

ABSTRACT

Title of Dissertation: **IDENTIFICATION AND SECRETION OF EFFECTORS FROM THE *PSEUDOMONAS SYRINGAE* TYPE III SECRETION SYSTEM**

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The *hrp* pathogenicity island (PAI) of *Pseudomonas syringae* encodes a type III secretion system (TTSS) that translocates virulence proteins, called effectors, into plant cells. The whole array of effectors of different *P. syringae* strains and their activities inside the host are not known. Furthermore, the manner in which effectors are selected for secretion, and how the process is regulated are not clear in *P. syringae*. This study identified a novel effector from *P. syringae* pv. *syringae* Psy61 using a genomic screen. The effector was a 375 aa protein of 40.5 kDa that was

designated HopPsyL. A *hopPsyL::kan* mutant of Psy61 exhibited strongly reduced virulence in *Phaseolus vulgaris* cv. Kentucky Wonder, but did not appear to act as a defense response suppressor. The ectopically expressed gene reduced the virulence of *P. syringae* DC3000 transformants in *Arabidopsis thaliana* Col-0. HopPsyL appears to be a novel TTSS-dependent effector that functions as a host-species-specific virulence factor in Psy61. In addition, this study reports that TTSS-dependent effectors are subject to the proteolytic degradation by Lon that appears to be rate-limiting to secretion. TTSS-dependent secretion of these effectors could be detected from the Lon mutants. This study found that a primary role for chaperones in *P. syringae* appeared to be protection of effectors from Lon-mediated degradation prior to secretion. Distinct Lon-targeting and chaperone-binding domains were identified in at least one effector. The results imply that Lon is involved at two distinct levels in the regulation of the *P. syringae* TTSS: regulation of assembly of the secretion apparatus and modulation of effector secretion. Interestingly, degradation of *P. syringae* effectors was also retarded by the presence or expression of the *P. syringae* TTSS. The protection from Lon-mediated degradation was not due to the assembly of the TTSS. Rather, the results suggest the existence of a stabilizing factor harbored within the *hrp* cluster. This study proposed that this factor functions as a general chaperone for type III secretion in *P. syringae*.

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By

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Dedication

I would like to dedicate this dissertation, all the hard work and wonderful achievements to my husband, Bruce K. Brown, my parents Antonio and Constance Losada, and to my grandparents William and Lois Bohannon, who are with me all the time. These people have always made me strive to higher grounds and they partake in this accomplishment as much as I do. Thanks for all your love and support!

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Table of Contents

Dedication	ii
Acknowledgements.....	iii
Table of Contents.....	v
List of Tables	vii
List of Figures.....	viii
List of Abbreviations.....	x
Chapter 1: Introduction.....	1
Structure of type III secretion systems.....	2
Targeting of effectors to the TTSS	6
The process of type III secretion.....	7
The role of chaperones in TTSS	8
The notion of hierarchical secretion from the TTSS	14
Type III Secretion and Pathogenesis of <i>Pseudomonas syringae</i>	15
Type III effectors and secretion in <i>Pseudomonas syringae</i>	19
Statement of Purpose	20
Chapter 2: Identification of a novel <i>Pseudomonas syringae</i> Psy61 effector with virulence and avirulence functions by a HrpL-dependent promoter trap assay.....	23
Introduction.....	23
Results.....	25
Discussion.....	45
Materials and Methods.....	49

Chapter 3: Type III secretion chaperones of <i>Pseudomonas syringae</i> protect effectors from Lon-mediated degradation.	58
Introduction.....	58
Results.....	61
Discussion.....	86
Materials and Methods.....	94
Chapter 4: Identification of a generalized stabilizing factor within the <i>hrp</i> cluster .	103
Introduction.....	103
Results.....	106
Discussion.....	126
Materials and Methods.....	131
Chapter 5: General Conclusions and Future Directions	138
Bibliography	149

List of Tables

Table 1-1. Type III secretion components of <i>P. syringae</i> TTSS are conserved with the <i>Yersinia pestis</i> TTSS and flagellar biosynthesis apparatus.....	11
Table 2-1. The hypersensitive response is not inhibited by HopPsyL.....	42
Table 2-2. Conservation of <i>hopPsyL</i> among <i>P. syringae</i> strains.....	43
Table 2-3. Strains, plasmids and primers used in this study.....	51
Table 3-1. HrpL overexpression does not affect the induced HR.....	67
Table 3-2. Strains, plasmids and primers used in this study.....	95
Table 4-1. Strains and Plasmids.....	131
Table 5-1. The proposed secretion signal of <i>P. syringae</i> effectors is not conserved.	146

List of Figures

Figure 1-1. The type III secretion system of <i>P. syringae</i> injects virulence factors into host cells.....	3
Figure 1-2. The process of substrate docking and mounting in the flagellar TTSS. ...	9
Figure 2-1. Arrangement and Sequence of ORF54.	26
Figure 2-2. Translational Fusions Between the three possible open reading frames and <i>lacZ</i>	29
Figure 2-3. HopPsyL is a translocated effector with virulence and avirulence functions.....	31
Figure 2-4. Southern Hybridization confirms <i>hopPsyL</i> insertion.....	34
Figure 2-5. HopPsyL is required for virulence of Psy61 in <i>P. vulgaris</i> cv. Kentucky Wonder.....	36
Figure 2-6. Ectopic expression of HopPsyL reduces the virulence of DC3000 in <i>A. thaliana</i> Col-0.....	39
Figure 3-1. Secretion of AvrPto from <i>P. syringae lon</i> mutants.	62
Figure 3-2. Ectopic expression of <i>hrpL</i> does not bypass Lon regulation on the <i>hrp</i> cluster.....	65
Figure 3-3. Effect of Lon protease on stability of effectors in <i>P. syringae</i>	69
Figure 3-4. <i>E. coli Alon</i> mutants induce HR in half the time as wild-type.....	72
Figure 3-5. <i>P. syringae</i> effectors are secreted from an <i>E. coli lon</i> background.....	75
Figure 3-6. Lon affects the stability of <i>P. syringae</i> effectors in <i>E. coli</i>	77
Figure 3-7. Effectors are stabilized by their chaperone.	80
Figure 3-8. The C-terminus of HopPtoM targets the peptide for degradation.....	84

Figure 3-9. An MBP fusion to the C-terminus of HopPtoM is degraded.	87
Figure 4-1. AvrPto is more stable in the presence of the <i>hrp</i> cluster.....	107
Figure 4-2. Levels of AvrPto in cells expressing <i>hrp</i> operon mutants.	110
Figure 4-3. The <i>hrpA</i> and <i>hrpU</i> operons can stabilize AvrPto.	113
Figure 4-4. Far Western Analysis of effector interacting proteins.	118
Figure 4-5. Column binding experiments.	121
Figure 4-6. Crosslinking experiments.....	124
Figure 5-1. Substrate selection in the TTSS of <i>Pseudomonas syringae</i>	140

List of Abbreviations

CBD	Chaperone-binding domain
DSP	Dexamethasone sodium phosphate
IM	Inner membrane
<i>hrp</i>	Hypersensitive response and pathogenicity
HR	Hypersensitive Response
MBP	Maltose binding protein
NC	Needle complex
OM	Outer membrane
PAI	Pathogenicity Island
PCD	Programmed cell death
SSD	Substrate discrimination domain
TAIL-PCR	Thermal Asymmetric Interlaced-polymerase chain reaction
TTSS	Type III Secretion System
WT	Wild-type

Chapter 1: Introduction

Bacterial pathogens use very intricate and elegant methods to manipulate their hosts. These pathogens produce a wide variety of virulence factors that promote colonization and disease in the host, thereby allowing bacteria to multiply and spread. A common theme among bacterial pathogens is the secretion of these virulence factors to maximize their influence on the host. Thus far, five different secretion pathways have been discovered for the transport of factors out of the bacterial cell [157]. Among them, the Type III Secretion System (TTSS) is one of the major export mechanisms for virulence factors in Gram-negative pathogens. TTSS's are used to inject proteins directly into host and manipulate defense responses by interfering with cell signaling [40, 86]. TTSS are closely related to the apparatus for flagellar synthesis, and are commonly found among Gram-negative bacteria that cause disease on both animals and plants [40, 86]. TTSS contribute to pathogenicity [40, 86], but in some cases they were shown to be necessary for symbiotic relationships with hosts [157]. Pathogens that depend on TTSS for virulence are usually incapable of causing disease if the TTSS is absent or malfunctioning [40, 86, 89] presumably because they cannot deliver bacterial proteins into the host cell.

The current understanding of TTSS involves a translocation complex that traverses the bacterial membrane, produces a needle that can pierce the host cell membrane, and directly inject proteins, called effectors, into the cytoplasm of the host cell [86]

(See Figure 1-1). These delivered proteins can aid the bacterium in the evasion of defense responses, or alternatively in permitting the leakage of nutrients from the host cell. Although much effort is currently being placed in discovering the roles of some of these effectors and how they influence the host (see [26, 32, 42, 94] for reviews), many questions persist about the mechanisms of TTSS. For example, it is still not known what signals are used to target an effector to the secretion port, or once it is at the port how the effector is actually loaded into the system, or what triggers the system to physically transfer the effector into the host. Furthermore, although it is known that effectors interact with a cytosolic chaperone in the bacterium, it is not known how this interaction facilitates secretion. This study began as an attempt to understand some of the early steps in the selection of substrates for TTSS by using *Pseudomonas syringae* as a model system. The project later matured into a study of the impact on the TTSS of proteolytic degradation of effectors, and the specific question of why effectors interact with specific chaperones.

Structure of type III secretion systems

Structurally, the TTSS shares similarity to the inner membrane components of flagellar biosynthesis and to the pore-forming protein involved in filamentous-phage assembly [40, 86]. The system is thought to span the inner and outer membrane analogously to the flagellar basal body [101] and form a long needle that protrudes out of the bacterium and into the host [40, 86] (See Figure 1-1). For two species (*Salmonella* and *Shigella* spp) the structure of the TTSS needle-complex (NC) has

Figure 1-1. The type III secretion system of *P. syringae* injects virulence factors into host cells.

A schematic representation of the needle complex based on electron-microscopy images (see inset), with several ring structures that span the inner and outer membranes. The indigo protrusion is the 'needle' filament, which is thought to engage a bacterial pore-forming complex that can penetrate the plant cell wall and become lodged in the host cell membrane. The *hrp/hrc* gene cluster is portrayed below with the genes colored to represent their proposed location in the TTSS structure. The virulence factors/effectors wait to be transported through the export machinery. Some effectors may be bound by chaperones during this wait. This figure was adapted with permission from [180].

been visualized using electron microscopy [21, 110, 111]. It was characterized as a cylindrical structure composed of at least four substructures: (i) two inner membrane rings 40 nm in diameter that anchor the whole structure, (ii) two outer rings 20 nm in diameter which are in close contact with the peptidoglycan and outer membrane, (iii) a rod that links the two sets of rings, and finally (iv) a needle-like extension about 80 nm in length and 8 nm in diameter that protrudes from the base (Figure 1-1).

It is believed that once the system is organized, effectors arrive at the inner membrane rings of the TTSS, proceed to be loaded into the central channel of the translocon, and are then pushed through the needle out of the bacteria. This process is similar to what is thought to occur during flagellar biosynthesis [132]. There, the basal body forms a conduit through which the flagellin monomers can travel unfolded to the distal end of the flagellar propeller and elongate the structure [113]. Effectors, too, are thought to travel unfolded through the TTSS needle since higher-order protein structures cannot be secreted through the system [60]. In addition, studies of TTSS in *P. syringae* provided compelling evidence that secretion must occur through the lumen of the needle since effectors were only visible at the tip of the TTSS needle [98, 99].

Effectors are thus called because they can cause an effect in the host. The variety of functions and effects of effectors is beginning to be understood, but appear to involve the active suppression of defense responses, remodeling or rearrangement of the host cytoskeleton, and possibly changing the physiology of the host membrane to allow

the release of nutrients into the intercellular space [26, 42]. Effectors are divided into three functional domains. The first domain contains the N-terminal secretion signal discussed below. The second domain is the chaperone-binding domain, which is only present in the subset of effectors that interact with a cytoplasmic chaperone, also discussed below. The third domain is the activity domain which carries out the function of the protein once inside the host cell. The array of known functions and activities of effectors is well covered in other reviews, for example [26, 42].

Targeting of effectors to the TTSS

Targeting of secretion substrates to the TTSS is only beginning to be understood. Despite the increasing number of identified TTSS export substrates, no conserved secretion signal has been determined. So far, two different signals within effectors have been identified and shown to be required for proper translocation. The first signal is surrounded by controversy. This signal was shown to be sufficient for export of reporter constructs in *Yersinia*, *Erwinia*, and *Xanthomonas* [7, 9, 145]. Anderson *et al* [7] and Mudgett *et al* [145] propose that the signal is located in the 5' end of the mRNA. In their experiments, frameshift mutations that altered the sequence of the first 15 amino acids, but retained the remainder of the protein intact, led to reporter secretion. They argued that since no conserved protein sequence was necessary for secretion the signal was most likely a stem loop structure in the mRNA and secretion likely to be co-translational [9], as has been proposed for flagellar biosynthesis [104]. However, effector accumulation in the cytoplasm prior to

secretion [184] implies that there must also be a mechanism for post-translational secretion. Interestingly, Lloyd *et al* [124] showed that while frameshift mutations drastically reduced secretion of the reporter, changes in the mRNA sequence, but not in the amino acid sequence, had no effect on reporter secretion [124]. They proposed that the signal is contained in the N-termini of effectors and it consists of an amphipathic helical domain residing in the first 15-20 amino acids. Furthermore, they show that even a synthetic amphipathic helix could drive secretion of a reporter [125]. Thus, it is possible that both targeting mechanisms are not mutually exclusive, but may act simultaneously. Whether peptidic or mRNA, the signal contained in the first 15-20 amino acids can be recognized by heterologous TTSS, since plant pathogens can secrete mammalian pathogen effectors and vice versa [7, 77, 144, 164]. The second signal is between residues 20-70 and is dependent on the presence of a chaperone [34], as will be discussed in greater detail below.

The process of type III secretion

Perhaps as a consequence of the undefined nature of the secretion signal, the manner in which the secretion signal is relayed to the translocation apparatus is also unknown. How an effector is mounted to the secretion port and how it is pushed through the system are questions that remain unanswered. From the knowledge gathered in flagellar biosynthesis, the soluble components of the TTSS could be predicted to play an important role in substrate recognition and secretion. For

example, FliI forms a complex with FliH that interacts with export substrates bound by chaperones [143] (Figure 1-2). This complex can further interact with FlhA, and FlhB, two inner membrane components of the basal body of the secretory apparatus to provide the initial docking of the substrate-chaperone complex [204] [183] (Figure 1-2). As this occurs, FliI most likely hydrolyzes ATP [58], a process that could energize the initiation of translocation, as well as a conformational change that ultimately dismounts the soluble complex from the membrane. Dismount would leave the pore open for secretion of other substrate complexes. FliK, which has been implicated in substrate selection and as a switch mechanism for substrate specificity can be determined, also interacts with inner membrane components [106, 142]. The process appears to be similar for the virulence TTSS since there are homologs to most of the soluble components and inner membrane proteins of flagellar biosynthesis, excluding FliK (see Table 1-1). In addition, the ATPase of *Yersinia pestis*, *Shigella flexneri* and enteropathogenic *Escherichia coli* have been shown to be also capable of interacting with inner membrane components or chaperone-effector complexes [65, 96, 102].

The role of chaperones in TTSS

Recently, many studies have dealt with the identification and characterization of chaperones in TTSS. Chaperones have been shown to be necessary for secretion of

Figure 1-2. The process of substrate docking and mounting in the flagellar TTSS.

FliJ acts as a chaperone for an export substrate. The FliJ—substrate complex interacts with the cytoplasmic components FliI (the ATPase) and FliH (the ATPase regulator). The whole complex interacts with inner membrane components FlhA and FlhB, to dock the FliJ—substrate complex on the TTSS. This interaction most likely induces ATP hydrolysis by FliI, which could provide the necessary energy for translocation. After the substrate has been pushed through the system, FliJ is released and the FliI—FliH complex is prepared to energize the translocation of other substrates.

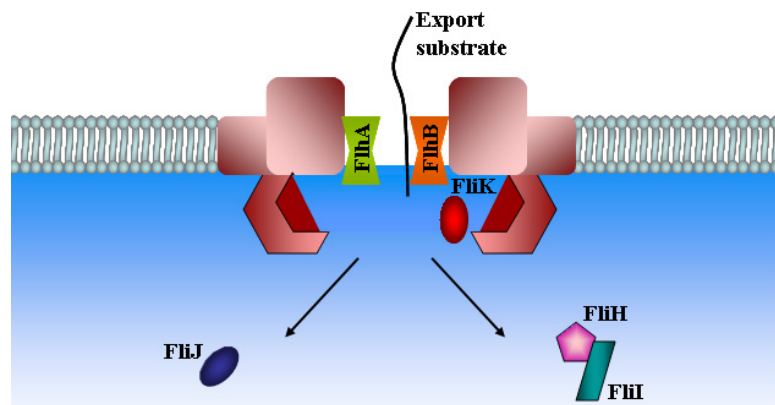
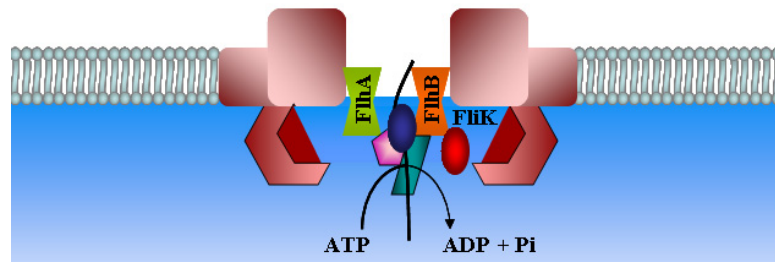
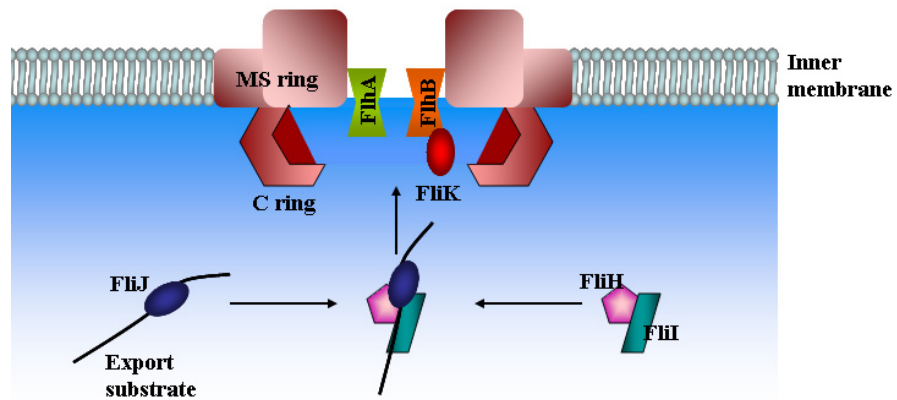


Table 1-1. Type III secretion components of *P. syringae* TTSS are conserved with the *Yersinia pestis* TTSS and flagellar biosynthesis apparatus.

Pseudomonas syringae	Yersinia pestis	Flagellar biosynthesis ^a	Cellular location
HrcC	YscC		Outer membrane (OM)
HrpQ	YscD	FliG	Inner membrane (IM)
HrcJ	YscJ	FliF	IM/OM
HrpE	YscL	FliH	Cytoplasmic
HrcN	YscN	FliI	Cytoplasmic/ATPase
HrcQ	YscQ	FliN	IM
HrcR	YscR	FliP	IM
HrcS	YscS	FliQ	IM
HrcT	YscT	FliR	IM
HrcU	YscU	FlhB	IM
HrcV	YscV	FlhA	IM
	YscW		OM lipoprotein
HrpD	YscK		Cytoplasmic
HrpO	YscO		Secreted
HrpP	YscP		Secreted
	YscF		Secreted, needle subunit
	YscE		Cytoplasmic
	YscG		Cytoplasmic
HrpB	YscI		Secreted
	YscX		Secreted
	YscY		Cytoplasmic
HrpA			Secreted
HrpF			Cytoplasmic
HrpG			Cytoplasmic
HrpT			OM
HrpK			Cytoplasmic
HrpL			Cytoplasmic
HrpJ			Cytoplasmic
HrpV			Cytoplasmic
HrpR/HrpS			Cytoplasmic
HrpW			Secreted
HrpZ			IM
		FliO	IM
		FliM	IM

their corresponding effector [59, 156]. The reason for this necessity is not clear. In some instances, the chaperone was shown to bind to the translocation domain and thus permit secretion. The chaperone is thought to interact with a signal contained within the N-terminus of the effectors. The interaction between the chaperone and the effector was sufficient to drive secretion of an effector, even in the absence of the first secretion signal [34]. These results imply that there must be a recognition step after the first signal has been processed. This fact, along with some evidence that some chaperone-effector interactions form similar structures (see below) led to the hypothesis that the TTSS can recognize three-dimensional structures as the signal for secretion [20]. Secretion using this signal, however, is absolutely dependent on the presence of the chaperone [24]. Some effectors are not secreted at all in the absence of their corresponding chaperone even when the first signal is present [117, 155], thus underscoring the importance of chaperones in the process of secretion.

An alternative manner in which chaperone may be necessary for secretion is by maintaining effectors in a “secretion competent” structure. For instance, two chaperones were shown to form coiled-coil motif with their substrates that prevents higher order structures that might “clog up” the secretion port [19, 179].

Interestingly, the manner in which both of these chaperones interact with their corresponding effector was very similar [20]. These results led to the exciting hypothesis that the chaperone-effector complex would form a three-dimensional structure that could act as the elusive secretion signal [20]. This hypothesis was similar to the mechanism in which flagellar components are recognized and secreted

[83, 132]. However, it does not seem that all chaperone-effector interactions form the same structure [148]. Moreover, the crystal structure of two type III effectors not associated with chaperones, AvrB and AvrPphB, do not overlap with any of the structures mentioned above [115, 205]. Therefore, although this hypothesis provides a novel concept in chaperone function, it may not fully describe the phenomenon.

Another way in which chaperones have been implicated in secretion is through the regulation of transcription of effector genes. For example, SicA has been shown to be required for the expression of *sopE*, a *Salmonella* effector [186]. SicA was later shown to act as a co-factor of InvF (an AraC/XylS family activator) and drive the expression of *sigDE* [49, 50]. SycH in *Yersinia* and FlgN from flagellar biosynthesis, in contrast, are not transcription co-factors; instead, they have a positive impact on effector gene transcription by sequestering transcriptional inhibitors YscM1, YscM2, and FlgM. Upon binding of the chaperone to these negative regulators, secretion through the TTSS is possible and thus the repression is relieved [4, 30]. Lastly, chaperones may be necessary for secretion by virtue of their stabilizing role on effectors. By binding to their respective effector, chaperones can sequester and protect it from proteolysis, resulting in higher levels of effector [44]. A higher abundance of effectors in the cytoplasm might result in a greater chance of secretion, thereby increasing the levels of effectors in culture supernatants.

Paradoxically, as more data have been gathered about the function of chaperones, their exact roles are becoming harder to define. For example, at first chaperones were

proposed to inhibit premature aggregation and folding of effectors in the bacterial cytoplasm to prevent possible enzymatic activity and maintain effectors competent for secretion [59, 156, 179]. However, later studies showed that the enzymatic activities of different effectors were still detectable even in the presence of the chaperone [20, 130], challenging the notion that effectors do not fold in the cytoplasm. Another contradiction was found when YopE was secreted in the absence of its chaperone only when its chaperone-binding domain was deleted, indicating that somehow the chaperone was necessary for relieving an inhibition of secretion [24]. Therefore, there is not one universally recognized function for chaperones, but rather it is generally accepted that their importance is based on multiple functions.

The notion of hierarchical secretion from the TTSS

What has become recently apparent, however, is that chaperones are not only involved in permitting an effector to be secreted, but also in determining when that effector is produced and/or secreted [59, 156]. With these data a model was created that proposes an ordered sequence for effector secretion, and states that chaperones are responsible for establishing this hierarchy [24]. Hierarchical secretion through the TTSS was not a novel concept. For flagella, this ordered secretion had been proposed to explain how the system changes from secreting membrane components, to filament components and finally to placing the cap on the flagellum [112, 196]. In pathogens, the importance of hierarchical secretion lies in the idea that in order to invade and colonize a host, a highly regulated order of events must occur. For example, a

pathogen initially might want to induce host cell death or leakage, but does not want secondary and delayed defense alarms to be activated.

The fact that chaperones were also involved in a feedback regulation of effector gene transcription and other methods of posttranscriptional regulation supported the model of hierarchical secretion [4, 29, 50, 198]. It appears, then, that chaperones can dictate when an effector is transcribed, translated, and whether the TTSS apparatus recognizes and secretes the effector [20]. This model, however, does not take into account the important observation that chaperones can stabilize effectors in the bacterial cytoplasm, nor does it deal with the fact that most effectors in plant pathogens are not associated with chaperones.

Type III Secretion and Pathogenesis of Pseudomonas syringae

Pseudomonas syringae is a fluorescent pseudomonad in the γ -subgroup of proteobacteria that facultatively infects a wide range of plant species. A typical symptom of *P. syringae* infection is an initial “water soaking” (darkening of the tissue) at the site of infection (indicative of altered membrane physiology) followed by slowly developing programmed cell death, and in the some cases, a spreading chlorosis (yellowing of the tissue as chlorophyll is broken down) [17, 122]. Most agriculturally important plant species are susceptible to at least one *P. syringae* strain. However, individual *P. syringae* strains usually have a very limited host range and only cause disease in a small subset of plant species. Some strains can only infect a

few varieties of a single plant species [82].

P. syringae occurs naturally on plant surfaces, and becomes pathogenic if the bacteria invade the intercellular space of the leaf mesophyll of susceptible plants [82, 122]. This invasion is aided by wounding and leaf wetting [134]. Although not much is known of the molecular interaction between the bacterium and the plant cell, it appears that the bacterium adsorbs to the host cell surface, multiplies, and begins to produce virulence factors that contribute to symptom formation [134]. Common virulence factors of *P. syringae* strains are type III effector proteins [87, 100], extracellular polysaccharides [118, 165], derivatized peptide exotoxins [16, 74], and several plant growth hormones and cytokinins [67]. Over several days, bacterial populations in infected tissue can reach as high as 10^9 cells / g fresh weight of leaf tissue, but bacterial cells are found only in the intercellular spaces of the tissue.

Resistance or susceptibility of a plant host to a specific *P. syringae* strain depends on the timing of the plant defense response to the initial colonization by the bacterium. In a susceptible plant, recognition of the pathogen and response to the infection occurs slowly [87]. As a result, the bacterial population is able to spread into new tissue before the cellular defense responses of colonized cells are activated, thus enabling the pathogen to spread continuously. Eventually, large areas of leaves and other tissues become infected and necrosis develops due to a slowly induced oxidative burst and programmed cell death in the host [47, 54]. In contrast, a resistant plant is able to initiate a rapid cellular defense response during the initial colonization

and is able to prevent the spread of the infection. The bacteria elicit a cascade of cellular defense responses in the cells of the resistant plant within 1-2 h that result in an oxidative burst and later programmed cell death (PCD). PCD typically develops within 12 hours of infection [92, 109], a phenomenon called the hypersensitive response (HR).

When the pathogenicity of *P. syringae* was first characterized, a genetically clustered set of transposon mutations were isolated that abolished virulence [121]. This region was called the *hypersensitive response and pathogenicity (hrp)* cluster because it was necessary for both the induction of HR and for disease [87, 120]. This cluster was shown to be sufficient to enable non-pathogenic bacteria to elicit a cellular response in plants [85]. The *hrp* cluster includes 7 operons containing 26 genes that encode a TTSS and its dedicated regulatory system. Among TTSSs of mammalian pathogens, the *Yersinia* Ysc proteins are the closest homologs to the conserved Hrc products of the *hrp* cluster (see Figure 1-1 and Table 1-1). Nine of these twelve genes are conserved components of all TTSS necessary for proper assembly and secretion through the translocation apparatus [23].

Expression of the *P. syringae* TTSS is environmentally regulated in such a manner that it is activated during pathogenesis [87]. Within the *hrp* cluster three genes, *hrpR*, *hrpS*, and *hrpL* positively regulate the expression of genes responsible for plant pathogenesis. Previous work demonstrated the interaction between HrpR and HrpS activated the expression of *hrpL* in yeast models [91, 199]. In turn, it has been shown

that the activation of *hrpL* then induces transcription of all other *hrp* operons including, *hrpK*, *hrpJ*, *hrpU*, *hrpC*, *hrpA*, *hopPsyA*, other genes in the *hrp* PAI, and effector genes dispersed throughout the genome [201] [33, 62, 76, 206]. HrpL is an alternative sigma factor related to FliA, the alternative sigma factor involved in expression of class III flagellar genes [199] [153]. Promoters positively regulated by HrpL are highly conserved between *P. syringae* strains. The HrpL-dependent promoter consists of a 12 base pair sequence, are critical to the formation of the binding motif of HrpL-dependent promoter [199]. According to this model, genes involved in the pathogenesis process appear to be under the control of a single transcription factor.

Among the negative regulators of the *P. syringae* TTSS are Lon protease and HrpV. Lon is involved in the degradation of HrpR, and therefore, the assembly of the secretion apparatus [25]. Lon mutants are capable of hypersecreting effectors and eliciting plant defense responses in about one half the time required for wild-type cells. Conditions that mimic the environment in plant tissue (amino acid starvation) suppress Lon-mediated degradation of HrpR, which would allow the HrpR/HrpS complex to form. Interestingly, Lon has also been implicated in the regulation of TTSS from the SPI-1 system in *Salmonella typhimurium* [22, 182], suggesting that this mode of negative regulation might be conserved among Gram-negative pathogens with a TTSS. HrpV also negatively regulates *hrp* expression, but this mechanism remains unknown [161].

Type III effectors and secretion in *Pseudomonas syringae*

Because the main role of the *P. syringae* TTSS is to translocate effector proteins directly into the plant cell host cytoplasm, identification of effectors is of great importance. However, finding *P. syringae* effectors has been comparatively tricky because they are difficult to detect by biochemical methods [88, 188, 203] or by using molecular reporters. Thus far the only reporter useful to detect translocated effectors in *P. syringae* strains is the avirulence domain of AvrRpt2 [144], which itself is an effector. The ability to identify effectors from various strains of *Pseudomonas syringae* would provide tools to characterize common and necessary factors for pathogenesis, and would also provide a way to study the manner in which pathogens adapt and evolve according to the hosts they can infect.

Recently, bioinformatics and genetic methods for identifying effectors have revealed many novel candidate effectors (see [26, 39, 73] for review). Analyses of the genomes of two *P. syringae* strains have indicated that individual strains encode a large number of effectors, at least five times more than mammalian pathogens. By searching for HrpL-dependent promoters and proteins that fit a set of effector characteristics, the genome of *P. syringae* DC3000 was found to encode 58 known or likely effectors [39, 73, 158] and B728a is predicted to express at least 30 probable effectors [73]. The variety of effectors produced by each *P. syringae* strain could reflect the complex interactions with plant cells, which have cell walls, chloroplasts,

large vacuoles, or alternatively be due to the unique requirements to infect various hosts. An interesting result from the genomic analyses of *P. syringae* was that the genes for the vast majority of effectors were not associated with genes for a candidate chaperone [39, 73]. However, as in other species, the known chaperones found in *P. syringae* strains were necessary for the secretion of effectors [14, 189, 193].

Statement of Purpose

When I began my research at Dr. Steven Hutcheson's laboratory five years ago, I was intrigued by the controversy surrounding the TTSS export signal. Two studies had been published suggesting that the secretion signal for effectors of mammalian and plant pathogens might be contained in the mRNA [7, 9]. Many investigators were not convinced by the data, and a later study showed that the mRNA sequence could be altered without effect on secretion of effectors, but the the proper amino acid sequence was absolutely necessary [124]. From studies demonstrating that mammalian and plant pathogen effectors could be secreted from heterologous TTSS [7, 77], it was proposed that the secretion signal must be at least partially conserved between all effectors. However, no consensus sequence could be detected among the known effectors. It was possible that the number of effectors studied until then had been too limited to form a large enough statistical group. Thus, in order to determine whether there existed a conserved secretion signal in *Pseudomonas syringae* effectors that could be extrapolated to other TTSS, I proposed to find and characterize novel effectors in Psy61, and identify possible sequence and/or feature similarities that

could act as the candidate secretion signal. The first part of this dissertation describes the studies carried out for this purpose.

The second part of my study, discussed in Chapter 3 and 4, was focused on determining the mechanisms of early substrate selection for TTSS in *Pseudomonas syringae*. When I first arrived in Dr. Hutcheson's laboratory, it was known that secretion by *P. syringae* appeared to be more tightly regulated than in mammalian pathogens [89], but the reasons for this tight regulation were not understood. The initial observation that a Lon protease null mutant of *P. syringae* could hypersecrete proteins (Lisboa, K. and S.W. Hutcheson, unpublished results) prompted me to study whether some of the hypersecreted proteins were TTSS effectors, and if so, the manner in which the tight regulation of secretion was bypassed. Given that proteases degrade proteins, I was interested in determining if Lon protease was directly responsible for the degradation of effectors in *P. syringae*, and in this manner regulated secretion through the system. Furthermore, the roles of chaperones in secretion of effectors were beginning to be elucidated for other TTSS, but not in *P. syringae*. One of these roles was the capacity to stabilize effectors in the bacterial cytoplasm. Therefore, I investigated whether the role of chaperones in the *hrp* TTSS was to protect the effectors from Lon-mediated degradation to allow secretion.

As a result of the initial studies delving into the roles of Lon in the regulation of TTSS from *P. syringae*, I discovered that effectors were stabilized when the *hrp* regulon was expressed. These results led me to explore the possibility of a

generalized stabilizing factor that could prevent Lon mediated degradation of effectors, similarly to chaperones, and thereby allow secretion. The interaction between this generalized factor and effectors could also explain how and when substrates were selected for secretion in the *hrp* TTSS. These studies are discussed in chapter 4.

Chapter 2: Identification of a novel *Pseudomonas syringae* Psy61 effector with virulence and avirulence functions by a HrpL-dependent promoter trap assay.

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Introduction

Identification of the effectors produced by *P. syringae* strains was of high priority to clarify the secretion signal guiding effectors to the TTSS, and to understand *P. syringae* pathogenesis in general. This task, however, has been comparatively difficult. Initially, genomic libraries of one strain were screened for *avr* genes that alter the host range of another strain [73, 114, 178, 191]. The products of *avr* genes were subsequently shown to encode translocated effector proteins [69, 119, 159]. In some cases, proteins detected in the growth medium have also been found to be secreted by the TTSS [203]. A randomly generated fusion to a truncated effector lacking the native secretion domain has also been used to identify effectors [76]. Most recently, *in silico* approaches were used to screen the genome of *P. syringae* pv. *tomato* DC3000, a pathogen of tomato and *Arabidopsis thaliana*, and the bean pathogen *P. syringae* pv. *syringae* B728a for genes that match a set of postulated effector characteristics [158] or carry conserved promoter sequences unique to the *hrp* regulon [62, 206]. These studies indicate that *P. syringae* strains carry numerous effector genes that are distributed throughout the genome as individual loci [62, 76, 108, 158, 206], clustered with the pathogenicity island (PAI) as

apparent “integron-like” modules [5, 33, 52, 89] and/or are found in plasmid-borne gene clusters [11, 97].

Although these approaches have established catalogs of the effectors secreted by a few *P. syringae* strains, the effectors produced by most strains are still uncharacterized. Thus, the effectors that define the host range of the majority of strains have yet to be established. Genome analysis of multiple strains is limited by its cost, and most other previously employed methods are laborious. Due to the limitations of these approaches, a more generally applicable method for screening previously uncharacterized *P. syringae* strains for effector genes was developed in our laboratory.

Because the genes for all known or candidate effectors of *P. syringae* require the alternative sigma factor HrpL for expression [62, 201, 206], an inexpensive plate assay was devised to identify HrpL-dependent promoter fusions to a promoterless ‘*lacZYA*’ cassette based on the Lac phenotype of *E. coli* transformants carrying an arabinose-inducible *hrpL* construct, pSHL4K. The inducible *hrpL* construct was used to distinguish HrpL-dependent promoter fusions, which produce a Lac⁺ phenotype only in the presence of arabinose, from constitutively expressed promoters of other genes. The promoter-trap assay was used to perform a partial screen of the *P. syringae* pv. *syringae* Psy61 genome. Twenty-two HrpL-dependent promoter fragments were identified (Pak, K. Senior Thesis, Sussan, T. personal communication). These fragments revealed promoters for previously characterized *hrp* PAI operons of Psy61, effector genes originally identified in other *P. syringae* strains, and a candidate gene for a novel

effector. This candidate gene was shown here to produce a translocated effector that is necessary for virulence of Psy61 in bean plants but elicits defense responses in other plants. This work has been published in [128].

Results

Identification of ORF54

From the genomic analysis carried out previously in the lab (Pak, K. Senior Thesis, Sussan, T. personal communication), a potential ORF, ORF54, was identified 23 bp downstream of the deduced HrpL-dependent promoter (Figure 2-1). ORF54 was preceded by a strong candidate ribosome binding site. The predicted ORF54, however, extended past the end of the initially cloned fragment. TAIL-PCR [123] and Inverse PCR [152] were used to determine the remaining sequence of the ORF. ORF54 was found to encode a leucine-rich (12.8%) 375 aa protein with a predicted molecular weight of 40.5 KDa and a predicted pI of 8.74 (Figure 2-1) (Genbank accession number AY349161). The deduced ORF54 product exhibited characteristics similar to other type III effectors, including an amphipathic N-terminus, high serine and isoleucine content in the first 50 aa, and few cysteines in the polypeptide [76, 158]. Other than an apparent chloroplast localization signal and two deduced transmembrane domains (residues 289-307 and 349-369) in the carboxy-terminal region of the protein, no other similarities to the deduced ORF54 product were detected in the databases.

To confirm the expression of ORF54, translational fusions between each open reading

Figure 2-1. Arrangement and Sequence of ORF54.

Sequence of the *hopPsyL* locus in Psy61. Bases labeled in bold-type are the candidate *hrpL*-dependent promoter. The open reading frame is italicized, and the translation is underneath. *hopPsyL* encodes a leucine-rich (12.8%) 375 aa protein, predicted molecular weight of 40.5 KDa and pI of 8.74. The Genbank accession number is AY349161. The asterisk denotes the stop codon TGA. Nucleotide sequence was obtained by primer walking using synthetic oligonucleotides at the CBR sequencing facility at the University of Maryland.

AGGTGGCGGTGGAGTCGGCGTAACGGCAAGACGCCCTTCAGTGACGGACAG 51
 AGACCTGTCTGTATT**GGAACC**GCTGTCTGCGTCGGGT**CCACCA**AGGGGTTT 102
hopL: TAGGCCATGAGACCCGCCATGAACCCCATTCGTAATTCTCCGTCTTTCATC 153
 +1: M N P I R N S P S F I
hopL: CCGCCCGCTCATTTCGCCGGCAACTCAAGCGCCATCGCCTGGCACTGCGCTG 204
 +1: P P A H S P A T Q A P S P G T A L
hopL: CATTCTGCAGTGGTTCAGTCGCGACAGCAAGGCCGTAGCGCAGCTGCGCAAT 255
 +1: H S A V V S R D S K A V A Q L R N
hopL: GAGGGCGCGCGCGCAATAAACTGGATGCACAAGGGCACTCGCCTCTGGAC 306
 +1: E G A R A N K L D A Q G H S P L D
hopL: GTCCTCGACACTATGCGTGATATCGACGAGCGCAGCCGTTCCAGTCTGCGC 357
 +1: V L D T M R D I D E R S R S S L R
hopL: ATGGCATTACTGCAGTCACTCAATCCGACTGCGCAACTGGGCTATAACCAAG 408
 +1: M A L L Q S L N P T A Q L G Y T K
hopL: CCCGAGGCTCTGCACGGAACGCCCTGGGGCCTGAAAATCCTGCAGTCGGGA 459
 +1: P E A L H G T P W G L E I L Q S G
hopL: GCGCTCAGGGGCGGTGTCAATGATGCAAAGGGTGGAAACCCAGTCTCTTGAA 510
 +1: A L R G G V N D A K G G T Q S L E
hopL: GGCAAGGTGTTTTTCTCTGATCGAACGCGGGAGAGTGCGAGCGCTGAAACC 561
 +1: G K V F F S D R T R E S A S A E T
hopL: ACTCGTGCAGACTTNCGCAGCAAGCCCAGAGTCTATGCGAGAGNGAGGGG 612
 +1: T R A D X R S K P R V Y A R G E G
hopL: ATGCATCCCAGCAATGCTTACTCGCGCGCTCAGCAACACCGAATGGCGCAA 663
 +1: M H P S N A Y S R A Q Q H R M A Q
hopL: GTGATCCTGCATGCGCTGGACAACGGCAGATCGCTCTCCACCAATGAACTC 714
 +1: V I L H A L D N G R S L S T N E L
hopL: GCGCCATCGATCGAAGTGAGCAGCCCAGAGACGCTGTATATCGAAGGTGCT 765
 +1: A P S I E V S S P E T L Y I E G A
hopL: GCGTGGCTGCAACGTTTACTGCATGGCGGGTACATCAATAAATTAGGCGGG 816
 +1: A W L Q R L L H G G Y I N K L G G
hopL: CTCCCATTCAATGCCCACTGGGCGAGCATCTGGACTCACTGAGGTTA 867
 +1: L P F I N A P L G E H L D S L R L
hopL: CCTGGCTCGATTGAGTTGAGAGTCGATGGGCAGGTAAAGAAGTTACAGGGT 918
 +1: P G S I E L R V D G Q V K K L Q G
hopL: GAAGAGCTGAATCGCTTTTATCATCAGGCGGCGAGTGAAGTGCAGCGTTCT 969
 +1: E E L N R F Y H Q A A S E L Q R S
hopL: CTGGAAAATGGCAAGGCCCTTACCTGGGTTTGTGTAACAAGGGAGCCATT 1020
 +1: L E N G K A P Y L G L L N K G A I
hopL: GTTCCACTGGTGTTCGGTTTCGAGAAAATCAACAACCTGTCTACGCATGAA 1071
 +1: V P L V F G F E K I N N L S T H E
hopL: ATCAAATTACGCTCGAAAACCACACAGCACTCCTATCAGGATACCGAGCAC 1122
 +1: I K L R S K T T Q H S Y Q D T E H
hopL: CCACTTGCCGGAAGTCCGGAGAATGGTGGGAAGCTGAAAGAAGTGGAGGTG 1173
 +1: P L A G S P E N G G K L K E V E V
hopL: CGTAGCCTGGGTGATTTTCGCCACATTGTGCCTGGGGTGGCGGGTCAAGGG 1224
 +1: R S L G D F A T L C L G C A G Q G
hopL: GCTTTGAATTGCCTACCGACATAGTCGTGCGTGTTAAAGGCCAAAAAAGCC 1275
 +1: A L N C L P T *
 AAAAAGCTCAGTATCTGGACGCGCAACAAATACAGGCATTTTCGGCAAACC 1326
 TGGCAGCTCAAGTGGCCGAGCAGGCAAAGGGGAAGCCTCTGGGNACGCTCC 1377
 CTTTGCATCAGTTGCAGGAGAATCAATTCTCGACTGCGGGCTGGCGATTTA 1428

frame in the region and *lacZ* were constructed in pMLB1034. Only the fusion to the deduced ORF54 expressed appreciable β -galactosidase activity in induced SLR400 (pSHL4K) (Figure 2-2A). To verify the deduced size of the gene product, an amino-terminal 6xHis fusion to ORF54 was constructed in pQE30. The predicted 40 kDa ORF54 product was detected in immunoblots of DH5 α (pLL54Q30) probed with anti-6xHis antibody (Figure 2-2B).

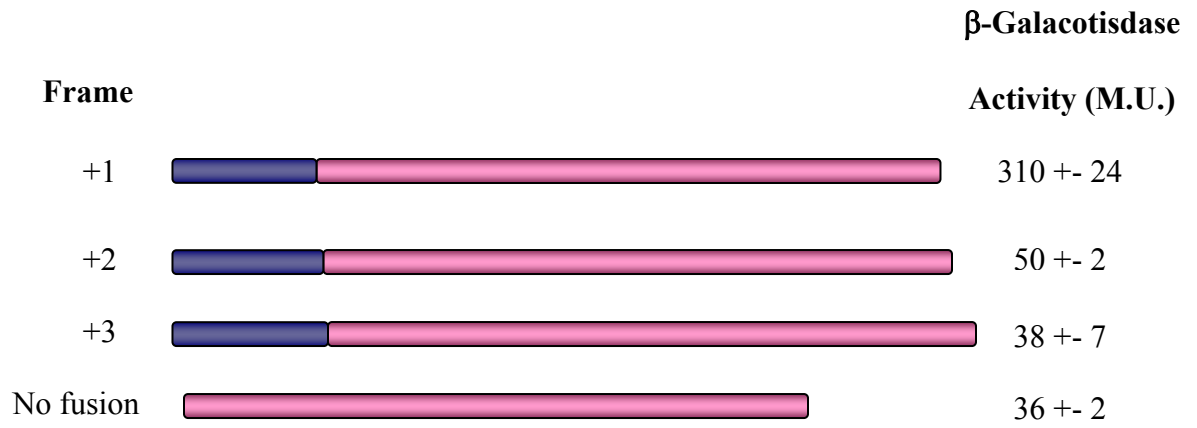
The ORF54 product is translocated into plant cells.

The structural and genetic features of ORF54 suggested that this protein could be an effector translocated by the *hrp* TTSS. To test this hypothesis, translational fusions between the amino terminal 46 codons of ORF54 and the carboxy terminus of *AvrRpt2* were created in pDSK519 and tested in the effector translocation assay described previously [73, 76, 146]. In this assay, the avirulence domain of *AvrRpt2* lacking the ability to be secreted (*AvrRpt2*) was fused to the potential secretion signal of the candidate effector. If secretion and translocation of *AvrRpt2* occurs, an Rps2-specific HR would be observed only in Rps2⁺ plants. Pto DC3000 (pLL54AvrRpt2K) expressing the fusion elicited a classic hypersensitive response (HR) in RPS2⁺ *A. thaliana* (Figure 2-3A). The inoculated tissue of *rps2* plants developed disease symptoms by 42 h in the form of purple, expanding lesions (Figure 2-3A). A null response was detected when these fusions were expressed in the Pto DC3000-A9 (*hrpA:: Ω*) mutant that is incapable of type III secretion (Figure 2-3A). These results are consistent with the TTSS-dependent translocation of the ORF54 product into plant cells and its identification as an effector.

Figure 2-2. Translational Fusions Between the three possible open reading frames and *lacZ*.

- A. PCR products carrying the region with the *hrpL*-dependent promoter, the ribosome-binding site and the first 120, 121, or 122 bp of *hopPsyL* were cloned into pMLB1034, which generated a translational fusion to '*lacZ* in three distinct reading frames. The constructs were assayed for β -galactosidase activity in *E. coli* SLR400 carrying pSHL1K, which expressed HrpL from an arabinose dependent promoter. The expression of *hrpL* was induced with 0.02% arabinose. Lac activity was measured following the procedures of Miller [139] and expressed as Miller Units.
- B. *hopPsyL* was amplified by PCR using specific primers (See Table 2-3) and cloned into pQE30 as a *Bam*HI- *Hind*III fragment in frame with the 6xHis tag. The expression of HopPsyL_{6xHis} was induced with 1mM IPTG for 4 hours in DH5 α . Whole cell extracts were resolved in a 12% SDS-PAGE and HopPsyL_{6xHis} was visualized using the anti-His antibody and ECL chemiluminescence kit. L, HopPsyL; M, protein marker.

A.



B.

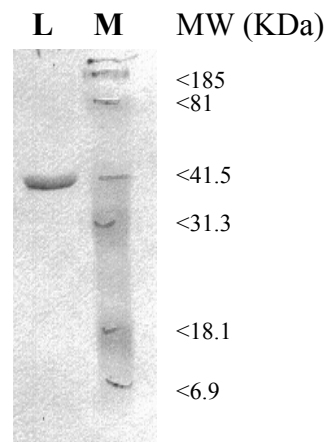
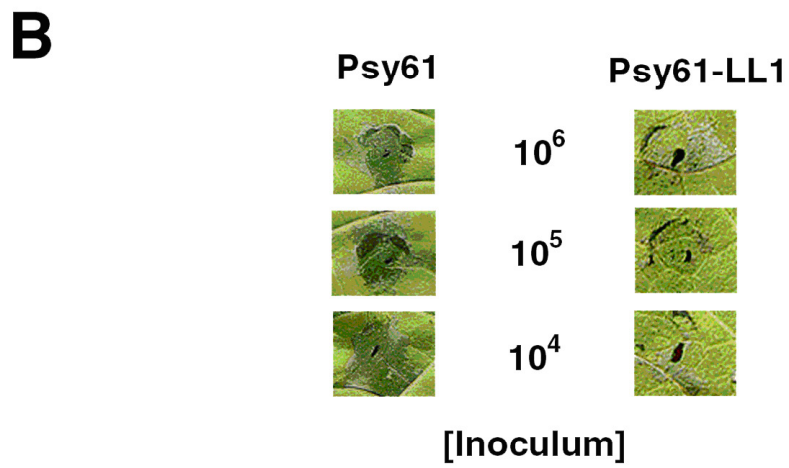
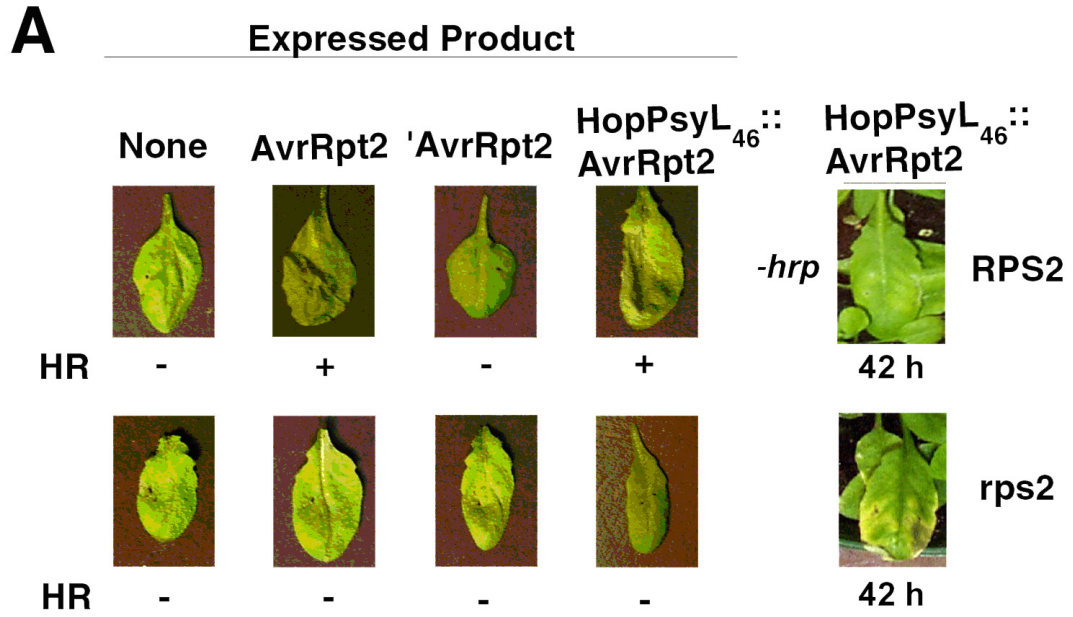


Figure 2-3. HopPsyL is a translocated effector with virulence and avirulence functions.

(A) AvrRpt2 translocation assay. A truncated AvrRpt2 protein (80-255 aa, 'AvrRpt2) was fused to the N-terminal 46 aa of HopPsyL (HopPsyL₄₆::AvrRpt2). Plasmids expressing the indicated proteins were introduced into DC3000 or DC3000-A9 (-*hrp*). *Arabidopsis thaliana* Col-0 (RPS2) or *rps2*⁻ leaves were infiltrated with bacterial suspensions at 10⁸ cfu/ml and evaluated for hypersensitive response (HR) after 18 h or disease symptoms after 42 h. Col-0 leaves showing the HR (+) appeared wrinkled at 18 h, *rps2*⁻ leaves showing disease appeared yellow and necrotic at 42 h. (B) Responses of *P. vulgaris* cv. Kentucky Wonder to *P. syringae* Psy61 and the *hopPsyL*::kan mutant, Psy61-LL. Leaves were inoculated at the indicated inoculum and photographed at 36 h. Note the lesions observed in tissue inoculated with Psy61 at 10⁵ and 10⁴ CFU/ml that are absent with the same levels of Psy61-LL1. (C) Ectopic expression of HopPsyL in *P. syringae* pv tomato DC3000 abolishes symptom development in *A. thaliana*. DC3000 was transformed with plasmids expressing the indicated protein. Bacterial suspensions of 10⁵ cfu/ml were used to inoculate *A. thaliana* Col-0 plants. The photographs were taken at 72 h after inoculation and were representative of developed symptoms. Note the necrosis and water soaking symptoms in leaves where HopPsyL was not expressed, indicative of a resistant plant response. These symptoms were absent where HopPsyL was expressed.



The ORF54 product was therefore designated as HopPsyL.

***hopPsyL* is necessary for virulence in *Phaseolus vulgaris* cv Kentucky Wonder.**

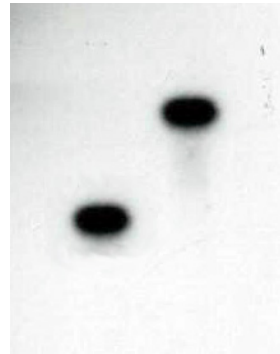
To determine the role of HopPsyL in the virulence of Psy61, a *hopPsyL::kan* insertion was constructed in Psy61 by allelic exchange to create Psy61-LL1. The mutation was confirmed by Southern hybridization analysis (Figure 2-4). Psy61 has recently been reported to be a pathogen of *Phaseolus vulgaris* [52]. *P. vulgaris* cv. Kentucky Wonder was susceptible to Psy61 infection as demonstrated by the appearance of water-soaking lesions (symptom score of 4 [93]) and necrosis observed at all inoculum levels by 36 hr (Figure 2-3B, Psy61). A 10^5 -fold increase in bacterial populations detected 3 days post-inoculation (Figure 2-5). The mutant strain, in contrast, was considerably reduced in virulence. Plants inoculated with Psy61-LL1 showed minimal water-soaking symptoms (Figure 2-3B, Psy61-LL1). Red-brown necrotic response, typical of resistance [93], developed near the inoculation site by 36 h at high inocula and macroscopic symptoms did not develop in tissue inoculated with less than 10^6 CFU/ml (Figure 2-3B, Psy61-LL1). Detected populations of Psy61-LL1 were 10^2 lower than in the parent strain at 3 days (Figure 2-5). Complementation of the mutant with a plasmid-borne *hopPsyL* restored virulence to the strain as indicated by the population levels detected in tissue (Figure 2-5). The failure of the *hopPsyL::kan* mutant to develop disease symptoms and the reduced growth in the previously susceptible host indicate that HopPsyL is important for Psy61 virulence in this bean cultivar.

Figure 2-4. Southern Hybridization confirms *hopPsyL* insertion.

A. A ^{32}P -labelled probe was generated by PCR amplification from Psy61 genomic DNA with primers 54-549E and 54-1247B (see Table 2-3). Chromosomal DNA from Psy61 and Psy61-LL1 was digested with *Bam*HI and transferred to a charged nylon membrane. Membranes were allowed to hybridize to the labeled probe and washed under low stringency conditions [167]. Bands that hybridized to the probe were visualized by autoradiography. The shift in size from the wild-type to the mutant is consistent with the insertion of the 2.9 Kb plasmid into the chromosome.

A.

Psy61 Psy61-LL1

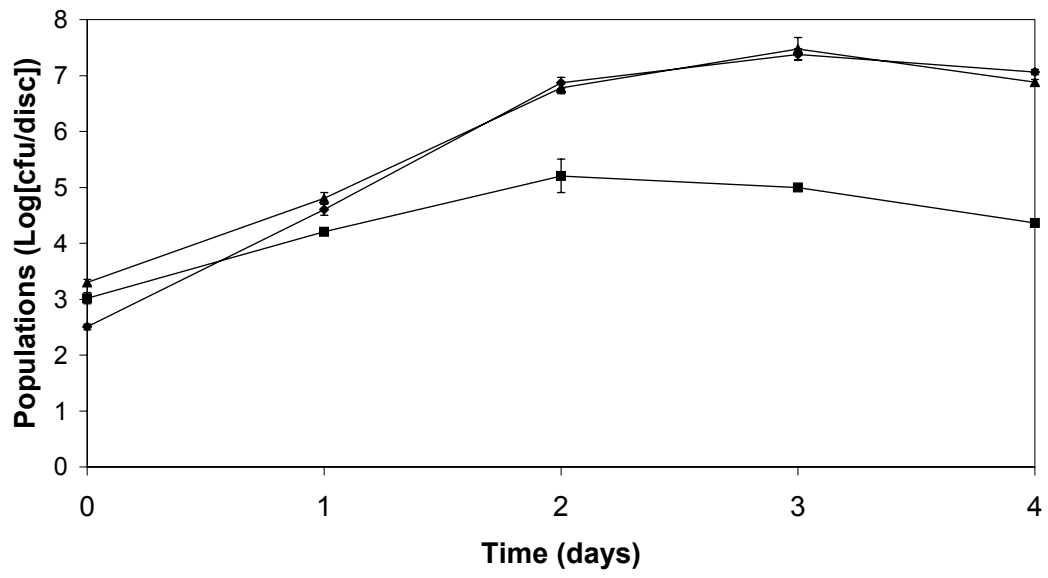


← 6.3 Kb

← 3.4 Kb

Figure 2-5. HopPsyL is required for virulence of Psy61 in *P. vulgaris* cv. Kentucky Wonder.

P. vulgaris leaves were inoculated with a 10^5 cfu/ml suspension of Psy61 (wild-type, ▲), Psy61-LL1 (*hopPsyL::kan*, ■), or the complemented strain Psy61-LL1 expressing HopPsyL (◆). The bacterial populations were monitored every 24 h by mascerating a 38 mm² leaf disk in 100 µl of M63 media. The suspensions were diluted to appropriate bacterial concentrations and bacteria were enumerated after 24 – 30 h. The values reported are the mean of 6 replicates. Error bars represent the standard deviation. The experiment was repeated three times with similar results.



HopPsyL acts as an avirulence determinant in *Arabidopsis thaliana*.

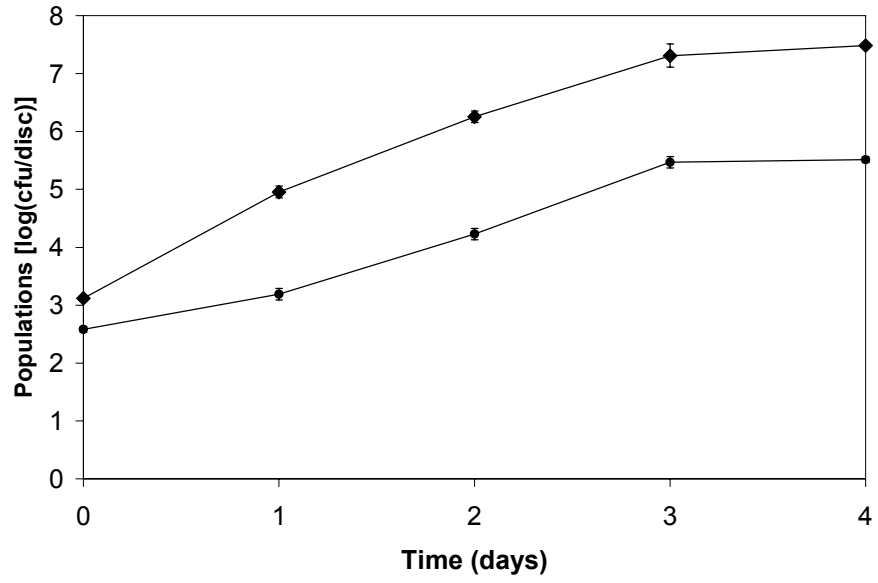
Ectopic expression of effectors in non-native strains has been previously shown to affect virulence of the recipient *P. syringae* strains [177, 191]. The region carrying *hopPsyL* was amplified from Psy61 genomic DNA and cloned into the broad host range cosmid pLAFR3 to create pLL54L. When *A. thaliana* Col-0 was inoculated with Pto DC3000 (pLL54L) at 10^7 cfu/ml, an HR developed by 18 h. A null response was observed at lower inoculum levels (Figure 2-3C, HopPsyL). In contrast, leaves inoculated with the virulent Pto DC3000 (pLAFR3) developed typical disease symptoms by 42 h (Figure 2-3C, None). To quantify the effect of HopPsyL on the virulence of Pto DC3000, populations of Pto DC3000 (pLAFR3) and Pto DC3000 (pLL54L) were monitored in inoculated *A. thaliana* Col-0 leaves. Whereas DC3000 (pLAFR3) populations increased 10^4 -fold during the 4 day assay period, those of DC3000 (pLL54L) increased only 500-fold (Figure 2-6).

HopPsyL does not suppress the HR.

Various TTSS-dependent effectors of *P. syringae* strains have been recently identified that facilitate pathogenicity of the source strain by an apparent suppression of host defense responses [1, 27, 55, 185]. Several lines of evidence argue against HopPsyL

Figure 2-6. Ectopic expression of HopPsyL reduces the virulence of DC3000 in *A. thaliana* Col-0.

A. thaliana leaves were inoculated with a 10^5 cfu/ml suspension of DC3000 carrying either the empty vector pLAFR3 (◆), or a construct in pLAFR3 expressing HopPsyL (●). The bacterial populations were monitored every 24 h as in Figure 2-5. The values reported are the mean of 6 replicates. Error bars represent the standard deviation. The experiment was repeated three times with similar results.



suppressing host defense responses. Unlike HopPtoD2, which could delay the timing of the HR during an incompatible host-pathogen interaction [27, 55], HopPsyL did not alter development of the HR of DC3000 in *P. vulgaris* cv Kentucky Wonder. Bacterial suspension (10^8 cfu/ml) of DC3000 (pLAFR3) or DC3000 (pLL54L) were used to inoculate *P. vulgaris* cv Kentucky Wonder leaves and the appearance of HR was checked every 2 hours for the first 16 h. No differences in phenotype or development of the HR elicited were detected (Table 2-1). In addition, several non-host plants of Psy61 (see Materials and Methods) were challenged with high inocula of Psy61 or Psy61-LL1 and the timing and manifestation of the elicited HR were studied. The induced HR was indistinguishable in all the hosts studied. Taken together, these results suggest that HopPsyL does not suppress plant defense responses, but instead acts by another mechanism to facilitate parasitism of Kentucky Wonder by Psy61.

Distribution of *hopPsyL* among *P. syringae* strains.

To determine the distribution of *hopPsyL* alleles among *P. syringae* strains, a PCR screen was performed using the primers 54-549 and 54-1247. Diagnostic fragments indicative of the presence of *hopPsyL* were amplified from six of 10 *P. s. pv. syringae* strains tested (each with distinct host range) (Table 2-2), but not from any of the *P. syringae* pvs. *tomato*, *maculicola*, or *phaseolicola* strains screened. Sequence of the amplified fragments exhibited greater than 99% identity with the Psy61 *hopPsyL* (data not shown).
To

Table 2-1. The hypersensitive response is not inhibited by HopPsyL.

Time (h) ¹	Psy61 ²	Psy61-LL1 ²
2	nr	Nr
4	nr	Nr
6	2	2
8	2-3	2-3
14	3-4	3
18	4	4
20	4	4
22	4-5	4-5
24	5	5

1. Leaves of *Phaseolus vulgaris* cv. Kentucky Wonder were inoculated with a 10^8 cfu/ml suspension of Psy61 or the *hopPsyL::kan* mutant, Psy61-LL1. The induced hypersensitive response (HR) was measured every 2 hours for water soaking as initial evidence of initiation of HR, and then for tissue collapse and necrosis.
2. The symptoms were scored using the following scale: nr, no reaction; 2, glazing; 3, water soaking; 4, tissue collapse; 5, desiccation and necrosis.

Table 2-2. Conservation of *hopPsyL* among *P. syringae* strains.

Strain ¹	Detection by:		Response in Kentucky Wonder ⁴
	PCR ²	Hybridization ³	
<u>Psy</u>			
61	+	+	D
B3A	+	+	D
B76	+	+	Null
5D417	+	+	Null
B362	+	+	D
B382	+	+	Null
3097	+	+	Null
Ps-1 Bean	-	-	na
B460	-	-	na
Ps-1	-	-	na
1053	-	-	na
WYN 108	-	-	na
S-4B-1	-	-	na
<i>E. coli</i>	-	-	
DH5 α	-	-	na

¹ Strains and hosts were described in [53]. Other *P. syringae* pathovars were tested but resulted in a lack of either amplification or signal from the different assays. Those strains are as follows: *pv. maculicola* #1, #5, and #10; *pv. phaseolicola* NK343, B130, and BK378; *pv. tomato* T1,4355, 3523, B76, B88, B118, B121, B122, B19, and DC3000.

² PCR analysis was performed using primers 54-549 and 54-1247 and an annealing temperature of 55° C. A positive sign indicates specific amplification of a 700 bp

fragment. Psy61 was used as a positive control, and DH5 α as a negative control.

³ Hybridization experiments were performed using a 698 bp probe generated using primers 54-549 and 54-1247 and room temperature washes in 1x SSC. A positive sign indicates that hybridization of the probe could be detected in autoradiograms.

⁴ Overnight cultures were diluted into fresh media and allowed to grow until the OD₆₀₀ reached 1.0. The cells were collected, washed in M63, and resuspended at 10⁶ cfu/ml. The suspension was used to inoculate *Phaseolus vulgaris* cv Kentucky Wonder. The symptoms were scored daily up to 5 days. D, slowly developing necrosis typical of disease observed at 36 h; d, slight symptoms of disease by 72 h; Null, no response detected during the 5 day assay period; na, not tested.

screen for more divergent alleles, the 698 bp fragment carrying the amino terminal portion of *hopPsyL* was amplified from Psy61 genomic DNA and used to probe the genomes of the selected strains for presence of an *hopPsyL* allele by low stringency hybridization. Hybridization results mirrored those from the PCR screen. Those strains that produced the indicative PCR product hybridized to the probe whereas the other strains did not. An ortholog of *hopPsyL* was not detected in the DC3000 and B728A genomes. Of the strains carrying HopPsyL, only B632, originally isolated from diseased beans (J. DeVey, personal communication) and B3A, isolated from a peach variety, were apparent pathogens of Kentucky Wonder (Table 2-2). The contribution of HopPsyL to this virulence has not been established. The observation that only a few strains carrying a *hopPsyL* allele are pathogens of bean indicates that HopPsyL does not act epistatically to other effectors to enable virulence in bean.

Discussion

The pathogenicity and host range of *P. syringae* strains have been linked to the expression of strain-specific arrays of effectors that are translocated into host cells by the *hrp* TTSS. By utilizing the previous observations that all known effectors expressed by *P. syringae* strains are components of the *hrp* regulon [62, 201, 206], and therefore are dependent upon HrpL for transcription, an *E. coli*-based HrpL-dependent promoter trap screen was developed to identify candidate effector genes expressed by a strain. The screen was successful in identifying several effectors previously characterized in other strains. Of primary interest in this study was the promoter active fragment KP54. This

fragment carried a near consensus HrpL-dependent promoter but the associated coding sequence lacked similarity to other genes. HrpL-dependent activity of the fragment was verified in Psy61 and the ORF identified directly downstream of the HrpL-dependent promoter was shown to encode HopPsyL, a 40.5 kDa protein with no homologs in the databases. HopPsyL was translocated into host cells by the *hrp* TTSS and facilitated Psy61 virulence in *P. vulgaris* cv. Kentucky Wonder. When ectopically expressed in the *A. thaliana* pathogen DC3000, HopPsyL acted as an avirulence determinant, reducing the growth of the expressing strain and suppressing symptom development.

The mechanisms through which HopPsyL contributes to parasitism and disease in Kentucky Wonder remain unknown. No enzymatic or metabolic function could be assigned to HopPsyL but the structural features suggest that it may localize to chloroplasts as suggested for several other effectors of *P. syringae* strains [73, 76]. HopPsyL is unique in that it also contains an apparent ankyrin repeat motif at residues 36-64. Ankyrin-like domains are thought to function in protein-protein interactions, and are present in some plant defense response genes, such as PR1 [170]. HopPsyL also has a comparatively high Leu content that could be indicative of protein-protein interactions. The *Yersinia* effector YopM, and *Salmonella* effectors SspH1 and SspH2 also have high Leucine content and carry Leucine-rich repeats [56] [138] which are used in the pathogenicity of both organisms. However, leucine rich repeats were not identified in HopPsyL by the REP, REPRO or Radar algorithms [10, 66, 79]. The presence of two separate TM domains suggests that this effector could localize to a membrane. Unfortunately, a function for HopPsyL during pathogenesis could not be identified.

In contrast to several recently characterized effectors [1, 27, 55, 78], HopPsyL does not appear to be a general suppressor of plant defense responses. For instance, expression of the locus in the native Psy61 or in Pto DC3000 did not affect the ability of the strain to elicit the HR or related responses in resistant plants. HopPsyL does not appear to act epistatically to other effectors to facilitate pathogenesis. Namely, ectopic expression of *hopPsyL* in Pto DC3000 did not expand its host range to include Kentucky Wonder, and most strains of *P. syringae* that carry a homolog of *hopPsyL* were not pathogens of Kentucky Wonder. One explanation for the role of HopPsyL in pathogenicity could be that it acts as a virulence gene required for Psy61 to replicate in Kentucky Wonder. Alternatively, HopPsyL could act to mask the activity of another *avr* product specifically active in Kentucky Wonder, similarly to the suppression of AvrPphF activity in Canadian Wonder by AvrPphC [185]. Consistent with this hypothesis, inactivation of *hopPsyL* in Psy61 resulted in an apparent conversion to an incompatible interaction in Kentucky Wonder. Irrespective of the mechanism of action, HopPsyL can be added to a growing set of *P. syringae* effectors that are required for virulence in one host but elicit defense responses in another host.

Unfortunately, the great number of discovered *P. syringae* effectors has not led us any closer to the clarification of the type III secretion signal. Petnicki-Ocweija, *et al.* [158] postulated a set of common characteristics shared among type III effectors. These characteristics include: 1) the first 50 amino acids of known effector proteins have a high serine content compared to a set of random housekeeping

proteins in *P. syringae* [76]; 2) the N-termini of most Hops are amphipathic and rich in polar amino acids; 3) they have an aliphatic amino acid (Ile, Val, or Leu) or Pro at the third or fourth position; 4) they have no acidic amino acids (Asp or Glu) in the first 12 amino acids; 5) the fifth position is rarely Met, Ile, Leu, Phe, Tyr, or Trp; 6) cysteine residues are rarely found after the fifth position (among the N-terminal 50 residues); and 7) there are no more than three consecutive residues consisting of Met, Ile, Val, Phe, Tyr or Trp [158]. Genomic searches for ORFs with these characteristics have yielded many potential *hop* genes [76, 158]. Although some of these ORFs were confirmed to encode Hops [169], most of the effector proteins identified thus far, including HopPsyL, do not share all of the postulated characteristics. Interestingly, HopPtoV did not share any of the characteristics [169], but was still shown to be secreted and translocated [169, 193]. Identifying more *P. syringae* effectors does not seem to be the answer for the secretion signal riddle. Instead, bioinformatic and biochemical methods such as those using engineered synthetic peptides [125] might aid in fully clarifying the signal.

In summary, the HrpL-dependent promoter trap screen provides a relatively inexpensive, high throughput assay for candidate HrpL-dependent promoters that is independent of the role of the gene product in pathogenesis, and can be used to survey previously uncharacterized *P. syringae* strains for effector genes. The ability to identify the set of effectors produced by a strain should facilitate comparative analyses of *P. syringae* strains to identify the factors controlling pathogenicity and host range. By applying this screen to Psy61, we were able to isolate five effectors, including a novel locus required for virulence of the source strain, but the survey of the Psy61 genome is far from

complete. *P. syringae* strains are predicted to express up to 60 effectors [73]. In DC3000, a trial promoter trap screen led to the identification of a translocated protein tyrosine phosphatase that modulates several defense responses [27] and sequences obtained during the characterization of the promoter-active fragment carrying the *shcA* promoter facilitated characterization of the exchangeable effector loci carried by 30 *P. syringae* strains [33]. Thus, the conservation of *hrpL* among *P. syringae* strains [168] indicates that this assay should be broadly applicable to all *P. syringae* strains.

Materials and Methods

Bacterial Stains and Media.

Strains and plasmids used in this study are described in Table 2-3. Bacteria were routinely grown on King's medium B [12]. Plasmids were propagated in *E. coli* DH5 α . *E. coli* strains were grown at 37°C, and *Pseudomonas syringae* strains were grown at 25°C. LB, MacConkey and M63 minimal salts media were used for culture of *E. coli* strains [12]. M63 medium was supplemented with 1mM MgSO₄ and 1% fructose. The following antibiotics were added at the indicated concentrations (in μ g/ml): ampicillin, 200; kanamycin, 50; spectinomycin, 100, tetracycline, 25; nalidixic acid, 50; rifampicin, 200; and chloramphenicol; 30. L-arabinose concentration in agar media was 0.01%, unless stated otherwise.

General DNA manipulations.

Restriction enzymes were purchased from Invitrogen (Bethesda, MD). T4 DNA ligase was acquired from New England Biolabs (Beverly, MA) and used according to the manufacturer's recommendations. Basic manipulations were done using standard procedures [167]. PCRs were performed using a Hybaid PCR Sprint™ thermal cycler with 50 µl reaction volumes. Unless indicated otherwise, *Pwo* polymerase (Boehringer-Mannheim) was used to amplify fragments for cloning.

Construction of genomic library.

Genomic DNA isolations were performed following the CTAB protocol [167], and DNA concentration was adjusted to 1.5 µg/µl. Genomic DNA (30 – 40 µg) was digested with 1 unit of *Sau3A* for 2, 5, 10, 15, and 30 min at 37°C. Partially digested DNA was fractionated by agarose gel electrophoresis, and fragments of desired sizes were isolated from gels using the Prep-A-Gene™ kit (Bio-Rad, Hercules, CA). Isolated fragments were ligated to *Bam*HI digested pRG970 at a molar ratio of 5:1 using T4 DNA ligase at 4°C.

β-galactosidase assays.

β-galactosidase activity in bacterial cells was estimated by the procedures of Miller [139].

Table 2-3. Strains, plasmids and primers used in this study.

Strain or plasmid	Genotype or phenotype	Reference or source
<i>Strains</i>		
<i>E. coli</i>		
DH5 α	<i>endA1 hsdR17</i> (r _k ⁻ m _k ⁻) <i>supE44 thi-1 recA1</i> <i>gyrA96 relA1</i> Δ (<i>argR-lacZYA</i>) U169 ϕ 80 <i>lacZDM15</i>	Invitrogen
MC4100	F' <i>araD139</i> Δ (<i>argF-lacZYA</i>) U169 <i>rpsL150</i> <i>relA1 flb-5301 ptsF25 deoC1</i>	[31]
SLR400	<i>araD139</i> Δ (<i>ara leu</i>)7697 derivative of MC4100	S. Benson
<i>P. syringae</i>		
A9	<i>hrpA</i> mutant, HR ⁻ , Rif ^r , Kan ^r	[194]
DC3000	Wild-type, Rif ^r , HR ⁺ Tomato and <i>Arabidopsis</i> pathogen	[46]
Psy61	Wild-type, Nal ^r , HR ⁺ bean pathogen	[15, 52]
Psy61-2070	<i>hopPsyA::TnphoA</i> , Nal ^r , Kan ^r	[84]
Psy61-2074	<i>hrpL::TnphoA</i> , Nal ^r , Kan ^r	[84]
Psy61-LL1	<i>hopPsyL::pUC18K</i> , Nal ^r Kan ^r	This work
Plasmids		
pDSK519	Broad-host range vector, IncQ Kan ^r	[107]

pJBAvrRpt2-600	<i>avrRpt2</i> cloned into pDSK600	[27]
pLAFR3	IncP1, Tet ^r	[177]
pLL54L	1.3-kb PCR product containing <i>hopPsyL</i> cloned into pLAFR3	This work
pLL54 ₄₆₋₁₇₀ K	0.5-kb PCR product containing codons 46— 146 of <i>hopPsyL</i> cloned into pUC18K	This work
pLL54AvrRpt2K		This work
pLL54 ₄₆ ::AvrRpt2K	N-terminal 46 aa of HopPsyL fused to 'AvrRpt2 in pDSK519	This work
pLL54Q30	1.2-kb PCR product containing ORF54 cloned into pQE30	This work
pMLB1034	' <i>lacZYA</i> , for creating translational fusions, Amp ^r	[173]
pMPM-K6	AraC ⁺ , P _{BAD} -MCS, Kan ^r , Sp ^r :Ω, OriV _{P15A}	[136]
pQE30	Am ^r , mcs:his	Qiagen
pRG970	IncP, Sp ^r , promotorless <i>lacZ</i> and <i>gusA</i> in opposite orientations	[187]
pSHL4K	P _{BAD} : <i>hrpL</i> fusion in pMPM-K6, Km ^r , ΔΩ:Sp ^r	[27]
pTS54R	1.2-kb genomic fragment from Pss61 cloned into pRG970	This work
pUC18K		

Primers

54-549E	GGAATTCGGCAACGCATGATTGAG
54-1247B	CGGGATCCAGCGATCTGCCGTTGTCCA
54-739E	GGAATTCGTCGCGACAGCAAGGCCGTA
54-739B	CGGGATCCTACGGCCTTGCGTCGCGAC
54-1098	AACACCTTGCCTTCAAGAGA
54-1029	GCTCAGGGGCGGTGTCAATG
54-549X	GCTCTAGAGGCAACGCATGATTGAG
54q30S	TCCCCGGGAACCCATTCGTAATTCT
54-1871S	ACGCGTTTGCCGAAAATGCCTGTAT
54-Rpt ₄₆ -SapI	GATGCTCTTCACCCTACGGCCTTGCTGTGCGG
AvrRpt2 ₄₀₈ SapI	GATGCTCTTCAGGGAAGCACGAGACGGGCGGT
AvrRpt2-1028X	GCTCTAGATAGGGACCAAAAAGCCAGAC
970-5	CCACAGCCGTCGGAGT
970-3	ACGCCAGGGTTTTCCCAGTCA

Inverse PCR.

Inverse PCR was performed using the procedures of [152]. Psy61 genomic DNA (1 µg) was digested with *Sst*I and self ligated. The ligation mixture was diluted 100X and used as template for PCR using primers 54-739B and 54-1029 and ProofPro™ DNA polymerase (Continental Lab Products) following the manufacturer's protocol. These primers were located 100-200 bp internal to the previously sequenced portion of ORF54, such that any specific amplification product could be identified by the presence of these known flanking sequences. Thermal cycling was carried out using a 53°C annealing temperature and an extension time of 6 min. Amplified fragments were gel purified and nucleotide sequence obtained at the University of Maryland Sequencing facility.

TAIL-PCR.

The Psy61 genome was used as template for TAIL-PCR following the protocols of Liu and Whittier [123]. Primer 54-549E was used for the primary PCR reaction with the degenerate primer AD3. For the secondary and tertiary nested PCR reactions, primers 54-739E and 54-1029 were used, respectively. The PCR product was purified using Qiaquick PCR purification kit (Qiagen) and nucleotide sequence obtained at the University of Maryland sequencing facility.

Construction of Psy61-LL1.

The region of *hopPsyL* corresponding to codons 40-170 was amplified by PCR from genomic Psy61 DNA using primers 54-739E and 54-1247B, digested with *EcoRI* and *BamHI* and ligated into pUC18K which does not replicate in Psy61. The resulting plasmid was transformed into Psy61. Nal^r Kan^r integrants were selected and gene disruption confirmed by PCR analysis and Southern hybridization.

Construction of pLL54Q30 and size determination of HopPsyL.

hopPsyL was amplified using primers 54q30S and 54-1871S, and cloned into pQE30 as a *SmaI* fragment. To determine the size of HopPsyL, DH5 α (pLL54Q30) was grown to OD_{600} 0.2, IPTG was added to 2mM and HopPsyL expression was induced for 2 hr. The cells were harvested and resuspended in 50 μl SDS-loading buffer. Samples were run on a 12% SDS-PAGE gel, and blotted onto PVDF membranes. The samples were probed using a monoclonal anti-His antibody (Novagen, WI), and visualized using a goat anti-mouse HRP conjugate and an ECL chemiluminescence kit (Amersham-Pharmacia, NJ).

AvrRpt2 translocation assay.

AvrRpt2 translocation assays were performed as described previously [76] with minor changes. The amino terminal region of *hopPsyL* was amplified from genomic Psy61 DNA using primer 54-549 and reverse primer 54-Rpt2₄₆-*SapI* to generate a fragment carrying the first 46 codons of *hopPsyL*. *avrRpt2* was amplified using primers AvrRpt2-

408*SapI* and AvrRpt2-1028X. These fragments were digested with *SapI* and ligated. The ligation mixture was used as a template for a PCR amplification of the fusion using primers 54-549X and AvrRpt2-1028X. The resulting PCR product was extracted from agarose gels using the QIAspin kit (Qiagen, CA) and cloned into pDSK519 as an *XbaI* fragment. Clones with the correct orientation were transformed into DC3000 and transformants screened for phenotype in wild-type or *rps2 A. thaliana* leaves.

Virulence assays.

A. thaliana Col-0 and *P. vulgaris* cv. Kentucky Wonder plants were inoculated with 10^5 cfu/ml suspensions of the indicated strains unless indicated otherwise. *P. vulgaris* cv Kentucky Wonder plants were scored daily using the five point scale described previously [93]. Bacterial populations were monitored as described previously [18]. For HR phenotype in *N. tabacum*, *A. thaliana*, or *P. lunatus* leaves, bacteria were grown to 10^9 cfu/mL in KB and serially diluted to 10^8 , 10^7 , and 10^6 . Dilutions were infiltrated into leaves and HR was scored after 18 hours. The plants used were: *N. tabacum* cv. Samsun, *Phaseolus lunatus* cv Roma, and *A. thaliana* accessions Col-0, Col-0/*rps2*, Tsu-1, Leesburg, Shadhana, Ws and Dijon.

PR1 induction assay

Leaves of *A. thaliana* Col0:PR1/GUS plants [172] were inoculated with bacterial suspensions (10^6 cfu/ml). After 48 h single leaf disks were excised and used for GUS

assay with the fluorogenic substrate 4-methyl-umberlliferyl- β -D-glucuronide (ICN Biochemicals). Reactions were incubated 30 minutes at 37° C, and the fluorescence was measured using a TKO 100 fluorometer (Hoefer Scientific). The units of activity were calculated using a standard curve.

Colony Hybridizations.

A 32 P-labelled probe was generated by PCR amplification from Psy61 genomic DNA with primers 54-549E and 54-1247B in a reaction containing 25 μ Ci of γ - 32 P dATP. *P. syringae* colonies were transferred to charged nylon membranes, lysed and allowed to hybridize to the labeled probe under low stringency conditions[167]. Colonies that hybridized to the probe were visualized by autoradiography.

Chapter 3: Type III secretion chaperones of *Pseudomonas syringae* protect effectors from Lon-mediated degradation.

Introduction

The array of effectors translocated by the TTSS of each pathogen is distinct [26, 39, 45]. For plant pathogenic bacteria, effectors have been associated with the suppression of defense responses and enhanced nutrient leakage in susceptible plants, or alternatively with the activation of programmed cell death in resistant plants [26, 48, 73, 176]. In mammalian pathogens, effectors have been shown to suppress or activate innate defense responses, regulate the inflammatory response and cause cytoskeletal rearrangements in host cells [45]. The number of effectors produced by pathogenic strains varies from as few as nine in *Yersinia* [41] to an estimated more than sixty for individual *P. syringae* strains [39, 73]. Interestingly, effectors from both groups of pathogens can be enzymes, such as protein tyrosine phosphatases and cysteine proteases [13, 27, 55, 103, 151, 154, 171], but in *P. syringae* strains the function for most has not been established.

Despite the variety of effectors produced by plant and mammalian pathogens, the mechanism of effector secretion is thought to be similar for all TTSS. A cryptic N-terminal secretion signal directs effectors to the secretion port [44] [162]. Both co-translational and post-translational mechanisms have been proposed to be involved in this process [7, 9, 124, 125, 162]. Once at the secretion port, effectors appear to be

introduced and directed through the central channel of the secretion in an ATP-dependent process [160, 197]. The size restriction of the secretion channel ($\sim 20\text{\AA}$) [21, 111] predicts that effectors are likely to be in an unfolded state during the secretion process. Consistent with this model, peptide structures with diameters greater than the $\sim 20 - 30\text{\AA}$ obstructed the secretion channel [60].

Regulation of effector secretion is complex and involves multiple tiers as observed in flagellar biosynthesis [175]. In many cases, contact with a host cell activates a regulatory network to express the genes required for assembly of the TTSS [2, 40] [89]. Transcription and/or translation of these genes is thought to occur in a specific order to assemble the translocation apparatus similarly to the flagellar biosynthesis apparatus [35]. Culture conditions mimicking the host environment have been used to bypass the contact-dependent regulation of the TTSS of many pathogens such as *Yersinia pestis* [61] and *P. syringae* [188, 202]. A second tier of regulation results in the expression of genes whose products are substrates for type III secretion [40, 89]. In some species, effectors do not appear to be secreted simultaneously [24, 37, 51]. Therefore, an additional level of regulation within the second regulatory tier has also been proposed that establishes a hierarchy for effector secretion [24]. The exact manner in which the hierarchy is determined is as yet unclear, but appears to involve a family of proteins called TTSS chaperones [59, 156, 198].

P. syringae strains express a TTSS encoded by the *hrp* pathogenicity island that is closely related to its counterpart found in strains of *Yersinia* spp [38, 89]. Analysis of

the regulatory network associated with the *hrp* TTSS showed that HrpR and HrpS are truncated enhancer binding proteins that interact to form an activation complex for the *hrpL* promoter [90]. HrpL, an alternative sigma factor, can recognize a conserved promoter sequence to direct the expression of all known genes encoding components of the *hrp* translocation apparatus as well as the translocated effectors and their cognate chaperones [62, 199, 201]. Expression of the TTSS of *P. syringae* is minimal under nutritionally-rich growth conditions, but is activated *in planta* or under conditions that mimic the host environment, such as acidic minimal salts media (i.e. inducing conditions) [202]. During a search for a postulated negative regulator of the *hrp* TTSS, Lon protease was shown to mediate the turnover of HrpR to regulate expression of *hrpL* [25]. Regulated proteolysis by Lon functions in several regulatory networks in eubacteria, and Lon has also been associated with the degradation of abnormally folded proteins [70]. Interestingly, *lon* mutants of *P. syringae* exhibited substantially higher protein secretion and induced plant defense responses faster than the wild type strain [25]. Ectopic expression of HrpRS by several groups, however, has not reproduced this phenotype, suggesting that Lon could have an additional role in the regulation of type III secretion in *P. syringae* strains.

Here we report that Lon-mediated regulation of the *P. syringae* TTSS also involves regulated proteolysis of effectors prior to secretion that can be suppressed by the cognate chaperone. Domains targeting effectors for degradation were identified. The data advances our understanding of the essential role of chaperones in protecting effectors from degradation prior to secretion and provides a potential mechanism for

hierarchical secretion of effectors.

Results

***P. syringae lon* mutants hypersecrete the TTSS-dependent effector AvrPto**

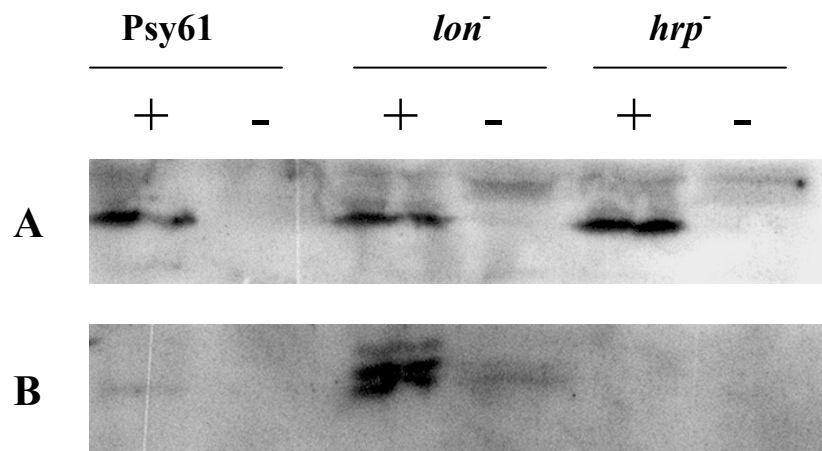
During the initial studies, other members of the lab noticed *lon* mutants could elicit the early visible symptoms of the HR more rapidly than wild-type *P. syringae*. Additionally, the mutants secreted higher amounts of specific proteins into cell supernatants. The data suggested that these mutants were specifically secreting higher amounts of effectors. To determine if the *lon::Tn* mutants could secrete effectors in the absence of plant host, *avrPto* was ectopically expressed using a vector's P_{lac} promoter and secretion of AvrPto into culture filtrates was monitored. As reported previously [188], secretion of AvrPto by the wild-type *P. syringae* was difficult to detect. Levels of AvrPto in immunoblots of concentrated culture filtrates from Psy61 (pAVRPTO6H-600) were nearly undetectable (Figure 3-1). Culture filtrates of the *lon::Tn* mutant KL11 (pAVRPTO6H-600), however, were estimated to contain at least 100-fold higher levels of AvrPto than its Lon⁺ parent strain after densitometry analysis.

Secretion through the *hrp* TTSS is regulated beyond HrpL

To determine whether the role of Lon in the regulation of the *hrp* TTSS is limited to the control of *hrpL* expression, we attempted to bypass the known regulatory activity of Lon by ectopic expression of HrpL. If the role of Lon were solely to regulate *hrpL*

Figure 3-1. Secretion of AvrPto from *P. syringae lon* mutants.

Levels of AvrPto were monitored in Psy61, Psy61-KL11 (*lon*::Tn) and a TTSS secretion mutant, Psy61-2082 (*hrcN*::Tn) expressing AvrPto from pAvrPto6H-600 (+), or the empty vector pDSK600 (-). Overnight cultures were diluted in fresh rich media and grown to an OD₆₀₀ of 1.0. The cells were collected, washed and resuspended in *hrp*-inducing media (M63Fructose, pH 5.5) at an OD₆₀₀ of 0.6 and grown for an additional 4 hours. The cells from a 250 µL sample of the culture were collected and resuspended in SDS-PAGE loading buffer. Culture filtrates of the remaining culture were obtained by centrifugation and concentrated 50X using Millipore Ultra-free centrifugal filter devices. Ten µg of protein were loaded and separated in an SDS-PAGE and levels of anti-AvrPto reactive proteins monitored in cell lysates (A) and culture filtrates (B) using immunoblots. AvrPto migrated as an 18 kDa protein.



expression, then vector-directed expression of *hrpL* should result in the same phenotype as a Δlon mutant. The induction of effector-dependent programmed cell death in resistant hosts and the secretion of effectors into culture supernatants was studied in wild-type and *Lon*⁻ strains carrying a *hrpL* construct expressed from the *lacUV5* promoter of pDSK600 (pMLL600). A similar construct expressing the *lacZYA* operon (pLLlac600) exhibited equal activities in both strains. The steady state level of LacZ activity in *P. syringae* pv tomato DC3000 (pLLlac600) was 346±12 Miller units whereas 375±34 units were measured in the DC3000 *lon::Tn* mutant JB7 (pLLlac600). This similarity indicates that this expression system is insensitive to the activity of *Lon*. Concentrated culture supernatants of DC3000 (wild-type), JB7, or the DC3000 *hrpA::kan* mutant A9 carrying pMLL600 were monitored for AvrPto secretion using immunoblots. As before (Figure 3-1), secretion of AvrPto could be detected at low levels in culture filtrates of DC3000, but up to 50-fold higher levels were detected by densitometry in culture filtrates of the JB7 *lon* mutant (Figure 3-2). Ectopic expression of HrpL, however, did not affect AvrPto secretion from the wild-type cells or the *lon* null mutant as levels of AvrPto in culture filtrates of strains carrying a HrpL expression system were indistinguishable from their counterparts lacking the HrpL construct. Similarly, ectopic expression of HrpL did not affect the development of the HR in leaves of *Nicotiana tabacum* (Table 3-1) whereas the *Lon* mutant elicited a more rapid HR as reported previously [25]. The observation that ectopic expression of HrpL could not bypass the *Lon*-mediated repression of the *hrp* TTSS activity implied that *Lon* has a second activity in the regulation of the *hrp* TTSS.

Figure 3-2. Ectopic expression of hrpL does not bypass Lon regulation on the hrp cluster.

Levels of AvrPto were monitored in culture filtrates of DC3000 (wild-type), JB7 (*lon::Tn*) and a secretion mutant, A9 (*hrpA::Tn*) expressing *hrpL* from pMLL600, or carrying the empty vector pDSK600. Overnight cultures were diluted into M63 Fructose with 1% casein hydrolysate and grown to an OD₆₀₀ of 1.0. The cells were collected and washed in M63Fructose and diluted to an OD₆₀₀ of 0.6 in *hrp*-inductive media (M63Fructose, pH 5.5) and grown for 6 hours. The supernatants were concentrated 50X in centrifugal devices and ten µg of total protein from each sample were fractionated by electrophoresis in a 12% SDS-polyacrylamide gel. Levels of anti-AvrPto reactive proteins were monitored using immunoblots. AvrPto migrated at 18 kDa, and apparent levels were higher in the *lon* mutant.

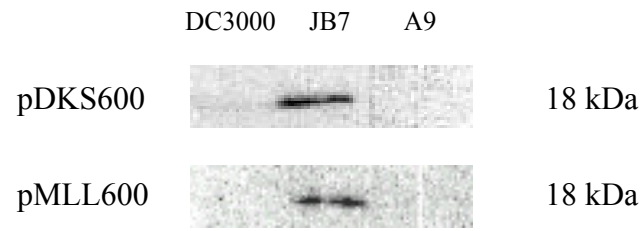


Table 3-1. HrpL overexpression does not affect the induced HR.

Time (h) ¹	DC3000, empty	DC3000 +
	vector ²	HrpL ²
2	nr	nr
4	nr	nr
6	nr	nr
14	++	++
16	++	++
18	+++	+++
20	+++	+++
22	+++	+++
24	++++	++++

1. Leaves of *Nicotiana tabacum* cv. Samsun were inoculated with a 10^8 cfu/ml suspension of DC3000 (empty vector) or DC3000 (pMLL600 expressing HrpL). The induced HR was measured every 2 hours for water soaking as evidence of initiation of HR, and then for tissue collapse and necrosis.
2. The symptoms were scored using the following scale: nr, no reaction; ++, water soaking; +++, tissue collapse; +++++, necrosis.

***P. syringae* effectors are subject to Lon degradation**

The role of Lon in the proteolysis of abnormally folded proteins [70] raised the possibility that Lon could directly target AvrPto and other TTSS effectors for degradation, since they are predicted to be in an unfolded state prior to secretion [60, 179]. The stability of AvrPto was compared in DC3000 (pMLAvrPto600) and JB7 (pMLAvrPto600) expressing AvrPto from the Lon-insensitive pDSK600 expression system described above. The amounts of AvrPto after inhibition of translation were measured by probing whole-cell extracts with anti-AvrPto sera. The stability of AvrPto in translationally inhibited cells appeared to be substantially higher in the *lon* mutant. The estimated half-life of AvrPto was 8 min in DC3000, but was greater than 30 min in JB7 (Figure 3-3). Notable differences in the initial levels of AvrPto were detected between Lon⁺ and Lon⁻ strains. Roughly five times more AvrPto could be detected in JB7 when compared to DC3000 (Figure 3-3). Since the rate of synthesis would be expected to be equivalent in the two strains, the difference in accumulation of AvrPto is likely attributable to faster degradation of AvrPto in the presence of Lon. In contrast, the levels of a similarly expressed bacterial alkaline phosphatase (BAP-FLAG) were indistinguishable between the two strains (Figure 3-3) and the half-life of BAP-FLAG was found to be greater than 30 minutes in either DC3000 or JB7 (Figure 3-3).

To assess whether other effectors might also be targets for Lon-mediated degradation, the stability of selected effectors was monitored under *hrp*-inducing

Figure 3-3. Effect of Lon protease on stability of effectors in *P. syringae*.

Levels of AvrPto, HopPsyA, AvrRpt2 and BAP-FLAG detected in immunoblots.

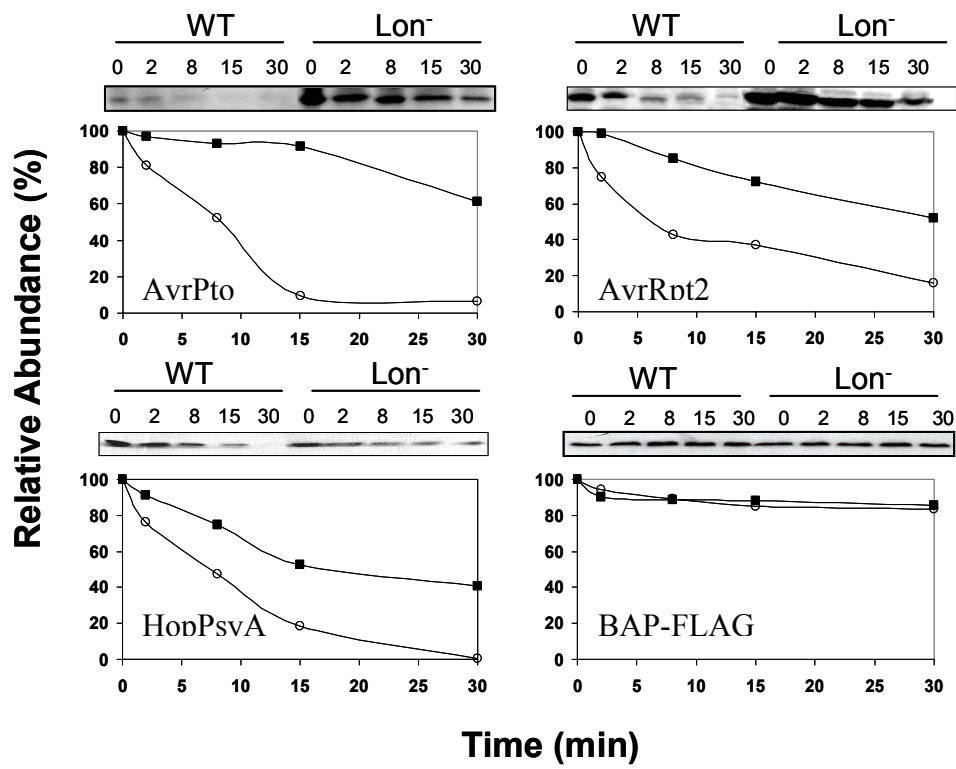
Overnight cultures of DC3000 (wild-type) or JB7 (*lon::Tn*) ectopically expressing the effectors were diluted into M63 fructose media and grown at 25° C to an OD₆₀₀ of 0.6.

Translation was stopped by the addition of tetracycline to a final concentration of 200 µg/ml, and 250 µl samples were taken at the times (min) indicated above. The cells

were collected by centrifugation and resuspended in 50 µl of SDS-PAGE loading buffer to lyse the cells. Whole-cell lysates were separated in a 12% SDS-PAGE, and immunoprobed with anti-AvrPto, anti-AvrRpt2, anti-HopPsyA or anti-FLAG sera.

Levels of effectors were estimated in scanned images using NIH Image 1.59 and normalized to total cells. The abundance of effectors (DC3000, open circles; JB7, closed squares) was calculated relative to their initial amounts at time 0 min.

Estimated levels from single experiment are shown, but similar results were obtained in at least 3 additional experiments.



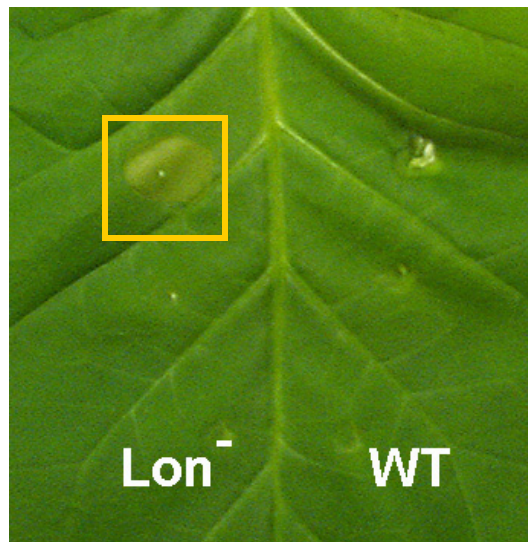
conditions known to enhance expression of effectors. Using polyclonal antibodies raised against the purified polypeptides (gifts of S. Heu, and J. Greenberg, respectively), HopPsyA from Psy61 and AvrRpt2 from *P. syringae* pv. maculicola ES4326 were also found to be unstable in Lon⁺ *P. syringae* cells. As with AvrPto, the initial levels of both HopPsyA and AvrRpt2 appeared to be 2-5 times higher in the Lon⁻ mutants than in wild type strains (Figure 3-3) and the observed half-lives of both effectors were considerably longer in the *lon* null mutant. HopPsyA exhibited an apparent half-life of 6 min in the wild-type strains that increased to >30 min in the Δlon mutant. Similarly, the half-life of AvrRpt2 increased 3-fold in the Δlon mutant.

Lon modulates *hrp* TTSS in an *E. coli* background.

Previous studies had shown that *E. coli* MC4100 expressing a the *hrp* cluster carried by pHIR11 could elicit effector-dependent responses in tobacco leaves [85], and therefore, assemble a functional TTSS [6]. Because a *P. syringae lon* allele could complement a mutation in its *E. coli* counterpart [25], inactivation of *lon* in *E. coli* should modulate effector levels and stability similarly to that observed in *P. syringae*. The MC4100 derivatives SG22622 (wt) and SG22623 (Δlon) were transformed with pHIR11 and their ability to elicit an effector-dependent response in tobacco plants was tested. As observed in *P. syringae*, Lon⁻ SG22623(pHIR11) elicited a visible programmed cell death response in 14 hrs, whereas Lon⁺ SG22622(pHIR11) took over 30 hrs to produce a similar response (Figure 3-4). These results suggest that the role of Lon in the regulation of the *hrp* TTSS is similar in *E. coli*.

Figure 3-4. *E. coli* Δlon mutants induce HR in half the time as wild-type.

Overnight cultures of *E. coli* SG22622 (wild-type) and SG22623 (Δlon) carrying pHIR11 (cosmid clone expressing a functional *hrp* cluster) were diluted in M63 Fructose with 1% casein hydrolysate and grown at 37 °C to an OD₆₀₀ of 1.0. The cells were collected and washed with fresh media, and resuspended to 10⁹ cfu/ml. *Nicotiana tabacum* cv Samsun leaves were infiltrated with roughly 100 µl of the suspension and scored for visible signs of a hypersensitive response (HR) during the first 30 h. Note that *lon* mutants could induce the HR as indicated by water soaking and tissue collapse by 14 h (highlighted area), whereas wild-type required greater than 30 h to produce a similar response. The picture was taken 20 h post-infiltration.



To determine if inactivation of *lon* also caused enhanced secretion of effectors from the *E. coli* transformants, levels of ectopically-expressed AvrPto, AvrRpt2 and HopPsyA were monitored in culture filtrates. The effectors could be detected at low levels in culture filtrates of Lon⁺ SG22622 (pHIR11) transformants (Figure 3-5). AvrPto, AvrRpt2 and HopPsyA, however, were detected in culture filtrates of the Lon⁻ SG22623 (pHIR11) and were not obvious in culture filtrates of SG22623 (pLAFR3), a vector control. These results suggest that the role of Lon in the regulation of effector secretion is similar in the *E. coli* and *P. syringae* systems.

Lon affects stability of effectors in *E. coli*

To assess whether Lon plays a role in the stability of effectors in *E. coli*, the half-lives of AvrPto, HopPsyA, and AvrRpt2 were determined in translationally-inhibited Lon⁺ SG22622 and Lon⁻ SG22623 as before. For all three of these effectors, a 2- to 5- fold increase in their observed half-life was detected in the *lon* null mutant as observed in *P. syringae* (Figure 3-6). However, similar to what occurred in *P. syringae*, the initial amounts of the polypeptides were significantly lower in the wild-type than the *lon* mutant cells. The abundance of effectors in the wild-type cells was between 20 and 40% of that observed in the Δlon cells (Figure 3-6).

To determine if the stability of effectors from other *P. syringae* strains was also affected by Lon, HopPtoG from *P. syringae* pv. tomato 5846 and HopPsyE1 from *P.*

Figure 3-5. *P. syringae* effectors are secreted from an *E. coli lon* background

Levels of effectors secreted from *E. coli* in immunoblots. AvrPto, HopPsyA, and AvrRpt2 were ectopically expressed from pDSK600, pYXSS or pDSK519 in *E. coli* SG22622 (wild-type) or SG22623 (*Δlon*) carrying either pLAFR3 or pHIR11 expressing a functional *hrp* TTSS. Overnight cultures were diluted into M63 Fructose with 1% casein and cells were grown 5 h at 37°C. The supernatants were concentrated as in Figure 3-1 and protein levels determined using the micrBSA protein assay kit (Pierce, Ca). Ten μg of total protein were loaded and separated in an 12% SDS-PAGE and anti-AvrPto, anti-HopPsyA or anti-AvrRpt2 reactive proteins were monitored using immunoblots. AvrPto migrated at 18 kDa, HopPsyA migrated at 39 kDa, and AvrRpt2 migrated at 28 kDa.

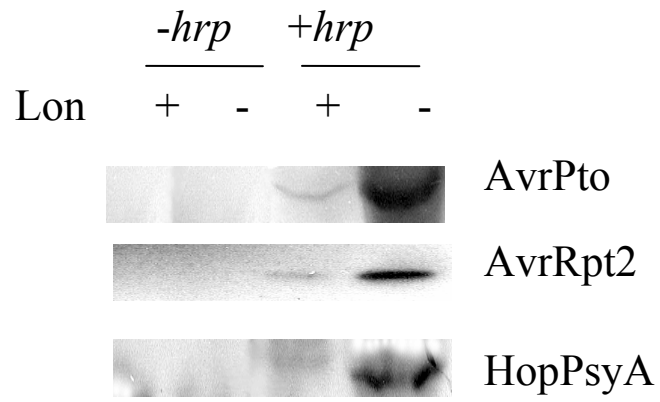
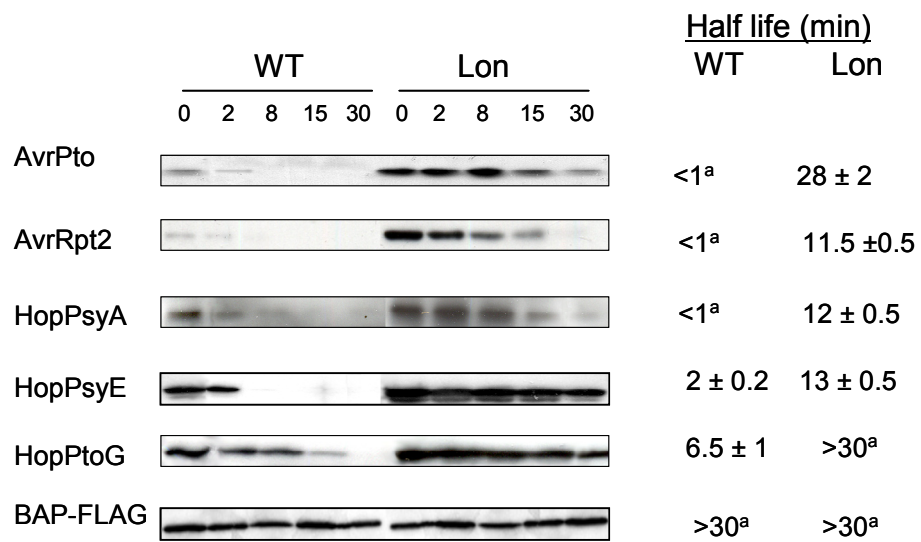


Figure 3-6. Lon affects the stability of *P. syringae* effectors in *E. coli*.

E. coli SG22622 (WT) or SG22623 (Lon⁻) carrying AvrPto, AvrRpt2 or HopPsyA expressed as in Figure 3-3, and HopPtoG, or HopPsyE expressed from pTrcHis2 were grown to an OD₆₀₀ of 0.6 and translation was stopped by the addition of excess chloramphenicol (200 µg ml⁻¹). Whole cell lysates were treated as in Figure 3-3, and the levels of effectors were monitored in immunoblots using NIH Image 1.59. The half lives were estimated in immunoblots using appropriate polyclonal or anti-His antibodies as in Figure 3-3. Equivalent results were obtained in three different experiments.



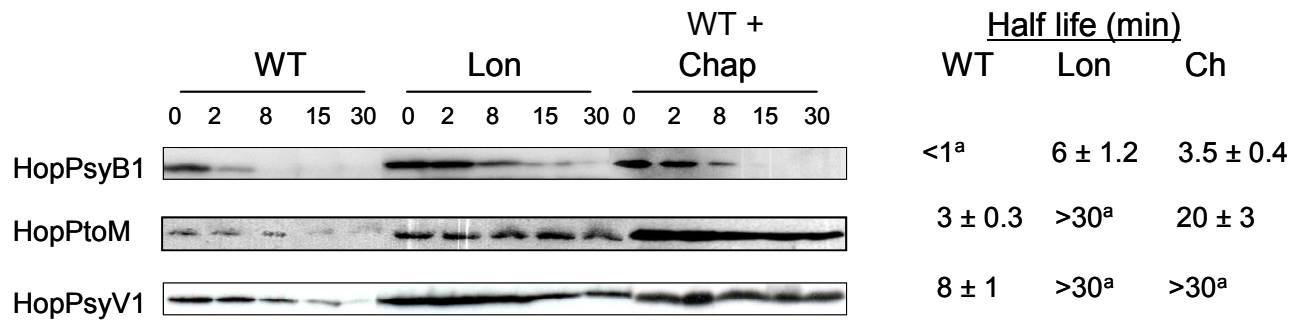
syringae pv *syringae* DH015 were cloned into pTrcHis2 to generate a C-terminal 6xHis epitope tag and the stability of each fusion was monitored in SG22622 and SG22623 using anti-His antibodies. A comparison between the half-life of native AvrPto and 6xHis-tagged AvrPto in *P. syringae* showed that the fusion did not alter the stability of AvrPto (8.5 vs. 9 min, respectively). In all cases, the measured stability of the epitope-tagged effector was 2-4-fold higher in Δlon mutants (Figure 3-6). These results suggest that effectors are generally susceptible to degradation by Lon prior to secretion, irrespective of the source and host strain. The effectors appeared to fall into two general categories of stability: effectors had half-lives shorter than 2 minutes, and those with half-lives of >6 minutes (Figure 3-6).

Chaperones stabilize effectors

To investigate the effect of chaperones on the stability of *P. syringae* effectors, the half-lives of HopPsyB1, HopPtoM and HopPsyV1 were determined in Lon⁺ SG22622 or Lon⁻ SG22623 in the presence or absence of their cognate chaperones, ShcB1, ShcM, and ShcV1, respectively. Each chaperone was cloned individually into pDSK519 such that expression was directed from the *lac* promoter and the construct was transformed into the *E. coli* strains carrying one of the previously mentioned effector constructs. In each case, the co-expression of the chaperone had a stabilizing effect on its cognate effector as expected. The $t_{1/2}$ of HopPsyB1 increased from under 1 min in SG22622 (pLLhopB1-Trc)(pDSK519) to 5 min in SG22622 (pLLhopB1-Trc)(pLLshcB-1D) (Figure 3-7). This effect was only observed in Lon⁺ SG22622. HopPsyV1 was also stabilized by its apparently dedicated chaperone. Expression of

Figure 3-7. Effectors are stabilized by their chaperone.

Estimation of half-lives of effectors in the presence of chaperones. Overnight cultures of *E. coli* ectopically expressing HopPsyB1, HopPsyV1, or HopPtoM carrying their cognate chaperone (Chap) or empty vector in SG22622(WT) or SG22623 (Lon) were diluted and grown in LB at 37°C until they reached an OD₆₀₀ of 0.6 and the half life of each effector was estimated as in Figure 3-3. The results are representative of three experiments.



ShcV1 increased the half-life of HopPsyV1 to >30 min which is comparable to its $t_{0.5}$ in Lon⁻ SG22623. Likewise, the half-life of HopPtoM increased from 5 min in the absence of its chaperone to >20 minutes when ShcM was expressed (Figure 3-7). The effects of these chaperones were specific, as expression of a heterologous chaperone did not affect the stability of the effectors HopPsyB1 or HopPsyV1 (Figure 3-7). The results suggest that a primary role for chaperones in TTSS could be protection of effectors from Lon-mediated degradation.

The Lon-Targeting Domain of HopPtoM Does Not Overlap Its Chaperone-Binding Domain

The manner in which chaperones protect effectors from degradation is unknown. Other Lon-degraded proteins, such as SulA and UmuD, are targeted to Lon by specific amino or carboxy terminal motifs [68, 95]. It is possible that a TTSS chaperone protects its cognate effector by masking a Lon-targeting motif. Alternatively, the chaperone could be directly inhibiting Lon activity or forming a complex with the effector that is immune to degradation. To test the possibility that the chaperone directly inhibits Lon, the activity of RcsA was monitored in strains expressing the chaperones ShcB1, ShcV1 or ShcM from pDSK519. RcsA is a transcriptional activator that regulates the expression of *cpsB*, and whose activity is regulated by Lon [72]. A chromosomal *cpsB::lacZ* reporter that is only expressed when RcsA is not degraded by Lon was used to monitor Lon activity. Consistent with the specificity of activity for the chaperones, none of the chaperones had an effect on the Lac phenotype of the indicator strains, indicating that the chaperones do

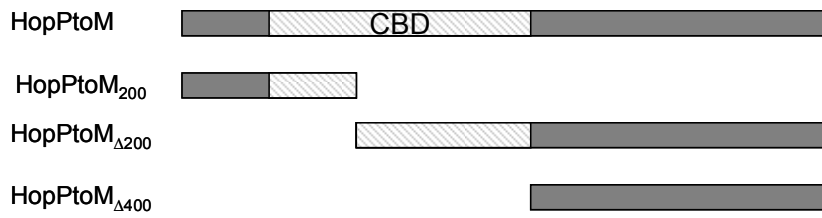
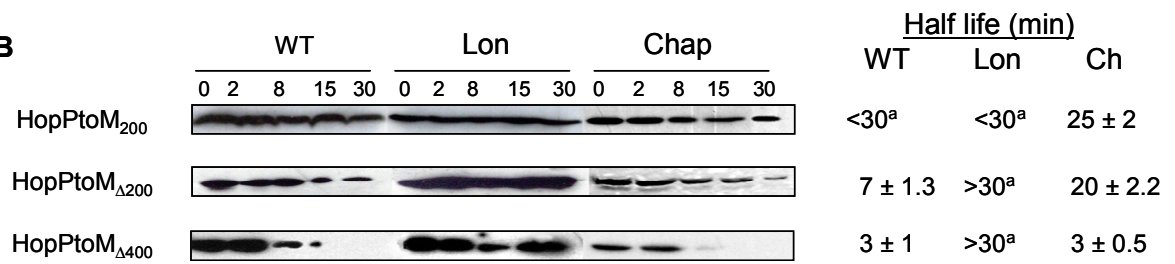
not generally inhibit Lon activity.

The apparent chaperone-binding domain (CBD) of HopPtoM had previously been localized to an internal 300 aa domain located between residues 100-400 [14]. To determine if the Lon-targeting domain of HopPtoM overlaps this apparent CBD, His-tagged derivatives of HopPtoM were created that carried either amino or carboxy terminal deletions that mirrored those used in the previous analyses (Figure 3-8a). One of the constructs ($\Delta 400$) contained the fragment encoding the polypeptide domains that did not interact with ShcM, whereas the other two constructs ($\Delta 200$ and N200) contained coding sequence for regions that did interact [14]. When the constructs were expressed in Lon⁺ cells, the constructs expressing the C-terminus of the peptide were degraded (Figure 3-8b). Among these, the $\Delta 400$ truncation lacking any of the CBD exhibited a short half-life (3 min). In contrast, the construct that expressed only the N-terminus, N200, which includes a portion of the CBD, was not degraded by Lon as the half-lives were equal in both strains (>30 min)(Figure 3-8b). These results suggest that the Lon-targeting domain of HopPtoM is located in the C-terminal 312 amino acids, and does not overlap with the CBD.

To verify that the C-terminus of HopPtoM could target the peptide for Lon-mediated degradation, a fusion between HopPtoM $_{\Delta 400}$ and the maltose binding protein (MBP) was constructed and tested for sensitivity to Lon. MBP has previously been shown to be immune to degradation by Lon, and a similar assay was used before to determine

Figure 3-8. The C-terminus of HopPtoM targets the peptide for degradation.

A. Schematic diagram of HopPtoM truncations used in this study. CBD, chaperone binding domain. B. N-terminal truncations lacking the first 200 or 400 amino acids (HopPtoM $_{\Delta 200}$, and HopPtoM $_{\Delta 400}$, respectively) or a C-terminal truncation expressing only the first 200 amino acids (HopPtoM $_{200}$) were expressed in SG22622 (WT) and SG22623 (Lon) or SG22622 carrying pShcMD (Chap). The half lives of each derivative were estimated as in Figure 3-3 using polyclonal antibodies raised against HopPtoM. Equivalent results were obtained in three different experiments. The reduced levels of HopPtoM $_{\Delta 400}$ observed in the 8 minute sample from the *lon* mutant was not observed in the other replicates.

A**B**

the Lon-targeting motif in Sula [95]. A MBP:LacZ fusion was expressed in SG22622 and SG22623 and its half-life determined to be roughly 35 minutes in both backgrounds (Figure 3-9). In contrast, when HopPtoM $_{\Delta 400}$ was fused to MBP, it was degraded in the wild type background with a half-life of 8.5 min (Figure 3-9). This construct was only slowly degraded in the Δlon mutant background as its half-life was 24 minutes. Taken together these results support the hypothesis that the Lon-targeting domain of HopPtoM is located in the C-terminus, and that it is sufficient to target polypeptides for Lon-mediated degradation.

Discussion

Lon has been previously shown to regulate assembly of the hrp-encoded TTSS in *P. syringae* strains through its effects on the activity of the *hrpL* promoter [25]. Regulated proteolysis of the transcriptional activator HrpR controlled the activity of the *hrpL* promoter, thereby modulating expression of the Hrp regulon. Interestingly, the *lon* mutants also secreted several effectors consistent with a TTS⁺ phenotype that could not be reproduced by ectopic expression of *hrpRS* or *hrpL*. This suggested that Lon had an additional activity in the regulation of *hrp* TTSS. Consistent with this hypothesis, eight distinct effectors isolated from several *P. syringae* strains were found to be unstable in Lon⁺ *P. syringae* and *E. coli* strains, but were relatively stable in the corresponding Lon⁻ mutants. Targeting domains for Lon-mediated degradation were identified in two effectors and chaperones were shown to suppress

Figure 3-9. An MBP fusion to the C-terminus of HopPtoM is degraded.

A The $\Delta 400$ or N200 truncations of HopPtoM (Figure 3-8) were amplified by PCR using specific primers and used to create fusions with the C-terminus of MBP in the vector pMAL-p2x (New England Biolabs) following manufacturer's instructions. Cells expressing any of MBP::LacZ or the fusion proteins were induced for 2.5 h and translation was stopped using chloramphenicol (200 $\mu\text{g}/\text{mL}$) and levels of the fusions estimated in 250 μl samples at the indicated times. The relative amounts of the fusion proteins were monitored in immunoblots using anti-MBP antibodies, and the half lives were estimated from densitometry analyses.

A



B



Half life (min)

>30

8.5 ± 1.2

>30

the degradation of the cognate effector in Lon⁺ cells. Thus it appears that Lon has a dual function in the regulation of the *hrp* TTSS of *P. syringae* strains by: 1) regulating the assembly of the TTSS and expression of effectors through regulated proteolysis of HrpR [25]; and 2) controlling the accumulation of effectors prior to secretion through turnover.

Lon belongs to a family of cytosolic ATP-dependent proteases that are highly conserved among prokaryotes and Archea and are also found in eukaryotic organelles (reviewed in [195]). Like other bacterial energy-dependent proteases, Lon forms a multimeric complex that couples ATP-dependent protein unfolding with an endopeptidase activity [190] [195] to rapidly degrade targeted proteins. Each of these activities is assigned to independent domains separated by a “sensor and substrate-discrimination” domain (SSD) which functions in substrate selection. Each of the functional domains is conserved in the *P. syringae* Lon homolog and the *P. syringae* Lon can complement its *E. coli* counterpart [25]. *P. syringae lon::Tn* mutants exhibited enhanced cell length and UV sensitivity similar to Lon null mutants of other bacteria. Thus, *P. syringae* Lon likely functions similarly to its homologs of other bacteria.

Lon is well known for its involvement in the degradation of unstable regulatory proteins, such as FlhC/FlhD, SulA and RcsA/RcsB [36, 71, 181]. Another principal activity of Lon is the degradation of misfolded or abnormal proteins, such as those that occur due to the incorporation of irregular amino acids or temperature-sensitive

mutations [70]. Because TTSS-linked effectors are predicted to be in an unfolded state prior to secretion [59, 156, 179], they would be strong candidates for degradation by proteases such as Lon. Among the effectors studied, five had apparent half-lives of less than 2 min in Lon⁺ strains whereas three others had apparent half-lives longer than 6 min. Consistent with the observed degradation rates, steady state levels of effectors expressed from a *lacUV5* promoter were 20 to 50% of the levels detected in Lon⁻ mutants. Since levels of LacZ expressed from the same promoter were indistinguishable between Lon⁺ and Lon⁻ cells, the rate of synthesis for these ectopically-expressed effectors is likely to be equivalent in Lon⁺ and Lon⁻ cells. The differences in the steady state levels, thus, are consistent with the observed differences in the rate of decay. The instability of the tested effectors in Lon⁺ *P. syringae* and *E. coli* cells and the absence of proteolytic degradation products in immunoblots suggest the direct degradation of effectors by Lon.

TTSS chaperones are usually small acidic proteins that can interact with one or more effectors. In the absence of their cognate chaperones, some effectors are not readily secreted, and consequently chaperones have been proposed to act either as secretion pilots or as factors that maintain effectors in a “secretion-competent” state [59, 156]. For example, the crystal structure of the *Salmonella* effector SptP interacting with its chaperone SycP revealed that SptP was maintained in an “unfolded” state in which the effector is wrapped around the chaperone [179]. A similar structure was resolved for the YopE-SycE complex [20]. Another proposed role of chaperones has been to block enzymatic or toxic activity of effectors within the bacterial cytoplasm. For

example, binding of the chaperone SycD neutralizes the intracellular toxicity of YopB when expressed in *E. coli* [150]. More recently, chaperones have been proposed to control the timing of effector secretion as mentioned above. This activity was first identified for SicA, the *Salmonella* chaperone for SopA, which interacts with the TTSS transcriptional activator InvF to promote the expression of virulence factors [186]. In addition, other chaperones, such as FlgN, can control the expression of class III flagellar genes by activating the secretion of FlgM [105]. The *Yersinia* chaperone SycD/LcrH can interact with YopD to regulate the expression of YopQ [8], and IpgC, the chaperone for IpaB and IpaC, can bind to MxiE and activate the transcription of at least two effectors in *Shigella* [135].

Our results are consistent with other studies proposing that chaperones act as stabilizing agents for their effectors. In *Yersinia* and *Salmonella* strains, cytoplasmic levels of some effectors have been higher in the presence of their cognate chaperone consistent with protection from proteolytic degradation [63, 64]. Lon-mediated degradation of the tested effectors could be suppressed by the cognate chaperone. The effect was specific to a chaperone-effector interaction as only the cognate chaperone stabilized the effector. It is likely, then, that one of the major roles for chaperones is in fact to protect effectors from Lon-mediated degradation. Consistent with this hypothesis, the chaperones had little effect on the half-lives of effectors in strains lacking Lon. In contrast to many effectors of mammalian pathogens, most effectors expressed by *P. syringae* strains do not have an obvious cognate chaperone. In *P. syringae* DC3000, only 7 out of 60 postulated effectors have been associated

with a chaperone. Why some effectors have a chaperone, and most do not is an enigma.

The mechanism by which *P. syringae* chaperones protect effectors from Lon-mediated degradation does not appear to be due to the masking of the targeting motif in the case of HopPtoM. ShcM has been reported to interact with an internal domain of HopPtoM located between residues 100-300 [14] whereas the Lon-targeting domain in HopPtoM was shown here to be located in the carboxy terminal domain and does not overlap with the previously established chaperone binding domain. Most likely, this chaperone-effector complex has a Lon-insensitive conformation. Crystal structures of other chaperone-effector interactions have revealed a high degree of secondary structure [19, 57, 179]. Since unfolding of the substrate is an essential part of Lon proteolysis [190], the formation of a stable complex structure with the chaperone would inhibit its degradation by Lon.

It is becoming increasingly clear that, at least in mammalian pathogens, chaperones play a crucial role in determining the hierarchy of effector secretion [59, 156, 198]. Whether the chaperones in *P. syringae* also have a similar role is an interesting question. Differential degradation of effectors prior to secretion, however, could provide a mechanism for controlling the hierarchy of effector secretion. Since suppression of proteolysis through inactivation of Lon increased the secretion of effectors from the *hrp* TTS, it appears that proteolytic degradation of effector precursors is rate limiting to effector secretion (but other effects on the secretion

system itself [22] can not be completely excluded). Effectors with longer half-lives are likely to be secreted at higher levels than those with short half-lives and would accumulate more rapidly in host cells.

In conclusion, we found that Lon plays an important role not only in the regulation of assembly of the TTSS, but also in controlling the secretion of effectors through that system. The impact of Lon on effector stability may not be unique to *P. syringae* effectors. A stabilizing effect of chaperones has been reported in other systems [63] [34] [64]. YopE and SptP, for example, are readily detectable in the presence of their corresponding chaperones, SycE and SicP respectively, but only a small percentage of that amount is present in the absence of the chaperone [34] [64]. The mechanism for effector degradation and chaperone stabilization has not been established in these systems but seems likely to be due to Lon-mediated degradation. Lon has been shown to influence the assembly and activity of the SPI TTSS of Salmonella [22, 182]. Here we propose a model in which effectors would have two distinct fates in the bacterial cell: secretion through the TTSS, or degradation by Lon that is most consistent with the post-translational mechanism for secretion of effectors by the TTSS. Differential turnover of effectors could provide a mechanism for the hierarchical secretion of effectors.

Materials and Methods

Bacterial Stains and Media.

Strains and plasmids used in this study are described in Table 3-2. Bacteria were routinely grown on King's B medium [12]. Plasmids were propagated in *E. coli* DH5 α . *E. coli* strains were grown at 37°C, and *Pseudomonas syringae* strains were grown at 25°C. LB and M63 minimal salts media were used as described previously [167]. M63 medium was supplemented with 1mM MgSO₄ and 1% fructose (M63F). The following antibiotics were added where needed at the indicated concentrations (in micrograms per milliliter): ampicillin, 200; kanamycin, 50; spectinomycin, 100, tetracycline, 25; nalidixic acid, 50; rifampicin, 200; and chloramphenicol; 30.

General DNA manipulations.

Restriction enzymes were purchased from Invitrogen (Bethesda, MD). T4 DNA ligase was acquired from New England Biolabs (Beverly, MA) and used according to the manufacturer's recommendations. Basic manipulations were done using standard procedures. PCRs were performed using a PCR Sprint thermal cycler (Hybaid, Ashford, UK) with 50 μ l reaction volumes. Unless indicated otherwise, *ProofPro* polymerase (Continental Lab Products, San Diego) was used to amplify fragments for cloning.

Table 3-2. Strains, plasmids and primers used in this study.

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
<i>E. coli</i>		
DH5 α	<i>endA1 hsdR17 (r_k- m_k-) supE44 thi-1 recA1 gyrA96 relA1 Δ(argR-lacZYA) U169ϕ80lacZDM15</i>	Invitrogen
SG22622	<i>cpsB::lacZ Δara malP::lacI^f</i>	S. Gottesman
SG22623	SG22622 Δ lon-510	S. Gottesman
SLR400	<i>araD139 Δ(ara leu)7697</i> derivative of MC4100	S. Benson
TOP10	F ⁻ <i>mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔ M15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG</i>	Invitrogen
<i>Pseudomonas syringae</i>		
A9	DC3000 <i>hrpA</i> mutant, HR ⁻ , Rif ^r , Kan ^r	[194]
DC3000	Wild-type, Rif ^r , HR ⁺ Tomato and <i>Arabidopsis</i> pathogen	[46]
JB7	DC3000 <i>lon::Tn</i> , Rif ^r Kan ^r	[25]

Psy61	Wild-type, Nal ^r , HR ⁺ Weak bean pathogen	[15]
Psy61-KL11	Nal ^r Kan ^r , <i>lon</i> ::Tn	[25]
Plasmids		
pAvrRpt2D	1.0 kb PCR product containing <i>avrRpt2</i> cloned into pDSK519	This work
pDSK519	Broad-host range vector, IncQ Kan ^r	[107]
pDSK600	Broad-host range vector, IncQ Sp ^r	[147]
pFLAG-CTS-BAP	Bacterial Alkaline Phosphatase in pFLAG- CTC	Sigma-Aldrich
pHIR11	pLAFR3 derivative carrying <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrp/hrc</i> cluster, Tc ^r	[85]
pHopPsyB1trc	<i>hopPsyB1</i> cloned into pTrcHis2	This work
pHopPsyEtrc	<i>hopPsyE</i> cloned into pTrcHis2	This work
pHopPtoGtrc	<i>hopPtoG</i> cloned into pTrcHis2	This work
pHopPtoMtrc	<i>hopPtoM</i> cloned into pTrcHis2	This work
pHopPtoM _{Δ400} trc	C-terminal 1836 bp of <i>hopPtoM</i> cloned into pTrcHis2	This work
pHopPtoM _{Δ200} trc	C-terminal 1536 bp of <i>hopPtoM</i> cloned into pTrcHis2	This work
pHopPtoM ₂₀₀ trc	N-terminal 600 bp of <i>hopPtoM</i> cloned into pTrcHis2	This work

pHopPtoM Δ 400MAL	MBP fusion to the C-terminal 1836 bp of <i>hopPtoM</i> in pMAL-p2x	This work
pHopPsyV1trc	<i>hopPsyV1</i> cloned into pTrcHis2	This work
pLAFR3	Tc ^r , IncP1	[177]
pLLlac600	HindIII-PstI fragment from pRG970, subcloned into pDSK519 and then the Lac operon excised with EcoRI and cloned into pDSK600 , Sp ^r , Lac+	This work
pMAL-p2x	MBP:: <i>lacZ</i> fusion	New England Biolabs
pMLAvrPto600	0.45 kb PCR cloned as <i>EcoR1-HindIII</i> into pDSK600	[25]
pMLL600	0.4-kb PCR product containing <i>hrpL</i> cloned into pDSK600	This work
pSGAS	3.6-kb fragment containing <i>hopPsyA</i> in pYXSS	[80]
pSHAMB	<i>hopPsyA</i> cloned into pMLB1034	[80]
pShcB1D	0.5 kb PCR product cloned into pDSK519	This work
pShcMD	0.5 kb PCR product cloned into pDSK519	This work
pShcV1D	0.5 kb PCR product cloned into pDSK519	This work

Construction of 6xHis protein fusions.

The genes for individual effectors were amplified from chromosomal DNA from the indicated strain using the following primers: *hopPsyB1* (Psy B5) ATGAACCCGATACAAACG; TTCCAACCTGAATGCCGG, *hopPtoM* (Pto DC3000) ATGATCAGTTCGCGGATC; ACGCGGGTCAAGCAAGCC, *hopPsyE1* (Psy W4N15) ATGAGACCTGTCGGTGGG; GACCTTATAAGACAGGAC, *hopPsyV1* (Psy B728a) ATGAATATCTCAGGTCCG; AGGCTTGCCCCGGACCCT, and *hopPtoG* (Pto DH015) ATGAGACCCGTCGGTGGA; ATCAGCGCCAACAATCGG, The products were cloned into pTrcHis2 using the TOPO TA cloning kit (Invitrogen, Carlsbad) following manufactures instructions. The fusion was verified by sequencing and by immunodetection as described by the manufacturer.

Cloning of effector chaperones.

shcPsyB1 and *shcPsyV1* were amplified from genomic DNA using the following primers: ; GCTCTAGACAGTTCGGGATTGACAGG; CGGAATTCGACAGACGCTGGAATACGG; and CTCTAGAACTGGACATGACGCTGGA; GCTCTAGAATCGAATAGTCCCCGCCA, respectively. The PCR products were cloned into pDSK519 a *XbaI* or *BamHI* fragments. *shcPtoM* was cloned into pTrcHis2 using the primers ATGACCAACAATGACCAG and CTGGAATCTCCCAGGAGC, and subsequently amplified using the primers GCTCTAGAGAATAAACCATGGCCCTT and

GCTCTAGAGATTTAATCTGTATCAGG to clone into pDSK519 or pBAD33 as an *Xba*I fragment. Inserts were verified by restriction analysis and sequencing.

Cloning of HopPtoM truncations.

N-terminal truncations of HopPtoM were amplified using the forward primers M Δ 25 CAGCACGATACTGTTCCC, M Δ 200 GCCGGTCGTGCAAGCAAG, M Δ 400 CTGAAAAGCGAACACGGT, and the reverse primer ACGCGGGTCAAGCAAGCC. C-terminal truncations were generated using the forward primer ATGATCAGTTCGCGGATC, and the reverse primers M100aa ACTACCGATCAACAAGCG, and M200aa GTATTCGCCAAGGGCAGT. All amplification products were cloned into pTrcHis2 and inserts were verified by sequencing and by immunodetection following manufacturer's directions.

Maltose binding protein (MBP) fusions.

The 3' 936 bp of *hopPtoM* were amplified using the primers GCTCTAGATTAACGCGGGTCAAGCAA and GCTCTAGAAAAAGCGAACACGGTGAG, digested with *Xba*I and ligated to pMAL-p2x (New England Biolabs, Beverly MA) to create a C-terminal fusion to MBP following manufacturer's instructions. The 5' 150 bp, and the 3' XX bp of *hopPsyB1* were amplified using the primers GCTCTAGATTCCAACCTGAATGCCGG; GCTCTAGACCGCAGGACAGGACAAGC, digested with *Xba*I and cloned into pMAL-p2x.

β-galactosidase assays.

β-galactosidase activity in bacterial cells was estimated by the procedures of Miller [139].

Plant assays.

P. syringae DC3000, JB7 and A9 were transformed with pMLL600 or pDSK600. Overnight cultures grown at 25 C were harvested and diluted in M63 minimal media. *Nicotiana tabacum* cv Samsun leaves were syringe infiltrated with a 10⁸ cfu/ml solution of the different strains as described previously [85]. Infiltrated leaf panels were scored for responses beginning 2 h after inoculation and monitored for 24 h. For *E. coli*, SG22622 and SG22623 were transformed with pHIR11, pHIR11-2082, or pLAFR3 and used to inoculate *N. tabacum* plants in the same way as *P. syringae*.

Secretion of effectors from bacterial cultures.

Culture supernatants were collected as described previously [25]. Briefly, *P. syringae* DC3000, JB7 and A9 carrying pMLL600 or pDSK600 cells were grown in KB overnight. Cells were harvested, diluted into fresh KB medium containing selecting antibiotics and 2mM IPTG and grown to an OD₆₀₀ 0.6. Cells were harvested, washed once with M63 and transferred to 50 mL M63F, at an OD₆₀₀ of 0.6. After 6 hours, cells in a 500μL sample were harvested and cell pellets were resuspended in SDS-PAGE sample buffer for analysis of proteins in whole cell lysates. An identical sample was collected and it was resuspended in 10% SDS to calculate protein concentrations. Culture filtrates of the remaining culture was obtained by

centrifugation and concentrated 50X using MilliPore Ultra-free centrifugal filter devices with a 5 KDa exclusion limit. Total protein concentration in whole cell lysates was measured using the BCA Total Protein Assay kit (Pierce, Rockford). Total protein concentration in supernatant samples were measured using the MicroBCA Assay Kit (Pierce).

E. coli SG22622 or SG22623 carrying pHIR11 or pLAFR3 and ectopically expressing AvrPto or HopPsyA from pDKS600 or pYXSS, respectively, were grown overnight in KB medium with proper antibiotics. One ml of overnight culture was used to inoculate 50 ml of M63 media containing fructose and 1% casein hydrolysate and cultures were grown to an OD₆₀₀ of 1.0. Supernatants and whole cell lysates were collected in the same manner as *P. syringae*.

Immunoblots.

Ten µg of total protein from every sample were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was carried out using a polyclonal antibody raised against AvrPto, AvrRpt2, HopPsyA or HopPtoM at 1:3000 dilution. Commercial antibodies, anti-His (Novagen, San Diego), anti-FLAG (Sigma, St. Louis), anti-MBP (New England Biolabs, Beverly) and secondary antibodies conjugated to horse radish peroxidase were used following manufacturer's recommendations. Cross-reactive proteins were visualized using the ECL chemiluminescence kit (Amersham-Pharmacia, Piscataway).

Stability of Effectors.

Overnight cultures of *P. syringae* strains expressing AvrPto, HopPsyA or AvrRpt2 were diluted into M63 media containing fructose as the carbon source to an OD₆₀₀ of 0.6 and incubated for 4 h at 25°C. After incubation, tetracycline (200 µg ml⁻¹) was added to inhibit translation. Cells were harvested at specified time points, lysed in sample buffer and fractionated in 12% SDS-PAGE gels. For *E. coli*, SG22622 or SG22623 were grown overnight at 37°C, diluted into fresh M63F containing 1 % casein hydrolysate and grown to an OD₆₀₀ of 0.6. After incubation, chloramphenicol (200 µg ml⁻¹) was added and samples were collected as in *P. syringae*. The half-life effectors were calculated from the exponential decay in levels estimated in scanned images using NIH Image 1.59. Each experiment was repeated at least three times.

Chapter 4: Identification of a generalized stabilizing factor within the *hrp* cluster

Introduction

Although the general mechanism for assembly of the TTSS is considered to be conserved in both mammalian and plant pathogens, secretion through *P. syringae* TTSS is considerably different from that in mammalian pathogens. Firstly, the *hrp* TTSS appears to regulate secretion more tightly than in mammalian pathogens. For example, levels of effectors in supernatants from *P. syringae* cultures appear to be 10 to 100-fold lower than observed in cultures from mammalian pathogens. In addition, secretion of reporter constructs used in mammalian pathogens has proven very difficult in *P. syringae*. Secondly, it has been proposed that *P. syringae* produces significantly more effectors than mammalian pathogens [39, 43, 62, 73]. Lastly, a majority of the effectors from *P. syringae* are not associated with a chaperone, whereas in mammalian pathogens more than half of the effectors have a dedicated chaperone. So far, only three chaperones have been described for *P. syringae* [14, 189, 193] and a total of four more have been postulated according to their localization and characteristics [62, 76].

The exact role of TTSS chaperones remains elusive. By binding to their respective effector, chaperones can sequester and protect them from proteolysis, resulting in higher levels of effectors in the cytoplasm [63, 64]. It has been postulated that the three-dimensional structure of the chaperone/substrate complex is recognized

by the TTSS [20] to trigger the secretion of an effector. Most chaperones have been defined because they are either necessary for secretion of their specific effectors, physically interact with the effectors, or they are involved in stabilizing effectors in the cytoplasm [34]; [64, 137, 141]. Chaperones have also been implicated in the regulation of transcription of effector genes by acting as either activators or by preventing transcriptional repression [49, 104, 126]. Taken together, these data suggest that chaperones can be involved not only in stabilizing and targeting effectors to the translocation complex, but may also play an important role in controlling which effectors are produced and secreted.

We observed previously that effectors were degraded by Lon protease, and that chaperones had the ability to protect effectors from this degradation. We proposed that the differential stability of effectors in *P. syringae* could be a method for determining hierarchical secretion of effectors (See Chapter 3). In our model, chaperones would permit the rapid accumulation of an effector in the cytoplasm, and in this manner aid secretion. Considering the important roles of chaperones, it is incongruous that *P. syringae* only contains 7 chaperones for its 60 known or candidate effectors [39, 73]. It is possible that not all the chaperones carried in the *P. syringae* genome have been identified. However, it is important to note that the genes for most chaperones in *P. syringae* and other species are found adjacent or in close proximity with their corresponding effector [14, 33, 59, 156, 189, 193]. None of the candidate effectors are adjacent to ORFs that exhibit the expected properties of a TTSS chaperone [59, 62, 76, 156, 206]. In addition, it would be expected that a *hrp*

chaperone would be coordinately regulated by HrpL along with the effectors. And yet, none of the candidate proteins found in the extensive studies of HrpL-dependent genes from DC3000 [62, 76, 206] have the predicted chaperone characteristics either.

It is possible, then, that in *P. syringae* an alternative to individualized chaperones to stabilize effectors evolved. One alternative is that each of the seven characterized chaperones interacts with a large number (> 8) of effectors. Many TTSS chaperones in mammalian pathogens can interact with more than one substrate [148, 149, 155, 192], although interaction has been limited to two effectors. A serendipitous observation from the results of experiments carried out for Chapter 3, showed that the stability of effectors was also affected by the presence or expression of the *hrp* cluster. These experiments gave birth to the hypothesis that there exists a factor within the *hrp* cluster that is involved in enhancing effector stability, and could therefore be a candidate for a generalized chaperone, as opposed to the specific chaperones currently accepted. This generalized chaperone could function analogously to individual chaperones of TTSS in mammalian pathogens, and could be an adaptation to the large number of effectors necessary for virulence on plant hosts for *P. syringae*. This study aimed to test the existence of a generalized chaperone in *P. syringae*, whether this factor fits into the description and roles of known chaperones.

Results

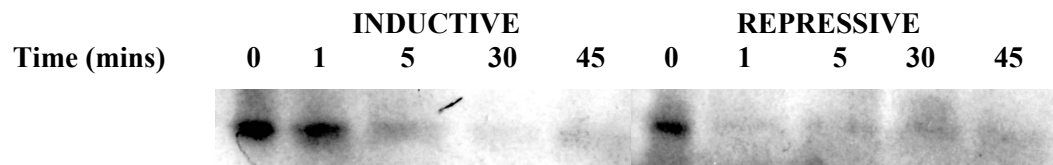
AvrPto is more stable in the presence of the *hrp* cluster.

My previous work had shown that the stability of effectors was affected by the presence of Lon protease (Chapter 3). I proposed that this sort of degradation was limiting to TTSS from *P. syringae*. Because wild-type cells were capable of secretion only when the TTSS was induced, we hypothesized that effectors would be protected from Lon-mediated degradation under these conditions in order to be secreted. We studied the abundance and stability of effectors under *hrp*-inducing or repressive conditions (See Materials and Methods). No difference could be detected between AvrPto's cytoplasmic abundance under *hrp*-inducing or repressive conditions (Figure 4-1A, time 0). Since a difference in the apparent half-lives had been detected previously in Lon mutants of *E. coli* and Psy61 compared to wild-type (See chapter 3), the same experiment was performed in Psy61 under *hrp* repressive and inductive conditions. When the cells were grown under *hrp*-inducing conditions, the half-life of AvrPto was about 12 minutes, whereas it was close to 3 minutes under repressive conditions (Figure 4-1A). In addition, the steady state levels of the effector AvrPto were also studied in *E. coli* cells with or without pHIR11, which encodes for the whole *hrp* cluster. It was found that in the presence of pHIR11, estimated levels of AvrPto levels were at least 5-fold higher than in cells without pHIR11 (Figure 4-1B). Combined, these results provide evidence for a stabilizing factor for AvrPto contained within the *hrp* cluster

Figure 4-1. AvrPto is more stable in the presence of the *hrp* cluster.

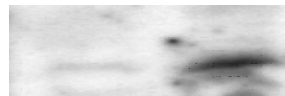
- A. An overnight culture of *P. syringae* Psy61 (wild-type) expressing AvrPto from pDSK600 was diluted into fresh media and grown to an OD₆₀₀ of 1.0. The cells were collected and resuspended in either inducing (M63 Fructose) or repressive (M63 Fructose with 1% casein) media to an OD₆₀₀ of 0.6 and grown at 25° C for 4 additional hours. Translation was stopped by the addition of tetracycline to a final concentration of 200 µg/ml, and the levels of AvrPto were estimated in 250 µl samples. The cells were collected at the indicated times and resuspended in 50 µl SDS-PAGE loading buffer. Samples were fractionated in a 15% SDS-PAGE, and blotted to nitrocellulose and probed using anti-AvrPto sera.
- B. AvrPto was expressed from the Lac promoter in pDSK600 in *E. coli* SG22622 (wild-type) in the presence of either pHIR11 (+hrp) or pLAFR3 (-hrp). Cells were grown to an OD₆₀₀ of 0.8, and levels of AvrPto were estimated in a 0.5 ml aliquot as described above.

A.



B.

hrp - +



that is only expressed under *hrp*-inducing conditions in Psy61, suggesting this factor is part of the *hrp* regulon.

Search for the stability factor within the *hrp* cluster

Because the stability of effector proteins seemed to be affected by the expression of the *hrp* cluster, it was reasonable to predict the existence of a stabilizing factor within the cluster. The stability of AvrPto was tested in an *hrpL* mutant, lacking the alternative sigma factor responsible for *hrp* gene expression. If the stability factor was part of the *hrp* cluster, then the levels of AvrPto in the *hrpL*::Tn strain would be decreased compared to the wild-type *hrp* cluster because the factor would not be expressed. The steady state levels of AvrPto were studied in strains carrying the wild type pHIR11 or the transposon-mutated derivative. *E. coli* SG22622 (Lon+) were transformed with pMLAvrPto600, which expresses a 6xHis tagged AvrPto, and either pHIR11 or pHIR11-2084 (*hrpL*::Tn). The cells were grown to mid-log phase and the whole cell extracts were assayed for the amount of AvrPto in immunoblots. The levels of AvrPto were decreased in the *hrpL* mutant cells (Figure 4-2), validating the hypothesis that the stabilizing factor is contained in the *hrp* cluster. The difference in levels was not due to a loading difference since the levels of BAP-FLAG were equivalent in these strains (Figure 4-2).

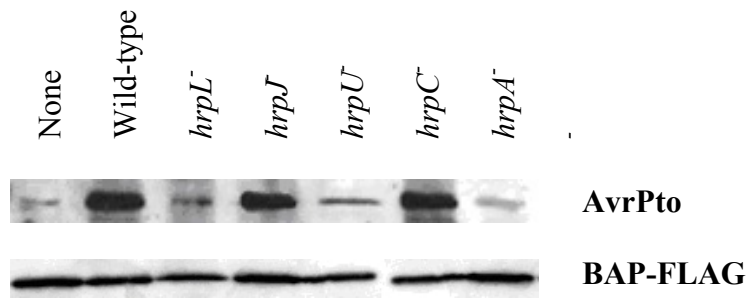
It was possible that the enhanced stability of the effectors was due to the compartmentalization of the effector into the TTSS and, thus, a physical separation from Lon protease. If this were the case, any mutation that resulted in an abnormal

Figure 4-2. Levels of AvrPto in cells expressing *hrp* operon mutants.

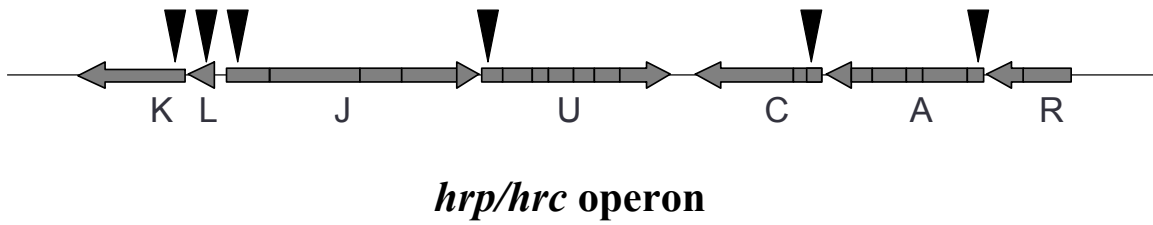
- A. Overnight cultures of *E. coli* SG22622 (wild-type) carrying different pHIR11 transposon mutants (containing mutations in different operons of the *hrp* TTSS) and ectopically expressing AvrPto and BAP-FLAG were diluted into fresh media until they reached an OD₆₀₀ of 1.0. The cells were washed and resuspended in M63 fructose with 1% casein to an OD₆₀₀ of 0.6, and grown for 4 hours. A 0.5 ml aliquot was centrifuged and the cells were resuspended in 50 µl of SDS-PAGE sample buffer. An identical sample was taken but resuspended in 50 µl 10% SDS to measure the total protein amounts using the BCA kit (Amersham-Pharmacia, NJ). Ten µg of total protein from the samples were separated in a 15% SDS-PAGE and immunoprobed with anti-AvrPto antibodies. The blots were stripped with 10M urea and reprobed with anti-FLAG antibodies.
- B. Schematic representation of the *hrp/hrc* operons and the locations for the transposon insertions (triangles) used in the different pHIR11 derivatives.

A.

***hrp* Genotype**



B.

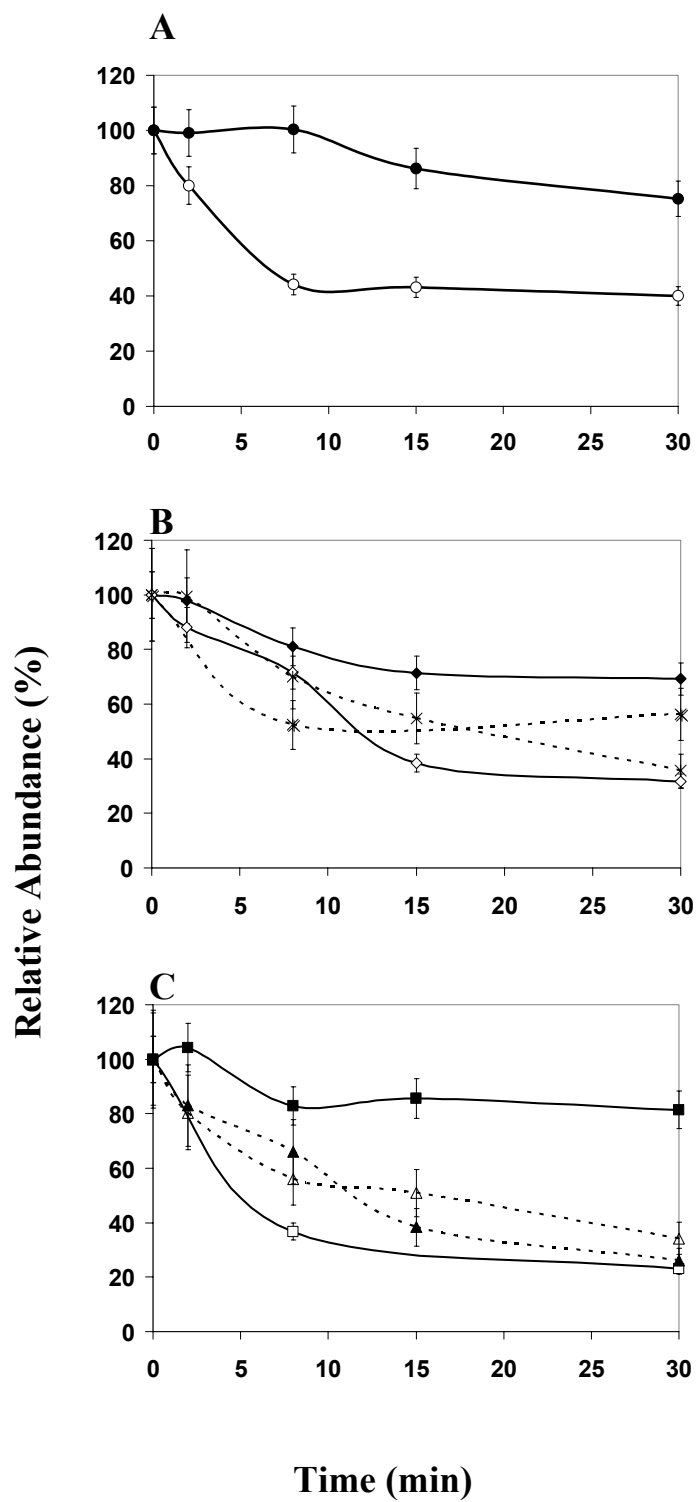


TTSS, would also result in an inability to protect effectors from degradation. Alternatively, it was possible that there existed a chaperone-like molecule that protected effectors from Lon-mediated degradation. If this were the case, then only the mutation in one specific gene would abolish the protective effect of expressing the *hrp* cluster. To differentiate between these alternatives, various pHIR11 mutants were used to study their impact on the stability of AvrPto. These mutants included the insertion of a polar transposon in each of six identified *hrp* operons, except *hrpRS*. The results showed that the stability of AvrPto was still enhanced in four of the mutants, suggesting that the effect of the *hrp* cluster was not due to a sequestering of the effector into the secretion apparatus, but could be due to the production of a stabilizing agent. Two mutants, pHIR11-2090 (*hrpU*) and pHIR11-2093 (*hrpA*) consistently showed a decreased amount of AvrPto when assayed (Figure 4-2), suggesting that the stabilizing factor was contained in one or both of these operons. The levels of AvrPto in the *hrpK* operon mutant also appeared to be decreased (~50% of wild-type, data not shown), however, these levels varied in the different trials carried out, and therefore were not further pursued.

Attempts were made to verify the stabilizing effect of the *hrpA* and *hrpU* operons. Each operon was cloned into pCR-XL and co-expressed with AvrPto. Both the *hrpA* and *hrpU* operons were expressed from the Lac promoter present in pCR-XL and induced with IPTG. When expressed at the same time as AvrPto, both operons were able to stabilize the effector (Figure 4-3A-B) to similar levels as pHIR11, but

Figure 4-3. The *hrpA* and *hrpU* operons can stabilize AvrPto.

A. Wild-type *E. coli* SG22622 carrying pAvrPto600 were transformed with either pLAFR3 (empty vector, open circles) or the cosmid clone pHIR11 (carrying a functional *hrp* cluster, closed circles). Overnight cultures were diluted in fresh M63 Fructose with 1% casein hydrolysate and grown to an OD₆₀₀ of 0.6. Translation was stopped by the addition of chloramphenicol to a final concentration of 200 µg/ml, and 250 µl samples were taken at the times (min) indicated. Cells were collected and resuspended in SDS-PAGE loading buffer. Whole-cell lysates were separated in a 12% SDS-PAGE, and levels of AvrPto were estimated as before. The abundance of AvrPto was measured relative to its initial amounts at time 0 min. **B.** The levels of AvrPto in cells carrying inactive pHIR11-2093 (*hrpA*::Tn, solid line, open squares); a construct expressing the entire *hrpA* operon in pCR-XL (solid line, closed squares); *hrpA*, *hrpZ*, and *hrpB* genes of the *hrpA* operon in pCR-XL, pTop*hrpAXL* (dashed line, open triangles); or *hrcJ*, *hrpD*, and *hrpE* genes of the *hrpA* operon in pCR-XL, pBottom*hrpAXL* (dashed line, closed triangles). **C.** The levels of AvrPto were estimated also in cells expressing inactive pHIR11-2090 (*hrpU*::Tn, solid line, open diamonds); a construct expressing the entire *hrpU* operon in pCR-XL (solid line, closed diamonds); *hrcP*, *hrpQ_a*, *hrcQ_b*, and *hrcR* genes of the *hrpU* operon in pCR-XL, pTop*hrpUXL* (dashed line, star); or *hrcS*, *hrc*, *hrcT*, and *hrcU* genes of the *hrpU* operon in pCR-XL, pBottom*hrpUXL* (dashed line, double stars). Experiments were repeated five times with similar results obtained in three of these attempts.



variability was observed. The experiments were performed a total of 5 times, but only 3 out of those gave the reported results. The other 2 trials resulted in opposite outcomes in which AvrPto was not stabilized at all by the presence of the operon (data not shown). The outcome may be a result of inconsistencies in expression from the *lac* promoter in the pCR-XL system or a problem with the experimental approach.

Identification of the gene encoding the stabilizing factor.

In order to identify the gene responsible for the stability, half of the genes from each operon was subcloned individually into pCR-XL and their effects on AvrPto were studied. Neither half of the *hrpA* operon had the ability to stabilize AvrPto (Figure 4-3C). The results from the *hrpU* operon are very similar. Neither the 5' nor 3' genes of the operon could consistently stabilize AvrPto (Figure 4-3D). The experiments suggest that there was a problem with the expression of the cloned genes, or more than one gene product is necessary for the stabilizing effects observed on AvrPto. Alternatively, the approach used was improper for this study and could not be relied upon to identify the stabilizing factor in the *hrp* cluster. As a consequence, biochemical approaches were attempted to ascertain the nature of the stabilizing factor.

Further attempts to identify the stabilizing factor.

Building on the hypothesis that the generalized stabilizing factor would be acting in a similar fashion to a chaperone that physically interacts with its effector, three different biochemical methods were used to try to identify proteins which interact with AvrPto and other effectors: Far Westerns, crosslinking, and column binding experiments. Each of these methods probes for interacting proteins, that, if found, would have to be characterized using peptide sequencing or mass spectrometry analysis. The following experiments were performed on at least two effectors, however only representative results from HopPsyV1 are shown.

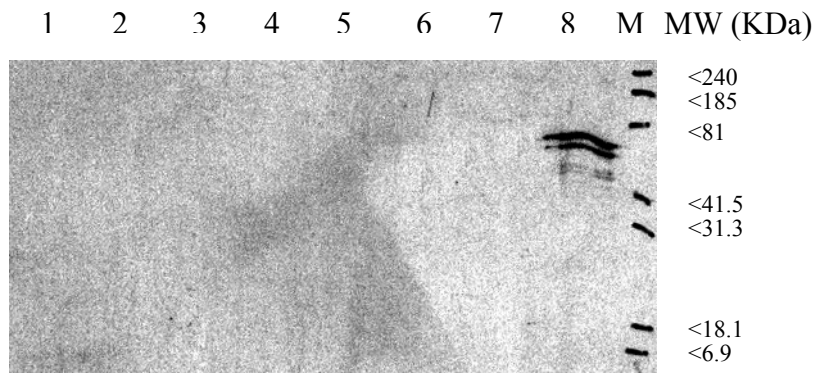
Far Westerns. Far-Western blotting is a method of probing a nitrocellulose or PVDF membrane, containing immobilized proteins, with another protein to detect specific protein-protein interactions. The method involves transferring cell extracts to a membrane, allowing a 8-24 h refolding period, and then overlaying the blot with a soluble protein that may bind to one or more immobilized proteins on the blot. After the overlay, interacting proteins are visualized by immunodetection based on the probing protein. A band would appear on the blot at the location where the interacting protein was located. For these experiments 6xHis tagged effectors were purified using Ni-NTA columns and used as the probing protein. Out of five effectors, two, AvrPto and HopPsyV1, were purified at high enough concentrations to carry out the experiments. The purified effectors were used to probe blots of whole cell extracts from different strains and growth conditions, including *P. syringae* grown under *hrp*-inducing and non-inducing conditions, *P. syringae hrpL* mutants, and *E. coli* either expressing or not the *hrp* cluster carried in pHIR11. As a positive

control an *E. coli* strain that expresses ShcV1 was used. ShcV1 is the predicted chaperone for HopPsyV1 and it's expected to interact physically with its effector. Neither AvrPto nor HopPsyV1 stably associated with another protein (Figure 4-4). The experiments were performed with 10X more effector than recommended in other methods [167], but no interactions could be detected. The major limitation of this method is that it relied on a very strong physical interaction between the effector and the stabilizing factor. It is possible that the interaction between this generalized factor and each effector is in reality very weak, and thus, this approach could not be used. Because the effector used as a probe would be washed away easily during the procedure it would be impossible to identify interacting proteins properly. Consequently, other more sensitive approaches were used.

Column-binding experiments. These experiments were performed by immobilizing the effectors on a Ni-NTA column by virtue of a 6xHis epitope, and passing whole cell extracts through the column. Those proteins that interact with the effector would remain immobilized in the column until both proteins are eluted. The experiments were tried with AvrPto, HopPsyV1, HopPsyB1 and HopPtoG. Both HopPsyV1 and HopPsyB1 are proposed to interact with their chaperones ShcV1 and ShcB1, respectively, and these interactions were used as controls for detecting protein-protein interactions. Only the representative results from HopPsyV1 are shown, but similar results were obtained with all the effectors. The effectors were adequately

Figure 4-4. Far Western Analysis of effector interacting proteins.

HopPsyV1 was purified from *E. coli* SG22622 wild type cells using the Ni-NTA agarose slurry (Qiagen, CA) following manufacturer's instructions. Ten µg of purified protein were diluted in interaction buffer and incubated with a blot of whole cell extracts for 2 hours. The blot was washed once with wash buffer (10mM Tris-HCl, 0.01% Tween-20) and the blot was probed with anti-His antibodies and recommended by the manufacturer. Lanes 1: SG22623 (Δlon) pLAFR3 (empty vector); 2: SG22623, pHIR11 (expressing a functional *hrp* TTSS); 3: SG22623, pShcV1D (expressing ShcV1 chaperone); 4: *P. syringae* Psy61 (wild-type), inducing conditions; 5: Psy61, repressive conditions; 6: Psy61-2084 (*hrpL*::Tn, *hrp*⁻), inducing conditions; 7: *P. syringae* DC3000 (wild-type), inducing conditions; 8: purified HopPsyV1 (as a positive control for the immunoblot). Similar results were achieved with AvrPto. HopPsyV1 ran as a doublet.

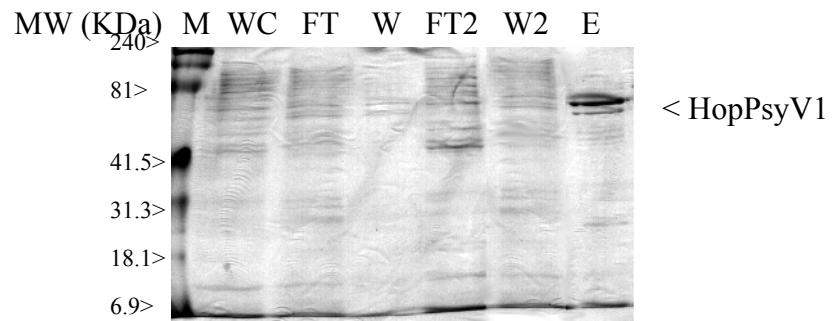


immobilized on the column as is evident because very low amounts of HopPsyV1 of other effectors were detected in the flow through or wash fractions of the column (Figure 4-5A). The effectors were not eluted from the column by the whole cell extracts from Psy61 or the secondary washes (Figure 4-5A). The buffers used were sufficient to elute the effectors, as is evident because of the high concentration of HopPsyV1 in the eluate fraction (Figure 4-5A). HopPsyV1 was always purified as two bands at around 69 KDa and 73 KDa, this molecular weight shift might be the result of the loss of the peptide added by cloning the protein into the pTrcHis2 vector. However, too many additional proteins could be detected in the elution after application of whole-cell extracts from Psy61, even under the most stringent of wash buffers (Figure 4-5A). These results did not make it possible to identify a single protein that was directly interacting with the effector. Moreover, the eluates of different stains all resulted in the same protein species (Figure 4-5B). Even cell extracts of a strain expressing the cognate chaperone, ShcV1, did not result in a band at the expected 18 KDa, suggesting that the observed bands were not specifically interacting with the effector, but were some form of contamination. Regardless of the wash protocol followed, the eluate was always contaminated. The most obvious limitation to this approach was that it did not appear to be functioning adequately. The controls used to test the interaction between an effector and its cognate chaperone did not result in the expected purification of the chaperone. Those results suggest that the method was not fine-tuned, and needs to be optimized.

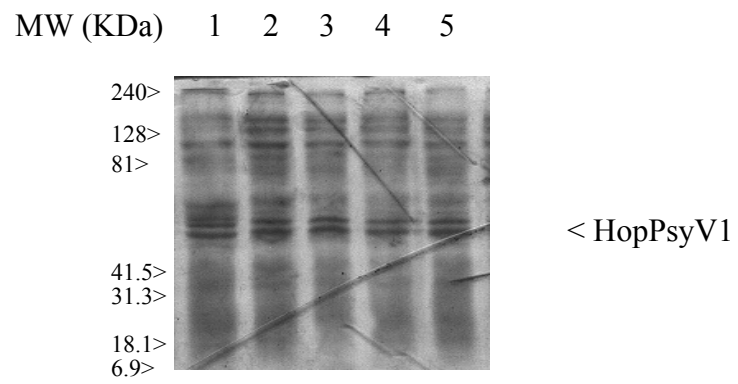
Figure 4-5. Column binding experiments.

- A. A whole cell lysate (WC) of *E. coli* SG22622 (wild-type) expressing HopPsyV1 was mounted on a Ni-NTA agarose column (Qiagen, CA) as recommended by the manufacturer. The lysate was allowed to pass through the column by gravity (FT) and then washed three times with a stringent buffer (W) containing 50 mM imidazole. A cleared lysate from Psy61 grown under inducing conditions created as recommended by the manufacturer (Qiagen, CA) was passed through the column (FT2). The column was washed once again with a mild buffer (W2) containing only 10 mM imidazole and eluted with elution buffer (E) containing 250 mM imidazole as recommended by the manufacturer. The results presented are from one experiment but it is representative of three attempts.
- B. Whole cell extracts were treated as before, except that cell lysates from the *lon* null cells SG22623 carrying pLAFR3 (lane 1), pHIR11 expressing a functional *hrp* TTSS (lane 2), or the wild type cells SG22622 carrying pShcV1D which expressed the ShcV1 chaperone (lane 3), pLAFR3 the empty vector (lane 4), or pHIR11 with the *hrp* TTSS (lane 5) were passed through the column and the eluates were run on a SDS-PAGE side by side. No difference in the proteins detected could be observed. Similar results were obtained with all other effectors tested.

A.



B.

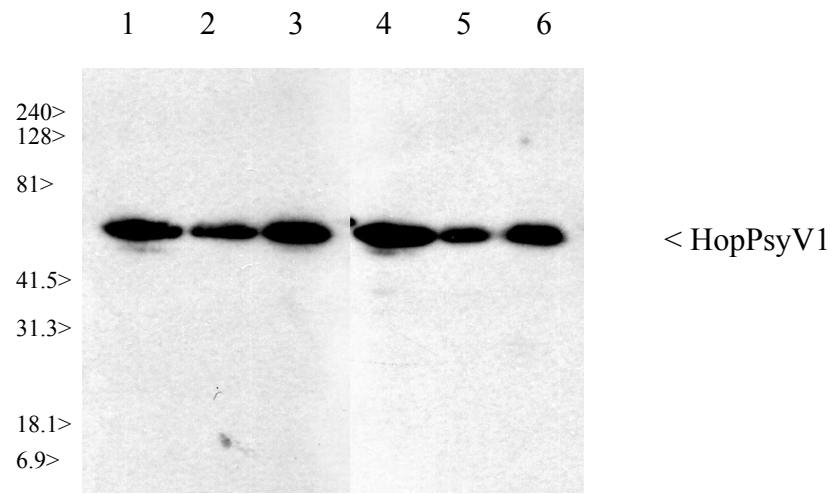


Cross-linking experiments. Two cross-linking agents were used to covalently link proteins that are in close proximity. If there are proteins that physically interact with the effectors, then the crosslinking would result in a species with a higher molecular weight than the effector. Formaldehyde and DSP were used as described in other methods [167]. After crosslinking, cell extracts were either fractionated by SDS-PAGE and immunoblotted, or the potential effector-interacting protein complex was purified on the Ni-NTA columns as described before. These experiments were performed on AvrPto and HopPsyV1. As a positive control, ShcV1 was expressed and its interaction with HopPsyV1 was monitored. For neither of these proteins or with either of the methods was there a detectable weight shift that could be attributed to interaction with another protein (Figure 4-6). As was the case for the Far Westerns, the major limitation of this approach was that it depended on a very intimate and strong interaction between the effector and the proposed generalized stability factor. If this interaction does not occur, then no proteins could be cross-linked to the effector. These results suggest that the methods used were not properly optimized for the protein-protein interaction under scrutiny, and that the positive controls used were insufficient to adequately troubleshoot the protocols used.

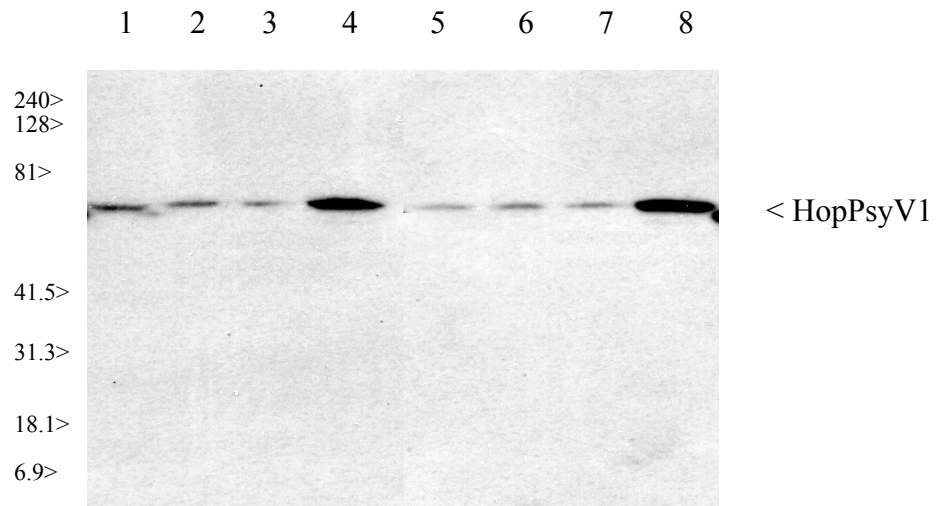
Figure 4-6. Crosslinking experiments.

- A. Overnight cultures of SG22623 (Δlon) carrying pHIR11 that expresses a functional *hrp* TTSS (lanes 1 and 4), the empty vector, pLAFR3 (lanes 2 and 5), or pShcV1D expressing the ShcV chaperone (lanes 3 and 6) and co-expressing HopPsyV1 were diluted into fresh LB media and grown to an OD_{600} 0.6. DSP to 1 mM was added to one half of the culture (lanes 4 – 6) and the cells were incubated for two additional hours. A 0.5 ml aliquot sample was centrifuged and the pellet was resuspended in 50 μ l of SDS-PAGE loading buffer. The samples were fractionated by SDS-PAGE and immunoblotted with anti-His antibodies.
- B. SG22623 (Δlon) cells were treated as above except that formaldehyde (10 mM) was used for crosslinking instead of DSP and the incubation time was reduced to 30 min. After the incubation 6xHis tagged moieties were purified using the Ni-NTA agarose slurry and analyzed by immunoblots. Similar results were obtained with AvrPto. Lanes 1 and 5: no vector; lanes 2 and 6: pLAFR3; lanes 3 and 7: pHIR11; and lanes 4 and 8: pShcV1D.

A.



B.



Discussion

Secretion through the TTSS of *P. syringae* is different from mammalian pathogens in various ways. Most notably, the number of proteins which are substrates for TTSS in *P. syringae* is very high (>60) compared to less than 15 for other pathogens [39, 43, 62, 76]. All effector genes are controlled by the alternative sigma factor, HrpL, and there are no known mechanisms for regulation of their transcription and/or translation. In *P. syringae*, however, chaperones appear to be associated with a very small minority of these effectors [62, 75]. These facts pose a dichotomy in *P. syringae*: either chaperones are not important for its TTSS; or *P. syringae* has found an alternative method for carrying out the roles of individual chaperones. The observation presented here that effectors can be generally stabilized by expression of the *hrp* regulon in *P. syringae*, provides some evidence that indeed there is a need for at least one of the chaperone activities prior to secretion. In addition, the recent studies that show that three chaperones, ShcA, ShcM and ShcV, are necessary for the secretion of their corresponding effectors [14, 189, 193] underlines the necessity for chaperone functions in the *hrp* TTSS. It is likely, then, that *P. syringae* requires chaperone functions for secretion, but developed a novel way to achieve these functions.

The results obtained in the course of this study suggest that there is a factor within the *hrp* cluster that can complete the stabilizing function of a TTSS chaperone. Effectors

appeared to be more abundant and had longer apparent half lives when the *hrp* regulon was expressed in *P. syringae*. Those effectors were also stabilized in *E. coli* cells expressing the *hrp* cluster, suggesting that the protein(s) responsible for stabilization was encoded by one of those genes. I proposed that this factor could act as a generalized chaperone that could protect effectors from proteolysis, and also allow their secretion through the TTSS. This proposed generalized chaperone would act analogously to other TTSS chaperones. Firstly, it was necessary for the cytoplasmic stabilization of effectors. Secondly, it would be regulated as part of the *hrp* regulon by HrpL. Thirdly, it would physically interact with the effectors and form either a “secretion competent” structure [179], or alternatively, a three dimensional structure that would be recognized by the secretion apparatus [20]. Lastly, the generalized chaperone would be necessary for proper secretion of the effectors.

The initial attempts to identify the generalized chaperone were based on its ability to stabilize effectors in the cytoplasm. To this end, various transposon mutations of the operons in the *hrp* cluster were used to find mutants where the abundance of AvrPto was decreased when compared to the wild-type *hrp* cluster. The approach was useful to identify two operons that had the ability to stabilize AvrPto, the *hrpA* and *hrpU* operons. The *hrpA* operon consists of *hrpA*, *hrpZ*, *hrpB*, *hrpJ*, *hrpE* and *hrpD*. As shown in Table 1-1 (page 27), HrpA (the main component of the TTSS needle [163]), HrpZ (an HR elicitor), and HrpB are all secreted proteins, and might not function as the generalized stability factor. However, HrpD, HrpE, and HrpJ are all predicted to

be cytoplasmic, and at least HrpD has been shown to also associate with membranes [140]. Most interestingly, however, is the fact that HrpE is the FliH/YscL homolog (Table 1-1). FliH and YscL have been proposed to interact with FliI and YscN, the ATPases involved in flagellar biosynthesis and *Yersinia* TTSS [96, 204]. FliH appears to interact with chaperone/substrate complexes and seems to be involved in the initial docking of flagellar export substrates [183] (see Figure 1-2, pg. 26). In *Yersinia*, YscL interacts with YscK [96], which is the HrpD homolog (Table 1-1). Thus, it is possible that the stability factor in *P. syringae* could indeed be one of these docking proteins. The function of HrpJ is still unknown, and therefore could also be an excellent candidate for the stability factor. In the future, experiments that directly address the ability of HrpD, HrpE, and HrpJ to interact with specific effectors should be conducted.

The *hrpU* operon consists of *hrcU*, *hrcR*, *hrcS*, *hrcT* and *hrcQ_a*/*hrcQ_b* and is mostly responsible for the formation of the inner membrane structure of the TTSS [89].

Inner membrane components of the flagellar TTSS are known to interact with chaperone/substrate complexes [204], and are also involved in the initial docking onto the system [183]. Therefore, it is most likely that the stability factor is one of the soluble components expressed from the *hrpA* operon. Possibly, in the absence of the proper inner membrane components, the soluble complex formed between the effector and the soluble components dissolves quickly, and hence the effector would be prone to Lon-mediated degradation.

Further analyses were carried out to decipher the gene(s) responsible for the

stabilization, but none of the results was sufficiently convincing to pinpoint the nature of the stabilizing factor. The major limitation of the genetic approach was the lack of sensitivity of the antibody used during the study. The antibody was cross-reactive with too many proteins which made it difficult to determine the actual levels of AvrPto in the blots, and therefore, unreliable. In the future, these experiments should be performed using more precise antibodies, such as anti-HopPsyA and anti-HopPtoM which were recently acquired. In addition, these experiments should also be conducted using the native *P. syringae* background, since it would be more accurate than the *E. coli*-based screen. There are transposon mutants in all the operons tested in this study, and therefore, a complete screen of the *hrp* cluster could be performed.

The second set of experiments attempted in this study focused on the identification of a protein that could physically interact with effectors. Based on other TTSS, the stabilizing factor could act as a chaperone that has multiple substrates, such as SycD, IpgC, and SycH in *Shigella*, and *Yersinia* spp. [148, 149, 155, 192]. Three different biochemical methods for identifying protein interactors were used. All of these attempts were based on the premise that the stability factor could physically interact with the effectors. In some cases, the methods were modified to fit the conditions necessary for even weak interactions. Since the positive controls did not yield the expected results these experiments remain inconclusive. More effort should be dedicated to enhance the effectiveness of these methods and to obtain the proper results with the control reactions in order to find the stabilizing factor.

It is essential to keep in mind that the generalized stability factor may influence other processes that may stabilize the effectors. For example, the stability factor may be involved in the post-translational modification of the effector, therefore protecting it from degradation. However, none of *hrp* the gene products is predicted to have an enzymatic activity capable of modifying the effectors, and hence it is unlikely that the stabilizing factor is involved in such a modification. Alternatively, it may regulate protease activity and in this manner protect effectors. Other results comparing proteolysis in *P. syringae* between *hrp*-inducing and *hrp*-repressing conditions have shown that there is no difference in the general turnover of proteins (Bretz, unpublished results). These results suggest that the activity of proteases is the same under both conditions and supports the idea that effectors are differentially protected under the two different conditions. The assay has not been carried out specifically for Lon, and so it is possible that the affinity of Lon for the effectors is different under the two situations. Since the stability factor could be directly interacting with the effector, or involved in other mechanisms that change the affinities of Lon, it is vital that both genetic and biochemical approaches with enhanced sensitivity be used to discover the stability factor.

In conclusion, during this study various attempts were conducted to identify a possible generalized stability factor for TTSS in *P. syringae*. This stability factor would accomplish the roles of other TTSS chaperones by acting on a wide range of effectors. Although several approaches were used, the nature and function of the

stability factor still remains to be discovered.

Materials and Methods

Bacterial Stains and Media.

Strains and plasmids used in this study are described in Table 4-1. Bacteria were routinely grown on King's B medium [12]. Plasmids were propagated in *E. coli* DH5 α . *E. coli* strains were grown at 37°C, and *Pseudomonas syringae* strains were grown at 25°C. LB and M63 minimal salts media were used as described previously [167]. M63 medium was supplemented with 1mM MgSO₄ and 1% fructose (M63F). The following antibiotics were added where needed at the indicated concentrations (in micrograms per milliliter): ampicillin, 200; kanamycin, 50; spectinomycin, 100, tetracycline, 25; nalidixic acid, 50; rifampicin, 200; and chloramphenicol; 30.

Table 4-1. Strains and Plasmids.

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
<i>E. coli</i>		
DH5 α	endA1 hsdR17 (rk- mk-) supE44 thi-1 recA1 gyrA96 relA1 Δ (argR-lacZYA) U169 ϕ 80dlacZDM15	Invitrogen
SG22622	cpsB::lacZ Δ ara malP::lacIq	S. Gottesman
SG22623	SG22622 Δ lon-510	S. Gottesman

SLR400	araD139 Δ (ara leu)7697 derivative of MC4100	S. Benson
TOP10	F- mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 recA1 ara Δ 139 Δ (ara-leu)7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
<i>Pseudomonas syringae</i>		
Psy61	Wild-type, Nal ^r , HR ⁺ Weak bean pathogen	[15]
Psy61-KL11	Nal ^r Kan ^r , lon::Tn	[25]
Plasmids		
pAvrRpt2D	1.0 kb PCR product containing <i>avrRpt2</i> cloned into pDSK519	This work
pDSK519	Broad-host range vector, IncQ Kan ^r	[107]
pDSK600	Broad-host range vector, IncQ Sp ^r	[147]
pFLAG-CTS-BAP	Bacterial Alkaline Phosphatase in pFLAG-CTC	Sigma-Aldrich
pHIR11	pLAFR3 derivative carrying <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrp/hrc</i> cluster, Tc ^r	[85]
pHIR11-2074	<i>hrpL</i> :Tn derivative of pHIR11	[84]
pHIR11-5134	<i>hrpK</i> :Tn derivative of pHIR11	[84]
pHIR11-2075	<i>hrpJ</i> :Tn derivative of pHIR11	[84]
pHIR11-2084	<i>hrpU</i> :Tn derivative of pHIR11	[84]
pHIR11-2090	<i>hrpC</i> :Tn derivative of pHIR11	[84]

pHIR11-2093	<i>hrpA</i> :Tn derivative of pHIR11	[84]
pHopPsyB1trc	<i>hopPsyB1</i> cloned into pTrcHis2	This work
pHopPsyEtrc	<i>hopPsyE</i> cloned into pTrcHis2	This work
pHopPtoGtrc	<i>hopPtoG</i> cloned into pTrcHis2	This work
pHopPtoMtrc	<i>hopPtoM</i> cloned into pTrcHis2	This work
pHopPsyV1trc	<i>hopPsyV1</i> cloned into pTrcHis2	This work
pLAFR3	Tc ^r , IncP1	[177]
pLLHrpAXL	<i>hrpA</i> operon cloned into pCR-XL	This work
pLLHrpUXL	<i>hrpU</i> operon cloned into pCR-XL	This work
pLLBottomHrpAXL	5' genes of <i>hrpA</i> operon cloned into pCR-XL	This work
pLLBottomHrpUXL	5' genes of <i>hrpU</i> operon cloned into pCR-XL	This work
pLLTopHrpAXL	5' genes of <i>hrpA</i> operon cloned into pCR-XL	This work
pLLTopHrpUXL	5' genes of <i>hrpU</i> operon cloned into pCR-XL	This work
pMLAvrPto600	0.45 kb PCR cloned as <i>EcoR1-HindIII</i> into pDSK600	[25]
pMLL600	0.4-kb PCR product containing <i>hrpL</i> cloned into pDSK600	This work
pSGAS	3.6-kb fragment containing <i>hopPsyA</i> in pYXSS	[80]
pSHAMB	<i>hopPsyA</i> cloned into pMLB1034	[80]
pShcB1D	0.5 kb PCR product cloned into pDSK519	This work

pShcMD	0.5 kb PCR product cloned into pDSK519	This work
pShcVID	0.5 kb PCR product cloned into pDSK519	This work

General DNA manipulations.

Restriction enzymes were purchased from Invitrogen (Bethesda, MD). T4 DNA ligase was acquired from New England Biolabs (Beverly, MA) and used according to the manufacturer's recommendations. Basic manipulations were done using standard procedures. PCRs were performed using a PCR Sprint thermal cycler (Hybaid, Ashford, UK) with 50 µl reaction volumes. Unless indicated otherwise, *ProofPro* polymerase (Continental Lab Products, San Diego) was used to amplify fragments for cloning.

Cloning of operons into pCR-XL

The regions containing the *hrpA* and *hrpU* operons, including their ribosome binding site, were amplified from Psy61 genomic DNA using the following primers: *hrpA* complete operon A1 (TACCGTCGCATCAAGGAAT) and A2 (CCATTTTCGCCGCAAGA); 5' region A1 and A3 (GCAGGCCCGTTCTCTTCGT); 3' region A4 (ACGAAGAGAACGGGCCTGC) and A2, *hrpU* complete operon U1 (GATCCTCGACCACTTAGCA) and U2 (GGTACGCGTATGGCTAAAC); 5' region U1 and U3 (AACTACAGTTTGTCGGCGTTG); 3' region U4 (CAACGCCGACAAACTGCAG) and U2. The products were cloned into pCR-XL using the TOPO TA cloning kit (Invitrogen, Carlsbad) following manufacturer's

instructions. The inserts were verified by sequencing.

Immunoblots.

Ten μg of total protein from every sample were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was carried out using a polyclonal antibody raised against AvrPto at 1:3000 dilution. Commercial antibodies, anti-His (Novagen, San Diego), anti-FLAG (Sigma, St. Louis), and secondary antibodies conjugated to horse radish peroxidase were used following manufacturer's recommendations. Cross-reactive proteins were visualized using the ECL chemiluminescence kit (Amersham-Pharmacia, Piscataway).

Stability of Effectors.

Overnight cultures of *P. syringae* strains expressing AvrPto, HopPsyA or AvrRpt2 were diluted into M63 media containing fructose as the carbon source to an OD_{600} of 0.6 and incubated for 4 h at 25°C. After incubation, tetracycline (200 $\mu\text{g ml}^{-1}$) was added to inhibit translation. Cells were harvested at specified time points, lysed in sample buffer and fractionated in 12% SDS-PAGE gels. For *E. coli*, SG22622 or SG22623 were grown overnight at 37°C, diluted into fresh M63F containing 1 % casein hydrolysate and grown to an OD_{600} of 0.6. After incubation, chloramphenicol (200 $\mu\text{g ml}^{-1}$) was added and samples were collected as in *P. syringae*. The half-life effectors were calculated from the exponential decay in levels estimated in scanned images using NIH Image 1.59. Each experiment was repeated at least three times.

Purification of Effectors.

C-terminally 6xHis tagged effectors were purified using the Ni-NTA agarose slurry (QIAGEN, CA) under native conditions as recommended by the manufacturer.

Far Western Blots.

Ten μg of whole cell lysates from Psy61 and Psy61-2084 under inducing or repressing conditions as described by [200] were separated by SDS-PAGE and transferred to a PVDF membrane. Blots were incubated with up to 10 μg of purified effector in binding buffer (10 mM MgSO_4 , 10mM Tris-Cl pH 7.8) for two hours. Blots were washed with PBS buffer once for 10 min, and then immunoprobed using anti-His antibody as recommended by the manufacturer.

Column Binding.

Whole cell lysates of cells expressing 6xHis tagged effectors were prepared following the instructions for native isolation of proteins (Qiagen, CA) with the exception that after four washes the proteins were not eluted. Instead, a 3 ml sample of a whole cell lysate of Psy61, Psy61-2084 (inducing and repressing conditions), SG22623(pHIR11) or SG22623(pLAFR3) was allowed to pass through the column in 0.5 ml aliquots. The column was then washed with wash buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole pH 8.0) three times and eluted with elution buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 250 mM imidazole pH 8.0). 250 μl samples were taken after the flow-

through and washes to detect any leaking of the bound effector. After elution, proteins were separated by SDS-PAGE, and either stained with Coomassie Brilliant Blue or immunoprobed for the effector to verify the proper functioning of the column.

Crosslinking experiments.

Cells were grown to an OD₆₀₀ of 0.8 at which point either formaldehyde (10 mM) or DSP (1 mM) were added. Cells were incubated for an additional 30 min with formaldehyde or two hours with DSP. Whole cell extracts were created by taking a 0.5 ml aliquot, centrifuging, and resuspending the pellet in 50 µl of SDS-PAGE sample buffer and separating them by SDS-PAGE and immunoprobng for the effector. Alternatively, after the crosslinking, cell lysates were created under native and denaturing conditions and incubated with the Ni-NTA agarose slurry for 1 hour and treated as recommended by the manufacturer. Eluted proteins were detected by immunoblots with anti-His antibodies.

Chapter 5: General Conclusions and Future Directions

This work was successful in identifying and characterizing a novel effector in *P. syringae* Psy61, HopPsyL. This effector was shown to be necessary for virulence in *Phaseolus vulgaris* cv Kentucky Wonder, and was an avirulence determinant on many non-host plants. In addition, effectors were found to be hypersecreted and more stable in strains lacking Lon protease. From those experiments, a novel model on how *P. syringae* regulates secretion of effectors through its TTSS was proposed. The model stipulates that the cytoplasmic abundance of effectors is limiting to secretion, and suggests that an order for effector secretion could be established depending on the relative abundances of different effectors. This study found that effectors were protected from Lon-mediated degradation by their corresponding chaperones, therefore clarifying the role of chaperones in *P. syringae*. The study also found evidence for a generalized chaperone used in *P. syringae* secretion in order to stabilize effectors prior to secretion. This general chaperone could be another method for imposing hierarchy in effector secretion, and an adaptation to the great number of effectors present in the *P. syringae* genome.

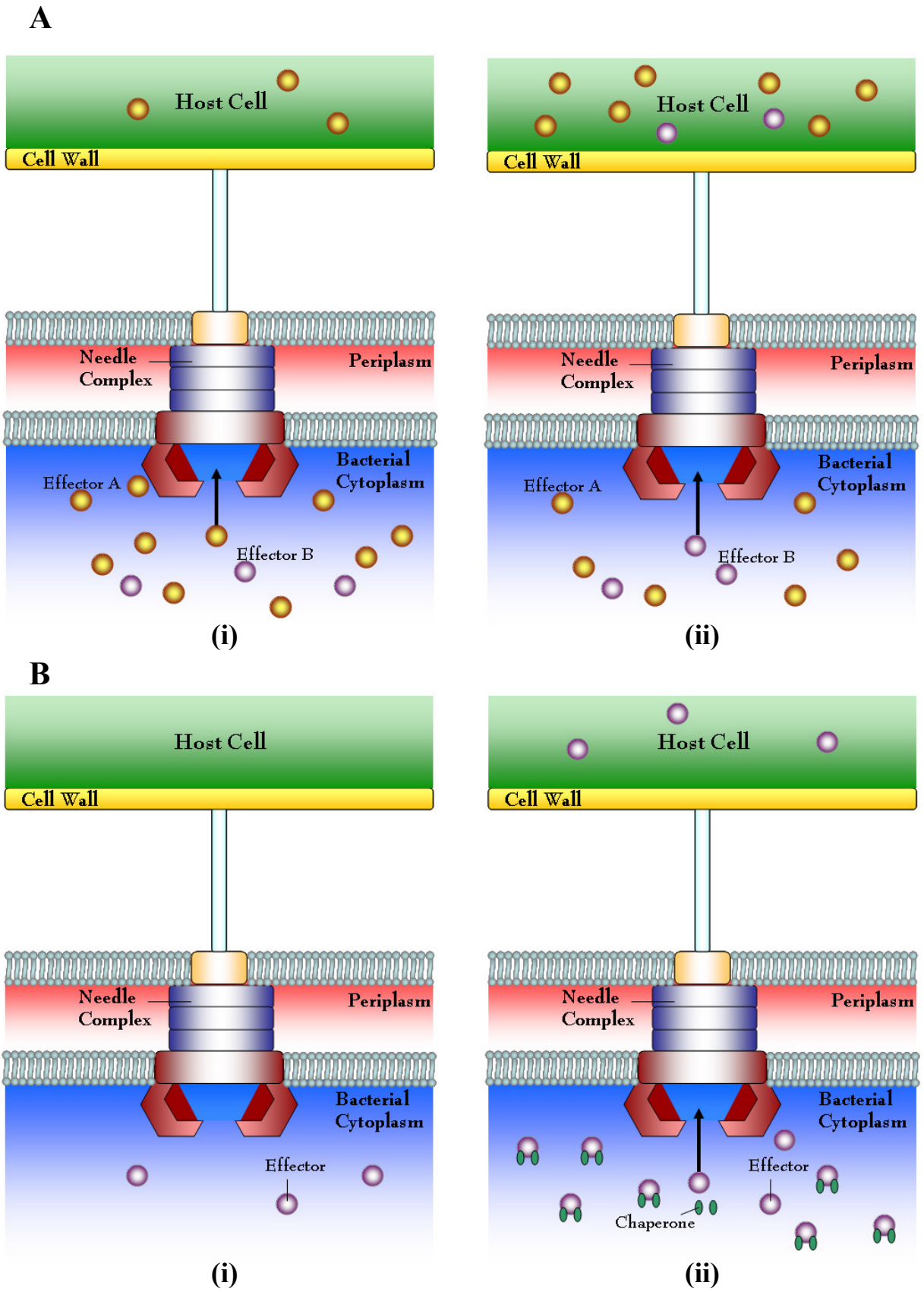
One of the major impediments of studying secretion through the *P. syringae* TTSS was that the process is very tightly regulated. I found that *lon* mutants were capable of hypersecreting effectors (Chapter 3) [25], and that bypassing the regulation of the *hrp* TTSS by ectopically expressing HrpL did not result in the same phenotype (Chapter

3). The hypersecretion was due to an enhanced amount of the effector in the cytoplasm, mainly because effectors were not degraded by Lon in that strain (Chapter 3). These results led me to propose that secretion was limited by the cytoplasmic amount of effectors available, and that selection of secretion substrates in *P. syringae* could be dictated by the comparative abundance of each effector in the cytoplasm (Figure 5-1a). This model was novel in the field and could help to explain the manner in which a hierarchy of effector secretion could be established in *P. syringae*. The model implies that effectors present at high concentrations would be secreted more readily, and would accumulate in the host more rapidly than those at low concentrations. The model is exciting because up to now, the only method proposed for imposing an order of secretion has been through chaperones. However, most TTSS have fewer chaperones than effectors, and thus it was unclear how the order could be established for effectors that don't appear to have a cognate chaperone. The model is especially important for plant pathogens that have only a small number of chaperones compared to the vast number of effectors [76], and thus could explain how those effectors are selected for secretion.

I found that chaperones positively affected the stability of effectors in the *P. syringae* cytoplasm by protecting them from Lon-mediated degradation. Therefore, I was able to clarify the role of these chaperones in this system. The results fit in with my proposed model of effector secretion, since an effector that interacts with its chaperone is more stable and present at higher amounts in the cytoplasm, and therefore, could be more easily secreted through the TTSS (Figure 5-1b).

Figure 5-1. Substrate selection in the TTSS of *Pseudomonas syringae*.

- A. (i) Two effectors wait to be secreted. Effector A is more stable and more abundant, and therefore is secreted more readily than Effector B. (ii) The relative abundance of Effector B rises and becomes a proper substrate for secretion through the system. Thus Effector B is secreted later than Effector A, and it takes a longer time for accumulation of Effector B in the host cell.
- B. (i) The effector is not stable without its chaperone, and consequently its cytoplasmic abundance is limited and not sufficient for proper secretion. (ii) In the presence of its chaperone, the effector is stabilized and begins to accumulate in the bacterial cytoplasm and is secreted through the TTSS.



Interestingly, effectors interacting with chaperones were also susceptible to Lon protease to different degrees, suggesting that here chaperones might also be involved in imposing a secretion hierarchy as in the *Yersinia* and flagellar biosynthesis TTSS [3, 198].

Many tests need to be conducted, however, in order to understand if the model I proposed actually describes what occurs in *P. syringae*. For instance, eight effectors were analyzed in this study, which is only a fraction of the total number of effectors produced by *P. syringae* strains. Moreover, most of the sampled effectors belonged to different strains. The observation that there exist two categories of effectors due to their difference in stability might be a consequence of the small sample used for the study, and might not reflect what really occurs in the bacterial cell. More effectors, from a single strain, should be analyzed to determine if the model is still valid. It still remains to be shown whether secretion through the *hrp* TTSS is ordered in a similar fashion to *Yersinia* or flagellar biosynthesis [142, 198]. However, the available technology is not sensitive enough for this type of analysis, since secretion from *P. syringae* is very limited and reporter constructs cannot be used effectively to analyze the amount of secretion. There might be the possibility of using the adenylate cyclase reporter assay as recently published [169]. Although the assay appears to be quantitative in nature [169], it is still not known whether it is sufficiently sensitive to detect very low levels of translocated effectors, or whether it can differentiate between early and late secretion. Furthermore, if the model we propose is applicable to other Gram-negative pathogens, particularly mammalian pathogens which appear

to secrete elevated amounts of effectors, then it could be possible to assay the existence of “early” and “late” secretion substrates in those species, and their correlation with Lon-mediated degradation.

A contradiction within our proposed model was that effectors that have very short half lives, and therefore do not accumulate in the bacterial cytoplasm, are still secreted through the TTSS, albeit at an unknown rate. For example, HopPsyB has been shown to be secreted and translocated through the system [33] despite its short half life (>3 min). Our observation that AvrPto and other effectors were stabilized when the *hrp* regulon was expressed provided evidence of the existence of a generalized stability factor that could potentially be in charge of stabilizing effectors. This generalized stability factor would accomplish the roles of chaperones, and possibly form the three-dimensional structure used for targeting an effector to the secretion port, as discussed later. The proposed factor could have distinct affinities for different effectors, that might allow it to act as the regulator of substrate selection, much like FliK in flagellar biosynthesis [106, 142], or Spa32 in *Shigella flexneri* [133]. This stability factor, however, still remains to be identified, and its real role in TTSS from the *hrp* system needs to be elucidated. More genetic and biochemical attempts to identify the generalized stability factor should be carried out as discussed in greater detail in Chapter 4.

I determined that the protection granted by the chaperone ShcM on its cognate effector, HopPtoM, was not a result of masking of the Lon-targeting motif. The C-

terminal motif leading HopPtoM to Lon-mediated degradation did not appear to overlap the chaperone-binding domain. The chaperone probably protected the effector because of the formation of some Lon-insensitive structure. Interestingly, this structure might also function as the elusive TTSS signal, as discussed below. It would be of great interest to locate the Lon-targeting motif of HopPtoM and other effectors more precisely. It is possible that the identified motif would be different between “early” and “late” secretion substrates, and therefore would provide us with the molecular basis for the difference, and a possible mechanism for imposition of the proposed secretion hierarchy. The targeting sequence from Sula [81, 95] was not present in HopPtoM or other effectors, and no consensus sequence that might target effectors to Lon could be discerned. To clearly define the Lon-targeting motif, further experiments using the MBP fusion strategy should be attempted. Both truncations of the C-terminal domain of HopPtoM, and N- and C-terminal truncations of other TTSS effectors should be studied.

The results from my studies, namely that the secretion from *P. syringae* is limited by Lon-mediated degradation of effectors, also helps clarify the controversy surrounding the TTSS secretion signal. My results imply that the secretion signal is contained in the peptide, and not the mRNA as had previously been proposed [7, 9, 145]. If the signal was in the form of mRNA, then it would be likely that secretion occurred co-translationally, and would imply that there exists a mechanism for the ribosome to get in close contact with the TTSS, the idea known as “co-translational translocation” [9]. If translocation occurs at the same time as secretion, then there would be no necessity

for the cytoplasmic accumulation of effectors, as observed here and elsewhere [184]. In addition, the degradative effects of Lon protease should not have an impact on the secretion of effectors, as I observed. It is fascinating that the field of flagellar biosynthesis had been through a very similar situation trying to explain their secretion signal [105, 131], and to date both signals remain uncertain [132, 162].

Since this study was initiated, three *P. syringae* genomes have been partially or completely sequenced [28]; (www.tigr.org; www.jgi.doe.gov), and several studies have attempted to identify novel effectors [27, 33, 62, 76, 128, 158, 206]. One of these studies [158], postulated a set of rules that defined the peptidic secretion signal of *P. syringae* effectors. The rules postulate that the N-termini of effectors are generally basic amphipathic helices with several restrictions on the amount and location of non-polar and cysteine residues. A survey I conducted of thirteen known or candidate effectors revealed that the majority did not fit these characteristics. HopPsyL, the novel effector identified in Chapter 2, did not fit three of the postulated characteristics (Table 5-1), and other effectors long known to be secreted and translocated also failed at least two of these rules (Table 5-1). Indeed, proteins lacking any of those characteristics were recently shown elsewhere to be secreted and translocated [169]. Thus, a consensus sequence that could function as a secretion signal has not been identified by me or others [62].

It is obvious to me that the secretion signal is more complicated than what we have

Table 5-1. The proposed secretion signal of *P. syringae* effectors is not conserved.

Effector	Rule 1 ^a	Rule 2	Rule 3	Rule 4	Rule 5	Rule 6
HopPsyA	No	Yes	No	Yes	Yes	Yes
HopPsyL	No	Yes	Yes	No	No	Yes
AvrPphE	Yes	Yes	Yes	No	Yes	Yes
AvrPphC	Yes	Yes	Yes	No	Yes	Yes
HopPtoB1	No	Yes	Yes	No	Yes	Yes
HopPtoB2	No	Yes	Yes	No	Yes	Yes
AvrXcT	Yes	Yes	Yes	Yes	Yes	Yes
HopPsyE1	Yes	Yes	Yes	No	Yes	Yes
HopPsyC1	Yes	Yes	Yes	Yes	Yes	Yes
HopPsyV1	Yes	Yes	No	No	No	Yes
HopPtoV	No	No	No	No	No	No
HopPtoG	Yes	Yes	No	No	Yes	Yes

a. Rule 1: Ile, Leu, Val, Ala or Pro in position 3 or 4, but not both.

Rule 2: Position 5 is rarely Met, Ile, Leu, Phenylalanine, Tryptophan, or Tyrosine.

Rule 3: Asp or Glu not present in first 12 positions.

Rule 4. Cys is rarely present in after position 5.

Rule 5. First 50 residues form an amphipathic helix rich in polar amino acids except Ser or Gln.

Rule 6. No more than 3 consecutive residues consisting of Met, Ile, Leu, Val, Phe, Tyr or Trp in first 50 amino acids.

thought up to now. It is likely that the secretion signal is actually a three-dimensional structure produced as a result of the interaction between the N-termini of effector proteins and one or more of the soluble components of the TTSS. To date, almost all the data about the secretion signal has come from the *Yersinia* TTSS [7-9, 20, 29, 34, 60, 116, 124, 127, 174, 184]. I believe that it is important to study the signal from many different species, since the similarities and slight differences between their TTSS might shed some light onto the actual nature of the signal. Some of the studies that have attempted to elucidate the signal used alanine scanning of the first 15 codons of the effector peptide [9]. Inserting an alanine might not have sufficiently disrupted the possible interactions with soluble components, and therefore I think it is necessary to carry out studies that more aggressively interrupt the N-terminus of various effectors. These studies could use highly polar amino acids, in particular acidic residues, since the N-termini have been proposed to be basic [158]. Alternatively, the studies could incorporate proline residues which would severely interfere with the secondary and tertiary structures of the peptides.

For those effectors that interact with a chaperone, it is likely that the interaction leads to either the supramolecular structure required for the secretions signal, similar to what Birtalan, *et al.* propose [20], or allows the proper contact with the soluble components of the system, like FliJ or CesT [65, 141, 183]. An expensive and time-consuming approach, but which ultimately may be the only way of clarifying the issue of the secretion signal, is resolving the three-dimensional structures of more effectors and effector/chaperone complexes interacting with the soluble components

of the TTSS, including those from flagellar biosynthesis. These structures may help reveal the spatial organization of the secretion signal. These approaches must all be combined with different bioinformatic tools that are becoming more capable of predicting hidden similarities.

The knowledge of the secretion signal would be beneficial for several reasons. A thorough understanding of the secretion signal could allow us to predict other effectors in genomic data from both plant and animal pathogens, in similar fashion to how the knowledge of the *hrpL*-dependent promoters allowed identification of effectors in *P. syringae* [62]. Elucidating the whole artillery of each pathogen could help us better prevent and treat diseases in animals, humans, and plants. For example, pharmaceuticals could be artificially produced that inhibit or prevent the function of one or more of the bacterial effector proteins. An interesting novel application of understanding the secretion signal has been the production of fusion proteins for therapeutic functions. For example, the TTSS-dependent delivery of the fusion products between YopE and Listeriolysin O or p60 from *Listeria monocytogenes* are currently being studied as a novel immunization method against listeriosis [166]. Other therapeutic applications have recently been initiated with tumor-seeking, attenuated *Salmonella*, such that direct delivery of toxic proteins and/or surface-localized antigens could be delivered into tumor cells to attack or induce an immune response against the tumor [129]. I believe that this form of “inverted pathogenicity” [166] holds great promise for future therapeutic treatments, not to mention the awesome irony of the whole process!

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