IDENTIFICATION AND CHARACTERIZATION OF VIRULENCE FACTORS VIA THE ELUCIDATION OF REGULATORY NETWORKS IN THE FIRE BLIGHT PATHOGEN *ERWINIA AMYLOVORA*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Pathology – Doctor of Philosophy

2013

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF NOVEL VIRULENCE FACTORS VIA THE ELUCIDATION OF REGULATORY NETWORKS IN THE FIRE BLIGHT PATHOGEN *ERWINIA AMYLOVORA*

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Erwinia amylovora is a Gram-negative bacterium and the causal agent of fire blight, a destructive disease of rosaceous species such as apple and pear. The genetic determinants of fire blight disease development include a diverse array of virulence mechanisms including biofilm formation and the type III secretion system (T3SS). The activity of these virulence factors in *E. amylovora* is coordinated by a complex system of regulatory elements that interact with all stages of gene expression and function. As such, the characterization of virulence regulators is a powerful tool for understanding host-microbe interactions via the identification of downstream virulence mechanisms. In *E. amylovora*, multiple virulence regulators control disease development including the alternative sigma factor HrpL. HrpL is conserved in many bacterial plant pathogens and plays a vital role the transcription of both structural and translocated components of the T3SS. While the role of HrpL in regulating the T3SS is indispensible for pathogenesis, the HrpL regulon also includes a large number of uncharacterized genes that do not have predicted roles in type III secretion. Because the HrpL regulon is a quintessential virulence regulon, uncharacterized genes regulated by HrpL are likely to play important roles in disease development. Using a combined approach including microarray expression analyses, bioinformatics, and mutagenesis, six novel virulence factors were identified including a biofilm regulator NlpI (EAM_3066), a transcriptional regulator YdcN (EAM_1248) and a HrpL-regulated gene cluster including EAM_2938.

ACKNOWLEDGMENTS

I would like to extend my sincerest thanks to my advisor Dr. George W. Sundin for his enthusiastic support and advice during my tenure at Michigan State University. I would also like to thank the members of my committee, Dr. Ray Hammerschmidt, Dr. Brad Day and Dr. Sheng Yang He for their valuable time and consideration during the creation of this document. In addition, I want to thank the Department of Plant Pathology and my colleagues in the Student Phytopathological Organization for Research and Education. Their contribution to creating an engaged, creative, and welcoming academic community at Michigan State University have not gone unnoticed and are greatly appreciated. Finally, my most heartfelt thanks go to my friends and family whose constant love and support have made this work possible.

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1.1 Introduction

Fire blight is a highly destructive disease of rosaceous tree species caused by the Gram-negative enteric bacterium *Erwinia amylovora*. *E. amylovora* is distinguished as the first bacterium identified as a causal agent of a plant disease (Denning 1794; Winslow et al., 1920). Since the first reported observation of fire blight in 1794 in the northeastern United States, *E. amylovora* has spread globally with fire blight currently occurring in Europe, the Middle East, New Zealand and Japan (Bonn and van der Zwet 2000).

E. amylovora infects a broad range of species within the Rosaceae including the subfamily Spiraeoideae as well as the genus *Rubus* (Mann et al., 2013). These hosts include economically important crops such as apple and pear in addition to quince, hawthorn, cotoneaster and *Rubus* species blackberry and raspberry.

E. amylovora cells are disseminated by water, wind, insects and human activity (van der Zwet and Beer 1999). Flowers represent the primary infection court and bacterial populations can exceed 1,000,000 cells on the stigma surface (Wilson and Lindow 1992). *E. amylovora* invades hosts via nectarthodes and moves systemically within the vascular system and cortical parenchyma (Sjulin and Beer 1978; Koczan et al., 2009). Colonization of xylem elements occludes water transportation resulting in the most characteristic signs and symptoms of fire blight: desiccation, the shepherd's crook and bacterial ooze. Ooze is rich in polysaccharides, sap and bacterial cells and serves as an important source of inoculum (Geider 2000). While young tissues exhibit increased susceptibility to infection, *E. amylovora* is capable of infecting many host tissues including flowers, shoots, leaves, fruits as well as rootstocks (Pusey and Smith

2008). Infections of rootstocks can kill host trees and, when environmental conditions are conducive, fire blight epidemics can result in major economic losses (Longstroth 2000).

In the United States, estimated annual costs attributable to fire blight infection are approximately US$ 100 million and consequently, successful management of *E. amylovora* populations is imperative (Norelli et al., 2003). Currently, the antibiotic streptomycin serves as the most effective and commonly utilized chemical control of fire blight. However, due to widespread use, streptomycin resistance in *E. amylovora* has developed in the United States and other countries including Canada, Israel and New Zealand (McManus et al., 2002; McGhee et al., 2011). While less effective controls are available, such as oxytetracycline and copper sulfate, development of new *E. amylovora* control strategies is increasingly urgent.

Since transposon mutagenesis experiments conducted by Steinberger and Beer (1988) revealed the first genetic determinants required for fire blight disease development, the identification and characterization of *E. amylovora* virulence determinants has progressed rapidly. This chapter summarizes our current understanding of the molecular basis for fire blight development and emphasizes non-canonical virulence factors that play a vital, but under-described role in pathogenesis.

1.2 Structure and function of the type three secretion system

Fire blight development of *E. amylovora* is characterized by the function of two main pathogenicity factors: the exopolysaccharide amylovoran and the type III secretion system (T3SS) (Oh and Beer 2005; Khan et al., 2012; Zhao and Qi 2011). The T3SS is a macromolecular protein complex consisting of three structural components: the basal body, the pilus and the translocon (Buttner and He 2009). The basal body spans the Gram-negative

bacterial cell wall and contains an ATPase to provide energy for the translocation process (Kubori et al., 1998). Anchored to the basal body is a hollow pilus that extends into extracellular space and is distally capped by a pore-forming protein complex called the translocon (Buttner and Bonas 2002). The translocon associates with host membranes and allows pathogen effectors to translocate across extracellular space directly into the host cytoplasm in a single step (Buttner and Bonas 2002; He and Jin 2003). Post-translocation, pathogen effectors reprogram host cells to create an environment conducive to bacterial survival and proliferation (Alfano and Collmer 2004; Deslandes and Susana Rivas 2012).

In *E. amylovora*, the T3SS is encoded on a pathogenicity island consisting of approximately 62 kilobases of genomic DNA and 60 genes (Oh et al., 2005). The T3SS was discovered via transposon mutagenesis that resulted in *E. amylovora* strains unable to elicit hypersensitive response (HR) and pathogenicity (*hrp*) in non-host plants and hosts respectively (Steinberger and Beer 1988). The T3SS basal body includes nine proteins that are highly conserved between *E. amylovora* and other bacterial pathogens of animals and plants (Oh and Beer 2005). These proteins include the ATPase HrcN and HrcU, a protein known to regulate secretion hierarchy in other type III systems, including flagella (Pozidis et al., 2003; Lorenz and Buttner 2011). The *hrp* pilus is constructed from numerous HrpA subunits and the translocon, while less characterized than counterparts in animal bacterial pathogens, is likely composed of the harpins HrpN and HrpW as well as HrpK (Jin and He 2001; Bocsanczy et al., 2008). The *hrp* T3SS in *E. amylovora* also requires multiple helper proteins such as HrpJ which is required for HR elicitation in tobacco as well as for disease in host species and functions in regulating the secretion of both HrpN and HrpW (Nissinen et al., 2007).

The role of type III secretion in *E. amylovora* is to enable the translocation of pathogen effectors directly into host cells. While effector repertories in bacterial pathogens such as *Pseudomonas syringae* pv. *phaseolicola* 1448A have been reported to include as many as 27 candidates, *E. amylovora* only encodes five known effector genes (Vencato et al., 2006; Nissinen et al., 2007; McNally et al., 2012). By far, DspA/E represents the most important effector of *E. amylovora* as null mutants are non-pathogenic in immature pear and apple shoots (Barny et al., 1990; Bogdanove et al., 1998). Interestingly, while required for pathogen growth and disease development, *dspA/E* mutants maintain population numbers *in planta*. Conversely, *E. amylovora* strains generally defective in type III secretion are unable to persist in compatible host species and populations quickly decline (Zhao et al., 2005). This suggests that additional constituents of the T3SS secretome in *E. amylovora* are important in mediating successful host-microbe interactions.

1.3 Analyses of T3SS effector repertoire

Amongst the small repertoire of *E. amylovora hrp* type III effectors, DspA/E is the most characterized virulence factor mediating *E. amylovora*-host interactions. DspA/E is part of the AvrE-family of effectors with homologs in many phytobacterial pathogens (Lorang and Keen 1995; Bogdanove et al., 1998; Ham et al., 2006). While AvrE-class effectors in pathogens including *P. syringae* pv. *tomato* DC3000 are believed to function redundantly with other effectors including HopM1, DspA/E in *E. amylovora* is absolutely required for pathogenicity (Bogdanove et al., 1998; Kvitko et al., 2009).

DspA/E is secreted by the T3SS (Bogdanove et al., 1998b; Nissinen et al., 2007). While direct efforts to localize DspA/E *in planta* using co-immunofluorescence and gold labeling

confocal microscopy have failed to identify DspA/E in the host cytoplasm (Boureau et al., 2006), indirect measurements of protein translocation, including fusions with adenylate cyclase (CyaA), suggest that DspA/E is translocated into host cells (Bocsanczy et al., 2008; Triplett et al., 2009; Oh et al., 2010).

Translocation of DspA/E depends on a number of factors. Along with a functional T3SS and N-terminal amino acid residues within DspA/E, a class of proteins known as chaperones play an important role in DspA/E localization and function (Triplett et al., 2009, Oh et al., 2010). Type III secretion chaperones are small proteins that facilitate the secretion of some T3SS substrates. *E. amylovora* produces at least three such chaperones including Esc1, Esc3 and DspF, the cognate chaperone of DspA/E (Oh and Beer 2005). In *E. amylovora*, *dspF* mutants are severely attenuated in virulence and display reduced levels of DspA/E translocation (Gaudriault et al., 2002; Triplett et al., 2009). Factors responsible for residual DspA/E translocation in *dspF* mutants remain undescribed. Interestingly, DspA/E exhibits several chaperone binding sites, and multiple chaperones, including DspF and Esc1, interact with DspA/E via yeast two-hybrid screening (Triplett et al., 2009, Triplett et al., 2010; Oh et al., 2010). The interaction between DspA/E and multiple type III secretion chaperones highlights a new model for T3SS effector regulation, and accumulating evidence suggests that the pool of T3SS chaperones, most importantly DspF, collectively play an important role in regulating type III secretion hierarchy in pathogens like *E. amylovora*.

To date, DspA/E has not been associated with gene-for-gene interactions but the role of DspA/E in mediating disease development has been explored in a number of host and non-host pathosystems. Primary host responses attributed to DspA/E include suppression of callose deposition and cell death. Elicitation of callose deposition during host-microbe interactions is a

characteristic of plant innate immunity and is typically associated with salicylic acid (SA)-mediated defense responses (Boller and He 2009). During compatible interactions, DspA/E mutants generate more callose deposition than wild-type *E. amylovora* implicating DspA/E as a suppressor of innate immunity (Debroy et al., 2004; Boureau et al., 2011). Conversely, interactions with non-host *Arabidopsis thaliana* are not affected by DspA/E and elicitation of callose deposition only requires a functional T3SS (Degrave et al., 2008). Interestingly, while DspA/E does not modulate callose in *A. thaliana*, inducible expression of DspA/E in transgenic *A. thaliana* can suppress callose deposition in response to *P. syringae* pv. *tomato* lacking a functional T3SS (Degrave et al., 2013).

DspA/E is also attributed with inducing cell death, however the nature of DspA/E-mediated cell death is subject to debate. In compatible host interactions, DspA/E is a pathogenicity factor required for necrotic disease development (Bogdanove et al., 1998). Agrobacterium-mediated transient expression of DspA/E in apple also causes necrotic lesion formation (Boureau et al., 2006). In comparable transient expression assays, DspA/E also triggers cell death in multiple non-host species including yeast, tobacco and *A. thaliana* (Boureau et al., 2006; Oh et al., 2007; Degrave et al., 2008). Notably, Degrave et al., (2013) found that while production of DspA/E in transgenic *A. thaliana* induced marker gene expression indicative of SA signaling activity, SA itself was dispensable for DspA/E-mediated cell death. While this information suggests that DspA/E may function in a toxin-like capacity, in *Nicotiana benthamiana* DspA/E-induced cell death was shown to require SGT1, a host factor necessary for programmed cell death (Oh et al., 2007). During infection of SGT1-silenced *N. benthamiana*, *E. amylovora* exhibited higher population numbers compared to wild-type tobacco

(Oh et al., 2007). This indicates that host cell death does not promote *E. amylovora* growth and argues against DspA/E promoting virulence via inducing cell death (Oh et al., 2007).

DspA/E may function as a toxin or as an elicitor of HR, simultaneously or separately in a host-specific manner. Elucidating the molecular mechanisms underpinning host responses to DspA/E may have important implications for future fire blight control strategies.

The function of DspA/E and AvrE-family effectors is a major area of research in the molecular plant-microbe interactions community. To date, four apple proteins have been shown to interact with DspA/E via yeast two-hybrid assays and *in vitro* western blotting (Meng et al., 2006). These proteins have been termed DIPMs (DspA/E-interacting proteins from *Malus*) and represent a class of plant signal transducers termed leucine-rich repeat (LRR) receptor-like kinases (RLK). LRR-RLKs transmit signals from the plant apoplast via the ligand-binding potential of extracellular LRR domains and reprogram host cells accordingly by intracellular RLK-initiated phosphorylation signal cascades (Gómez-Gómez and Boller 2000; Nurnberger and Kemmerling 2006). In support of DspA/E translocation and intracellular function, during the host-microbe interaction DspA/E binds DIPM proteins through RLK domains only and full DspA/E-DIPM interactions require RLK kinase activity in yeast two-hybrid assays (Meng et al., 2006). Unfortunately, DIPMs appear to be constitutively expressed in *E. amylovora* host species regardless of susceptibility (Meng et al., 2006) and the significance of DspA/E-DIPM interactions remains to be determined.

Besides DspA/E, *E. amylovora* is known to produce a suite of additional effectors including Eop1, $HopX1_{Ea}$, $HopPtoC_{Ea}$, and $AvrRpt2_{Ea}$ (Zhou et al., 2005; Nissinen et al., 2007; McNally et al., 2012). Of these, most notable is $AvrRpt2_{Ea}$. Analyses of $avrRpt2_{Ea}$ mutants with a range of apple varieties revealed that $avrRpt2_{Ea}$ exhibits the characteristics of an

avirulence gene during interactions with *Malus* X *robusta* 5 (Mr5) (Voigt et al., 2013). A large screen exploring compatibility between different *E. amylovora* strains and Mr5 found that the degree *E. amylovora* strains could necrotize Mr5 could be traced to a single nucleotide polymorphism in *avrRpt2*$_{Ea}$ (Voigt et al., 2013). In contrast, during interactions with immature pear fruit, AvrRpt2$_{Ea}$ appears to function as a virulence factor (Zhou et al., 2006). These results suggest a role for AvrRpt2$_{Ea}$ in contributing to virulence in one plant-microbe interaction while functioning as an avirulence factor in a second.

While DspA/E and AvrRpt2$_{Ea}$ contribute to *E. amylovora* pathogenicity and virulence, Eop1 and HopX1$_{Ea}$ are implicated as host range limiting factors (Asselin et al., 2011, Bocsanczy et al., 2012). As described above, the host range of *E. amylovora* spans a number of rosaceous genera. In 2011, Asselin et al., observed that Eop1, a YopJ-family effector, exhibits significant genetic variation between *E. amylovora* CFBP 1430 and *E. amylovora* ATCC BAA-2158, a *Rubus*-infecting isolate. Though Eop1 has no role in virulence of pear or HR elicitation by *E. amylovora* CFBP 1430, Eop1 from *Rubus*-infecting *E. amylovora* ATCC BAA-2158 restricted disease development in immature pear when expressed in both *E. amylovora* strains (Asselin et al., 2011). Conversely, the Eop1 allele native to *E. amylovora* CFBP 1430 increased virulence on pear when expressed in the *Rubus* isolate *E. amylovora* ATCC BAA-2158 (Asselin et al., 2011).

Like Eop1, HopX1$_{Ea}$, an AvrPphE homolog of *Pseudomonas syringae*, is not required for virulence (Bocsanczy et al., 2012). However, over-expression of HopX1$_{Ea}$ in *E. amylovora* CFBP 1430 caused delayed disease development in pear fruits and apple shoots while a

chromosomal deletion of *hopX1*$_{Ea}$ retarded HR development in the non-host *Nicotiana tabacum* (Bocsanczy et al., 2012). This indicates that HopX1$_{Ea}$ quantitatively contributes to avirulence and may limit host range as exemplified by Eop1. Interestingly, inoculation of *E. amylovora* strains over-expression *hopX1*$_{Ea}$ in *N. benthamiana* suppresses HR development (Bocsanczy et al., 2012). Continued dissection of the differential responses of non-host *Nicotiana* species to HopX1$_{Ea}$ may help elucidate the avirulence role of HopX1$_{Ea}$ during compatible interactions with apple and pear.

1.4 Harpins and translocation

Harpins represent a class of glycine-rich, heat-stable, cysteine-lacking proteins unique to the T3SS of plant pathogenic bacteria (Wei et al., 1992). *E. amylovora* produces two harpins: HrpN and HrpW (Wei et al., 1992; Kim and Beer, 1998). HrpN is distinguished as a pathogenicity factor and the first protein identified as a cell-free elicitor of the hypersensitive response (Wei et al., 1992). *In vitro* and *in planta* expression analyses suggest that *hrpN* is transcribed at early stages of the host-bacterium interaction (McNally et al., 2012; Sarowar et al., 2011; Pester et al., 2012) and that the HrpN protein is secreted via the T3SS into the host apoplast (Perino et al., 1999, Nissinen et al., 2007). Fusions of the HrpN protein to the translocation reporter CyaA also indicate low levels of HrpN translocation into tobacco and apple cells (Bocsanczy et al., 2008; Boureau at al., 2011). The significance of HrpN translocation remains to be determined.

During disease development, HrpN is hypothesized to function as a T3SS translocator. Translocators associate with the distal tip of the T3SS pilus, exhibit pore-formation abilities in

host membranes, and facilitate the delivery of pathogen effectors into the host cytoplasm (Buttner and He 2009). To date, no T3SS translocators for plant pathogens have been demonstrated to directly associate with T3SS pili, but indirect evidence indicates that HrpN does interact with host and non-host membranes causing changes in ion flux (Wei and Beer 1992; El-Maarouf et al., 2001; Reboutier et al., 2007a; 2007b). Interestingly, ion fluxes triggered by purified HrpN are species specific and opposite in host apple and non-host *Arabidopsis thaliana* (Reboutier et al., 2007a). This species specificity may play an important role is successful disease development versus HR elicitation. In addition, the role of HrpN as a translocator is suggested by its requirement for wild-type levels of DspA/E translocation into *N. tabacum* (Bocsanczy et al., 2008). Low levels of HrpN translocation also require functional *hrpN* (Boureau et al., 2011).

As plant membrane reactions to HrpN are species specific, host and non-host downstream responses exhibit equal variability. Non-host interactions with HrpN are characterized by the HR and induction of systemic acquired resistance (SAR) (Wei et al., 1992; Peng et al., 2003). HR elicitation in tobacco requires functional *hrpN* and purified HrpN produces dose-dependent cell death in *A. thaliana* cell cultures (Barny 1995; Sinn et al., 2008). Interestingly, HrpN-induced HR can be suppressed in tobacco by AvrPtoB, an effector of *P. syringae* known to block the HR (Oh and Beer 2005). Sinn et al. (2008) conducted research that revealed multiple intriguing details regarding HR elicitation in tobacco and virulence in immature pears. Sinn and colleagues found that high concentrations of *E. amylovora* (1×10^8 CFU/ml) cells do not require functional HrpN for HR induction in tobacco indicating that additional elicitors in *E. amylovora* may play a larger role in plant-microbe interactions. The group also created *hrpN* mutant alleles that are required to generate HR and virulence when expressed in a *E. amylovora hrpN* null

mutants. Conversely, these same *hrpN* mutant alleles were not required for HR elicitation when produced in *Escherichia coli* and infiltrated into tobacco as a cell-free suspension. This suggests that additional factors contribute to HrpN-mediated HR elicitation by *E. amylovora* and that HrpN virulence and avirulence functions may be inseparable during compatible host-microbe interactions. In addition to HR, HrpN also triggers salicylic acid-dependent SAR in non-host and host plants. Transient expression of HrpN in tobacco increased resistance to the necrotrophic fungus *Botrytis cinerea* while exogenous application of HrpN to apple trees has been shown to induce resistance to blue mold and *Venturia* spp. scab diseases (de Capdeville et al., 2003; Barbosa-Mendes et al., 2009; Percival et al., 2009).

The molecular response of host and non-host species to HrpN is characterized by production of reactive oxygen species, callose deposition, mitogen-activated protein kinase signaling, and defense marker gene expression (Baker et al., 1993; Adam et al.,, 1999; Dong et al., 1999; Peng et al., 2003; Boureau et al., 2011). Recent analyses suggest that HrpN is not required for elicitation of the oxidative burst in apple (Boureau et al., 2011). *E. amylovora hrpN* mutants and purified HrpN are not involved in callose deposition in *A. thaliana* (Degrave et al., 2008). Conversely, investigations found that HrpN does elicit callose deposition in apple and that *in trans* production of *hrpN* was capable of triggering callose deposition in a *dspA/E* and *hrpN* double-mutant (Boureau et al., 2011). Together with information implicating DspA/E as a callose suppressor, a model is emerging that HrpN facilitates DspA/E translocation while DspA/E inhibits HrpN-mediated callose deposition.

HrpW is a second harpin produced by *E. amylovora* representing a larger group of plant pathogenic translocators exhibiting C-terminal pectate lyase domains (Kim and Beer 1998). Like HrpN, HrpW is secreted by the T3SS, elicits HR on non-host species, and purified HrpW

triggers dose-dependent cell death of *A. thaliana* cell culture (Kim and Beer 1998; Nissinen et al., 2007; Reboutier et al., 2007b). Unlike HrpN, *hrpW* mutants do not exhibit a virulence phenotype. HrpW does not induce callose deposition in apple and, while HrpW contributes to DspA/E translocation rate, DspA/E-CyaA translocation is not statistically affected by *hrpW* mutations (Bocsanczy et al., 2008; Boureau et al., 2011). Intriguingly, *hrpW* mutants induce more electrolyte leakage than wild-type *E. amylovora* in *A. thaliana* and *N. tabacum* and data from Reboutier et al., (2007b) suggest that the primary function of HrpW is to ameliorate HrpN-induced host defense responses as mixing purified HrpW with purified HrpN decreases HrpN-triggered cell death, oxidative burst, and host membrane changes in conductivity during treatment of *A. thaliana* cell suspensions (Gaudriault et al., 1998; Degrave et al., 2008). Collectively, research into the functions of HrpW, HrpN and DspA/E suggests that mediating HrpN-induced host defense responses may play a large role in disease development by *E. amylovora*.

In addition to harpin translocators like HrpN and HrpW, *E. amylovora* also produces a third putative translocator, HrpK. Little is known about HrpK but, like HrpW, it is not required for disease development. Likewise, HrpK is also secreted via the T3SS and null mutants exhibit slower DspA/E-CyaA translocation than wild-type *E. amylovora* (Nissinen et al., 2007; Bocsanczy et al., 2008). Despite overall slower DspA/E translocation in *hrpK* mutants, total DspA/E secretion may be greater than wild-type *E. amylovora* suggesting that HrpK may function as a negative regulator of translocation (Bocsanczy et al., 2008). It remains to be determined if HrpK function regulates HrpN-induced host defense responses as do HrpW and DspA/E.

1.5 Additional virulence factors affecting fire blight pathogenesis

For successful disease development by *E. amylovora* during compatible host interactions, *E. amylovora* synthesizes the exopolysaccharides levan and amylovoran (Sjulin and Beer 1978; Geier et al., 1993). While levan mutants are attenuated in virulence and biofilm production, amylovoran is a pathogenicity factor (Bellemann and Geider 1992; Koczan et al., 2009; Zhao et al., 2009). Originally considered a toxin, amylovoran is currently hypothesized to facilitate fire blight development via the occlusion of water-conducting xylem elements during *E. amylovora* biofilm formation (Sjulin and Beer 1978). This hypothesis is supported by observations that decreasing amylovoran viscosity decreases xylem blockage (Sijam et al., 1985). Amylovoran also is an important component of the *E. amylovora* biofilm and as such aids in mediating survival to environmental stresses like copper and presumably antimicrobial compounds produced during infection (Ordax et al., 2010). The dual importance of amylovoran production and type III secretion is highlighted by the observation that co-inoculation of immature pears with amylovoran and T3SS *E. amylovora* mutant strains can rescue the loss-of-pathogenicity phenotype exhibited by either strain inoculated independently (Zhao et al., 2009a). Notably, co-inoculation of either mutants with *Rubus*-adapted *E. amylovora* strain MR1 failed to complement their respective phenotypes suggesting fundamentally unsolved issues surrounding *E. amylovora* host-specificity (Zhao et al., 2009a).

Rubus-adapted *E. amylovora* strains are also distinguished from Spiraeoideae-infecting genotypes by lipopolysaccharide (LPS) biosynthesis (Rezzonico et al., 2012). LPS are large molecules composed of lipids and polysaccharides and serve as important components of the outer membrane of Gram-negative bacteria. LPS protects bacteria from antimicrobial compounds while also serving as important elicitors of host defense (Ingle et al.,, 2006). The

LPS ligase WaaL represents an important difference between LPS biosynthetic gene clusters in the two *E. amylovora* pathovars as their respective WaaL proteins share modest to no homology at the amino acid level (Rezzonico et al., 2012). In Spiraeoideae-infecting *E. amylovora* Ea1189, WaaL was characterized as an important virulence factor required for tolerance to antimicrobial compounds and twitching motility (Berry et al., 2009).

Erwinia tasmaniensis Et1/99 is a non-pathogenic relative of *E. amylovora* and consequently a useful source for identifying putative virulence factors via genome wide comparisons of the two Erwinia species. Exemplifying this concept is the lack of an amylovoran biosynthetic gene cluster in the genome of *E. tasmaniensis* Et1/99 (Kube et al., 2008). In addition to the amylovoran discrepancy, *E. amylovora* produces a number of verified virulence factors not found encoded in the *E. tasmaniensis* genome. These include the *srl* sorbitol metabolism operon, a putative toxin encoded by the *hsv* gene cluster, as well as the protease PrtA (Kube et al., 2008).

In rosaceous species, sorbitol is utilized for carbohydrate transportation. The first gene of the *srl* operon, *srlA*, is co-expressed with the T3SS in minimal media and mutations affecting the *srl* operon caused reduced virulence or loss-of-pathogenicity after inoculation into apple seedlings (Aldridge et al., 1997; McNally et al., 2012). The gene cluster *hsvABC* shares sequence homology with genes involved in the peptide biosynthesis of phaseolotoxin in *P. syringae* pv. *phaseolicola* (Oh et al., 2005). While no phaseolotoxin activity nor small toxin-like peptide has been discovered in *E. amylovora* to date, mutations in *hsvABC* display attenuated virulence in apple shoots (Oh et al., 2005). PrtA is a metalloprotease secreted via the type I secretion system (Zhang et al., 1999). While PrtA is required for full virulence in apple seedlings, the mechanism underlying PrtA expression remains unknown (Zhang et al., 1999).

Iron is an important cofactor for many proteins and consequently of vital nutritional importance. *E. amylovora* synthesizes the siderophore desferrioxamine E (DFO) as well as the TonB-dependent ferrioxamine receptor FoxR (Kachadourian et al., 1996; Dellagi et al., 1998). DFO is also important for protection against oxidative stress (Venisse et al., 2003). In *E. amylovora*, *dfoE* and *foxR* mutants exhibit severe inhibition of flower infection highlighting iron scarcity *in planta* during host interactions (Dellagi et al., 1998).

Another important nutritional factor is the vitamin thiamine. In *E. amylovora*, the ubiquitous plasmid pEA29 encodes thiamine biosynthesis genes ThiOSGF (McGhee and Jones 2000). *E. amylovora* strains cured of pEA29 are less virulent than wild-type *E. amylovora* and exhibited decreased amylovoran production (Falkenstein et al., 1989; McGhee and Jones 2000).

Survival *in planta* requires that *E. amylovora* not only meet nutritional demands but also persist in and colonize host extracellular spaces characterized by the presence of preformed phytoanticipins and induced antimicrobial compounds. Along with protective biofilm matrices and the LPS ligase WaaL, *E. amylovora* also utilizes a tripartite system consisting of the multidrug efflux pump AcrAB and the outer membrane secretin TolC (Burse et al., 2004; Al-Karablieh et al., 2009). In *E. amylovora*, *acrB* and *tolC* mutants are non-pathogenic or acutely reduced in shoot virulence and both strains are unable to persist *in planta* (Burse et al., 2004; Al-Karablieh et al., 2009). When challenged with apple leaf extracts, the growth of the *acrB* mutant was inhibited in agar diffusion assays and both AcrB and TolC are required for survival in the presence of a wide range of plant phytoalexins (Burse et al., 2004; Al-Karablieh et al., 2009). Taken together, these data suggest that survival *in planta* is an active process and that AcrAB and TolC function collaboratively to mediate *E. amylovora* survival via the export of host defense compounds.

Table 1.1 Genes identified as virulence determinants in *Erwinia amylovora*

Gene	Accession	Description	Function	Reference
Movement				
eae	EAM_0301	invasin	attachment	Koczan 2011
hofC	EAM_0729	type IV pilus assembly	attachment	Koczan 2011
crl	EAM_0898	curlin activator	attachment	Koczan 2011
fliH	EAM_1494	flagellar assembly	motility	Cesbron 2006
fimD	EAM_2951	type I pilus usher	attachment	Koczan 2011
Metabolism				
foxR	EAM_0358	TonB-dependent siderophore receptor	iron acquisition	Dellagi 1998
dfoA	EAM_0360	desferrioxamine synthesis	iron acquisition, oxidative burst survival	Dellagi 1998
srlE	EAM_0522	PTS sorbitol-specific transporter	sorbitol metabolism	Aldridge 1997
srlD	EAM_0524	sorbitol-6-phosphate dehydrogenase	sorbitol metabolism	Aldridge 1997
scrA	EAM_1596	PTS system sucrose-specific transporter	sucrose metabolism	Bogs & Geider 2000
scrY	EAM_1597	sucrose porin	sucrose metabolism	Bogs & Geider 2000
galE	EAM_2161	UDP-glucose 4-epimerase	galactose metabolism	Metzger 1994
Growth				
0344	EAM_0344	translation factor	translation	Wang & Beer, 2006
carB	EAM_0661	carbamoylphosphate synthase	pyrimidine and arginine synthesis	Wang & Beer, 2006
pyrD	EAM_1366	dihydroorotate dehydrogenase	pyrimidine synthesis	Wang & Beer, 2006
mltE	EAM_1530	lytic murein transglycosylase	cell elongation and division	Zhao 2005
cysB	EAM_1869	HTH-type transcriptional regulator	cysteine synthesis	Wang & Beer, 2006
purM	EAM_2441	phosphoribosylaminoimidazole synthetase	purine synthesis	Wang & Beer, 2006
guaB	EAM_2465	inosine-5'-monophosphate dehydrogenase	purine synthesis	Eastgate 1997
tyrA	EAM_2620	bifunctional chorismate mutase	prephenate synthesis	Wang & Beer, 2006
pyrB	EAM_3054	aspartate carbamoyltransferase	pyrimidine synthesis	Wang & Beer, 2006

Table 1.1 (cont'd)

Exopolysaccharides				
amsE	EAM_2167	glycosyltransferase	amylovoran synthesis	Bellemann & Geider, 1992
amsD	EAM_2168	glycosyltransferase	amylovoran synthesis	Bellemann & Geider, 1992
amsC	EAM_2169	oligosaccharide repeat unit polymerase	amylovoran synthesis	Bellemann & Geider, 1992
amsB	EAM_2170	glycosyltransferase	amylovoran synthesis	Bellemann & Geider, 1992
amsA	EAM_2171	tyrosine-protein kinase	amylovoran synthesis	Bellemann & Geider, 1992
amsI	EAM_2172	tyrosine-phosphatase	amylovoran synthesis	Bugert & Geider, 1995
amsH	EAM_2173	amylovoran export	amylovoran synthesis	Bugert & Geider, 1995
amsG	EAM_2174	UDP-galactose-lipid carrier transferase	amylovoran synthesis	Bugert & Geider, 1995
lscC	EAM_3468	levansucrase	levan biosynthsis	Geier 1993
Host interactions				
waaL	EAM_0086	lipid A core - O-antigen ligase	lipopolysaccharide synthesis	Berry 2009
avrRpt2	EAM_0423	type III cysteine protease effector	host interactions	Zhao 2006
acrB	EAM_1016	multidrug efflux transporter	*in planta* survival	Burse 2004
hopX1	EAM_2190	type III effector	avirulence	Bocsanczy 2012
dspF	EAM_2871	type III effector chaperone	type III secretion	Gaudriault 2002
dspE	EAM_2872	type III avrE-like effector	host interactions, pathogenicity	Barny 1990
eop1	EAM_2875	type III peptidase effector	avirulence	Asselin 2011
hrpN	EAM_2877	type III secretion harpin/translocator	type III secretion	Wei 1992
hrpT	EAM_2879	type III secretion lipoprotein	type III secretion	Kim 1997
hrcC	EAM_2880	type III secretion outer membrane pore	type III secretion	Kim 1997
hrpE	EAM_2883	type III secretion apparatus protein	type III secretion	Kim 1997
hrcJ	EAM_2885	type III secretion inner-membrane protein	type III secretion	Kim 1997
hrpB	EAM_2886	type III secretion protein	type III secretion	Kim 1997
hrpA	EAM_2887	type III pilus protein	type III secretion	Kim 1997
hrpJ	EAM_2895	type III secretion regulator	type III secretion	Nissinen 2007
hrcV	EAM_2896	type III secretion inner membrane protein	type III secretion	Wei & Beer, 1993

Table 1.1 (cont'd)

hrcN	EAM_2898	type III secretion system ATPase	type III secretion	Wei & Beer, 1993
hrcT	EAM_2904	type III secretion apparatus protein	type III secretion	Bogdanove 1996
hsvC	EAM_2908	biotin carboxylase	toxin synthesis	Oh 2005
hsvB	EAM_2909	biotin carboxylase	toxin synthesis	Oh 2005
hsvA	EAM_2910	amidinostransferase	toxin synthesis	Oh 2005
tolC	EAM_2988	outer membrane efflux	*in planta* survival	Al-Karablieh 2009
prtD	EAM_3366	type I secretion system ATPase	type I secretion	Zhang 1999
Unknown				
ydcN	EAM_1248	transcriptional regulator	unknown	McNally 2012
2937	EAM_2937	hypothetical protein	unknown	Wang & Beer, 2006
2938	EAM_2938	hypothetical protein	unknown	McNally 2012
nlpI	EAM_3066	tetratricopeptide repeat lipoprotein	unknown	McNally 2012

Accession numbers representing genes involved in *Erwinia amylovora* virulence and pathogenicity are annotated in accordance with the genome of *E. amylovora* ATCC 49946 (accession NC_013971).

The contents presented in chapter two can also be found in:

McNally, R.R., Toth, I., Cock, P., Pritchard, L., Hedley, P., Morris, J., Zhao, Y.F., and Sundin, G.W. (2012). Genetic characterization of the HrpL regulon of the fire blight pathogen *Erwinia amylovora* reveals novel virulence factors. Mol. Plant Pathol. 13, 160-173.

Chapter 2: Genetic characterization of the HrpL regulon of the fire blight pathogen *Erwinia amylovora* reveals novel virulence factors

2.1 Introduction

The enterobacterium *Erwinia amylovora* is the causal agent of fire blight, a devastating disease affecting apple, pear and other rosaceous plants. *E. amylovora* pathogenesis is dependent upon the production of a functional type III secretion system (T3SS), the type III effector DspA/E, and the exopolysaccharide amylovoran (Oh and Beer 2005). A T3SS is utilized by Gram negative bacterial plant pathogens and functions in the delivery of pathogen-derived effector proteins into the host cytoplasm (Buttner and He 2009). Type III effectors translocated into host cells function to suppress host defenses and promote infection (Hogenhout et al., 2009). Translocation of the *E. amylovora* type III effector DspA/E is required for pathogenesis and exemplifies the role of the T3SS in the development of fire blight (Barny et al., 1990; Bauer and Beer 1991; Bocsanczy et al. 2008; Triplett et al., 2009).

Structural, secreted, and translocated components of the T3SS are encoded by hypersensitive response and pathogenicity (*hrp*) genes located within a pathogenicity island (PAI) in the *E. amylovora* genome (Barny et al., 1990; Bauer and Beer 1991; Zhao et al., 2009a). Other genes encoding type III effectors such as *avrRpt2*$_{Ea}$ are located elsewhere in the genome (Zhao et al., 2006). In plant pathogens such as *E. amylovora* and *Pseudomonas syringae*, the ECF-family alternate sigma factor HrpL coordinates the transcription of T3SS genes (Innes et al., 1993; Shen and Keen 1993; Wei and Beer 1995; Chatterjee et al., 2002a).

The regulatory signals culminating in *hrpL* activation begin with environmental stimuli including unknown plant factors. A specific minimal medium (hrpMM), is used *in vitro* to

mimic conditions of the plant apoplast (Wei et al., 1992). While *hrp*-inducing stimuli may or may not be communicated via the two component signal transduction system *hrpXY* (Wei et al., 2000; Zhao et al., 2009b), the NtrC-family σ^{54} enhancer protein HrpS is a pathogenicity factor in many T3SS-dependent phytobacteria and is required for *hrpL* transcription in *E. amylovora* and other plant pathogens including *P. syringae* and other enteric plant pathogens in the genera *Dickeya*, *Pantoea* and *Pectobacterium* (Xiao et al., 1994; Wei et al., 2000; Hutcheson et al., 2001; Chatterjee et al., 2002b; Merighi et al., 2003; Yap et al., 2005). In addition to *hrpS*, σ^{54} and integration host factor (IHF) are also required for *hrpL* transcription in *Pectobacterium carotovorum* subsp. *carotovorum* Ecc71 (Chatterjee et al., 2002b).

The *hrp* promoter is a *cis*-element required for HrpL-mediated transcriptional activity (Innes et al., 1993; Shen and Keen 1993; Xiao and Hutcheson 1994; Wei and Beer 1995). In *Pantoea agglomerans* pv. *gypsophilae* 824-1 and *Dickeya dadantii* 3937, HrpL is known to exhibit RNA polymerase-dependent binding to the *hrp* promoter (Nissan et al., 2005; Yang et al., 2010). While functional *hrp* promoters exhibit sequence variability (Nissan et al., 2005; Vencato et al., 2006), conserved motifs have allowed for accurate prediction of genes subject to positive HrpL regulation (Fouts et al., 2002; Zwiesler-Vollick et al., 2002; Ferreira et al., 2006; Vencato et al., 2006; Yang et al., 2010).

Among HrpL-regulated genes, virulence factors not directly related to type III secretion have also been implicated as constituents of the HrpL regulon: most notably *P. syringae* pv. *tomato* DC3000 phytotoxins syringomycin and coronatine (Fouts et al., 2002; Sreedharan et al., 2006). In *E. amylovora*, the *hrp* pathogenicity island encodes putative phaseolotoxin-like biosynthetic proteins required for systemic fire blight development (Oh et al., 2005). Additional virulence roles for HrpL are also suggested by type III secretion-independent *hrpL* mutant

phenotypes including increased peroxidase activity and hypermotility (Faize et al., 2006; Cesbron et al., 2006).

Global analyses of gene regulation in bacteria have been greatly facilitated by the availability of microarrays. To date, genome-wide microarray analyses of the HrpL regulon have been conducted in *P. syringae* pv. *tomato* DC3000 and *D. dadantii* 3937 (Ferreira et al., 2006; Lan et al., 2006; Yang et al., 2010). In this study, an *E. amylovora* ATCC 49946 microarray was designed and validated and, combined with bioinformatic *hrp* promoter modeling, sought to characterize HrpL-mediated gene expression across the genome of *E. amylovora* Ea1189. Relevant to this study, it was hypothesized: (i) that microarray data comparing gene expression of wild-type (WT) *E. amylovora* to its corresponding *hrpL* mutant would reveal differential transcription of T3SS genes in *hrp*-inducing medium; (ii) that this microarray experiment would identify novel components of the HrpL regulon broader than T3SS genes; and (iii) that novel HrpL-regulated genes will play a quantifiable role in fire blight pathogenesis by *E. amylovora*.

2.2 Results

HrpL in *Erwinia amylovora* Ea1189 is a pathogenicity factor

In conformity with previous reports in *E. amylovora* Ea321 (Wei and Beer 1995), the *hrpL* chromosomal deletion mutant Ea1189Δ*hrpL* was non-pathogenic when inoculated into immature pear fruit (Fig 2.1). Percent necrosis measurements at four and six days post inoculation (dpi) revealed a complete inhibition of disease development and exemplified the role of *hrpL* as a pathogenicity factor during host infection (Fig 2.1). The *hrpL* mutant strain

was successfully complemented *in trans* with pRRM1, a clone of *hrpL*, restoring full virulence in the Ea1189Δ*hrpL* mutant (Fig 2.1).

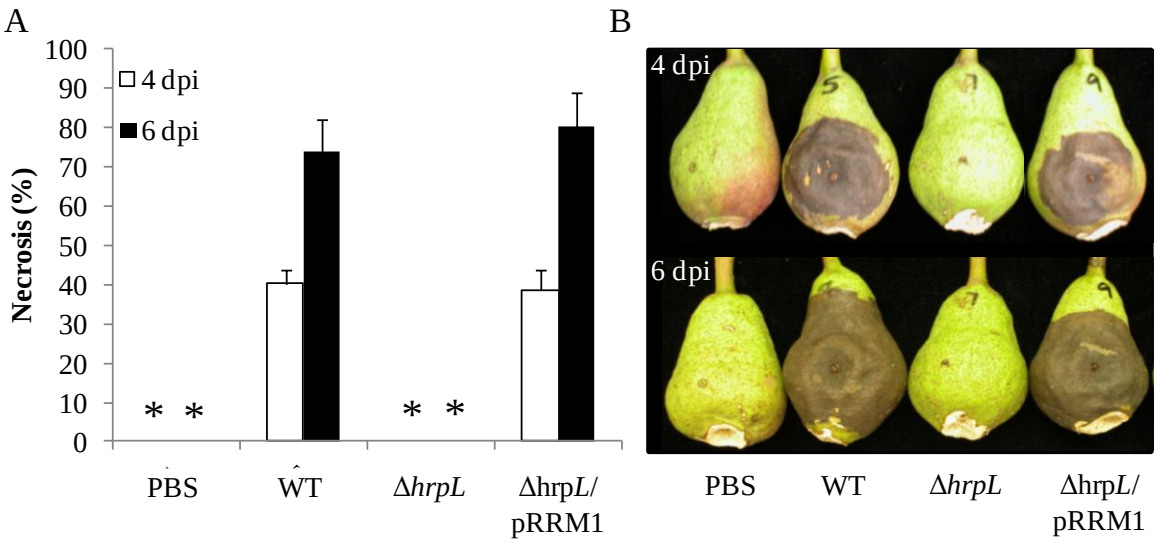

Figure 2.1 Immature pear fruit virulence assay. Pear fruits were inoculated with WT Ea1189, Ea1189Δ*hrpL* and Ea1189Δ*hrpL*/pRRM1 complemented *in trans* with full-length hrpL. Percent necrosis was observed and recorded four and six days post inoculation (dpi). WT Ea1189 represents a full virulence positive control. Phosphate-buffered saline (PBS) was used as a negative control. (A) In quantitative measurements of percent necrosis and (B) qualitative imagery of inoculated pears, the Ea1189Δ*hrpL* deletion mutant was non-pathogenic at four and six dpi. Complementation with plasmid-borne *hrpL* (pRRM1) fully restored pathogenicity to Ea1189Δ*hrpL* at all time points measured. *, Indicates results are significantly different (P-value < 0.05) from WT results at same day post-inoculation. Error bars represent standard error. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Microarray analyses reveal differential gene expression in Ea1189 and Ea1189Δ*hrpL*

Microarrays represent a genomics tool useful for the rapid identification of differentially-regulated genes. To begin characterization of the HrpL regulon, was an oligonucleotide microarray developed encompassing the annotated genes of the fire blight pathogen *E. amylovora* ATCC 49946. Since the two sequenced *E. amylovora* genomes exhibit more than 99.99% sequence conservation (Smits et al., 2010) and Agilent arrays utilize long 60mer oligonucleotide probes, it was surmised that this microarray would be applicable to working with any *E. amylovora* strain. Based on preliminary quantitative real time (qRT)-PCR to explore HrpL-mediated gene expression, strains were induced in hrpMM for 6 and 18 hours, total RNA was isolated from WT Ea1189 and Ea1189Δ*hrpL* and subjected to microarray analysis. Results indicated differential gene expression in Ea1189Δ*hrpL* relative to WT Ea1189. In total, 24 genes were found to be differently regulated with fold-change expression ratios greater than 1.5 (Fig 2.2). Of these, 19 genes exhibited direct or indirect positive regulation by HrpL and five genes were negatively regulated. The majority of genes exhibiting HrpL-mediated regulation were identified from RNA extracted after six hours post inoculation (hpi) in hrpMM. At 18 hpi, only five genes showed differential expression between WT Ea1189 and Ea1189Δ*hrpL* (Table 2.1). No gene indentified in our microarray analysis displayed HrpL-dependent transcript accumulation at both 6 and 18 hpi, suggesting that the characteristics of the HrpL regulon change dramatically over time (Table 2.1). No differential expression was observed from the microarray probes for plasmid encoded genes.

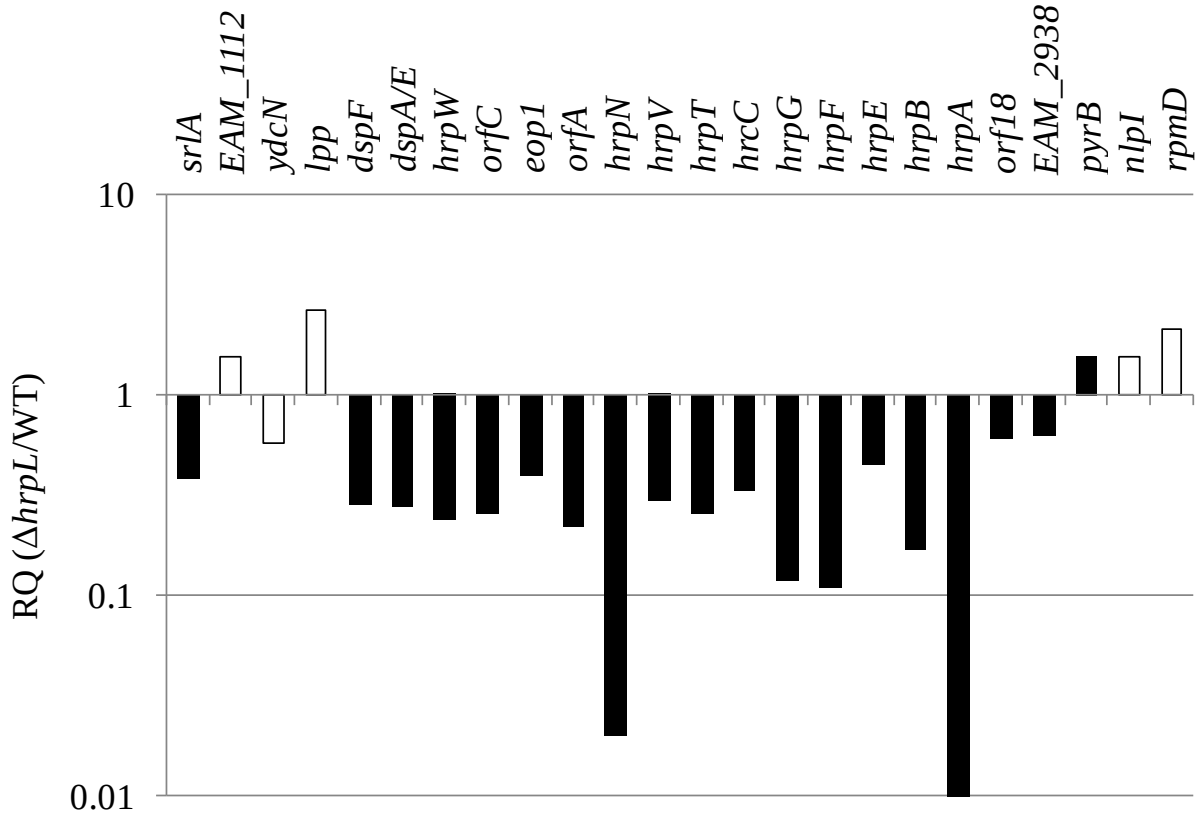

Figure 2.2 Microarray expression profile of in vitro HrpL regulon comparing RNA extractions from WT Ea1189 and Ea1189Δ*hrpL* after inoculation in hrpMM for 6 (black bars) and 18 (white bars) hours. Transcript abundance was increased for 19 genes and decreased for five genes in the presence of *hrpL*. Relative quantification (RQ) values satisfied P-values < 0.05 and expression ratios > 1.5X cut-off.

Table 2.1 Results of *E. amylovora* WT/Δ*hrpL* microarray analysis 6 and 18 hpi in hrpMM

Accession	Gene	Fold Δ (WT/Δ*hrpL*)	Description	qRT-PCR RQ	Et 1/99 ortholog
6 hpi					
EAM_2887	*hrpA**	100	T3SS pilus	0.58±0.1	+
EAM_2877	*hrpN**	40.0	T3SS translocator	0.74±0.1	+
EAM_2882	*hrpF**	9.43	T3SS protein		+
EAM_2881	*hrpG*°	8.55	T3SS protein		+
EAM_2886	*hrpB*°	5.81	T3SS protein		+
EAM_2876	*orfA*°	4.50	T3SS chaperone		+
EAM_2873	*hrpW**	4.20	T3SS translocator		+
EAM_2879	*hrpT*°	3.82	T3SS protein		+
EAM_2874	*orfC*°	3.77	T3SS chaperone		+
EAM_2872	*dspA/E**	3.55	T3SS effector	1.01±0.2	+
EAM_2871	*dspF*°	3.40	T3SS chaperone		+
EAM_2878	*hrpV*°	3.32	T3SS protein		+
EAM_2880	*hrcC*°	2.96	T3SS protein		+
EAM_0521	*srlA*	2.56	sorbitol permease		–
EAM_2875	*eop1*°	2.48	T3SS effector		+
EAM_2883	*hrpE*°	2.22	T3SS protein		+
EAM_2912	*orf18*	1.63	hypothetical protein	0.71±0.2	–
EAM_2938	*EAM_2938*	1.57	membrane protein	0.66±0.1	–
EAM_3054	*pyrB*	-1.54	carbamoyltransferase	0.71±0.1	+
18 hpi					
EAM_1248	*ydcN*	1.76	XRE transcriptional regulator	1.77±0.1	+
EAM_3066	*nlpI*	-1.53	Tetratricopeptide lipoprotein	0.57±0.1	+
EAM_1112	*EAM_1112*	-1.55	hypothetical protein	1.56±0.0	–
EAM_3180	*rpmD*	-2.11	50S ribosomal protein L30		+
EAM_1656	*lpp*	-2.64	membrane lipoprotein	1.40±0.0	+

*, HrpL-regulated has known *hrp* promoter 5´ adjacent to open-reading frame; °, gene is constituent of HrpL-regulated operon in T3SS PAI; +/– , presence/absence of predicted *E. amylovora* ortholog in *Erwinia tasmaniensis* Et1/99 with E-value less than 1.00E-04 in NCBI protein database. Gene annotations are in accordance with *E. amylovora* ATCC 49946 genome (Sebaihia et al., 2010).

Positive Regulation by HrpL in microarray analysis

The majority of genes displaying HrpL-mediated regulation in hrpMM 6 hpi encode components of the T3SS, including structural components of the T3SS such as *hrcC*, the harpins *hrpN* and *hrpW*, effectors *dspA/E* and *eop1*, and type III chaperones *dspF* and *orf*C. Of all genes under HrpL regulation, the pilus subunit gene *hrpA* exhibited the greatest fold change in transcript abundance in Ea1189Δ*hrpL* relative to WT Ea1189 (Table 2.1). This is compatible with the large number of HrpA subunits presumably required for functional T3SS pilus formation (He and Jin 2003).

While T3SS components are transcribed from known *hrp* promoters (Wei and Beer 1995), our microarray data revealed four individual genes (*srlA*, *orf18*, *EAM_2938* and *ydcN*) at different genomic locations that were up-regulated by HrpL and all lacked recognizable *hrp* promoters (Table 2.1). *srlA*, *orf18* and *EAM_2938* transcription was HrpL-dependent in hrpMM 6 hpi (Table 2.1). The sorbitol permease *srlA* is a known virulence factor utilized during apple shoot infection (Aldridge et al., 1997). *orf18* is predicted to encode a VirK-like protein with signal peptides that, though located within the T3SS PAI of *E. amylovora* remains uncharacterized and lacks a canonical *hrp* promoter upstream of the translational start site (Oh et al., 2005). *EAM_2938* encodes an uncharacterized membrane protein. The only gene identified as being positively regulated by HrpL in hrpMM at 18 hpi is *ydcN*, a XRE family transcriptional regulator predicted via sequence similarity to an uncharacterized *Pantoea* sp. At-9b protein Pat9b_1215 (accession NC_014837.1; E-value 3e-38) using NCBI protein-protein BLAST (Table 2.1). *ydcN* also lacks a *hrp* promoter, and collectively these results suggest that indirect HrpL regulation takes place, and that the HrpL regulon of *E.*

amylovora is part of a larger signaling network interconnected to broader gene regulatory networks.

Negative regulation by HrpL in microarray analysis

While functionally implicated as a positive regulator of genes important for pathogenesis and hypersensitive response elicitation, genome-wide analyses of the HrpL regulon in other bacterial plant pathogens have also identified negative HrpL-mediated gene expression (Ferreira et al., 2006; Lan et al., 2006; Yang et al., 2010). Of the 24 genes identified in our microarray study as components of the HrpL regulon, five genes demonstrated negative regulation by HrpL, i.e. showed an increased transcript abundance in Ea1189Δ*hrpL* (Table 2.1). The majority of these genes were differentially expressed in hrpMM 18 hpi, indicating that positive HrpL-mediated gene expression is reduced over time *in vitro* (Table 2.1). Genes negatively regulated by HrpL in hrpMM 18 hpi include a lipoprotein with tetratricopeptide repeats designated *nlpI* (Table 2.1). The only gene negatively regulated by HrpL in hrpMM at 6 hpi is the aspartate carbamoyltransferase catalytic subunit *pyrB* (Table 2.1).

Identification of *hrp* promoters in *E. amylovora* using hidden Markov modeling

To complement our microarray results the HrpL regulon was additionally analyzed for the occurence and distribution of *hrp* promoters based on an alignment of 99 known promoter regions from *Erwinia* and *Pseudomonas* species using T-Coffee multiple alignment software (Notredame et al., 2000). The consensus motif of this alignment (GGAAC-N18/N19-ACNNA) (Fig 2.3A) is less conserved than recent *hrp* promoter alignments

reported in *P. syringae* alone (Fouts et al., 2002; Ferreira et al., 2006). While comparisons of *hrp* promoter sequence alignments across genera did not reveal stark differences between promoter elements, the -10 motif of *P. syringae hrp* promoters exhibited additional conserved residues (Fig 2.3). Therefore, to identify novel *hrp* promoters in *E. amylovora*, a hidden Markov model (HMM) was created from a multiple sequence alignment of 54 *Erwinia*, *Pectobacterium*, *Pantoea* and *Dickeya hrp* promoters (Fig 2.3C) and tested against the genome of *E. amylovora* ATCC 49946 using HMMer 2.3.2 biosequence analysis software (Eddy 1998). Thirty *hrp* promoters were predicted with a bit-score classifier threshold of 8.5 (Table 2.2). Eleven of the predicted *hrp* promoters are positioned upstream of open reading frames or operons encoding T3SS components including all known structural, secreted, and translocated T3SS proteins. Of a further 19 putative *hrp* promoters, *hsvA*, *orf12* and *orf19* are located on the T3SS PAI, although their respective contributions to type III secretion and translocation are unknown (Oh et al., 2005).

The remaining candidate *hrp* promoters are not oriented to transcribe mRNA related to type III secretion (Table 2.2) and may be involved in regulating the expression of other virulence factors consistent with observations in *E. amylovora* that *hrpL* mutants exhibit increased peroxidase activity relative to type III secretion null strains and that *hrpL* mutants are also hyper-flagellated and more motile than WT *E. amylovora* (Cesbron et al., 2006; Faize et al., 2006).

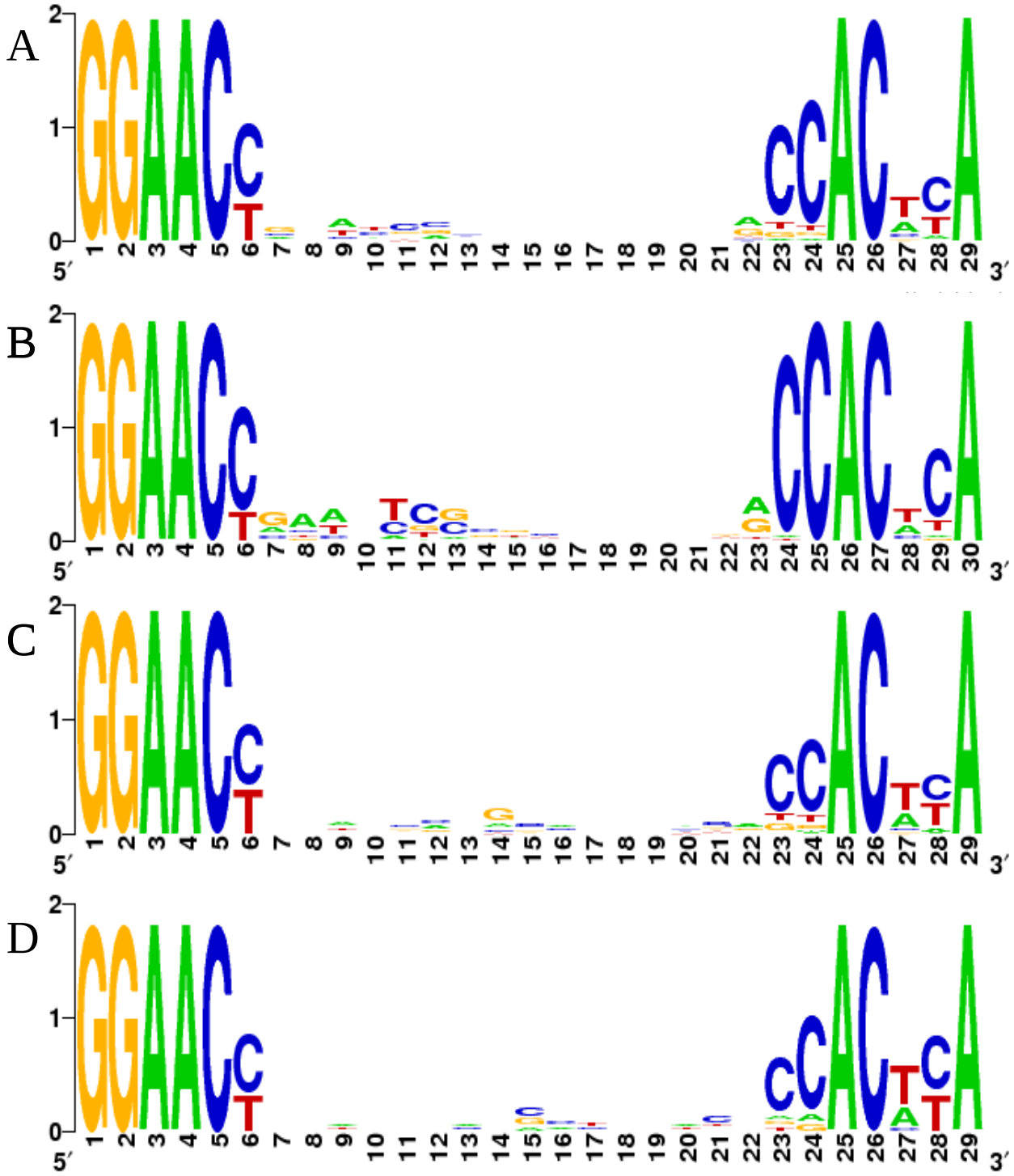

Figure 2.3 T-Coffee multiple sequence alignment of *hrp* promoters. Weblogo was used to visualize alignments of (A) 99 *hrp* promoters from phytopathogenic pseudomonads and

Figure 2.3 (cont'd) *Erwinia* species, (B) 45 *hrp* promoters collected from *P. syringae* pathovars, (C) 54 *hrp* promoters collected from *Erwinia*, *Pectobacterium*, *Dickeya* and *Pantoea* spp. and (D) 13 *hrp* promoters from *E. amylovora*. *hrp* promoter motifs are broadly conserved across phytopathogenic bacteria excluding additionally conserved cytosine residues 5´ of -10 motif in *Pseudomonas* spp.

Verification of microarray and HMMer data with quantitative real time-PCR

To validate *hrpL* gene expression patterns observed in our microarray analysis and to verify the HrpL-dependent activity of novel *hrp* promoters predicted using HMM 2.3.2, 20 genes based on results from the microarray and HMMer analyses were selected for expression profiling. RNA was extracted from WT Ea1189 and Ea1189Δ*hrpL* after induction in hrpMM for 6 and 18 hours and subjected to qRT-PCR analysis. qRT-PCR expression results in triplicate confirm HrpL-mediated transcript accumulation for 18 genes (Table 2.2). These results are in agreement with our microarray data and highlight 8 novel *hrp* promoters predicted via hidden Markov modeling. Based on 22 hrp promoters experimentally verified with microarray expression data and qRT-PCR, the conserved *hrp* promoter in *E. amylovora* is 5´-GGAAC-$N_{(16-20)}$-ACNNC-3´ and averages 88 nucleotides 5´ adjacent of a HrpL-regulated gene (Table 2.2). These characteristics are largely consistent with previously reported *hrp* promoter motifs with the exception of the conjugative transfer gene *traF* and a predicted exported lipoprotein *yfiM*, both of which exhibit -35 and -10 promoter motifs separated by an abnormal number of nucleotides, 16 and 20 respectively (Table 2.2).

Table 2.2 Results of hrp promoter modeling and qRT-PCR analysis

Annotation	HMM (E-value)	Description	qRT-PCR RQ	Et 1/99	Position (nt from ORF)	nt b/t motifs
Type III Secretion						
eop2	6.0E-05	T3SS helper	0.60±0.1	–	-139	18
*hrpA**	2.5E-04	T3SS pilus	0.58±0.1	+	-058	18
*hrpF**	4.9E-04	T3SS protein		+	-033	19
hrpJ	9.7E-04	T3SS protein	0.57±0.1	+	-035	18
*hrpW**	1.2E-03	T3SS translocator		+	-059	18
*hrpN**	1.2E-03	T3SS translocator	0.74±0.1	+	-077	18
hrpK	1.5E-03	T3SS protein	0.57±0.1	–	-036	18
$hopC_{Ea}$	1.8E-03	T3SS effector	0.57±0.1	–	-031	18
$avrRpt2_{Ea}$	2.8E-03	T3SS effector	0.61±0.1	–	-032	18
*dspA/E**	4.6E-03	T3SS effector	1.01±0.2	+	-042	18
eop3	6.5E-03	T3SS effector	0.54±0.1	–	-551	19
Other						
yfiM	5.0E-04	exported lipoprotein	0.50±0.1	+	-173	16
orf19	7.2E-04	lysozyme inhibitor	0.40±0.0	+	-076	18
invG	8.3E-04	non-*hrp* T3SS protein		+	-122	20
*EAM_2938**	8.9E-04	membrane protein	0.66±0.1	–	-126	18
traF	1.2E-03	conjugation protein	0.53±0.1	+	-156	20
EAM_1012	1.3E-03	hypothetical protein	0.64±0.1	–	-043	18
ybhH	1.3E-03	hypothetical protein	0.58±0.1	–	-211	18
cysJ	1.6E-03	sulfite reductase		+	-013	16
orf12	1.7E-03	N-acyltransferase	0.50±0.1	+	-031	18
aroQ	2.2E-03	chorismate mutase	0.48±0.1	–	-083	19
fimD	3.2E-03	fimbrial usher	0.96±0.2	+	-034	17
hsvA	3.4E-03	amidinotransferase	0.38±0.1	–	-098	18
ompA	4.4E-03	membrane protein		+	-079	22
yceG	4.8E-03	chorismate lyase		+	-014	15
terC	5.1E-03	membrane protein		+	-678	16
yjbB	5.9E-03	symporter		+	-114	16
yaiL	6.2E-03	hypothetical protein		+	-115	16
fliN	6.3E-03	flagellar motor switch	0.55±0.1	+	-199	18
yafS	6.5E-03	methyltransferase		+	-283	24

*, identified as HrpL-regulated in WT/Δ*hrpL* microarray analysis; HMM, hidden Markov model; +/– , presence/absence of predicted *E. amylovora* ortholog in *E. tasmaniensis* Et1/99 with E-value less than 1.00E-04 in NCBI protein database; nt b/t motifs, number of nucleotides between the -35 and -10 promoter regions.

Mutational analyses of HrpL-regulated genes

While HrpL is required for the transcriptional promotion of the T3SS (Wei and Beer 1995; Chatterjee et al., 2002a; Innes et al., 1993; Shen and Keen 1993), the HrpL regulon is also implicated in additional regulatory activities such as regulating motility (Cesbron et al., 2006; Ortiz-Martin et al., 2010). To determine the biological relevance of genes regulated by HrpL several chromosomal mutations were generated in Ea1189. These mutants were assayed for phenotypes related to factors important for fire blight pathogenesis including virulence, biofilm formation, and swarming motility. Ea1189Δ*EAM_2938*, Ea1189Δ*ydcN* and Ea1189Δ*nlpI* were all significantly less virulent than WT Ea1189 in immature pear assays measured four and six dpi (Fig 2.4A; Fig. 2.4B). *EAM_2938*, *ydcN*, and *nlpI* have not been shown previously to contribute to the virulence of *E. amylovora*. *orf18*, a HrpL-regulated constituent of the T3SS PAI in *E. amylovora*, appeared not to play a quantifiable role in virulence (Fig 2.4B). The *ydcN* mutant strain was complemented with pRRM2, a clone of *ydcN*, restoring virulence in the Ea1189Δ*ydcN* mutant. The addition of pRRM3 to Ea1189Δ*EAM_2938* and pRRM4 to Ea1189Δ*nlpI* did not result in complementation of the cognate mutant phenotypes likely due to pleiotropic effects.

While known to play a role in regulation of the T3SS (Innes et al., 1993; Shen and Keen 1993; Wei and Beer 1995; Chatterjee et al., 2002a), previous examinations of HrpL function have also demonstrated that the HrpL regulon includes genes whose activity is not directly related to type III secretion (Yap et al., 2005; Cesbron et al., 2006; Faize et al., 2006; Sreedharan et al., 2006). To better understand the diversity of phenotypes observed in Δ*hrpL* strains, mutants in our selected HrpL-regulated genes were assayed for alterations in their biofilm formation and motility phenotypes. To examine swarming motility, mutants of

Ea1189Δ*EAM_2938*, Ea1189Δ*orf18*, Ea1189Δ*ydcN* and Ea1189Δ*nlpI* were measured two and four dpi. To assay biofilm formation these mutants were also cultured in the presence of glass cover-slips for 48 hours and stained with crystal violet prior to spectrophotometric analysis. In both assays, Ea1189Δ*ydcN* and Ea1189Δ*nlpI* demonstrated increased biofilm formation and decreased swarming motility relative to WT Ea1189 (Fig 2.4C; Fig 2.4D).

Analysis of the *EAM_2938* gene

In response to observations that *EAM_2938* is positively regulated by HrpL (Fig 2.2; Table 2.1), contains a novel *hrp* promoter (Table 2.2), and that the mutant Ea1189Δ*EAM_2938* exhibits a strong loss-of-virulence phenotype (Fig 2.4A; Fig 2.4B), the *EAM_2938* gene was subjected to bioinformatic analyses. Using the operon finding package FGENESB (Tyson et al., 2004), *EAM_2938* is predicted as the first open-reading frame in an operon upstream of *EAM_2937*, *EAM_2936* and *EAM_2935* (Fig 2.5). To determine whether *hrpL* can direct the transcriptional activiation of genes downstream of *EAM_2938*, qRT-PCR was conducted to study gene expression of genes downstream of *EAM_2938* using RNA extractions from WT Ea1189 and Ea1189Δ*hrpL* after induction in hrpMM for six hours. Results indicate that *EAM_2938*, *EAM_2937*, *EAM_2936* and *EAM_2935* are all differentially regulated by HrpL suggesting that *EAM_2938* may be the first gene in a HrpL-regulated operon (Fig 2.5). Based on protein-protein NCBI database searches, *EAM_2937* encodes a disulfide bond-forming inner membrane protein, *EAM_2936* encodes a phytochelatin synthase-like protein and the EAM_2935 protein is a predicted γ-glutamyltranspeptidase (Fig 2.5.). The EAM_2938 and EAM_2937 proteins are expected to be membrane localized with putative transmembrane regions predicted via Dense Alignment

Surface (DAS) analytical server (Fig 2.5). SignalP 3.0 indicates that the phytochelatin synthase-like gene *EAM_2936* encodes a putative exported protein due to the presence of a signal peptide (Fig 2.5).

EAM_2938 represents a novel virulence factor in Ea1189. To determine the distribution of EAM_2938 orthologs among phytopathogenic bacteria, the NCBI protein database was searched for amino acid sequences with significant sequence conservation. Interestingly, the presence of *EAM_2938* appears unique to *E. amylovora* relative to other bacterial plant pathogens (Fig 2.5). Similarly, the downstream genes *EAM_2937* and *EAM_2936* are also not broadly conserved in known plant pathogens, excluding the closely related species *E. pyrifoliae* (Fig 2.5). In addition, GC-profile software calculated that *EAM_2938* exhibits 42% GC content (Fig 2.5). The average GC content of the sequenced genome of *E. amylovora* is 53.6% (Smits et al., 2010; Sebaihia et al., 2010) suggesting that *EAM_2938* (42%), as well as *EAM_2937* (45%) and *EAM_2936* (49%), may have been recently acquired by the *E. amylovora* genome.

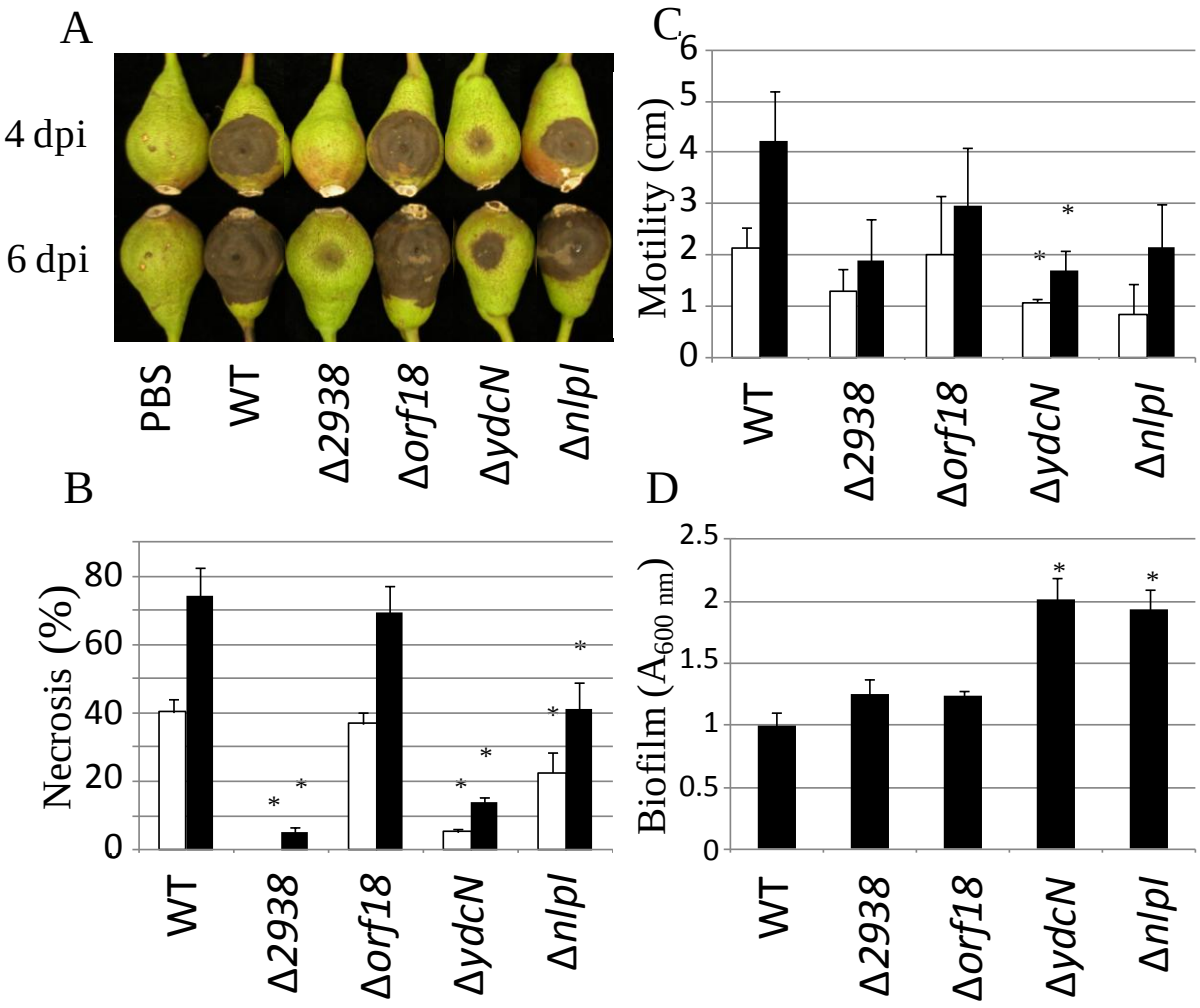

Figure 2.4 Phenotypic characterization of HrpL-regulated mutant strains in Ea1189.

(A) Symptoms of Ea1189Δ*EAM_2938*, Ea1189Δ*orf18*, Ea1189Δ*ydcN* and Ea1189Δ*nlpI* in immature pear fruit at 4 and 6 days post-inoculation and (B) % area of necrosis in immature pear fruit 4 (white) and 6 (black) days post-inoculation. Quantification of (C) cellular motility 2 (white) and 4 (black) days post-inoculation and (D) biofilm formation of Ea1189Δ*EAM_2938*, Ea1189Δ*orf18*, Ea1189Δ*ydcN* and Ea1189Δ*nlpI*. *, Indicates results were significantly different from WT Ea1189 at same time point at $P < 0.05$. Error bars represent standard error.

	2938	2937	2936	2935
%GC	42	45	49	54
RQ	0.66±0.07	0.59± 0.07	0.56±0.06	0.57±0.06
E-value	4e-07/ XBJ1_3905•	9e-45/ XNC1_3917#	7e-63/ SVI_1964○	0.0/ PC1_0062■

Figure 2.5 *EAM_2938* gene cluster including GC content (%GC) of individual genes relative to *E. amylovora* (53.6%), qRT-PCR relative quantification (RQ) of WT/Δ*hrpL* transcript abundance, and E-values and homologs determined using NCBI protein BLAST analysis. *, exhibits putative transmembrane domains predicted using DAS method software; ‡, displays signal peptide, predicted using SignalP 3.0; •, homolog found in *Xenorhabdus bovienii* SS-2004 (accession NC_013892) ; #, homolog found in *Xenorhabdus nematophila* ATCC 19061 (accession NC_ 014228); ○, homolog found in *Shewanella violacea* DSS12 (accession NC_ 014012); ■, homolog found in *Pectobacterium carotovorum* PC1 (accession NC_ 012917).

2.3 Discussion

In this study, the HrpL regulon of the fire blight pathogen *E. amylovora* was explored using a combination of techniques including microarray, bioinformatics and qRT-PCR, and identified 39 genes that exhibited HrpL-dependent transcriptional activity. Mutational analyses of constituents of the HrpL regulon revealed novel virulence factors with differential biofilm formation and motility phenotypes. Our results suggest that the HrpL regulon is interconnected with downstream signaling networks and that HrpL-regulated genes, in addition to those with predicted roles in type III secretion, are important in fire blight pathogenesis.

As in previous analyses of the HrpL regulon in other plant pathogenic bacteria including *P. syringae* pv. *tomato* DC3000 and *D. dadantii* Ech3937 (Fouts et al., 2002; Ferreira et al., 2006; Yang et al., 2010), the HrpL regulon in *E. amylovora* encompasses genes regulated directly (via the *hrp* promoter) as well as indirectly. Of the 24 genes identified in our microarray, 16 genes were directly up-regulated in the presence of functional *hrpL*. All genes identified in our microarray analysis that appeared to be directly regulated by HrpL, excluding *EAM_2938*, have been characterized or have predicted roles in type III secretion. These results exemplify the role of *hrpL* in coordinating the expression of type III secretion genes and are in accordance with previous genome-wide demonstrations of the role of *hrpL* in type III secretion (Fouts et al., 2002; Ferreira et al., 2006; Lan et al., 2006; Yang et al., 2010).

The type III effector repertoire of *P. syringae* pv. *phaseolicola* 1448A was reported to include 27 candidates (Vencato et al., 2006). Our combined results, including *hrp* promoter modeling and qRT-PCR, demonstrate the existence of only five effector-like genes (*eop1*, *eop3*, *avrRpt2*$_{Ea}$, *dspA/E*, and *hopPtoC*$_{Ea}$) subject to direct HrpL regulation (Table 2.2). Translocation of DspA/E is known to be required for pathogenesis of *E. amylovora*, but the comprehensive role of DspA/E in facilitating disease development remains elusive (Triplett et al., 2009). *avrRpt2*$_{Ea}$ exhibits homology to AvrRpt2 in *P. syringae* pv. *tomato* and is a known virulence factor in pears that is capable of eliciting the HR in Arabidopsis RPS2 when heterologously expressed from *Pst* DC3000 (Zhao et al., 2006). Both *dspA/E* and *hopPtoC*$_{Ea}$ are induced in an immature pear fruit IVET screen but mutations in *hopPtoC*$_{Ea}$ do not result in a quantifiable virulence defect or reduction in colonization in immature pear virulence

assays (Zhao et al., 2005). Eop1 and Eop3 are YopJ and HopX homologs, respectively and, along with DspA/E and *AvrRpt2*$_{Ea}$, were identified in mass spectrometric analysis of the T3SS-dependent secretome of *E. amylovora* ATCC 49946 (Nissinen et al. 2007). Mutations in *eop1* have no effect on virulence (Asselin et al., 2006), while the functional role of *eop3* remains uncharacterized.

Hidden Markov modeling for *in silico* genome-wide identification of conserved *cis*-elements is a tested strategy for predicting genes under direct *hrp* promoter-mediated transcription by HrpL (Ferreira et al., 2006; Vencato et al., 2006). To find other genes directly up-regulated by HrpL, a hidden Markov model was assembled from an *Erwinia* spp. *hrp* promoter alignment. Using HMMer 2.3.2, our model identified 30 putative *hrp* promoters in the genome of *E. amylovora* ATCC 49946, including all know components of the T3SS. Using qRT-PCR verification of hrpMM-induce WT Ea1189 and Ea1189Δ*hrpL* RNA extracts, 19 *hrp* promoters were verified as HrpL-regulated, 7 of which represent novel components of the HrpL regulon of Ea1189 not identified using microarray analysis presumably due to increased qRT-PCR sensitivity toward low copy-number transcripts.

EAM_2938 represents a novel HrpL-regulated gene first identified as being differentially expressed in our microarray analysis of WT Ea1189 and Ea1189Δ*hrpL*. HrpL-mediated up-regulation of *EAM_2938* was confirmed using qRT-PCR and promoter modeling identified a candidate *hrp* promoter 126 nucleotides up-stream of the translational start site of EAM_2938. Most notably, a chromosomal deletion of *EAM_2938* severely attenuated virulence in immature pear fruits. Three open-reading frames downstream of *EAM_2938* were also HrpL-regulated suggesting that *EAM_2938, EAM_2937, EAM_2936* and *EAM_2935* may constitute a novel HrpL-regulated operon. *EAM_2938, EAM_2937* and

EAM_2936 are not broadly conserved in other bacterial plant pathogens and exhibit differential GC content suggesting that the *EAM_2938* gene cluster may be a recently acquired virulence determinant(s) in Ea1189, and understanding the function of this cluster is, therefore important for a better understanding of fire blight development by *E. amylovora*. Interestingly, the EAM_2937 protein (GeneBank: DX936506), is a putative inner membrane protein that was identified by Wang and Beer (2006) via a signature-tagged mutagenesis screen as a pathogenicity factor in apple shoot infection providing additional evidence that the HrpL-regulated *EAM_2938* gene cluster is an important component of pathogenesis by *E. amylovora*.

Two additional *hrp* promoters were also identified via bioinformatics and experimentally confirmed using qRT-PCR including the conjugative transfer protein *traF* and a chorismate mutase *aroQ*. While both genes remain uncharacterized, mass spectrometric analysis of the *in vitro* secretome of *E. amylovora* previously indentified HrpL-dependent *in vitro* secretion of TraF (Nissinen et al., 2007). Our data support this observation and collectively suggest that functional *hrp* promoters in *E. amylovora* can exhibit a non-canonical number of nucleotides between the -35 and -10 conserved *hrp* promoter motifs. In *E. amylovora* and *D. dadantii*, chorismate mutase gene expression was indentified using *in vivo* expression techniques (Yang et al., 2004; Zhao et al., 2005), and a signature-tagged mutagenesis screen indentified chorismate mutase as an *E. amylovora* pathogenicity factor required for apple shoot infection (Wang and Beer 2006). Chorismate mutase is part of the shikimate metabolic pathway and may be required for normal growth and development but recent analyses indicate that chorismate mutase may contribute to plant-nematode and plant-bacteria interactions (Jones et al., 2003; Degrassi et al., 2010).

Promoter modeling is a useful tool for identifying genes directly regulated by HrpL via its cognate *hrp* promoter. While *hrp* promoter-driven gene expression is a prerequisite for many plant-bacteria interactions, genome-wide microarray analysis allows for the detection of indirectly regulated genes. The HrpL regulon is known to include indirectly as well directly regulated genes (Ferreira et al., 2006; Lan et al., 2006; Yang et al., 2010). Our microarray analysis uncovered eight genes that appear to be indirectly up- and down-regulated by functional *hrpL*. The XRE family transcriptional regulator *ydcN* exhibited indirect up-regulation in Ea1189 strains. In hrpMM at 18 hpi, *ydcN* was the only gene exhibiting positive HrpL-mediated expression. XRE transcription factors are broadly conserved across bacterial species and bind DNA, generally resulting in the repression of target gene expression (Gerstmeir et al., 2004; Barragan et al., 2005; Kiely et al., 2008). Consequently, YdcN may connect the HrpL regulon to other signaling networks suppressing the transcription of genes 18 hpi. Phenotypic analysis of Ea1189Δ*ydcN* revealed a strong attenuation of virulence in immature pear, a decrease in motility and hyper-biofilm formation.

In our microarray analysis of the HrpL regulon, five genes appear indirectly down-regulated. For the 50S ribosomal protein *rpmD*, this is consistent with Lan et al., (2006) who reported that ribosomal proteins represent the largest group of HrpL down-regulated genes in *P. syringae* pv. DC3000. Aspartate carbamoyltransferase *pyrB* also exhibited HrpL-mediated down regulation. In a transposon mutagenesis screen for *E. amylovora* virulence factors, Wang and Beer (2006) identified *pyrB* as a pathogenesis factor required for disease activity in greenhouse apple shoots. The down-regulation of a pathogenicity factor by HrpL 6 hpi suggests disease development by *E. amylovora* is temporal in nature requiring specific

pathogenicity and virulence factors at different stages of infection. Like *pyrB*, the lipoprotein *nlpI* was also down-regulated by HrpL in hrpMM 18 hpi. When inoculated into immature pear, Ea1189Δ*nlpI* displayed a quantifiable decrease in virulence. Further phenotypic characterization of the *nlpI* mutant strain found that, like Ea1189Δ*ydcN*, Ea1189Δ*nlpI* exhibits reduced motility and increased biofilm formation. In *Escherichia coli*, *nlpI* is a confirmed outer membrane protein with conserved tetratricopeptide repeats (Wilson et al., 2005; Teng et al., 2010). In a screen for *E. coli* mutant strains with abnormal extracellular DNA phenotypes, *nlpI* was identified as a negative regulator of extracellular DNA export (Sanchez-Torres et al., 2010). Extracellular DNA has been increasingly recognized as an important component of biofilm matrices, and continued analysis of Ea1189Δ*nlpI* may help understand the role of extracellular DNA in plant pathogenesis. It was previously determined that biofilm formation is critical to *E. amylovora* virulence and to cell migration within apple xylem (Koczan et al., 2009). To date *nlpI* is the first HrpL down-regulated gene to be implicated in disease development. Of note, the HrpL regulon is suppressed in nutrient-rich media (Wei et al., 1992) and characterization of HrpL-mediated gene expression in rich medium may identify additional genes down-regulated by HrpL involved in adaptation to nutrient-rich host niches such as flower nectarines; an important infection court for fire blight development. Collectively, *nlpI*, *ydcN* and *EAM_2938* represent novel virulence factors in *E. amylovora*.

Microarray technology enables the simultaneous characterization of an entire transcriptome in response to different environmental stimuli. A total of 24 genes were differentially regulated in response to the presence of *hrpL*, including nine genes unrelated to the T3SS. Hidden Markov modeling and bioinformatics support our findings and further

allowed us to identify 15 novel predicted *hrp* promoters, seven of which were verified as responsive to functional *hrpL* via qRT-PCR. Taken together these data suggest that the HrpL regulon of *E. amylovora* encompasses more than just T3SS regulation and may communicate directly or indirectly with other signaling networks to coordinate gene expression during pathogenesis.

2.4 Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2.3. All bacterial strains used in this study were cultured in Luria Bertani broth (LB) unless otherwise noted. All strains were grown at 28°C in a shaking incubator. Where appropriate, media were supplemented with 50 μg ml^{-1} ampicillin, 20 μg ml^{-1} chloramphenicol, or 12 μg ml^{-1} oxytetracycline.

Deletion mutagenesis

Nonpolar chromosomal mutants were generated in *E. amylovora* using the phage λ Red recombinase system previously described (Datsenko and Wanner 2000). Briefly, *E. amylovora* strain Ea1189 was transformed with the helper plasmid pKD46 encoding recombinases red β, γ, and exo. Ea1189/pKD46 was grown overnight at 28°C in a shaking incubator, reinoculated in LB broth supplemented with 0.1% L-arabinose, and cultured for 4-6 hr to exponential phase OD_{600} = 0.8. Cells were made electrocompetent and stored at -80°C. Recombination fragments encoding acetyltransferase cassettes flanked by 50-nucleotide arms homologous to target genes were synthesized using polymerase chain reaction (PCR) with plasmid pKD3 as template. Recombination fragments were purified and concentrated using a PCR purification kit (Qiagen;

Valencia, CA) and electroporated into competent Ea1189. Putative mutants were screened on selective LB agar medium amended with chloramphenicol. Single-gene recombinatorial deletion was confirmed using PCR (Table 2.3) and functional complementation.

DNA manipulation and cloning

Restriction enzyme digestion, T4 DNA ligation, and PCR amplification of genes were conducted using standard molecular techniques (Sambrook et al., 1989). DNA extraction, PCR purification, plasmid extraction, and isolation of DNA fragments from agarose were performed with related kits (Qiagen, Valencia, CA). All DNA was sequenced at the Research Technology Support Facility at Michigan State University. To complement mutant strains, primer pairs were designed with restriction sites for double digestion and directional ligation into pBBR1MCS3 (Kovach et al., 1995). Final constructs were transformed into competent Ea1189 by electroporation and screened on LB agar plates amended with oxytetracycline.

Table 2.3 Bacterial Strains, plasmids and primers used in chapter 2

Strains and plasmids	Relevant characteristics[a]	Source or reference
Escherichia coli strain		
DH5α	F- 80dlacZ ΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(rK–mK+) deoR thi-1 supE44 gyrA96 relA1 λ-	Invitrogen, Carlsbad, CA, USA
Erwinia amylovora strains		
Ea1189	Wild type	Burse et al., 2004
Ea1189Δ*hrpL*	*hrpL* deletion mutant, Cm-R	This study
Ea1189Δ*EAM_2938*	*EAM_2938* deletion mutant, Cm-R	This study
Ea1189Δ*ydcN*	*ydcN* deletion mutant, Cm-R	This study
Ea1189Δ*nlpI*	*nlpI* deletion mutant, Cm-R	This study
Ea1189Δ*orf18*	*orf18* deletion mutant, Cm-R	This study
Plasmids		
pBBR1-MCS3	Tc-R, broad host-range cloning vector	Kovach 1995
pRRM1	Tc-R, pBRR1-MCS3 containing *hrpL*	This study
pRRM2	Tc-R, pBRR1-MCS3 containing *ydcN*	This study

Table 2.3 (cont'd)

pRRM3	Tc-R, pBRR1-MCS3 containing *EAM_2938*	This study
pRRM4	Tc-R, pBRR1-MCS3 containing *nlpI*	This study
pKD3	Amp-R, CmR, mutagenesis cassette template	Datsenko and Wanner 2006
pKD46	Amp-R, expresses λ red recombinase	Datsenko and Wanner 2006
Primers		
hrpLKO.F	5´-GGAGCAAGCCATGACAGAAATT CACCTGCAAACAACTGAATCAACA TCGGGTGTAGGCTGGAGCTGCTTC-3´	This study
hrpLKO.R	5´-TTAAGAAAATACTGACTGTTTCA GCGTGACGCGCGCACGCGACAGAC GTGCATATGAATATCCTCCTTA-3´	This study
EAM_2938KO.F	5´-ATGTATATGTATTTACCCTTTTTA CTAGGCGCAGGCATTGTTTTTAGTA TGTGTAGGCTGGAGCTGCTTC-3´	This study
EAM_2938KO.R	5´-CTAGAACGAAAGGAGAAGCGCA GTGGTGGCATGAACTTTCAACCACG CGACATATGAATATCCTCCTTA-3´	This study
EAM_orf18KO.F	5´-ATGATCAAAAAAACACTTCTTGC CGCTATGTTCGCGGGTACATGCAGT ACGTGTAGGCTGGAGCTGCTTC-3´	This study
EAM_orf18KO.R	5´-TTATGGCGCGGTGTAAAATTGCG CGCCGCTGCCGATTTTGCAGTTCCA GCCATATGAATATCCTCCTTA-3´	This study
EAM_3066KO.F	5´-ATGAAGCCATTTTTGCGCTGGTG TCTCGTTGCGACGGCTTTATCGTTG GCGTGTAGGCTGGAGCTGCTTC-3´	This study
EAM_3066KO.R	5´-CTATTGCTGGTCAGATTCTGATA AATCGTCTTGTGTCTGGCCGAGTAG CGCATATGAATATCCTCCTTA-3´	This study
EAM_1248KO.F	5´-TTGCTCAATCGCCTGTGCCACCT CTATGGCTTCACCCTGTCACGCTTG TTGTGTAGGCTGGAGCTGCTTC-3´	This study
EAM_1248KO.R	5´-TCATGGGCGAGCCACCACCAGC GCATAGTGCGCCCCCGTGGCCTCC GGCGCATATGAATATCCTCCTTA-3´	This study
hrpL(sacI).F	5´-GTGCATGAGCTCAGCAGTTGTC ATTGTGTGGTGC-3´	This study
hrpL(kpnI).R	5´-ACTGACGGTACCGTAAACATTGTT TACCTGATTAGGCTG-3´	This study
ydcN(kpnI).F	5´-ATGCTAGAGGTACCCTTGCTCAA TCGCCTGTGCCAC-3´	This study

Table 2.3 (cont'd)

ydcN(sacI).R	5´-TCGATTCGGAGCTCTCATGGGCGA GCCACCACC-3´	This study

[a] Cm-R, Tc-R and Amp-R indicates resistance to chloramphenicol, oxytetracycline and ampicillin, respectively

Virulence assay

The virulence of Ea1189 strains was determined using a standard immature pear fruit assay as described previously (Zhao et al., 2005). In brief, bacterial strains were cultured overnight, washed, and resuspended in 0.5x phosphate buffered saline (PBS) to 1 x 10^3 to 1 × 10^4 CFU/ml. Immature pear fruits (*Pyrus communis* L. cv. Bartlett) were surface sterilized with 10% bleach, dried in laminar flow hood, and pricked with a needle prior to application of 2 µl bacterial suspension. Inoculated pears were incubated at 28°C in humidified chambers. Symptoms were recorded 4 and 6 days post inoculation. The experiments were repeated three times with six replications per experiment. Virulence was determined quantitatively via percent necrosis, calculated as surface area of pear relative to surface area of necrotic and water soaked tissue.

Microarray design

An oligonucleotide microarray was designed at the James Hutton Institute (JHI, formerly SCRI), and synthesized by Agilent Technologies, Inc. (Palo Alto, CA). Each slide contains 8 arrays and each array has nearly 15,000 spots, containing our probes in triplicate. The main *E. amylovora* ATCC 49946 genome (accession NC_013971, Sebaihia et al. 2010) had 3483 target sequences (annotated genes and pseudo-genes), plus a further 483 target genes or simple gene predictions from five sequenced plasmids: plasmids 1 and 2 for the same strain (accessions

NC_013972, NC_013973, Sebaihia et al. 2010), pEL60 and pEU30 (accessions NC_005246, NC_005247, Foster et al., 2004), and pEI70 (Spanish strain *Erwinia amylovora* IVIA1614-2a, unpublished, Lopez and Llop, personal communication).

Up to five sense orientation candidate probes per target were designed with the Agilent eArray webtool, using temperature matching methodology, a preferred probe melting temperature of 80°C, no 3' bias, and a target length of 60 bp. Any short probes were later extended to 60 bp using the Agilent linker. BLASTN (Altschul et al., 1997) and Biopython (Cock et al., 2009) were used to identify potential cross-hybridization in order to rank the candidate probes. Selecting one probe per genome target, and up to five probes per plasmid target, allowed all our probes to be present in triplicate.

RNA isolation and microarray execution

Wild type Ea1189 (WT) and *hrpL* (Δ*hrpL*) mutant strains were cultured overnight in LB broth at 28°C in shaking incubator and collected the following day via centrifugation at 2,300 x g in microcentrifuge. Each strain was washed once in *hrp*-inducing minimal medium (HrpMM) (Huynh et al., 1989) before resuspension in HrpMM to 0.6 OD_{600}. Strains were then incubated for 6 and 18 hours at 180-200 RPM in shaking incubator at 18°C. RNA was extracted from 1 ml resultant cultures using SV Total RNA Isolation System (Promega; Madison, WI) as described by manufacturer. All RNA was quantified using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies; Wilminton, DE) and quality checked using an RNA 6000 Nano Kit on a 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA). Fluorescent labeling of total RNA was performed as described previously (Venkatesh et al., 2006) using the following experimental array design on a single 8x 15k format slide: 1. WT 6h rep 1 (Cy3), Δ*hrpL* 6h rep 1 (Cy5); 2.

Δ*hrpL* 6h rep 2 (Cy3), WT 6h rep 2 (Cy5); 3. WT 6h rep 3 (Cy3), Δ*hrpL* 6h rep 3 (Cy5); 4. Δ*hrpL* 6h rep 4 (Cy3), WT 6h rep 4 (Cy5); 5. Δ*hrpL* 18h rep 1 (Cy3), WT 18h rep 1 (Cy5); 6. WT 18h rep 2 (Cy3), Δ*hrpL* 18h rep 2 (Cy5); 7. Δ*hrpL* 18h rep 3 (Cy3), WT 18h rep 3 (Cy5); 8. WT 18h rep 4 (Cy3), Δ*hrpL* 18h rep 4 (Cy5). This design incorporated a dye-swap and balanced labeling of all samples. Levels and efficiencies of labeling were estimated using a NanoDrop spectrophotometer. Microarray hybridization, washing and scanning were performed in the JHI Sequencing and Microarray Facility as described (Stushnoff et al., 2010). Microarray images were imported into Agilent Feature Extraction (v.9.5.3) software and aligned with the appropriate array grid template file (021826_D_F_20081029). Intensity data and QC metrics were extracted using the recommended FE protocol (GE2-v5_95_Feb07). Entire FE datasets for each array were loaded into GeneSpring (v.7.3) software for further analysis. The experimental design and all microarray data are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/, accessions: Microarray #A-MEXP-2000, Dataset #E-TABM-1137).

Microarray analysis

Data were normalized using default settings for two-channel arrays: data were transformed to account for dye-swaps and data from each array were normalized using the Lowess algorithm to minimize differences in dye incorporation efficiency. Unreliable data flagged as absent in all replicate samples by the FE software were discarded. Significantly changing gene lists were generated from combined replicate datasets for each timepoint using volcano plot filtering (fold-change ratio >1.5x; Students *t*-test p-value < 0.05). None of these

represented potentially ambiguous probes (e.g. psuedo-genes or plasmid genes) which would have required a more careful investigation.

Bioinformatics

Nucleic and amino acid sequences were managed using the Lasergene® 7.2.0 software suite (DNASTAR, Madison, WI) and annotated in agreement with the *E. amylovora* ATCC 49946 genome (Sebaihia et al., 2010). Similarity of DNA and protein sequences was determined using cognate BLAST programs at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997). Gene of interest were analyzed for GC content with GC-Profile (http://tubic.tju.edu.cn/GC-Profile) (Gao and Zhang 2006) and putative transmembrane domains using the DAS - Transmembrane Prediction server http://www.sbc.su.se/~miklos/DAS) (Cserzo et al., 1997). Signal peptides were predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004). All nucleic and amino acid alignments were created using T-Coffee multiple sequence alignment software (http://www.tcoffee.org/homepage.html) (Notredame et al., 2000). Weblogo 2.8.2 (http://weblogo.berkeley.edu) was used to visualize multiple sequence alignments (Crooks et al., 2004).

Hidden Markov modeling of *hrp* promoter

To identify HrpL cis-regulatory elements in the genome of *E. amylovora*, sequence training sets were assembled including 54 *hrp* promoters from *Erwinia*, *Pectobacterium*, *Dickeya* and *Pantoea* species. Promoter sequences were 28 or 29 bp in length and flanked by canonical "-35" and "-10" HrpL-binding sites (Table 2.4). Sequences were aligned with T-coffee multiple

alignment software and the resulting alignment was used to construct a hidden Markov model with the hmmbuild and hmmcalibrate functions of HMMer 2.3.2 (Eddy 1998). Our HrpL promoter model was then tested against the genome of *E. amylovora* ATCC 49946 (NCBI: NC_013971) (Sebaihia et al., 2010) using the HMMer function hmmsearch, with a bit-score classifier threshold of 8.5. Predicted *hrp* promoters were then sorted empirically based on three criteria, 1) location in intergenic space, 2) orientation relative to annotated genes in *E. amylovora* ATCC 49946, and 3) e-values less than *hrp* promoters with known HrpL-dependent activity. Reported results were experimentally verified using quantitative real time (qRT)-PCR. The hidden Markov model described above is available upon request.

Table 2.4 Sequences used to construct hidden Markov *hrp* promoter models

Name	Sequence	Reference
DspE_Ep	5´-GGAACCGGTTGCAGAGAAATGCAACATA-3´	Shrestha 2008
DspE_Pa	5´-GGAACCAGAGGGGGGAAATGACCACTTA-3´	Holeva 2004
DspE2_Ep	5´-GGAACCTTCCCGGCGTTTACCTCGACTAA-3´	Shrestha 2008
DspE_Ea	5´-GGAACCGGTTGCAGAGAATTGCAACATA-3´	Wei et al., 2000
Eop2_Ea	5´-GGAACCCGATAGAAATCATTACCACTCA-3´	Oh & Beer 2005
Eop3_Ea	5´-GGAACCTTCGTTGACTTTTTTTCCACTTA-3´	Oh & Beer 2005
HrcV_Ea	5´-GGAACCGCGCGCCAGCCTGACACAACCCA-3´	NCBI database
HrpA_Dd	5´-GGAACCACCTCCGCATATCTCCTACTTA-3´	NCBI database
HrpA_Ep	5´-GGAACCGATCGAAACTGTCCGCCACTTA-3´	Shrestha 2008
HrpA_Ps	5´-GGAACCGCAATAAAAGACTCACCACTAA-3´	NCBI database
HrpA_Ea	5´-GGAACCGATCGAAACTGCCCGCCACTTA-3´	Wei et al., 2000
HrpF_Dd	5´-GGAACCGCCGCCACTCCCCGGCCACACA-3´	NCBI database
HrpF_Ps	5´-GGAACCGTTTTGCACTTTCTGCCACTCA-3´	NCBI database
HrpJ_Ea	5´-GGAACCGATGCGTCAATCGCACCACACA-3´	Wei &Beer 1995
HrpJ_Eh	5´-GGAACCGCAGCGTTAATCCCACCACACA-3´	NCBI database
HrpJ_Ep	5´-GGAACCGATGCGCTAATCGCACCACACA-3´	Shrestha 2008
HrpJ_Pa	5´-GGAACCCATCCTTTTCTGCGTCCACACA-3´	Holeva 2004
HrpJ_Pc	5´-GGAACCCATCCTTTTCTGCGTCCACACA-3´	NCBI database
HrpJ_Ps	5´-GGAACCGGAGCATAAAACACGCCACATA-3´	NCBI database
HrpN_Dd	5´-GGAACCGTTTCACCGTCGGCGTCACTCA-3´	NCBI database
HrpN_Ea	5´-GGAACCAGAGCGGAATAACCAGCACTCA-3´	Wei &Beer 1995

Table 2.4 (cont'd)

HrpN_Ep	5′-GGAACCAGAGCGGGATAACCAGCACTCA-3′	Shrestha 2008
HrpN_Pa	5′-GGAACCACGCAGGCAAGAAAATCACTTA-3′	Holeva 2004
HrpN_Ps	5′-GGAACCAGCACCGCGATAGCGGCACTTA-3′	NCBI database
HrpS_Ep	5′-GGAACCGGAAAAGACACGCTGGCACGAA-3′	NCBI database
HrpW_Ea	5′-GGAACCCTGTCAACGCCAAACCCACTCA-3′	Wei et al., 2000
HrpW_Ep	5′-GGAACCCTGCCAACGCCACACCCACTTA-3′	NCBI database
HrpW_Pa	5′-GGAACCACATCACACCGCTCTTCACTTA-3′	Holeva 2004
HsvA_Ea	5′-GGAACCCGACTTCGTTTAAGCACACACA-3′	Oh & Beer 2005
Orf1_Dd	5′-GGAACCGCATTACCATGTTGATTACGAA-3′	NCBI database
Orf2_Ps	5′-GGAACCACAGAATGAATAGAAACCACAAA-3′	NCBI database
OrfU1_Ea	5′-GGAACCGGAAAAGACACGCTGGCACGAA-3′	Wei &Beer 1995
???_Dd	5′-GGAACTCCCCCCGGTAGTCGACTACCTA-3′	NCBI database
DspE2_Ea	5′-GGAACTGAACACAAGGCGGCAGTACACA-3′	NCBI database
Eop4_Ea	5′-GGAACTTTATTACACGGTAATTCACTCA-3′	Oh & Beer 2005
HrpA_Pa	5′-GGAACTCCTCCAGCCCGATCTCTACTTA-3′	Holeva 2004
HrpA_Pc	5′-GGAACTCACTGACCCGCTCTCCCACTTA-3′	NCBI database
HrpF_Ep	5′-GGAACTCCGCCGCGCCCCATCCCACTCA-3′	Shrestha 2008
HrpF_Pa	5′-GGAACTGCACGCCAGGGTTAACCACTCA-3′	Holeva 2004
HrpF_Pc	5′-GGAACTGCACGCTTAGGTTAACCACTCA-3′	NCBI database
HrpF2_Ea	5′-GGAACTCCGCCACGCCCGAACCCCACTCA-3′	Wei &Beer 1995
HrpKrc_Ea	5′-GGAACTCAAAACTCCTTCACACGACATA-3′	Oh * Beer 2005
HrpN_Pc	5′-GGAACTGAGCAGGCAAGAAAATCACTTA-3′	NCBI database
HrpX_Ps	5′-GGAACTTGTAGAGTCGGACAGTTCACTTA-3′	NCBI database
Orf_12_Eh	5′-GGAACTTATACGGGGCACACACCACTTA-3′	NCBI database
Orf1_Ps	5′-GGAACTGAATAACTTCCTAGGCGACAAA-3′	NCBI database
Orf12_Ea	5′-GGAACTATTACCTGCCGTTCGCCACCTA-3′	Oh & Beer 2005
Orf12_Ep	5′-GGAACTTTTAGCTGCCGTTTCGCCACCCA-3′	NCBI database
Orf12_Ps	5′-GGAACTTAATGCCACATCTCGCCACTTA-3′	NCBI database
Orf16_Ep	5′-GGAACTCTACCCCGCTAAAGCACACACA-3′	NCBI database
Orf17_Ep	5′-GGAACTCAAAACTCCGCCAGACGACACA-3′	NCBI database
Orf19_Ea	5′-GGAACTTTTCTCTGCCTGTCACCACTCA-3′	Oh & Beer 2005
Orf7_Ps	5′-GGAACTTAATGCCACATCTCGCCACTTA-3′	NCBI database
WstE_Ps	5′-GGAACTATCCACGCAAACTCACCACACA-3′	NCBI database
AvrE1_Pst	5′-GGAACCGGTCGCTGCGCTTTGCCACTCA-3′	Fouts et al., 2002
HrpW	5′-GGAACCGGTCGCTGCGCTTTGCCACTCA-3′	Fouts et al., 2002
AvrPpiG1	5′-GGAACCACGGTAGCTTAGCTGACCACTCA-3′	Fouts et al., 2002
HrpC_Pss	5′-GGAACCGCTCCACCTGTTTGCTCCACTCA-3′	Fouts et al., 2002
HrpC_ Pst	5′-GGAACCGCTCGGCGGGTTTGCTCCACTCA-3′	Fouts et al., 2002
AvrE2_ Pst	5′-GGAACCCGCTGGCATTGCATGCCACTCA-3′	Fouts et al., 2002
Orf07755	5′-GGAACCCTGCGCAGGTCATTGACCACTCA-3′	Fouts et al., 2002

Table 2.4 (cont'd)

HrpR20	5´-GGAACCGGACGAGGCTTTTTACCACTCA-3´	Fouts et al., 2002
AvrPto_ Pst	5´-GGAACCGATCCGCTCCCTATGACCACTCA-3´	Fouts et al., 2002
AvrPphB_phas	5´-GGAACCGAATGGGTCAGATGGACACTTA-3´	Fouts et al., 2002
HrpR57	5´-GGAACCGATCCGGTTGCCTGGCCACTCA-3´	Fouts et al., 2002
HrpZ_Pss	5´-GGAACCGATTCGCGGACACATGCCACCTA-3´	Fouts et al., 2002
HrpZ_phas	5´-GGAACCGATTTAAGGGTCGTTACCACCTA-3´	Fouts et al., 2002
HrpZ_ Pst	5´-GGAACCGTATCGCAGGCTGCTGCCACCTA-3´	Fouts et al., 2002
HrpR36	5´-GGAACCGTAACGGCGAGCGTGCCACGTA-3´	Fouts et al., 2002
HrpJ_Pss	5´-GGAACCGAACCGCGTCAATGACCCACTCA-3´	Fouts et al., 2002
HrpY_phas	5´-GGAACCAACTCGCACGCAAAACCACACA-3´	Fouts et al., 2002
HrpK_Pss	5´-GGAACCAACTCGCACGCAAAACCACACA-3´	Fouts et al., 2002
hrpK_ Pst	5´-GGAACCAACTTGCACCTTCAACCACACA-3´	Fouts et al., 2002
AvrPphD_phas	5´-GGAACCCAAGAGCCCTTGCGACCACACA-3´	Fouts et al., 2002
AvrD_lac	5´-GGAACCAAATCCGTCCCAAAGGCCACACA-3´	NCBI database
AvrD_ Pst	5´-GGAACCAAATCCGTCCCAAAGGCCACACA-3´	Fouts et al., 2002
AvrD_phas	5´-GGAACCAAATCCGTCCCAAAGGCCACACA-3´	Fouts et al., 2002
AvrD_glycinia	5´-GGAACCAAATCCGTCCCAAAGGCCACACA-3´	Fouts et al., 2002
AvrRpt2_ Pst	5´-GGAACCCATTCATTGTTTGGAACCACCAA-3´	Fouts et al., 2002
HrpR31	5´-GGAACCGCATCACGTCTTGAACCACAGA-3´	Fouts et al., 2002
HrpR40	5´-GGAACCGATTTCGATGAGTCGCCACACA-3´	Fouts et al., 2002
HrpR34	5´-GGAACCGCCTCGAGCAGAGGCTCCACTCA-3´	Fouts et al., 2002
AvrB_glycinia	5´-GGAACCTAATTCAGGGTAAATGCCACACA-3´	Fouts et al., 2002
HrpF09	5´-GGAACCAGATCTCGTTGCTTGCCACCAA-3´	Fouts et al., 2002
HopPsyA_Pss	5´-GGAACCTTATCGGGAAAATTTGCCACCCA-3´	Fouts et al., 2002
HrpR25	5´-GGAACCGAATCCATATTTCGACCACCCA-3´	Fouts et al., 2002
AvrPphF_phas	5´-GGAACCAGCTACATAGGTATGACCACTGA-3´	Fouts et al., 2002
HrpF03	5´-GGAACCTCACGCTTAGTGATGACCACGCA-3´	Fouts et al., 2002
HopPtoA	5´-GGAACCGTCAACCGATCCGGGACCACACA-3´	Fouts et al., 2002
AvrPto2	5´-GGAACTCTTTCCTGCTCTTTTGCCACACA-3´	Fouts et al., 2002
HrpR55	5´-GGAACTCTTTCCCTGCGCTTTCCACTCA-3´	Fouts et al., 2002
AvrPpiC2_pisi	5´-GGAACTGAACCGCTTATGAAACCACTCA-3´	Fouts et al., 2002
AvrPpiC2	5´-GGAACTGAACCGCTTATGAAACCACTCA-3´	Fouts et al., 2002
HrpF43	5´-GGAACTGCAACGTTGTTTCGGTCACTCA-3´	Fouts et al., 2002
HrpJ_tomato	5´-GGAACTGATCCGGGACCGTGACCCACTCA-3´	Fouts et al., 2002
HrpA	5´-GGAACTCATCACCGCGAATCGCCACTCA-3´	Fouts et al., 2002
AvrPpiA1_pisi	5´-GGAACTCATTTTCTTTTAAAACCACACA-3´	Fouts et al., 2002
HrpU_ Pst	5´-GGAACTGAAATCGATGCTCGACCACTTA-3´	Fouts et al., 2002
HrpU_Pss	5´-GGAACTGAAATCGATCCTCGACCACTTA-3´	Fouts et al., 2002

qRT-PCR and cDNA synthesis

To validate gene expression patterns identified in our microarray experiment and explore the HrpL regulon as revealed using bioinformatic techniques, qRT-PCR was performed using RNA isolations independent of samples used for microarray hybridization. RNA was extracted as described above. First-strand cDNA synthesis was conducted with 1 µg total RNA and Taqman Reverse Transcription Reagents (Applied Biosystems; Foster City, CA) under manufacturer's protocol. Residual RNA was degraded with RNase H (Invitrogen; Carlsbad, CA). After determining primer efficiencies, 20 µl qRT-PCR reactions were made using SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA). Primers were designed using Primer Express 3.0 (Applied Biosystems; Foster City, CA) and sequences are listed in Table 2.5. All reactions were conducted with a StepOne Plus Real-Time PCR System (Applied Biosystems; Foster City, CA) and amplification was carried out with one polymerase activation cycle at 95°C for 10 min followed by 40 cycles: denaturing at 95°C for 15 s and annealing at 60°C for 1 min. Nonspecific primer activity was monitored using a dissociation curve and resulting threshold cycles (CT) were determined using StepOne Software 2.1 (Applied Biosystems; Foster City, CA) software. All reactions were normalized against endogenous control *gyrA* (Takle et al., 2007) and CT data was quantitatively analyzed via the comparative CT method to generate relative fold change values comparing wild type and Ea1189Δ*hrpL* transcript abundance. Each qRT-PCR analysis was done in triplicate and repeated three times.

Table 2.5 Quantitative Real-Time PCR primers used in chapter 2

Name	Sequence	Source
EAM_2935.F	5´-CGCCGTTACGCTGTCAGTAC-3´	This study
EAM_2935.R	5´-CCGGCCAGGATATTGATCAT-3´	This study
EAM_2936.F	5´-TCATTGCCGCGCTTAAGC-3´	This study
EAM_2936.R	5´-GCCCCTAAAGGCGAAAAGTG-3´	This study
EAM_2937.F	5´-GTGCGGCTTTACTGCTGACA-3´	This study
EAM_2937.R	5´-AGTGCTAAGGCACCGAAAATG-3´	This study
EAM_1012.F	5´-AGGCTTTAACAGTGATGATTTGATGT-3´	This study
EAM_1012.R	5´-ACGATCCGTCAGGTAATTATCACA-3´	This study
EAM_2911(hrpK).F	5´-GCGATAATCCGGACGGAAA-3´	This study
EAM_2911(hrpK).R	5´-AAATCATTGATGTCGCGATCTG-3´	This study
EAM_2912(orf18).F	5´-GCATTACCCACCCCGCTTA-3´	This study
EAM_2912(orf18).R	5´-GGCCTTCACAACGGTCAAAT-3´	This study
EAM_2895(hrpJ).F	5´-TGCGCCATACGCTGTTACAG-3´	This study
EAM_2895(hrpJ).R	5´-TCAGCCCCTTTTTCCTTGTG-3´	This study
EAM_2572(fliN).F	5´-AAGAGCTGCTGCGTTTAAGTCA-3´	This study
EAM_2572(fliN).R	5´-CCCTGAGCAATTAAATAGCCGTTA-3´	This study
EAM_423(avrRpt2Ea).F	5´-CACCAGCCTCGTCAATCAGA-3´	This study
EAM_423(avrRpt2Ea).R	5´-CGAAACATCCGCCAAAAGAT-3´	This study
EAM_2697(hopCEa).F	5´-CCTGACCAGCACAGCACTTC-3´	This study
EAM_2697(hopCEa).R	5´-CCAGCGCGTATCGTTTATCTG-3´	This study
EAM_2611(yfiM).F	5´-GAGTGGACTGGGCGGGATA-3´	This study
EAM_2611(yfiM).R	5´-GGCTCGGGCTCCAGTTCT-3´	This study
EAM_2887(hrpA).F	5´-AAGACGCTGGAATCAGCAATG-3´	This study
EAM_2887(hrpA).R	5´-TGCCATCCAGGATCGAGTTC-3´	This study
EAM_2872(dspE).F	5´-CGCAACATCGGAACCATTAA-3´	This study
EAM_2872(dspE).R	5´-TGCGACCTGCGGATTAGC-3´	This study
EAM_2780(eop2).F	5´-GCGTCCGGCAATTCCTTTAT-3´	This study
EAM_2780(eop2).R	5´-CCGGCTTGAGGGAAAAACTT-3´	This study
EAM_2189(eop3).F	5´-GCTCCGGAGGTCTGGTTTG-3´	This study
EAM_2189(eop3).R	5´-TGCTGTCCATGCCGAAAAT-3´	This study
EAM_2910(hsvA).F	5´-CCGCAGATCCCGGATATTTT-3´	This study
EAM_2910(hsvA).R	5´-GGATCCAGTTGGACGACATGT-3´	This study
EAM_1472(ybhH).F	5´-GAAGAATAGCGACGGCTCCTT-3´	This study
EAM_1472(ybhH).R	5´-ATCACAGTTTTCATTTCCCTGACTAA-3´	This study
EAM_2906(orf12).F	5´-ATGTTAACCATATCCGCCAGAAG-3´	This study
EAM_2906(orf12).R	5´-CCGCTGTCATCATCGATAAAGA-3´	This study
EAM_2913(orf19).F	5´-GCTGTCCGCTTACGCACAA-3´	This study
EAM_2913(orf19)RP	5´-CAACGGATGTTTTCCAACCAT-3´	This study
EAM_2195(aroQ).F	5´-ATGGCGACGTTAACCCAAAA-3´	This study
EAM_2195(aroQ).R	5´-CAAGATGGTTGTTCGCTTTGTATC-3´	This study
EAM_0268(traF).F	5´-CGCTACCGTTTGCCTTTGTT-3´	This study

Table 2.5 (cont'd)

EAM_0268(traF).R	5′-GCATCTGCGATTCCCTGTAAC-3′	This study
EAM_2938.F	5′-TAGGCGCAGGCATTGTTTTT-3′	This study
EAM_2938.R	5′-ACTTTCAACCACGCGAGGAT-3′	This study
EAM_2877(hrpN).F	5′-GGTGAAAACCGATGACAAATCA-3′	This study
EAM_2877(hrpN).R	5′-ATGCCCTTGGCTTTGTTGAA-3′	This study
EAM_2951(fimD).F	5′-GGGCCAACCAAAGCATCA-3′	This study
EAM_2951(fimD).R	5′-TAGCTGTCATCGCCATAGAAACC-3′	This study
EAM_2640(recA).F	5′-CTAACGCGGGCAACTTCTTG-3′	This study
EAM_2640(recA).R	5′-TCAGGCTTGTCGTCCTGGTT-3′	This study
EAM_2630(ffh).F	5′-CTGGCCAGCAAACTGAAGAAG-3′	This study
EAM_2630(ffh).R	5′-CCATCAGGCTGGCCATACC-3′	This study
EAM_2264(gyrA).F	5′-GCGATCAGTTCGGTGACAAA-3′	This study
EAM_2264(gyrA).R	5′-AGGGTGACAACCACATCTTCCT-3′	This study
EAM_3054(pyrB).F	5′-ACCGATATGCTGGACGAAAAA-3′	This study
EAM_3054(pyrB).R	5′-TCTGCACGCGCGTCATATAC-3′	This study
EAM_1248(ydcN).F	5′-GGATAGTCAAAGCCTCTGGTACGA-3′	This study
EAM_1248(ydcN).R	5′-CCGTGCTTCAACCAGTTCAG-3′	This study
EAM_1112.F	5′-AATAAATCTGCGACAACCACAT-3′	This study
EAM_1112.R	5′-ATCGTTTGCATGGTAAGCAA-3′	This study
EAM_3066(nlpI).F	5′-GTTTTGGCCGTTCCTTTGC-3′	This study
EAM_3066(nlpI).R	5′-CGCGTTCATCATCGGTTAAA-3′	This study
EAM_1656.F	5′-CGGTAATCCTGGGTTCAACTCT-3′	This study
EAM_1656.R	5′-TGGTCAACTTTTGCGTTCAGA-3′	This study

Biofilm formation *in vitro* crystal violet assay

The biofilm formation abilities of Ea1189 strains were explored using a modified crystal violet assay described previously (O'Toole et al., 1999; Koczan et al., 2009). Ea1189 strains were grown overnight to 2 x 10^8 CFU ml^{-1}. Cultures were diluted to 0.2 OD_{600} in 0.5x LB broth and 25 µl suspension was combined with 2 ml sterile 0.5x LB broth in 24-well polystyrene plate (Corning, New York City). To quantify biofilm formation, a glass coverslip was added to each plate well at a 30° angle to maximize surface contact with inoculated medium. Plates were then incubated at 28°C for 48 hrs. Following incubation, culture suspensions were removed, replaced with 10% crystal violet and allowed to stain at room temperature for 1 hr. Crystal violet

was then decanted and glass coverslips and plate wells were washed gently three times with water before air drying overnight. For qualitative analysis, crystal violet pigment accumulation representing biofilm formation was observed and recorded from plate well walls and coverslips. For quantitative analysis, crystal violet was resolubilized from glass coverslips using 200 µl of 40% methanol, 10% glacial acetic acid. A Safire microplate reader (Tecan, Research Triangle Park, NC) was then utilized at an absorbance of 600 nm to quantify solubilized crystal violet via spectrophotometry. The biofilm formation abilities of each Ea1189 strain were assayed three times with 12 replicates per assay.

Motility assay

To assess swarming motility of Ea1189, wild type and mutant strains were cultured overnight to 2 x 10^8 CFU/ml, collected by centrifugation, and washed once with 0.5x PBS. Each sample was then resuspended to 0.2 OD_{600} in 0.5x PBS and 10 µl diluted sample was added to the center of swarming agar plates (10g tryptone, 5g NaCl, 3g agar per liter water) as previously described (Hildebrand et al., 2006; Skerker et al., 2005). Swarming diameters were recorded for each sample after 24 and 48 hpi at 28°C. The assay was repeated three times with 10 replicates per experiment.

Chapter 3: YdcN is a negative regulator of *nlpI* and links the HrpL regulon to biofilm formation in *Erwinia amylovora*

3.1 Introduction

Erwinia amylovora is the causal agent of fire blight and is one of the most significant pathogens affecting apple and pear production world-wide (Norelli et al., 2003). While *E. amylovora* exhibits an array of virulence mechanism, the current model for fire blight disease development emphasizes the role of two pathogenicity factors: the type three secretion system (T3SS) and biofilm formation (Khan et al., 2012). The T3SS is hypothesized to facilitate interactions with herbaceous tissues near the site of infection early during pathogenesis. Biofilm formation contributes to the colonization of the host vasculature and to systemic fire blight and biofilm-related genes appear to be needed late during disease development (Koczan et al., 2009).

To better understand the array of virulence factors deployed, *in vitro* microarray analysis and bioinformatic modeling were used to identify the regulon of HrpL, a master regulator of the T3SS (McNally et al., 2012). While transcript accumulation in Ea1189Δ*hrpL* relative to wild type Ea1189 confirmed the role of HrpL in regulating the T3SS at early time points, analyses of the HrpL regulon revealed two notable characteristics (McNally et al., 2012). 1) More than 60% of the HrpL regulon had no predicted role in type III secretion and 2) HrpL failed to regulate the T3SS at later time points (McNally et al., 2012). In order to develop a more comprehensive understanding of virulence mechanisms in *E. amylovora,* a suite of HrpL-regulated genes were selected for analysis based on criteria including a lack of predicted T3SS function and HrpL-mediated expression not co-regulated with components of the T3SS (McNally et al., 2013).

Multiple non-T3SS genes were found to exhibit HrpL-dependent transcriptional activity including *ydcN* (EAM_1248) and *nlpI* (EAM_3066) (McNally et al., 2013). Both genes were regulated by HrpL 18 hours post-inoculation (McNally et al., 2013). HrpL positively regulated *ydcN* while negatively impacting *nlpI* transcript abundance (McNally et al., 2013). YdcN is a predicted xenobiotic response element (XRE)-family transcriptional regulator while NlpI is a uncharacterized lipoprotein.

As *ydcN* is a predicted transcriptional regulator, Ea1189Δ*ydcN* was subject to microarray and quantitative PCR analyses to describe the YdcN regulon and to characterize the role of YdcN within a broader HrpL signaling network. Results indicate that YdcN negatively regulates *nlpI* transcription and that both genes are involved in biofilm formation when assayed in a continuous-flow biofilm system.

3.2 Results

The transcriptional regulator YdcN negatively regulates *nlpI* transcript abundance.

Two genes, *ydcN* and *nlpI*, were previously shown to exhibit HrpL-mediated transcriptional activity at 18 hpi in HrpMM (McNally et al., 2012). While *ydcN* was positively regulated by HrpL, *nlpI* was negatively regulated (McNally et al., 2012). Amino acid sequence analyses comparing YdcN with constituents of the NCBI protein database revealed that YdcN is a predicted transcriptional regulator (Sebaihia et al., 2010). In order to describe the regulon of YdcN and to determine if the HrpL regulon is in fact a network comprised in part of interconnected transcription factors, WT Ea1189 and Ea1189Δ*ydcN* were cultured in hrpMM for 18 hours. RNA was isolated and transcript abundance in the two strains was compared using a custom microarray designed to represent the annotated genes of *E. amylovora* ATCC 49946

(McNally et al., 2012). Of the 3966 target sequence represented by the *E. amylovora* microarray slide, *nlpI* was the only gene found to be differentially regulated between the transcriptomes of WT Ea1189 and Ea1189Δ*ydcN* (Table 3.1).

Table 3.1 *in vitro* YdcN microarray

Annotation	Gene	Fold change (WT/Δ*ydcN*)	P < 0.01
EAM_3066	*nlpI*	0.347864	0.00352

Microarray expression profile of *in vitro* YdcN regulon comparing RNA extractions from wild type Ea1189 and Ea1189Δ*ydcN* after inoculation in hypersensitive response and pathogenicity (hrp)-inducing minimal medium (hrpMM) for 18 hours. Transcript abundance of *nlpI* was decreased in the presence of *ydcN*. Values satisfied P < 0.05

To confirm the YdcN microarray results, Ea1189Δ*ydcN* and WT Ea1189 were culture as before and RNA was extracted. RNA samples were subject to qRT-PCR to measure *nlpI* transcript abundance. Three separate reference genes, *recA*, *gyrA* and *ffh* were employed to independently standardize the results qRT-PCR. Results indicated that YdcN is a negative regulator of *nlpI* (Fig 3.1).

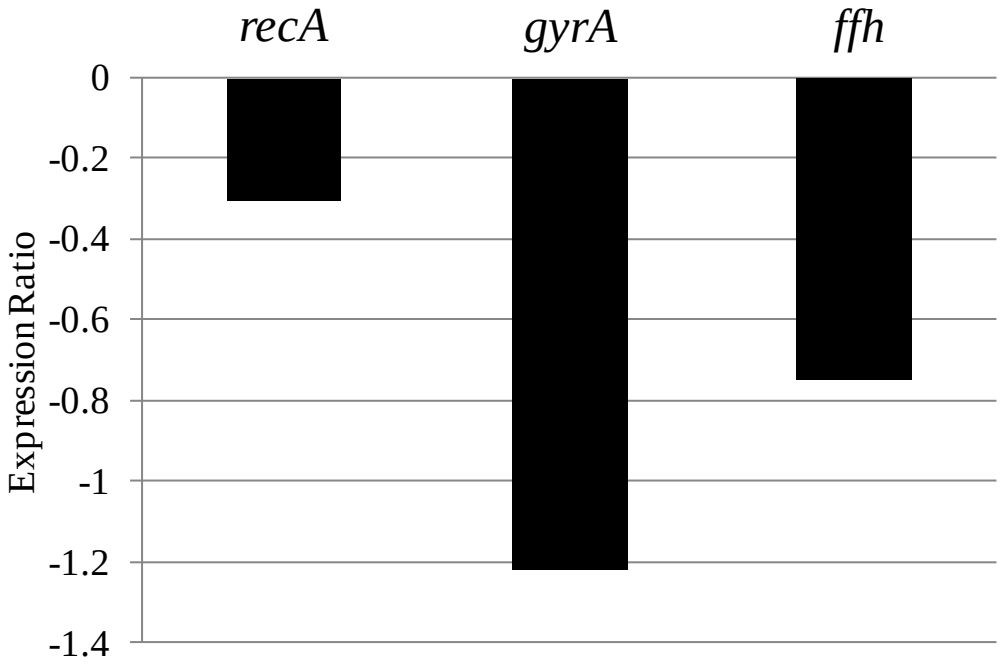

Figure 3.1 Relative expression of *nlpI* comparing WT Ea1189 and Ea1189Δ*ydcN*. RNA was extracted 18 hpi in hrpMM and *nlpI* transcript abundance was measured in Ea1189 and Ea1189Δ*ydcN*. Results were compared and calibrated against three separate reference genes; *recA*, *gyrA* and *ffh* and analyzed using expression software as described previously (Zeng et al., 2013).

NlpI and YdcN regulate biofilm formation in flow cell analysis

In a previous analysis of the HrpL regulon, HrpL-dependent transcription of the T3SS did not temporally overlap with regulation of *nlpI* and *ydcN* (McNally et al., 2012). To explore the possibility that the virulence functions of NlpI and YdcN are independent of the T3SS, Ea1189Δ*nlpI* and Ea1189Δ*ydcN* were assayed to determine their biofilm properties in a continuous-flow biofilm system. WT Ea1189, Ea1189Δ*ydcN*, and Ea1189Δ*nlpI* were transformed with pMP2444 expressing green fluorescent protein (GFP). GFP-labeled strains

were subjected to flow cell analysis for 48 hours in 0.5X LB medium. Results revealed that YdcN promotes biofilm formation while NlpI is a biofilm inhibitor (Fig 3.3, Fig 3.4)

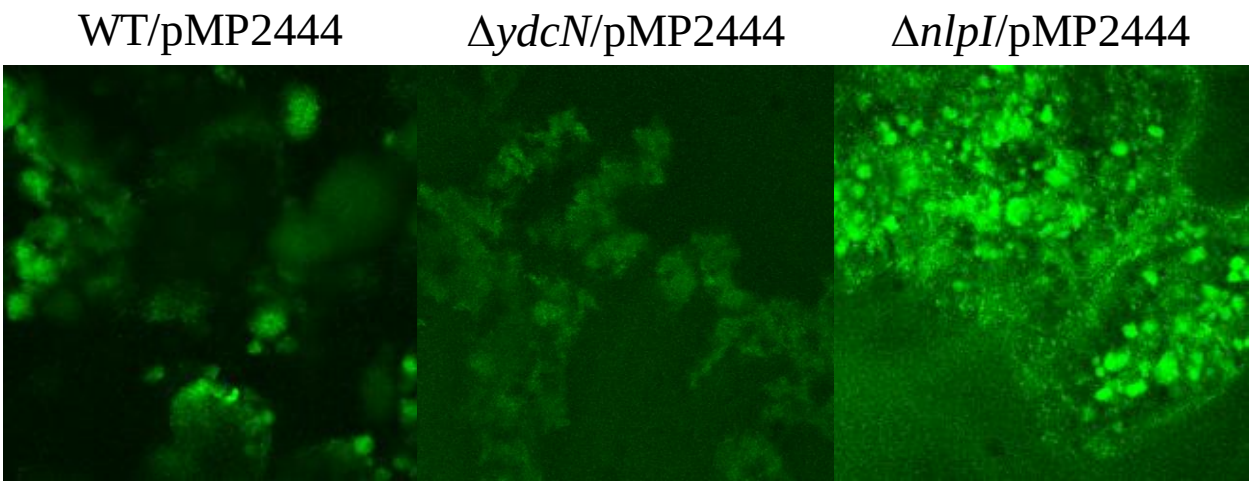

Figure 3.2 Confocal laser scanning microscopic visualization of *E. amylovora* biofilms. WT Ea1189, Ea1189Δ*ydcN* and Ea1189Δ*nlpI* labeled with GFP and imaged after growth for 48 hours in a continuous-flow biofilm system. Images were recorded at 100X magnification.

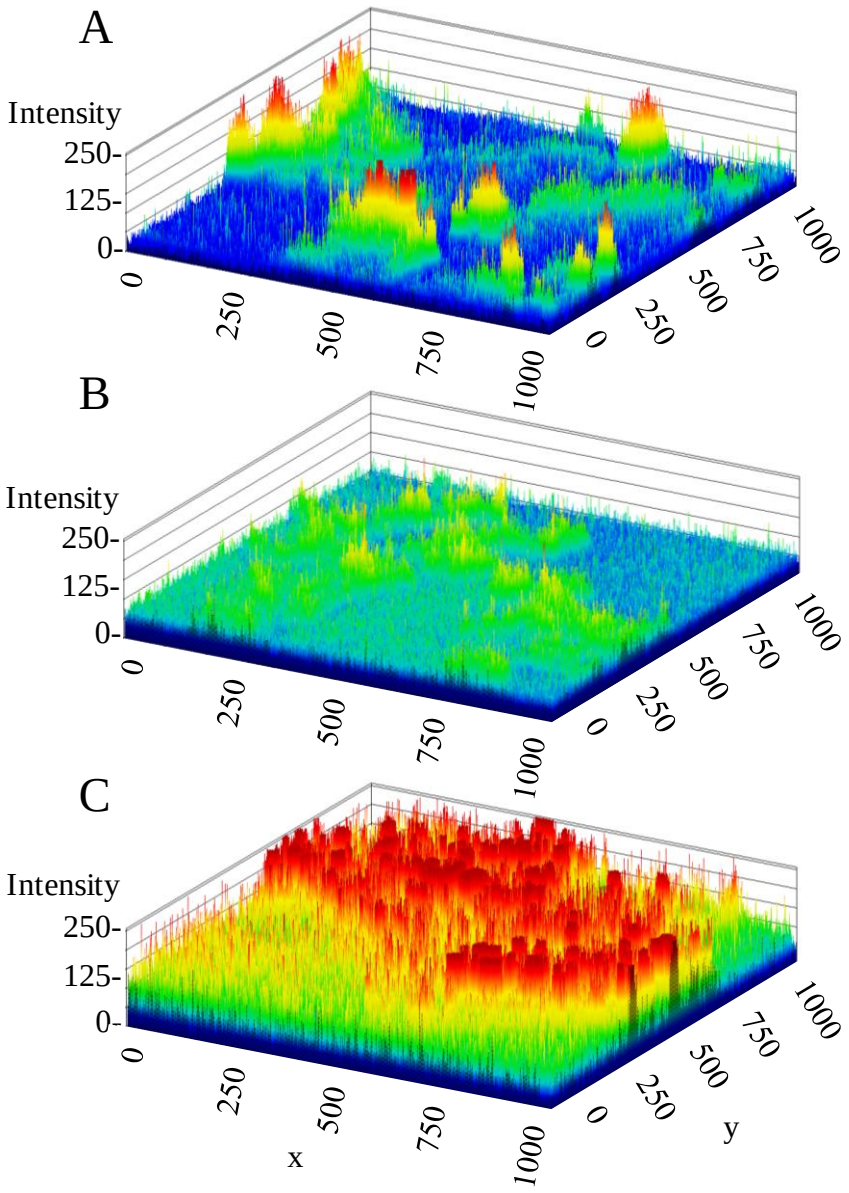

Figure 3.3 Three-dimensional image analysis of *E. amylovora* biofilms. Ea1189 strains labeled with GFP were cultured for 48 hours in a continuous-flow biofilm system. GFP fluorescence was recorded and intensity (z) represents cell aggregation in a given area (x and y). (A) WT Ea1189 represents typical biofilm formation. (B) Ea1189Δ*ydcN* exhibits highly reduced levels of cell aggregation and biofilm formation. (C) Ea1189Δ*nlpI* produces more biofilm formation than WT Ea1189.

3.3 Discussion

E. amylovora employs an array of virulence mechanisms. To date, 56 virulence factors have been verified experimentally to contribute to fire blight disease development by *E. amylovora* (Table 1.1). Of these, approximately 28% represent structural or secreted components of the T3SS.

In a previous analysis of the HrpL regulon, HrpL-dependent transcription of the T3SS was observed 6 hpi in HrpMM. At 18 hpi however, no T3SS transcriptional activity was distinguishable in WT Ea1189 versus Ea1189Δ*hrpL*. At 18 hpi HrpL instead regulated a distinct suite of Ea1189 genes including *nlpI* and *ydcN* (McNally et al., 2012). The revelation that HrpL regulates a number of genes without known functions at time points after the T3SS was inactive prompted interest in how constituents of the HrpL regulon change over time.

Results of expression analyses exploring *nlpI* transcription in WT Ea1189 and Ea1189Δ*ydcN* revealed that YdcN negatively regulates the expression of the lipoprotein NlpI. Taken together with information that HrpL also negatively regulates NlpI while positively regulating YdcN (McNally et al., 2012) and evidence suggests that HrpL negative regulation of NlpI is mediated by the transcriptional regulator YdcN.

Biofilm experiments also indict that NlpI is a negative regulator of biofilm formation as Ea1189Δ*nlpI* has a hyper-biofilm phenotype after flow cell analysis. NlpI homologs in *Salmonella enterica* and *Escherichia coli* have also been reported to repress biofilm formation. In S. *enterica* NlpI inhibits curli biosynthesis while *E. coli* data suggests that NlpI suppresses biofilm formation via the suppression of extracellular DNA secretion (Sanchez-Torres et al., 2010; Rouf et al., 2011). While the mechanism governing NlpI-mediated regulation of biofilm

formation in *E. amylovora* remains uncharacterized, a model is emerging that implicates HrpL in a previously unknown role in regulating biofilm formation late in infection (Fig 3.4).

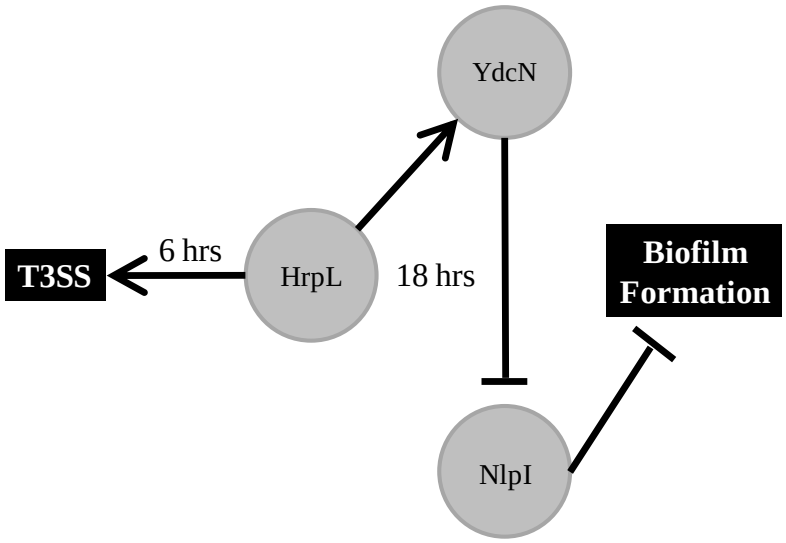

Figure 3.4 Model for HrpL-mediated regulation of biofilm formation.

3.4 Experimental procedures

Bacterial strains and growth conditions

E. amylovora strains were cultured in Luria broth (LB) in a shaking incubator at 28°C. For biofilm formation assays, strains were grown in 0.5X LB medium. Where appropriate, culture media were supplemented with antibiotics including 50 μg ml^{-1} ampicillin, 20 μg ml^{-1} chloramphenicol, 15 μg ml^{-1} gentamicin and 12 μg ml^{-1} tetracycline.

DNA manipulation and sequencing

Chromosomal mutants were synthesized via the phage λ Red recombinase system as described by Datsenko and Wanner (2000). Chloramphenicol-amended LB agar medium was used to

screen putative mutants and single-gene recombinatorial deletion was confirmed using PCR and functional complementation. Standard molecular techniques such as restriction enzyme digestion, T4 DNA ligation, and PCR amplification followed established protocols (Sambrook et al., 1989). DNA and plasmid extraction and DNA isolation and purification were performed with related kits (Qiagen, Valencia, CA). DNA sequencing was conducted at the Research Technology Support Facility at Michigan State University.

RNA isolation and qRT-PCR

Bacterial strains were cultured in *hrp*-inducing minimal medium (Huynh et al., 1989). RNA was isolated using an RNeasy minikit method (Qiagen, Valencia, CA). TaqMan reverse transcription reagents were used for cDNA synthesis and SYBR green master mix used for qRT-PCR (Applied Biosystems, Foster City, CA). Oligonucleotide primer sequences are listed in Table 3.2 and *recA* (EAM_2640), *gyrA* (EAM_2264) and *ffh* (EAM_2630) were used as endogenous controls for data analysis (Takle et al., 2007). Data were collected and analyzed using a StepOne Plus real-time PCR system (Applied Biosystems).

Microarray design and execution

An oligonucleotide microarray was designed and synthesized as previously described (McNally et al., 2012). WT Ea1189 and Ea1189Δ*ydcN* were washed and resuspended in hrpMM to 0.6 OD600. Strains were induced for 18 hours in a shaking incubator at room temperature and RNA was extracted using an SV Total RNA Isolation System (Promega, Madison, WI, USA). RNA quantity and quality was determined using a Nano-Drop ND-100 spectrophotometer (NanoDrop Technologies; Wilminton, DE) and RNA quality was checked

using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Microarray hybridization and data analysis was conducted as previously described (McNally et al., 2012; Wang et al., 2012) Gene expression value was determined as significant when the p-value was less than 0.05 and the expression ratio exceeded a 2.5X expression ratio.

Confocal laser scanning microscopic imagining of *E. amylovora* biofilms

Biofilm formation was three-dimensionally examined using a flow cell apparatus (Stovall Life Sciences, Greensboro, NC) as described previously (Koczan et al., 2009). Strains were labeled with green fluorescent protein via the transformation of the plasmid pMP2444 into electro-competent Ea1189 strains. A Zeiss 510 Meta ConfoCor3 LSM confocal laser scanning microscopy was utilized to observe Ea1189 biofilm formation (Carl Zeiss Microimaging GmbH) and a three-dimensional image that measuring intensity was produced at 100X using LSM image browsing software (Carl Zeiss Microimaging GmbH).

Chapter 4: HrcU and HrpP interact in the fire blight pathogen *Erwinia amylovora* and regulate type III secretion

4.1 Introduction

The type III secretion system (T3SS) is an important pathogenicity factor in many host-pathogen interactions. The T3SS is a common feature of Gram-negative bacterial pathogens and function to facilitate the translocation of bacterial effector proteins into eukaryotic host cells were they suppress host defense responses and facilitate colonization and disease development (Galan and Collmer 1999). Consequently, type III secretion has been the focus of intensive research in animal pathogens like *Yersinia pestis, Escherichia coli, Salmonella enteric* as well as plant pathogens such as *Xanthomonas campestris* and *Pseudomonas syringae*.

The T3SS is a complex proteinaceous machine consisting for more than 20 components (Burger et al., 2010; Buttner 2012). Eleven T3SS proteins are conserved between plant and animal pathogenic bacteria and among these, eight are additionally conserved with the flagellum, an evolutionary relative (He et al., 2004; Desvaux et al., 2006). The structure of T3SS is frequently subdivided into three main parts including the basal body, filament, and translocon.

The core components of the T3SS are highly conserved and constitute the membrane-spanning basal body. The nomenclature associated with conserved T3SS protein families is based on the Ysc proteins of *Yersinia* spp (Bogdanove et al., 1996). The T3SS basal body is comprised of the outer membrane secretin YscC, a periplasmic inner rod YscI as well as an inner membrane export complex including proteins YscQ, YscV, YscN and YscU (Buttner 2012). Functions of the basal body include creating a conduit for protein secretion across the bacterial cell envelope, energizing T3S via ATP hydrolysis, and regulating type III secretion via protein-

protein interactions with T3SS substrates and regulators (Muller et al., 2006; Lorenz and Buttner 2009; Lorenz et al., 2012; Hartmann and Buttner 2013).

The T3SS filament is referred to as a needle or pilus in animal and plant bacterial pathogens, respectively, and is composed of repeating subunits that extend from the basal body serving as a hollow conduit across extracellular space (Roine et al., 1997; Jin and He 2001). Delivery of pathogen effectors into the host cytoplasm is ultimately mediated via proteins called translocators that associate distally with the T3SS needle and form a protein complex call the translocon (Mueller et al, 2005; Mueller et al., 2008). The T3SS translocon exhibits pore-forming abilities and facilitates effector translocation across the eukaryotic plamsa membrane (Lee et al., 2001; Kvitko et al., 2007; Bocsanczy et al., 2008). Extracellular components of the T3SS are secreted by the T3S basal body and are more species-specific than components of the basal body (Jin and He 2001) . For example, the length of T3SS needles ranges from 40 to 80 nm in animal pathogenic bacteria whereas plant pathogenic T3S pili can be more than 2 µm long (Buttner 2012). In addition, plant bacterial pathogens utilize a unique class of translocators called harpins that can form pores in membranes and frequently exhibit distinct pectate lyase domains (Lee et al., 2001, Kvitko et al., 2007).

Because effector translocation necessitates a functioning multipartite machine, the secretion of T3SS structural components and effectors has been assumed to be hierarchical. Recent analyses have confirmed the hierarchical nature of type III secretion (Williams et al., 1996; Minamino and Macnab 2000; Sorg et al., 2007, Lorenz and Butter 2011). Characterization of this hierarchy has revealed multiple substrate classes. Early substrate are involved in needle or pilus formation and middle substrates represent translocators and harpins. Effectors are categorized as late substrates and secreted after the assembly of a functioning T3S system.

An array of factors have be implicated in regulating T3SS hierarchy (Buttner 2012). Among these are both secretion signals, encoded within the amino acid sequence of T3SS substrates, and chaperones that enable substrate localization and secretion. In *Yersinia enterocolitica*, a fusion protein composed of the translocator LcrV and the N-terminal secretion signal of the effector YopE lead to LcrV co-secretion with *Y. enterocolitica* effectors (Sorg et al., 2007). In *Xanthomonas campestris*, the T3S chaperone HpaB is required for the secretion of late substrates and prevents the translocation of the pilus protein XopA and the translocator HrpF (Buttner et al., 2004).

While a complex phenomenon, a family of proteins termed YscU/FlhB proteins may represent the most characterized regulator of type III secretion hierarchy (Williams et al., 1996; Minamino and Macnab 2000; Sorg et al., 2007, Lorenz and Butter 2011). The YscU/FlhB family describes YscU from *Yersinia* spp. and the flagellar protein FlhB as well as EscU from *E. coli*, Spa40 from *Shigella flexneri*, and HrcU from plant bacterial pathogens such as *X. campestris* and *P. syringae*.

YscU/FlhB proteins exhibit four N-terminal transmembrane domains that play a structural role in the inner membrane export apparatus of the T3SS basal body (Berger et al., 2010; Diepold et al., 2012). Type III secretion is completely inhibited in *yscU/flhB* null mutants (Allaoui et al., 1994, Hirano et al., 1994, Lorenz and Butter 2011). The C-termini of YscU/FlhB proteins however encode a cytoplasmic domain involved in regulating T3SS hierarchy (Allaoui et al., 1994; and Macnab 2000; Lorenz and Butter 2011). This domain is required for conformational changes in YscU/FlhB proteins via autoproteolytic cleavage at an Asp-Pro-Thr-His (NPTH) motif (Ferris et al., 2005; Sorg et al., 2007; Zarivach 2008). The NPTH motif is conserved in all YscU/FlhB homologs and point mutations in the NPTH motif are typically

associated with phenotypes including 1) decreased secretion of middle and/or late T3S substrates, 2) increased secretion of early T3S substrates, and 3) changes in T3S filament length (Hirano et al., 1994; Sorg et al., 2007). While FlhB and YscU exemplify the aforementioned mutant phenotypes, point mutations in the NPTH motifs of EscU from *E. coli* and HrcU from *X. campestris* inhibit the secretion of middle and late T3S substrates while not affecting early substrate secretion or needle and pilus length (Zarivach 2008; Lorenz and Butter 2011).

Due to the location of YscU/FlhB proteins at the basal body-cytoplasm interface and due to their role in regulating type III secretion hierarchy, YscU/FlhB proteins display numerous protein-protein interactions (Minamino and MacNab 2000, Lorenz et al., 2008, Riordan and Schneewind 2008, Lorenz and Buttner 2011). HrcU from *X. campestris* has been demonstrated to interact with at least seven other T3S proteins including other components of the basal body and HpaC (Lorenz et al., 2008, Lorenz and Buttner 2009; Lorenz et al., 2012; Hartmann and Buttner 2013). HpaC, and HpaC homologs, differ from the YscU/FlhB protein family in that they share little amino acid sequence conservation. They are hydrophobic, globular and contain a Pro-X-Leu-Gly C-terminal motif (Agrain et al., 2005). Mutations affecting HpaC-like proteins frequently inhibit the ability of T3S systems to change substrate specificity during hierarchical type III secretion and consequently HpaC-like proteins are collectively referred to as T3S substrate specificity switches (T3S4) (Buttner 2012). T3S4 mutant phenotypes include 1) reduced secretion of late substrates, 2) increased filament length, and sometimes 3) increased secretion of early substrates (Minamino et al., 1999; Edqvist et al., 2003; Journet et al., 2003; Lorenz et al., 2008; Schulz and Buttner 2011). YscP from *Yersinia* spp., FliK from flagella, HrpP from *P. syringae* as well as HpaC all represent T3S4 proteins. Both FliK and HpaC have been demonstrated to directly bind the cytoplasmic domains of YscU/FlhB proteins and

phenotypes associated with NPTH domain mutations are attributed to loss of protein-protein interaction with T3S4 proteins (Minamino and Macnab 2000; Sorg et al., 2007; Lorenz et al., 2008; Schulz and Buttner 2011).

The Gram-negative plant pathogenic bacterium *Erwinia amylovora* is the causative agent of fire blight, a disease of rosaceous species including apple and pear. Disease development by *E. amylovora* requires a functioning T3S system (Oh and Beer, 2005). In *E. amylovora*, the T3S system is known to secrete at least 12 proteins including the harpins HrpN and HrpW as well as the effector DspE, a pathogenicity factor (Barny et al., 1990; Bauer and Beer, 1991; Nissinen et al., 2007). To date, little is known about how secretion hierarchy is regulated in *E. amylovora*. While HrpJ, a homologue of YopN in *Yersinia* spp., is required for secretion of translocators HrpN and HrpW, nothing is known about how *E. amylovora* regulates the substrate specificity of DspE, the most important component of the T3S system for fire blight disease development (Nissinen et al., 2007). In *E. amylovora* YscU/FlhB and TS34 proteins are represented by HrcU and HrpP, respectively. Here HrcU and HrpP are explored for roles in T3SS regulation in *E. amylovora*.

4.2 Results

HrcU exhibits a conserved NPTH motif required for pathogenicity

The NPTH motif in YscU/FlhB family proteins is the site of autoproteolytic cleavage and conformational change required for protein function (Ferris et al., 2005; Sorg et al., 2007; Zarivach 2008). This NPTH motif is conserved in all known YscU/FlhB proteins (Minamino and Macnab 2000; Lavander et al., 2002; Lorenz and Buttner 2011). Bioinformatic analysis of HrcU from *E. amylovora* using a dense alignment surface algorithm predicted that, like

YscU/FlhB homologs, HrcU encodes four transmembrane domains as well as a cytoplasmic C-terminal tail (Cserzo et al., 1997; Berger et al., 2010) (Fig 4.1). Using T-Coffee multiple alignment software, the amino acid sequence of HrcU was compared to multiple homologs in T3SS of plant and animal bacterial pathogens as well as in the flagellum (Notredame et al., 2000) (Table 4.2). The *E. amylovora* HrcU NPTH motif ($HrcU_{NPTH}$) was found to be conserved in *E. amylovora* and in all analyzed homologs (Fig 4.1).

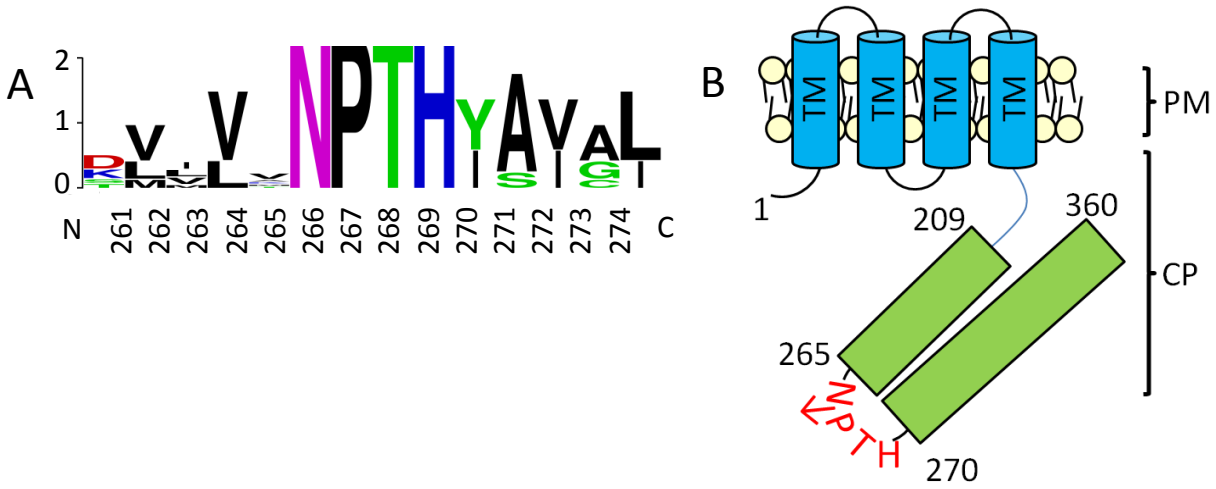

Figure 4.1 Bioinformatic analysis of HrcU from *E. amylovora*. (A) T-Coffee multiple sequence alignment of NPTH motif in amino acid sequences YscU/FlhB family proteins. Aligned was visualized using Weblogo software. The NPTH motif is conserved in *E. amylovora* and all homologs. (B) A schematic representation of HrcU from *E. amylovora*. Image depicts the plasma membrane (PM) localization of HrcU via transmembrane domains (TM) as well as the cytoplasmic (CP) tail of HrcU. The NPTH motif is labeled in red and the arrow represents the site of cleavage and conformational change reported in YscU/FlhB family proteins.

To determine the role of HrcU in disease development, a chromosomal deletion of *hrcU* (EAM_2905) was created in *E. amylovora* Ea1189. Ea1189Δ*hrcU* was confirmed to be nonpathogenic due to a lack of sympotom development 6 days post inoculation (dpi) in immature pears (Fig 4.1). *In trans* expression of *hrcU* via the plasmid pRRM5 was able to successfully complement the mutant strain restoring full virulence to Ea1189Δ*hrcU* (Fig 4.1).

To ascertain the importance of $HrcU_{NPTH}$ in *E. amylovora*, HrcU was subjected to site-directed mutagenesis. The asparagine residue of the NPTH motif is required for YscU/FlhB protein function in all assayed homologs (Sorg et al., 2007; Riordan and Schneewind 2008; Lorenz and Buttner 2011). Consequently, the conserved asparagine residue located at position 266 in the amino acid sequence of HrcU was mutated to encode a codon corresponding to alanine. This *hrcU* mutant allele ($HrcU_{\Delta N266A}$) was cloned into an expression vector creating pRRM6 (Heckman and Pease 2007).

To determine the role of $HrcU_{\Delta N266A}$ on host-microbe interactions with *E. amylovora*, Ea1189Δ*hrcU*/pRRM5 and Ea1189Δ*hrcU*/pRRM6 were inoculated into immature pear fruits. While plasmid-borne *hrcU* was able to re-establish wild type (WT) virulence levels to Ea1189Δ*hrcU*, Ea1189Δ*hrcU*/pRRM6 was unable to restore pathogenicity 6 dpi in immature pear fruits (Fig 4.2). This indicates that $HrcU_{NPTH}$ is required for HrcU function and that $HrcU_{NPTH}$ is necessary to mediate compatible host interactions.

$HrcU_{NPTH}$ is required for the elicitation of the hypersensitive response

The hypersensitive response (HR) is a hallmark of incompatible plant-microbe interactions. HR is characterized by rapid, localized programmed cell-death in response to pathogen-associated proteins frequently represented by T3SS substrates. HR elicitation in *E. amylovora* requires a functional T3S system (Steinberger and Beer 1988). *E. amylovora* Ea1189 strains were inoculated into *Nicotiana benthamiana* mesophyll tissue and, 16 hours post inoculation (hpi), results revealed that *E. amylovora* Ea1189 requires HrcU, and specifically $HrcU_{NPTH}$, for HR development (Fig 4.2). While WT Ea1189 and complemented Ea1189Δ*hrcU*/pRRM5 induced robust HR symptoms in *N. benthamiana*, Ea1189Δ*hrcU* and Ea1189Δ*hrcU* expressing $HrcU_{\Delta N266A}$ failed to trigger an incompatible defense response (Fig 4.2). As HR in response to *E. amylovora* infection requires T3S, these results suggest that the inability of $HrcU_{\Delta N266A}$ to complement Ea1189Δ*hrcU* is due to the role of $HrcU_{NPTH}$ in mediating type III secretion.

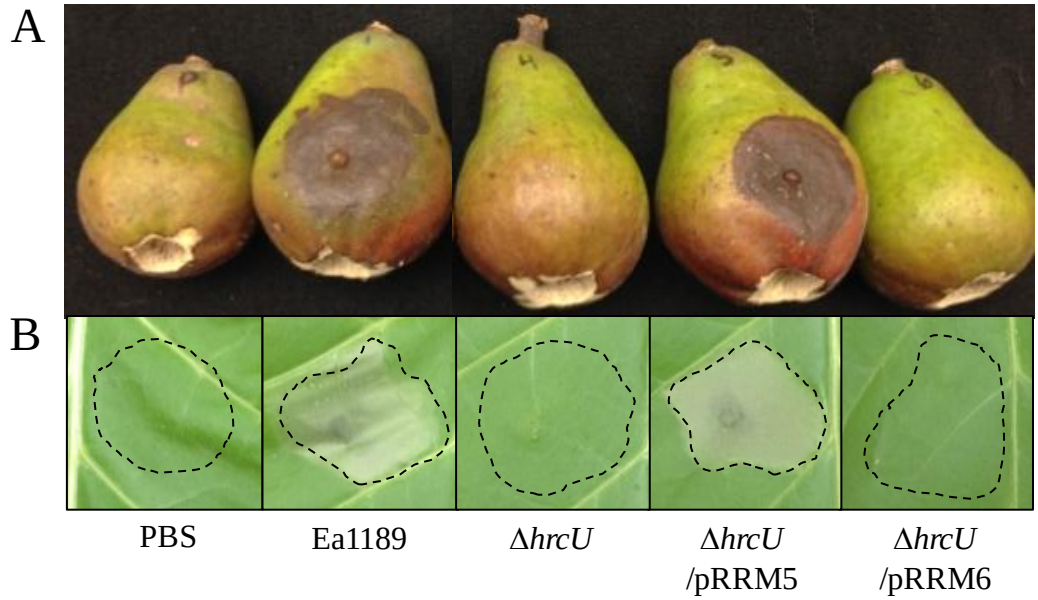

Figure 4.2 Phenotypic characterization of HrcU-related mutant strains in Ea1189. WT Ea1189, Ea1189Δ*hrcU* and Ea1189Δ*hrcU* stains expressing native HrcU from pRRM5 or $HrcU_{\Delta N266A}$ from pRRM6 were inoculated into (A) immature pear fruits and (B) *Nicotiana benthamiana*. Pear fruit necrosis was recorded 6 days post inoculation while the hypersensistive response in *N. benthamiana* was observed 16 hours post inoculation. pRRM6 was unable to complement the *hrcU* null mutation.

$HrcU_{NPTH}$ is required for the secretion of DspE

The T3SS effector DspE is a pathogenicity factor of *E. amylovora* and translocation of DspE is required for fire blight disease development (Barny et al., 1990; Bauer and Beer, 1991; Bocsanczy et al., 2008; Triplett et al., 2009). Mutations affecting the T3SS that result in a loss-of-pathogenicity phenotype are consequently hypothesized to be attributed to decreased DspE translocation by *E. amylovora*. To determine if $HrcU_{NPTH}$ is involved in regulating DspE

secretion, *E. amylovora* strains were incubated *in vitro* in *hrp*-inducing minimal medium (HrpMM) used to mimic conditions of the plant apoplast (Wei et al., 1992). Proteins were extracted 48 hpi and subjected to one-dimensional SDS-PAGE separation and western blot analysis. As predicted, an Ea1189 strain harboring native *hrcU* secreted DspE *in vitro* while Ea1189Δ*hrcU* failed to secrete any DspE protein (Fig 4). Likewise, an Ea1189 strain synthesizing $HrcU_{\Delta N266A}$ was also unable to secrete DspE (Fig 4). These results show that $HrcU_{NPTH}$ is required for DspE secretion *in vitro* and suggest that Ea1189Δ*hrcU* strains expressing $HrcU_{\Delta N266A}$ are nonpathogenic due to loss of DspE secretion.

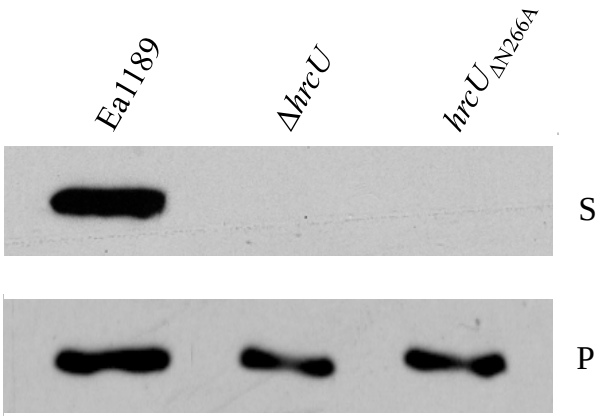

Figure 4.3 DspE secretion in Ea1189 strains. Secretion of a DspE-CyaA fusion protein was assayed *in vitro* in minimal medium. An anti-CyaA antibody was used to detect DspE-CyaA (~200 kDa) in the culture supernatant (S) and the cell pellet (P). Native HrcU and the $HrcU_{NPTH}$ domain were required for DspE secretion as $HrcU_{\Delta N266A}$ was unable to secrete DspE into the supernatant. HrcU did not affect the production of DspE in the cell pellet.

HrpP is required for pathogenicity and hypersensitive response induction

In YscU/FlhB family proteins, the NPTH motif is required for the regulation of type III secretion hierarchy (Allaoui et al., 1994; and Macnab 2000; Sorg et al., 2007; Lorenz and Butter 2011). T3SS hierarchy regulation is mediated via direct and indirect interactions with T3S4 proteins (Minamino and Macnab 2000; Sorg et al., 2007; Lorenz et al., 2008; Schulz and Buttner 2011). In *E. amylovora*, HrpP (EAM_2900) is a predicted T3S4 protein. Bioinformatic analyses of the HrpP amino acid sequence are in accordance with previous observations that T3S4 proteins are poorly conserved between species and that, in bacterial plant pathogens, T3S4 proteins are N-terminally truncated relative to homologs in bacterial animal pathogens and the flagellum (Buttner 2012) (Fig 5). Beginning at amino acid position 98 though, HrpP does exhibit a modified Pro-X-Leu-Gly motif that is characteristic of T3S4 domains (Deane et al., 2010) (Table 4.3; Fig 4.4).

To establish the role of HrpP in mediating plant-microbe interactions, a chromosomal deletion of HrpP was synthesized and relevant strains were inoculated into host and non-host plant species. Like Ea1189Δ*hrcU* strains expressing $HrcU_{\Delta N266A}$, Ea1189Δ*hrpP* was nonpathogenic 6 dpi in immature pear fruit and unable to elicit a HR in *N. benthamiana* (Fig 5).

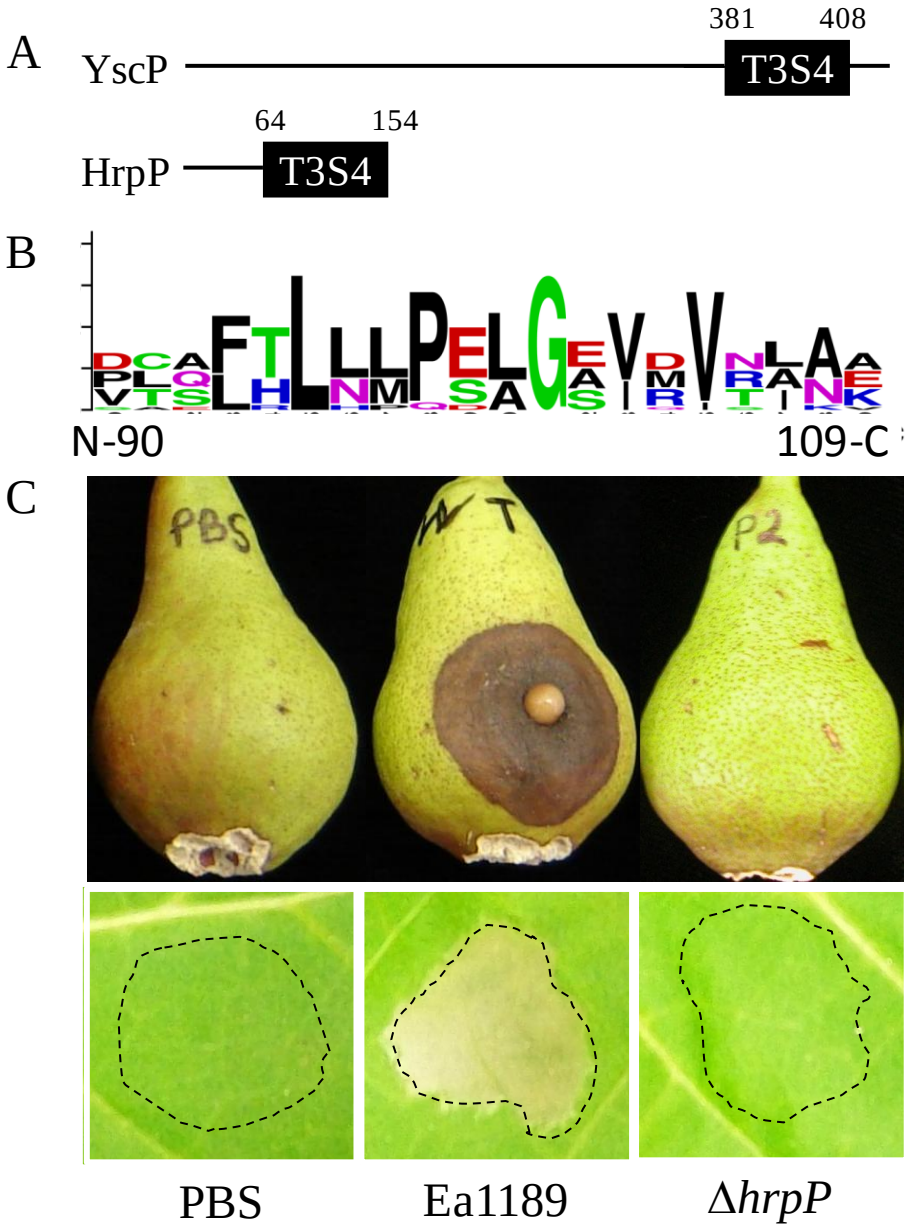

Figure 4.4 Bioinformatic and phenotypic analyses of HrpP from *E. amylovora*. (A) T-Coffee multiple sequence alignment of HrpP (EAM_2900) with the *Yersinia* spp. T3S4 protein YscP exemplify that HrpP in *E. amylovora* is markedly shorter than T3S4 proteins in animal bacterial pathogen and the flagellum. (B) Visualization of T3S4 protein sequence alignments with Weblogo software demonstrates that HrpP exhibits a conserved P-X-L-G motif

characteristic of T3S4 family proteins. (C) WT Ea1189 and Ea1189Δ*hrpP* inoculated into immature pear fruits

Figure 4.4 (cont'd) and *N. benthamiana*. Pear fruit necrosis was recorded 6 days post inoculation while the hypersensistive response in *N. benthamiana* was observed 16 hours post inoculation. HrpP is a pathogenicity factor required for HR elicitation.

HrcU and HrpP interact in *E. amylovora*

While not all T3S4 proteins have been observed to interact directly with YscU/FlhB counterparts, direct interactions have been recorded between the T3S4 proteins FliK and HpaC (Minamino and Macnab 2000; Lorenz et al., 2008). To explore the possibility of HrpP interactions with HrcU in *E. amylovora*, *hrpP* and *hrcU* constructs were cloned into Y2H vectors and assayed in *Saccharomyces cerevisiae* AH109 via survival on minimal medium and α-galactosidase activity. In the Y2H assay, *hrcU* alleles featuring the $HrcU_{\Delta N266A}$ point mutation were included along with N-terminal *hrcU* deletions (HrcU-CT). HrcU-CT constructs were included due to reported transmembrane domain interference with protein-protein interactivity in homologous YscU/FlhB proteins (Minamino and Macnab 2000; Lorenz et al., 2008). Alongside HrpP, the YopN-family protein HrpJ was also screened for the ability to interact with HrcU in yeast as HrpJ is a demonstrated regulator of T3SS hierarchy in *E. amylovora* (Nissinen et al., 2007).

In all cases, full length HrcU encoding N-terminal transmembrane domains were unable to interact with either HrpP or HrpJ (Fig 4.5). HrpJ exhibited a very weak interaction with both HrcU-CT and $HrcU\text{-}CT_{\Delta N266A}$ (Fig 4.5). Conversely, HrpP interacted strongly with HrcU-CT in Y2H experiments (Fig 4.5). While the $HrcU_{NPTH}$ motif was not required for interactions with

HrpP, HrcU-CT$_{\Delta N266A}$ displayed less α-galactosidase activity in the presence of HrpP than did HrcU-CT (Fig 4.5). This indicates that HrpP does interact with HrcU and that HrcU$_{NPTH}$-mediated conformational changes in HrcU affect HrpP binding in Y2H assays (Fig 6).

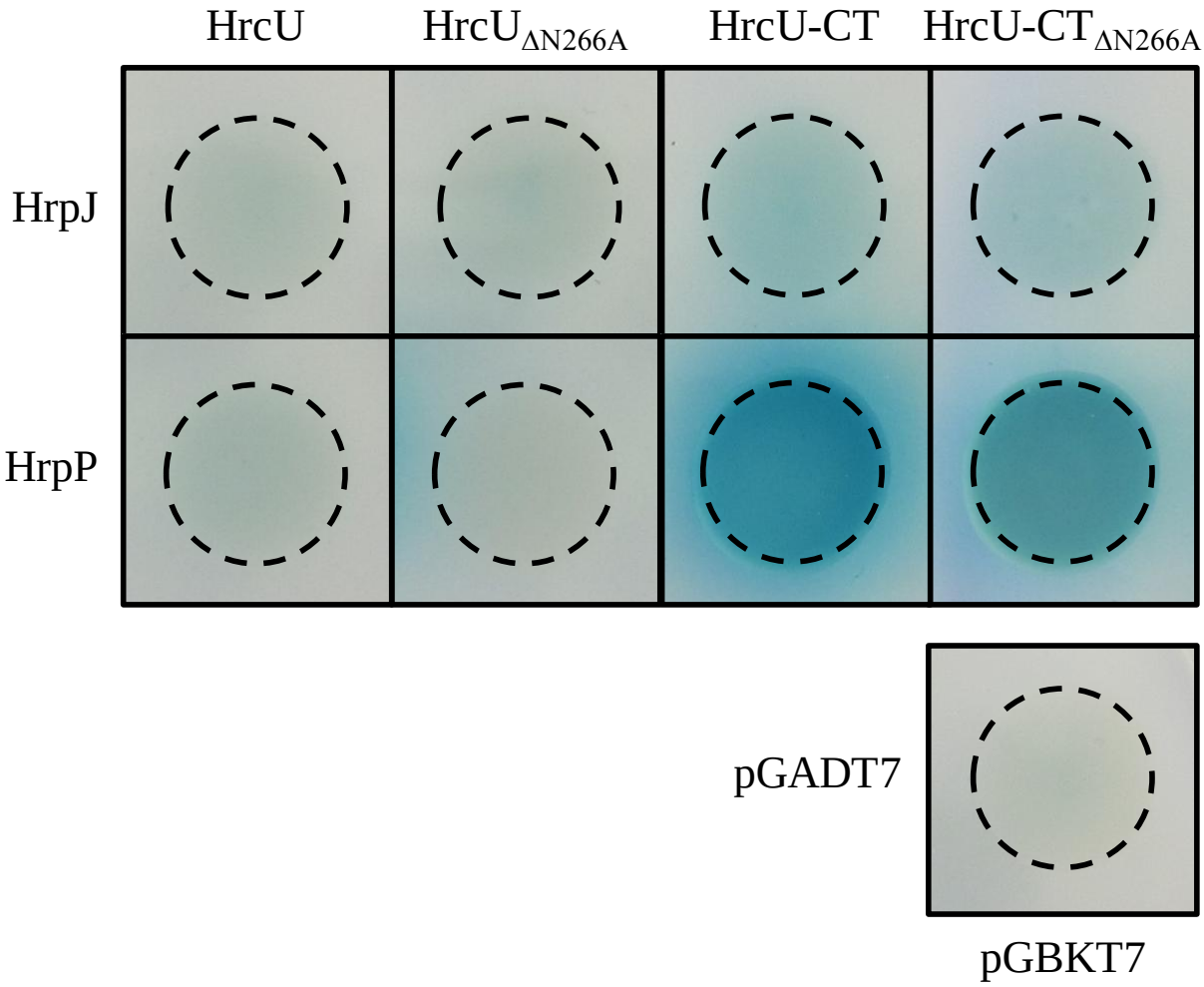

Figure 4.5 HrcU yeast two-hybrid (Y2H) interaction assays. Native HrcU and HrcU$_{\Delta N266A}$ are cloned into the Y2H bait vector pGBKT7 as full-length HrcU or as C-terminal truncations representing the cytoplasmic tail of HrcU (HrcU-CT). HrpJ and HrpP were expressed from the Y2H prey vector pGADT7. Both HrpJ and HrpP failed to interact with either full-length HrcU constructs. HrpJ in yeast exhibits weak pigmentation when co-transformed with both HrcU-CT

constructs. HrpP strongly interacts with WT HrcU-CT and HrcU-$CT_{\Delta N266A}$ although the HrpP-HrcU-$CT_{\Delta N266A}$ interacting is relatively impaired.

HrcU and HrpP regulate the T3S secretome of *E. amylovora*

To elucidate the roles of HrcU and HrpP in regulating T3S hierarchy, *E. amylovora* strains were incubated in liquid minimal medium for 48 hours. Supernatant was collected, concentrated and purified to remove biofilm polysaccharides (Nissinen et al., 2007). Ten µg of purified protein were separated via one-dimensional SDS-PAGE and visualized with silver nitrate. Results clearly demonstrated that $HrcU_{NPTH}$ and HrpP were required for wild type T3S secretion (Fig 4.6). $HrcU_{NPTH}$ and HrpP were clearly required for the T3S of the effector DspE as the secretomes of Ea1189Δ*hrcU* and Ea1189Δ*hrpP* failed to secrete a 200 kDa protein corresponding to full length DspE (Nissinen et al., 2007; Zeng et al., 2013) (Fig 4.6). HrpN, corresponding to a 40 kDa protein, was also absent in the secretomes of Ea1189Δ*hrcU* and Ea1189Δ*hrpP* (Fig 4.6). Interestingly, Ea1189 is reported to secrete an approximately 100 kDa low-abundance DspE fragment (Nissinen et al., 2007). While Ea1189Δ*hrpP* failed to secrete full-length DspE, the secretome of Ea1189Δ*hrpP* retained secretion of a protein probably corresponding to this DspE fragment (Fig 7).

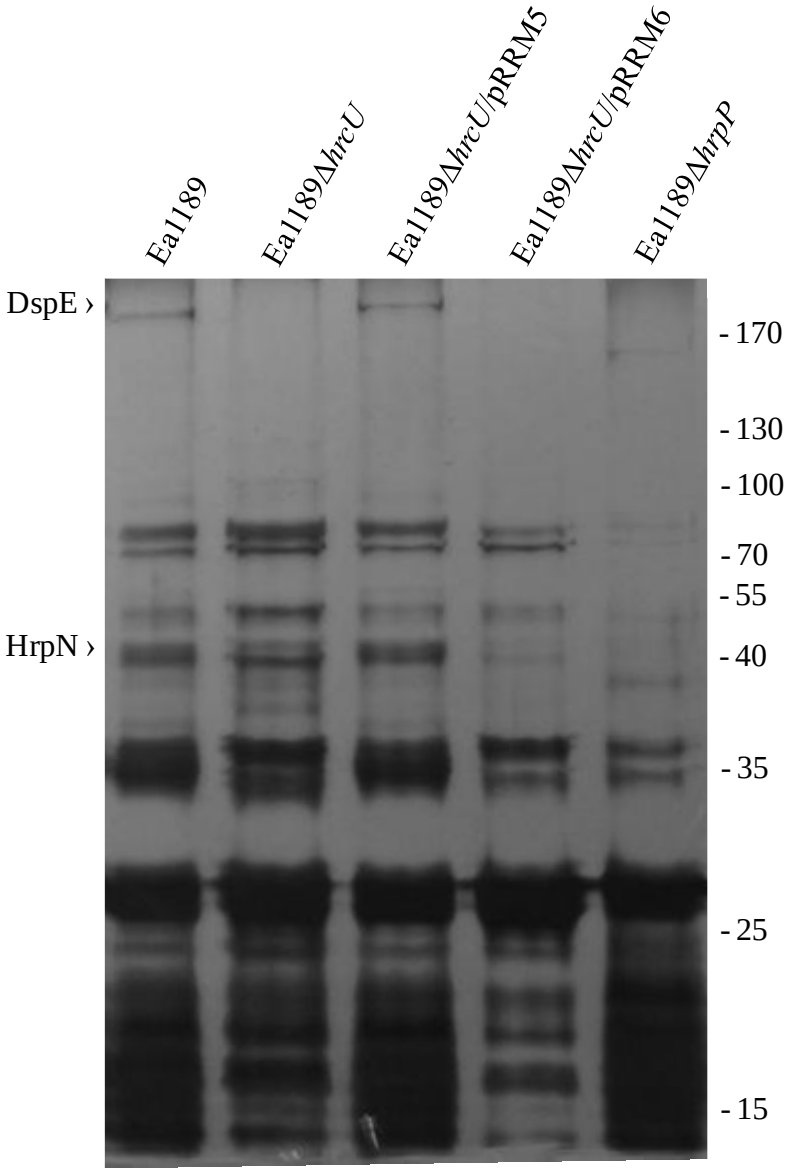

Figure 4.6 Effect of HrcU and HrpP on T3S secretome of *E. amylovora*. WT Ea1189, Ea1189Δ*hrcU* strains and Ea1189Δ*hrpP* cultured in minimal medium for 48 hours. 10 micrograms of protein sample separated in a 11% acrylamide gel using SDS-PAGE and visualized using silver nitrate staining. Ea1189Δ*hrpP* and Ea1189Δ*hrcU*/pRRM6 expressing $HrcU_{\Delta N266A}$ *in trans* dramatically impacted the T3SS secretome.

4.3 Discussion

In this study the roles of HrcU and HrpP in regulating the T3SS in *E. amylovora* Ea1189 was explored. Using site-directed mutagenesis, phenotypic analyses, Y2H assays and secretome visualization, HrcU and HrpP were shown to interact and mediate host-microbe interactions via the regulation of T3S secretion substrates like the effector DspE.

HrcU and HrpP were both confirmed to be pathogenicity factors in *E. amylovora*. Ea1189Δ*hrcU* and Ea1189Δ*hrpP* were both unable to cause disease in immature pear fruits. Likewise, Ea1189Δ*hrcU* and Ea1189Δ*hrpP* were also unable to elicit a hypersensitive response after inoculation into *N. benthamiana*. These results are in agreement with previous observations regarding HrcU in *P. syringae* and *X. campestris* (Charkowski et al., 1997; Lorenz and Buttner 2011). Interestingly, while HrpP in *E. amylovora* and *P. syringae* are both required for disease and HR induction, the T3S4 homolog HpaC is not a pathogenicity factor in *X. campestris* (Buttner et al., 2006; Morello and Collmer 2009).

The important influence of HrcU and HrpP in facilitating disease development is hypothesized to stem from roles in regulating type III secretion hierarchy. Here were report that HrpP and HrcU have dramatic effects of the T3SS secretome in *E. amylovora*. Western blot analysis revealed that DspE secretion required HrcU and HrcU-mediated secretion of DspE was dependent on the integrity of its conserved NPTH motif. In addition, the inability of *E. amylovora* mutant strains to elicit HR in tobacco indicates that secretion of HrpN, an HR elicitor, is likely impaired in Ea1189Δ*hrcU* and Ea1189Δ*hrpP* (Wei et al., 1992).

Other YscU/FlhB and T3S4 proteins exhibit different effects on T3S hierarchy regulation. In *Yersinia* spp., YscU and the T3S4 protein YscP regulate a T3 substrate specificity

switch from translocator secretion to effector secretion (Edqvist et al., 2003; Sorg et al., 2007). In *X. campestris* and the flagellum, HrcU and HrpP homologs regulate the secretion of early versus late substrates (Minamino et al., 1999; Minamino and Macnab 2000; Lorenz et al., 2008; Lorenz and Buttner 2009; Lorenz and Buttner 2011). Unlike *Yersinia* spp., *X. campestris* does not appear to recognize effectors and translocators as distinguishable T3SS substrates (Lorenz et al., 2008; Schulz and Buttner 2011).

In *P. syringae,* while HrcU has not been studied, HrpP has been shown to function atypically compared to other T34S family proteins. Like HpaC from *X. campestrix*, *hrpP* null mutants in *P. syringae* do not secrete translocators or effectors (Buttner et al., 2006; Morello and Collmer 2009). When mutated however, T34S proteins canonically over-secrete some early T3S substrates as a resulted of inhibited substrate specificity switching. Null *yscP* mutants in *Yersinia* spp. secrete more early T3S substrates like the pilus subunit YscF and the inner rod protein YscI (Edqvist et al., 2003; Wood et al., 2008). HpaC mutants hyper-secrete the inner rod protein HrpB2 (Lorenz et al., 2008). HrpP mutations in *P. syringae* however weakly secrete early T3S substrates like the pilus subunit HrpA and HrpP may and may be more accurately described as a post-translational activator of T3S as opposed to a T3S4 protein (Morello and Collmer 2009).

The regulatory function of YscU/FlhB proteins depend on a conserve NPTH amino acid motif (Hirano et al., 1994; Sorg et al., 2007; Zarivach 2008; Lorenz and Butter 2011). The cytoplasmic C-terminus of HrcU in *E. amylovora* encodes an NPTH motif. Notably, a site-directed mutation of HrcU resulting in the construct $HrcU_{\Delta N266A}$ was unable to complement Ea1189Δ*hrcU* suggesting the role of HrcU in mediating plant-microbe interactions requires the presence of an asparagine residue at position 266. Ea1189Δ*hrcU* strains expressing

$HrcU_{\Delta N266A}$ were nonpathogenic and unable to secrete the effector protein DspE. These phenotypes are likely linked as DspE secretion is required for disease development (Triplett et al., 2009). This result reinforces data highlighting the importance of the NPTH motif in HrcU as synonymous mutations in *X. campestris* also abolish disease development (Lorenz and Buttner 2011).

Here evidence is presented for the first time suggesting that HrcU and HrpP interact in *E. amylovora*. Considering that both HrcU and HrpP are pathogenicity factors in *E. amylovora* and as both Ea1189Δ*hrcU* and Ea1189Δ*hrpP* exhibit impaired DspE secretion, it is tempting to speculate that interactions between HrcU and HrpP may be important for their relative roles in pathogenicity. While HrpP interacted strongly with the cytoplasmic domain of HrcU during Y2H assays, HrpP also interacted with $HrcU_{\Delta N266A}$. This indicates that the important role of the NPTH motif in HrcU for regulating DspE secretion and disease development is not related to loss of HrpP-HrcU interaction.

Protein-protein interaction is not a ubiquitous feature among YscU/FlhB and T3S4 families (Morris et al., 2010; Lorenz and Buttner 2011). While synonymous mutation in HrcU from *X. campestris* abolish HrcU binding with the T3S4 protein HpaC, FlhB mutations affecting the NPTH motif in the flagellum do not impair binding to the T3S4 protein FliK (Morris et al., 2010; Schulz and Buttner 2011). Collectively these results highlight regulatory divergence between bacterial species the complicated relationship between YscU/FlhB and T3S4 proteins in regulating T3SS hierarchy and T3S.

In *E. amylovora* Ea1189, both HrcU and HrpP were found to be required for compatible as well as incompatible plant-microbe interactions. The status of HrcU as a pathogenicity factor is likely attributed to defects in type III secretion as Ea1189Δ*hrcU* was unable to secrete DspE.

Both Ea1189Δ*hrcU* phenotypes required an asparagine residue in a conserved NPTH motif as Ea1189Δ*hrcU* expressing $HrcU_{\Delta N266A}$ could not secrete DspE and was nonpathogenic.

4.4 Experimental procedures

Bacterial strains and growth conditions

Table 4.1 lists bacterial strains and plasmids used in this study. Unless otherwise referenced, bacterial strains were grown in Luria Bertani (LB) broth supplemented with 50 μg ml^{-1} ampicillin, 20 μg ml^{-1} chloramphenicol, 12 μg ml^{-1} oxytetracycline or 30 μg ml^{-1} kanamyacin where appropriate. All strains were cultured at 28°C in a shaking incubator.

Deletion mutagenesis

E. amylovora site-directed nonpolar chromosomal mutants were generated using the phage λ Red recombinase system previously described (Datsenko and Wanner 2000). Briefly, *E. amylovora* strain Ea1189 harboring pKD46, encoding recombinases red β, γ, and exo, was cultured overnight at 28°C in a shaking incubator. Strains were reinoculated with 0.1% L-arabinose in LB broth and cultured for four hours to exponential phase. Cells were made electrocompetent and stored at -80°C. Homologous recombination fragments encoding acetyltransferase cassettes were generated via polymerase chain reaction (PCR) using the plasmid pKD3 as a template. A PCR purification kit (Qiagen; Valencia, CA) was using to purify recombination fragments before electroporation into competent Ea1189. LB agar amended with chloramphenicol was used to screen putative mutants and single-gene recombinatorial deletion was confirmed using PCR and functional complementation.

DNA manipulation and cloning

Restriction enzyme digestion, T4 DNA ligation, and PCR amplification of genes were carried out using standard molecular techniques (Sambrook et al., 1989). DNA extraction, PCR purification, plasmid extraction, and isolation of DNA fragments from agarose were performed with related kits (Qiagen, Valencia, CA). All DNA was sequenced at the Research Technology Support Facility at Michigan State University. Double digestion and directional ligation into pBBR1MCS3 with PCR-generated gene sequences was utilized for mutant strain complementation (Kovach et al., 1995). Final constructs were transformed into competent Ea1189 by electroporation and screened on LB agar plates amended with oxytetracycline.

Table 4.1 Bacterial Strains, plasmids and primers used in chapter 4

Strains and Plasmids	Relevant characteristics[a]	Source or reference
Escherichia coli strain		
DH5α	F- 80dlacZ, ΔM15, Δ(lacZYA-argF)U169, endA1, recA1, hsdR17(rK–mK+), deoR, thi-1, supE44, gyrA96, relA1 λ-	Invitrogen, CA, USA
Yeast strain *Saccharomyces cerevisiae* AH109	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3 : : $MEL1_{UAS}$-$MEL1_{TATA}$-lacZ	James et al., 1996
Erwinia amylovora strains		
Ea1189	Wild type	Burse et al., 2004
Ea1189Δ*hrpL*	*hrpL* deletion mutant, Cm-R	McNally et al., 2012
Ea1189Δ*hrcU*	*hrcU* deletion mutant, Cm-R	This study
Ea1189Δ*hrpP*	*hrpP* deletion mutant, Cm-R	This study

Table 4.1 (cont'd)

Plasmids		
pBBR1-MCS3	Tc-R, broad host-range cloning vector	Kovach et al., 1995
pRRM5	Tc-R, pBBR1-MCS3 containing *hrcU*	This study
pRRM6	Tc-R, pBBR1-MCS3 containing *hrcU*ΔN266A	This study
pMHJ20	Amp-R, pWSK29 containing codons 2 to 406 of CyaA	Miao and Miller, 1999
pLRT201	Amp-R, pMJH20 expressing DspE(1-737)-CyaA	Triplett et al., 2009
pGADT7	LEU2, Amp-R, Y2H activation vector	Clontech, CA, USA
pRRM7	Amp-R, pGADT7 containing *hrpP*	This study
pRRM8	Amp-R, pGADT7 containing *hrpJ*	This study
pGBKT7	TRP1, Km-R, Y2H bait vector	Clontech, CA, USA
pRRM9	Km-R, pGBKT7 containing *hrcU*	This study
pRRM10	Km-R, pGBKT7 containing *hrcU*ΔN266A	This study
pRRM11	Km-R, pGBKT7 containing *hrcU*209-360	This study
pRRM12	Km-R, pGBKT7 containing *hrcU*209-360, ΔN266A	This study
pKD3	Amp-R, Cm-R mutagenesis cassette template	Datsenko and Wanner 2000
pKD46	Amp-R, expresses λ red recombinase	Datsenko and Wanner 2000
Primers		
HrcUKO.F	TTGGCAGAAAAAACGGAAAAACCCACGGC GAAAAAGCTACAGGATGCGCGGTGTAGGCT GGAGCTGCTTC	This study
HrcUKO.R	TTATTCGGCTTCCAGTTCGATCACCTCATCG GTTATCTGGTCTTCCAGCTCATATGAATATC CTCCTTA	This study
HrpPKO.F	ATGAATACATCGGGTTATTCTGACCGGCTG CCGCCCTCCCCGCGCCAAACGTGTAGGCTG GAGCTGCTTC	This study
HrpPKO.R	TCATGGCGCTTCTCCTTGCTCTATCGACAAC CGTACCGGGCCGTTGAGCGCATATGAATAT CCTCCTTA	This study
HrcU_N266A.B	TGCGTGGGTGCGACCAGCA	This study
HrcU_N266A.C	TGCGTGGGTGCGACCAGCA	This study
hrcU(KpnI).F	TACTTGATGAGGTACCTTGGCAGAAAAAAC GGAAAAACCC	This study
hrcU(N266A).R	AGATTAGTACGAATTCTTATTCGGCTTCCAG TTCGATCAC	This study
hrpJ(EcoRI).F	TGCAGAATTCAAAATTGCTCCCGTTTTACC	This study
hrpJ(BamHI).R	GCTAAACCCAGGACGGCGCTGTAAGGATCC	This study
hrpP(NdeI).F	TTAGCATATGAATACATCGGGTTATTCTGA CCGG	This study
hrpP(ClaI).R	TAGTATCGATTCATGGCGCTTCTCCTTGCTC	This study

Table 4.1 (cont'd)

hrcU(EcoRI).F	GAATTCTTGGCAGAAAAAACGGAAAAACC CACG	This study
hrcU(SalI).R	GTCGACTTATTCGGCTTCCAGTTCGATCACC TC	This study
hrcU_CT(EcoRI).F	TGCAGAATTCGTAGAAGAAGCCGACCTGCT GCTG	This study
hrcU_CT(SalI).R	GACTGTCGACTTATTCGGCTTCCAGTTCGAT CACCTC	This study

[a] Cm-R, Tc-R, Amp-R, Km-R indicates resistance to chloramphenicol, oxytetracycline, ampicillin and kanamycin

Bioinformatics

Lasergene® 7.2.0 software suite was used to manage nucleic and amino acid sequences (DNASTAR, Madison, WI). Genes were annotated in agreement with the *E. amylovora* ATCC 49946 genome (Sebaihia et al., 2010). Protein sequence conservation was determined using BLAST programs at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997). Putative transmembrane domains were predicted using the DAS - Transmembrane Prediction server (http://www.sbc.su.se/~miklos/DAS) (Cserzo et al., 1997). T-Coffee multiple sequence alignment software (http://www.tcoffee.org/homepage.html) was used to create all amino acid sequence alignments (Notredame et al., 2000). Multiple sequence alignments were visualized using Weblogo 2.8.2 (http://weblogo.berkeley.edu) (Crooks et al., 2004).

Table 4.2 YscU/FlhB family proteins used for sequence alignment

Protein	Accession	Bacterium
YscU	NC_004564.1	*Yersinia enterocolitica* A127/90
FlhB	NC_021176.1	*Salmonella enteric* Ty21a
Spa40	AY206439.1	*Shigella flexneri*
EscU	AE005174.2	*Escherichia coli* O157:H7
HrcU	NC_013971.1	*Erwinia amylovora* ATCC 49946
HrcU	NC_007508.1	*Xanthomonas campestris* pv. *vesicatoria* str. 85-10
HrcU	NC_004578.1	*Pseudomonas syringae* pv. *tomato* str. DC3000

Table 4.3 TS34 family proteins used for sequence alignment

Protein	Accession	Bacterium
YscP	NP_783677.1	*Yersinia enterocolitica* A127/90
FliK	YP_007925249.1	*Salmonella enteric* Ty21a
HrpP	YP_003539966.1	*Erwinia amylovora* ATCC 49946
HrpP	NP_791225.1	*Pseudomonas syringae* pv. *tomato* str. DC3000

Virulence and hypersensitive response assays

The virulence of Ea1189 strains was assayed using a standard immature pear fruit assay as described previously (Zhao et al., 2005). In brief, bacterial strains were cultured overnight, washed, and resuspended in 0.5x phosphate buffered saline (PBS) to 1×10^3 to 1×10^4 CFU/ml. Immature pear fruits (*Pyrus communis* L. cv. Bartlett) were surface sterilized with 10% bleach, dried in laminar flow hood, and pricked with a needle prior to application of 2 µl

bacterial suspension. Inoculated pears were incubated at 28°C in humidified chambers. Symptoms were recorded 6 days post inoculation. The experiments were repeated three times with six replications per experiment. To study elicitation of the hypersensitive response during incompatible interactions, *E. amylovora* strains were cultured over night in LB broth. Bacterial cells were collected via centrifugation and washed twice with 0.5X PBS. Cells were resuspended and adjusted to a final concentration of 1×10^7 CFU ml^{-1} in 0.5X PBS. 100 µl of cell suspension were in turn infiltrated into 9-week-old N. benthamiana leaves using a syringe and HR was observed 16 hpi.

Yeast two-hybridization

The bait vector pGBKT7 and the prey vector pGADT7 were used for yeast expression and Y2H screening (Clontech, Mountain View, CA, USA). A Frozen-EZ Yeast Transformation II Kit was used to create competent *Saccharomyces cerevisiae* AH109 and for cotransformation of bait and prey (Zymo Research Corporation, Orange, CA, USA). Transformants were selected on minimal SD agar amended with -Ade/-His/-Leu/-Trp dropout supplement and Mel1 α-galactosidase activity was detected using topically applied X-α-Gal at 4 ug ul^{-1} (Clontech, Mountain View, CA, USA).

Secretion assays

Strains were cultured overnight in 50 ml LB broth at 28°C. Cells were washed twice with 0.5X PBS, and resuspended in 50 ml minimal medium, pH 5.7 (Huynh et al. 1989). Strains were induced for 48 hours with shaking, collected by centrifugation, and the supernatant was filtered using 0.22 µm vacuum filtration (Millipore, Billerica, MA, USA). Filtrate was

supplemented with 0.5 mM phenylmethylsulfonyl fluoride and concentrated to approximately 500 µl using 10-kDa Amicon centrifugal filter units (Millipore, Billerica, MA, USA). For western blot analysis, proteins were analyzed using anti-CyaA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For secretome visualization, proteins were additionally purified to remove biofilm polysaccharides as previously described (Nissinen et al., 2007). Briefly, protein samples were extracted twice with 0.5 volume of water-saturated phenol and precipitated with by the addition of 5 volumes 100 mM ammonium acetate in methanol. After overnight incubated at -20°C, protein were extracted via centrifugation, resuspended in 50 ul water and reprecipitated in 500 µl of cold acetone. Samples were again incubated overnight at -20°C and protein pellets were collected by centrifugation and subsequent resuspension in 50 µl 5% acetic acid. 10 ug of each protein sample were separated via one-dimensional SDS-PAGE and visualized with a Pierce Silver Stain Kit (Thermo Fisher Scientific, Rockford, IL, USA).

Chapter 5: The Future of Erwinia amylovora research

The catalog of *E. amylovora* research is an important component of our understanding of plant-microbe interactions. *E. amylovora* was identified by Thomas Burrill as the first bacterium to cause a plant disease and subsequently named after the father of plant bacteriology, Erwin F. Smith. Understanding HR in non-host plants continues to a be a major endeavor among plant pathologists, and HrpN, an *E. amylovora* harpin and translocator, was the first protein identified to function as a cell-free elicitor of HR. While many avenues are available for critical *E. amylovora* research, continued analyses of major virulence factors such as DspE and HrpN will play an important role in fundamentally understanding fire blight disease development and critical questions remain to be answered.

What is the biological significance of DspE interactions with DIPMs? What are the underlying mechanism responsible for DspE-induced plant cell death? Is DspE-mediated cell death a product of host defenses exemplified by classic gene-for-gene interactions or does DspE-mediated cell death directly promote virulence typical of necrotrophic plant bacterial pathogens such as *Dickeya* spp. and *Pectobacterium* spp., two species closely related to *E. amylovora*. How does DspE suppress callose deposition in host interactions and what role does HrpN play in eliciting callose deposition? What host genetic determinants are responsible for defenses triggered by HrpN, directly or indirectly?

In addition, recent revelations that $AvrRpt2_{Ea}$ exhibits quantitative gene-for-gene interactions with the apple species *Malus* X *robusta* 5, together with information that Eop1 and $HopX1_{Ea}$ also contribute quantitatively to host range and avirulence, indicate that *E. amylovora* is an importantly unique plant pathogen (Asselin et al., 2011; Bocsanczy et al., 2012; Vogt et al.,

2013). Disease development by *E. amylovora* exhibits some qualities of biotrophic plant bacterial pathogens as well as some qualities found in necrotrophic bacteria. While *E. amylovora* requires a functional T3SS like *Pseudomonas* spp. and *Xanthomonas* spp., it does not utilize comparatively large effector repertoires nor does *E. amylovora* produce the cocktail of cell wall-degrading enzymes found in *Dickeya* spp. and *Pectobacterium* spp.

Continuing to develop an understanding of how *E. amylovora* causes disease will likely be bolstered greatly by genomic technologies. A recent analysis of the *E. amylovora* pan-genome found that, while strains of *E. amylovora* display unique host specificity, the pan-genome of *E. amylovora* is remarkably conserved (Mann et al.,, 2013). A recent comparison of 12 *E. amylovora* genomes found that 89% of the *E. amylovora* pan-genome is represented by coding sequences conserved between strains (Mann et al.,, 2013). This degree of conservation is markedly higher than that exhibited by other bacterial plant pathogens and will be of great significance for plant pathologists interested in studying host specificity and virulence (Mann et al.,, 2013).

In addition, the ultimate goal of fire blight research is to reduce the threat posed by *E. amylovora* to fruit production around the world. The recent identification of T3SS effectors that contribute to avirulence coupled with genomic data suggesting that host specificity in *E. amylovora* is likely due to a small number of genetic factors, means that continued research into *E. amylovora* pathogensis is poised to produced translational results in the foreseeable future. In light of the spread of antibiotic resistant *E. amylovora* populations, the development of apple and pear cultivars with durable fire blight resistance will be a welcome solution to one of plant pathology's oldest problems.

APPENDIX

Annotation	Gene	Ea273	EaCFBP1430	Ep1/96	Et1/99	Eb661	PcPC1	Ech703	PstDC3000
2930	bglE	0	1E-58	6E-53	3E-52	1E-21	1E-36	2E-18	0.51
2931	bglF	0	0	0	0	1E-50	0	5E-44	0.51
2932	bglI	0	3E-51	8E-44	3E-46	4E-31	3E-36	8E-33	6.4
2933	bglD	0	0	2E-89	1E-88	4E-96	8E-173	4E-101	0.12
2934	bglA	0	0	0	0	0	0	0	0.89
2935	**ggt**	**0**	**0**	**0**	**1E-120**	**8E-123**	**0**	**0**	**1E-145**
2936	**2936**	**0**	**2E-150**	**2E-146**	**0.91**	**0.27**	**1.9**	**04**	**2.8**
2937	**2937**	**0**	**2E-85**	**6E-80**	**X**	**0.54**	**0.57**	**X**	**0.45**
2938	**2938**	**0**	**1E-23**	**8E-21**	**2.1**	**8.7**	**4.4**	**1.1**	**2.7**
2939	2939	0	7E-55	01	1E-24	9E-04	8E-04	1E-05	8E-04
2940	ftnA	0	1E-103	3E-101	4E-95	2E-84	2E-71	6E-71	0.62
2941	2941	0	0	0	0	0	7E-178	1E-176	0.082
2942	phoC	0	0	2E-04	0	1E-152	1E-146	5E-130	3E-26

Figure A.1 EAM_2938 gene cluster and adjacent open-reading frames from *E. amylovora* ATCC 49946 with NCBI E-values representing homology to other Erwinia spp. strains and *P. syringae* pv. *tomato* DC3000. Homologs are represented in green. ΔEAM_2936, ΔEAM_2937, and ΔEAM_2938 have not homologs in non-pathogenic Erwinia species or Pst DC3000.

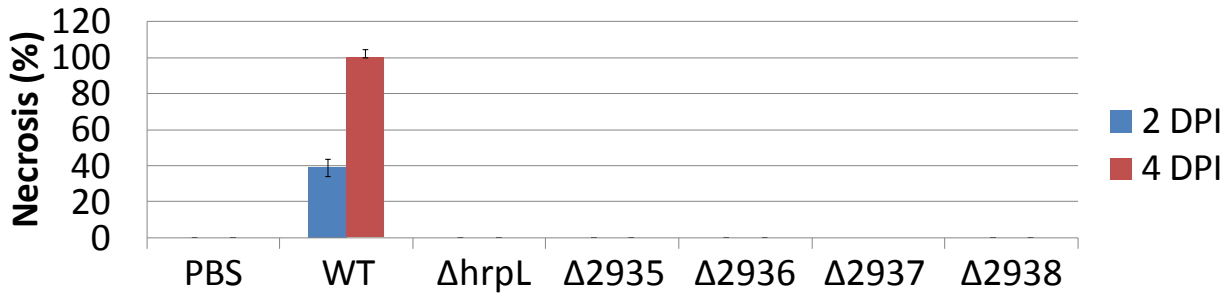

Figure A.2 Analysis of EAM_2938 cluster mutants for virulence in immature pear. Percent necrosis was measured 2 and 4 days post inoculation. All EAM_2938 cluster mutants are nonpathogenic.

Figure A.3 Analysis of EAM_2938 cluster mutants for ability to elicit the hypersensitive response (HR) in *N. benthamiana* 16 hpi. All EAM_2938 cluster mutants retain the ability to elicit HR indicating that type III secretion is not defective in EAM_2938 cluster mutants.

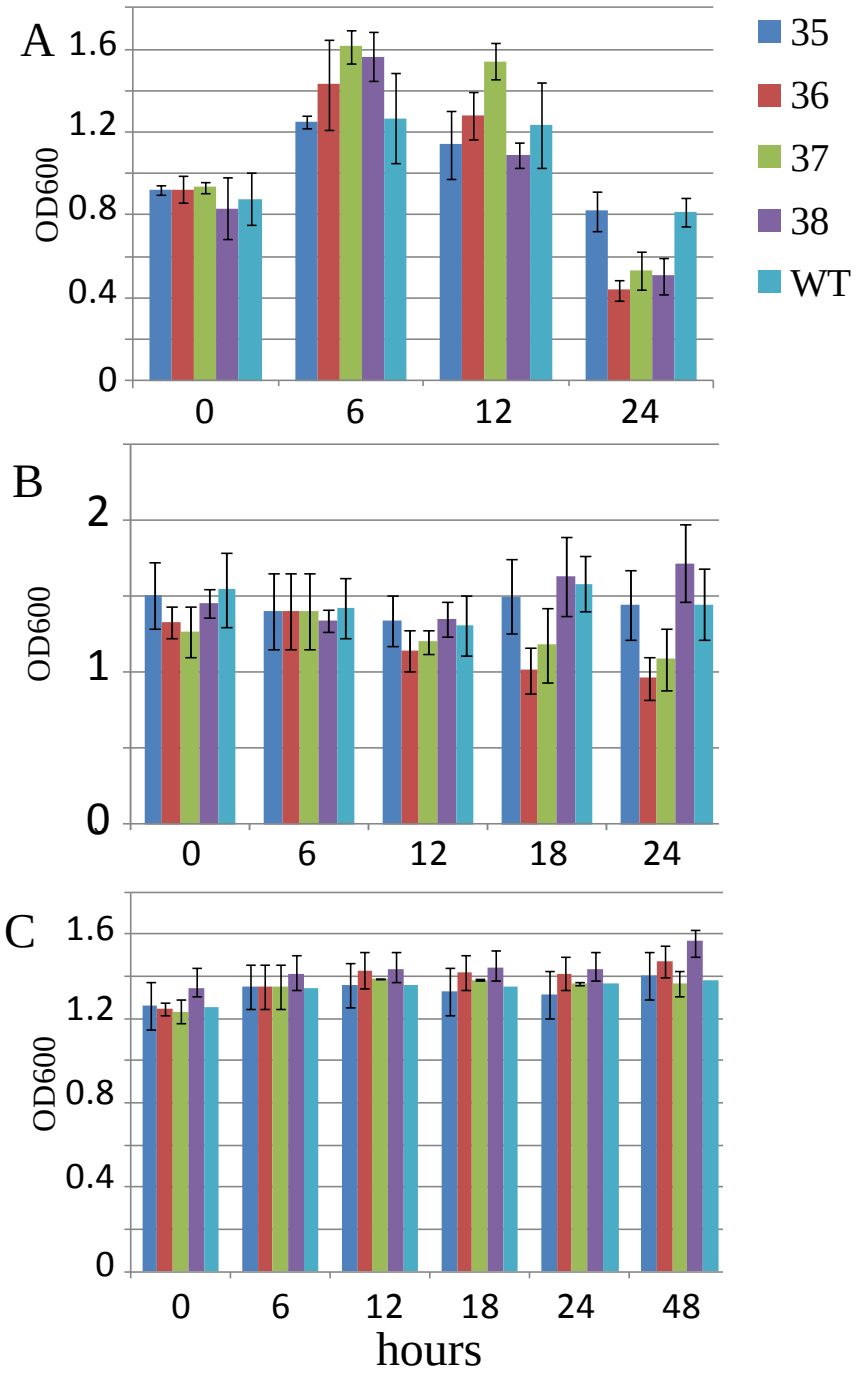

Figure A.4 WT Ea1189, ΔEAM_2935, ΔEAM_2936, ΔEAM_2937, and ΔEAM_2938 cultured in polystyrene wells with 500 ug/ml (A) phloretin, (B) narigenin and (C) quercitin. Optical desity was measured 600 nm.

Table A.1 WT/Δ*hfq* microarray

Name	Description	Fold change (WT/Δhfq)	p < 0.05
EAM_0074	ribosomal protein L33	3.26	0.02
EAM_0270	hypothetical protein	6.62	0.02
EAM_0308	ssDNA-binding protein	2.82	0.03
EAM_0445	biofilm stress and motility protein	2.84	0.02
EAM_0624	Nuclear pore complex protein	2.61	0.02
EAM_0933	hypothetical protein	3.2	0.04
EAM_1116	Cold shock-like protein	3.21	0.01
EAM_1428	biofilm regulator	3.65	0.04
EAM_1454	Ribosomal protein L32	3.68	0.05
EAM_1617	hypothetical protein	2.51	0.04
EAM_1714	hypothetical protein	3.31	0
EAM_1867	hypothetical protein	3.31	0.03
EAM_1883	hypothetical protein	3.22	0.03
EAM_1897	oligopeptide ABC transporter	2.92	0.03
EAM_1935	serine protein kinase	3.79	0.02
EAM_2617	Sigma 54 modulator	4.68	0.05
EAM_2679	rpoS sigma factor	3.53	0.03
EAM_2877	hrpN harpin	4.3	0.04

Microarray expression profile of in vitro *hfq* regulon comparing RNA extractions from wild-type (WT) Ea1189 and Ea1189Δhfq after inoculation in hypersensitive response and pathogenicity (hrp)-inducing minimal medium for 6 hours. Transcript abundance was decreased for 18 genes in the absence of *hfq*. Fold change values were significant at $P < 0.05$ with an expression ratiocut-off of >2.5.

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