGAGTTT	This study
hrpX reverse	ATCATTGCGATAGCCCAACATGCG	This study
hrpY forward	GACCCGGAGACGTTGCTGGC	This study
hrpY reverse	AACCCCTCTGCCACGCGCTA	This study

[a]Underlined bases indicate restriction sites in the primers. Ap^r, Cm^r, Gm^r, Km^r and Rif^r indicate ampicllin, chloramphenicol, gentamycin, kanamycin and rifampin resistance respectively.

Table 2: Promoter activity of *E. amylovora* 273 (Ea273) *hrpA* measured by the GFP promoter-reporter fusion plasmid (phrpA) in HIM and HIM supplemented with phenolic compounds and their analogs.

Phenolic compound (compound no.)[a]	**Avg MFI ±SD[b] at:**	
	6 h	**9 h**
Ea273 (pPROBE-gfp[AAV])	1.4±0.2	1.2±0.1
Ea273 (phrpA) :		
DMSO	1229.4±39.2	1024.3±52.9
TS24, *trans*-2-carboxycinnamic acid	361.1±76.1 *	332.2±19.6 *
TS30, methyl *trans*-cinnamate	674.8±252.1	668.5±31.2 *
TS32, cinnamyl alcohol	681.6±217.9	784.0±53.3 *
TS27, *trans*-4-aminocinnamic acid	255.6±7.4 *	132.1±8.5 *
TS26, *trans*-4-mercaptocinnamic acid	159.7±22.0 *	44.7±2.7 *
TS29, *trans*-4-formlycinnamic acid	285.1±40.8 *	218.3±9.9 *
TS28, *trans*-4-nitrocinnamic acid	14.4±16.9 *	4.0±0.3 *
TS101, methyl para-coumate	1443.4±49.4*	1227.8±41.6*
TS102, *trans*-4-hydroxycinnamaide	1134.0±36.7	766.4±94.8
TS103, *trans*-4-hydroxycinnamohydroxamic acid	206.9±3.9*	168.2±24.9 *
TS104, para-coumaryl alcohol	814.7±55.2 *	556.9±40.3 *
TS105, *trans*-2-(4-methoxyphenyl)-1-cyclopropanecarboxylic acid	345.3±11.9 *	200.2±11.0 *
TS106, ethyl *trans*-2-(4-hydroxyphenyl)-1-cyclopropanecarboxylate	1289.3±46.5	1110.9±67.5
TS107, *trans*-2-(4-hydroxyphenyl)-1-cyclopropanecarboxylic acid	1464.3±76.5*	1348.0±82.6 *
TS108, *trans*-4-phenylcinnamic acid	173.7±7.9*	79.4±1.8 *
TS109, *trans*-4-chlorocinnamide	172.9±20.5 *	90.6±12.0 *
TS110, *trans*-4-fluorocinnamic acid	279.2±5.3 *	152.4±0.7 *
TS111, *trans*-4-bromocinnamic acid	102.8±23.5 *	61.8±4.2 *
TS112, *trans*-4-dimethylaminocinnamic acid	87.0±4.0 *	37.3±3.7 *
DMSO	1476.7±63.8	1649.1±96.9
TS6, ortho - coumaric acid	443±16.9 *	437.8±21.1 *
TS1, *trans* -cinnamic acid	471.2±12.6 *	432.6±17.8 *
TS4, para- coumaric acid	920.6±29.1 *	993.8±43.3 *
TS5, meta-coumaric acid	1212±47.6	1439.1±97.9
TS33, salicylic acid	49.8±13.9 *	34.3±4.2 *
TS34, benzoic acid	48.1±5.4 *	32.6±1.9 *
DMSO	1242.1±27.0	1672.5±126.0
TS122, ethyl *trans*-2-(4-hydroxyphenyl)-ethenylsulfonate	2042.8±20.7 *	2900.6±120.1*
TS123, *trans*-2-(4-hydroxyphenyl)ethenylsulfonic acid tetra(n-butyl)ammonium salt	1073.4±107.7	1377.3±81.1
TS124, *trans*-4-hydroxymethylcinnamic acid	419.8±12.2 *	444.3±11.4 *
TS125, *trans*-4-methoxycinnamohydroxamic acid	215.5±6.5 *	230.0±4.4 *
TS126, *trans*-4-methoxycinnamyl alcohol	386.9±19.4 *	401.4±10.8 *
TS127, *trans*-3-indoleacrylohydroxamic acid	91.4±4.9 *	102.7±0.5 *
TS128, *trans*-4-bromocinnamohydroxamic acid	17.6±2.8 *	5.7±2.2 *

TS129, *trans*-2-hydroxycinnamohydroxamic acid	187.9±1.2 *	181.0±0.7 *
TS130, *trans*-3-hydroxycinnamohydroxamic acid	431.4±4.1 *	491.0±10.2 *
TS131, *trans*-3,4-dihydroxycinnamohydroxamic acid	1057.5±41.2 *	1515.1±39.1
TS132, *trans*-cinnamohydroxamic acid	145.7±1.2 *	147.8±4.2 *
TS133, *trans*-3-(4-hydroxyphenyl)acrylohydrazide	1716.5±33.1 *	2780.9±58.5 *
TS136, phenylpropiolic acid	261.7±1.0 *	307.0±9.3 *
TS134, benzhydroxamic acid	679.0±12.7 *	921.6±10.3 *
TS135, salicylhydroxamic acid	124.7±7.7 *	147.8±4.3 *
DMSO	1068.6±170.9	1157.8±58.7
TS8, hydrocinnamic acid	395.9±16.0 *	342.0±11.7 *
TS9, phenoxyacetic acid	342.7±24.8 *	269.9±15.2 *
TS10, *trans*-2-phenylcyclopropane-1-carboxylic acid	336.7±28.1 *	293.0±4.7 *
TS13, *trans*-3-(3-pyridyl)acrylic acid	468.3±7.7 *	245.7±3.1*
TS35, 3-(2-naphthyl) acrylic acid	178.9±9.7 *	89.0±6.2 *
TS12, *trans*-3-indoleacrylic acid	33.7±8.9 *	47.9±6.9 *
TS11, *trans*-3-(2-thienyl)acrylic acid	230.1±5.8 *	167.3±8.2 *
TS15, *trans*-2-methoxycinnamic acid	342.6±7.0 *	255.1±1.7 *
TS18, *trans*-2-methylcinnamic acid	303.8±8.7 *	262.5±9.3 *
TS21, *trans*-2-chlorocinnamic acid	225.7±35.8 *	192.7±8.6 *
TS14, *trans*-3-(4-imidazolyl)acrylic acid	874.6±23.1 *	839.2±17.6 *
TS2, 2,4-dihydroxycinnamic acid	1365.3±104.9	1479.8±72.9*
TS7, 3-(4-hydroxyphenyl)propionic acid	1093.1±62.8	1211.0±58.7
TS23, *trans*-4-chlorocinnamic acid	171.8±7.7 *	125.8±3.9 *
TS17, *trans*-4-methoxycinnamic acid	132.5±5.5 *	76.4±2.7 *
TS20, *trans*-4-methylcinnamic acid	208.5±2.7 *	145.4±3.7 *
TS3, 3,4-dihydroxycinnamic acid	783.4±80.2 *	787.3±25.2 *
TS22, *trans*-3-chlorocinnamic acid	332.9±16.9 *	238.3±8.7 *
TS16, *trans*-3-methoxycinnamic acid	265.3±24.4 *	198.5±20.0 *
TS19, *trans*-3-methylcinnamic acid	311.5±18.6 *	251.6±13.3 *
DMSO	1120.4±44.4	1499.8±30.9
TS25, *trans*-cinnamamide	1034.7±12.5	1378.1±14.6
TS31, *trans*-4-carboxycinnamic acid	257.7±14.3 *	187.1±8.4 *
TS100, ethyl *trans*-2-(4-methoxyphenyl)-1-cyclopropanecarboxylate	1090.5±20.6	1406.4±9.0
TS113, *trans*-4-trifluoromethylcinnamic acid	172.1±4.7 *	199.2±11.1 *
TS114, diethyl *trans*-2-(4-hydroxyphenyl)-vinylphosphonate	1371.3±16.1 *	2023.3±28.9*
TS115, *trans*-2-(4-hydroxyphenyl)-vinylphosphonic acid	1176.6±56.6	1636.9±53.3
TS117, para-coumarylamine	897.6±48.9 *	1372.6±48.6
TS118, N-(4-methoxycinnamyl)phthalimide	919.8±34.4	1240.0±29.7
TS119, *trans*-4-methoxycinnamylamine	285.9±44.2 *	570.0±68.4 *
TS120, ethyl *trans*-2-(4-methoxyphenyl)-ethenylsulfonate	982.1±12.8	1338.3±75.4
TS121, *trans*-2-(4-methoxyphenyl)ethenylsulfonic acid tetra(n-butyl)ammonium salt	977.1±20.4	1336.3±79.2
DMSO	1100.1±30.8	1351.9±62.4
TS160, *trans*-2-methylcinnamohydroxamic acid	122.7±1.9 *	95.7±3.7*
TS161, *trans*-3-(2-thienyl)acrylhydroxamic acid	132.4±13.2 *	145.0±11.7*

TS165, *trans*-2-[(4'-benzylcarbonyl]phenylcyclopropane-1-carboxylic acid	292.8±17.1*	229.4±15.0*
DMSO	1132.3±93.6	1295.0±86.5
TS137, N-methyl-4-hydroxycinnamamide	1064.1±99.3	1121.1±52.1
TS138, N-(2-hydroxyethyl)-4-hydroxycinnamamide	1474.5±30.4*	1907.0±39.8*
TS139, 3-phenylpropionohydroxamic acid	306.3±11.9*	286.3±10.0*
TS140, *trans*-4-phenylcinnamohydroxamic acid	101.7±17.1*	82.8±4.3*
TS141, *trans*-4-fluorocinnamohydroxamic acid	167.6±4.7*	119.0±7.1*
TS142, *trans*-4-methylcinnamohydroxamic acid	126.2±5.6*	106.0±13.6*
TS143, 2-phenoxyacetohydroxamic acid	228.7±4.6*	178.5±27.1*
TS144, 4-hydroxybenzoic acid	434.4±22.1*	424.7±24.8*
TS145, 3-hydroxybenzoic acid	559.4±23.6*	529.9±32.0*
TS146, *trans*-4-formylcinnamohydroxamic acid	414.3±31.6*	435.2±36.2*
TS147, *trans*-4-hydroxymethylcinnamohydroxamic acid	688.6±39.2*	746.8±96.3*

[a] HIM (*hrp* inducing minimal medium) was supplemented with 100 μM of the indicated compounds. The compounds were assayed two different times, with HIM supplemented with DMSO as the control treatment (indicated by DMSO) for each set of experiments.

[b] *E. amylovora* 273 cells carrying GFP reporter phrpA were used in this study. The promoter activities at 6 and 9 hours (hrs) of bacterial growth were determined. GFP mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values are representative of two experiments, and three replicates were used for each experiment. Asterisks indicate statistically significant differences in MFI between bacterial cells grown in HIM supplemented with DMSO and HIM supplemented with the different compounds ($P < 0.05$, Student's *t* test).

Table 3: Growth of *E. amylovora* in M9 supplemented with 100 µM of the inhibitors or inducers.

Inhibitors	**Absorbance (OD_{590})[a]**				
Compounds	**3hrs**	**6hrs**	**9hrs**	**12hrs**	**24hrs**
DMSO	0.24±0.001	0.40±0.002	0.42±0.004	0.50±0.010	0.75±0.005
TS1	0.24±0.001	0.40±0.005	0.41±0.001	0.49±0.003	0.74±0.010
TS6	0.23±0.002	0.39±0.002	0.41±0.004	0.49±0.006	0.70±0.007
TS8	0.24±0.003	0.42±0.002	0.44±0.004	0.51±0.007	0.77±0.006
TS9	0.25±0.006	0.41±0.003	0.43±0.004	0.48±0.009	0.72±0.008
TS10	0.26±0.012	0.45±0.001	0.45±0.001	0.55±0.006	0.74±0.007
TS11	0.25±0.001	0.43±0.000	0.43±0.006	0.51±0.005	0.75±0.006
TS12*	0.18±0.001	0.33±0.003	0.36±0.003	0.43±0.002	0.62±0.006
TS13	0.25±0.002	0.41±0.002	0.42±0.003	0.46±0.004	0.70±0.002
TS15	0.25±0.002	0.42±0.003	0.44±0.001	0.51±0.007	0.70±0.003
TS16	0.25±0.002	0.42±0.001	0.45±0.003	0.52±0.003	0.70±0.008
TS17	0.25±0.001	0.42±0.003	0.44±0.003	0.51±0.003	0.73±0.003
TS18	0.25±0.002	0.43±0.003	0.44±0.006	0.50±0.008	0.70±0.005
TS19	0.25±0.001	0.44±0.003	0.45±0.000	0.50±0.010	0.71±0.005
TS20	0.25±0.002	0.43±0.001	0.43±0.003	0.50±0.003	0.67±0.010
TS21	0.26±0.002	0.45±0.006	0.47±0.002	0.51±0.001	0.71±0.006
TS22	0.26±0.003	0.44±0.001	0.45±0.003	0.50±0.007	0.68±0.013
TS23	0.25±0.000	0.44±0.003	0.45±0.006	0.51±0.012	0.72±0.001
TS24	0.26±0.003	0.45±0.002	0.46±0.007	0.51±0.001	0.77±0.012
DMSO	0.26±0.000	0.36±0.003	0.44±0.003	0.51±0.004	0.70±0.009
TS25	0.27±0.002	0.36±0.003	0.44±0.003	0.50±0.000	0.72±0.007
TS26	0.27±0.002	0.37±0.001	0.45±0.002	0.54±0.003	0.81±0.005
TS27	0.27±0.002	0.36±0.003	0.44±0.001	0.54±0.004	0.74±0.006
TS28*	0.25±0.001	0.35±0.003	0.40±0.002	0.46±0.004	0.57±0.003
TS29	0.28±0.003	0.41±0.003	0.49±0.001	0.55±0.002	0.72±0.005
TS33	0.26±0.002	0.36±0.001	0.46±0.001	0.55±0.002	0.79±0.008
TS34	0.26±0.001	0.36±0.001	0.46±0.003	0.53±0.006	0.79±0.009
TS35	0.28±0.004	0.37±0.003	0.46±0.004	0.53±0.008	0.76±0.010
TS103	0.25±0.004	0.37±0.002	0.49±0.002	0.56±0.005	0.66±0.008
TS105	0.28±0.000	0.38±0.003	0.48±0.004	0.56±0.009	0.74±0.008
TS108	0.28±0.001	0.37±0.002	0.48±0.004	0.52±0.004	0.71±0.008
TS109	0.30±0.009	0.41±0.010	0.49±0.013	0.60±0.016	0.76±0.013
TS110	0.26±0.002	0.36±0.002	0.43±0.002	0.53±0.003	0.72±0.004
TS112	0.27±0.002	0.37±0.003	0.46±0.003	0.52±0.009	0.72±0.014
TS113	0.27±0.000	0.38±0.002	0.46±0.006	0.53±0.002	0.75±0.005
TS119	0.27±0.002	0.39±0.005	0.48±0.006	0.55±0.011	0.78±0.012
TS124	0.27±0.002	0.38±0.002	0.46±0.000	0.55±0.003	0.71±0.010
TS125	0.25±0.002	0.36±0.001	0.46±0.001	0.54±0.004	0.74±0.003
DMSO	0.20±0.002	0.28±0.004	0.34±0.001	0.39±0.003	0.60±0.007
TS126	0.20±0.001	0.28±0.003	0.34±0.006	0.39±0.003	0.60±0.014
TS127	0.17±0.001	0.24±0.003	0.30±0.003	0.33±0.001	0.43±0.003

TS128*	0.15±0.000	0.16±0.002	0.17±0.001	0.17±0.001	0.19±0.002
TS129	0.17±0.001	0.26±0.001	0.37±0.024	0.39±0.004	0.47±0.006
TS130*	0.18±0.001	0.26±0.001	0.33±0.002	0.38±0.002	0.46±0.002
TS132	0.17±0.000	0.24±0.001	0.30±0.001	0.34±0.001	0.53±0.007
TS135	0.19±0.003	0.27±0.001	0.33±0.004	0.36±0.002	0.57±0.004
TS136	0.20±0.002	0.27±0.002	0.33±0.002	0.37±0.002	0.57±0.002
TS139	0.19±0.002	0.28±0.003	0.34±0.005	0.38±0.003	0.59±0.007
TS140	0.22±0.005	0.28±0.001	0.32±0.003	0.36±0.004	0.51±0.005
TS141*	0.18±0.004	0.23±0.001	0.28±0.000	0.32±0.002	0.49±0.006
TS142	0.19±0.002	0.27±0.004	0.33±0.003	0.38±0.004	0.61±0.008
TS143*	0.18±0.001	0.24±0.003	0.28±0.002	0.30±0.002	0.48±0.003
TS144	0.19±0.001	0.27±0.001	0.33±0.003	0.38±0.002	0.61±0.013
TS145	0.20±0.000	0.28±0.001	0.34±0.004	0.38±0.002	0.59±0.005
TS146	0.19±0.001	0.27±0.002	0.34±0.003	0.38±0.002	0.59±0.005
TS147	0.20±0.001	0.26±0.002	0.33±0.003	0.39±0.002	0.58±0.005
DMSO	0.23±0.004	0.38±0.004	0.48±0.006	0.55±0.007	0.86±0.006
TS160*	0.19±0.000	0.27±0.001	0.35±0.006	0.40±0.003	0.67±0.001
TS161	0.23±0.001	0.36±0.003	0.47±0.002	0.55±0.004	0.86±0.003
TS165	0.23±0.001	0.40±0.004	0.51±0.003	0.61±0.001	0.85±0.013
Inducers					
DMSO	0.17±0.001	0.26±0.001	0.30±0.003	0.34±0.000	0.52±0.004
TS002	0.18±0.000	0.26±0.001	0.31±0.004	0.35±0.004	0.54±0.004
TS101	0.17±0.001	0.26±0.004	0.31±0.000	0.34±0.001	0.54±0.013
TS106	0.18±0.002	0.25±0.002	0.31±0.002	0.35±0.002	0.55±0.001
TS107	0.18±0.002	0.26±0.003	0.31±0.001	0.35±0.001	0.55±0.006
TS114	0.18±0.002	0.27±0.005	0.32±0.003	0.35±0.003	0.55±0.006
TS122	0.18±0.001	0.26±0.001	0.31±0.001	0.35±0.003	0.56±0.002
TS133	0.18±0.001	0.26±0.002	0.30±0.000	0.36±0.005	0.54±0.001
TS138	0.20±0.016	0.26±0.001	0.30±0.001	0.34±0.002	0.51±0.003

[a] indicates the standard deviation for the absorbance values. Asterisk indicates the compounds that affected the growth rate of Ea273.

Table 4: Expression of T3SS genes *hrpA*, *hrpL and hrpS* of *E. amylovora* 273 (Ea273) in HIM and HIM supplemented with DMSO or 100µM TMCA, BA or EHPES.

	Average MFI ± SD for growth in the HIM[a] at:		
	3 h	6 h	9 h
	Ea273(pNT-phrpA)		
Vector	2.9±1.0	2.7±0.9	2.1±0.3
DMSO	263.7±8.3	1242.1±27	1672.5±126
TMCA	56.2±4.1*	154±5.5 *	110.4±3.5 *
BA	10.1±0.2 *	40.5±5.4 *	33.1±2 *
EHPES	406.2±7.8*	2042.8±21*	2900±120*
	Ea273(pAT-phrpL)		
Vector	2.9±0.1	4.9±0.1	4.5±0.8
DMSO	47.2±8.2	325.0±33.2	491.6±19.5
TMCA	7.4±0.2*	48.9±3.4*	78.9±12.8*
BA	9.7±1.1*	11.2±0.4*	14.0±0.4*
EHPES	181.1±16*	743.3±8 *	1101.5±30*
	Ea273 (pAT-phrpS)		
Vector	3.8±0.2	3.9±0.2	5.1±0.1
DMSO	57.2±1.5	93.1±6.6	163.3±8.1
TMCA	35.1±0.2*	19.22±0.2*	25.9±0.3*
BA	15.2±0.9*	14.2±1.6*	15.1±0.2*
EHPES	108.3±4*	223.0±27*	342.2±11.6*

[a] *hrpA* promoter was cloned in pPROBE-*gfp*[AAV] (pNT) whereas promoter of *hrpL* as well as *hrpS* were cloned in pPROBE-AT (pAT). The promoter activities were compared at 3, 6 and 9 h of bacterial growth in TMCA, BA and EHPES. GFP mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values are representative of two experiments, and three replicates were used for each experiment. Empty vector controls (pNT and pAT) were also included for all the promoter activities tested. Asterisks indicate statistically significant differences in GFP intensity between bacterial cells grown in HIM (*hrp* inducing minimal medium) supplemented with DMSO and HIM supplemented with 100µM TMCA, BA and EHPES ($P<0.01$, Student's *t* test).

Figures

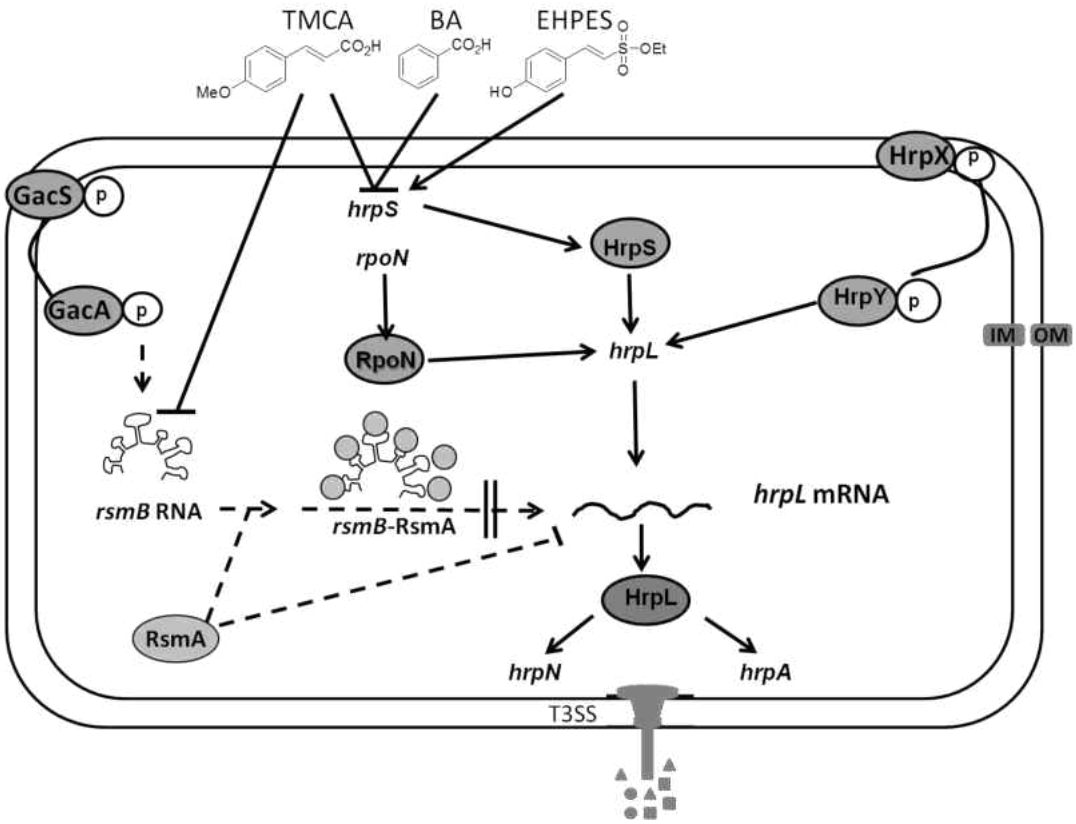

Figure1: **Schematic of the effect of compounds on T3SS regulation in *E. amylovora***. Solid lines indicate direct regulation (protein-protein interaction or direct binding to the promoter region) and dashed lines indicate indirect regulation or hypothetical regulatory links based on evidence shown in *E. amylovora* or other plant pathogenic bacteria. IM and OM stand for inner and outer membrane respectively. Based on genetic analysis in *E. amylovora* and biochemical evidences shown in *P. stewartii* (Merighi et al., 2003), HrpX/Y forms a two component system that activates the transcription of *hrpL* (Wei et al., 2000). HrpL activates the expression of the T3SS genes such as *hrpA* and *hrpN*(Kim et al., 1997; Xiao and Hutcheson, 1994). HrpS is a σ^{54} enhancer binding protein that activates the transcription of *hrpL*, independent of HrpXY (Wei et al., 2000; Wei and Beer, 1995). Based on the two component system study in *E. amylovora* and the evidences in *P. syringae* (Zhao et al., 2009; Chatterjee et al., 2003), it is suggested that in *E. amylovora,* GacAS two component system may activate transcription of *hrpS*. Also, based on previous work shown in *P. carotovorum* (Chatterjee et al., 2002) and the evidences of the functional homolog of *rsmB* found in *E. amylovora* E9 strain (Ma et al., 2001), *rsmB*-RsmA system may regulate the T3SS by affecting the stability of *hrpL* mRNA in *E. amylovora*. From this study we observed that TMCA, BA and EHPES alter the *hrpA* promoter activity majorly through HrpS-HrpL pathway. In addition, TMCA alters the T3SS expression through $rsmB_{Ea}$-$RsmA_{Ea}$ pathway.

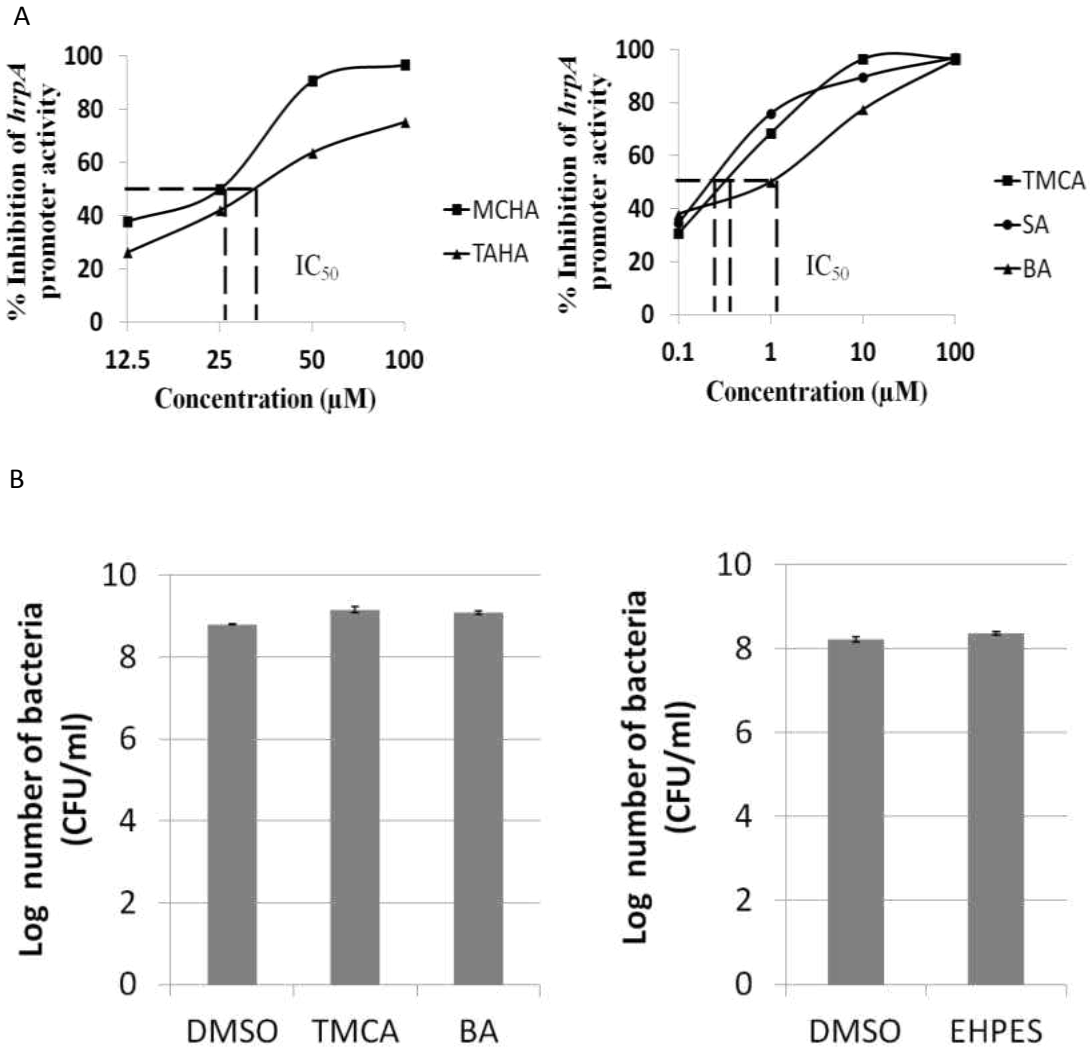

Figure 2: **A**. Effectiveness of selected compounds that inhibited *E. amylovora hrpA* promoter activity. Promoter-probe reporter fusion plasmid (phrpA) was examined by measuring GFP in the presence of the selected inhibitory compounds at respective concentrations. Dashed lines show the IC_{50} of these compounds that is required for the inhibition of 50% of the promoter activity of *hrpA* compared to the DMSO control. **B**. Comparison of number of *E. amylovora* cells carrying promoter-reporter fusion plasmid at 9 hours of growth in HIM supplemented with DMSO or 100µM TMCA, BA or EHPES by serial dilution plating method.

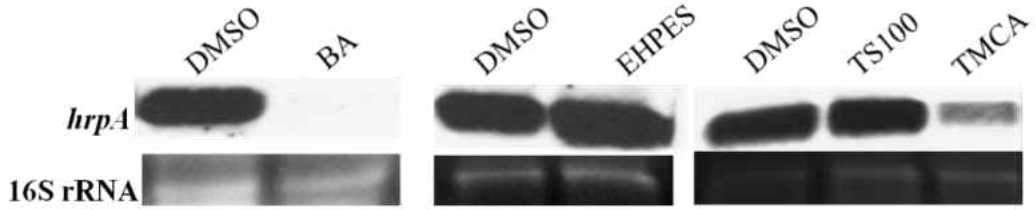

Figure 3: **Northern blot analysis of *hrpA* transcripts of *E. amylovora* 273**. RNA was isolated from cells harvested after 6 h of bacterial growth in HIM supplemented with DMSO and 100 µM BA, TMCA, and EHPES. 16S rRNA was used as an RNA loading control. The experiment was repeated three times with similar results.

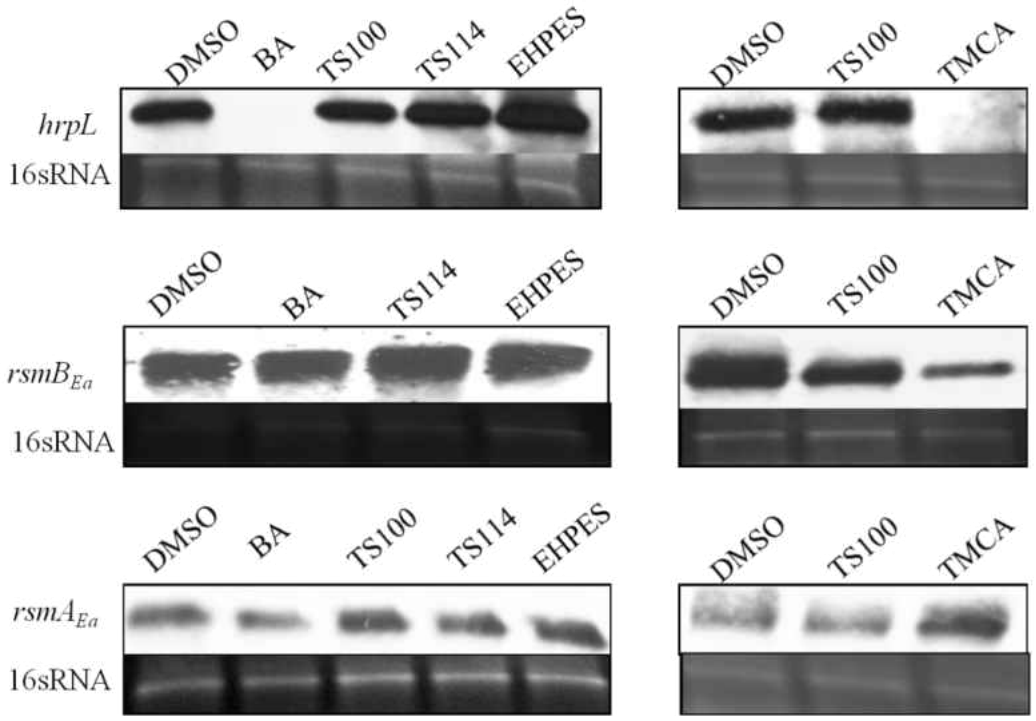

Figure 4: **Northern blot analysis of *hrpL*, *rsmBEa*, and *rsmAEa* transcripts of *E. amylovora* 273**. RNA was isolated from cells harvested after 6 h of bacterial growth in HIM supplemented with DMSO control and 100 µM TMCA, BA, and EHPES. 16S rRNA was used as an RNA loading control. The experiment was repeated three times with similar results. The image was spliced to conserve space.

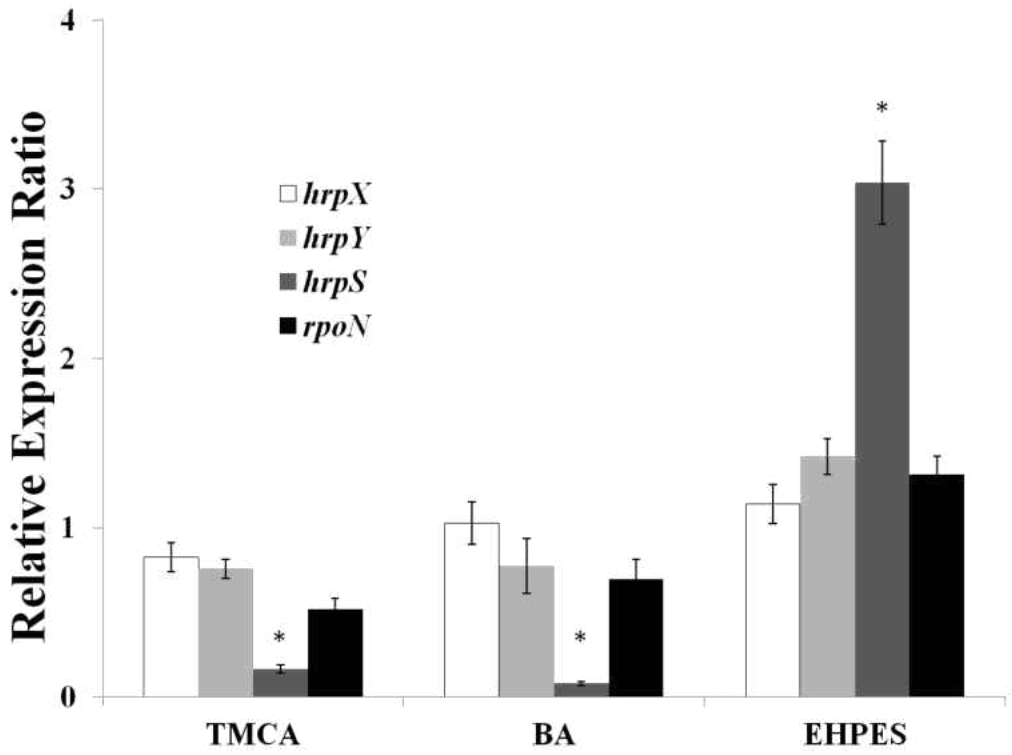

Figure 5: **Relative mRNA levels of *hrpX*, *hrpY*, *hrpS* and *rpoN E. amylovora* 273 in HIM supplemented with 100µM TMCA, BA and EHPES compared to mRNA levels in HIM with DMSO as determined by qPCR**. RNA was collected at 6 hrs of bacterial growth. There is no significant difference between HIM supplemented with DMSO and HIM supplemented with TMCA, BA or EHPES for gene *hrpX*, *hrpY* and *rpoN*. Levels of gene expression of *hrpS* are significantly different between HIM supplemented with DMSO and HIM supplemented with 100µM TMCA, BA and EHPES ($P < 0.001$) as indicated by asterisks. Five replicates were used in this experiment. The *P* value was calculated by the Relative Expression Software Tool (Pfaffl et al., 2000). The experiment was repeated three times with similar results.

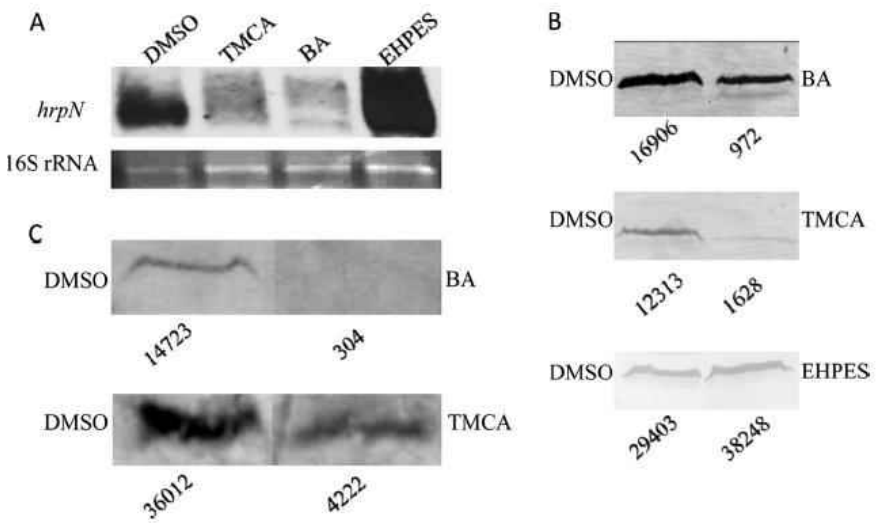

Figure 6: **Alteration of the T3SS effector *hrpN* by T3SS inhibitors and inducers in *E. amylovora***. **A**. Northern blot analysis of *hrpN*. RNA was isolated from cells harvested after 6 h of bacterial growth in HIM supplemented with DMSO control and 100 µM TMCA, BA, and EHPES. 16S rRNA was used as RNA loading control. The experiment was repeated twice with similar results. (**B** and **C**) *E. amylovora* cells were grown in HIM supplemented with DMSO(all blots) or 100µM BA, TMCA, or EHPES. Western blot analyses of total cell fractions and supernatant fractions were performed using an anti-HrpN polyclonal antibody. Numbers below the blots are relative intensities of secreted HrpN provided by ImageJ. The experiment was repeated three times with similar results.

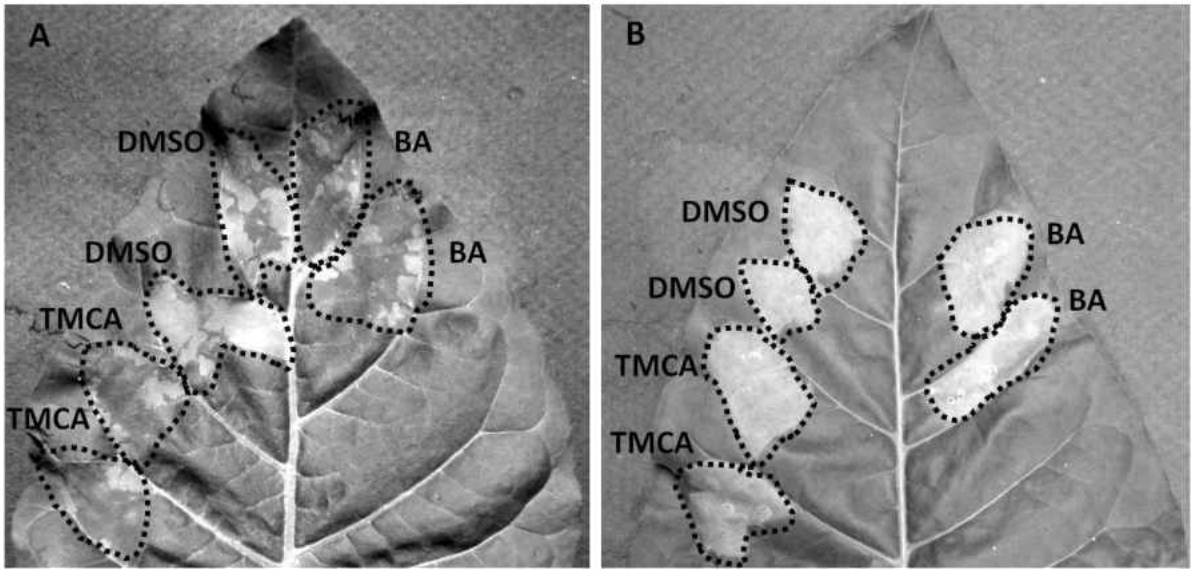

Figure 7: **TMCA and BA inhibit HR by targeting the T3SS**. **A**. Effect on HR development when BA and TMCA were coinfiltrated with *E. amylovora* 273 cells in *Nicotiana tabacum* cv. Xanthi leaves. **B**. Effect on HR development in tobacco leaves transiently expressing *hopQ1*. TMCA and BA (100 µM) were injected at different time points after the tobacco leaves had been infiltrated with *Agrobacterium tumefaciens* GV3101 carrying the effector gene *hopQ1* of *P. syringae* DC3000. In this particular picture, the compounds were injected at 6 h after the infiltration of *Agrobacterium*. The infiltrated areas are outlined. Both the experiments were repeated three times with similar results.

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CHAPTER 3

YcgR, a c-di-GMP receptor involved in the regulation of motility, biofilm formation and the Type III secretion system of *Dickeya dadantii* 3937

Abstract

Cyclic diguanylate (cyclic di-GMP or c-di-GMP) is a secondary messenger molecule involved in signal transduction in a wide variety of bacteria. *Dickeya dadantii* 3937 is a plant bacterial pathogen causing soft rot diseases on many economically important crops. Two EAL proteins of *D. dadantii*, EcpB and EcpC, regulate c-di-GMP-associated phenotypes, including motility and virulence, via the type three secretion system (T3SS) by acting as phosphdiesterases (PDE), which break down c-di-GMP. Additionally, YcgR, a PilZ-domain protein, was reported to function as a c-di-GMP binding protein and to negatively regulates motility. Our data showed that disruption of the *ycgR* gene in the Δ*ecpC* background of *D. dadantii* partially recovers motility toward wild type levels. Additionally, inactivation of the *ycgR* gene in the Δ*ecpB* background caused an increase in T3SS *hrpA* gene expression. Site-directed mutagenesis of the conserved residue in the PilZ domain suggested that Arg 124 in YcgR was essential for c-di-GMP binding. To our knowledge, this is the first report showing the c-di-GMP binding protein YcgR regulating the T3SS of a plant pathogen.

Introduction

Bacteria have an immense capability of adapting to different environments. This is mainly due to the presence of sensor proteins at the cell surface that act as the primary messengers. These messengers recognize various types of signals, which are transmitted to the target molecules in the cell, with the help of intracellular intermediates known as secondary messengers. For example, depending on environmental cues, bacteria can modify their cell surface such that it can either facilitate dispersion to a new environment or adhesion to a surface, including aggregation with members of their own or other species. Hence, secondary messenger systems can integrate many such sensory inputs and offer flexibility of recognition and response. Examples of secondary messengers include cyclic adenosine monophosphate (cAMP), cyclic di (3'-5')-adenylic acid (c-di-AMP), cyclic guanosine monophosphate (cGMP), guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and cyclic di (3'-5')-guanylic acid (c-di-GMP). Depending on the signals received, these second messengers control a wide range of biological processes in bacteria such as alternative carbon utilization, cell size control, cyst formation and stringent response to nutrient limitation (Gomelsky, 2011).

c-di-GMP is a common secondary messenger found in bacteria. The cellular levels of the c-di-GMP are controlled through the opposing activities of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). The GGDEF domain of DGCs catalyzes the synthesis of c-di-GMP from GTP, whereas EAL or HD-GYP domains in different classes of PDE catalyze c-di-GMP degradation to pGpG and GMP. Some proteins contain both GGDEF and EAL or occasionally, HD-GYP domains. Although a few bifunctional enzymes have been described, for the majority of GGDEF–EAL domain proteins either one or the other of the domains is active. Many

bacterial genomes encode multiple DGCs and PDEs. These proteins often contain other signaling domains, suggesting that their enzymatic activities may be responsive to different environmental cues (Hengge, 2002; Romling, 2011; Schirmer and Jenal, 2009).

Mutational analysis has shown that proteins with GGDEF, EAL or HD-GYP domains, through their c-di-GMP level modulating activities, regulate a wide range of functions in bacteria, including adhesion, biofilm formation, motility, synthesis of polysaccharides and synthesis of virulence factors in pathogens (Hengge, 2009; Romling, 2011; Schirmer and Jenal, 2009). Although there has been tremendous progress in elucidating the enzymology of c-di-GMP turnover and in uncovering c-di-GMP-dependent processes, much less is known about the molecular mechanisms of c-di-GMP action. A considerable research effort in recent years has addressed the mechanisms by which the c-di-GMP exerts such diverse influences in the cell. Bioinformatics studies came up with the first mechanistic insight by defining a protein domain that is called PilZ as a putative c-di-GMP receptor (Amikam and Galperin, 2006). This prediction was subsequently confirmed experimentally (Ryjenkov et al., 2006). It is now evident that cyclic di-GMP has a range of cellular effectors or receptors that include proteins with a PilZ domain, and, non-PilZ domain proteins such as transcription factors, enzymatically inactive GGDEF, EAL or HD-GYP domain proteins, and RNA riboswitches (Ryan et al., 2012; Breaker, 2011). In this manner, cyclic di-GMP can exert a regulatory influence at the transcriptional, post-transcriptional, and post-translational level.

c-di-GMP was discovered as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus*. Based on the bioinformatics analysis, the C-terminal domain of BcsA subunits of bacterial cellulose synthase complex was hypothesized to

bind c-di-GMP. This hypothesis was confirmed by demonstrating that c-di-GMP binds to *Escherichia coli* PilZ domain protein YcgR, and the *G. xylinus* PilZ domain protein, BcsA. It has been shown that YcgR controls the swimming speed in *E. coli* in response to the level of c-di-GMP by slowing flagellar rotation. It is speculated that this 'brake' effect is exerted by binding to motor proteins of the flagella, although different binding targets have been proposed (Boehm et al., 2010; Fang and Gomelsky, 2010; Paul et al., 2010).

In the plant pathogen *Dickeya dadantii* 3937, several GGDEF and EAL domains were identified. Two PDEs, EcpB and EcpC, were shown to regulate several cellular behaviors such as motility, biofilm formation, pectate lyase production, and type III secretion system (T3SS) expression (Yi et al., 2010). In this study, we looked for potential c-di-GMP receptors involved in regulation of the T3SS. Using Pfam, we identified two PilZ domain proteins, YcgR and BcsA, in *D. dadantii* 3937. We began with genetic analysis by deleting YcgR in PDE mutantstrains, Δ*ecpB* and Δ*ecpC*. Our data showed that disruption of the *ycgR* gene in the Δ*ecpC* background resulted in partial recovery of motility. Interestingly, the inactivation of the *ycgR* gene in the Δ*ecpB* background showed an increase of T3SS *hrpA* gene expression. Site-directed mutagenesis of the conserved residue in the PilZ domain suggested that Arg 124 in YcgR is essential for c-di-GMP binding. To our knowledge, this is the first report showing the c-di-GMP binding protein YcgR regulating the T3SS.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *D. dadantii* 3937 wild type and its mutant derivatives were grown in Luria–Bertani (LB) medium or T3SS inducing minimal medium (MM) at 28°C (Yang *et al*., 2007). When

required, antibiotics were added at the following concentrations: kanamycin 50 µg/ml; ampicillin 100 µg/ml; and gentamicin 20 µg/ml. The *D. dadantii* 3937 genome sequence can be retrieved from ASAP (https://asap.ahabs.wisc.edu/asap/home.php).

Mutant construction and complementation

Mutations of genes encoding GGDEF and/or EAL domain proteins were generated by allelic exchange (Metcalf et al., 1996). The flanking regions were amplified by PCR with specific primers (Table 1). The chloramphenicol and kanamycin cassettes were amplified from pKD3 and pKD4 respectively (Datsenko and Wanner, 2000). Three-way cross-over PCR was performed using the flanking regions and either the kanamycin or chloramphenicol cassette as templates. The PCR product was then digested with XhoI and NotI (New England BioLabs, MA), and cloned into pWM91 digested with the same enzymes. The resulting plasmid was transformed into *E. coli* S17-1 λ-*pir*, and then mobilized into *D. dadantii* 3937 by conjugation. Recombinants resulting from double cross-over events were selected by *sacB* and sucrose positive selection. Mutations were confirmed by PCR and sequencing. To construct plasmids for complementation and overexpression, the coding region of *ycgR* was cloned into a low-copy-number plasmid, pCL1920, at XbaI and SacI sites. In the resulting plasmids, pCL1920:*ycgR* and pCL1920:*ycgR*^{R124D}, the expression of *ycgR* and *ycgR*^{R124D} was controlled by its native promoter.

Site directed mutagenesis of PilZ domain protein

Single nucleotide substitutions in the coding sequence for the RRxxxR motif of the PilZ domain were performed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Primer sets "antisenseR124D" and "sense" were used

to generate *ycgR*^{R124D} (Table 1). These mutations altered the RRxxxR motif to RRxxxD. Substitutions were confirmed by DNA sequencing.

Promoter–*gfp* fusion plasmid construction and promoter activity assay

The GFP reporter plasmid pPROBE-AT was used to construct transcriptional fusions with the *hrpA, hrpN* and *hrpL* promoters. The promoter regions were amplified by PCR using forward and reverse primers incorporating BamHI and EcoRI sites, respectively (Table 1). The DNA fragments were digested with BamHI and EcoRI, and cloned into similarly digested pPROBE-AT. The resulting plasmids, pAT–*hrpA*, pAT-*hrpN* and pAT–*hrpL*, were mobilized into *D. dadantii* by conjugation using *E. coli* S17-1 λ pir as the donor strain. The promoter activity was evaluated by measuring GFP intensity using flow cytometry (Becton Dickinson, San Jose, CA) as previously described (Peng et al., 2006).

Bacterial motility assay

Swimming motility was examined on MG (mannitol 10 g/L, glutamic acid 2 g/L, KH_2PO_4 0.5 g/L, NaCl 0.2 g/L, $MgSO_4$ 0.2 g/L, pH 7.2) plates containing 0.2% or 0.4% agar. The center of the plates was inoculated with 10 µl of overnight bacterial cultures. All plates were incubated at 28°C, and the diameter of the radial growth was measured (Antúnez-Lamas et al., 2009).

Quantification of biofilm formation

Quantification of biofilm formation was followed from the method described by O'Toole and Kolter (1998). Briefly, overnight bacterial cultures were inoculated 1:100 in MM in 200 µl wells of 96 well flat bottom plate (Corning). The plate was incubated at 28°C for 48 h. Cells were stained with crystal violet (CV) for 15 min. The planktonic cells were removed by rinsing with H_2O. The CV-stained bound cells

were solubilized in 90% ethanol, and the absorbance was measured at A590 to quantify biofilm formation.

Pel activity assay

Extracellular pel activity of cells grown in MM supplemented with 1% polygalacturonic acid was measured by spectrometry (Matsumoto et al., 2003). Bacterial cultures were centrifuged at 15,800 *g* for 1 min and 10 µl of the supernatant was added to 990 µl of Pel reaction buffer (Matsumoto et al., 2003). Pel activity was monitored at A230 over a period of 3 min. Pel activity was calculated as previously described (Matsumoto et al., 2003). The assay was performed at least three times in triplicate.

YcgR expression and purification

A DNA fragment encoding the YcgR protein was amplified from *D. dadantii* 3937 chromosomal DNA by PCR using primers that incorporated NdeI (forward) and EcoRI (reverse) sites (Table 1). The fragment was digested with NdeI and EcoRI, and cloned into the pET21b expression vector (Merck KGaA, Darmstadt, Germany) digested with the same enzymes. The resulting plasmid, pETycgR, encoded YcgR with a C-terminal His_6-tag. The construct was confirmed by sequencing, and transformed into the expression host *E. coli* BL21 (DE3). For purification of the proteins, the following protocol was used. Briefly, expression of fusion proteins was induced by addition of isopropyl-thio-galactopyranoside (IPTG) at a final concentration of 0.5 mM and the bacterial cultures were then incubated at 16°C 12 h. The cells were chilled to 4°C and collected by centrifugation. The cell pellets were resuspended in phosphate buffered saline, followed by sonication. The crude cell extracts were centrifuged at 12,000 rpm for 25 min to remove cell debris. The supernatant containing the soluble proteins was collected and mixed with

preequilibrated Ni^{2+} resin (GE Healthcare, Piscataway, NJ, U.S.A.) for 3 h at 4°C, then placed into a column and extensively washed with buffer containing 30 mM Tris-HCl (pH 8.0), 350 mM NaCl, 0.5 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, and 30 mM imidazole. The proteins were subsequently eluted with buffer containing 300 mM imidazole. The purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

c-di-GMP binding analysis by Isothermal Titration Calorimetry Assay

c-di-GMP binding ability of YcgR was characterized by performing ITC using calorimeter ITC200 (MicroCal, Northampton, MA), following the manufacturer's descriptions. In brief, 2 µl c-di-GMP solution (500 µM) was injected at 2 min intervals via a 60 µl syringe into the sample cell containing 50 µM of His_6 tagged YcgR proteins with constant stirring, and the heat change based on these additions were recorded. Similar treatment was given to YcgR with point mutation, $YcgR^{R124D}$. The titration experiment was repeated at least three times, and the data were calibrated with a buffer control and fitted with the single-site model to determine the binding constant (Kd) using the MicroCal ORIGIN version 7.0 software. This experiment was performed at Dr. He's lab in China.

c-di-GMP measurement by Mass Spectrometry

An overnight culture was grown from a plate or freezer stock and sub-cultured for overnight in new media (1:1000 dilution). 40 ml of cultures were grown to mid- to -late log phase. The optical density OD_{600} of the cultures was measured. The cultures were spun down at 4000 rpm for 30 min and the resulting supernatant was removed by aspiration, and the pellet was resuspended in 100 µL of fresh cold extraction buffer (MeOH/Acetonitrile/Water 40:40:20 + 0.1 N Formic Acid) by pipetting and sonication. The cell lysates were incubated at -20 °C for overnight. The following

day, the cell lysates were centrifuged at max speed for 5 minutes to pellet the cellular debris. The resulting supernatant was transferred to a fresh 1.5 mL eppendorff tube. Samples were stored at -80 °C and shipped to the lab of Chris Waters at Michigan State University for the c-di-GMP measurement by mass spectrometry (MS). Three technical replicates were used in each experiment. c-di-GMP was quantified using liquid chromatography coupled with tandem mass spectrometry as previously described (Massie et al., 2012). 150 µL of each sample was evaporated in a vacuum manifold to remove the extraction buffer, and the remaining pellet was resuspended in 100 µL water. 10 µL was then analyzed on a Quattro Premier XE mass spectrometer (Waters) coupled with an Acquity Ultra Performance LC system (Waters). To quantify c-di-GMP, samples were compared to an 8-point standard curve of purified c-di-GMP (Biolog) ranging from 1.95 nM to 250 nM.

Results

Identification of c-di-GMP effector proteins

In our previous study, we found two PDEs in *D. dadantii* 3937, called EAL containing proteins, EcpB, and, EcpC. Deletion of these PDE genes resulted in increased biofilm formation, and reduced swimming motility, pectate lyase production, and type III secretion system expression (Yi, 2009, Yi et al., 2010). Compared to single deletion mutants of *ecpB* and *ecpC*, *ecpBC* double mutant had stronger effect on all the above mentioned phenotypes. In order to find the effectors that mediate these phenotypes in a c-di-GMP dependent manner, we searched for PilZ domain containing proteins by using Pfam program. We found two PilZ domain-containing proteins, YcgR and BcsA. In *E.coli*, YcgR is reported to play a role of c-di-GMP receptor that regulates motility under high c-di-GMP (Ryjenkov et al., 2006; Paul et al., 2010; Boehm et al., 2010), whereas BcsA plays a weaker role as a c-di-GMP receptor (Ryjenkov et al., 2006). In order to determine their role as c-di-GMP effectors in *D. dadantii* 3937, we first investigated the role of YcgR. Using the marker exchange method, *ycgR* was deleted in the Δ*ecpB*Δ*ecpC* double deletion mutant background. We then tested the biofilm formation, swimming motility, and pectate lyase production in this mutant (Fig. 1). We observed that the *ycgR* deletion led to partial restoration of normal phenotypes. In order to determine the effect on the T3SS expression, *hrpA* and *hrpL* promoter activity was measured. We deleted *ycgR* in single deletion mutants of *ecpB* and *ecpC*. We observed that Δ*ecpB*Δ*ycgR* deletion mutant recovered both *hrpA* and *hrpL* expression, whereas Δ*ecpC*Δ*ycgR* was unable to recover the activity of these promoters to the wild type levels (Fig 2A and B). Interestingly, Δ*ecpC*Δ*ycgR* deletion mutant recovered the swimming motility whereas Δ*ecpB*Δ*ycgR* was unable to recover it (Fig. 2C). These observations indicated that

YcgR may act as one of the c-di-GMP receptors in *D.dadantii* 3937. Additionally, it negatively co-regulates swimming motility and the T3SS (*hrpA* and *hrpL* promoter activities), whereas it positively regulates biofilm formation.

Arginine is important for YcgR mediated phenotypes

PilZ domain proteins harbor an RRxxxR motif which is crucial for the binding of c-di-GMP (Ryjenkov et al., 2006). The third arginine residue is required for c-di-GMP binding whereas the second one determines the affinity of its binding. Based on the preliminary results, we sought to determine the c-di-GMP binding ability of YcgR. Purified YcgR was utilized to perform isothermal titration calorimetry. Results show that YcgR binds to c-di-GMP at a 1:1 stoichiometric ratio, with an estimated dissociation constant (Kd) of 413±64 nM (Fig. 3). In order to characterize the binding of this effector, the third arginine in the RRxxxR motif of the PilZ domain was mutated to aspartate, $YcgR^{R124D}$. The mutated version of YcgR ($YcgR^{R124D}$) was unable to bind c-di-GMP (Fig. 3). In order to demonstrate the importance of this arginine in c-di-GMP dependent phenotypes such as motility and T3SS expression, $YcgR^{R124D}$ was tested for the recovery of these phenotypes. A plasmid carrying $ycgR^{R124D}$ was transformed into single deletion mutants of *ecpB* and *ecpC*. Thereafter, *hrpA* promoter activity was measured in these strains. We observed that when $ycgR^{R124D}$ was overexpressed in *ecpB* and *ecpC* mutants, *hrpA* promoter activity was similar to the empty vector control. At the same time, wild type *ycgR* overexpression led to further reduction in the *hrpA* promoter activity compared to the empty vector controls in the same mutants (Fig. 4B). This indicated that increased YcgR was bound to increased c-di-GMP in the *ecpB* and *ecpC* mutants which led to further reduction in *hrpA* promoter activity. Next, a point mutation ($ycgR^{R124D}$) was constructed in the Δ*ecpB*Δ*ecpC* at the native locus of *ycgR* in the chromosome using

allelic exchange. Δ*ecpB*Δ*ecpC*Δ*ycgR*^{R124D} showed a partial recovery of the motility similar to Δ*ecpB*Δ*ecpC*Δ*ycgR* (Fig. 2C), which further confirmed the importance of the arginine residue of YcgR in relation to swimming motility. The *ycgR*^{R124D} allele was cloned into a plasmid pCL1920 and overexpressed in the triple deletion mutant, Δ*ecpB*Δ*ecpC*Δ*ycgR*^{R124D}:Km, which did not result in any further reduction of motility compared to Δ*ecpB*Δ*ecpC*Δ*ycgR*. However, when wild-type *ycgR* was overexpressed in Δ*ecpB*Δ*ecpC*Δ*ycgR*^{R124D}:Km, swimming motility was reduced back to the level of the Δ*ecpB*Δ*ecpC* mutant (Fig. 3A). Taken together, these results demonstrate the importance of the arginine residue of YcgR in binding to c-di-GMP and in c-di-GMP-dependent control of motility and T3SS expression.

Intracellular levels of c-di-GMP increase in PDE mutants

In order to confirm that the phenotypic changes observed in the deletion mutants are due to the changes in the c-di-GMP levels, intracellular levels of c-di-GMP were measured in Δ*ecpB*, Δ*ecpC* and Δ*ecpB*Δ*ecpC* mutants. According to the MS results, c-di-GMP concentration is significantly higher in Δ*ecpB*Δ*ecpC* double mutant compared to the wild type or single deletion mutant of *ecpB* and *ecpC* (Fig. 5). The results indicate that the two PDEs, EcpB and EcpC regulate the motility and T3SS expression by modulating the intracellular levels of c-di-GMP.

Investigation of other c-di-GMP effectors regulating the T3SS

Based on homology search, BcsA is another PilZ domain protein found in *D. dadantii* 3937. In order to determine its role as a possible c-di-GMP effector involved in T3SS regulation, *bcsA* was deleted in the Δ*ecpB*Δ*ecpC* background and *hrpA* promoter activity was measured. Interestingly, *hrpA* promoter activity was further reduced compared to Δ*ecpB*Δ *ecpC* strain (Fig. 6). However, deletion of *bcsA* alone did not result in any reduction of *hrpA* expression compared to the wild type. In order

to confirm the result, expression of another T3SS gene, *hrpN* was measured. HrpN is the harpin protein secreted through the T3SS pilus composed of pilin protein, HrpA. A similar trend was observed for *hrpN* promoter activity in Δ*ecpB*Δ*ecpC*Δ*bcsA* strain (Fig. 6). Overall, these results indicated that BcsA may act as a positive regulator of the T3SS expression in a c-di-GMP-dependent manner.

Discussion

c-di-GMP was identified as an allosteric activator of the cellulose synthase complex in the fruit rotting bacterium *Gluconacetobacter xylinus* (Ross et al., 1987; Mayer et al., 1991). Since then there has been a tremendous progress in elucidating the enzymology of c-di-GMP turnover and in uncovering c-di-GMP-dependent processes. However, much less was known about the molecular mechanisms of c-di-GMP action. Based on the bioinformatics analysis, a conserved protein domain was first identified in the C-terminus of BcsA of *P. aeruginosa* which is involved in type IV fimbrial biogenesis (Alm et al., 1996; Amikam and Galperin, 2006). Subsequently, this domain was named as PilZ domain. Thereafter, several studies showed that these PilZ domain proteins, such as YcgR and BcsA, serve as c-di-GMP receptors in different bacteria. Moreover, it was shown that YcgR regulates motility in a c-di-GMP-dependent manner (Ryjenkov et al., 2006; Girgis et al., 2007, Ko and Park, 2000). Site-directed mutagenesis performed on YcgR implicated the most conserved residues in the PilZ domain directly in c-di-GMP binding. Further, two studies showed that YcgR regulates flagellum-based motility in a c-di-GMP-dependent manner by targeting the flagellar proteins. According to one model, when the c-di-GMP levels increase, the interaction between YcgR–c-di-GMP and the flagellar switch complex FliGMN is strengthened, which stabilizes the counterclockwise (CCW) conformation of the complex (Fang and Gomelsky, 2010; Paul et al., 2010). Another model suggested that, YcgR–c-di-GMP complex interacts with the MotA subunit of the stator (Boehm et al., 2010) and interferes with the stator-rotor energy transfer, which slows down the rotating flagellum, i.e., it acts as a brake. Taken together, it was concluded that YcgR is a motility-specific c-di-GMP receptor.

As with other enterobacteria, YcgR of *D. dadantii* 3937 also suppressed flagellar based motility (Fig. 1C). Interestingly, it also suppressed T3SS expression (Fig. 2A and B) and promoted biofilm formation (Fig. 1A) under high intracellular levels of c-di-GMP. *ycgR* deletion in Δ*ecpB* restores T3SS expression (*hrpA* and *hrpL*) to wild-type levels whereas *ycgR* deletion in Δ*ecpC* strain fails to do so (Fig. 2A). On the other hand, *ycgR* deletion in Δ*ecpB* did not recover the motility but was able to recover it in Δ*ecpC* strain (Fig. 2B). These results indicate that YcgR regulates the T3SS and motility in a c-di-GMP-dependent manner. In addition, YcgR responds differently to c-di-GMP generated by *ecpB* and *ecpC* deletion. This indicates that there may be a spatial effect leading to differential regulation of the phenotypes. A site-directed mutagenesis experiment revealed that the third arginine residue in the "RRxxxR" motif in the PilZ domain is important for YcgR-c-di-GMP complex formation, which in turn regulates the T3SS and motility (Fig. 4A and B). At this stage, the mechanism by which YcgR regulates T3SS of *D. dadantii* 3937 is unknown. It is possible that YcgR interacts with other proteins, such as transcriptional regulators of the T3SS, or the basal structural proteins of T3SS pili and regulates the T3SS at post-translational level. It is noteworthy to mention that YcgR regulates both the motility and the T3SS of *D. dadantii* 3937 in a similar fashion, i.e under high intracellular c-di-GMP levels. Studies suggested that similar trends for T3SS and motility expression were observed in *Salmonella enterica* serovar Typhi within macrophages (Iyoda et al., 2001; Faucher et al., 2005). It is possible that YcgR may be responsible for such co-regulation in *D. dadantii* 3937 which may allow successful invasion of the host.

Although YcgR plays a key role in c-di-GMP-dependent motility regulation in enterobacteria, other components are involved in this phenotype. Consistent with our

study, *ycgR* deletion in *yhjH* (*ecpC* in *D.dadantii*) mutants of *E. coli* is not able to completely restore the swimming ability to wild-type levels (Fang and Gomelsky, 2010). However, motility was fully restored by deletion of *ycgR* and the cellulose synthase gene *bcsA* (Zorraquino et al., 2013), whose product binds c-di-GMP via the C-terminal PilZ domain (Ryjenkov et al., 2006). That study suggested that extracellular cellulose may prevent flagellar rotation. In another study, mutation of *bcsA* reduced the accumulation of the T3SS-secretion protein HrpN (Jahn et al., 2011). In this study, we also investigated the possibility of another PilZ domain protein BcsA, as c-di-GMP effector involved in the T3SS regulation. Deletion of *bcsA* did not have any effect on *hrpA* and *hrpN* promoter activity, whereas its deletion in Δ*ecpB*Δ*ecpC* mutant led to further reduction of both *hrpA* and *hrpN* promoter activities (Fig. 6). Therefore, based on previous research and the results of this study, BcsA appears to positively regulate the T3SS in a c-di-GMP-dependent manner. .

In conclusion, we found that two different PDEs regulate the same c-di-GMP receptor that regulates motility and T3SS expression. It is worth to note that both positive and negative c-di-GMP receptors that regulate the T3SS were identified in this study. In our future studies, the ratio of c-di-GMP bound to BcsA will be determined. Identification of non-PilZ domain proteins would further give insight into the c-di-GMP-dependent regulation of the T3SS.

Table 1: Strains, plasmids and primers used in this study

Designation	Relevant characteristics or sequences (5'to 3')[a]	Reference or source
E.coli		
EC 100	F¯ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ– *rps*L *nup*G.	
S17-1 pir+	λ *-pir* lysogen of S17-1	Victor de Lorenzo
BL21 (DE3)	Protein expression host	Novagen
***D. dadantii* 3937**	Wild type, *Santpaulia* (African violet) isolate	Hugouvieux-Cotte-Pattat, N
Δ*ecpB*	Δ*ecpB*, ABF-20123 deletion mutant	This study
Δ*ecpC*	Δ*ecpC*, ABF-20364 deletion mutant	This study
Δ*ecpB*Δ*ecpC*	Δ*ecpB*Δ*ecpC,* ABF-20123 and ABF-20364 double mutant	This study
Δ*ycgR*	Δ*ycgR,* ABF-14564 deletion mutant	This study
Δ*ecpB*Δ*ycgR*	*ycgR* deletion in single deletion mutant of *ecpB*	This study
Δ*ecpC*Δ*ycgR*	*ycgR* deletion in single deletion mutant of *ecpC*	This study
Δ*ecpB*Δ*ecpC*Δ*ycgR*	*ycgR* deletion in double deletion mutant of *ecpB* and *ecpC*	This study
Δ*bcsA:km*	Δ*bcsA:km,* Km^r, ABF-17612 deletion mutant	This study
Δ*ecpB*Δ*ecpC*Δ*bcsA:km*	bcsA deletion in double deletion mutant of *ecpB* and *ecpC*	This study
Δ*ecpB*Δ*ecpC*Δ*ycgR*^{R124D}:Km	Site-directed mutation of *ycgR* in the chromosome of double deletion mutant of Δ*ecpB*Δ*ecpC* strain	This study
Plasmids		
pKD4	Km^r, template plasmid carrying Km resistance cassette	Datsenko and Wanner (2000)
pWM91	Suicide vector, Ap^r	Metcalf et al. (1996)
pPROBE-AT	Long life GFP Promoter-probe vector, Ap^r	Miller et al. (2000)
pFLP2	Flippase FRT excision vector, Ap^r	Hoang et al., 1998
pET21b	Overexpression and purification vector, Ap^r	Novagen
pET21b:*ycgR*	Overexpression of ycgR in expression vector	This study
pET21b:*ycgR*^{R124D}	Overexpression of ycgR^{R124D} in expression vector	This study
pCL1920	Low copy vector for complementation or overexpression, Sp^r	Lerner (1990)

pCL1920:*ycgR*	YcgR Overexpression construct, Sp^r	This study
pCL1920:ycgR^{R124D}	ycgR^{R124D} Overexpression construct, Sp^r	This study
P*hrpA*	Long life GFP Promoter-probe vector with PCR fragment containing *hrpA* promoter, Ap^r	Yang et al. (2008)
P*hrpL*	Long life GFP Promoter-probe vector with PCR fragment containing *hrpL* promoter, Ap^r	Yang et al. (2008)
P*hrpN*	Long life GFP Promoter-probe vector with PCR fragment containing *hrpN*promoter, Ap^r	Yang et al. (2008)
Primers		
bcsA-A-Xho1-for	ATAATACTCGAGGACGGATAACCGCCGTGCAA	This study
bcsA-B-rev	GAAGCAGCTCCAGCCTACACATGCAGGGTTTC CGTTGCCC	This study
bcsA-C-for	CTAAGGAGGATATTCATATGCGTCGATATTCC GCTGGCCC	This study
bcsA-D-Not1-rev	AATATTATGCGGCCGCCACGGAGACGCTGCTG GACA	This study
ycgR-A-XhoI	AATACTCGAGACCCATAAAGGCGGCATTTT	This study
ycgR-B	GAAGCAGCTCCAGCCTACACCATTTTATTACG CCTGGCGT	This study
ycgR-C	CTAAGGAGGATATTCATATGGGTGATCACCGA GCTGGAAT	This study
ycgR-D-NotI	AATATTATGCGGCCGCTGGCTTTCTGGGCATA AGTA	This study
ycgR-for-XbaI	ATCCTCTAGATCCTGGGGTTTTGGGACACT	This study
ycgR-rev-HindIII	TACATAAGCTTTGTACGACTTGCACGGTCCC	This study
ycgR-antisense R124D	GCAGGCGAGTTGATATCGAAAAAGTTACGGCG CTGG	This study
ycgR-Sense	CCAGCGCCGTAACTTTTTCGATATCAACTCGCC TGC	This study
ycgR-for-NdeI	ATATACATATGATGACGGTGGGGATGGAT	This study
ycgR-rev-EcoRI	ATTAGAATTCATGCGCAGCCGTTTGCGCTTTT	This study

[a]Underlined bases indicate restriction sites in the primers. Ap^r, Gm^r, Sp^r and Km^r indicate ampicllin, Gentamicin, spectinomycin and kanamycin resistance respectively.

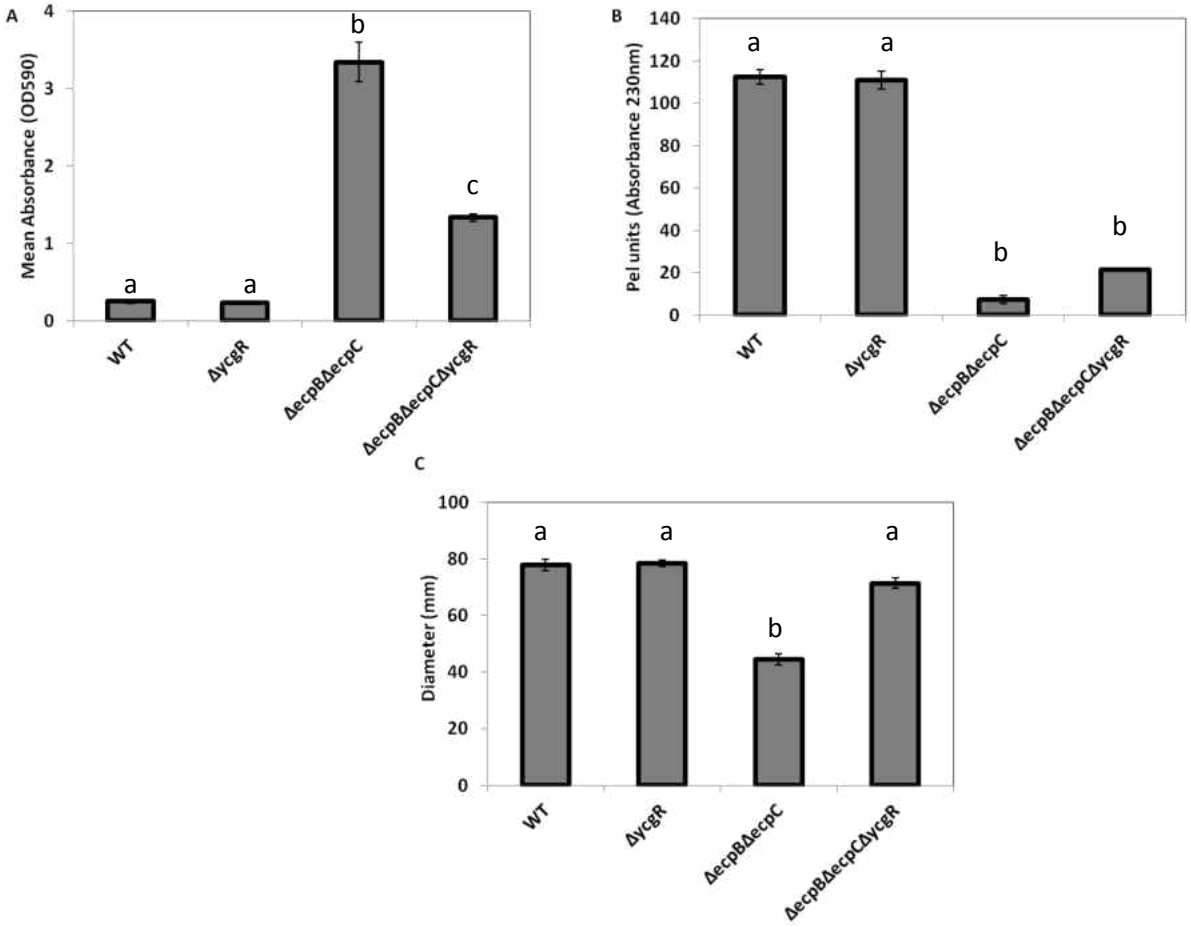
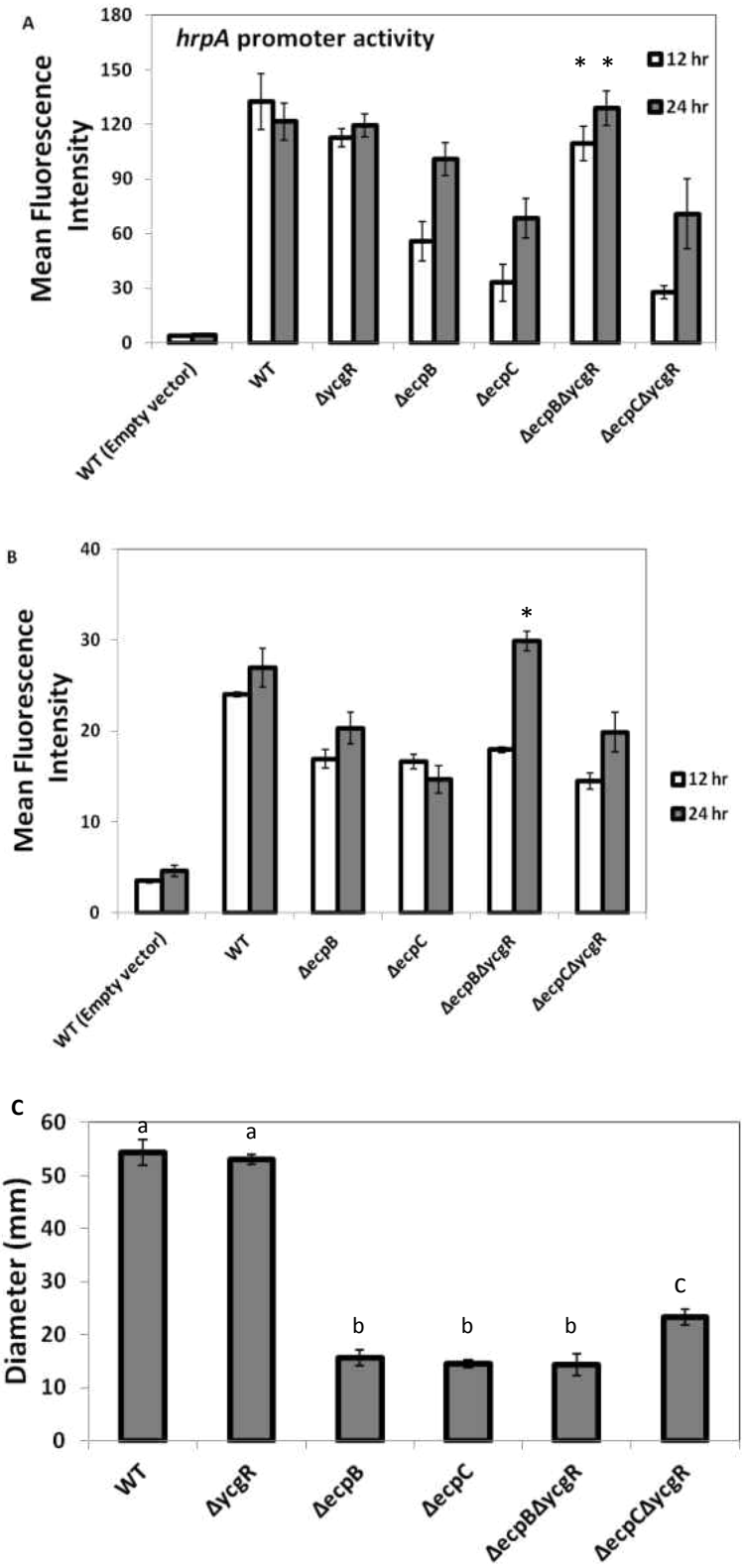


Figure 1: Phenotypic analysis of *D. dadantii* mutants of PilZ domain protein, YcgR. **A**. Biofilm formation of wild-type, Δ*ycgR*, Δ*ecpB*Δ*ecpC* and Δ*ecpB*Δ*ecpC*Δ*ycgR* mutant strains in MM. Lower case letters indicate statistically significant difference between different treatments $P < 0.05$ (Student's *t*-test). **B**. Pel activity in wild-type, Δ*ycgR*, Δ*ecpB*Δ*ecpC* and Δ*ecpB*Δ*ecpC*Δ*ycgR* mutant strains in MM. Pel activity was measured after 12 h induction in MM containing 1% polygalacturonic acid. **C**. Swimming motility was measured on MG plates containing 0.2% agar. Values on Y axis represent the diameter of the radial growth.


A
hrpA promoter activity
Mean Fluorescence Intensity
180
150
120
90
60
30
0
12 hr
24 hr
* *
WT (Empty vector)
WT
ΔycgR
ΔecpB
ΔecpC
ΔecpBΔycgR
ΔecpCΔycgR
B
Mean Fluorescence Intensity
40
30
20
10
0
*
12 hr
24 hr
WT (Empty vector)
WT
ΔecpB
ΔecpC
ΔecpBΔycgR
ΔecpCΔycgR
C
Diameter (mm)
60
50
40
30
20
10
0
a
a
b
b
b
c
WT
ΔycgR
ΔecpB
ΔecpC
ΔecpBΔycgR
ΔecpCΔycgR

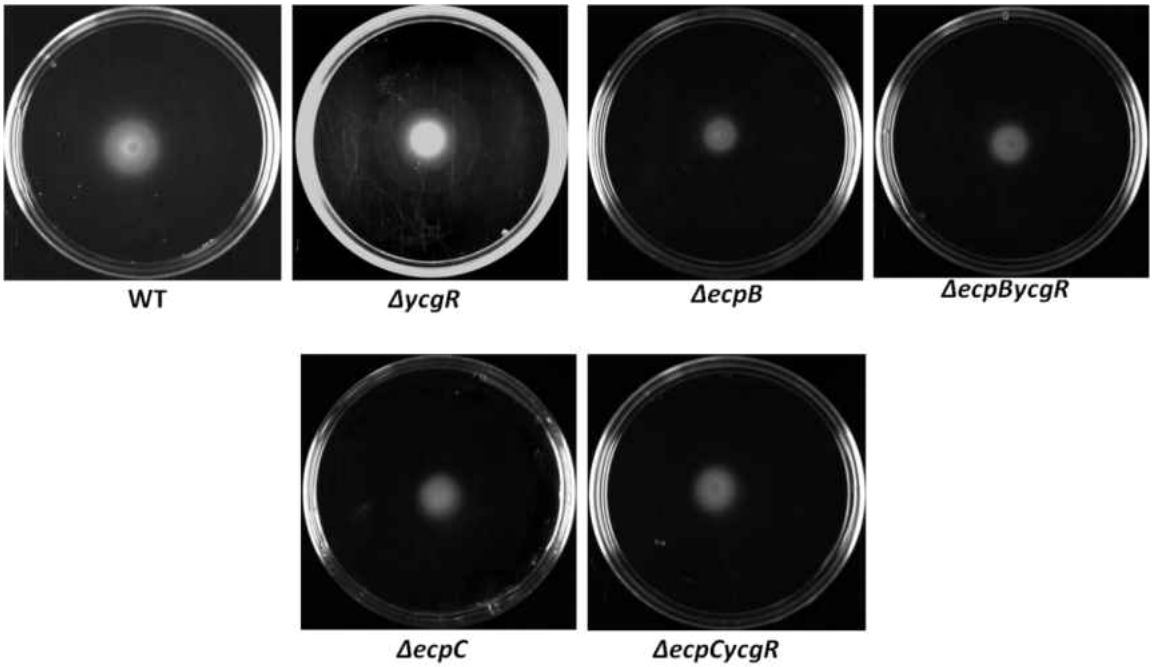


Figure 2: Phenotypic analysis of YcgR is the single mutants. **A**. and **B**. Promoter activity of *hrpA*, and *hrpL* in wild type, Δ*ycgR*, Δ*ecpB,* Δ*ecpC*, Δ*ecpB*Δ*ycgR* and Δ*ecpC*Δ*ycgR* mutant strains in MM. Values (Mean Fluorescence Intensity) of GFP were measured by flow cytometry. **C**. Swimming motility of the same strains was measured on MG plates containing 0.2% agar. Graphical presentation for motility was based on the pictures of the motility plates shown below it. Three technical replicates were used in this experiment. Each of these experiments was repeated three times and similar results were observed. Different letters and asterisks indicate statistically significant differences among treatments, $P < 0.05$ (Student's *t*-test).

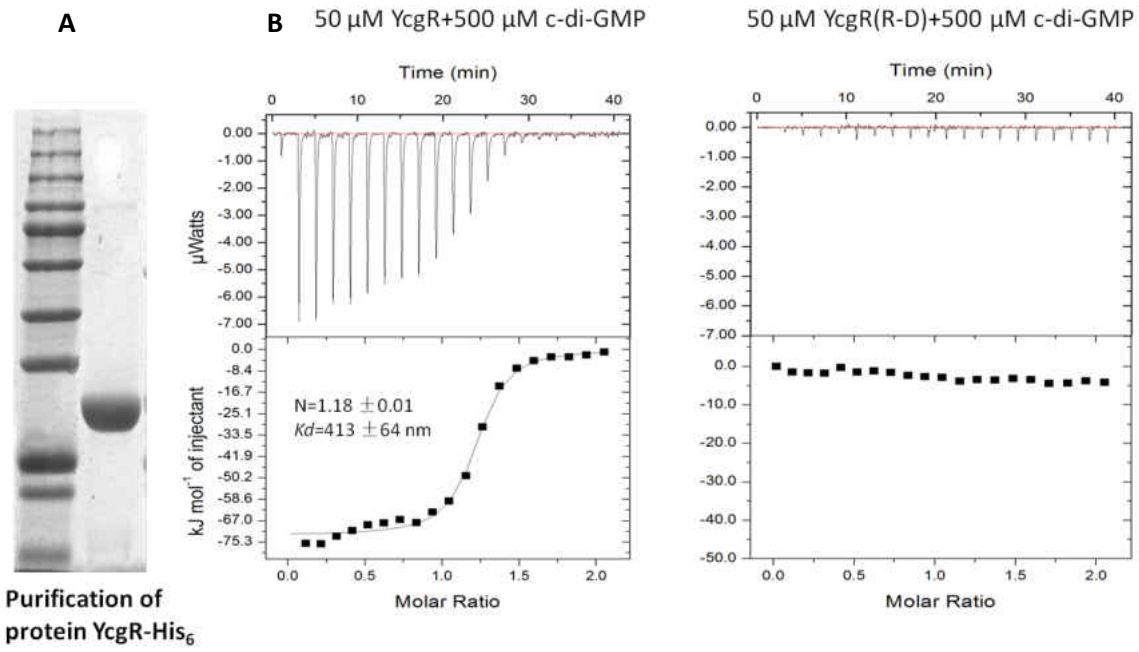
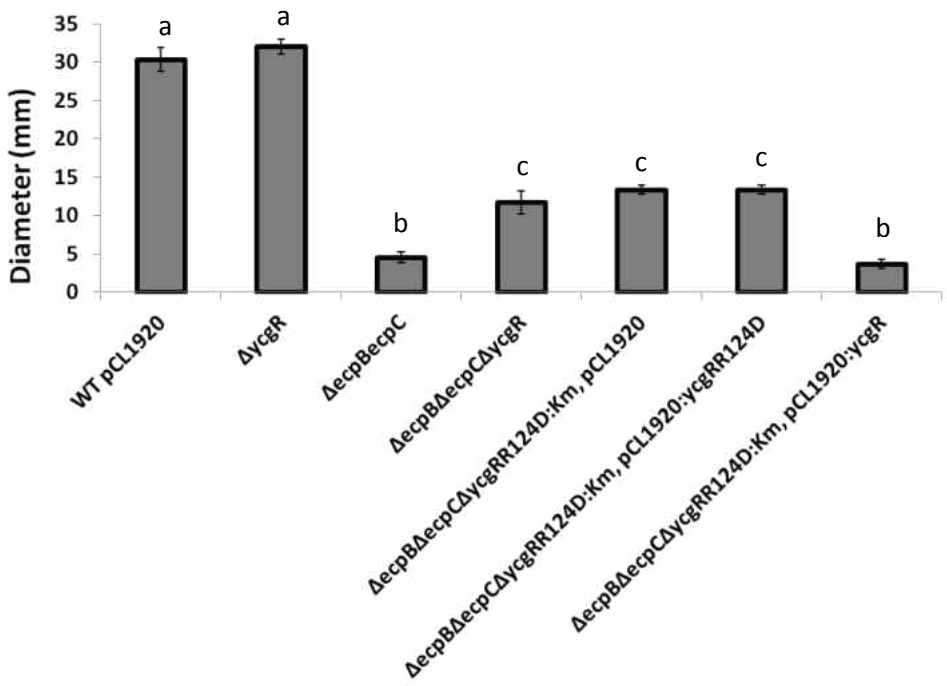


Figure 3: Binding analysis of YcgR A. SDS gel of YcgR-His_6 protein. **B**. Isothermal Titration Calorimetric assay of YcgR-His_6 protein with wild-type and point mutation versions of YcgR.

A


Diameter (mm)
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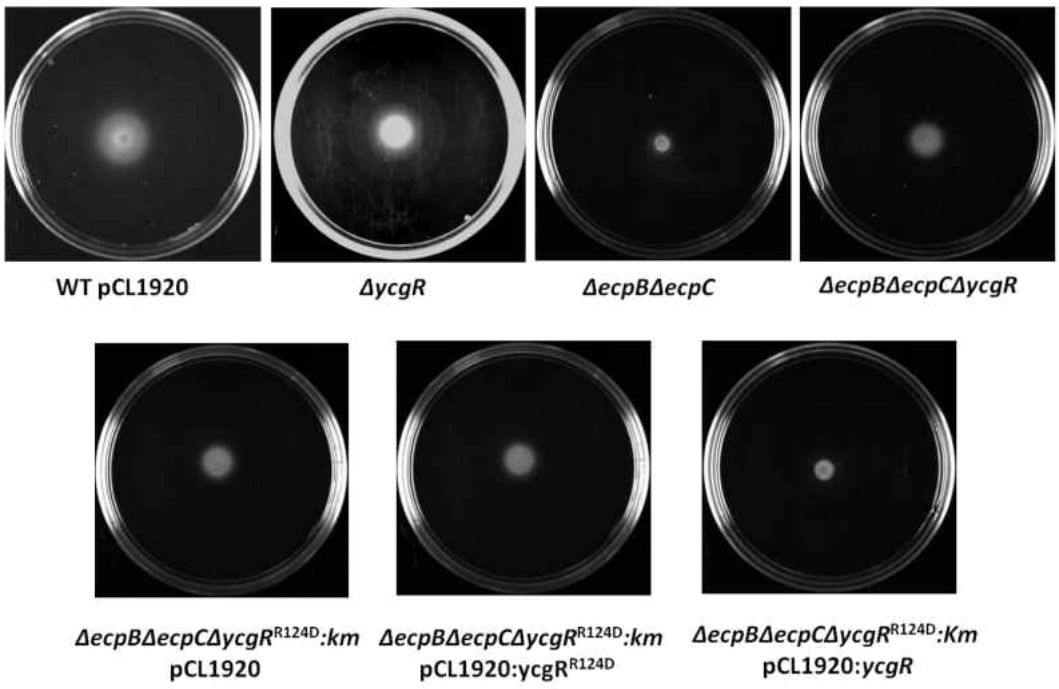
30
25
20
15
10
5
0
a
a
b
c
c
c
b
WT pCL1920
ΔycgR
ΔecpBecpC
ΔecpBΔecpCΔycgR
ΔecpBΔecpCΔycgRR124D:Km, pCL1920
ΔecpBΔecpCΔycgRR124D:Km, pCL1920:ycgRR124D
ΔecpBΔecpCΔycgRR124D:Km, pCL1920:ycgR


WT pCL1920
ΔycgR
ΔecpBΔecpC
ΔecpBΔecpCΔycgR
ΔecpBΔecpCΔycgR^{R124D}:km
pCL1920
ΔecpBΔecpCΔycgR^{R124D}:km
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ΔecpBΔecpCΔycgR^{R124D}:Km
pCL1920:ycgR

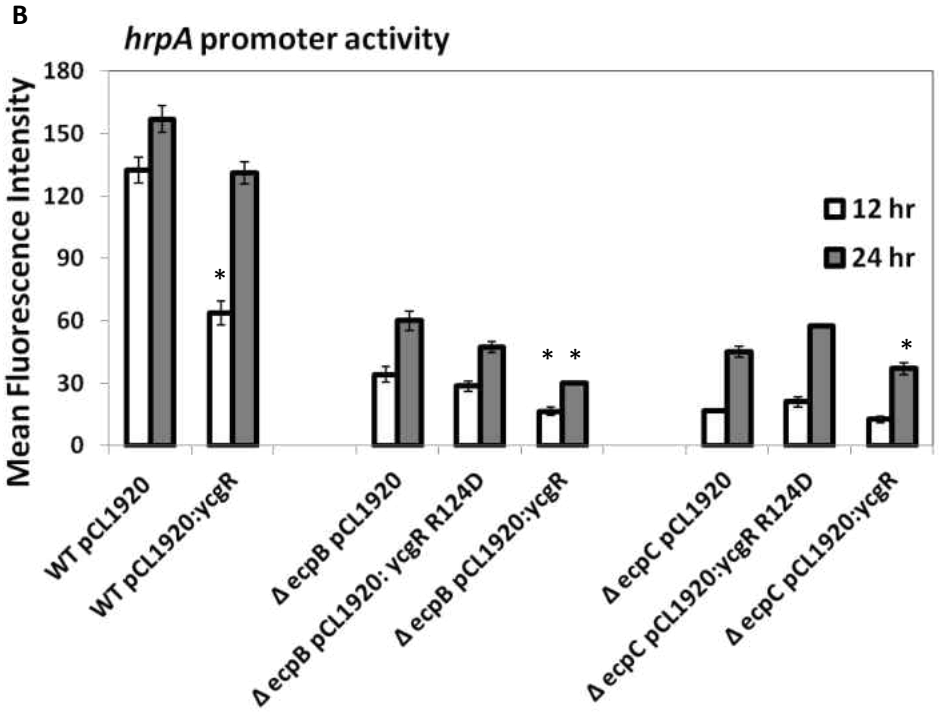

Figure 4: Phenotypic analysis of YcgR^{R124D}. **A**. Swimming motility assay of Δ*ecpB*Δ*ecpC*Δ*ycgR*^{R124D}:Km complemented with either pCL1920:*ycgR*^{R124D} or pCL1920:*ycgR*. Graphical presentation for motility was based on the pictures of the motility plates shown below it. **B**. Promoter activity of *hrpA* of Δ*ecpB* and Δ*ecpC* strains complemented with pCL1920:*ycgR*^{R124D} or pCL1920:*ycgR*. Three technical replicates were used in this experiment. Each of these experiments was repeated three times and similar results were obsevered. Different letters indicate statistically significant differences between treatments, $P < 0.05$ (Student's *t*-test).

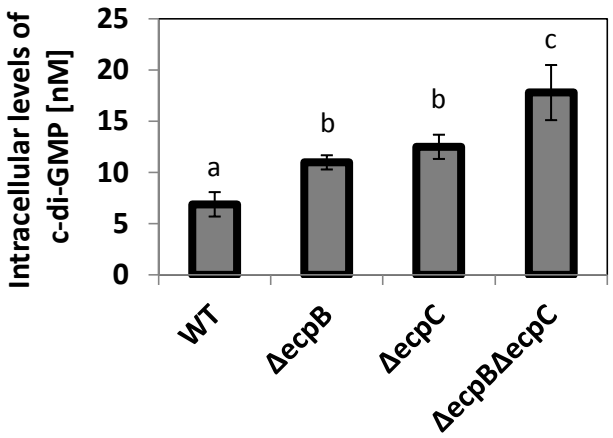

Figure 5: Measurement of intracellular levels of c-di-GMP in wildtype, Δ*ecpB*, Δ*ecpC*, Δ*ecpB*Δ*ecpC* strains. Three technical replicates were used in each experiment. The values are representatives of one experiment. Different letters indicate statistically significant differences between treatments, $P < 0.05$ (Student's *t*-test).

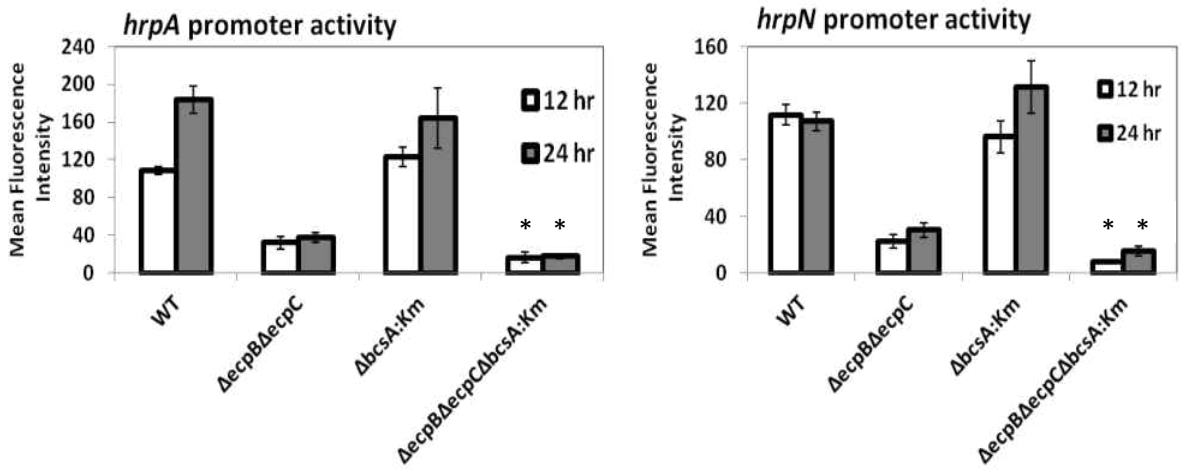

Figure 6: Promoter activity of *hrpA* and *hrpN* in wild type, Δ*ecpB*Δ*ecpC*, Δ*bcsA*:km and Δ*ecpB*Δ*ecpC*Δ*bcsA*:km strains. Three technical replicates were used in this experiment. Each of these experiments was repeated three times and similar results were observed. Asterisks indicate $P < 0.05$ (Student's *t*-test).

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CHAPTER 4

Role of Hfq and small RNA in the regulation of the Type III Secretion System in *Dickeya dadantii* 3937

Abstract

Hfq is a ubiquitous RNA-binding protein which is required for the fitness and virulence of several bacterial pathogens. *hfq* mutants are often found to have attenuated virulence. Hfq may impact the expression of several key genes in some organisms, including genes of type 3 secretion systems (T3SS). Hfq is known to work with a subset of small non-coding RNA molecules (sRNAs) in order to facilitate post-transcriptional regulation of the target mRNAs. sRNAs are integral components of the regulatory machinery for many bacterial species and are known to posttranscriptionally regulate key cellular processes. The *Dickeya dadantii* 3937 T3SS is a critical virulence component and the regulation of this system is tightly controlled at each step from transcription to translocation of effectors into host cells. However, the contribution of Hfq and sRNAs to the regulation of the T3SS in *Dickeya* has not been studied. The current study identified a role for the sRNA chaperone protein Hfq in the regulation of components of the T3SS in the necrotrophic soft-rot pathogen *D. dadantii* 3937. Here, we demonstrate the requirement for Hfq in T3SS regulation of *D. dadantii* 3937. In addition, the results suggest that Hfq may regulate the T3SS via GacA-RsmB-RsmA-HrpL regulatory pathway. Furthermore, a sRNA-ArcZ was identified to be required for the expression of the T3SS. Also, Hfq was found to modulate the c-di-GMP levels. Finally, the discovery of Hfq and sRNA that influences the synthesis of the T3SS adds an additional layer of regulation to this tightly controlled virulence determinant of *D. dadantii*.

Introduction

The small RNA (sRNA) chaperone Hfq is required as a mediator for the action of a subset of sRNA that act in *trans* by base-pairing with target mRNAs and inhibit their translation. Hfq is also known to affect the stability of several sRNAs. There has been a considerable increase in the evidence for such posttranscriptional control of gene expression in prokaryotes (Gottesman 2004; Valentin-Hansen et al., 2004). Structurally, Hfq is a hexameric ring-like protein that binds sRNA at its AU rich region, and the target mRNA at A-R-N triplet repeats. Therefore, Hfq provides a platform for the potential base-pairing of sRNA and mRNA that may range from 10 to 30 nucleotides (Waters and Storz, 2009; Maki et al., 2010).

Hfq is a highly conserved and abundant protein encoded in large number of bacterial species (Sun et al., 2002). Several reports indicate that Hfq is autoregulated (Vecerek et al., 2005) and its expression is also growth-phase dependent (Christiansen et al., 2004; McNealy et al., 2005). Earlier Hfq had been shown to be required for the synthesis of RpoS (Brown and Eliott, 1996; Bang et al., 2005), which is crucial for virulence in bacteria. Since then, Hfq mutants in several bacteria have been reported to have defects in multiple processes such as motility, EPS production, expression of protein secretion systems, quorum sensing, surface attachment and invasion of the host cell (Chao and Vogel, 2010). Mutants lacking Hfq are often sensitive to host defense mechanisms and highly attenuated in animal models. However, there is considerable variation in both the severity and extent of the phenotypes (Chao and Vogel, 2010). Therefore, Hfq has emerged as a global regulator that controls several key cellular processes in bacteria as well as a major determinant of virulence. For instance, *Salmonella typhimurium* Δ*hfq* mutant is weakened in its ability to invade the host or its ability to survive in the intracellular conditions (Sittka et al., 2007). Both

of these phenotypes are regulated by the type III secretion systems (T3SSs), which in turn require Hfq for their activation (Sittka et al., 2008). On the other hand, Hfq negatively regulates the T3SS and its effectors in enterohaemorrhagic *Escherichia coli* (EHEC) (Shakhnovich et al., 2009). Additionally, Hfq-mediated mRNA repression of regulatory proteins may be dependent on temperature or salt concentrations, as suggested for *virF* or *invE* in *Shigella sonnei* (Mitobe et al., 2008; Mitobe et al., 2009). In order to understand how Hfq controls the gene expression at the post-transcriptional level, several methods such as co-immunoprecipitation with Hfq, cDNA cloning, and deep sequencing analysis of tagged Hfq protein have been used to identify Hfq associated sRNAs and their mRNA ligands (Zhang et al., 2003; Sittka et al., 2008).

While most of the phenotypic studies of Δ*hfq* mutants have been reported in animal pathogens, there are only few such reports on plant pathogens (Schimdkte et al., 2012; Weiberg et al., 2013). In the current study, *Dickeya dadantii* 3937, a bacterial plant pathogen, was used as the model system to understand the effect of the Δ*hfq* mutation on the type III secretion system (T3SS). Interestingly, Hfq was found to affect the expression of RsmB, which modulates the activity of the RNA binding protein RsmA, which in turn regulates HrpL, the master regulator of the T3SS. In addition, a sRNA called ArcZ (also called RyhA) was found to be involved in the regulation of the T3SS. Finally, levels of a secondary messenger c-di-GMP were also found to be influenced by Hfq, which may be responsible for the inverse regulation between surface attachment and motility as observed in the *hfq* mutant of *D. dadantii* 3937.

Materials and Method

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *D. dadantii* 3937 wild type and its mutant derivatives were grown in Luria–Bertani (LB) medium or T3SS-inducing minimal medium (MM) at 28°C (Yang et al., 2007). When required, antibiotics were added at the following concentrations: kanamycin, 50 µg/ml; ampicillin, 100 µg/ml; and spectinomycin, 100 µg/ml. The *D. dadantii* 3937 genome sequence can be retrieved from ASAP (https://asap.ahabs.wisc.edu/asap/home.php).

Mutant construction and complementation

Mutations of genes encoding Hfq and ArcZ were generated by allelic exchange (Metcalf *et al*., 1996). The flanking regions were amplified by PCR with specific primers (Table 1). The kanamycin cassettes were amplified from pKD4 (Datsenko and Wanner, 2000). Three-way cross-over PCR was performed using the flanking regions and either the kanamycin or chloramphenicol cassette as templates. The PCR product was then digested with XhoI and NotI (New England BioLabs, MA), and cloned into pWM91 digested with the same enzymes. The resulting plasmid was transformed into *E. coli* S17-1 λ-*pir*, and then mobilized into *D. dadantii* 3937 by conjugation. Recombinants resulting from double cross-over events were selected by *sacB* and sucrose positive selection. Mutations were confirmed by PCR and sequencing. To construct plasmids for complementation and overexpression, the coding regions of *hfq* and the non coding region of *arcZ* along with their promoter regions were cloned into a low-copy-number plasmid, pCL1920.

Promoter–*gfp* fusion plasmid construction and promoter activity assay

The GFP reporter plasmid pPROBE-AT was used to construct transcriptional fusions with the *hrpA, hrpN* and *hrpL* promoters. The promoter regions were amplified by

PCR using forward and reverse primers incorporating restriction sites (Yang et al., 2008). The DNA fragments were digested with the same restriction sites as in the primers, and cloned into similarly digested pPROBE-AT. The resulting plasmids, pAT–*hrpA*, pAT-*hrpN,* pAT–*hrpL*, pAT-*gcpA*, pAT-*gcpD*, pAT-*ecpB* and pAT-*ecpC* were mobilized into *D.dadantii* by electroporation. The promoter activity was evaluated by measuring GFP fluorescence intensity using flow cytometry (Becton Dickinson, San Jose, CA) as previously described (Peng et al., 2006).

Bacterial motility assay

Swimming motility was examined on MG (mannitol 10 g/L, glutamic acid 2 g/L, KH_2PO_4 0.5 g/L, NaCl 0.2 g/L, $MgSO_4$ 0.2 g/L, pH 7.2) plates containing 0.2% or 0.4% agar. The center of the plates was inoculated with 10 µl of overnight bacterial cultures. All plates were incubated at 28°C, and the diameter of the radial growth was measured (Antúnez-Lamas et al., 2009).

c-di-GMP measurement by Mass Spectrometry

An overnight culture was grown from a plate or freezer stock and sub-cultured overnight in fresh media (1:1000 dilution). 40 ml of cultures were grown to mid- to -late log phase. The optical density OD_{600} of the cultures was measured. The cultures were spun down at 4000 rpm for 30 min and the resulting supernatant was removed by aspiration, and the pellet was resuspended in 100 µL of fresh cold extraction buffer (MeOH/acetonitrile/water 40:40:20 + 0.1 N formic acid) by pipetting and sonication. The cell lysates were incubated at -20 °C for approximately overnight. The following day, the cell lysates were centrifuged at max speed for 5 min to pellet the cellular debris. The resulting supernatant was transferred to a new 1.5 mL eppendorf tube. Samples were stored at -80 °C and shipped to the lab of Chris Waters at Michigan

State University for c-di-GMP measurement by mass spectrometry (MS). Three technical replicates were used in each experiment.

RNA extraction and real-time PCR analysis

D. dadantii cells were cultured in LB medium overnight at 28°C and sub-cultured in MM for 12 h. Total bacterial RNA was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA) and treated with Turbo DNA-*free* DNase kit (Ambion, Austin, TX). cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) from 0.6 μg of total RNA. The Real Master Mix SYBR ROX (5 PRIME, Gaithersburg, MD) was used for real-time PCR reactions to quantify the cDNA levels. Data were collected by the Opticon 2 system (Bio-Rad, Hercules, CA) and analyzed using delta-delta C_t method. A housekeeping gene *rplU* was used as an endogenous control for data analysis (Mah et al., 2003). The primer pairs used in this study are listed in Table 1.

RNA isolation and Northern blot analysis

D. dadantii cells grown in MM for 12 h were harvested and total RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, followed by DNase treatment with a Turbo DNA-free DNase kit (Ambion, Austin, TX). Each RNA sample was analyzed by Northern blot analysis using a NorthernMax kit (Ambion, Austin, TX) according to the manufacturer's instructions. Hybridization probes used to detect the mRNA of target genes were PCR amplified and labeled with biotin using a BrightStar Psoralen-Biotin kit (Ambion, Austin, TX). Signals were developed using the Bright-Star BioDetect kit (Ambion, Austin, TX). 5S rRNA was visualized under a UV transilluminator (Syngene, Frederick, MD) and used as an internal control for normalization of RNA.

Statistical analysis

Means and standard deviations of experimental results were calculated using Excel (Microsoft, Redmond, WA) and the statistical analysis was performed using a two-tailed *t*-test.

Results

Hfq positively regulates the expression of structural and effector genes of the T3SS

The *hfq* mutant of *D. dadantii* 3937 showed a significant decrease in the promoter activities of the T3SS structural and effector genes – *hrpA*, *hrpN* and *dspE* respectively (Yang et al., 2008) (Fig. 1A). HrpA makes up the T3SS pilus structure utilized for the translocation of the harpin protein HrpN, and the T3SS effector DspE. Interestingly, promoter activity of *hrpL*, the master regulator of the T3SS was unaltered (Fig. 1A). Since HrpL activates the expression of *hrpA*, *hrpN* and *dspE* genes (Yang et al., 2008), this indicated that the regulation of Hfq on *hrpL* may be at the post-transcriptional level. Therefore, we tested the levels of *hrpL* mRNA by qPCR. Indeed, the *hrpL* mRNA levels were reduced fivefold compared to their levels in the wild-type (Fig. 1B). Overall, our results suggested that Hfq positively regulates the T3SS of *D. dadantii* 3937 at the post-transcriptional level.

Hfq positively regulates the expression of RsmB

hrpL mRNA is degraded by an RNA-binding protein, RsmA (Yang et al., 2008; Cui et al., 1999), which is sequestered by an untranslated small RNA, RsmB. Since Hfq is a small RNA chaperone and it was found to regulate the *hrpL* at post-transcriptional level, the RsmB expression level was tested in the *hfq* mutant. Both the promoter activity (Fig. 2A) and the RNA level of RsmB were reduced in the *hfq* mutant (Fig. 2B). Additionally, overexpression of RsmB in the *hfq* mutant restored the promoter activities of *hrpA*, *hrpN* and *dspE* to the wild-type levels (Fig. 3A). This result indicated that, although Hfq regulates the expression of RsmB, the chaperone may not physically associate with this sRNA that modulates the protein activity. Furthermore, in order to explore the possibilities of the RsmB regulators as targets of

Hfq, mRNA levels of *gacA* were tested. RsmB is positively regulated by GacA, the response regulator of the two component system, GacA/S (Cui et al., 2001). qPCR results showed an almost tenfold reduction in *gacA* mRNA levels in the *hfq* mutant as compared to wild-type (Fig. 3B). Taken together, Hfq regulates expression of the T3SS via GacA-*rsmB*-RsmA pathway.

Hfq regulates sRNA, ArcZ – positive regulator of the T3SS

Several sRNAs were identified in the genome of *D. dadantii* 3937 using Rfam and blast search tools (data not shown). Among them, ArcZ is a well conserved small regulatory RNA that increases translation of RpoS (Argaman et al., 2001; Papenfort et al., 2009; Soper et al., 2010; Mandin et al., 2010). Moreover, Hfq is also required for the synthesis of RpoS, a stress sigma factor. Hence, we tested the levels of *arcZ* transcripts in *hfq* mutant. Reports have shown that the primary 120 nt *arcZ* transcript is processed to form a low-abundance 88 nt form and a stable 55 nt form derived from the 3' end of the primary transcript (Wassarman et al., 2001; Argaman et al., 2001; Soper et al., 2010; Mandin et al., 2010). The northern analysis revealed that *arcZ* transcripts were reduced dramatically in the *hfq* mutant. This result indicated that Hfq may be required for the stability of ArcZ (Fig. 4A). Further, *arcZ* mutant was examined for the T3SS expression levels. Similar to the *hfq* mutant, promoter activities of *hrpA*, *hrpN*, and, *dspE* were significantly reduced whereas that of *hrpL* was not altered (Fig. 4B). Together, these results suggested that Hfq regulates the expression levels of ArcZ which in turn positively regulates the expression of key T3SS genes.

Surface attachment and motility are inversely regulated by Hfq

Mutation of the *hfq* mutant can also affects motility and biofilm formation, which have a significant impact on the pathogenicity of the bacteria (Chao and Vogel,

2010). In *D. dadantii* 3937, *hfq* mutant exhibited decreased motility (Fig. 5B). On the other hand, *hfq* mutant cells were consistently found to adhere and form a ring on the walls of the flask when grown in MM media (Fig. 5A). These phenotypes have been reported to be regulated by c-di-GMP, a ubiquitous secondary messenger found in bacteria (Jenal and Malone, 2006). Therefore, intracellular levels of c-di-GMP were measured by mass spectrometry and were found to be significantly higher in the *hfq* mutant (Fig. 5C). In our previous study, increased c-di-GMP levels generated by elimination of two PDEs (EcpB and EcpC) down regulated the expression of the T3SS (Yi et al., 2010). In order to understand, if Hfq regulates the T3SS by modulating c-di-GMP levels in the cell, RsmB levels were measured in *hfq* mutant with the overexpression of one of the major phosphodiesterases (PDE), EcpC. According to the Northern analysis, there was no significant change exhibited in RsmB levels between *hfq* mutant and *hfq* mutant with the overexpression of EcpC (Fig. 5D). The result indicated that the effect of increased c-di-GMP in the *hfq* mutant on the T3SS expression is not through the Rsm system. Further, we tested the promoter activities of selected DGC genes (*gcpA* and *gcpD*) and PDE gens (*ecpB* and *ecpC*) as their mutants resulted in dramatic changes in the expression levels of the T3SS (unpublished data, Yi et al., 2010). Promoter activity of *gcpA* was increased in the *hfq* mutant. However, there was a decrease in the promoter activity of *gcpD*, *ecpB* and a subtle decrease in *ecpC* (Fig. 6).

Discussion

Several small non-coding RNA molecules (sRNAs) have been found to be integral components of the complex regulatory machinery for many bacterial species. They are known to post-transcriptionally regulate a variety of important bacterial functions such as metabolic and stress response, quorum sensing, production of virulence factors, and many more. This kind of post transcriptional regulation provides the bacteria with phenotypic plasticity under constantly changing environment. One such example is an sRNA RsmB that modulates the activity of the RNA binding protein, RsmA. The RsmA regulates several cellular processes by binding to the untranslated region of the target mRNAs which prevents translation and causes these transcripts to be degraded rapidly (Liu et al., 1995; Liu and Romeo, 1997; Baker et al., 2002). RsmB sequesters RsmA by means of the repeat sequences called GGA motifs in the stem-loop structure that have strong binding affinity to RsmA. RsmB neutralizes RsmA action by forming an inactive RsmB-RsmA ribonucleoprotein such that RsmA is no longer available for binding to its target mRNAs (Romeo, 2013). T3SS of *D. dadantii* 3937 is subject to post-transcriptional regulation by the Rsm system where RsmA binds to and promotes the degradation of the *hrpL* mRNA, the master regulator of the T3SS. Under appropriate environmental conditions RsmB sequesters RsmA and neutralizes its action (Fig. 7). As a result, the translation continues and HrpL protein activates the expression of the genes encoding the proteins that form the T3SS apparatus and the effector proteins that are translocated to the host cell through the T3SS pilus (Yap et al., 2005).

Another kind of sRNA is known to repress the translation of the target mRNAs by imperfect base-pairing to the leader sequence of the target mRNAs. A subset of these sRNAs is known to associate with a sRNA chaperone, Hfq (Vogel and

Luisi, 2011). The role of Hfq has been studied extensively in Gram negative bacterial pathogens, where *hfq* mutant results in dramatic phenotypes (Chao and Vogel, 2010) such as attenuated virulence. Furthermore, several reports have shown that Hfq is a negative regulator of the T3SS in Gram-negative bacteria (Shaknovich et al., 2009; Hansen and Kaper, 2009; Nakano et al., 2008). For example, Hfq is required by *Salmonella* and *Yersinia* for T3SS activity (Sittka et al., 2007; Ansong et al., 2009; Schiano et al., 2010; Koo et al., 2011; Lathem, 2012). In this study, we found that Hfq is also required for the expression of the T3SS in the plant pathogenic bacteria *D. dadantii* 3937. Furthermore, we found a sRNA, ArcZ, which is also required for the T3SS expression.

hfq mutants of *D. dadantii* 3937 exhibited a dramatic decrease in the expression of the T3SS structural and effector genes (Fig. 1A). Additionally, the *hfq* mutation was also found to affect the T3SS expression at the post-transcriptional level (Fig. 1B). Interestingly, expression of sRNA RsmB was found to be regulated by Hfq (Fig. 2). According to coimmunoprecipitation experiments (Zhang et al., 2003; Sittka et al., 2008), the RsmB sRNA does not interact with Hfq. In the current study, overexpression of RsmB in the *hfq* mutant recovered the expression levels to wildtype levels (Fig. 3A), which confirmed the previous findings that RsmB does not physically associate with Hfq in T3SS regulation of *D. dadantii* 3937. On the other hand, reduced level of *gacA* mRNA in the *hfq* mutant was found to be responsible for the reduced expression of RsmB in the *hfq* mutant (Fig. 3B). A study in *Salmonella* indicated that Hfq may activate the *hilD* mRNA encoding the master transcription factor of *Salmonella* pathogenicity island-1 (SPI-1), and overexpression of *hilD* in the *hfq* mutant restored effector synthesis and secretion (Sittka et al., 2008). DNA microarray study in the *algU* mutant of *Xylella fastidiosa* predicted that biofilm

formation in is regulated by *algU* through a complex Hfq/*rsmB*/*rsmA*-mediated system (Shi et al., 2007). At this stage, we cannot rule out that there may be other regulators of *rsmB* (Suzuki et al., 2006) that may also be regulated by Hfq. The CsrD protein, which recruits RNase E to degrade the sRNAs, is known to regulate the stability of RsmB (Suzuki et al., 2006). It is possible that Hfq may also regulate RsmB expression via CsrD.

Recently, Ysr141 a sRNA was shown to influence the synthesis of the T3SS in *Y. pestis* (Schiano et al., 2014). In our study we found a sRNA called ArcZ (a.k.a RyhA, SraH) whose expression levels are dependent on Hfq and which is also required for the expression of the T3SS (Fig. 4). Previously, ArcZ was shown to strongly bind to Hfq (Wassarman et al., 2001; Zhang et al., 2003; Pfeiffer et al., 2007) and is highly abundant in the stationary phase of bacterial growth (Mandin et al., 2010). Similar to the *hfq* mutant, the *arcZ* mutation did not alter the promoter activity of *hrpL*. Although it has not been tested in this study, it is likely that *hrpL* mRNA levels may be reduced in an *arcZ* mutant. There are examples where Hfq and a sRNA work together to activate the expression of genes at the post-transcriptional level by relieving auto-inhibitory loop in the structure of the target mRNA, such as the activation of *rpoS* by Hfq and sRNAs – DsrA, RprA and ArcZ (Soper et al., 2010). It is possible that sRNA ArcZ and Hfq together may activate *hrpL* expression in a similar fashion.

hfq mutants are known to exhibit pleiotropic effects in several bacteria. Interestingly, a population of *hfq* mutant cells was consistently found to be attached to the walls of the flask used for culturing bacteria (Fig. 5A). On the other hand, motility was significantly reduced in the *hfq* mutant (Fig. 5B). Previously, the *hfq* mutation has been reported to either promote or reduce biofilm formation (Attia et al.,

2008; Kulesus et al., 2008). Motility, which provides many pathogens an advantage in host cell colonization, was shown to require Hfq in several bacteria (Chao and Vogel, 2010). Both biofilm formation and motility are phenotypes commonly known to be regulated by secondary messenger c-di-GMP in several bacteria (Jenal and Malone, 2006) including *D. dadantii* 3937 (Yi et al., 2010). In support of these observations, intracellular levels of c-di-GMP were found to be increased in the *hfq* mutant (Fig. 5C). Additionally, c-di-GMP is known to down-regulate the expression of the T3SS (Yi et al., 2010). However, when EcpC was overexpressed in the *hfq* mutant, there was no change in RsmB levels (Fig. 5D) which indicated that the effect of c-di-GMP on the expression of the T3SS is not exerted via RsmB. Moreover, the result also suggested that there may be some DGCs or PDEs whose levels may be altered by Hfq. We found *gcpA*(DGC) promoter activity showed an increase in the *hfq* mutant whereas that of *ecpC* (PDE) showed a subtle decrease (Fig. 6). However, the mRNA levels of these DGCs and PDEs may provide a completely different scenario where Hfq may alter their levels in order to modulate the c-di-GMP levels (Bellows et al., 2012).

Expression of virulence factor T3SS is one of the most energy consuming cellular processes and hence it is tightly controlled. Although the T3SS of *D. dadantii* 3937 is regulated by two major regulatory pathways, such as HrpX/Y-HrpS-HrpL, and GacA/S-RsmB-RsmA pathways (Fig. 7), the regulation of the T3SS appears to be complex. This study shows that Hfq and sRNA ArcZ provide an additional layer of regulation of T3SS expression. Modulation of c-di-GMP levels by Hfq makes this regulation even more complex. Finally, identification of the common target mRNA(s) of Hfq and/or ArcZ will provide a clearer idea of the post-transcriptional control of T3SS expression.

Table 1: Strains, plasmids and primers used in this study

Designation	Relevant characteristics or sequences (5'to 3')[a]	Reference
E.coli		
EC 100	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ– *rps*L *nup*G.	
***D. dadantii* 3937**	Wild type, *Saintpaulia* (African violet) isolate	Hugouvieux-Cotte-Pattat, N
Δ*hfq*	Δ*hfq*, ABF-15209 deletion mutant	This study
Δ*arcZ:Km*	Δ*arcZ:Km*,Km^r, ABF-61315 deletion mutant	This study
Plasmids		
pKD4	Km^r, template plasmid carrying Km resistance cassette	Datsenko and Wanner, 2000
pWM91	Suicide vector, Ap^r	Metcalf et al., 1996
pFLP2	Flippase FRT excision vector, Ap^r	Hoang et al., 1998
pPROBE-AT	Long life GFP Promoter-probe vector, Ap^r	Miller et al., 2000
pCL1920	Low copy vector for complementation or overexpression, Sp^r	Lerner, 1990
pML123	RSF1010-derived expression and *lac*-fusion broad-host-range vector, Gm^r	Labes et al. 1990
pCL1920:*hfq*	Hfq overexpression construct, Sp^r	This study
pCL1920:*ecpC*	EcpC overexpression construct, Sp^r	This study
pML123:*rsmB*	pML123 derivative with PCR fragment containing full length *rsmB*, Gm^r	Zeng et al., 2010
P*hrpA*	Long life GFP Promoter-probe vector with PCR fragment containing *hrpA* promoter, Ap^r	Yang et al., 2008
P*hrpL*	Long life GFP Promoter-probe vector with PCR fragment containing *hrpL* promoter, Ap^r	Yang et al., 2008
P*hrpN*	Long life GFP Promoter-probe vector with PCR fragment containing *hrpN*promoter, Ap^r	Yang et al., 2008
P*dspE*	Long life GFP Promoter-probe vector with PCR fragment containing *dspE*promoter, Ap^r	Yang et al., 2008
P*gcpA*	Long life GFP Promoter-probe vector with PCR fragment containing *gcpA*promoter, Ap^r	This study
p*gcpD*	Long life GFP Promoter-probe vector with PCR fragment containing *gcpD*promoter, Ap^r	This study
P*ecpB*	Long life GFP Promoter-probe vector with PCR fragment containing *ecpB*promoter, Ap^r	This study
P*ecpC*	Long life GFP Promoter-probe vector with PCR fragment containing *ecpC*promoter, Ap^r	This study
Primers		
hfq-A-Xho1-for	ATAATACTCGAGCATCCAAATGATCCGCAGAG	This study

hfq-B-rev	GAAGCAGCTCCAGCCTACACAACAAAGAACC CTGTGGCCC	This study
hfq-C-for	CTAAGGAGGATATTCATATGCGCGTCCTTACC AGTTTACC	This study
hfq-D-Not1-rev	AATATTATGCGGCCGCCCCCTGTTCACGCTGCT TTT	This study
arcZ-A-XhoI-for	ATAATACTCGAGAGTTTCGGTAACATGGCAGG	This study
arcZ-B-rev	GAAGCAGCTCCAGCCTACACGCAAATATGGCG TCGTTTGT	This study
arcZ-C-for	CTAAGGAGGATATTCATATGCGGGGTCATTTT TTTTCAGC	This study
arcZ-D-NotI-rev	AATATTATGCGGCCGCGGCGAATGTGCTCAAA GATA	This study
hfq-for-SacI	GATCGAGCTCGTTATTTTAGGGCCACAGGG	This study
hfq-rev-XbaI	TCGATCTAGAAAAGTCGCTGAAATCAGCGC	This study
ecpC-for-SacI	TATAGAGCTCGCACCGGCGTCAGGGTTAAT	Yi et al., 2010
ecpC-rev-XbaI	CAGCTCTAGAAAGTTTTTTCGCATCCGCCG	Yi et al., 2010
Probes for Northern blot		
ryhA-for	GGTGTGTGTTGCCAGAAGATGGG	This study
ryhA-rev	AAGCCGGGGTGCGCGAATTA	This study
rsmB-for	GGTGTTCGTCTATAAACCGCG	This study
rsmB-rev	TAGTTCGTTTGCAGCAGTCC	This study
qPCR		
rplU-for	ACAACACCGAGTAAGCGAAGGTCA	Khokhani et al., 2013
rplU-rev	GCTTTAATCACGCCGCCTGAAACT	Khokhani et al., 2013
hrpL-for	GCAAGCGCGTATCGAACCGT	This study
hrpL-rev	ACTTCCAACGCATCGTCGCTG	This study
gacA-for	GTTGTTGGTGAGGCGCAGTG	This study
gacA-rev	CCGGCCTGCATCACTTTAGC	This study

[a]Underlined bases indicate restriction sites in the primers. Ap^r, Gm^r, $Sp^r$and Km^r indicate ampicllin, gentamycin, spectinomycin and kanamycin resistance respectively.

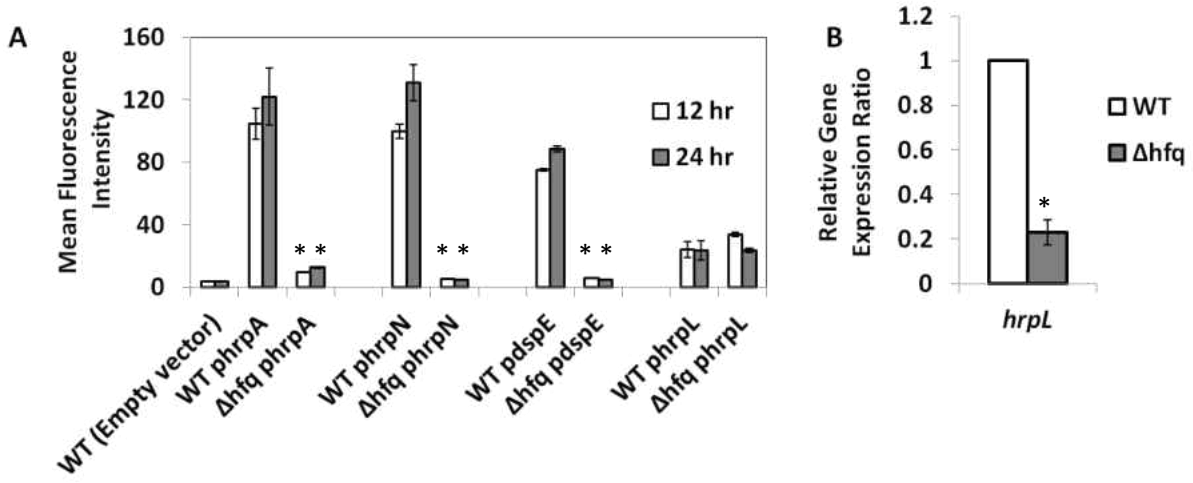


Figure 1: **Hfq positively regulates the expression of structural and effector genes of the T3SS of *D. dadantii* 3937**. **A**. Promoter activity of *hrpA*, *hrpN*, *dspE* and *hrpL* in wild type and Δ*hfq* strains in MM. Values (Mean Fluorescence Intensity) of GFP were measured by flow cytometry. Three technical replicates were used in each experiment. Each experiment was repeated three times and similar results were obsereved. Asterisks indicate $P < 0.05$ (Student's *t*-test). **B**. Relative mRNA levels of *hrpL* in *D. dadantii* 3937 in MM as measured by qPCR. RNA was collected at 12 h of bacterial growth. Levels of gene expression of *hrpL* are significantly lower than in the wild-type (WT) ($P < 0.05$) as indicated by asterisks. Three replicates were used in this experiment. The *P* value was calculated by the Relative Expression Software Tool (Pfaffl et al., 2000). The experiment was repeated three times with similar results.

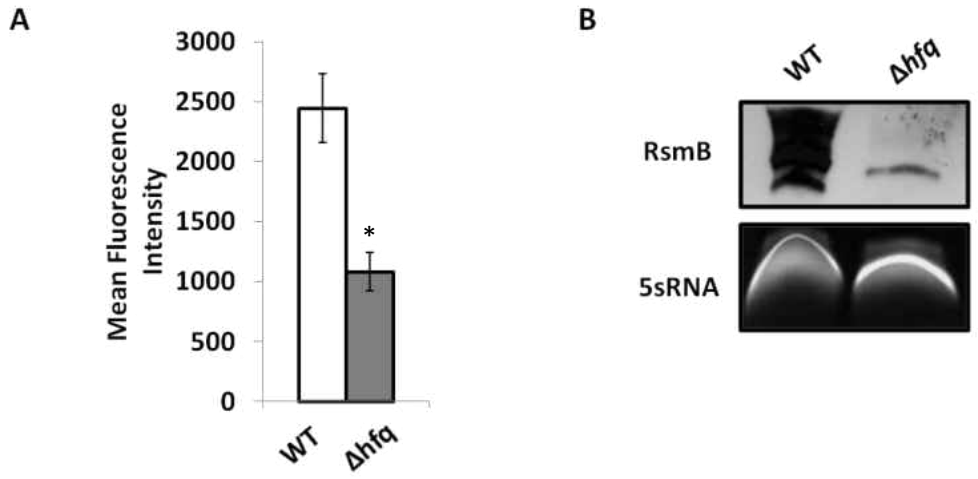


Figure 2: **Hfq positively regulates the expression of RsmB in *D. dadantii* 3937**. **A**. Promoter activity of *rsmB* in wild type and Δ*hfq* strains in MM. Values (Mean Fluorescence Intensity) of GFP were measured by flow cytometry. Three technical replicates were used in each experiment. Each experiment was repeated three times and similar results were observed. Asterisks indicate $P < 0.05$ (Student's *t*-test). **B**. Northern blot analysis of *rsmB* transcripts in wildtype (WT) and Δ*hfq* strains of *D. dadantii* 3937 using 6% PAGE gel. RNA was isolated from cells harvested after 12 h of bacterial growth in MM. 5S rRNA was used as RNA loading control. The experiment was repeated three times with similar results.

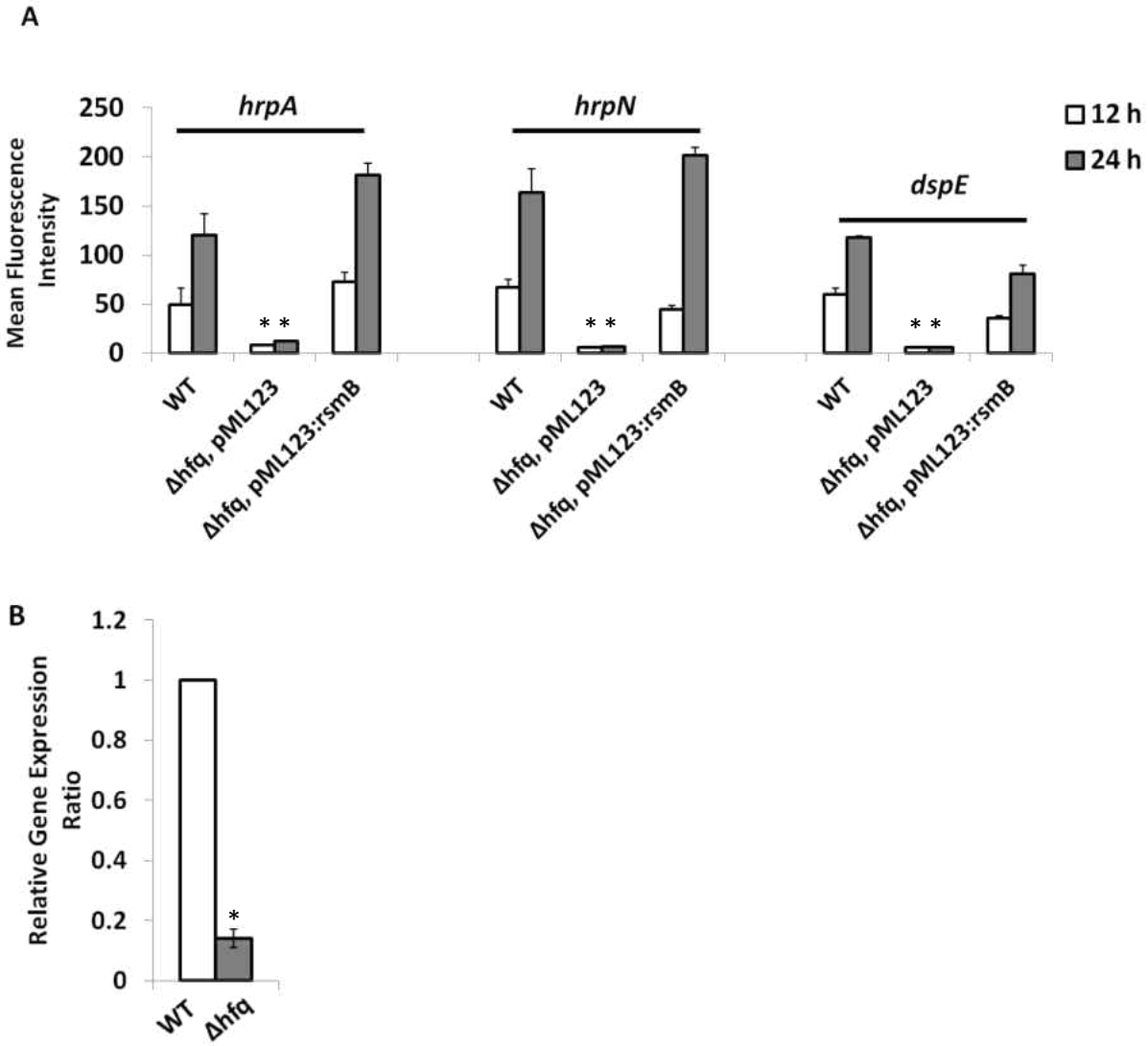


Figure 3: **Overexpression of RsmB in Δ*hfq* strain of *D. dadantii* 3937**. **A**. Promoter activity of *hrpA, hrpN and dspE* in wild-type, Δ*hfq* with pML123 or pML123:rsmB strains in MM. Values (Mean Fluorescence Intensity) of GFP were measured by flow cytometry. Three technical replicates were used in each experiment. Each experiment was repeated three times and similar results were observed. Asterisks indicate $P < 0.05$ (Student's *t*-test). **B**. Relative mRNA levels of *gacA* in *D. dadantii* 3937 in MM as measured by qPCR. RNA was collected at 12h of bacterial growth. Levels of gene expression of *hrpL* are significantly lower than the wildtype (WT) ($P < 0.05$) as indicated by asterisks. Three replicates were used in this experiment. The *P* value was calculated by the Relative Expression Software Tool (Pfaffl, 2000). The experiment was repeated two times with similar results.

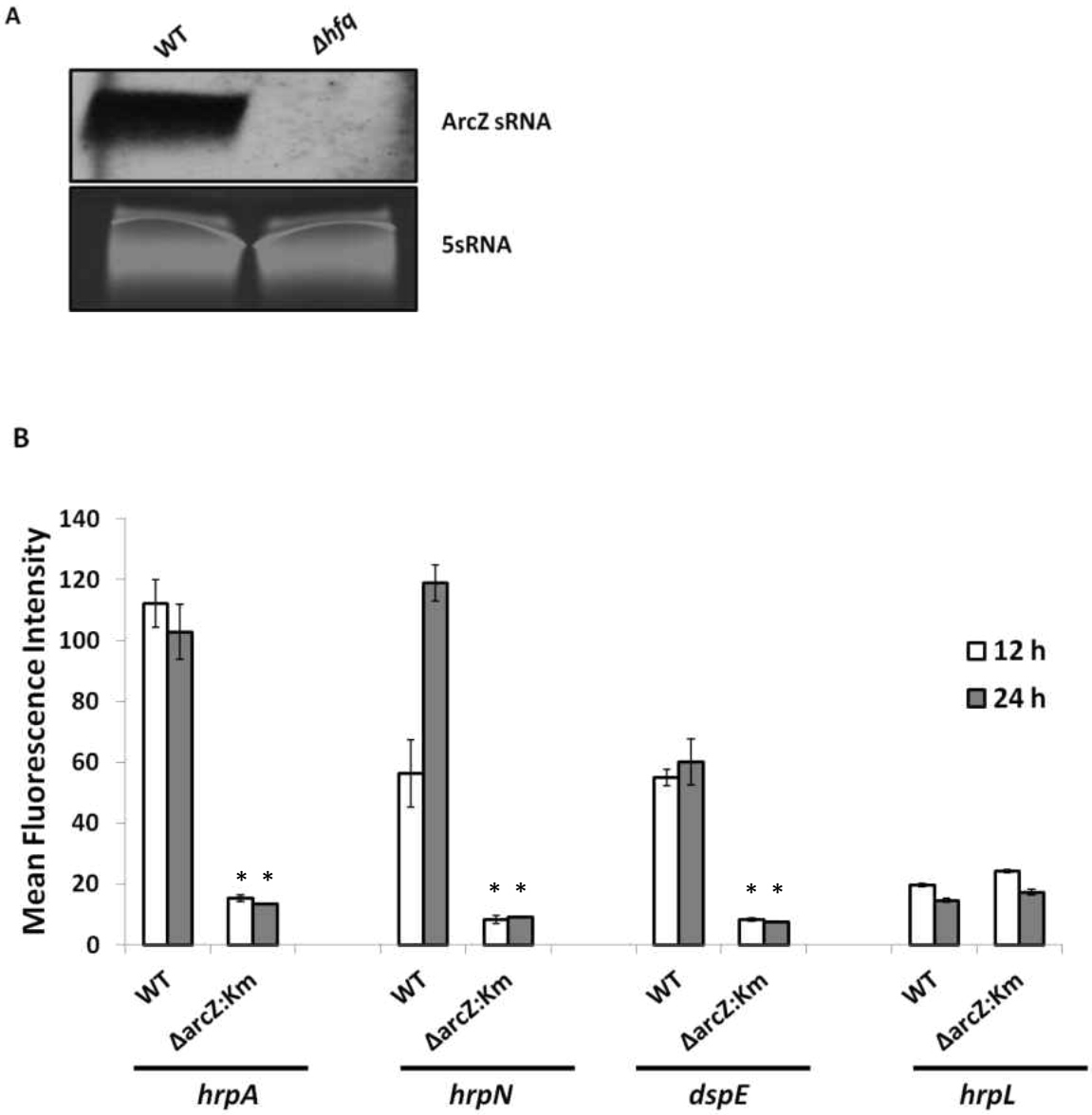
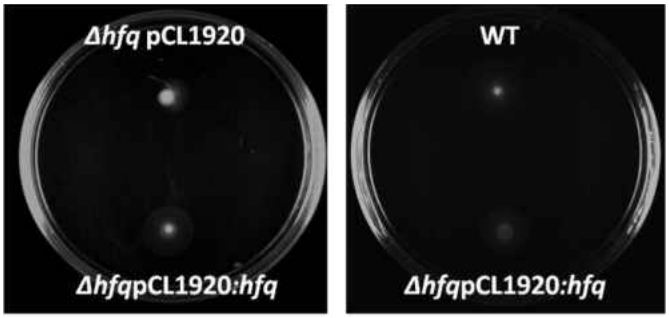


Figure 4: **sRNA ArcZ positively regulates the expression of the structural and effector genes of the T3SS in *D. dadantii* 3937**. **A**. Northern blot analysis of *arcZ* transcripts in wildtype (WT) and Δ*hfq* strains of *D. dadantii* 3937 using 6% PAGE gel. RNA was isolated from cells harvested after 12 h of bacterial growth in MM. 5S rRNA was used as RNA loading control. The experiment was repeated three times with similar results. **B**. Promoter activity of *hrpA*, *hrpN*, *dspE* and *hrpL* in wild type and Δ*arcZ:km* strains in MM. Values (Mean Fluorescence Intensity) of GFP were measured by flow cytometry. Three technical replicates were used in each experiment. The values are representatives of one experiment. Asterisks indicate $P < 0.05$ (Student's *t*-test).

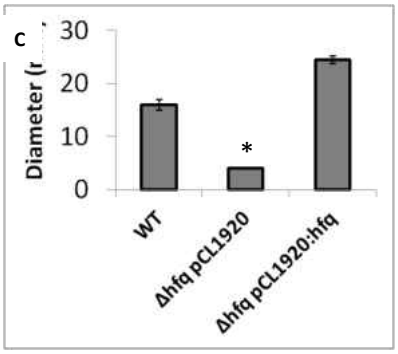
A

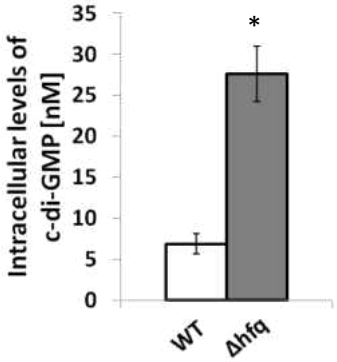

WT
Δhfq

B


Δhfq pCL1920
ΔhfqpCL1920:hfq
WT
ΔhfqpCL1920:hfq

C


Diameter
30
20
10
0
*
WT
Δhfq pCL1920
Δhfq pCL1920:hfq


Intracellular levels of c-di-GMP [nM]
35
30
25
20
15
10
5
0
*
WT
Δhfq

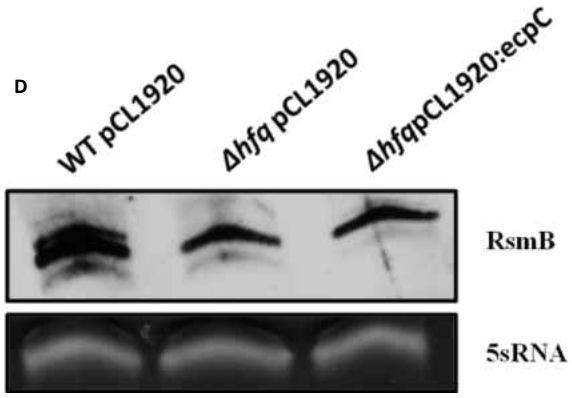


Figure 5: Phenotypic analysis of *hfq* mutant. **A**. Surface attachment increases in Δ*hfq* strains of *D. dadantii* 3937. Wild-type (WT) and Δ*hfq* strains were grown in MM for 12 h which resulted in increased attachment of the cells to the walls of the flasks. This experiment has been repeated at least three times with similar results. **B**. Swimming motility in WT, Δ*hfq* pCL1920 or pCL1920:*hfq* was measured on MG plates containing 0.2% agar. Values on Y axis represent the diameter of the radial growth. **C**. Measurement of intracellular levels of c-di-GMP in wildtype and Δ*hfq* strains. Three technical replicates were used in each experiment. Each experiment was repeated at least three times and similar results were observed. Asterisks indicate $P < 0.05$ (Student's *t*-test). **D.** Northern blot analysis of *rsmB* transcripts in wildtype (WT) with empty vector, pCL1920, Δ*hfq* with pCL1920 or pCL1920:*ecpC* strains of *D. dadantii* 3937 using 6% PAGE gel. RNA was isolated from cells harvested after 12 h of bacterial growth in MM. 5S rRNA was used as RNA loading control. The experiment was repeated two times with similar results

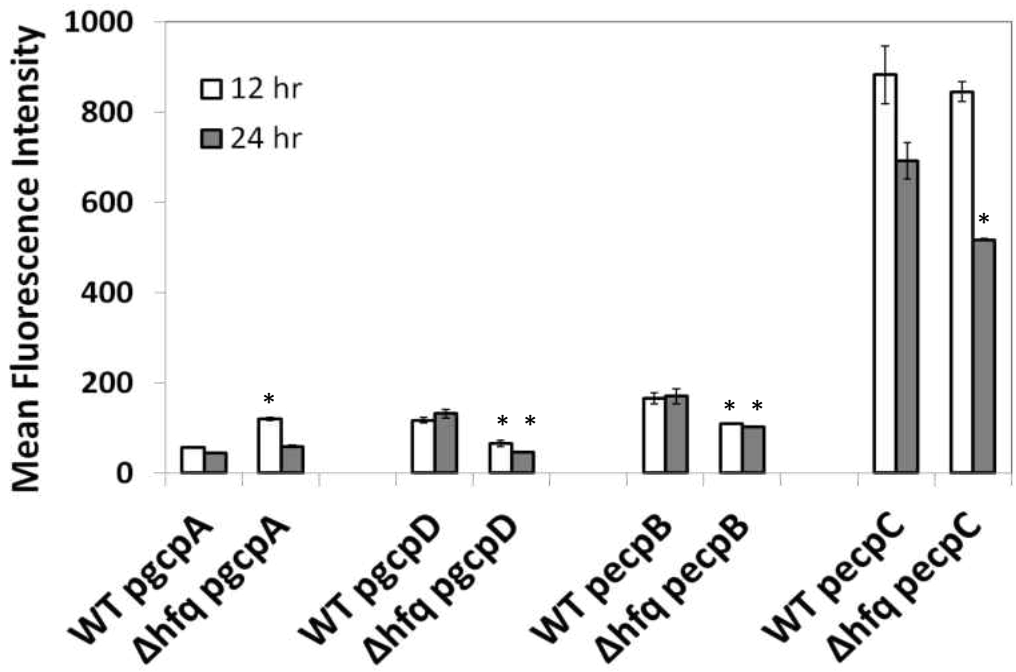


Figure 6: **Expression levels of diguanylate cyclases and phosphodiesterases are modulated in the *hfq* mutant of *D. dadantii* 3937**. Promoter activity of DGCs (*gcpA* and *gcpD)* and PDEs (*ecpB* and *ecpC)* in wild type and Δ*hfq* strains in MM. Values (Mean Fluorescence Intensity) of GFP were measured by flow cytometry. Three technical replicates were used in each experiment. Each experiment was repeated three times and similar results were observed. The values are representatives of one experiment. Asterisks indicate $P < 0.05$ (Student's *t*-test).

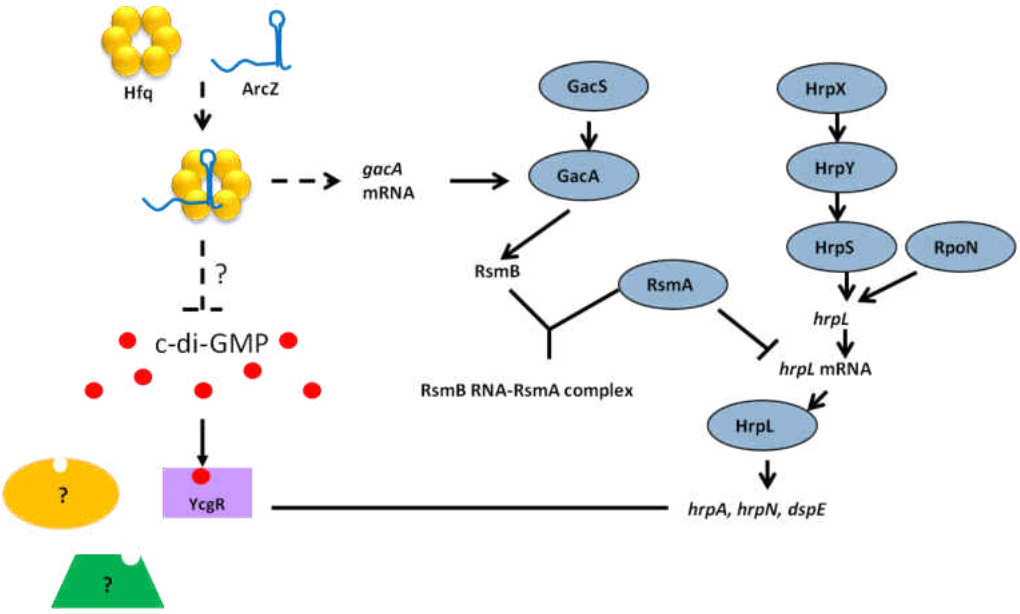


Figure 7: **Working model of Hfq-mediated regulation of the T3SS via sRNA ArcZ and c-di-GMP in *D. dadantii* 3937**. Proteins are indicated by colored circles, ovals, rectangles, and trapezium. Small red circles indicate c-di-GMP. Solid lines indicate direct regulation (protein-protein interaction or direct binding to the promoter region) and dashed lines indicate indirect regulation or hypothetical regulatory links based on evidence shown in bacteria. Based on genetic analysis in *D. dadantii* 3937, HrpX/Y forms a two component system that activates the transcription of *hrpS*. HrpS is a σ^{54} enhancer binding protein that activates the transcription of *hrpL*. HrpL activates the expression of the T3SS genes such as *hrpA*, *hrpN*, and, *dspE*. GacA/S two component system activates transcription of *rsmB*. RsmB is an untranslated sRNA that can sequester RsmA, an RNA binding protein. RsmB-RsmA system regulates the T3SS by affecting the stability of *hrpL* mRNA in *D. dadantii* 3937. In this study, Hfq was found to positively regulate the T3SS via RsmB-RsmA system. Additionally, Hfq modulated the c-di-GMP concentrations through an unknown mechanism.

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Concluding Remarks

In our study in *Erwinia amylovora* 273, we found two highly potent T3SS inhibitors which targeted the T3SS by affecting its regulators, such as HrpS and RsmB. These inihibitors also reduced the HR in tobacco by targeting the T3SS. Additionally, these inhibitors did not affect the growth rate of bacteria. Overall, the results indicated that T3SS is an attractive target for developing antimicrobial compounds that may have reduced selection pressure and hence lower the possibilities of developing resistance against the compounds.

In our other work in *Dickeya dadantii* 3937, a PilZ domain protein, YcgR was found to regulate the T3SS in c-di-GMP-mediated manner which was further confirmed by the site-directed mutagenesis experiments. sRNA chaperone, Hfq, and sRNA, ArcZ, both were found to positively regulate expression of T3SS genes. Apart from the known RsmB-RsmA regulation of *hrpL* mRNA, this study indicated the possibility of another layer of regulation of T3SS through Hfq-ArcZ action. Interestingly, Hfq positively regulated expression of RsmB which is required for expression of *hrpL*. c-di-GMP levels were also found to be modulated by Hfq. Together these findings suggest that Hfq may regulate T3SS in many ways in order to tightly regulate expression of T3SS. Firstly, under stress conditions, Hfq-ArcZ complex may regulate GacA which is required for the activation of *rsmB* and hence allow expression of T3SS. Secondly, Hfq-ArcZ may directly regulate *hrpL* expression at post-transcriptional level in order to activate expression of T3SS. Lastly, Hfq may also alter the expression of several DGCs and PDEs in order to modulate the cellular c-di-GMP levels. The ratio of the c-di-GMP receptors such as YcgR and BcsA may regulate expression of T3SS under specific environmental circumstances.

DEVANSHI KHOKHANI

Objective: I want to be a committed professional by having a qualitative and, result oriented approach towards the set objective of an organization. I want to reach the pinnacle and establish myself in my field with the best use of my ability, attitude and aptitude.

Educational qualification:

1. **Ph.D in Biological Sciences** (Microbiology Major), (UW-Milwaukee, 2014)
   Dissertation title: Small RNAs, Cyclic-di-GMP and Phenolic compounds regulate the Type III Secretion System in bacterial phytopathogens.
   Dissertation Advisor: Ching-Hong Yang, PhD.
2. **Masters of Science in Biochemistry** (M.S. University of Baroda, 2007)
   Dissertation title: Study of effect of population density on the growth rate of bacteria in their vegetative phase
   Dissertation Advisor: Shashikant Acharya, PhD.

3. **Bachelors of Science in Biochemistry and Vocational Biotechnology** (M.G. Science Institute, India, 2005)

Experience

A. **Research Experience**

Articles in Refereed Journals

A. Published articles

1. Li, Y., Hutchins, W., Wu, X., Liang, X., Zhang, C., Yuan, X., **Khokhani, D**., Chen, X., Che, Y., Wang, Q., Yang, C-H. 2014. Derivative of Plant Phenolic Compound Inhibits the Type III Secretion System of Dickeya dadantii via HrpX/HrpY Two-Component Signal Transduction and Rsm Systems. Accepted in Molecular Plant Pathology. April **2014**.
2. **Khokhani D.,** Zeng Q., Zhang C., Yamazaki A., Chen X., Yang C.H. Discovery of plant phenolic compounds that act as type three secretion system inhibitors or inducers of fire blight pathogen *Erwinia amylovora. Appl Environ Microbiol.* **2013** ;79(18):5424-36
3. Yamazaki, A., J. Li, Q. Zeng, **Khokhani D.**, W. C. Hutchins, A. C. Yost, E. Biddle, E. J. Toone, X. Chen, C.-H. Yang. (2012) Derivatives of plant phenolic compound affect the type III secretion system of *Pseudomonas aeruginosa* via a GacS/GacA two component signal transduction system. *Antimicrob Agents Chemother*. **2012** Jan; 56(1):36-43.

B. Presentations and Posters

1. Presentation-"Type III secretion system inhibitors and inducers" at School of Freshwater Sciences, UW- Milwaukee, 2013.
2. Poster presentation-"Identification of c-di-GMP receptors involved in regulation of the type III secretion system in bacterial phytopathogen *Dickeya dadantii*", Biological Sciences Symposium, UW-Milwaukee, 2013.

3. Poster presentation-"Inhibitors and inducers of the type III secretion system of *Erwinia amylovora",* American Phytopathological Society Meeting, 2012.
4. Poster presentation-"Type III secretion system Inhibitors and Inducers of phytopathogen *Dickeya dadantii*" Symposium, UW-Milwaukee, 2011.
5. Presentation –"Novel approaches for developing virulence-specific therapeutics" Symposium, UW- Milwaukee, 2010.

Work Experience:

1. I have worked on "Study of sRNA and c-di-GMP involvement in the regulation of the Type III Secretion System (T3SS) in *Dickeya dadantii* 3937. Other project involves identification of Inhibitors and Inducers of *Erwinia amylovora* 273." This work focuses on antivirulence therapy of a plant pathogen that causes disease in economically important plants like apple and pears.
2. Organized a Research Symposium at Department of Biological Sciences, 2013.
3. Participated in Workshop on "Advanced Molecular biology techniques" at Xavier Research Foundation" Ahmedabad, India, 2009.
4. Participated in Workshop on "Phytochemical extraction methods and its analysis by HPTLC", Xaviers Research Foundation", India, 2008.
5. Dissertation on "Study of effect of population density on the growth rate of bacteria in their vegetative phase", Masters, 2006-07.
6. As an observer at Gujarat Cancer & Research Institute, India, in different laboratories like receptor & growth factor laboratory, cell biology, immunohistochemistry, molecular endocrinology and biochemistry research division during first year of Bachelors, 2003.

B. Teaching Experience
Positions and Employment:

1. Teaching Assistant: (2009-2014)
    1. Elements of Biology (Fall and Spring, 2009-14)
    2. Experimental Microbiology (Spring-2011, 2012, 2013)
2. Trained undergraduate student for research work:
    1. Daniel Grienke (Summer 2013 and Fall 2013)
    2. Bryan Landrie (Spring 2012)
    3. Julia Keyes (Spring 2010, 2011 and Summer 2011)
    4. Kristen Timm (Summer 2010)

3. Full time Lecturer in St. Xavier's college, Ahmedabad, India.
    1. Courses taught: Vocational biotechnology course recognized by University Grants Commission. (2007-09)
    2. Along with being a lecturer, part of my duty also included research work on "Jatropha (bio diesel crop) *in vitro micro propagation*".

Awards and Honors

1. **Ruth Walker Grant-in-Aid Award** for "Outstanding Achievement in Biological Sciences" (2013-2014)
2. **Ruth Walker Grant-in-Aid Award** for "Outstanding Achievement in Biological Sciences" (2012)
3. **Student Travel Award** for the American Phytopathological Society Annual Meeting 2012 (Providence) from Department of Biological Sciences, UWM
4. **CSIR-NET Award** (Council of Scientific and Industrial Research- National Eligibility Test) during Masters Program (2006). This helped me to fetch good teaching opportunities in my career till now.