ABSTRACT

Title of Dissertation: ***PSEUDOMONAS SYRINGAE* PATHOGENESIS: REGULATION OF TYPE III SECRETION AND IDENTIFICATION OF A SECRETED EFFECTOR**

James R. Bretz, Doctor of Philosophy, 2004

Dissertation Directed By: **Dr. Steven Hutcheson, Professor, Department of Cell Biology and Molecular Genetics**

The *Pseudomonas syringae hrp* pathogenicity island encodes a type III secretion system (TTSS) which is used to translocate effector proteins into host cells to facilitate pathogenesis. Expression of the *hrp* TTSS is controlled by the alternative sigma factor, HrpL, whose expression in turn is positively controlled by two truncated enhancer binding proteins, HrpR and HrpS. Although a number of environmental conditions are known to modulate *hrp* TTSS expression, such as stringent conditions and pathogenesis, the mechanism by which the activities of these transcriptional factors are modulated had not been established. Both HrpR and HrpS were shown to be required for full expression of *hrpL*. *hrpRS* were shown to be expressed as an operon and a promoter was identified 5' to *hrpR*. The *hrpRS* promoter and coding sequence were found to be conserved among *P. syringae* strains. The *hrpRS* operon was constitutively expressed under conditions in which the *hrpL* promoter was inactive, indicating the involvement of a negative regulatory factor. Transposome (Tnp) mutagenesis was used to identify Lon protease as a negative regulator of *hrpL*

expression, suggesting an effect on HrpR and/or HrpS. HrpR was observed to be unstable in wild-type *P. syringae* strains grown in non-inductive media. The apparent half-life of HrpR increased more than 10-fold in the *P. syringae lon::Tnp* mutants or upon transfer to inductive (stringent) conditions. As a result, an interaction between factors involved in the stringent response, Lon protease, and *hrp* regulation was also investigated. The regulatory system described above was used to develop a HrpL-dependent promoter trap to identify effectors secreted by the Hrp TTSS. One of these potential effectors, HopPtoD2, was shown to encode a protein tyrosine phosphatase that was translocated into *Arabidopsis thaliana* cells via the *hrp*-encoded TTSS. A Δ*hopPtoD2* mutant exhibited strongly reduced virulence in *Arabidopsis thaliana*. Expression of *hopPtoD2* delayed the development of several defense-associated responses in infected plants. These results indicate that HopPtoD2 is a translocated effector with protein tyrosine phosphatase activity that modulates plant defense responses.

PSEUDOMONAS SYRINGAE PATHOGENESIS: REGULATION OF TYPE III SECRETION AND IDENTIFICATION OF A SECRETED EFFECTOR

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

To my father whose love of science led me down this road many years ago and instilled in me the desire to follow in his footsteps.

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There are numerous people who have contributed in both big and small ways to my graduate career here at Maryland. Although I would love to personally thank everyone who assisted me, accomplishing a goal as large as this required the help and support of too many to list here. Thanks to all of you for your help.

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List of Abbreviations

aa	amino acids
Amp	ampicillin
avr	avirulence
bp	base pairs
CC	coiled-coil domain
CCR	central conserved region
CEL	conserved effector locus
ECL	enhanced chemiluminescence
ECF	extracytoplasmic functions
EEL	exchangeable effector locus
EPS	extracellular polysaccharide
HR	hypersensitive response
hop	Hrp-dependent outer protein
hrc	*hrp* conserved
hrp	hypersensitive response and pathogenicity
Kan	kanamycin
KB	kilobase
kD	kilo Dalton
LAR	lymphocyte antigen-related protein
LRR	leucine rich repeat
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase

MAPKKK	MAPKK kinase
MU	Miller units
Nal	naladixic acid
NB	nucleotide binding domain
NO	nitric oxide
NTG	nitrosoguanidine
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAI	pathogenicity island
PCD	programmed cell death
PCR	polymerase chain reaction
*p*NPP	*para*nitrophenyl phosphate
PTP	protein tyrosine phosphatase
R	resistance gene/protein
Rif	rifampicin
ROS	reactive oxygen species
RT	reverse transcriptase
SA	salicylic acid
Spc	spectinomycin
TCA	trichloroacetic acid
Tet	tetracycline
TIR	Toll-like IL-1 receptor
Tnp	transposome

TTSS	type III secretion system
UAS	upstream activating sequence
ysc	*Yersinia* secretion
yop	*Yersinia* outer protein

Chapter 1: General Introduction

(Portions of this chapter have been published as a review in Infection and Immunity, 2004, 72:3697-705 (ref. 28).)

Pseudomonas syringae is a collection of biochemically related strains that can have distinct plant host ranges. *Pseudomonas syringae* is a fluorescent pseudomonad in the γ-subgroup of proteobacteria that facultatively infects a wide range of economically important plant species. *P. syringae* strains are known to cause blights, spots, and cankers in susceptible plants. 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 (PCD), and in the case of exotoxin producers, a spreading chlorosis (yellowing of the tissue due to chlorophyll breakdown) (17, 143). The disease usually does not kill the plant but diminishes the yield and marketability of the product. Susceptible plant species can be found in divergent taxonomic groups of flowering plants. 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. In fact, some strains can only infect a few varieties of a single plant species (98).

In many cases, asymptomatic epiphytic populations of the bacterium growing on exposed surfaces of the plants provide the inocula for the infection (98, 143). A single bacterial cell can be sufficient to initiate an infection. Invasion of the

mesophyll tissue (loosely packed cells with air-filled intercellular spaces necessary for gas exchange) is usually aided by leaf wetting and/or tissue wounding and by bacterial motility. Once the bacterium is in the intercellular spaces of the tissue, the bacterium adsorbs to a host cell's surface.

During the initial colonization of the tissue of a susceptible plant, the bacterium begins to multiply and produce virulence factors that contribute to symptom formation. Common virulence factors of *P. syringae* strains are extracellular polysaccharides (EPS; usually alginate but levan can also be produced), derivatized peptide exotoxins, and several plant growth hormones, such as indoleacetic acid (associated with cell volume control among other effects) and cytokinins (stimulate cell division but have other activities as well). The EPS forms a hygroscopic glycocalyx that may protect the bacterium from oxidative stress and aids in tissue colonization (124). The exotoxins inhibit specific enzymes in surrounding host cells, such as glutamine synthetase or ornithine carbamoyl transferase, to suppress some defense responses. Exotoxins can also directly alter membrane physiology to favor parasitism (17, 87, 250). Over several days, bacterial population density in infected tissue can reach as high as 10^9 cells / g fresh weight of leaf tissue. An important distinction from many mammalian pathogens is that *P. syringae* strains are noninvasive and remain external to the plant cell wall in the intercellular spaces of the tissue (143). This is a fundamental difference from many mammalian pathogens and impacts the nature of the interaction between the bacterium and its host and the strategy plant pathogens use to parasitize the host.

The timing of the plant defense response to the initial colonization by the bacterium is a determining factor in the outcome of an interaction. In a susceptible plant, the host cells are usually slow to recognize and respond to the infection (102). As a result, the infecting bacterial population is able to spread into new tissue before the cellular defense responses of the initially colonized cells are activated. Thus the pathogen is able to maintain a continuously expanding infection. Eventually, large areas of leaves and other organs/tissues become infected and necrosis develops due to a slowly induced PCD (61, 243). In contrast, a resistant plant is able to rapidly initiate a cellular defense response during the initial colonization of the tissue that functions to prevent further spread of the infection. A single adsorbed bacterium elicits a cascade of cellular defense responses in the host cell of the resistant plant within 1-2 h. An oxidative burst is commonly observed by 3-6 h, with PCD typically developing within 12 hours of infection. At high inocula, this rapid response is called the hypersensitive response (HR) and is visible as a localized necrotic lesion (102). Although the bacterial cells remain viable for several days, this response blocks further spread of the bacteria to surrounding tissue. In a susceptible plant, similar responses are not detected until at least 48-96 h post-infection (54).

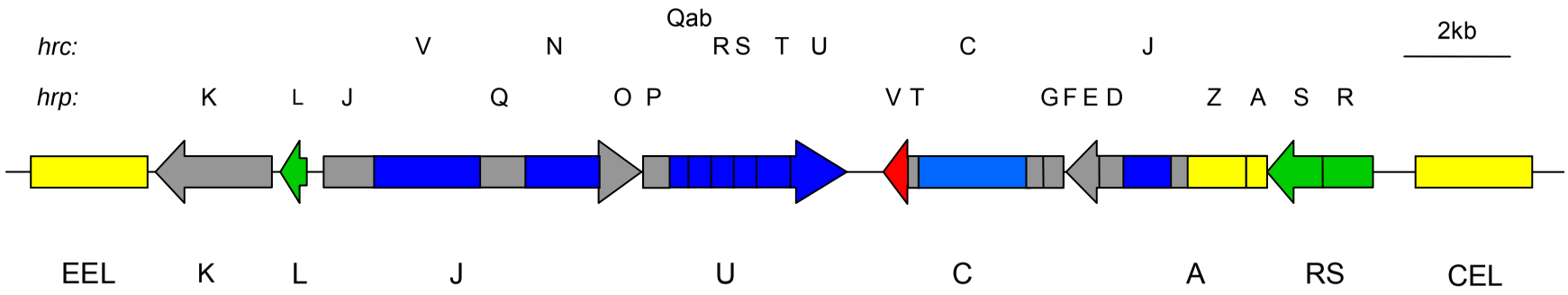
The Pseudomonas syringae Type III Secretion System

The ability to cause disease in susceptible plants and the induction of PCD (and the HR) in resistant plants have both been linked to the activities of a type III secretion

system (TTSS) encoded by the central conserved region (CCR) of the *P. syringae hrp* (hypersensitive response and pathogenicity) pathogenicity island (PAI)(Figure 1-1) (4, 73, 103). TTSSs are broadly conserved protein translocation systems found in bacterial pathogens of plants and mammals that utilize a needle-like secreton to translocate effectors that facilitate colonization and parasitism of susceptible hosts (51). The CCR of the *hrp* PAI includes 7 operons containing 26 genes that encode the TTSS and its dedicated regulatory network. Each of the 9 universally conserved components of the TTSS secreton is present in the *P. syringae* system (26). These nine proteins, called Hrc (*hrp* conserved), form the core of the TTSS. Among TTSSs of mammalian pathogens, the *Yersinia* Ysc proteins are the closest homologs to the conserved Hrc products. Twelve Ysc products share sequence similarity with counterparts of the *P. syringae* Hrp secreton (103).

The type III secretion process is similar to flagellar biogenesis. Secreted proteins (known as effectors) are loaded into a central channel of the TTSS secreton and exported from its distal end (51). The Hrp TTSS appears to be functionally interchangeable with its counterparts of other pathogenic bacteria. For example, secretion of *P. syringae*-encoded effectors by *Erwinia* and *Yersinia* TTSSs has been reported (7, 92). In *P. syringae*, a pilus is assembled from the structural protein HrpA and extends from the secreton to the plant cell (116, 117, 138). This pilus can be more than 2 μm long and appears to be flexible. Assembly of the HrpA

Figure 1-1: The *P. syringae hrp* pathogenicity island. The *hrp* pathogenicity island has a tripartite structure (4). The central conserved region (CCR) covers approximately 25 kb and contains the genes that encode for the TTSS and its dedicated regulatory machinery. Adjacent to the CCR are the exchangeable effector loci (EEL) and the conserved effector loci (CEL) which encode for proteins secreted through the Hrp TTSS. Gene names are listed above the figure while operon names are listed below the figure. The proteins encoded by the *hrc* (*hrp* conserved) genes are the universally conserved components of the TTSS and form the core of the TTSS secreton. Block arrows represent operons which are transcribed in the direction of the arrow. Genes colored blue encode components of the TTSS machinery. Yellow boxes indicate genes that encode for proteins that are secreted or translocated by the Hrp TTSS. Red and green colored genes indicated negative and positive regulators of *hrp* expression, respectively (see Figure 1-2). Genes colored grey encoded proteins with unknown function. The EEL and CEL are of variable size and are not drawn to scale.


Qab
hrc:
V
N
R S
T
U
C
J
2kb
hrp:
K
L
J
Q
O
P
V T
G F E D
Z
A
S
R
EEL
K
L
J
U
C
A
RS
CEL

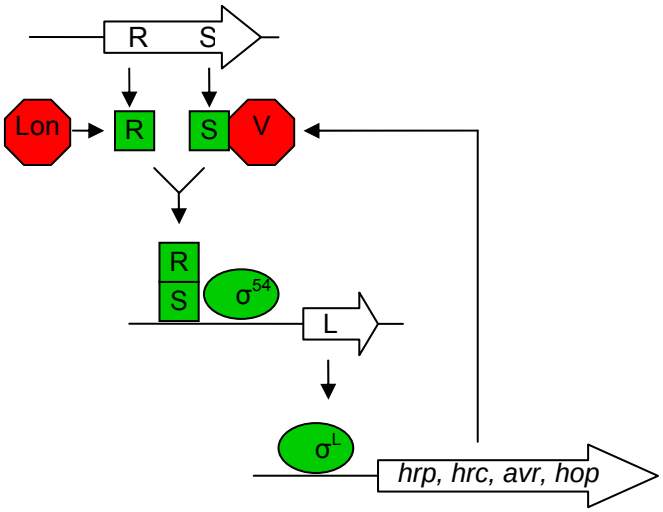
pilus is required for pathogenesis (231). Immunolocalization was used to demonstrate that effectors are extruded from the tip of the HrpA pilus (116, 117, 138).

Regulation of the hrp-encoded TTSS

Like many TTSSs, the expression of the *P. syringae* TTSS is environmentally regulated (103). During growth in rich media containing a broad spectrum amino acid source (such as casamino acids), expression of the *hrp* PAI is repressed. However, during pathogenesis *hrp* expression can be detected 1-2 hours after infiltration into the tissue (181, 240). In addition, *hrp* expression can be induced in culture media that is thought to mimic in planta conditions, such as an acidic minimal salts medium (227, 240, 245). It is not known precisely how environmental signals induce *hrp* expression. Evidence from *Ralstonia solanacearum* suggests that contact with a specific component of the host cell wall initiates assembly of its TTSS (3). Other pathogens such as *Yersinia* spp. have been shown to respond to physiological conditions (135).

Coordinating the assembly of the *hrp*-encoded TTSS is a complex regulatory system that shares some similarities to that controlling flagellar biogenesis (Figure 1-2). HrpR and HrpS, constitutively expressed from the *hrpRS* operon, are similar to enhancer binding proteins that are typically part of two component regulatory systems. However, HrpR and HrpS lack the usual amino terminal domain that

Figure 1-2: The *hrp* regulatory system. Expression of the *hrp* regulon is controlled by a complex regulatory cascade involving several factors. HrpR and HrpS are enhancer binding proteins that cooperatively interact to induce expression of *hrpL*. HrpL is an alternative sigma factor that activates expression of the *hrp*-encoded TTSS as well as effectors secreted via the *hrp* TTSS (*avr* and *hop* gene products). Lon protease negatively regulates *hrp* regulon expression by degrading HrpR and preventing full expression of *hrpL*. HrpV negatively regulates *hrp* expression via an unknown mechanism. Positive acting factors are green. Negative acting factors are red. Genes are represented by the open arrows.


R
S
Lon
R
S
V
R
S
σ54
L
σL
hrp, hrc, avr, hop

modulates activity and do not require a cognate sensor kinase for their activity. HrpR and HrpS instead appear to form a heteromeric complex which then activates the RpoN-dependent *hrpL* promoter (See Chapter 2)(105, 238). HrpL is an alternative sigma factor related to FliA, the alternative sigma factor involved in expression of class III flagellar genes. HrpL directs transcription of the operons encoding components of the TTSS as well as the genes encoding translocated effectors (49, 84, 238, 239). Regulated proteolysis of HrpR by Lon protease provides one mechanism for environmental regulation of *hrpL* expression, and therefore assembly of the secreton (See Chapter 3)(27). Conditions that mimic, in part, the environment in plant tissue (amino acid starvation) suppress Lon-mediated degradation of HrpR, thereby allowing the HrpR/HrpS complex to form. Another protein, HrpV, also negatively regulates *hrp* expression albeit via an unknown mechanism (179). The GacS/GacA two component system also has been shown to modulate *hrp* expression, as is the case with several mammalian pathogens (40).

Hrp secreted effectors

The primary function of the *hrp*-encoded TTSS appears to be the translocation of effectors into the plant cell cytosol to facilitate parasitism of the host (79, 178). Thus identification of the effectors expressed by a strain is critical to understanding the molecular interactions with the plant host. However, it has been comparatively difficult to identify the effectors produced by a *P. syringae* strain. As in *Salmonella* and *Yersinia*, secretion of *P. syringae* effectors through the *hrp* TTSS can occur

without translocation into the host cell but is several orders of magnitude lower than its mammalian counterparts. For example, the accumulation of Yop proteins in culture filtrates from *Yersinia* strains is obvious under inductive growth conditions (51). In *P. syringae*, only a few of the proteins secreted via the Hrp TTSS from just a couple strains can be easily detected by SDS-PAGE or in immunoblots under inductive conditions (227, 244). The proteins that can be detected, such as HrpW and HrpZ, do not appear to be translocated into plant cells, but instead may assist in early phases of the translocation process. HrpW has structural features that suggest it may degrade components of the plant cell wall and could facilitate penetration by the HrpA pilus (39). HrpZ has been shown to form pores in lipid bilayers (133) and could aid in the breaching of the host cell plasma membrane by the Hrp TTSS.

Because physical methods for identifying effectors could not be easily applied, most of the initially identified effectors from *P. syringae* strains were identified instead through phenotypic screens that attempted to isolate factors affecting the host range of strains (132). At least twenty distinct effectors which required the *hrp* TTSS for phenotypic activity were identified by this process in a variety of *P. syringae* strains. Given that these phenotypic screens searched for genes reducing virulence in an indicator plant (see below), an early name for effector genes was "avirulence (*avr*) genes." Although mechanistically inaccurate, the *avr* name has been retained in some cases for historical reasons (69). More recently identified effectors have been designated as *hop* genes (Hrp-dependent outer protein, analogous to *yop* genes) (227).

Although the gene products are structurally divergent, all effector genes have been found to be transcriptionally dependent upon HrpL for expression (49, 84).

Consistent with conservation of the TTSS secreton, effectors of *P. syringae* strains share some characteristics with the known effectors from mammalian pathogens. For instance, TTSS-secreted effectors are modular in nature, irrespective of the source. For the *P. syringae* effectors that have been analyzed for secretion signals, a cryptic secretion signal is located in the amino terminal 50 amino acids (49, 84, 90, 177). The phenotypic/biochemical activities of the effector are usually associated with a carboxyl terminal domain of the protein. Analogous to what has been observed for effectors associated with mammalian pathogens, some *P. syringae* effectors also have cognate chaperones, but most do not (51).

Analyses of the genomes of two *P. syringae* strains have revealed that individual strains encode a surprisingly large number of effectors. For example, by searching for HrpL-dependent promoters together with similarity searches for homologs of known effectors, the genome of *P. syringae* pv. *tomato* DC3000 (a pathogen of tomato and *Arabidopsis thaliana*) was found to encode 58 known or likely effectors (49, 84). Similarly, the genome of the bean pathogen *P. syringae* pv. *syringae* B728a includes genes for 29 probable effectors, of which 25 are at least partially conserved in DC3000 (84). The genes for some of these effectors were located in "effector loci" adjacent to the CCR of the *hrp* PAI (4, 38, 59), but the majority were encoded by genes distributed elsewhere in the genome (31, 126). Clusters of effector genes have

been identified on plasmids (73, 111), while other effectors have been associated with transposable elements (126). As most mammalian pathogens translocate considerably fewer effectors, the variety of effectors produced and apparently translocated into host cells by each *P. syringae* strain was unanticipated, but could reflect the complexity of plant cells, which contain chloroplasts and other unique features (39, 84, 90, 94, 133) distinct from mammalian cells. Alternatively, because plant pathogens encounter a larger number of potential hosts than mammalian pathogens, it is possible that they have acquired a larger set of secreted effectors. Interestingly, the genome of the plant pathogen *Ralstonia solanacearum*, which also uses a *hrp*-encoded TTSS, encodes at least 40 secreted effectors (189).

The variation in the host range of *P. syringae* strains can be attributed in part to differences in the effectors produced by the strains. Many effector genes appear to have been acquired via horizontal gene transfer. Plasmid-borne effector gene clusters and integron-like and transpositional redistribution mechanisms have been identified in *P. syringae* strains (38, 112, 126). Once acquired, effectors continue to evolve through the accumulation of point mutations. For example, single point mutations were identified in alleles of an effector gene found in closely related *P. syringae* strains that altered what variety of the susceptible plant species responded to the expressing strain (212). Thus, the host range of a strain can be attributed in part to the set of effector genes it expresses and to the activity of each individual effector.

Virulence Targets of Selected P. syringae Effectors

The cellular activities of several translocated effectors from various *P. syringae* strains have been identified and were found to be similar to the activities of effectors produced by *Yersinia* and *Salmonella* spp. Like their counterparts, each *P. syringae* effector appears to have a specific cellular target in the host to facilitate parasitism either by a direct manipulation of a physiological process in the host cell to aid in nutrient release and/or by the suppression of cellular defense systems. A common target of *P. syringae* effectors appears to be signal transduction pathways associated with the plasma membrane. In addition, some of these translocated effectors are post-translocationally processed in the host cell to become active.

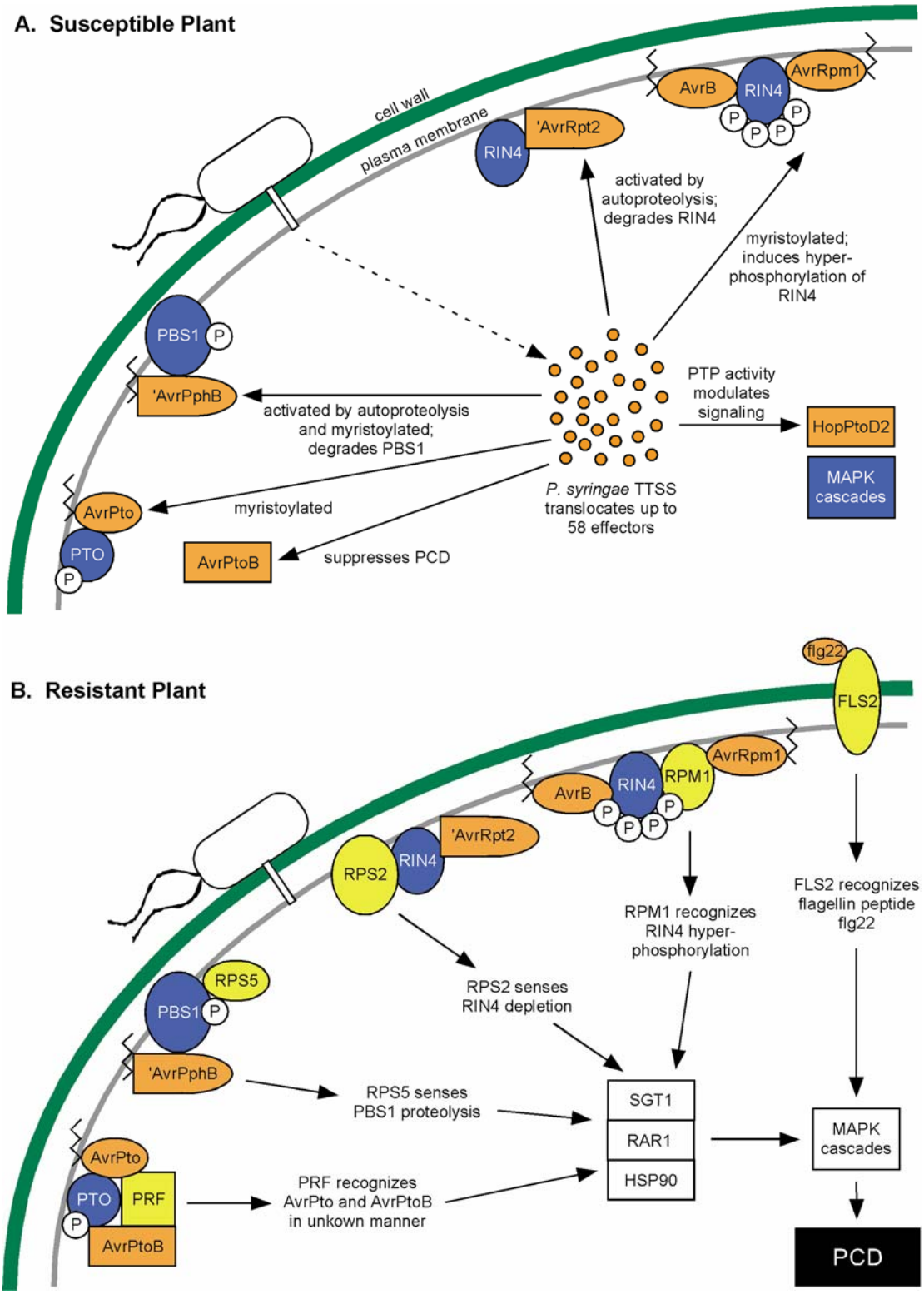
Translocated Cysteine Proteases

AvrPphB is an effector produced by some *P. syringae* strains that exhibits sequence similarity with the catalytic domains of YopT, Efa1, and other bacterial effectors that are members of this cysteine protease family (201). For mammalian pathogens, the cysteine protease effectors are essential virulence factors. For example, the translocation of the *Yersinia* YopT into the cytosol of host cells causes the degradation of RhoA, a small membrane bound GTPase found in the cytosol, and the disruption of the actin-based cytoskeleton (201). Efa1 is a related cysteine protease from enteropathogenic and enterohemorrhagic *E. coli* that may also cause cytoskeletal rearrangements through a similar mechanism (166). Similar to these

cysteine proteases, AvrPphB is required for virulence in susceptible hosts. After translocation into the host cell, AvrPphB is autocatalytically cleaved to expose an amino terminal myristoylation motif that is necessary for its activity (Figure 1-3). Upon myristoylation, the mature protein localizes to the plasma membrane of the host cell where ‘AvrPphB has been shown to physically interact with and to specifically cleave PBS1, a Ser/Thr kinase (200, 217). Consistent with its identification as a cysteine protease, the active site Cys is required for this activity (200). Unfortunately, the normal cellular function of PBS1 is unknown, but it is presumably a component of a signal transduction system which may affect membrane function. As will be discussed below, PBS1 is essential to the defense response of resistant plants.

More recently, AvrRpt2 was identified as a cysteine protease. No local sequence alignments could be detected, but the predicted secondary structure of the active domain aligned to the secondary structure of staphopain, a cysteine protease from *Staphylococcus epidermidis* (11, 150). Like AvrPphB and the other related cysteine proteases, AvrRpt2 is autocatalytically processed (Figure 1-3). In the host plant, AvrRpt2 is cleaved after amino acid 71 to yield a stable C-terminal product (163). This post-translocational processing requires at least one host factor (115). The cellular target of processed AvrRpt2 appears to be a host protein called RIN4, which is rapidly degraded in the presence of a catalytically active version of AvrRpt2. Inactivation of the predicted active site Cys blocked autocatalytic cleavage of AvrRpt2 and AvrRpt2-mediated degradation of RIN4 (12, 150). Like PBS1, the

Figure 1-3: A. *P. syringae* effector interactions with susceptible host plants. *P. syringae* injects effectors into the cytosol of the host plant using the Hrp TTSS. Once in the cytosol, the effectors target various host proteins to promote virulence. The cysteine proteases AvrPphB and AvrRpt2 are activated in the cytosol by autoproteolysis. AvrB, AvrRpm1, AvrPphB, and AvrPto are myristoylated and localize to the plasma membrane. AvrRpt2 also localizes to the plasma membrane. PBS1 and PTO autophosphorylate. AvrB and AvrRpm1 induce hyperphosphorylation of RIN4. **B.** *P. syringae* effector interactions with resistant plants. Resistant plants encode R proteins that monitor the cellular targets of the *P. syringae* effectors. Effector protein recognition initiates signal cascades that culminate in programmed cell death (PCD) of the responding cell. *P. syringae* TTSS-secreted effectors are orange. Plant-encoded target proteins are blue. Plant proteins involved in signaling are white. R proteins are yellow. Proteins thought to associate with the plasma membrane are represented by ovals. Cytosolic proteins are rectangular. Zigzags represent protein myristoylation. To date, no single *P. syringae* strain has been identified that encodes or secretes all of the effectors mentioned above. Likewise, no single host plant is known to express all of the indicated R genes. See text for description of effector activity.


A. Susceptible Plant
cell wall
plasma membrane
AvrB
RIN4
AvrRpm1
P
'AvrRpt2
RIN4
activated by autoproteolysis; degrades RIN4
myristoylated; induces hyper-phosphorylation of RIN4
PBS1
P
'AvrPphB
activated by autoproteolysis and myristoylated; degrades PBS1
PTP activity modulates signaling
HopPtoD2
MAPK cascades
AvrPto
PTO
P
myristoylated
P. syringae TTSS translocates up to 58 effectors
AvrPtoB
suppresses PCD
B. Resistant Plant
flg22
FLS2
AvrRpm1
AvrB
RIN4
RPM1
P
'AvrRpt2
RPS2
RIN4
FLS2 recognizes flagellin peptide flg22
RPM1 recognizes RIN4 hyper-phosphorylation
RPS2 senses RIN4 depletion
RPS5
PBS1
P
'AvrPphB
RPS5 senses PBS1 proteolysis
SGT1
RAR1
HSP90
MAPK cascades
AvrPto
PTO
PRF
P
AvrPtoB
PRF recognizes AvrPto and AvrPtoB in unkown manner
PCD

biochemical function of RIN4 in the host cell under normal physiological conditions is not known, but it is required for elicitation of defense responses in resistant plants.

Translocated Protein Tyrosine Phosphatases

HopPtoD2 is a chimeric effector whose amino terminal 140 amino acids share sequence similarity with the corresponding region of AvrPphD, a broadly conserved translocated effector (See Chapter 4)(29, 67). The carboxyl terminus contains a protein tyrosine phosphatase (PTP) active site domain similar to those in TTSS-secreted effectors of *Yersinia* and *Salmonella*. In *Yersinia*, the PTP activity of YopH dephosphorylates proteins involved in cytoskeleton development, thus inhibiting phagocytosis by macrophages (60). SptP, a PTP encoded by *Salmonella typhimurium,* also targets cytoskeletal components and inhibits activation of a *Salmonella*-induced mitogen-activated protein kinase (MAPK) in host cells (60, 141). HopPtoD2 was shown to be an active PTP that is translocated into plant cells by the Hrp TTSS. *hopPtoD2* knockout mutants were substantially less virulent in susceptible hosts. HopPtoD2 appears to suppress defense responses by delaying the onset of the reactive oxygen burst and PCD. Although a precise cellular target for this translocated PTP has not been identified, *P. syringae* HopPtoD2 likely functions similarly to SptP (Figure 1-3) by targeting a defense-associated MAPK cascade (67). HopPtoD2 and its effects on *P. syringae* virulence will be discussed in more detail in Chapter 5.

Other effectors with known cellular targets: AvrPto and AvrPtoB

Another effector, AvrPto, enhances symptom development and slightly increases growth in populations of a *P. syringae* strain infecting susceptible tomato plants (37, 198). Upon translocation into host cells by the *hrp* TTSS, AvrPto is myristoylated or palmitoylated at the amino terminus to target AvrPto to the plasma membrane (Figure 1-3)(199). AvrPto was found to specifically interact with PTO, a Ser/Thr kinase similar to the IRAK-1 kinase of mammals (174). PTO, in turn, interacts with several PTO-interacting (PTI) proteins that seem to have a function in signaling (e.g., PTI1 is a kinase and PTIs 4, 5, and 6 are transcription factors) (174). These data suggest that PTO may function in a phosphorelay cascade necessary for a defense response.

By using PTO as the bait in a yeast two hybrid screen, a second effector that also interacts with PTO, AvrPtoB, has been identified in the *avrPto*-expressing *P. syringae* strain (Figure 1-3). AvrPtoB does not share any significant similarity with AvrPto and is much larger (128). Post-translocational modification of AvrPtoB has not been observed. However, AvrPtoB appears to promote virulence by acting as a general inhibitor of defense-associated PCD (1). AvrPtoB inhibited PCD induced by another *P. syringae* effector, a fungal elicitor of PCD, and even the mouse pro-apoptotic protein Bax. Ectopic expression of AvrPtoB inhibited development of PCD in yeast as well. Recently four other *P. syringae* secreted effectors have been shown to suppress PCD in plants and yeast (114), indicating that PCD suppression may be a common pathogenic mechanism for *P. syringae.* The specific mechanism by which

AvrPtoB and these other effectors inhibit PCD has not been established. Interestingly, both AvrPto and AvrPtoB also enhance *P. syringae* virulence in plant species that do not carry a homolog of PTO. Therefore, it is likely that both proteins target at least one other plant protein.

Other P. syringae TTSS-secreted effectors

For the majority of effectors translocated from *P. syringae* strains, the cellular fate, biochemical activity, and role in virulence have not been established. Some effector polypeptides have apparent chloroplast or mitochondrial localization signals, suggesting that their cellular targets are located in these organelles (84, 90). Like the above effectors, many seem to function in both pathogenicity in susceptible plants and elicitation of defense responses in resistant plants. Insertional inactivation of most *P. syringae* effectors, however, only slightly affects virulence, suggesting a redundancy of effector activity in host cells (73, 84).

Host Components Affecting Effector Phenotype

Disease resistance in plants and the phenotype of an effector can be linked in many cases to the genotype of the host. Closely related plant varieties can differ in their susceptibility to a particular pathogen and can differentially respond to a particular effector. For example, it is common for one variety of a plant species to be susceptible to a strain of *P. syringae* whereas another variety of the same plant

species will be stably resistant to that same strain. Genetic analysis of these varieties usually reveals that the resistant variety carries at least one genetically dominant "resistance (R) gene" that confers the ability to respond defensively to that *P. syringae* strain. Analysis of the corresponding *P. syringae* strain reveals a phenotypically dominant effector gene (e.g., the aforementioned *avr* or *hop* genes) that is required to elicit a defense response in the host variety expressing its cognate R gene (69).

Because of their role in disease resistance in plants, a number of R genes have been cloned and characterized (93). The most abundant and best studied class consists of cytoplasmic proteins composed of a central nucleotide binding (NB) motif and a leucine rich repeat (LRR) at the carboxyl terminus. These are structurally similar to NOD1 and to portions of Apaf1 involved in caspase activation and the initiation of apoptosis (PCD) in humans (211). It is thought that specificity of an R gene product resides in the LRR region in most, but not all, cases. The amino terminus of this group of R proteins contains either a TIR (Toll-like IL-1 receptor) or a coiled-coiled (CC) domain that are thought to function in signal transduction. In the *A. thaliana* genome, there are approximately 150 members of the NB-LRR protein family that could be R genes (158). Other classes of potential R genes have also been identified. A few include transmembrane domains whereas others have extracellular LRR domains as expected for a typical membrane-associated receptor (93).

Perception of P. syringae Effectors by Resistant Hosts

For many bacterial pathogens, activation of the defensive PCD response in resistant plants requires a functional TTSS and is linked to specific R genes. Many of the initially identified effectors were isolated due to their ability to elicit R-gene-dependent responses. Most early models had predicted that the translocated effectors would be ligands for receptors encoded by R genes (72, 102). However, direct interactions could not be detected in many cases. Further analysis suggested that in these cases recognition of an effector by an R gene product might be indirect: instead of detecting the presence of the effector itself, the R gene product was monitoring the status of the particular cellular target for the effector (54, 99). In this way, R gene products form a surveillance system that detects pathogen induced alterations in cellular targets to initiate defense responses.

For example, a direct interaction of the cysteine protease AvrRpt2 with its cognate R-gene product RPS2 could not be detected using several sensitive methods. Instead, the effect of AvrRpt2 activity on its cellular target RIN4 appeared to be the eliciting signal. RIN4 has been shown to physically interact with RPS2 at the plasma membrane (12, 137, 150) , and thus could be an intermediate in the recognition process (Figure 1-3). Initiation of RPS2-dependent resistance was correlated with proteolysis of RIN4 by AvrRpt2. Furthermore, RNAi-mediated depletion of RIN4 leads to a constitutive PCD phenotype in RPS2 plants and overexpression of RIN4 suppresses AvrRpt2-mediated elicitation of PCD in RPS2 plants (150). Thus, it

appears that AvrRpt2-directed elimination of RIN4 signals RPS2-mediated resistance. In this way, RPS2 senses RIN4 levels in cells and “guards” whatever role RIN4 plays in the host cell.

A slightly different process appears to occur during the induction of a defense response in plants expressing RPM1. RPM1 confers resistance to *P. syringae* strains expressing either AvrRpm1 or AvrB (20). Although the biochemical activity of these secreted effectors is not known, the mechanism by which they induce PCD in host cells is better understood. RPM1 is a peripheral plasma membrane protein that, like RPS2, also interacts with RIN4 (151). In contrast to AvrRpt2, however, AvrRpm1 and AvrB appear to induce phosphorylation of RIN4 via an unidentified kinase (Figure 1-3). This phosphorylation likely alters the activity of RIN4 to allow AvrRpm1 and AvrB to act as virulence factors in susceptible plants that lack the RPM1 allele (151). In resistant plants, however, RPM1 appears to detect or respond to the phosphorylated form of RIN4 to induce PCD. In this case, the R gene product appears to be sensing effector-dependent modification of the cellular target for the effector.

In an analogous manner, the R gene product RPS5 appears to monitor PBS1, a Ser/Thr kinase that is the cellular target for the cysteine protease AvrPphB. Proteolysis of PBS1 by AvrPphB is required for RPS5-mediated induction of defense responses (200, 230) (Figure 1-3). Following cleavage by AvrPphB, the residual PBS1 derivative retains kinase activity and is autophosphorylated. Inactivation of the

kinase activity of the PBS1 derivative blocks RPS5-dependent induction of a defense response (200). This suggests that a phosphorylated derivative or another phosphorylated substrate for 'PBS1 might interact with RPS5 to elicit the defense response.

Parallels also exist between the mechanism by which resistant plants detect the presence of AvrPto and AvrPtoB and the recognition of AvrPphB. The induction of PCD in resistant plants by AvrPto and AvrPtoB is dependent upon two host factors: PTO, the Ser/Thr kinase mentioned previously as the apparent cellular target of AvrPto, and PRF, a comparatively large (~210 kD) CC-NB-LRR protein (190). PRF is highly conserved in a wide variety of plant species and its structural properties are similar to the largest class of R gene products. Plants that have a deletion in PRF are more susceptible to *P. syringae* strains that express AvrPto and/or AvrPtoB, suggesting that PRF may be the R gene product that responds to AvrPto and AvrPtoB (174). The kinase activity of PTO is required for AvrPto-and AvrPtoB-induced PCD as well as signaling via the PTI proteins (174). This suggests that the kinase activity of PTO generates a phosphorylated intermediate that could be the actual ligand for PRF (Figure 1-3).

Irrespective of their mechanism of activation, NB-LRR proteins transduce signals through at least two different pathways to elicit a defense response. Mutations in either pathway render the plant susceptible to disease. CC-NB-LRR proteins appear to signal through one pathway whereas TIR-NB-LRR proteins activate a separate

pathway (93). The components of these pathways appear to be unique to plants. However, signals from both pathways converge on three proteins that have been well characterized in mammalian cells. Homologues of RAR1 and SGT1 are implicated in cell cycle control and development in other eukaryotes. In plants, the corresponding homologs interact with each other and are required for disease resistance mediated by many, but not all, CC-NB-LRR and TIR-NB-LRR R proteins (93, 99). SGT1 is part of an ubiquitylation complex in yeast that targets proteins for degradation. There is evidence that ubiquitylation could be an important component of defense signaling in plants (99). A third protein, the chaperone HSP90, also interacts with RAR1 and SGT1 and is required for R-mediated defense responses (219). As a result, it appears that the RAR1/SGT1/HSP90 complex is a convergence point for signal transduction mediated through both classes of NB-LRR proteins. The precise mechanism by which RAR1, SGT1 and HSP90 interact to mediate signaling has not been established. Many of the defense responses have also been linked to MAPK cascades as mentioned above (170).

Effector-mediated activation of any of these pathways leads to a wide range of responses in the adjacent cells of the plant. Changes in membrane permeability, increased production of reactive oxygen species (ROS) and nitric oxide (NO), activation of protein kinases, and transcriptional reprogramming are just some of the responses induced by pathogens in resistant plants (54, 93, 170). One of the earliest plant responses to pathogen infection is a change in membrane permeability that leads to an influx of calcium and an efflux of potassium (193). Calcium then initiates a

series of additional responses, possibly via calcium-dependent protein kinases. Both ROS production and PCD are dependent upon an increase in cytosolic calcium (170). An oxidative burst is typically observed 3-6 hours after infection and has been attributed to an increase in activity of a calcium-dependent NADPH oxidase. ROS and NO, in turn, stimulate salicylic acid (SA) production. The ROS, NO, and SA can act as secondary messengers to induce the neighboring cells to modify their cells walls, produce antimicrobial products, and activate defense gene expression (54).

Specific Objectives

P. syringae interactions with plant host cells are complex and involve many factors that help to regulate pathogenesis as well as parasitize the host cell. The interplay of these factors and how they affect pathogenesis is at the core of *P. syringae* biology. The overall objective of this research was to investigate factors that affected *P. syringae* pathogenesis. In particular, the specific objectives were:

1. Establish the role of HrpR and HrpS in the regulation of the *P. syringae hrp* regulon,
2. Identify factors that negatively regulate the *hrp* regulon,
3. Establish a mechanism by which environmental signals are transduced into the *hrp* regulon,
4. Identify and characterize effectors secreted by the *hrp* TTSS.

Chapter 2: HrpR and HrpS interact to regulate *hrp*-encoded type III protein secretion in *Pseudomonas syringae*

(The data presented in this chapter have been published in the Journal of Bacteriology, 2001, 183:5589-98 (ref. 105).)

Introduction

The colonization of plant tissue and elicitation of active defense responses by *P. syringae* strains have both been linked to the type III secretion system (TTSS) encoded by the *hrp* pathogenicity island (PAI). Like many other TTSSs, expression of the *P. syringae hrp* regulon is environmentally regulated. The operons carrying the *hrp/hrc* genes encoding structural components of the TTSS as well as the genes for secreted effector proteins, such as *avr* and *hop* genes, form the *hrp* regulon. *hrp* regulon expression is low during growth in most rich media containing broad spectrum amino acid sources and is induced during pathogenesis or by culture in an acidic minimal salts medium (181, 227, 240). The acidic minimal salts medium is thought to mimic conditions found in planta. It is unclear at present whether *hrp/avr* genes are regulated by host cell contact or via nutritional or physiological signals related to the growth conditions.

Several transcription factors have been identified that mediate the environmental regulation of the *P. syringae hrp* regulon. The primary transcription factor controlling expression of most *hrp, hrc, hop,* and *avr* genes is the alternative sigma

factor HrpL (238), a member of the ECF family of sigma factors (145). A HrpL-dependent promoter consensus sequence was identified (239) that is present in all known HrpL-dependent promoters (103) and is a required *cis*-acting element associated with transcription initiation (109, 191, 203). Related sigma factors controlling TTSSs have been identified in *Erwinia* strains carrying closely related group I *hrp* clusters (HrpL) (233) and in *Bordetella* (Trs) (246).

Because HrpL is the primary transcription factor controlling expression of *hrp* regulon genes, regulation of *hrpL* transcription may in part control the environmental regulation of the *hrp* regulon. HrpR and HrpS have been reported to be positive-acting regulators of *hrpL* expression (238). HrpR and HrpS are both unusual members of the enhancer binding family of proteins (58, 85, 86, 238) that normally function as response regulators of two-component regulatory systems (173). Most enhancer-binding proteins are typically modular, consisting of a large regulatory receiver domain (AB) , a central domain (C) involved in the interaction with σ^{54}, and an enhancer/upstream activating sequence (UAS) binding domain (D) (162, 173). Similar to other enhancer binding proteins, HrpR/HrpS retain the σ^{54} interaction (C) and DNA binding domains (D) (162, 172). However, unlike most enhancer binding proteins involved in two component systems, HrpR and HrpS do not contain the receiver domain that functions in phosphorylation-dependent modulation of response regulator activity (see (204)). Thus, HrpR and HrpS are similar to the stress response regulator PspF which also lacks these domains (119, 120).

The mechanism by which HrpR and HrpS regulate *hrpL* promoter activity has not been established. Xiao *et al* (238) reported that *hrpL* promoter activity in *E. coli* transformants was dependent upon the expression of both *hrpR* and *hrpS* and suggested that an interaction between the two proteins may be required to activate expression of the σ^{54} -dependent *hrpL* promoter. Grimm *et al* (85) reported that *hrpS* expressed from a plasmid-borne construct could rescue the ability of a *hrpR*::Tn*5* mutant of *P. syringae* NPS3121 to elicit the hypersensitive response in tobacco leaves. An apparent *hrpS* transcript was detected that appeared to initiate near a minimal σ^{54} promoter consensus sequence internal to the *hrpR* coding sequence. HrpS was thus proposed to function independently of HrpR to activate expression of the *hrp* regulon in *P. syringae* strains (85). Other bacteria carrying closely related group I *hrp* PAIs found in *Erwinia* strains carry an apparent HrpS homolog (127, 232) but not a HrpR homolog. Since other aspects of type III secretion in *Erwinia* strains appear to be similar to that of *P. syringae*, the role of *hrpR* in the regulation of group I *hrp* PAIs was unclear (101, 232). The purpose of the following experiments was to elucidate the role of HrpR and HrpS in the regulation of the *P. syringae hrp* regulon. The results indicate that HrpR and HrpS are expressed as an operon and form a stable heteromeric complex to regulate the σ^{54}-dependent *hrpL* promoter in *P. syringae* strains.

Experimental Procedures

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this work are listed in Table 2-1. *E. coli* strains were grown at 37ºC in King's B broth unless otherwise noted (9). *P. syringae* strains were grown in King's B Broth or M63 minimal salts medium supplemented with glucose, fructose, and/or 1% Casamino acids as indicated in the text. Antibiotics were included when indicated at the following concentrations (μg/ml): ampicillin (Amp), 200; kanamycin (Kan), 50; nalidixic acid (Nal), 50; rifampicin (Rif), 200; spectinomycin (Spc), 100; and tetracycline (Tet), 25.

General DNA manipulations: Basic manipulations were done using standard procedures (192). Restriction enzymes and related reagents were purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer's recommendations. Ligations were performed using T4 DNA Ligase (New England Biolabs, Beverly, MA). Plasmid DNA isolations and gel extractions were performed using kits manufactured by BioRad (Hercules, CA). Polymerase Chain Reactions (PCR) were performed using a Hybaid PCRSprint Thermal Cycler and employed either Taq (Invitrogen) or ProofPro (Continental Lab Products, San Diego, CA) polymerases.

Table 2-1: Bacterial Strains and Plasmids.

Bacterium or plasmid	Genotype or Description	Source or Reference
Strains		
Escherichia coli DH5α	EndA1 *hsd*R17 ($r_{k-}m_{k-}$) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 (*arg*F-*lacZ*YA) U169o80d*lacZ*DM15	Invitrogen
E. coli MC4100	F'Δ(*argF-lacZYA*)U169	(35)
P. syringae pv. *syringae* Psy61	Weak bean pathogen, Nal^R, HR^+	(100)
P. syringae pv. *tomato* DC3000	Tomato and *Arabidopsis* pathogen, Rif^R	(234)
Plasmids		
pDRR1R	690 bp *Bst*Y1 fragment from Psy61 cloned into pRG970 to create P_{hrpRS}-*lacZ*	(105)
pDSK519	incQ, Kan^R	(122)

Bacterium or plasmid	Genotype or Description	Source or Reference
pDSK600	incQ, Spc^R, triple *lacUV5* promoter, mcs	(164)
pJBR6R	697 bp PCR product amplified from DC3000 and cloned into pRG970 to create P_{hrpRS}-*lacZ*	This work
pJBR7R	1087 bp PCR product amplified from DC3000 and cloned into pRG970 to create P_{hrpS}-*lacZ*	This work
pNTRS3D	2 kb PCR product amplified from Psy61 genomic DNA containing the *hrpRS* coding region ligated as a *Xba*I/*Hin*dIII fragment into pDSK519	(105)
pRG970	incP, Spc^R, promoterless '*lacZYA* for constructing transcriptional fusions	(226)

Bacterium or plasmid	Genotype or Description	Source or Reference
pTSR4R	1087 bp PCR product amplified from Psy61 and cloned into pRG970 to create P_{hrpS}-*lacZ*	(105)
pYXL1R	340 bp PCR product amplified from Psy61 and cloned into pRG970 to create P_{hrpL}-*lacZ*	(238)

Sequence Analysis. Sequences were aligned using CLUSTALW at the BCM search launcher (www.searchlauncher.bcm.tmc.edu)(43, 206). Sequence data for *P. syringae* pv. *tomato* were obtained from The Institute for Genomic Research website at www.tigr.org (31). Accessions for DNA sequences used in these analyses are shown in Figures 2-2 and 2-3. Promoters were identified using the Neural Network Promoter Prediction Algorithm (www.fruitfly.org/seq_tools/promoter)(182).

Construction of *hrpR'-lacZ* and *hrpS* '-*lacZ* promoter fusions. To construct the reporter constructs using DC3000 sequences, the primers DC715 (5'-CGGATATCGACCGCTTTGCCAGTATCC) and DC1412 (5'-CGGGATCCTGATGACCCGCTGATAATGC) were used to amplify the R6 fragment and DC985 (5'- CGGATATCTCCGCTTGCCACCCACCA) and DC2045 (5'- CGGGATCCCTCGTCCAGATCATCCTCAA) were used to amplify the R7 fragment from DC3000 genomic DNA. These fragments were digested and subsequently ligated into *Sma*I-*Bam*H1 digested pRG970 as *Eco*RV-*Bam*H1 fragments to create pJBR6R carrying the *hrpRS* promoter and pJBR7R carrying the putative *hrpS* promoter.

RNA Extraction from *P. syringae* cells. RNA was isolated from cells grown for 3 hr in M63 medium pH 5.5 containing fructose as the carbon source using hot Trizol (Invitrogen) extraction as recommended by the manufacturer.

Primer Extension. Protocol modified from Mellies *et al* (156) and Grimm *et al* (85). RNA was extracted as previously described. Incubations for the primer extension were performed in a PCRSprint Thermal Cycler (Hybaid). Primer DC25609R (5'-CGTCGTTATCAATGTCTGTGCTC)(10 pmol) was end-labeled with T4 polynucleotide kinase using 50 µCi [γ^{32}-P] ATP (Amersham Biosciences, Piscataway, NJ). End-labeled primer (2 µl) was added to 10 µg whole cell RNA isolated from either Psy61 or *E. coli* MC4100 (pDRpR1R) and brought up to 12 µl with DEPC-treated water. After denaturation at 90ºC for 10 minutes, 4 µl 5X First Strand Buffer, 2 µl 0.1 M DTT and 1 µl 10 mM dNTPs were added. Primer annealing was performed at 63ºC for 2 minutes and then at 54ºC for 90 minutes. Following annealing, 200 units of SuperScript II Reverse Transcriptase (RT) (Invitrogen) were added to each reaction and extension was performed at 50ºC for 90 minutes. Following inactivation of the RT at 70ºC for 15 minutes, RNA was digested with RNase (10 mg/ml) at 37ºC for 20 minutes. DNA was precipitated with 100% EtOH, washed with 70% EtOH and the pellet was resuspended in 8 µl dH_20 and 4 µl stop solution. To identify the transcription start point, the region upstream of *hrpR* was sequenced using the same primer as used in the extension and purified pDRR1R as template. Sequencing reactions were labeled with [α^{32}-P] ATP using the dsDNA Cycle Sequencing System from Invitrogen. Extension products and sequencing reactions were analyzed on an 8% polyacrylamide sequencing gel and analyzed by autoradiography.

β-galactosidase assays. β-galactosidase activity in bacterial cells was estimated by the procedures of Miller (160).

Results

***hrpRS* is expressed as an operon.** To determine whether promoters were associated with *hrpR* and/or *hrpS*, fragments 5' to the *hrpR* and to the *hrpS* coding sequences from *P. syringae* pv. *syringae* Psy61 (R1, R4; Figure 2-1) or *P. syringae* pv. *tomato* DC3000 genomic DNA (R6 & R7) were cloned into the low copy number plasmid pRG970 to create transcriptional fusions to *'lacZYA*. The resulting constructs were confirmed by sequence analysis and transformed into Psy61 or DC3000. Cells were assayed for β-galactosidase activity during mid-log phase growth in the inductive M63 Fructose medium. Promoter activity was detected from the constructs carrying 259 bp upstream of the *hrpR* coding sequence, irrespective of the host bacterium (Table 2-2). Strains carrying this construct exhibited greater than 30-fold higher β-galactosidase activity than background. In contrast, little promoter activity was detected from the 1087 bp constructs that included the predicted HrpR-dependent regulatory site (R Box), the potential *hrpS* promoter (85), the *hrpRS* intergenic region and the coding sequence for the first 13 aa of *hrpS*. β-galactosidase levels in strains carrying these constructs expressed less than 40 Miller units of β-galactosidase activity. Similar results were obtained when these constructs were tested in *E. coli* MC4100.

Figure 2-1: Features of the *hrpRS* region and constructs used in experiments. Map of the *hrpRS* region is shown at the top. Shaded boxes represent deduced coding sequence for *hrpR* (R) and *hrpS* (S) (58, 238). The location of the *hrpRS* promoter (P_R) is shown by the arrow. The HrpR box (white bar) and *hrpS* promoter (black bar) are positioned as proposed by Grimm *et al* (85). Fragments used in the promoter experiments are indicated by the labeled lines that represent the portion of the mapped region carried by the fragment. Promoter fusions are shown by the arrowhead with the left face representing the left end of the fragment. Transcriptional fusions to '*lacZ* are indicated by the labeled box. Fragments R1, R3 and R4 were amplified from Psy61 while fragments R6 and R7 were amplified from DC3000.

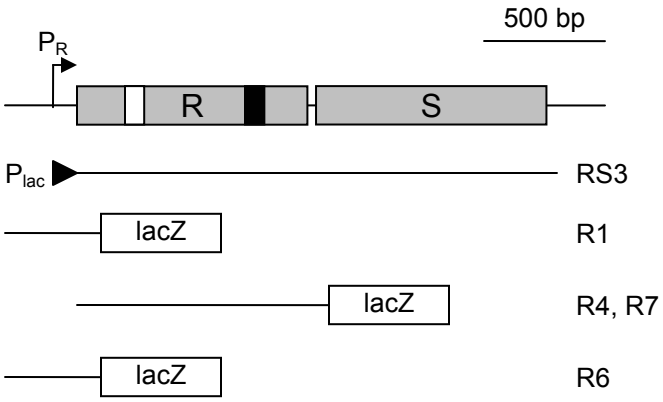

500 bp
P_R
R
S
P_{lac}
RS3
lacZ
R1
lacZ
R4, R7
lacZ
R6

Table 2-2: Activity of *hrpR*, *hrpS*, and *hrpL* promoter constructs in *P. syringae* pv. *syringae* Psy61, *P. syringae* pv. *tomato* DC3000 and *E. coli* MC4100.

Strain	*hrpRS* expressed[1]	β-galactosidase Activity from Promoter Fusion[2]:			
		None[3]	*hrpR*[4]	*hrpS*[5]	*hrpL*[6]
Psy61	-	3±1	417±16	31±1	348±16
	+	3±1	615±38	40±1	3469±184
DC3000	-	4±1	126±2	7±3	57±4
	+	6±1	147±4	9±1	216±8
MC4100	-	1±1	439±23	6±1	5±1
	+	1±1	364±19	5±1	271±5

[1] Indicated strains carrying pDSK519 (-) or pNTRS3D to ectopically express *hrpRS* (+).

[2] Strains carrying the indicated reporter construct were grown overnight to an OD600 of 1.0, harvested, washed and used to inoculate inductive M63 Fructose pH5.5 medium for *P. syringae* strains or KB medium for *E. coli* MC4100. After 6 hr growth, β-galactosidase activity was determined by the procedures of Miller (160). The data are reported as the mean of a single experiment done in triplicate. The error represents the standard deviation. Each experiment was repeated at least three times with similar results.

[3] Indicated strain carrying pRG970

[4] Psy61 carrying pDRR1R, DC3000 carrying pJBR6R, or MC4100 carrying pDRR1R

[5] Psy61 carrying pTSR4R, DC3000 carrying pJBR7R or MC4100 carrying pTSR4R

[6] Indicated strain carrying pYXL1R

Although the activity of the *hrpR* promoter construct indicates that *hrpR* was expressed in *P. syringae* strains during the conditions employed in the preceding experiments, additional experiments were performed using a plasmid-borne *hrpR* expression system. Consistent with previous results (238), vector-directed expression of *hrpRS* in Psy61 or MC4100 caused at least a 10-fold increase in *hrpL* promoter activity (Table 2-2), showing that the *hrpRS* expression system was functioning under the experimental conditions employed. In DC3000, a five-fold increase in *hrpL* expression was observed when HrpR and HrpS were over-expressed. Promoter activity of neither the *hrpR* promoter nor the putative *hrpS* promoter was substantially affected by the presence of the *hrpRS* expression system (Table 2-2). Levels of activity were similar irrespective of the presence of the *hrpRS* construct. Comparable results were obtained when a *hrpR* construct was employed (T. Sussan and S. Hutcheson, unpublished data). The absence of promoter activity in strains carrying the putative *hrpS* promoter even in the presence of expressed *hrpR* argues that the postulated HrpR-dependent *hrpS* promoter may be only weakly active in Psy61 and is inactive in DC3000 and *E. coli*, irrespective of the expression of *hrpR*. To determine if a transcript extends from *hrpR* into *hrpS,* S. Hutcheson performed RT-PCR on total RNA extracted from Psy61 cells. These experiments showed that a transcript is produced by these *P. syringae* strains containing the coding sequences for both *hrpR* and *hrpS* (105).

Conservation of *hrpRS* region in *P. syringae* strains. To determine if the *hrpRS* regulatory sequences might be unique to the strains examined above, the nucleotide

sequences of the *hrpRS* region of several *P. syringae* strains were compared. Within the region carried by the R4 construct, the Psy61 *hrpR* coding sequence exhibited 84% identity at the nucleotide level with the *P. syringae* pv. *phaseolicola hrpR* sequence. For comparison, the *hrpS* coding sequence retained 81% identity. The postulated HrpR box (Figure 2-2A) and the predicted *hrpS* promoter regions (Figure 2-2B) were also conserved in all strains examined. Highest divergence was detected in the noncoding intergenic region between *hrpR* and *hrpS* (Figure 2-2C). Although large for an intergenic region, this 45-50 bp region lacked motifs known to function as transcriptional terminators. The retention of major features of the region and the absence of significant sequence divergence in the region argue that regulation and expression of *hrpRS* are likely to be similar in all *P. syringae* strains, irrespective of their host range.

Expression of the *hrpRS* operon initiates at a putative σ^E promoter located 113 bp upstream of the HrpR start codon. The data presented above indicated that *hrpRS* was expressed as an operon and was expressed under similar conditions regardless of source strain. These data imply that a similar mechanism may be responsible for expression of *hrpRS* in each strain and that the *hrpRS* promoter region may be conserved between strains. To determine if the region upstream of the *hrpRS* operon was conserved between strains, the 200 bp upstream of the HrpR translational start codon were aligned (Figure 2-3). This analysis revealed an interesting dichotomy. The first 125 bp immediately upstream of the HrpR start codon were nearly 77% identical while the remaining 75 bp shared only 36% identity. The

Figure 2-2: Conservation of the *hrpR* coding sequence. Sequences were aligned using the ClustalW 1.8 program (43) using data in accessions U03853, U03852, NZ_AABP02000003, NC_004578, and NC_005773. **Panel A.** The proposed HrpR-responsive *hrpS* promoter element. The dashed lines represents the HrpR box proposed by Grimm *et al* (85); **Panel B.** The postulated σ^{54}-dependent *hrpS* promoter element. Conserved promoter elements (plus symbols) and the transcriptional initiation site (*) identified by Grimm *et al* (85) are shown; **Panel C.** The *hrpRS* intergenic region. The deduced ribosome binding site for *hrpS* is indicated by the "~" symbols.

A.

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                          ---------                    ---------------
Psy61    431 CGAGCAGGACAGGGCCCTTTGTCGGCATGAACTGTGCGGCCATCCCCGAGTCGTTGGCAG
B728a    169 CGAGCAGGACCGGGCCTTTTGTCGGCATGAACTGTGCGGCCATCCCCGAATCGTTGGCAG
DC3000   431 CCAGCAGGTCGGGACCCTTTGTGGGCATGAACTGCGCCGCCATTCCCGAGTCGCTGGCAG
1448A    361 CCAGCAGGTCCGGGCCCTTTGTCGGCATGAACTGCGCCGCCATCCCCGAATCGTTGGCAG

               --------------                 ---------
Psy61    491 AGAGTCAGCTATTCGGCGTGGTCAATGGTGCGTTCACCGGCGTGTGCCGCGCTCGCGAGG
B728a    229 AGAGCCAGTTGTTCGGCGTGGTCAATGGTGCGTTCACCGGCGTGTGTCGGGCTCGCGAGG
DC3000   491 AGAGCCAGTTATTCGGTGTGGTCAACGGTGCATTCACCGGCGTATGCCGGGCTCGCGAGG
1448A    421 AAAGCCAGTTATTCGGTGTCGTCAATGGCGCATTTACCGGTGTCTGCCGCGCTCGCGAGG
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B.

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                                      ++          ++          *
Psy61   1007 CGGTCGAAGCGCGTGACCCGGTAACAGGACTGCGCATGCAGATGCGCGTCATCGAGAAAA
B728a    748 CGGTCGAAGCGCGTGACCCGGTAACAGGGCTGCGCATGCAGATGCGCGTCATCGAGAAAA
DC3000  1007 CTGTGGAAGCGCTTGACCCTGCCACGGGGCTGCGCACGCAAATGCGCATCATCGAGAAAA
1448A    937 CAATGGATGCGCGCGATCCGGTGACAGGTCTGCGCATGCAGATGCGCGTGATCGAAAAGA
```

C.

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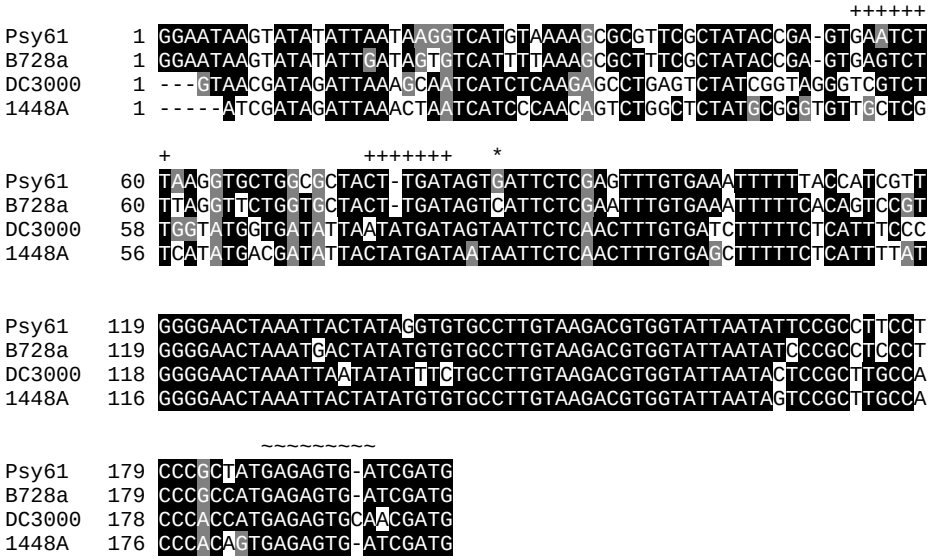
                                                        ~~~~~
Psy61   1207 TGAATCGCCGCTGCGCTCG---CGCAAC--GCCGATGCCATTGGAGTCATGAAATG
B728a    943 TGAATCGCTGCTGCGCTCG---CGCAAC--GCTGATGCCATTGGAGTCATGAAATG
DC3000  1210 TGA--CGCAGGTGTGGTTA---TCCAAC--GTTGATGTCCTGGGGGTCATGGGATG
1448A   1112 TAGATCGCAGCAACGCTGGAGTCCAAAAAGCTTATGTCTCGGGGGTCATGTGATG
```

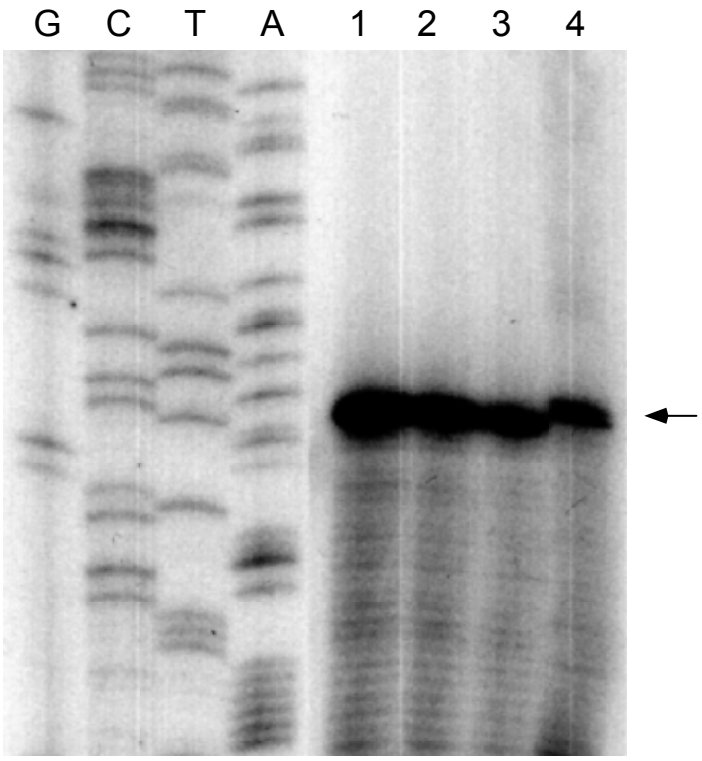
Figure 2-3: Conservation of the *hrpRS* promoter. Sequences were aligned using the ClustalW 1.8 program (43) and data in accessions U03853, U03852, NZ_AABP02000003, NC_004578, and NC_005773. Predicted σ^E promoter element is indicated by the plus symbols. Transcriptional start as determined by primer extension (see Figure 2-4) is marked with the asterisk (*). The deduced ribosome binding site for *hrpR* is indicated by the "~" symbols.


Psy61 1 GGAATAAGTATATATTAATAAGGTCATGTAAAAGCGCGTTCGCTATACCGA-GTGAATCT
B728a 1 GGAATAAGTATATATTGATAGTGTCATTTTAAAGCGCTTTCGCTATACCGA-GTGAGTCT
DC3000 1 ---GTAACGATAGATTAAAGCAATCATCTCAAGAGCCTGAGTCTATCGGTAGGGTCGTCT
1448A 1 -----ATCGATAGATTAAACTAATCATCCCAACAGTCTGGCTCTATGCGGGTGTTGCTCG
Psy61 60 TAAGGTGCTGGCGCTACT-TGATAGTGATTCTCGAGTTTGTGAAATTTTTTACCATCGTT
B728a 60 TTAGGTTCTGGTGCTACT-TGATAGTCATTCTCGAATTTGTGAAATTTTTTCACAGTCCGT
DC3000 58 TGGTATGGTGATATTAATATGATAGTAATTCTCAACTTTGTGATCTTTTTCTCATTTCCCC
1448A 56 TCATATGACGATATTACTATGATAATAATTCTCAACTTTGTGAGCTTTTTCTCATTTTAT
Psy61 119 GGGGAACTAAATTACTATAGGTGTGCCTTGTAAGACGTGGTATTAATATTCCGCCTTCCT
B728a 119 GGGGAACTAAATGACTATATGTGTGCCTTGTAAGACGTGGTATTAATATCCGCCTCCCT
DC3000 118 GGGGAACTAAATTAATATATTTCTGCCTTGTAAGACGTGGTATTAATACTCCGCTTGCCA
1448A 116 GGGGAACTAAATTACTATATGTGTGCCTTGTAAGACGTGGTATTAATAGTCCGCTTGCCA
Psy61 179 CCCGCTATGAGAGTG-ATCGATG
B728a 179 CCCGCCATGAGAGTG-ATCGATG
DC3000 178 CCCACCATGAGAGTGCAACGATG
1448A 176 CCCACAGTGAGAGTG-ATCGATG

conserved nature of the region directly upstream of *hrpR* indicates that it plays an important role in *hrpRS* expression, possibly by encoding the *hrpRS* promoter. To determine the location of the *hrpRS* promoter, primer extension analysis was used to identify the location of transcription initiation for the *hrpRS* operon of Psy61. Transcription was shown to initiate 113 bp upstream of the ATG translation start site for HrpR (Figure 2-4). In *E. coli* carrying the *hrpRS* promoter on pDRR1R, transcription initiated 114 bp upstream of the start codon for HrpR. Interestingly, transcription initiation occurred just downstream of the border between the divergent and conserved sequences mentioned in Figure 2-3. Further analysis revealed a putative σ^E promoter directly upstream of the transcription initiation site in Psy61 (Figure 2-3). Noting that transcription initiates in the conserved region and that *hrpRS* expression patterns are similar between Psy61 and DC3000, it appears likely that the *hrpRS* promoter is found in this location for at least both these strains. The significance of the divergent sequence upstream of the putative *hrpRS* promoter is not known, but may play a role in strain-specific regulation of *hrpRS* expression.

Maximal Activation of the *hrpL* promoter requires both HrpR and HrpS. As demonstrated above, HrpR and HrpS function as positive-acting regulatory factors for the *hrpL* promoter. To determine whether HrpR or HrpS could individually function as activators of the *hrpL* promoter, S. Jin cloned the coding sequences for *hrpR* and *hrpS* together with their native ribosome binding sites into pDSK600 or pLAFR3 such that the cloned genes were expressed from vector P_{lac} promoters. These studies found that significantly higher levels of *hrpL* promoter activity were

Figure 2-4: Primer extension analysis of the *hrpRS* promoter region. Primer extension reactions were performed using whole cell RNA extracted from Psy61 (lanes 1-3) or MC4100 (pDRR1R) (lane 4). RNA was extracted from cultures incubated in the *hrp*-inductive M63 media with fructose (lane 1) or the *hrp*-repressive M63 media with fructose and casamino acids (lane 2) or KB media (lanes 3 and 4). Sequencing reaction was performed using purified pDRR1R and primer DC25609R, the same primer as in the extension. The sequence on the left is the reverse complement of the region upstream of *hrpR*. Transcriptional start point is identified by the arrow and is 113 bp upstream of the HrpR start codon in Psy61 (see Figure 2-3). In *E. coli*, transcription of the *hrpRS* operon on pDRR1R initiates 114 bp upstream of the HrpR start codon.


G C T A 1 2 3 4

detected in strains expressing: i) *hrpR* and *hrpS* expressed from separate plasmids; ii) a reconstructed *hrpRS* operon; and iii) *hrpRS* as a native construct (105). As these activities were all more than 37-fold higher than the activity induced by *hrpS* alone, these results indicated that maximal activation of the *hrpL* promoter requires expression of both *hrpR* and *hrpS*.

HrpR and HrpS physically interact. One possible interpretation for the requirement of both HrpR and HrpS in the activation of the *hrpL* promoter is that the two proteins physically interact. S. Hutcheson and T. Sussan used a yeast two hybrid assay (16, 75) and column binding experiments to demonstrate that HrpR and HrpS could interact (105). These results indicated that a strong physical interaction formed between the two proteins (105).

Discussion

The *hrp*-encoded TTSS is central to the pathogenicity of *P. syringae* strains. Although the characterization of the regulatory system controlling assembly of the *hrp*-encoded TTSS is still incomplete, it is clear that expression of *hrp* genes in *P. syringae* is coordinated by the activity of HrpL. HrpL is an alternative sigma factor required for transcription of the operons encoding structural elements of the TTSS as well as the genes for the secreted effector proteins (103). As the only factor presently thought to affect HrpL activity is protein turnover, expression of *hrpL* is likely to be critical to the assembly of the *hrp*-encoded TTSS of *P. syringae*. The results

presented above indicate that the expression of *hrpL* is controlled in part at the transcriptional level by the interaction of two unusual enhancer-binding proteins, HrpR and HrpS.

HrpR and HrpS retain most of the structural features conserved in other members of the enhancer binding protein family that function in transcriptional regulation of σ^{54}-dependent promoters (162, 172). Consistent with these features, HrpR and HrpS activated the σ^{54}-dependent *hrpL* promoter (238). This promoter contains a σ^{54} promoter consensus sequence (238) and transcription of *hrpL* initiates 12 bp downstream of this promoter motif (S. Heu and S. Hutcheson, unpublished results). *hrpL* expression in *P. syringae* pv. *maculicola* was reported to be dependent upon *rpoN* (96, 97).

In contrast to other known enhancer binding proteins, both HrpR and HrpS were required for maximal activation of the *hrpL* promoter. *hrpS* expressed from a strong promoter on a multicopy number plasmid could only function as a weak activator of *hrpL* promoter activity (105). This activity was less than 2.5% of the activity detected when both *hrpR* and *hrpS* were expressed in a cell irrespective of the promoter construct used to drive expression. As the proposed HrpR-linked *hrpS* regulatory sequences internal to *hrpR* (85) were physically separated from *hrpS* in these experiments, it appears unlikely that HrpR directly influences transcription of *hrpS* in these constructs. The simplest explanation for these results is that both proteins are required to fully activate the *hrpL* promoter. The observation that HrpS

can act as a weak activator of the *hrpL* promoter provides an explanation for the reported plant response positive phenotype of a *P. syringae hrpR* mutant carrying a *hrpS* expression construct (85). Relatively little *hrp* expression appears to be necessary to assemble the *hrp*-encoded TTSS (240). Ectopic expression of *hrpS* would have induced at least some expression of the *hrp* regulon and thus allowed the *hrp*-encoded TTSS to be assembled.

Consistent with the requirement for both proteins in the activation of *hrpL* expression, *hrpR* and *hrpS* were shown to be expressed as an operon. The only fragment from the *hrpRS* region with significant promoter activity was 5' to *hrpR* and a transcript encompassing both *hrpR* and *hrpS* was detected by RT-PCR analysis. Although some sequence divergence was detected in the *hrpRS* region, most involved silent codon substitutions. The conservation of the *hrpRS* region argues that *hrpRS* are transcribed as an operon in all *P. syringae* strains. Primer extension analysis revealed a σ^E-like promoter 113 bp upstream of the HrpR start codon in Psy61. While it appears that *hrpRS* is transcribed as an operon in all strains, regulation of *hrpRS* expression in distinct strains may be different as sequences upstream of the *hrpR* promoter were not well conserved.

A requirement for both HrpR and HrpS in the activation of the *hrpL* promoter could indicate that HrpR either activates HrpS or forms a stable complex with HrpS. In either model, HrpR and HrpS would be expected to physically interact. The yeast two hybrid experiments demonstrated that a physical interaction between HrpR and

HrpS can occur. This apparent strong interaction was confirmed in column binding experiments. Another enhancer binding protein, NtrC, has been proposed to form a homodimer that upon phosphorylation assembles into a larger oligomeric activator complex (237). Dimerization involves the C-terminus of the protein (129). The ability of HrpR and HrpS to form a stable complex during column binding experiments in the absence of a target promoter suggests that these proteins form a heteromeric complex prior to activation of the *hrpL* promoter. Similar to NtrC the formation of this complex could involve the C-terminal domain of HrpR (105).

HrpR and HrpS lack the 130 aa receiver (AB) domain that is typically found in most other members of the protein family(162, 173). The receiver domain has been proposed to be a repressor of ATP hydrolysis in the absence of kinase-mediated phosphorylation or binding of a regulatory effector molecule (204). The absence of the receiver domains argues that HrpR and HrpS do not require posttranslational modification, such as phosphorylation or the binding of an effector molecule, to activate the target promoter. Consistent with this hypothesis, vector-directed expression of *hrpRS* as minimal coding sequence produced a functional activator complex in *E. coli* transformants. HrpR and HrpS are thus functionally similar to *E. coli* PspF (119, 120) or truncated derivatives of DctD (134) and XylR (176). These proteins lack the AB receiver domain and are also constitutively active. The activity of HrpR and HrpS would thus appear to be independent of a direct posttranslational modification mechanism, such as phosphorylation, but posttranslational modification by a broadly conserved mechanism cannot be fully excluded (see Chapter 3).

Regulation of the *P. syringae hrp* PAI shares some similarities to the regulatory system controlling flagellar biosynthesis. Flagellar biosynthesis has been proposed to be a form of a TTSS. Three classes of promoters have been identified for genes involved in the assembly of flagella (44). At the top of the regulatory system is the class 1 promoter for *flhCD*. Once expressed, FlhC and FlhD interact to form a FlhD/FlhC complex that then activates expression of class 2 promoters. FliA, expressed from a class 2 promoter, functions as an alternative sigma factor to direct expression of class 3 promoters. Like FlhD/C, HrpR and HrpS are expressed as an operon and form a complex. However, there is little if any sequence similarity between FlhD/FlhC and HrpR/HrpS. At present the only known target for the HrpR/HrpS complex appears to be the *hrpL* promoter but other HrpR/HrpS-dependent promoters may exist in cells. HrpL is a sigma factor related to FliA that directs expression of the HrpL-dependent regulon. These later promoters would be analogous to the class 3 promoters of flagellar biosynthesis. Although the HrpR/HrpS-HrpL regulatory system is superficially similar to the FlhD/C-FliA regulatory system, the genes controlled at each level of these regulatory systems are distinct. In flagellar biosynthesis, the genes encoding the TTSS are considered to be class 2 operons, although there is some influence of FliA on their expression (44, 152), whereas the *hrp* TTSS counterparts could be considered to be equivalent to the class 3 operons.

The HrpR/HrpS regulatory system also shares some similarity to the RcsB/RcsA system regulating capsular biosynthesis in several bacterial species (81). RcsB interacts with RcsA to regulate *cps* expression. RcsB is part of a two-component regulatory system involving RcsC. The RcsB/RcsC system can activate low level expression of the *cps* genes but acts "synergistically" with RcsA. RcsA is present at limiting levels in which RcsA levels are regulated by turnover mediated by Lon protease. HrpS appears to be able to activate low level expression of the *hrp* regulon but requires HrpR for maximal activity. A similar situation occurs in the regulation of *Erwinia amylovora hrp* genes. The *Erwinia* HrpS can initiate expression of the *hrpL* promoter but requires HrpX for maximal activity (232). HrpX is an enhancer binding protein that is part of a classic two component regulatory system involving a phosphorelay. As mentioned above there is no evidence at present to indicate that HrpR or HrpS functions as part of a two component regulatory system, and in contrast to *rcsA* and *rcsBC*, *hrpRS* are expressed as an operon.

Unresolved at present is the mechanism by which environmental signals generated during pathogenesis are transduced to alter *hrp* expression. The proposed regulatory system appears to represent a regulatory cascade in which expression of the *hrp* regulon could be controlled by the expression of *hrpRS* in a manner analogous to the role of PspF in the regulation of stress genes in *E. coli* (120) and *flhCD* in flagellar biosynthesis (44). *hrpS* transcript levels have been reported to be repressed in DC3000 during growth in repressive media (231). Other results suggest that *hrpRS* expression is constitutive in several strains because *hrpRS* transcripts could be

detected by primer extension and RT-PCR, irrespective of the growth conditions. Interestingly, significant differences in the *hrpR* promoter were observed between *P. syringae* strains as described above. This opens the possibility of strain-specific regulation of *hrpRS* expression. In contrast to the *hrpR* promoter, the *hrpL* promoter was observed to be environmentally regulated (103). This argues that additional factors must mediate the environmental regulation of the *hrp* cluster in addition to HrpR/HrpS, and HrpL.

Chapter 3: Lon protease functions as a negative regulator of type III protein secretion in *Pseudomonas syringae*

(The data presented in this chapter have been published in Molecular Microbiology, 2002, 45:397-409 (ref. 27).)

Introduction

Like the TTSS of most pathogenic bacteria, expression of the *hrp* encoded TTSS in *P. syringae* is environmentally regulated (107, 181, 227, 240). Expression of the *hrp* regulon is minimal during growth in media containing a complete amino acid source, such as casamino acids (240). During pathogenesis, enhanced expression of the *hrp* regulon genes can be detected beginning 1-2 h after infiltration into the tissue (181, 240). It is not known what environmental signals induce *hrp* expression during pathogenesis. However, growth in an acidic minimal salts medium, which is thought to mimic conditions in planta, can also induce expression of the *hrp* regulon (107, 240, 245). Stringent conditions seem to be a significant requirement for induced expression of the *hrp* regulon (240).

The primary transcriptional factor controlling expression of the *hrp*–encoded TTSS in *P. syringae* is the alternative sigma factor, HrpL (238). HrpL is a member of the extracytoplasmic functions (ECF) family of transcription factors (145) and recognizes a conserved promoter sequence upstream of all known HrpL-dependent genes (70, 239). Transcription of *hrpL*, in turn, is controlled by a σ^{54}-dependent *hrpL* promoter (96, 238) that requires HrpR and HrpS for activity (Chapter 2). HrpR and HrpS,

expressed from the *hrpRS* operon, are unusual members of the enhancer binding protein family (172). Both HrpR and HrpS lack the modulating receiver domain found in most other members of the protein family and do not appear to be part of a two component regulatory system (238). Instead, HrpR and HrpS physically interact to activate *hrpL* promoter activity (Chapter 2). Thus expression of the *hrp* regulon is controlled by an apparent regulatory cascade in which the *hrpRS* promoter controls the expression of the *hrpRS* operon, the HrpR/HrpS complex activates expression of the *hrpL* promoter and HrpL directs transcription of the remaining genes in the *hrp* regulon (Figure 1-2)(105, 238). In addition to the positive transcriptional factors described, HrpV has been proposed to be a negative regulator of the *hrp* regulon (179). Ectopic expression of *hrpV* suppressed expression of the *hrp* regulon. HrpV activity could be reversed by ectopic expression of *hrpRS*. HrpV has no known homologs in the databases.

The mechanisms by which environment signals are transduced into this apparent regulatory cascade to control expression of the *hrp* regulon during pathogenesis were not obvious from the transcriptional factors mediating expression of the *hrp* regulon. The purpose of the following experiments was to identify factors involved in environmental regulation of the *hrp* regulon. The experiments demonstrated that environmental regulation of the *hrp* regulon occurs primarily at the level of the *hrpL* promoter and involves Lon protease-mediated degradation of HrpR. Lon-associated degradation of HrpR was reduced during conditions inductive to *hrp* regulon expression. Lon mutants were found to elicit rapid plant responses.

Experimental Procedures

Bacterial strains and plasmids. Strains and plasmids are described in Table 3-1. Oligonucleotide primers are listed in Table 3-2. *Pseudomonas syringae* strains were routinely grown at 25˚C in KB broth (9) or M63 minimal salts medium (205) containing 1mM $MgSO_4$ and 0.2% fructose and/or 1% casein hydrolysate as indicated in the text. *E. coli* strains were grown at 37˚C in KB media. Antibiotics were added as indicated to the media at the following concentrations [μg/ml]: ampicillin (Amp), 100; kanamycin (Kan), 50; nalidixic acid (Nal), 50; rifampicin (Rif), 200; spectinomycin (Spc), 100; and tetracycline (Tet), 100.

General DNA manipulations. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich, St. Louis, MO) method (10). Plasmid DNA was isolated using a kit manufactured by BioRad (Hercules, CA). Restriction enzymes and related reagents were purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer's directions. Ligations were performed using T4 DNA Ligase (New England Biolabs, Beverly, MA) according the manufacturer's directions.

Electroporation. Electrocompetent cells were prepared in 10% glycerol. Plasmid DNA was transformed into competent cells using a BioRad Gene Pulser

Table 3-1: Bacterial Strains and Plasmids.

Bacterium or plasmid	Genotype or Description	Source or Reference
Strains		
Escherichia coli DH5α	EndA1 *hsd*R17 ($r_{k-}m_{k-}$) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 (*arg*F-*lacZ*YA) U169o80d*lacZ*DM15	Invitrogen
E. coli MC4100	F'Δ(*argF-lacZYA*)U169	(35)
E. coli SG22622	MC4100 derivative, *cpsB::LacZ*	S. Gottesman
E. coli SG22623	MC4100 derivative; *cpsB::lacZ lon::Tn10*	S. Gottesman
P. syringae pv. *syringae* Psy61	Weak bean pathogen, Nal^R, HR^+	(100)
P. syringae pv. *syringae* Psy61-2082	Psy61 derivative, *hrcN*::Tn*phoA*	(100)

Bacterium or plasmid	Genotype or Description	Source or Reference
P. syringae pv. *syringae* KL5, KL11, KL26, KL32	Tnp mutants of Psy61, *hrp* constitutive, *lon*::Tnp, Kan^R	This work
P. syringae pv. *syringae* KLW	Tnp mutant of Psy61, *hrp* inducible, Kan^R	This work
P. syringae pv. *tomato* DC3000	Tomato and *Arabidopsis* pathogen, Rif^R	(234)
P. syringae pv. *tomato* JB7	Tnp mutant of DC3000, *hrp* constitutive, *lon*::Tnp, Kan^R	This work
Plasmids		
pBluescript SK^+	ColE1, Amp^R	Stratagene
pDRR1R	690 bp *Bst*Y1 fragment cloned into pRG970 to create P_{hrpRS}-*lacZ*	Chapter 2
pDSK600	incQ, Spc^R, triple *lacUV5* promoter, mcs	(164)
pFLAG-MAC	N-terminal FLAG-tag expression vector, Amp^R	Sigma

Bacterium or plasmid	Genotype or Description	Source or Reference
pJBLONIL	pLAFR3 carrying a 2697 bp PCR fragment with the Psy61 *lon* gene	This work
pJBR26	*Eco*RI-*Hind*III restriction fragment containing His-HrpR from pSHR4Q30 cloned into pDSK600	This work
pJBS25	*Eco*RI-*Hind*III restriction fragment containing His-HrpS from pSH23Q30 cloned into pDSK600	This work
pKPA3R	*Hrp*A promoter-*lacZ* fusion in pRG970	(104)
pLAFR3	incP-1 cosmid vector, Tet^R	(210)
pQE30	N-terminal 6xHis tag expression vector, Amp^R	Qiagen

Bacterium or plasmid	Genotype or Description	Source or Reference
pRG970	incP, Spc^R, promoterless '*lacZYA* for constructing transcriptional fusions	(226)
pRG970ΔHIII	pRG970 derivative lacking the 3 kb *Hind*III fragment to create a constitutively expressed *aadA2-lacZ* fusion	This work
pSHR4Q30	0.9 kb PCR product cloned into pQE30, P_{lac}-6xHis-'*hrpR*	This work
pSHS23Q30	0.9 kb PCR product cloned into pQE30, P_{lac}-6xHis-'*hrp*S	(105)
pTSR4MAC	1087 bp PCR product cloned into pFLAG-MAC, FLAG-'*hrpR*	(27)
pYXL1R	340 bp PCR product cloned into pRG970, P_{hrpL}-*lacZ*	(238)

Table 3-2: Oligonucleotide Primers.

Primer Name	Sequence (5'-3')
LB16	ATCTACGAAAACAACGAGCC
LB257	CTTCATGATTTCGCCTCTAC
LB258	CGGGATCCTAGAGGCGAAATCATGAAGAC
LB548	GTGGTTCGGTAGAGCGTTTT
LB755	ACCAGGCGACCAGGCTCA
LB999	CCACAACGAAATCGAAGAGC)
LB1534	CGACAAGATGGGCAGCGA
LB2140	TTCGCTCGGGGATGTGATG
LB2249	GCGTGTCGTGTTTCTCGTG
LB2476	CGTGCGTGATTTGAAGGAGA
LB2743	GGCTTGCCAGTGATTGCTTT
LB2955	CCCAAGCTTCAAAGCCAACCAAAACAACC
LB3091	TCTCTATGCCTTTGCCCGCT

electroporation unit set at 2000V, 25 μF capacitance, and 200 ohm resistance. After 1 h outgrowth, cells were plated onto selective media.

β -galactosidase activity. Psy61 derivatives were screened for β-galactosidase activity on KB agar plates after application of X-gal top agar. Plates with visible colonies were overlaid with molten 0.75% water top agar containing 0.07% X-gal and incubated at 4˚C. The plates were scored for β-galactosidase activity after 24 h. *E. coli* strains were screened for β-galactosidase activity on MacConkey agar supplemented with 1% lactose.

For quantitative estimation of β-galactosidase activity, cell cultures were grown overnight in the indicated medium with selecting antibiotics. Cells were harvested, transferred to fresh medium and adjusted to an OD_{600} of approximately 0.5-1.0. After incubation for up to 3 hours, β-galactosidase activity was determined as described by Miller (160).

Transposome mutagenesis. Transposome mutagenesis was conducted using an EZ::TN™ Transposome™ mutagenesis kit purchased from Epicentre Technologies (Madison, WI). The transposome (Tnp) was introduced into the cells by electroporation as described above and after outgrowth for the recommended time, mutants were selected on media containing Nal, Spc and Kan, for Psy61 or Rif, Spc, and Kan for DC3000. Only one $LacZ^+$ transformant was chosen from each transformation for further study.

Plant Assays. Overnight cultures grown at 25˚C were harvested and diluted in sterile distilled water. Tobacco leaves were syringe infiltrated in parallel with 10^6-10^9 cells as described previously (100) and incubated at 25˚C. Infiltrated leaf panels were scored for responses beginning 2 hours after inoculation. Virulence of DC3000 was determined in *Arabidopsis thaliana* ecotype Columbia (Col0) leaves infiltrated with 10^5 cells/ml. Populations were monitored using the leaf disk assay of Bertoni and Mills (18).

Curing of pKPA3R from the mutants. Bacterial cells carrying the incP-1 reporter construct pKPA3R were made electrocompetent in 10% glycerol and transformed with the incP-1 plasmid pLAFR3. Tet^R transformants were grown in KB Tet broth culture and transferred daily to fresh media. Periodically the cultures were screened for Nal^R Tet^R Spc^S colonies for Psy61-derived strains or for Rif^R Tet^R Spc^S colonies for DC3000-derived strains. To cure the resulting derivatives of pLAFR3, selection for pLAFR3 was dropped and cultures serially grown in KB Nal or KB Rif media, respectively. Nal^R Kan^R Tet^S (Psy61) or Rif^R Kan^R Tet^S (DC3000) colonies were picked for subsequent analyses.

Mapping of transposome insertions. To construct genomic libraries of mutants, isolated genomic DNA was partially digested with *Eco*RI and ligated into *Eco*RI-digested pBluescript SK^+. The resulting library was transformed by electroporation into Electromax DH5α (Invitrogen) cells and Amp^R Kan^R transformants selected.

Plasmids from transformants were isolated and nucleotide sequence obtained using primers KAN-2 FP1 and KAN-2 RP1 provided with the EZ::TN^{TM} kit.

DNA Sequencing and Analysis. Plasmid DNA was sequenced at the University of Maryland Biotechnology Institute using an ABI Model 3100 Automated Sequencer. The sequence of the regions adjacent to the Tnp insertion sites allowed 1578 bp of the 2397 bp ORF to be assembled. The remaining sequence was determined by primer walking using primers LB16, LB257, LB 548, LB755, LB999, LB1534, LB2140, LB2249, LB2476, LB2743, and LB3091. Raw sequence data were assembled using MacDNASIS Pro v3.0. Sequences were aligned using CLUSTALW at the BCM search launcher (www.searchlauncher.bcm.tmc.edu)(43, 206). Sequence data for *P. syringae* pv. *tomato* were obtained from The Institute for Genomic Research website at www.tigr.org (31). Accessions for DNA sequences used in these analyses are shown in Figure 2B. Promoters were identified using the Neural Network Promoter Prediction Algorithm (www.fruitfly.org/seq_tools/promoter) (182). The sequence for the Psy61 has been submitted to GenBank under accession AF447727.

Construction of Psy61 *lon* clone. The *P. syringae lon* gene was amplified from Psy61 genomic DNA using primers LB258 and LB2955. After PCR, the fragment was digested with *Bam*HI and *Hind*III and gel purified using the Prep-A-Gene DNA Purification Master Kit (BioRad). The resulting fragment was ligated into *Bam*HI/*Hind*III-digested pLAFR3 and transformed into Electromax DH5α cells (Invitrogen). Plasmids were isolated from Tet^{R} colonies and inserts confirmed by

PCR. One plasmid carrying Psy61 *lon* (pJBLON1L) was transformed into electrocompotent *E. coli* SG22622 or SG22623.

UV Sensitivity. Overnight KB cultures were adjusted to an OD_{600} of 1.0, harvested and cells resuspended in equal volume 0.9% NaCl. 5 ml aliquots of cells were irradiated at 250 mW/cm^2 in uncovered petri plates for the indicated time. Initial and surviving cells were determined by plate counts.

Stability of HrpR and HrpS. Overnight cultures of SG22622 or SG22623 expressing pSHS23Q30 or pSHR5Q30 were diluted into fresh medium and incubated for one hour at 37˚C. Following the addition of 1 mM IPTG, cultures were incubated at 37˚C until OD_{600} ~1.0. For *P. syringae* strains, overnight cultures of strains expressing pJBR26-600 or pJBS25-600 were diluted into the indicated medium and incubated for 4-6 h at 25˚C. After incubation, Tet (500 μg/ml) was added to block translation. Cells were harvested at the specified time points, lysed in sample buffer and fractionated by SDS-PAGE in 15% polyacrylamide gels.

Immunoblots. Proteins fractionated by SDS-PAGE were electroblotted onto PVDF or nitrocellulose membranes in Tris-Glycine buffer pH 8.3 containing 20% MeOH. Membranes were then blocked with 5% dry milk in PBS-Tween20 and incubated with anti-RGSHis antibody (Qiagen) in 3 % BSA in PBS-Tween20 for 1 h at room temp. Membranes were washed 2 x in PBS-Tween20 and incubated with a 1:3000 dilution of anti-mouse IgG-horseradish peroxidase conjugant (BioRad) in 5 % dry

milk in PBS-Tween20 for 1 hr at RT. Membranes were washed 3 x in PBS-Tween20 and once in PBS and immunoreactive proteins detected by using an ECL detection kit (Amersham Biosciences, Piscataway, NJ).

Results

The *hrpRS* promoter exhibits high basal activity during growth in media non-inductive to *hrp* expression. To determine if the *hrpRS* promoter is environmentally regulated as postulated, a plasmid-borne reporter construct carrying the *hrpRS* promoter fused to '*lacZ* was monitored for activity in *P. syringae* Psy61 and *P. syringae* pv. *tomato* DC3000 grown under *hrp* inductive or non-inductive conditions. Reporter constructs carrying *hrpRS*'-'*lacZ* fusions exhibited high basal expression irrespective of the growth conditions. Psy61 (pDRR1R) typically expressed 250-300 Miller units (MU) of activity during logarithmic growth in the *hrp* non-inductive KB or M63F Cas media (Table 3-3). Expression levels detected were 40-50% those observed in inductive media. Similar results where obtained for DC3000 (Table 3-4). Expression of a control construct (pRG970ΔHIII) carrying a '*lacZ* transcriptional fusion to the constitutively expressed *aadA2* promoter was unaffected by the growth conditions, indicating that the observed effects were unlikely to be artifacts of the reporter system. Constitutive expression of the *hrpRS* promoter was confirmed by RT-PCR. The *hrpRS* transcript could be detected at nearly equivalent levels irrespective of the growth conditions (S. Hutcheson, unpublished results). In

Table 3-3: Activity of the *hrpRS* and *hrpL* promoters in Psy61 during growth in *hrp* non-inductive and inductive media.

Growth Medium[1]	Media Type[2]	Promoter Activity (MU)[3]		
		hrp RS	*hrpL*	Control
KB	N	300±9	26±1	1525±28
M63F Cas	N	242±31	27±1	1458±114
M63F	I	585±14	214±4	1483±70

[1] Medium used for broth culture of the indicated bacterial strains. KB, King's medium B; M63F Cas, M63 minimal salts medium contain 0.2% fructose and 1% casein hydrolysate, pH7.0; M63F, M63 minimal salts medium containing 0.2% fructose, pH5.5.

[2] Non-inductive (N) or inductive (I) for *hrp* expression as determined previously (240).

[3] Psy61 carrying the indicated plasmid-borne promoter fusion were grown to an OD_{600} of 0.5-1.0, harvested and the β-galactosidase activity determined by the procedures of Miller (160). Accumulated β -galactosidase activity, reported in Miller Units (MU) is used as an estimate of promoter activity. The *hrpRS* promoter was carried by pDRR1R. The *hrpL* promoter fusion was provided by pYXL1R. The Lac^+ control was transformed with pRG970ΔHIII carrying a constitutively expressed transcriptional fusion between *aadA2* and '*lacZ.*

Table 3-4: Activity of the *hrpRS* and *hrpL* promoters in DC3000 during growth in *hrp* non-inductive and inductive media.

Growth Medium[1]	Media Type[2]	Promoter Activity (MU)[3]		
		hrp RS	*hrpL*	Control
KB	N	87±3	12±1	1345±9
M63F Cas	N	92±5	25±1	1276±27
M63F	I	128±10	104±4	1291±156

[1] Medium used for broth culture of the indicated bacterial strains. KB, King's medium B; M63F Cas, M63 minimal salts medium contain 0.2% fructose and 1% casein hydrolysate, pH7.0; M63F, M63 minimal salts medium containing 0.2% fructose, pH5.5.

[2] Non-inductive (N) or inductive (I) for *hrp* expression as determined previously (240).

[3] DC3000 carrying the indicated plasmid-borne promoter fusion were grown to an OD_{600} of 0.5-1.0, harvested and the β-galactosidase activity determined by the procedures of Miller (160). Accumulated β -galactosidase activity, reported in Miller Units (MU) is used as an estimate of promoter activity. The *hrpRS* promoter was carried by pDRR1R. The *hrpL* promoter fusion was provided by pYXL1R. The Lac^+ control was transformed with pRG970ΔHIII carrying a constitutively expressed transcriptional fusion between *aadA2* and '*lacZ.*

contrast, the *hrpL* promoter exhibited only modest activity during growth in non-inductive media but showed 10- to 20-fold increases in activity during growth in inductive media.

Isolation of *hrp* constitutive mutants. The observations that the *hrpRS* operon was constitutively expressed coupled with the previous observations that HrpR and HrpS were fully active as transcriptional factors once expressed (Chapter 2) suggested that some form of negative regulation must modulate the activity of HrpR/HrpS. If the *hrp* regulon is subject to one or more forms of negative regulation, it should be possible to isolate mutants constitutively expressing the *hrp* regulon.

In collaboration with K. Lisboa, EZ::TN™ transposome (Tnp) mutagenesis was performed on Psy61 in an attempt to identify the postulated negative regulator(s). Tnp mutagenesis was carried out on Psy61 (pKPA3R) cells carrying a plasmid-borne HrpL-dependent *hrpA* promoter fused to '*lacZ*. The resulting mutants were screened for a Lac^+ phenotype on *hrp* repressive media. Of the 15,378 Kan^R Psy61::Tnp mutants screened, twenty one independent mutants from separate transformations were identified that consistently exhibited a $LacZ^+$ phenotype during growth on the non-inductive KB medium. A similar frequency of apparent *hrp* constitutive mutants was detected after Tnp mutagenesis of DC3000 (pKPA3R). The mutants retained diagnostic markers of the parent strains, such as antibiotic resistance and ability to elicit the HR in tobacco leaves. No significant change in the virulence of the DC3000 Tnp mutant JB7 was detected. With the exception of an unexplained spike in

population size experience by both strains on day 2, populations of JB7 in inoculated *Arabidopsis thaliana* ecotype Columbia were indistinguishable from the populations detected in tissue inoculated with DC3000 (Figure 3-1).

In the parental strain as well as in the control Tnp mutant KLW that exhibited wild-type *hrp* expression, basal expression of the *hrpA'-lacZ* reporter construct used to monitor *hrp* regulon expression resulted in the accumulation of less than 10 Miller units (MU) of β-galactosidase activity during logarithmic growth in the non-inductive M63F Cas medium (Table 3-5). In contrast, a representative *hrp* constitutive mutant, KL11 (pKPA3R), exhibited greater than 250 MU, consistent with the $LacZ^+$ phenotype observed on X-gal plates. The LacZ activity detected in the mutant grown under non-inductive conditions was equivalent to the activities observed in the wild-type strain grown in inducing media. Growth of the KL11 mutant in inducing media caused a further, but modest, stimulation of reporter expression. Levels detected in inducing media were roughly 50% higher than basal expression observed in the KL11 (pKPA3R) strain grown on non-inducing media. Strains cured of the reporter construct reverted to the wild-type $LacZ^-$ phenotype and only 9 MU of basal β-galactosidase activity were detected in the cured KL11 strain. Similar results were obtained with 3 other cured Psy61::Tnp mutants.

***hrp* constitutive mutants carry insertions in a *P. syringae lon* homolog.** To identify the mutated genes in the *hrp* constitutive mutants, Tnp carrying fragments were selected from genomic libraries of mutants (Psy61-KL5, KL11, KL26, and

Figure 3-1: Populations of DC3000 (closed symbols) and the *hrp*-constitutive Tnp mutant JB7 (open symbols) are indistinguishable in *Arabidopsis thaliana*. *A. thaliana* ecotype Columbia (Col-0) leaves were infiltrated with 10^5 CFU/ml. Bacterial populations were monitored over 5 days using the leaf disc assay developed by Bertoni and Mills (18).

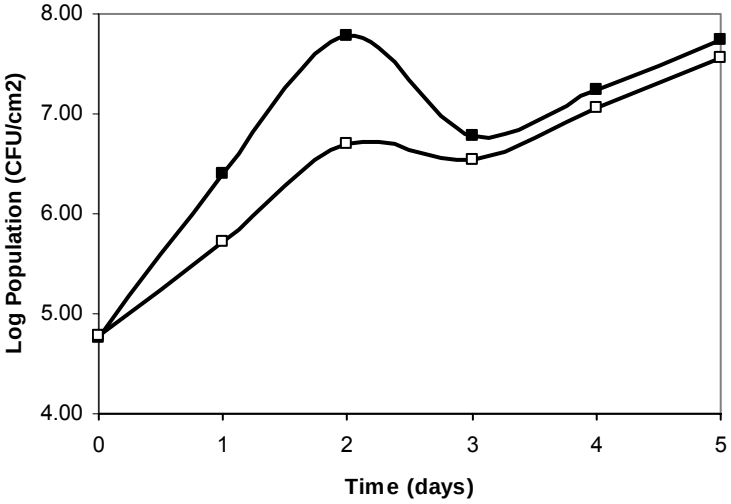

8.00
7.00
6.00
5.00
4.00
Log Population (CFU/cm2)
0
1
2
3
4
5
Time (days)

Table 3-5: β-galactosidase activity of the apparent *hrp* constitutive mutants.

Strain	Media Type[1]	β-galactosidase Activity (MU)[2]	
		Original	Cured[3]
Psy61	N	10±1	8±1
	I	258±4	ND[4]
KLW[5]	N	2±1	19±1
	I	256±6	ND
KL11[6]	N	256±6	9±1
	I	393±17	ND

[1] M63F Cas media was used for the non-inductive (N) conditions and M63F (pH 5.5) media was used for the inductive (I) conditions.

[2] The indicated strains carrying pKPA3R (original) or cured of the reporter construct (cured) were grown to an OD_{600} of 0.5-1.0, harvested and the β-galactosidase activity determined by the procedures of Miller (160). Accumulated β -galactosidase activity, reported in Miller Units (MU) is used as an estimate of promoter activity.

[3] Strains were cured of the pKPA3R plasmid as described in the Experimental Procedures.

[4] ND, not determined

[5] *hrp*-inducible Tnp mutant.

[6] *hrp*-constitutive Tnp mutant. Similar results were obtained with Tnp mutants KL5, KL26, and KL32.

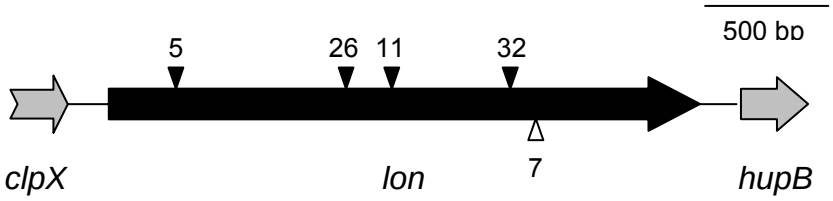
KL32) and the regions flanking the Tnp insertion sites sequenced. The Tnp insertions were found to be independent insertions into a single open reading frame (ORF; Figure 3-2A).

The complete ORF from Psy61 encoded a 798 aa, 88.8 kD deduced product that exhibited 94% identity and 96% similarity across its entire length with Lon protease from *P. fluorescens* (235)(Figure 3.2B). Strong similarities were also detected to the Lon proteases of *P. aeruginosa* (213), *E. amylovora* (63) and *E. coli* (45). A homolog was also identified in the partially completed *P. syringae* pv. *tomato* DC3000 genome that exhibited 98% similarity/97% identity with that of Psy61. All known Lon protease domains were conserved in the *P. syringae* homologs. The ATP binding domain (45), the catalytically active Ser 674 (6) and the substrate discriminator domain (64) were present. As observed in the *E. coli*, *P. aeruginosa* and *E. amylovora* genomes, homologs of *clpX*, encoding another energy-dependent protease (82), and *hupB*, producing a histone-like protein (56), were positioned adjacent to the *P. syringae lon* homologs. An apparent noncoding region of 207 bp separated *clpX* and *lon*. *lon* was separated from *hupB* by an apparent noncoding region of 150 bp that included an apparent *hupB* promoter.

Although the coding sequence for the *P. syringae lon* homolog was highly conserved, the 207 bp intergenic region expected to carry a conserved heat shock-inducible *lon* promoter (45) retained little similarity with that of its counterparts in *P. fluorescens* (235), *P. aeruginosa* (213) and *E. coli*. Minimal sequence similarity could be

Figure 3-2: Properties of the *P. syringae lon* locus. **A.** Map of the *lon* locus as determined by sequence analysis. The nucleotide sequence of 3747 bp was determined as in the Experimental Procedures. Open reading frames (ORFs) within the region are shown by block arrows. The filled triangles indicate the location of Tnp insertions identified in the mutants KL5, KL11, KL26 and KL32 and the open triangle shows the Tnp insertion site in JB7 as determined by sequence analysis of flanking sequences. A 2397 bp ORF was identified within the sequenced region that encodes a 798 amino acid protein and is preceded by typical ribosomal binding site (AGAGG). A weak σ^{70} promoter (TTGCATT N17 TTTTGT) is located 121 bp upstream of the start codon for the ORF. Similarities of the deduced product to Lon proteases are shown in part B. Homologs to *clpX* and *hupB* were identified as indicated. The C-terminal 191 aa of ClpX exhibited 89% similarity/82% identity with its homolog of *P. aeruginosa*. The deduced *P. syringae* HupB product retained 93% similarity/87% identity with *P. aeruginosa* HupB. **B.** Similarity of the Psy61 Lon protease to its homologs of other bacteria. GenBank accession numbers and percent identity (I) and similarity (S) as determined by the BLAST algorithm (5) are shown. The E values reported by the BLAST algorithm for all sequences shown were 0.

A.



B.

Strain	Accession/ Reference	Length (AA)	% identity	% similarity
Psy61	AAM97840	798	-	-
DC3000	AA057193	798	97	98
P. fluorescens	AAF65564	798	94	96
P. aeruginosa	AAG05192	798	87	92
E. coli	AAA24078	784	69	82
E. amylovora	CAA54779	784	68	81

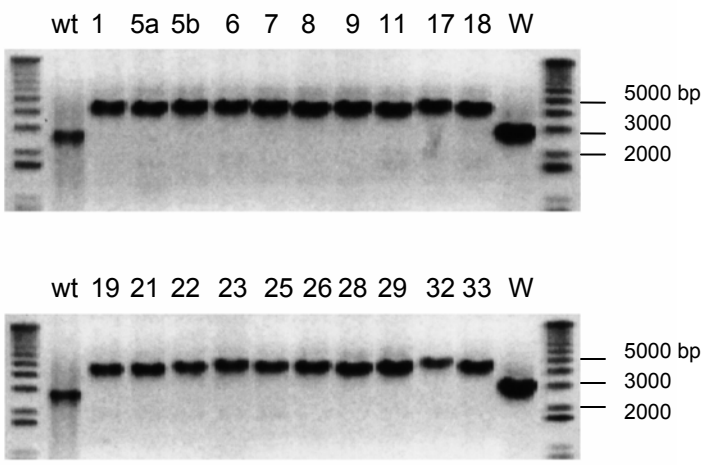
detected for the region extending from the stop codon for the *clpX* homolog to 9 bp upstream of the deduced ribosome-binding site for the *P. syringae lon* homolog for both Psy61 and DC3000. A weak candidate σ^{70}–dependent promoter was identified in this region. As a result, the regulation of *P. syringae lon* expression may be distinct from its counterpart in *E. coli* (See Chapter 4).

To determine if any of the other *hrp* constitutive mutants isolated in the original screen contained insertions in *lon*, the mutants were screened for insertions by PCR using primers LB258 and LB2955. A Tnp insertion into *lon* was detected in the 21 Psy61::Tnp hrp constitutive mutants and in the DC3000::Tnp *hrp* constitutive mutant JB7 (Figure 3-3). Sequence analysis of JB7 confirmed insertional inactivation of *lon* in this strain (Figure 3-2A).

The *P. syringae hrp* constitutive mutants exhibit phenotypes typical of Δ*lon* mutants. Lon protease is an ATP-dependent protease that has been associated with regulation of RcsA-dependent synthesis of the extracellular polysaccharide colanic acid in enteric bacteria (80) and can affect cell size and UV sensitivity by its effect on SulA levels (161, 195). In *P. syringae* strains, regulation of alginate biosynthesis is independent of Lon protease and RcsA (123). As expected, mucoidy of the *P. syringae lon*::Tnp mutants was indistinguishable from that of the parental strains after growth on KB agar medium or on M63 minimal salts medium containing 1% casamino acids and 5% glucose, fructose or sucrose as the carbon source. K. Lisboa and S. Hutcheson demonstrated that the *P. syringae lon*::Tnp mutant cells did

Figure 3-3: PCR analysis of *P. syringae hrp*-constitutive mutants. To determine if the *hrp* constitutive mutants contained a Tnp insertion in *lon*, the mutants were screened using intact cell PCR and the primers LB258 and LB2955. In wild-type strains, these primers amplify a 2.7 kb fragment. A Tnp insertion at this site yields a 3.9 kb product. Strain name is listed above each lane. Wild-type (wt) strains are either Psy61 or DC3000, respectively. **A.** *hrp* constitutive mutants derived from Psy61. Strain numbers are preceded by the prefix KL. KLW is the *hrp* inducible Tnp mutant, **B.** *hrp* constitutive mutants derived from DC3000. Strain numbers are preceded by the prefix JB.

A.


wt 1 5a 5b 6 7 8 9 11 17 18 W
5000 bp
3000
2000
wt 19 21 22 23 25 26 28 29 32 33 W
5000 bp
3000
2000

B.

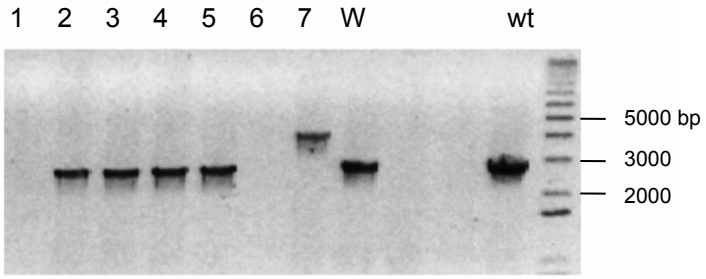
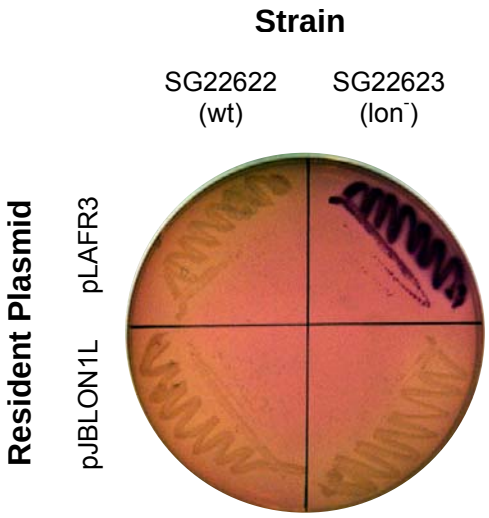

1 2 3 4 5 6 7 W wt
5000 bp
3000
2000

exhibit increased cell length compared to the parent strain and also exhibited enhanced sensitivity to UV light as observed in *E. coli lon* mutants (K. Lisboa and S. Hutcheson, unpublished results) (195).

***P. syringae lon* is functionally equivalent to *E. coli lon*.** To determine if the *P. syringae lon* homolog was functionally equivalent to its *E. coli* counterpart, the *P. syringae lon* gene was cloned into the low copy number plasmid, pLAFR3. The resulting plasmid carrying the Psy61 *lon* homolog (pJBLON1L) was then transformed into *E. coli* strains SG22622 (Lon^+) or SG22623 (Lon^-)(gifts of S. Gottesman) and transformants screened for Lac phenotype. SG22623 exhibits a Lac^+ phenotype due to increased expression of the RcsA-dependent *cpsB*::*lacZ* fusion (153). When transformed with the plasmid expressing the cloned *P. syringae lon*, this strain reverted to the wild-type Lac^- phenotype similar to that of the Lon^+ SG22622 strain (Figure 3-4). The strong sequence similarity, apparent Lon^- phenotype of the *hrp* constitutive mutants, and the complementation of an *E. coli lon* mutant by the Psy61 *lon* construct are consistent with the identification of the Psy61 Lon protease as a negative regulator of the *hrp* regulon. As has been observed by others, overexpression of *lon* is often lethal (46). As a result, attempts to directly complement the *P. syringae lon*::Tnp insertions were unsuccessful due to instability of the cloned construct in *P. syringae* strains.

Figure 3-4: The Psy61 *lon* gene can complement an *E. coli Δlon* mutant. The Psy61 *lon* gene was amplified by PCR and cloned into the plasmid pLAFR3 as described in Experimental Procedures. The resulting plasmid, pJBLON1L, was transformed into *E. coli* SG22622 (Lon^+, *cpsB::lacZ*) or SG22623 (Lon^-, *cpsB::lacZ*) and transformants were scored for Lac phenotype on lactose MacConkey plates.


Strain
SG22622
(wt)
SG22623
(lon⁻)
Resident Plasmid
pLAFR3
pJBLON1L

The activity of the *hrpL* promoter is enhanced in *P. syringae lon*::Tnp mutants. To determine at which level Lon protease influences the expression of the *hrp* regulon, promoters from each level of the *hrp* regulatory system were tested for activity in a representative *P. syringae lon*::Tnp mutant. The KL11 mutant cured of the original reporter construct was transformed with the plasmids: pDRR1R carrying a *hrpRS*'-'*lacZ* fusion; pYXL1R carrying a HrpR/HrpS-dependent *hrpL*'-'*lacZ* fusion; or pKPA3R carrying a HrpL-dependent *hrpA*'-'*lacZ* fusion. Transformants were monitored for β-galactosidase activity during logarithmic growth in non-inductive media. The activity of the *hrpRS* promoter construct in KL11 was only 1.5-2.0-fold higher than that observed in parent Psy61 (Table 3-6). A much stronger effect, however, was observed on the activity of the *hrpL* promoter. Psy61 (pYXL1R) typically expresses 25-35 MU of activity during growth on non-inductive media. The *lon* mutant KL11 (pYXL1R), in contrast, expressed between 6- to 10-fold higher β-galactosidase activity than observed in Psy61 (pYXL1R). Expression of the HrpL-dependent *hrpA* promoter was similarly enhanced in KL11. Basal β-galactosidase activity detected in wild-type Psy61 (pKPA3R) was 10-24 MU of activity, whereas KL11 (pKPA3R) expressed a 10-fold higher level. Since constitutive expression of *hrpL* would in turn increase expression of the HrpL-dependent *hrpA* promoter, these observations are consistent with the conclusion that Lon protease affects the activity of the *hrpL* promoter.

HrpR and HrpS levels are higher in Δ*lon* mutants of *E. coli.* Because the HrpR/HrpS-dependent regulation of the *hrpL* promoter shares some similarities with

Table 3-6: Activity of *hrp* promoter fusions in *P. syringae lon* mutants.

	Promoter Activity (MU)[1]			
Strain	*hrp RS*	*hrpL*	*hrpA*	Control
Psy61	205±17	35±1	24±1	1535±20
KLW[2]	253±4	30±2	10±1	1502±20
KL11[3]	367±22	180±6	207±1	1395±46

[1] *P. syringae* strain carrying the indicated plasmid-borne promoter fusion were grown in the non-inductive M63F Cas media to an OD_{600} of 0.5-1.0, harvested, and the β-galactosidase activity determined by the procedures of Miller (160). Accumulated β -galactosidase activity, reported in Miller Units (MU) is used as an estimate of promoter activity. The *hrpRS* promoter was carried by pDRR1R. The *hrpL* promoter fusion was provided by pYXL1R. The *hrpA* promoter fusion was provided by pKPA3R. The control was transformed with pRG970ΔHIII carrying a constitutively expressed transcriptional fusion between *aadA2* and '*lacZ*.

[2] *hrp* inducible Tnp mutant

[3] *hrp* constitutive Tnp mutant

the RcsA/RcsB-dependent regulation of *cps* gene expression and the FlhD/C-mediated regulation of flagellar biosynthesis (Chapter 2), both of which are potentially influenced by Lon activity (47, 83), the effect of Lon on HrpR and HrpS levels was examined. Levels of FLAG-tagged HrpR (pTSR4MAC) ectopically expressed from a vector promoter were at least 2-fold higher in total cell lysates of Lon^- SG22623 than in Lon+ SG22622 (Figure 3-5). A similar effect was detected on His-tagged HrpS (pSHS23Q30). To determine the half-life of the two proteins in *E. coli*, the stability of the two proteins was monitored. In Lon^+ SG22622 (pTSR4MAC), FLAG-HrpR had an apparent half-life of 5 min whereas in SG22623 FLAG-HrpR exhibited an apparent half-life of >30 min (Figure 3-6). His-HrpS was more stable than FLAG-HrpR in the Lon^+ SG22622 (half-life > 8 min). The His-tagged DHFR (dihydrofolate reductase) control and the FLAG-tagged BAP (bovine alkaline phosphatase) control were equally stable in both strains (half-lives > 30 min), suggesting that the epitope tag did not stimulate the observed Lon-mediated degradation directly.

High turnover of HrpR detected in Lon^+ *P. syringae* cells grown in non-inductive media. To determine the effect of Lon protease on the stability of HrpR and HrpS in *P. syringae* strains, the fragments carrying the His-HrpR and His-HrpS fusions from pSHR5Q30 and pSHS23Q30 were transferred into pDSK600 to create pJBR26-600 and pJBS25-600 for ectopic expression of HrpR and HrpS, respectively, from vector P_{lac} promoters in *P. syringae* strains. (In the *E. coli* strains, stability of His-HrpR and His-HrpS expressed from these constructs was similar to the stability exhibited by the

Figure 3-5: Levels or HrpR and HrpS in *E. coli* cells are linked to the activity of Lon protease. Overnight cultures of *E. coli* strains SG22622 (lon^+) or SG22623 (lon^-) carrying pTSR4MAC or SHS23Q30 were diluted into fresh media and induced with IPTG. Cells were incubated until OD_{600}=1.0. Whole cell lysates were prepared and 10μg total protein was analyzed by SDS-PAGE. HrpR and HrpS levels were estimated in immunoblots using NIH Image 1.59. Relative levels of protein are shown below each image.

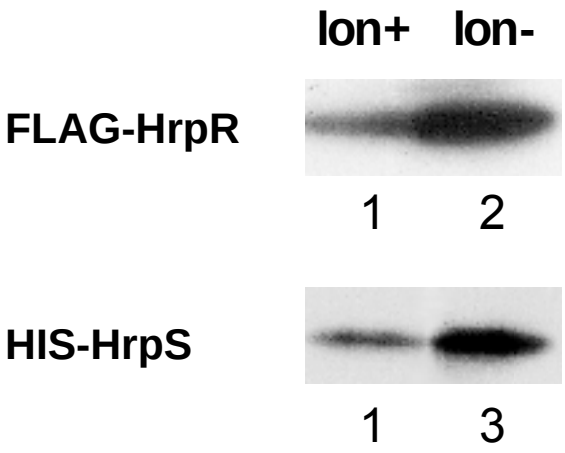
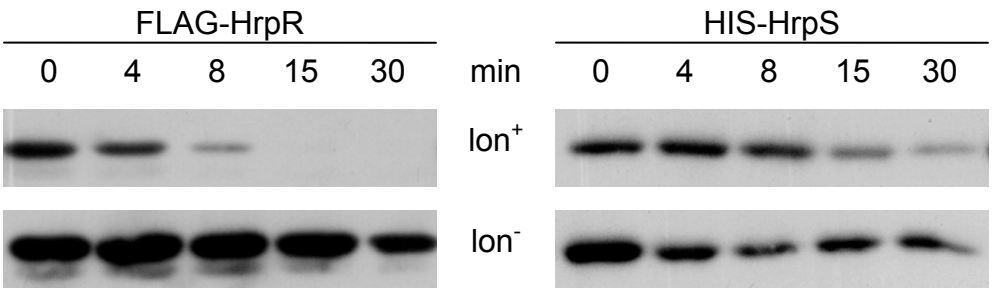
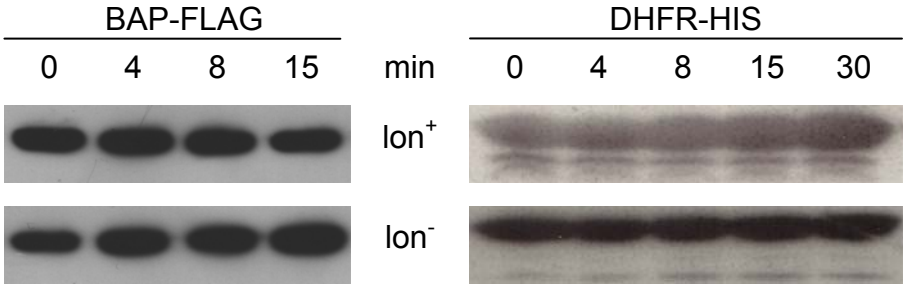

lon+
lon-
FLAG-HrpR
1
2
HIS-HrpS
1
3

Figure 3-6: Effect of Lon protease on the stability of HrpR and HrpS in *E. coli*. Overnight culture of *E. coli* strains SG22622 (Lon^+) and SG22623 (Lon^-) carrying pTSR4MAC (FLAG-HrpR) or pSHS23Q30 (His-HrpS) were diluted and induced with IPTG. Cells were incubated at 37ºC until OD600=1.0 and translation stopped by the addition of spectinomycin. Whole cell lysates were prepared at the indicated times and residual levels of HrpR and HrpS determined using immunoblots. Levels were quantified and plotted versus time to estimate half-lives. **A.** FLAG-HrpR was rapidly degraded in the wild-type Lon^+ stain, exhibiting an apparent half-life of approximately 5 minutes. In the Lon^- strain, HrpR had an apparent half-life of >30 minutes. His-HrpS exhibited a half-life of approximately 8 minutes in the Lon^+ strain and 30 minutes in the Lon^- strain. **B.** The control strains expressed BAP-FLAG and DHFR-His from the plasmid pATS-BAP and pQE16, respectively. Both exhibited apparent half-lives of >30 minutes irrespective of the presence of Lon.

A.


FLAG-HrpR
HIS-HrpS
0 4 8 15 30
min
0 4 8 15 30
lon+
lon-

B.


BAP-FLAG
DHFR-HIS
0 4 8 15
min
0 4 8 15 30
lon+
lon-

proteins expressed from the constructs used above (Figure 3-7).) Stability of HrpR and HrpS were then monitored in Lon^+ Psy61 and DC3000 or in Lon^- KL11 and JB7 transformants. Consistent with the effects of non-inductive and inductive media on *hrpL* promoter activity, growth medium affected the stability of HrpR. The His-HrpR fusion was unstable in the Lon^+ transformants grown in non-inductive media in which minimal expression of the *hrpL* promoter was detected. The apparent half-life of the His-HrpR fusion in Psy61 or DC3000 grown under these conditions was 3-6 min (Figure 3-8). In contrast, HrpR appeared to be relatively stable in cells grown in inductive media. The half-life of HrpR expressed in either Psy61 or DC3000 during growth in M63F medium was greater than 35 min. The effect of medium on HrpR stability was absent in the *lon*::Tnp mutants. In the *lon*::Tnp mutants, KL11 or JB7, the apparent half-life of the His-HrpR construct was greater than 35 min, irrespective of the growth conditions. Thus the observed turnover of HrpR during non-inductive conditions is associated with regulated proteolysis mediated by Lon.

In contrast, the His-HrpS fusion was relatively stable in the tested strains, irrespective of the growth medium or the presence of Lon. The half-life of His-HrpS in Psy61, KL11, DC3000 and JB7 was greater than 35 min during growth in a non-inductive medium (Figure 3-9). The observation that the His-HrpR fusion exhibited Lon-associated degradation whereas the His-HrpS fusion did not argues that the observed turnover was not an artifact of the fusions employed. Although a role for the epitope tags in targeting these proteins for degradation cannot be fully excluded, the effect of Lon protease on the activity of the *hrpL* promoter coupled with the

Figure 3-7: Effect of Lon protease on the stability of HrpR and HrpS in *E. coli*. Overnight culture of *E. coli* strains SG22622 (Lon^+) and SG22623 (Lon^-) carrying pJBR26-600 (His-HrpR) or pJBS25-600 (His-HrpS) were diluted and induced with IPTG. Cells were incubated at 37ºC until OD600=1.0 and translation stopped by the addition of tetracycline. Whole cell lysates were prepared at the indicated times and residual levels of HrpR and HrpS determined using immunoblots. Levels were quantified and plotted versus time to estimate half-lives. **A.** His-HrpR was rapidly degraded in the wild-type Lon^+ stain, exhibiting an apparent half-life of approximately 5 minutes. In the Lon^- strain, HrpR had an apparent half-life of >35 minutes. **B.** His-HrpS exhibited a half-life of approximately 10 minutes in the Lon^+ strain and 30 minutes in the Lon^- strain.

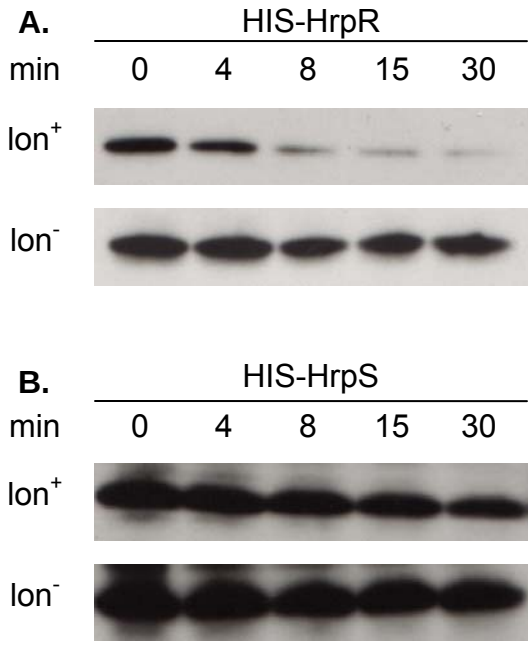

A.
HIS-HrpR
min
0
4
8
15
30
lon+
lon-
B.
HIS-HrpS
min
0
4
8
15
30
lon+
lon-

Figure 3-8: Effect of Lon protease on the stability of HrpR in *P. syringae*. **A.** Levels of HrpR detected in immunoblots. Overnight cultures of Psy61 (Lon^+), KL11 (Lon^-), DC3000 (Lon^+), or JB7 (Lon^-) carrying pJBR26-600 were diluted into M63 Fructose medium with (M63F Cas) or without (M63F) 1% casein hydrolysate. Cells were incubated at 25ºC until OD_{600}=1.0 and translation stopped by the addition of excess tetracycline. Whole cell lysates were prepared at the times (min) indicated above the lane and residual levels of HrpR determined using immunoblots. His-HrpR migrated as a 43 kD protein. **B**. Estimation of half-lives in Psy61 and KL11. Levels of His-HrpR in Psy61 (Squares) or KL11 (Diamonds) grown in M63F Cas (filled symbols) or M63F (open symbols) were quantified in scanned images using NIH Image 1.59. Estimated levels from a single experiment are shown but comparable results were obtained in at least 3 independent experiments. His-HrpR was calculated to have an apparent half-life of 3 minutes in the wild-type Lon^+ strain grown in the non-inductive M63F Cas medium. In the Lon^- strain grown under either growth condition or in the wild-type strain grown in the inductive M63F medium, His-HrpR had an apparent half-life of greater than 35 minutes. **C.** Estimation of half-lives in DC3000 and JB7. Levels of His-HrpR in DC3000 (Squares) or JB7 (Diamonds) grown in M63F Cas (filled symbols) or M63F (open symbols) were quantified in scanned images using NIH Image 1.59. Estimated levels from a single experiment are shown but comparable results were obtained in at least 3 independent experiments. His-HrpR exhibited an apparent half-life of 5.8 min in Lon^+ cells grown in M63F Cas whereas the half-life was more than 45 min in the Lon^- JB7 or M63F grown DC3000 cells.

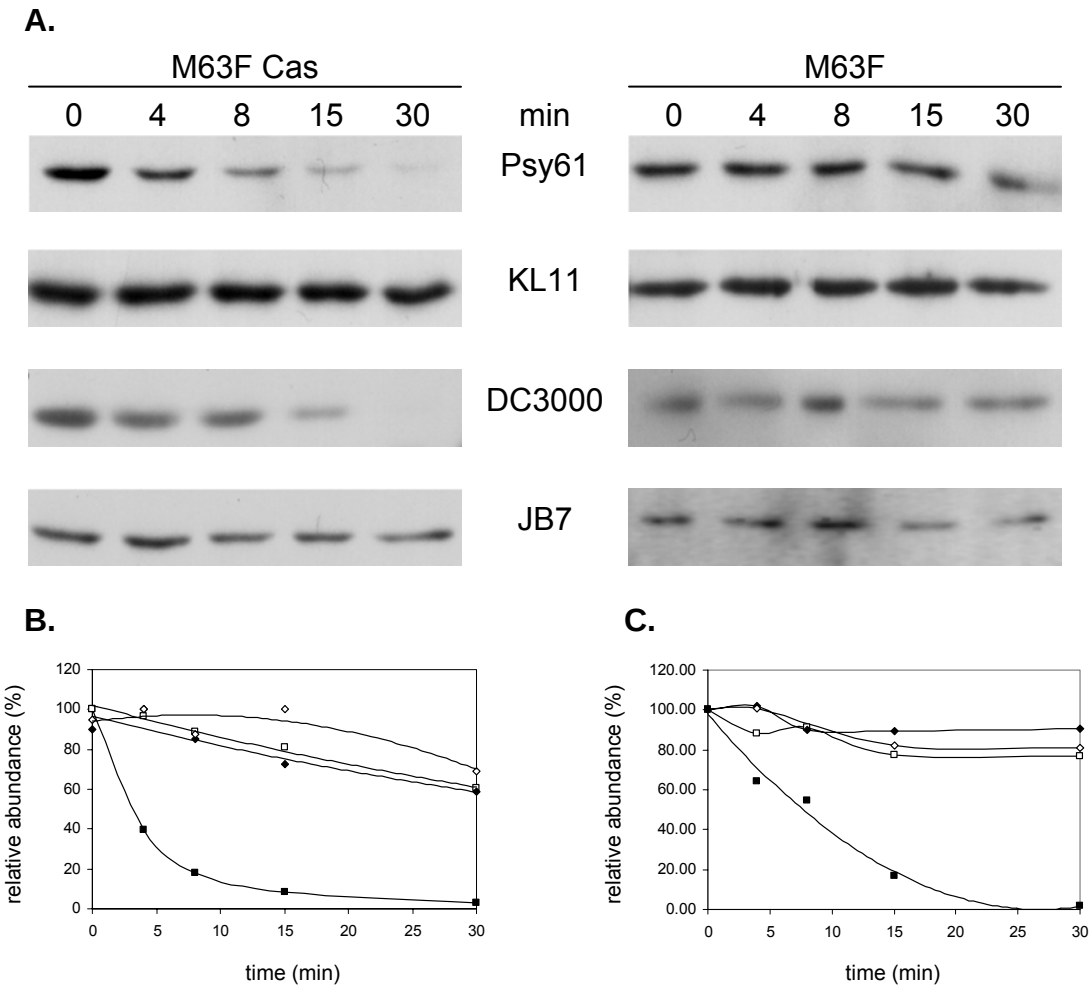
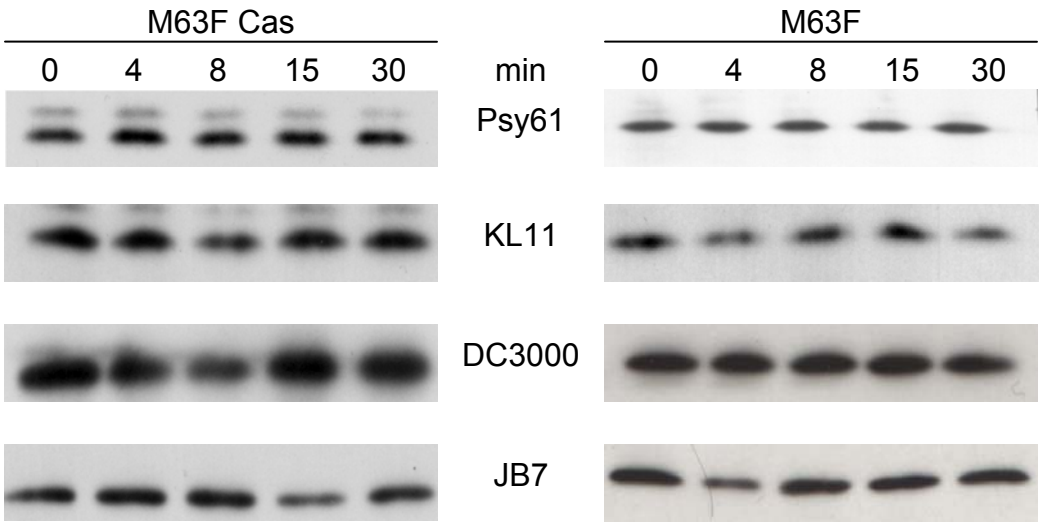

A.
M63F Cas
M63F
0 4 8 15 30
min
0 4 8 15 30
Psy61
KL11
DC3000
JB7
B.
relative abundance (%)
time (min)
C.
relative abundance (%)
time (min)

Figure 3-9: Effect of Lon protease on the stability of HrpS in *P. syringae*. Levels of His-HrpS were monitored in Psy61, KL11, DC3000 and JB7 grown in M63F Cas or M63F medium. Overnight cultures were diluted into the indicated medium and incubated at 25ºC until OD_{600}=1.0. Translation was stopped by the addition of excess tetracycline. Whole cell lysates were prepared at the times (min) indicated above the lane and residual levels of HrpS determined using immunoblots. His-HrpS migrated as a 39 kD protein. In all cases the apparent half-lives were greater than 35 min.

A.



apparent instability of HrpR in strains grown in non-inductive media are consistent with the conclusion that Lon protease-mediated degradation of HrpR has a critical function in the environmental regulation of the *hrp* regulon.

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

During our initial studies on the *hrp* constitutive mutants we noticed that early visible symptoms of the HR were elicited more rapidly by the *lon*::Tnp mutants. The initial stages of tissue necrosis could be detected by 3-3.5 h after inoculation with greater than 10^7 CFU/ml whereas the wild-type strain required at least 6 hr before a response could be observed (K. Lisboa, unpublished results). Consistent with the rapid development of visual symptoms of the HR, tissue inoculated with a *hrp* constitutive mutant was leaky to electrolytes by 3.5 h whereas the wild-type Psy61 strains required 6 h or more before a similar response was observed.

Constitutive *hrp* expression in the *P. syringae lon* mutants and rapid development of the HR suggests that the *hrp*-encoded TTSS may show enhanced activity under these conditions. To determine if the *lon*::Tnp mutants could secrete effectors in the absence of plant host, another member of the lab monitored secretion of an epitope tagged effector. Secretion of AvrPto by wild-type *P. syringae* was difficult to detect (139, 140). However, culture filtrates of the *lon*::Tnp mutant KL11 expressing epitope tagged AvrPto contained at least 100-fold higher levels of AvrPto than its Lon^+ parent strain. These results indicate that Lon-mediated degradation of HrpR is a major limiting factor for type III protein secretion from *P. syringae*.

Discussion

The ability of *P. syringae* to elicit defense responses in resistant plants and pathogenesis in susceptible plants has been linked to a TTSS and protein effectors encoded by the environmentally-regulated *hrp* regulon (Chapter 2)(48, 103). The induction of *hrp* regulon expression during pathogenesis had been shown previously to be dependent upon the unusual enhancer binding proteins HrpR and HrpS and the alternative sigma factor HrpL, but the mechanism for modulating the activity of these proteins during non-inductive conditions had not been established. The *hrpRS* operon was observed to be constitutively expressed, whereas the HrpR- and HrpS-dependent *hrpL* promoter was environmentally regulated, thereby requiring a mechanism to negatively regulate HrpR and/or HrpS activity. The results presented here indicate that Lon protease negatively regulates TTSS in *P. syringae* strains by controlling HrpR levels through regulated proteolysis.

The *P. syringae* Lon protease appears to be functionally equivalent to its counterparts in other bacteria. Key structural features are conserved and it could complement an *E. coli* Δ*lon* mutant. Lon protease is an ATP-dependent serine protease implicated in degradative turnover of several regulatory proteins in other bacteria (80, 159) such as SulA (161), RcsA (81), FlhD /FlhC (47) and perhaps σ^H (235). The enhanced cell length and UV sensitivity of the P. syringae *lon*::Tnp mutants indicates that Lon functions in the regulation of at least SulA in *P. syringae.* In addition, Lon protease

appears to negatively regulate the *hrp*-encoded TTSS of *P. syringae* strains through proteolytic turnover of the HrpR. Insertional inactivation of *lon* in Psy61 and DC3000 resulted in constitutive expression of the *hrpL* promoter and substantially reduced degradation of HrpR. Since both HrpR and HrpS are required for maximal expression of the *hrpL* promoter (Chapter 2), Lon-associated degradation of HrpR would reduce the expression of the *hrpL* promoter, thereby explaining the increased activity observed in the *lon*::Tnp mutants.

The activity of Lon appears to be regulated in a manner consistent with the observed environmental regulation of the *hrp* regulon. Lon-associated degradation of HrpR was detected during growth in media known to be non-inductive for *hrp* regulon expression but was minimal during growth of the wild-type strains in inductive media. The reduced degradation of HrpR during growth in *hrp* inducing media indicates that regulated proteolysis is required for the expression of the TTSS in *P. syringae* strains. Consistent with this conclusion, a representative *hrp* regulon promoter was expressed in *lon*::Tnp mutants at levels nearly equivalent to that observed in the wild-type strain during growth in *hrp* inductive media.

The data presented here indicate that Lon protease plays a significant role in regulation of *P. syringae* pathogenesis and type III secretion. Although the mechanism regulating Lon-mediated degradation of HrpR is unclear, it appears likely that this process is linked to the stringent conditions required for expression of the *hrp* regulon (240)(See Chapter 4). Whether Lon may also play a similar role in the

regulation of the TTSSs found in other pathogenic bacteria remains to be seen, but appears likely. The recent reports implicating Lon in *Salmonella* virulence (24, 25, 220, 221) seem to indicate that Lon-mediated regulation of TTSSs may be a common mechanism of regulating virulence factors among Gram negative pathogens.

Chapter 4: Other factors affecting *P. syringae hrp* regulation: a continuing study.

Introduction

Lon-mediated degradation of HrpR appears to be responsible for environmental regulation of the *hrp* regulon (Chapter 3). Specifically, *hrp* expression in *P. syringae* has been linked to stringent conditions (181, 245). Under *hrp*-inducing (stringent) conditions, HrpR is stabilized and the *hrp* regulon is expressed. The induction of *hrp* expression by stringent conditions would require a corresponding reduction and/or change in the activity of Lon protease for the HrpR-dependent *hrpL* promoter to be induced. This change in Lon activity could be due to a change in Lon's proteolytic activity or to a change in the Lon's mechanism for targeting potential substrates.

The stringent response is a cellular response to amino acid starvation (36). When a bacterial culture is switched from a rich media to a nutrient poor media, cells respond by repressing ribosomal genes required for rapid growth and by activating genes that help replenish depleted metabolites. The cell actively degrades otherwise stable proteins to release free amino acids for use during translation of essential proteins (159). In *P. syringae*, conditions similar to the stringent response (i.e. amino acid starvation) activate *hrp* expression.

In *E. coli*, the stringent response has been linked to the activity of three proteins: RelA, Ppx, and Ppk. RelA is closely associated with ribosomes and produces the signaling molecule (p)ppGpp when it senses uncharged tRNAs and idling ribosomes (36). (p)ppGpp inhibits the exopolyphosphatase Ppx from degrading polyphosphate to inorganic phosphate (131). However, the polyphosphokinase Ppk continues to produce polyphosphate. As a result, polyphosphate accumulates. Polyphosphate binds to Lon protease and directs it to degrade ribosomal proteins and other non-essential proteins (130). Lon protease is one of the chief general proteases encoded by bacterial cells. In this way, Lon protease frees up amino acids to use in the translation of essential proteins needed to survive starvation.

The following experiments were designed to identify the mechanism by which environmental signals are transduced through Lon protease and HrpR to alter the expression of the *hrp* regulon. In particular, I attempted to identify factors that linked *hrp* regulation with the stringent response. While these experiments are preliminary, the results do provide valuable information concerning the overall model of *hrp* regulation.

Experimental Procedures

Bacterial strains and plasmids. Strains and plasmids are listed in Table 4-1. *P. syringae* strains were routinely grown at 25˚C in KB broth (9) or M63 minimal salts medium (205) containing 1mM $MgSO_4$, 0.2% fructose, and/or 1% casein hydrolysate

Table 4-1: Bacterial Strains and Plasmids.

Bacterium or plasmid	Genotype or Description	Source or Reference
Strains		
Escherichia coli DH5α	EndA1 *hsd*R17 ($r_{k-}m_{k-}$) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 (*arg*F-*lacZ*YA) U169o80d*lacZ*DM15	Invitrogen
P. syringae pv. *syringae* Psy61	Weak bean pathogen, Nal^R, HR^+	(100)
P. syringae pv. *syringae* KL11	Tnp mutant of Psy61, *hrp* constitutive, *lon*::Tnp, Kan^R	Chapter 3
P. syringae pv. *tomato* DC3000	Tomato and *Arabidopsis* pathogen, Rif^R	(234)
P. syringae pv. *tomato* JB7	Tnp mutant of DC3000, *hrp* constitutive, *lon*::Tnp, Kan^R	Chapter 3
Plasmids		
pBlueScript SK^+	ColE1, Amp^R	Stratagene

Bacterium or plasmid	Genotype or Description	Source or Reference
pBS-Kan	pBlueScript derivative containing *kan* cassette from EZ-Tn <Kan-2> Tnp	M. Howard, Univ. Of Maryland
pDRR1R	690 bp *Bst*Y1 fragment cloned into pRG970 to create P_{hrpRS}-*lacZ*	(105)
pDSK519	incQ, Kan^R	(122)
pJBL5R	294 bp fragment from DC3000 cloned into pRG970 to create P_{hrpL}-*lacZ*	This work
pJBlon1R	241 bp fragment cloned into pRG970 to create P_{lon}-*lacZ*	This work
pJBrelA5D	2372 bp PCR product amplified from Psy61 and cloned into pDSK519	This work
pKnockout-G	ColE1, Amp^R, Gm^R, *mob*	(236)
pKO-tet	Derivative of pKnockout-G containing the *tet* gene from pBR322	This work
pKPA3R	Psy61 *hrpA* promoter fusion to *lacZ* in pRG970	(27)
pRG970	incP, Spc^R, promoterless '*lacZYA* for constructing transcriptional fusions	(226)

Bacterium or plasmid	Genotype or Description	Source or Reference
pRG970ΔHIII	pRG970 derivative lacking the 3 kb *Hind*III fragment to create a constitutively expressed *aadA2::lacZ* fusion	(27)
pYXL1R	340 bp PCR product cloned into pRG970, P_{hrpL}-*lacZ*	(238)

as indicated in the text. *E. coli* strains were grown at 37˚C in KB media. Antibiotics were added as indicated to the media at the following concentrations [μg/ml]: ampicillin (Amp), 100; kanamycin (Kan), 50; nalidixic acid (Nal), 50; rifampicin (Rif), 200; and spectinomycin (Spc), 100.

General DNA manipulations. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich, St. Louis, MO) method (10). Plasmid DNA was isolated using a kit manufactured by BioRad (Hercules, CA). Restriction enzymes and related reagents were purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer's directions. Ligations were performed using T4 DNA Ligase (New England Biolabs, Beverly, MA) according the manufacturer's directions.

Electroporation. Electrocompetent cells were prepared in 10% glycerol. Plasmid DNA was transformed into competent cells using a BioRad Gene Pulser electroporation unit set at 2000V, 25 μF capacitance, and 200 ohm resistance. After 1 h outgrowth, cells were plated onto selective media.

β -galactosidase activity. *P. syringae* Tnp derivatives were screened for β-galactosidase activity on KB agar plates after application of X-gal top agar. Plates with visible colonies were overlaid with molten 0.75% water top agar containing 0.07% X-gal and incubated at 4˚C. The plates were scored for β-galactosidase activity after 24 h. For quantitative estimation of β-galactosidase activity, cell

cultures were grown overnight in the indicated medium with selecting antibiotics. Cells were harvested, transferred to fresh medium and adjusted to an OD_{600} of approximately 0.5-1.0. (p)ppGpp (Sigma) was added to the culture media in the concentrations indicated. After incubation for up to 3 hours, β-galactosidase activity was determined as described by Miller (160).

Transposome mutagenesis. Transposome mutagenesis was conducted using an EZ::TN™ Transposome™ mutagenesis kit purchased from Epicentre Technologies (Madison, WI). The transposome (Tnp) was introduced into the Psy61 (pKPA3R) or DC3000 (pJBL5R) cells by electroporation as described above. After outgrowth for the recommended time, mutants were selected on media containing Nal, Spc and Kan, for Psy61 or Rif, Spc, and Kan for DC3000.

Mapping of transposome insertions. To construct genomic libraries of the Tnp mutants, isolated genomic DNA was partially digested with *Eco*RI and ligated into *Eco*RI-digested pBluescript SK^+. The resulting library was transformed by electroporation into Electromax DH5α cells and Amp^R Kan^R transformants selected. Plasmids from transformants were isolated and nucleotide sequence obtained using primers KAN-2 FP1 and KAN-2 RP1 provided with the EZ::TN™ kit. Plasmid DNA was sequenced at the University of Maryland Biotechnology Institute using an ABI Model 3100 Automated Sequencer. Sequence data for *P. syringae* pv. *tomato* DC3000 were obtained from The Institute for Genomic Research website at www.tigr.org (31).

General Protein Turnover. Overnight cultures of the indicated strains were diluted into 3 ml KB and incubated for an additional 1-3 h at 25˚C. Cells were labeled by the addition of 5μCi [^{14}C]-leucine and incubated an additional 3 h at 25˚C. Samples were split into 1.2 ml aliquots and washed with either M63F Cas or M63F. Cell pellets were resuspended in 1.2 ml M63F Cas or M63F containing 300 μg/ml non-radioactive leucine. Samples were assayed as described by Sussman and Gilvarg (216) and Kuroda *et al* (131). Briefly, 180 μl of culture were removed at the indicated times, mixed with 20 μl 50% trichloroacetic acid (TCA), and placed on ice for 30 minutes. After centrifugation, 180 μl of the supernatant was mixed with 20 μl 20 mg/ml BSA. Following centrifugation, radioactivity of an 180 μl sample of the supernatant was measured in scintillation fluid by a LKB Wallac 1290 Rackbeta Liquid Scintillation Counter. The TCA soluble counts were converted to the percentage of the total initially incorporated counts and graphed versus time.

Construction of pJBrelA1D. The Psy61 *relA* gene was amplified from Psy61 genomic DNA using primers DC185 (5'-CGGGATCCGCCGTAGGGAAGGTAAGCA) and DC2557 (5'-CGGAATTCCTCCCGTATAAAAACAGCAGT) and cloned into pDSK519 as a *Bam*HI/*Eco*RI fragment.

Construction of pJBlon1R. A 241 bp fragment containing the Psy61 *lon* promoter was amplified from Psy61 genomic DNA using primers LB16 (5'-

TCCCCCCGGGATCTACGAAAACAACGAGCC) and LB257 (5'-CGGGATCCCTTCATGATTTCGCCTCTAC). The resulting fragment was digested with *Xma*I and *Bam*HI and cloned into pRG970.

Construction of pJBL5R. The DC3000 *hrpL* promoter was amplified from DC3000 genomic DNA using primers JB003 (5'-GCGATATCGATAGGCGGAGCGACGATT) and JB004 (5'-GCGGATCCCTGACGGGGCTGGGTTGA). The resulting 294 bp fragment was digested with *Bam*HI and *Eco*RV and ligated into *Bam*HI/*Sma*I digested pRG970.

Results

Expression of the Psy61 *lon* promoter is unaffected by culture condition. In *Erwinia amylovora, P. fluorescens, P. aeruginosa,* and *E. coli*, expression of the *lon* gene is regulated by a heat shock dependent promoter (45, 63, 213, 235). It is thought that an increase in *lon* expression during times of stress will lead to increased proteolysis and a subsequent increase in free amino acids. To determine if similar regulation were occurring in *P. syringae,* the *lon* promoter from Psy61 was cloned into pRG970 to create a transcriptional fusion with *'lacZYA*. This construct (pJBlon1R) was transformed into Psy61 and expression of the P_{lon}-*lacZ* fusion was monitored. Strains carrying this construct expressed 60-90 Miller units (MU) of activity in *hrp*-repressive media, such as KB or M63F with casamino acids (Table 4-2). A similar level of activity was observed in the *hrp*-inductive M63F media. When

Table 4-2: Expression of the *P. syringae* pv. *syringae* Psy61 *lon* promoter is unaffected by culture conditions.

Promoter[1]	Promoter Activity (MU)[2]			
	KB	M63F + Cas	M63F	KB + HS[3]
lon	87±1	60±1	64±5	179±5
control	1433±24	1685±27	1277±22	1599±22

[1] *lon:'lacZ* promoter fusion was provided by pJBlon1R. The constitutively expressed control was provided by pRG970ΔHIII, which contains a P_{spec}-*lacZ* fusion

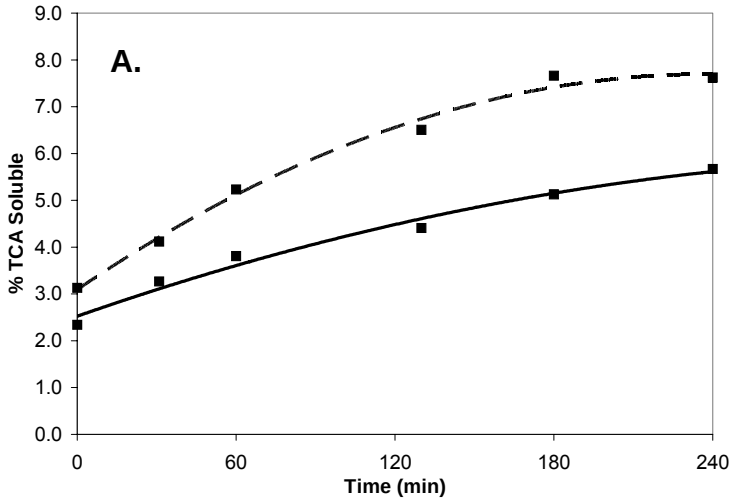
[2] Psy61 carrying the indicated reporter construct was grown to an OD_{600} of approximately 0.5 in the indicated media. Accumulated β-galactosidase activity, reported in Miller units, was used to estimate reporter activity (160). The data are reported as the mean of a single experiment done in triplicate. The error represents the standard deviation.

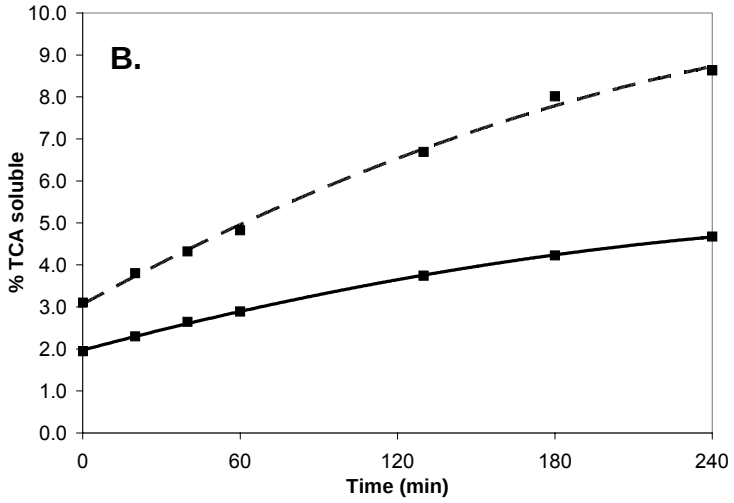
[3] HS, heat shock.

the cells were grown in KB and heat shocked for 30 m, a modest 2-3 fold increase in activity of the *lon* promoter was observed. Expression of the constitutively expressed control was unaffected by culture condition. While *lon* expression may be affected by heat shock conditions, it does not appear that the observed changes in *lon* expression under *hrp* inducing conditions explain the observed changes in HrpR stability.

A change in general protein turnover is not responsible for the change in Hrp R stability. The observed change in HrpR stability may be attributable to a change in the activity of Lon protease. Because Lon is the primary protease involved in degrading misfolded proteins (80), Lon activity may control the rate of general intracellular protein turnover, as in *E. coli* (131, 159, 216). To determine if a change in the rate of general protein turnover was responsible for the observed change in HrpR stability, *P. syringae* cultures growing in the *hrp*-repressive KB media were pulsed with [^{14}C]-leucine. The cells were washed and resuspended in either M63F and unlabelled leucine (*hrp*-inductive) or M63F Cas and unlabelled leucine (*hrp* repressive). Protein turnover was monitored by measuring the amount of TCA soluble counts over time. For wild-type *P. syringae,* the initial rate of general protein turnover following transfer to the new medium was higher under *hrp*-inductive conditions (Figure 4-1), as observed in *E. coli* (131, 216). Similar results were obtained in both Psy61 and DC3000 strains. For each strain, growth in *hrp* inducing media caused the overall rate of general protein turnover to increase, while the rate of

Figure 4-1: A change in general protein turnover does not appear responsible for the change in HrpR stability. Intracellular protein turnover was measured by labeling cells with [^{14}C]-leucine during growth in *hrp*-repressive KB media. Cells were washed and resuspended in either M63 minimal salts media with fructose and unlabelled leucine (dashed line) or M63 minimal salts media containing fructose, unlabelled leucine, and casamino acids (solid line). At the indicated times, protein was extracted from cell cultures using trichloroacetic acid. Trichloroacetic acid soluble counts are expressed as a percentage of the total initially incorporated counts. **A.** Psy61. **B.** DC3000.


A.
% TCA Soluble
Time (min)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
60
120
180
240


B.
% TCA soluble
Time (min)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
60
120
180
240

HrpR degradation decreased. As a result, it appears unlikely that a change in general intracellular protein turnover is responsible for the change in HrpR stability.

DC3000 encodes homologues to *E. coli* proteins involved in the stringent response. Because Lon mediated protein degradation in *E. coli* is affected by the stringent response, the following experiments were designed to determine if the stringent response also affected Lon activity and HrpR stability in *P. syringae*. The three proteins known to modulate the stringent response in *E. coli* are RelA, Ppk, and Ppx. By using the sequence of these proteins as probes, homologues of RelA, Ppk, and Ppx were identified in the genomic sequence of DC3000 (Table 4-3). Each of the three proteins was at least 30% identical and 50% similar to its *E. coli* counterpart. Another *E. coli* enzyme, SpoT, is also involved in (p)ppGpp synthesis. SpoT is not involved in the stringent response but is primarily involved in maintaining basal (p)ppGpp levels (36). DC3000 also encodes a homologue to SpoT which is 53% identical and 72% similar to the *E. coli* protein (Table 4-3). These four DC3000 proteins were much more closely related to their homologues in other Pseudomonads. In fact, all four of the proteins were at least 93% identical to their homologues encoded by the closely related plant pathogen *P. syringae* pv. *syringae* B728a and greater than 74% identical to homologues encoded by the human pathogen *P. aeruginosa* PAO1.

Because *P. syringae* carries homologues of *relA, ppx,* and *ppk,* the most direct method to link the stringent response to *hrp* regulation would be to create mutations

Table 4-3: *P. syringae* pv. *tomato* DC3000 homologues to *E. coli* RelA, Ppk, Ppx, and SpoT.

Protein[1]	DC3000		*E. coli*		B728a[2]		PAO1[3]	
	Accession Number	L[4]	L	I / S[5]	L	I / S	L	I / S
RelA	AAO55214	744	744	49/68	747	96/97	747	85/91
Ppk	AAO58676	736	688	34/54	736	93/93	690	83/92
Ppx	AAO59675	500	513	41/59	526	98/99	506	74/87
SpoT	AAO53627	701	702	53/72	701	99/99	701	89/95

[1] *E. coli* protein linked to stringent response. The putative DC3000 homologue was identified by using the *E. coli* protein to search the complete DC3000 genome sequence available at www.tigr.org (31).

[2] *P. syringae* pv. *syringae* B728a

[3] *P. aeruginosa* PAO1

[4] protein length (L) (AA)

[5] Percent identity (I) and percent similarity (S) were determined by the BLAST algorithm at www.ncbi.nlm.nih.gov (5)

in *relA*, *ppk*, and *ppx*. However, all attempts to create mutations in these genes were unsuccessful. Several attempts to create mutants via marker exchange mutagenesis were employed. The first involved cloning an internal region of each gene into the plasmids pKnockout-G, pKO-tet, or pBS-Kan (236). These constructs allow for the insertional mutagenesis of the target gene via homologous recombination. Following transformation, recombinants are chosen by selecting for the appropriate antibiotic. In each case, analysis of the potential mutants revealed that the target gene was not inactivated, indicating that non-homologous recombination had occurred elsewhere in the genome. An alternative strategy was also attempted to create a gene deletion rather than an insertional mutant. For this strategy, approximately 1 kb of flanking sequence upstream and downstream of the target gene was cloned consecutively into pBlueScript SK^+. A kan cassette was then cloned between the two regions. The new construct was transformed into DC3000 and kanamycin resistant colonies were selected. As was the case for the insertional mutants above, analysis of the potential mutants indicated that a wild-type copy of the gene was still present.

Artificial induction of the stringent response does not affect *hrp* expression in Psy61. The lack of mutations in *relA*, *ppk*, and *ppx* required the use of an artificial means of inducing the stringent response. Because the cellular response to amino acid starvation involves the accumulation of (p)ppGpp, the cellular levels of this molecule can be manipulated by over-expression of *relA* (196, 218). The *relA* gene from Psy61 was cloned behind the *lac* promoter of the expression vector pDSK519. This construct was transformed into Psy61 strains containing the vector based

hrpA:'lacZ promoter fusion used in previous chapters. Promoter activity was monitored by measuring accumulated β-galactosidase activity. Over-expression of *relA* did not induce *hrp* expression under repressive conditions, nor did it affect *hrp* expression under inductive conditions (Table 4-4). Regardless of whether *relA* was overexpressed, the *hrpA* promoter was inactive under *hrp*-repressive conditions but active under *hrp*-inductive conditions.

In another attempt to artificially induce the stringent response in *P. syringae,* (p)ppGpp was added to the culture media (218). As above, the *hrpA:'lacZ* reporter plasmid was used to monitor *hrp* expression in Psy61 cells. The *hrpA* promoter was not expressed in the repressive KB media as previously observed (Table 4-5). Nor was the *hrpA* promoter expressed when exogenous (p)ppGpp was added at either 200 or 890 μM. The *hrpA* promoter was expressed in the *hrp*-inducing M63F media. Expression of the constitutively expressed positive control was unaffected by culture conditions. Because a (p)ppGpp-induced promoter such as the *Pu* promoter from *P. putida* (34) was not included in these experiments, it was not possible to determine whether the (p)ppGpp gained entry into the cell.

Identification of *hrp* constitutive mutants. Whether or not a link between the stringent response and *hrp* regulation truly exists, other factors are certainly involved in *hrp* regulation. Other data suggested the presence of at least one additional regulatory determinant. During preliminary experiments to determine if *hrp* constitutive mutants could be identified, NTG mutagenesis of Psy61 had identified

Table 4-4: Over-expression of *relA* in *P. syringae* pv. *syringae* Psy61 does not affect *hrp* expression.

Promoter[1]	*relA*[2]	Promoter Activity (MU)[3]	
		KB	M63F
hrpA	-	11±1	272±4
	+	22±1	125±1
control	-	1509±10	952±45
	+	749±18	559±21

[1] *hrpA:'lacZ* promoter fusion was provided by pKPA3R. The constitutively expressed control was provided by pRG970ΔHIII.

[2] Psy61 carrying either pJBrelA5D (+) or pDSK519 (-) .

[3] Psy61 carrying the indicated plasmids was grown to an OD_{600} of approximately 0.5 in KB or M63 fructose. Accumulated β-galactosidase activity, reported in Miller units, was used to estimate reporter activity (160). The data are reported as the mean of a single experiment done in triplicate. The error represents the standard deviation. Similar results were obtained in at least three experiments.

Table 4-5: Addition of (p)ppGpp to the media does not induce *hrp* expression in *P. syringae* pv. *syringae* Psy61.

Promoter[1]	Promoter Activity (MU)[2]			
	KB	KB + 200 μM (p)ppGpp	KB + 800 μM (p)ppGpp	M63F
hrpA	4±1	4±1	4±1	100±1
control	1249±33	1227±19	1277±28	NT[3]

[1] The *hrpA* promoter fusion was provided by pKPA3R. The constitutively expressed control was provided by pRG970ΔHIII.

[2] Psy61 carrying the indicated reporter construct was grown to an OD_{600} of approximately 0.5 in the indicated media. Exogenous (p)ppGpp was added to the media in the indicated concentrations. Accumulated β-galactosidase activity, reported in Miller units, was used to estimate reporter activity (160). The data are reported as the mean of a single experiment done in triplicate. The error represents the standard deviation.

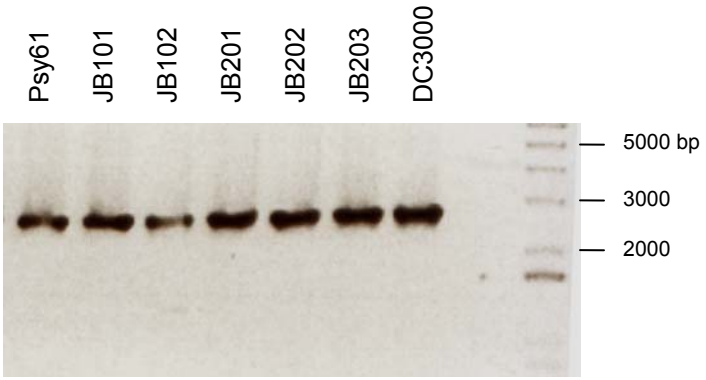
[3] NT, not tested.

apparent *hrp* constitutive mutants that were not linked to *hrpRS, hrpL,* or *hrpV* (N. Theraja and S. Hutcheson, unpublished results). To determine if the NTG mutants mapped to *lon,* primers flanking *lon* were used to amplify the region by PCR. Sequence analysis of the PCR products revealed wild-type sequence indicating that these mutations were not internal to *lon.* In addition, during the transposome (Tnp) screen of both Psy61 and DC3000, *hrp*-constitutive mutants were identified that did not link to *lon.* Several other mutants that did not map to *lon* in these screens displayed an intermediate Lac phenotype. Therefore, it appeared likely that other factors were involved in mediating the environmental regulation of the *hrp* regulon.

In an attempt to identify other factors involved in negative regulation of the *hrp* regulon, the Tnp mutagenesis screen of Psy61 and DC3000 from Chapter 3 was continued. As before, Tnp mutants were screened for constitutive expression of a *hrp:'lacZ* promoter fusion. In a continuation of the Psy61 screen, approximately 1100 more Tnp mutants were screened. Of these mutants, 8 were *hrp* constitutive mutants. A new Tnp screen of DC3000 was also initiated. With the assistance of Taki Roussis, approximately 3600 colonies were screened, 11 of which displayed the *hrp* constitutive phenotype. In both these screens, colonies with intermediate Lac phenotypes were chosen in addition to the standard Lac^+ colonies in order to broaden the search for negative regulators of *hrp* expression.

Surprisingly, none of these new *hrp* constitutive mutants mapped to *lon* (Figure 4-2). To identify the *hrp* constitutive mutants, Tnp carrying fragments were selected from

Figure 4-2: PCR analysis of *P. syringae hrp*-constitutive mutants. To determine if the *hrp* constitutive mutants contained a Tnp insertion in *lon*, the mutants were screened using intact cell PCR and the primers LB258 and LB2955. In wild-type strains, these primers amplify a 2.7 kb fragment. A Tnp insertion at this site yields a 3.9 kb product. Strain name is listed above each lane. Strains JB101 and JB102 are Psy61 derivatives. The other Psy61 *hrp* constitutive mutants, JB103-JB108, were not tested. Strains JB201, JB202, and JB203 are DC3000 Tnp mutants. DC3000 *hrp* constitutive mutants TK01-TK08 were screened by Taki Roussis and did not contain an insertion in *lon.*


Psy61
JB101
JB102
JB201
JB202
JB203
DC3000
5000 bp
3000
2000

genomic libraries of select mutants. The sequence of the Tnp carrying subclones was obtained and compared to the DC3000 genomic sequence using the BLAST algorithm. Sequence analysis of the mutants revealed that at least 6 different genes had been interrupted by the Tnp (Table 4-6). The strongest *hrp* constitutive mutant was mapped to the *sucD* gene which encodes for the alpha subunit of the succinyl-CoA synthetase enzyme. Four other mutants contained Tnp insertions in genes related to the stringent response. JB102 and JB202 contained mutations in the *P. syringae ppx* gene while JB201 and JB203 contained mutations in *relA.* TK04 had the weakest homology to a DC3000 gene, but could still be mapped to the heat shock gene *htpG.* When compared to the DC3000 genome, all other mutants had an E-value of 0.0. Unfortunately, two of the mutants appeared to have a mutation in two different genes found in different regions of the DC3000 genome. When the sequences obtained from both TK01 and TK07 were analyzed, two hits with an E-value of 0.0 were identified. Therefore, it appears that either these mutants have two independent Tnp insertions, or at least two distinct Tnp mutants were mixed in the same culture. The sequence for the remaining mutants has not yet been determined.

Discussion

At the beginning of this chapter, two models were proposed that could explain the observed changes in HrpR stability. The first posited that a decrease in *lon* expression and/or Lon activity accounted for the increased stability of HrpR under stringent conditions. If either were affected by stringent conditions, *lon* expression or

Table 4-6: *hrp*-constitutive mutants identified by transposome mutagenesis.

Strain Name	Gene[1]	Accession Number	E-value[2]
Psy61 derivatives			
KL1-22[3]	*lon*	AF447727	
JB101	*sucD* (succinyl-CoA synthetase, alpha subunit)	AE016863[4]	0.0
JB102	*ppx*	AE016875[4]	0.0
JB103	ND[5]	ND	ND
JB104	ND	ND	ND
JB105	ND	ND	ND
JB106	ND	ND	ND
JB107	ND	ND	ND
JB108	ND	ND	ND
DC3000 derivatives			
JB7[6]	*lon*	AE016869	
JB201	*relA*	AE016861	0.0
JB202	*ppx*	AE016875	0.0
JB203	*relA*	AE016861	0.0
TK01[7,8]	FKBP type peptidyl-prolyl *cis*-trans isomerase	AE016860	0.0
	polyamine ABC transporter (ATP binding protein)	AE016857	0.0

Strain Name	Gene[1]	Accession Number	E-value[2]
TK02[7]	ND	ND	ND
TK03[7]	ND	ND	ND
TK04[7]	*htpG*, putative heat shock protein	AE016863	4.7
TK05[7]	ND	ND	ND
TK06[7]	ND	ND	ND
TK07[7,8]	*gltB* (glutamate synthase, large subunit)	AE016874	0.0
	putative lipoprotein	AE016857	0.0
TK08[6]	ND	ND	ND

[1] the gene interrupted by the Tnp was identified by comparing the sequence of the mutant to the complete DC3000 genome sequence available at www.tigr.org (31).

[2] as determined by the BLAST algorithm at www.ncbi.nlm.nih.gov (5).

[3] see Chapter 3 for description, identified in collaboration with K. Lisboa.

[4] accession number of DC3000 homologue.

[5] ND, not determined: no sequence data or insufficient sequence data to make identification.

[6] see Chapter 3 for description.

[7] identified in collaboration with Taki Roussis.

[8] sequences from TK01 and TK07 align with DC3000 genomic sequence from at least two different areas of the genome and indicate that these strains are contaminated by at least two unique Tnp mutant strains.

Lon activity would be expected to decrease to account for the observed stability of HrpR under *hrp*-inducing conditions. In *E. coli,* the opposite is thought to occur. As mentioned above, *lon* expression is regulated by a heat shock promoter that is induced during stressful conditions in *E. coli* (45). Similarly, Lon activity increases during the stringent response to provide more free amino acids for the starving cell (36). In *P. syringae,* it appears that *lon* is expressed constitutively. Likewise, it does not appear that Lon proteolytic activity, as measured by general protein turnover, is affected by culture conditions either. As observed in *E. coli* (216), the rate of general protein turnover appears to increase under *hrp*-inducing conditions in Lon^+ *P. syringae* cells. When taken together, these data indicate that another mechanism must account for the change in HrpR stability under *hrp*-inducing conditions.

The second model presented at the beginning of this chapter suggested that other factors affected Lon-mediated degradation of HrpR. The most obvious factors to investigate first were those known to be involved with the stringent response in *E. coli.* As mentioned previously, the stringent response has been tied to Lon protease via the affect of polyphosphate on Lon substrate targeting. Attempts to create insertional mutant or knockouts of RelA, Ppk, and Ppx were unsuccessful. It is unclear why these mutation strategies were not successful but anecdotal evidence from others suggests that mutations involving homologous recombination in DC3000 are difficult to create (A. Sreedharan and L. Losada, personal communications). Some of the experiments attempted to establish a direct link between *hrp* expression and the stringent response in *P. syringae* by using an artificial means of inducing the

stringent response. Unfortunately, in each case the data were inconclusive, and a direct link between these two processes could not be identified. Over-expression of *relA* did not cause an increase in *hrp* expression. In *E. coli* and *Pseudomonas putida,* over-expression of *relA* leads to an increase in intracellular (p)ppGpp levels and to changes in gene expression patterns (196, 218). Although the genomes of DC3000 and *P. putida* are at least 68% similar (31), it's unclear whether (p)ppGpp has the same effects in *P. syringae.* Addition of exogenous (p)ppGpp to the culture media also had no effect on *hrp* expression (218). Steady-state intracellular levels of (p)ppGpp are estimated to be between 10 and 30 μM. These levels increase up to 20 fold during the stringent response (36). Even when supplied at levels that should be sufficient to induce the stringent response (890 μM), (p)ppGpp did not induce *hrp* expression. Unfortunately, the intracellular concentration of (p)ppGpp was not determined and it was therefore impossible to determine how much (if any) of the (p)ppGpp actually made it into the cell.

A non-targeted screen for other factors affecting *hrp* regulation was more fruitful. This new Tnp search identified at least six new candidate genes that may encode negative regulators of the *hrp* regulon. None of the new mutants were in *lon.* This was quite surprising considering that in the original screen of Psy61 described in Chapter 3, all 21 Lac^+ Tnp mutants were mapped to *lon.* Compared to the first two screens (Chapter 3), the frequency of *hrp*-constitutive mutants in these screens was slightly higher. This is probably due to the fact that the criteria for selecting

candidate mutant colonies was less stringent than the original screen and mutants with an intermediate Lac phenotype were included in the new screen presented here.

Several of the new *hrp* constitutive mutants mapped to genes whose relationship to *hrp* regulation was not immediately apparent. For instance, the mutant with the strongest Lac phenotype, JB101, mapped to the *sucD* gene. *sucD* encodes the alpha subunit of the succinyl-CoA synthetase. This enzyme is involved in substrate-level phosphorylation and converts succinyl-CoA to succinate during the citric acid cycle (136). A mutation in *sucD* would result in decreased intracellular levels of succinate. The other mutant whose function in *hrp* regulation was unclear was TK07. The mutation in TK07 was mapped to either a putative lipoprotein or *gltB*, which encodes for glutamate synthase. Glutamate synthase converts α-ketoglutarate and glutamine to glutamate (136). Similar to the *sucD* mutation, a mutation is *gltB* would result in a decrease in the intracellular concentration of glutatmate. It is interesting to note that both succinate and glutamate have been shown to suppress *hrp* expression (107). Therefore, it appears that neither *sucD* nor *gltB* are directly involved in *hrp* regulation, but rather "trick" the cell by producing two molecules that are *hrp* repressive.

A possible link to the stringent response was identified during the search for new *hrp* constitutive mutants. Two of the new *hrp*-constitutive mutants were identified as the stringent factors *relA* and *ppx*. These two mutants were unique in that they represent gene deletions rather than insertions. Deletions are unusual for Tnp mutagenesis, but

are known to occur with other transposons (52). Another rather unique aspect of these mutants is that JB102 and JB202 appear to be identical at the sequence level even though they are from different strains (Psy61 and DC3000, respectively). Despite these anomalies, the *relA* and *ppx* mutants may provide the crucial link between the stringent response and *hrp* regulation in *P. syringae.*

It is not entirely clear how these newly identified factors integrate into the established *hrp* regulatory system. Although more experimentation will be necessary to test these predictions, it is possible to hypothesize their functions. If the stringent factors interact with Lon protease in a similar way in both *E. coli* and *P. syringae,* RelA and Ppk would be expected to be positive regulators of the *hrp* regulon because they would promote the accumulation of polyphosphate. The polyphosphate would in turn direct Lon to degrade ribosomal proteins instead of HrpR. In this model, Ppx would be a negative regulator of the *hrp* regulon because it would lead to a decrease in intracellular levels of polyphosphate and HrpR. Interestingly, the Tnp screen described here also identified RelA as a negative regulator of *hrp* expression. RelA is the (p)ppGpp synthetase involved in the stringent response. In addition to inhibiting the enzymatic activity of Ppk, (p)ppGpp also interacts with RNA polymerase to increase or decrease the expression of stringently controlled promoters (36, 41). In this way, the starving cell can up regulate expression of genes necessary for survival and down regulate expression of nonessential genes. In *P. syringae,* (p)ppGpp may also have pleiotropic effects on *hrp* expression. When interacting with Ppk, (p)ppGpp acts as a positive regulator of the *hrp* regulon by promoting the

accumulation of polyphosphate. However, (p)ppGpp may also act as a negative regulator by decreasing the expression of genes necessary for *hrp* expression. As a result, RelA would be both a positive and negative regulator of the *hrp* regulon. Whether SpoT, the other (p)ppGpp synthetase encoded by DC3000, plays a role similar to RelA in *P. syringae hrp* regulation is not known, but is currently under investigation.

Chapter 5: A translocated protein tyrosine phosphatase of *Pseudomonas syringae* pv. *tomato* DC3000 modulates plant defense response to infection.

(The data presented in this chapter have been published in Molecular Microbiology, 2003, 49:389-400 (ref. 29).)

Introduction

The host range of *P. syringae* strains appears to be controlled, in part, by the ability of the host to mount a cellular defense response (54, 102). In resistant plants, primary defense responses to *P. syringae* strains are usually rapid, commonly detectable within the first few hours of the interaction, and frequently culminate in programmed cell death (PCD) observable in the responding cells 6 h after inoculation. When artificially high inocula are used, this defense-associated programmed cell death leads to a localized necrotic response in inoculated tissue known as the hypersensitive response (HR). Primary responding cells undergoing PCD release active oxygen (15) and nitric oxide (57) that stimulate additional responses in adjacent cells (14). These additional responses can involve enhanced secondary metabolism, accumulation of anti-microbial compounds, and induction of pathogenesis-related proteins, such as PR1, in the adjacent cells. Mitogen-activated protein kinases (MAPKs) have been implicated in the early phases of these defense responses (170, 247). The combined activities of these defense responses are thought to limit the spread of the pathogen. In contrast, host defense responses to *P. syringae* infection in susceptible plants are slow, thereby allowing the infection to progress before host cells respond.

The induction of the cellular defense responses in resistant plants and pathogenesis in susceptible plants has been linked to the activities of a type III secretion system (TTSS) encoded by the *P. syringae hrp* pathogenicity island (PAI) (4, 103). The primary function of the *hrp* TTSS appears to be the translocation of effector proteins into the cytosol of host cells through the needle-like HrpA pilus (116). As mentioned previously, a complex regulatory system has been partially characterized in *P. syringae* strains that utilizes HrpL, a member of the ECF family of alternative sigma factors (238), to direct expression of the TTSS as well as most of the known TTSS-dependent effectors during pathogenesis (Chapters 2 and 3)(239). In cells of resistant plants, effectors translocated by the *hrp*-encoded TTSS can be recognized by cytosolic receptors either encoded by resistance genes or associated with resistance gene products to initiate PCD in the responding cell (54, 102). In susceptible plants, the effectors are thought to facilitate parasitism of the host cells by opening pores in the plasma membrane (133), or by functioning in an unknown manner to suppress other types of cellular defense responses (1, 42, 111, 113, 114).

Because the effectors translocated into host cells by *P. syringae* strains appear to control host range, many different groups have attempted to identify effectors produced by individual strains. Assays screening for altered host range of transformants carrying a genomic library of another strain (209), surveys of secreted proteins found in culture filtrates (227) and screens for translocated fusions to ‘AvrRpt2 (90) have been used to identify translocated effectors. In the strains in

which the *hrp* PAI has been sequenced, some putative effectors have been identified by their inclusion in the conserved and exchangeable effector loci associated with the *hrp* PAI (4, 38, 59). Potential effectors have also been identified in the *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a genomes by searching for HrpL-dependent promoter sequences (70) or potential type III secretion signals (90, 177). These results indicate that individual *P. syringae* strains may express as many as 58 genes for effector proteins that contribute to the pathogenicity of each strain (Chapter 1)(49, 54). Some of these effectors appear to be widely distributed among *P. syringae* strains, whereas others are found in only a few strains. Recently, biochemical activities for some *P. syringae* effectors have been identified. AvrPto and AvrPtoB have been shown to interact with the IRAK-like Ser-Thr kinase, Pto (128, 197, 222) and to suppress defense responses (1). A family of proteins that includes AvrPphB and AvrRpt2 has been shown to be cysteine proteases (201). AvrB, AvrRpm1, and AvrRpt2 have been shown to interact with and alter RIN4, a plant-encoded virulence target with unknown function (12, 150, 151). Several other effectors have been shown to suppress PCD (114). The mechanisms by which most other effectors function in host cells have not been established.

The purpose of these experiments was to identify and characterize effectors secreted by the Hrp TTSS of *P. syringae* pv. *tomato* DC3000. Recently, other members of the lab developed a method to identify candidate effectors genes by screening genomic libraries of *P. syringae* strains for promoters dependent upon HrpL for expression (106, 148). During preliminary trials of this HrpL-dependent promoter trap assay, the

DC3000 genome was partially surveyed for candidate effector genes. This assay led to the identification of a modular effector with an amino terminal TTSS-dependent secretion domain similar to that of AvrPphD and a carboxyl terminal domain that functions as a protein tyrosine phosphatase (PTP). This novel effector appears to have a specific role in DC3000 virulence by suppressing activation of host defense responses.

Experimental Procedures

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 5-1. Other *P. syringae* strains reported in Table 5-6 are described in Charity *et al* (38). *P. syringae* strains were routinely grown at 25˚C in KB broth or in M63 minimal salts medium containing 1mM $MgSO_4$ and 0.2% fructose. *E. coli* strains were grown at 37˚C in KB media. Antibiotics were added as indicated to the media at the following concentrations [μg/ml]: ampicillin (Amp), 100; kanamycin (Kan), 50; rifampicin (Rif), 200; and spectinomycin (Spc), 100.

General DNA manipulations. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich, St. Louis, MO) method (10). Plasmid DNA isolations and gel extractions were performed using kits manufactured by BioRad (Hercules, CA). Restriction enzymes and related reagents

Table 5-1: Bacterial Strains and Plasmids.

Bacterium or plasmid	Genotype or Description	Source or Reference
Strains		
Escherichia coli DH5α	EndA1 *hsd*R17 ($r_{k-}m_{k-}$) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 (*arg*F-*lac*ZYA) U169o80d*lac*ZDM15	Invitrogen
E. coli SLR400	*Δara, Δlac*	S. Benson, University of Maryland
Pseudomonas syringae pv. *syringae* Psy61	Weak bean pathogen, Nal^R, HR^+	(100)
P. syringae pv. *tomato* DC3000	Tomato and *Arabidopsis* pathogen, Rif^R	(234)
P. syringae pv. *tomato* A9	DC3000 derivative, *ΔhrpA*, HR^-	(231)
P. syringae pv. *tomato* JB4	DC3000 derivative, *ΔhopPtoD2*	This work

Bacterium or plasmid	Genotype or Description	Source or Reference
Plasmids		
pBlueScript SK^+	ColE1, Amp^R	Stratagene
pDSK600	incQ Spc^R, triple *lacUV5* promoter	(164)
pJBAvrRpt2	Full length AvrRpt2 cloned into pDSK600	This work
pJBHopPtoD2	Full length HopPtoD2 cloned into pDSK600	This work
pJBHopPtoD2':AvrRpt2	171 amino terminal amino acids of HopPtoD2 fused to the carboxy terminal effector domain of AvrRpt2 cloned into pDSK600	This work
pJBHopPtoD2ΔC	321 amino terminal amino acids of HopPtoD2 cloned into pDSK600, does not contain PTP domain	This work
pJB*ΔhopPtoD2*	pBlueScript SK^+ derivative, used for marker exchange mutagenesis, Amp^R Kan^R	This work

Bacterium or plasmid	Genotype or Description	Source or Reference
pMPM-K6	Contains P_{BAD} promoter to create arabinose-dependent transcriptional fusions, Kan^R	(154)
pRG970	incP Spc^R, promoterless *'lacZYA* for constructing transcriptional fusions	(226)
pSHL4K	pMPM-K6 derivative containing an arabinose inducible HrpL	(29)

were purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer's recommendations. Ligations were performed using T4 DNA Ligase (New England Biolabs, Beverly, MA). Polymerase Chain Reactions (PCR) were performed using a Hybaid PCRSprint Thermal Cycler and employed either Taq (Invitrogen) or ProofPro (Continental Lab Products, San Diego, CA) polymerases. Oligonucleotide primers used in this study are listed in Table 5-2.

Electroporation. Electrocompetent cells were prepared in 10% glycerol. Plasmid DNA was transformed into competent cells using a BioRad Gene Pulser electroporation unit set at 2000V, 25 μF capacitance, and 200 ohm resistance. After 1 h outgrowth, cells were plated onto selective media.

DNA Sequencing and Analysis. Plasmid DNA or gel-purified PCR products were sequenced at the University of Maryland Biotechnology Institute. Raw sequence data were assembled using MacDNASIS Pro v3.0 and analyzed using BLAST algorithms (5). Sequences were aligned using CLUSTALW (www.searchlauncher.bcm.tmc.edu)(43, 206). Sequence data for *P. syringae* pv. *tomato* DC3000 were obtained from The Institute for Genomic Research website at www.tigr.org (31).

HrpL-dependent Promoter Trap Assay. DC3000 genomic DNA was digested with *Sau*3A, fractionated into 2-4 kb fragments, and ligated into *Bam*HI digested pRG970. The genomic library was electroporated into *E. coli* SLR400 (pSHL4K) and plated

Table 5-2: Oligonucleotide Primers.

Primer Name	Sequence (5'-3')
431-8	ACGCCAGGGTTTTCCCAGTCA
431-9	ATTGCCCGGCTTTCTTGTAACG
DC1048R	TTCTGTCGTTTGAGGATTACTT
DC1183	CGGCAGCATCGGGGTCAG
DC1522R	CCCAAGCTTAGCGCGAGAAACACTAAAGG
DC2037R	TCGCCCCGGGGGCTGACGAAAGGCATTGG
DC2050R	CACATGGCGCAGGAACTCG
DC285	CATCACTGGTACGCAACGCT
DC3497	TCGCCCCGGGCCCAGCCCTTTAGTGTTTCT
DC37	CGGAATTCCCAATGCCTTTCGTCAGCC
DC4631R	GCTCTAGAGATTGCTGCCCATACACTGC
DC598R	GATGCTCTTCACCCTAAGAGGACACGATTCATACC
DC929R	AACGGGATATGGATGTCTGC
DC971	TACCGTGGGCTGGAAAAGAA
DC974	CGGAATTCGCAGCCTCATTTCCAACATC
K1140R	TCGCCCCGGGCGCCACGGTTGATGAGAG
K12	TCGCCCCGGGACACATCTCAACCATCATCG
Rpt1028R	CCCAAGCTTTAGGGACCAAAAAGCCAGAC
Rpt131	CGGAATTCAACCACCAACGGACGACTTA
Rpt409	GATGCTCTTCAGGGAAGCACGAGACGGGCGGT

onto MacConkey agar containing 1% lactose and 0.02% arabinose. Following overnight incubation at 37°C, Lac^+ colonies were purified on KB and screened for arabinose-dependent Lac phenotypes on MacConkey lactose agar. Fragments from those colonies exhibiting an arabinose-dependent phenotype were amplified from pRG970 using primers 431-8 and 431-9 and the nucleotide sequence was obtained.

Construction of pJBHopPtoD2 and pJBHopPtoD2ΔC. *hopPtoD2* was amplified via PCR using primers DC37 and DC1522R and cloned into pDSK600 as an *Eco*RI/*Hind*III fragment. The region encoding for the first 321 codons of *hopPtoD2* was amplified by PCR using the primers DC37 and DC1048R and ligated into pDSK600 as an *Eco*RI/*Hind*III fragment to create pJBHopPtoD2ΔC.

Plant Assays. Overnight cultures grown at 25°C were harvested, washed, and diluted in sterile distilled water. To assay for the HR, *N. tabacum* L. cultivar Samsun leaves were infiltrated in parallel with bacterial suspensions of 10^6-10^9 CFU/ml using a syringe and incubated at 25°C. Infiltrated leaf panels were scored hourly for water soaking/tissue collapse beginning 2 hours after inoculation (27). Virulence of DC3000 was determined in *A. thaliana* ecotype Columbia (Col-0) leaves infiltrated with 10^5 cells/ml. Bacterial populations were monitored using the leaf disk assay (18).

Phosphatase Assays. The indicated *P. syringae* strains were grown in KB medium overnight. Cells were diluted into fresh KB medium and grown to an OD_{600} of 1.0.

Cells were harvested, suspended in M63 medium containing fructose and incubated at 25°C with shaking for an additional 4 hrs. The cells were removed by centrifugation and the supernatant concentrated 50-fold using Millipore Ultra-free centrifugal filter devices with 10 kD exclusion limits. Protein concentration in culture filtrates was determined using Pierce BCA Protein Assay kit (Pierce, Rockford, IL) and samples equivalent to 100 mg total protein were used in the *p*NPP assay. PTP activity was assayed at room temperature in *p*NPP assay buffer, 20 mM *p*NPP, and where indicated, 1mM sodium orthovanadate, as described previously (74). The hydrolysis of *p*NPP was detected by the increase in absorbance at 410 nm.. To measure tyrosine-specific dephosphorylation of phosphorylated peptides, whole cell extracts of *E. coli* strains expressing pDSK600 or pJBHopPtoD2 were assayed for activity using a commercial PTP Assay Kit according to the manufacturer's instructions (Sigma-Aldrich). For both assays, lymphocyte antigen-related protein (LAR) PTP was used as a control.

AvrRpt2-linked Translocation Assay. A HopPtoD2':'AvrRpt2 fusion was created using the procedures of Guttman and Greenberg (89). The region encoding for the first 171 codons of *hopPtoD2* was amplified by PCR using the primers DC37 and DC598R, while '*avrRpt2* was amplified using primers Rpt409 and Rpt1028R. The amplified fragments were digested with *Sap*I, ligated, and the desired fusion was amplified using primers DC37 and Rpt1028R. The resulting fusion was cloned as an *Eco*RI/*Hind*III fragment into pDSK600 to create pJBHopPtoD2':'AvrRpt2. The fusion was confirmed by sequencing. The positive control plasmid, pJBAvrRpt2,

carrying the full length *avrRpt2* gene was created using primers Rpt131 and Rpt1028R. *A. thaliana RPS2* or *A. thaliana rps2* (A gift of B. Staskawicz) leaves were inoculated in triplicate with 10^8 CFU/ml of the indicated strains using syringe-mediated infiltration and monitored for development of disease symptoms and/or HR production. The experiment was repeated 3 times with identical results.

Construction of *ΔhopPtoD2* mutant JB4. The regions flanking the *hopPtoD2* locus of DC3000 were sequentially cloned into pBlueScriptSK+ (Strategene; La Jolla, CA). The primers DC974 and DC2037R were used to amplify the upstream region, and the primers DC3497 and DC4631R were employed to amplify the downstream region. A *kan* cassette was amplified using primers K12 and K1140R from the EZ::TN™ <Kan2> transposon (Epicentre Technologies, Madison, WI) and ligated to the two flanking regions as a *Xma*I fragment. The resulting plasmid, pJBΔ*hopPtoD2*, was transformed into DC3000 and kanamycin resistant mutants were created by marker exchange. Insertional replacement of *hopPtoD2* in JB4 was verified by PCR employing primers DC974 and DC4631R.

Active Oxygen Assay. Tobacco suspension cells (*N. tabacum* cv. Hicks) were prepared (13) and resuspended in 25 ml assay buffer (0.5 mM MES (pH 6.0)) to a final concentration of 0.5 mg fresh weight/ml. Exogenous H_2O_2 was added to a final concentration of 50 μM to overcome the antioxidant activities of the suspension cultured cells. Overnight cultures of *P. syringae* grown at 25°C were harvested, washed with sterile dH_2O and added to the suspension cells to a final OD_{600} of 0.05.

P. syringae-inoculated cell cultures were incubated at 25°C and 0.4 ml samples were assayed in triplicate every 30 minutes to determine hydrogen peroxide levels. Hydrogen peroxide levels were determined using a Berthold 953 Luminometer (Bad Wildbad, Germany) as described previously (13). Each reaction mixture contained 0.72U peroxidase and 77.6 μM luminol.

PR1 gene expression assay. *A. thaliana* Col-O:PR1-GUS leaves were infiltrated with 1 x 10^6 CFU/ml of the indicated strain and leaf samples were removed after 48 h (202). GUS activity was assayed using the fluorometric substrate 4-methylumbelliferyl-β-D-glucuronide (ICN Biochemicals) and a TKO 100 Fluorometer (Hoefer Scientific) and quantified using a standard curve.

Detection of *hopPtoD* alleles in *P. syringae* strains. The genomes of *P. syringae* strains were initially surveyed for presence of each of the three identified *hopPtoD* homologs by intact cell PCR. The ability to amplify an 867 bp fragment from the 3' end of *hopPtoD1* using primers DC1183 and DC2050R was used to indicate the presence of *hopPtoD1*. A 763 bp fragment amplified by primers DC285 and DC1048R was used to indicate the presence of *hopPtoD2*. Amplification of a 630 bp fragment spanning the region between the IS52-like element and the 3' region of *hopPtoD3* using primers DC971 and DC929R was indicative of the presence of *hopPtoD3*. To verify the presence of the *hopPtoD2*, the 763 bp fragment amplified by primers DC285 and DC1048R was labeled with [α^{32}-P] dCTP to generate a probe

specific to *hopPtoD2*. Strains were surveyed for homologs by colony hybridization (10).

Results

Identification of an unusual *avrPphD* homolog in *P. syringae* pv. *tomato* DC3000 by a HrpL-dependent promoter trap assay. To evaluate the effectiveness of the HrpL-dependent promoter trap assay (Figure 5-1) in identifying promoters for effector genes, a partial screen of the *P. syringae* pv. *tomato* DC3000 genome was attempted. *E. coli* transformants carrying an arabinose-inducible '*hrpL* construct (pSHL4K) and a DC3000 genomic library fused to the promoterless '*lacZYA* cassette of pRG970 were screened for arabinose-dependent Lac phenotypes. In 5 of the 7 colonies exhibiting arabinose-dependent Lac^+ phenotypes, sequence analysis of the insert revealed HrpL-dependent promoter sequences (Table 5-3)(239). For two of these inserts, the HrpL-dependent promoter appeared to control the DC3000 orthologs of the *P. syringae* pv. *syringae* (*Psy)* B728A *hrpW* and *P. syringae* pv. *phaseolicola (Pph)* 1302A *avrPphE*. The three remaining clones carried a strong candidate HrpL-dependent promoter sequence that subsequently was found to be 5' to an IS52-inactivated homolog of *avrPphD* that consisted of a 711 codon open reading frame (ORF) with a 1210 bp IS52-like insertion at codon 169 (Figures 5-2 and 5-3). This previously unreported *avrPphD* homolog was located in an unannotated portion of the DC3000 genome, and excluding the IS52 sequence, exhibited 86% identity (I) and 90% similarity (S) to AvrPphD (8). This locus was designated here as *hopPtoD3*.

Figure 5-1: HrpL-dependent promoter trap assay. A *P. syringae* pv. *tomato* DC3000 genomic library fused to the promoterless *'lacZYA* cassette of pRG970 was created by digesting DC3000 genomic DNA with *Sau*3A and ligating 2-4 kb fragments into *Bam*HI digested pRG970. The resulting genomic library was electroporated into *E. coli* SLR400 (*Δara, Δlac*) containing an arabinose-inducible *'hrpL* construct expressed from the plasmid pSHL4K. Lac^+ transformants were selected by plating onto MacConkey agar containing lactose, arabinose, kanamycin, and spectinomycin. To identify colonies containing potential HrpL-dependent promoters, these Lac^+ colonies were screened for arabinose-dependent Lac phenotype on MacConkey lactose agar with or without arabinose. Fragments from those colonies exhibiting an arabinose-dependent phenotype (gray box) were amplified from pRG970 using PCR and the nucleotide sequence was obtained. Open boxes represent genes expressed from the individual plasmids. The P_{BAD} promoter is represented by the bent arrow.

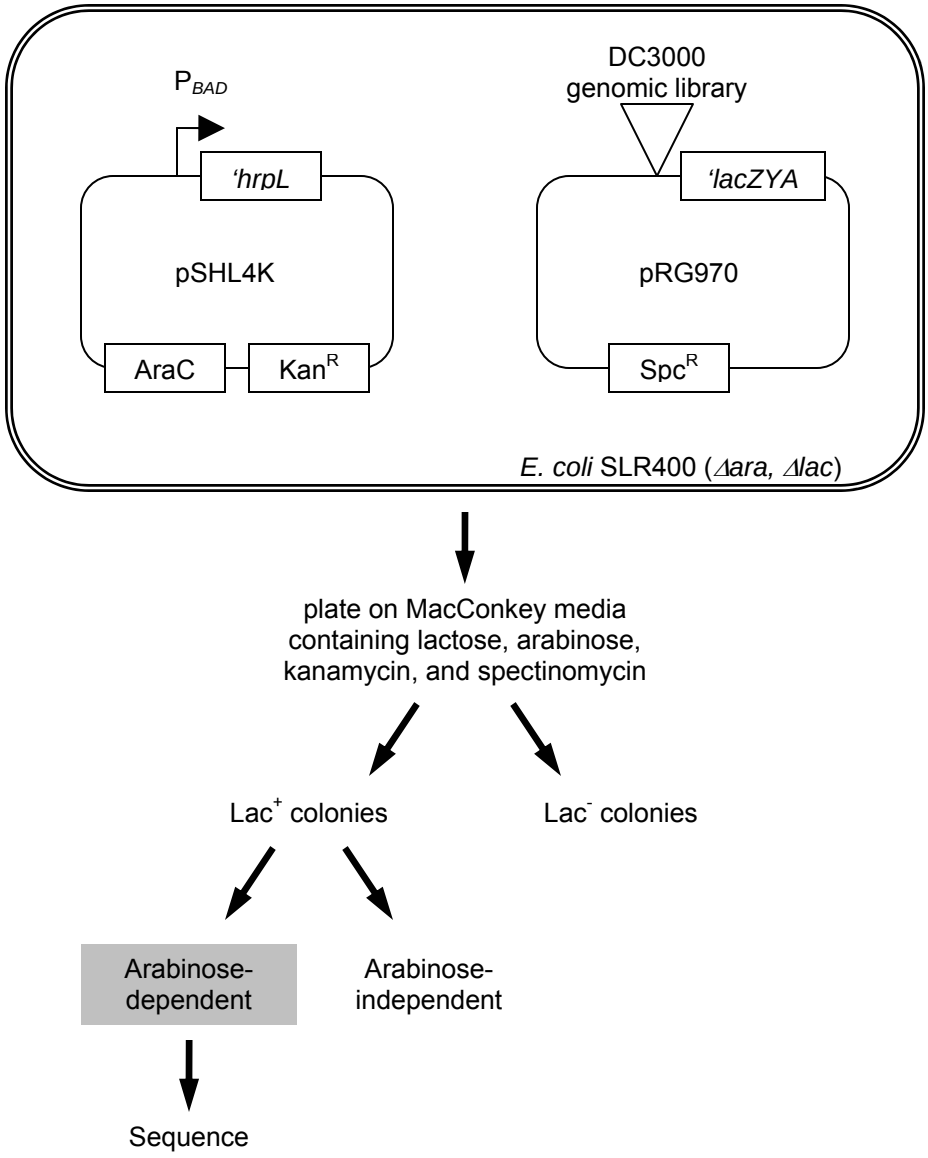

P$_{BAD}$
'hrpL
pSHL4K
AraC
Kan^R
DC3000
genomic library
'lacZYA
pRG970
Spc^R
E. coli SLR400 (Δara, Δlac)
plate on MacConkey media
containing lactose, arabinose,
kanamycin, and spectinomycin
Lac$^+$ colonies
Lac$^-$ colonies
Arabinose-
dependent
Arabinose-
independent
Sequence

Table 5-3: HrpL-dependent Promoters Identified in *P. syringae* pv *tomato* DC3000 Promoter-Trap Screen.

Clone(s)	Gene[1]	HrpL-dependent promoter[2,3]
pJB2-1	*hrpW*	GGAACCcG..N14..CCACtcA
pJB13-5	*avrPphE*	GGAACTgA..N13..CgACatA
pJB13-1, pJB5-3, pJB5-6	unknown	GGAACCcA..N13..CCACatA

[1] as identified by BLASTX search (5)

[2] apparent HrpL-dependent promoter identified by similarity to consensus sequence GGAACCNA..N13/14..CCACNNA

[3] identical nucleotides are capitalized

Figure 5-2: *P. syringae* pv. *tomato* DC3000 AvrPphD alleles. The deduced protein sequence from the uninterrupted ORF of *hopPtoD3,* the apparently wild-type allele HopPtoD1, and the amino terminal domain of HopPtoD2 were aligned against the original AvrPphD sequence from *P. syringae* pv. *phaseolicola*. The carboxyl-terminal domain of HopPtoD2 contains the PTP active site and does not align with any of the other DC3000 AvrPphD alleles. The IS52 insertion site in HopPtoD3 is marked by "<>". Proteins were aligned using CLUSTALW (43). Identical residues are framed in black while similar residues are marked in gray.

```
AvrPphD      1 MNPLRSIQHNITTPPISGGQPLDAVGPQAQ--QSHPKRISPSQLSQSAHQALERLS--AN
HopPtoD1     1 MNPLRSIQHNIATPPISGGQPLDAVGPQAQ--QSHPKRISPSQLSQSAHQALERLS--AN
HopPtoD3     1 MNPLQSTQHSITTPLISGGRPLDAVGPQAQ--QSHPKRISPSQLSPGAHQALKRPS--AN
HopPtoD2     1 MNPLQPIQHSITNSQMSGGQQLEAEGSQAHNSYSHPDRISLSQLSQSAHLALDHLSTQPN

AvrPphD     57 AEHQRLASLVRNALQDGTFQFQSSNHTQVTYKASICLPADT------DTVRTDHLINNEL
HopPtoD1    57 AEHQRLASLVRNALQDGTFQFQSSNHTQVTYKASICLPADT------DTVRTDHLINNEL
HopPtoD3    57 AEHQRIASLVRNALQDGTLQFQSSNDKQVTYKAPVCLPADTSTDTDTDTVRTERLINNEL
HopPtoD2    61 TDHQRVASLVRNAVQDGKFQLQSSNDTQVTYKTSVCPPANA------DTMGAAHLINNEL

                                                              <>
AvrPphD    111 TVQARLNDQSEYDIVSAHLHGSSKAISFDVPSPPPAHGSASSVLSERTHLGMSSVLSQDA
HopPtoD1   111 TVQARLNDQSEYDIVSAHLHGSSKAISFDVPSPPPAHGSASSVLSERTHLGMSRVLSQDA
HopPtoD3   117 TVQARLNDQSEYDIVSAHLHGSSRSISFDVPSPPPAHGSASSALSERTHLGMSRVLSQDA
HopPtoD2   115 TVQARLNDQLEYDIVSAHLYGPSEAISI--------------------------------

AvrPphD    171 VDSSSLETPLVSSPDHSRPPSQPKPVHIGSVRRDSGSLVSDNPVVQALLSFVQADQAFPP
HopPtoD1   171 VDSSSLETPLLSSPDHSRPPSQPKPVHIGSVRRDSGSLVSDNPVVQALLSFAQADQAFPP
HopPtoD3   177 VDGSGLESPLLSSRAHSRPPSQPEPVHIGSVRRDSGSLVSDNPVVHALLAFAQADQAFPP
HopPtoD2       ------------------------------------------------------------

AvrPphD    231 QAASIAGVQLEMRSRRDIEQALEELKGAFTVEKAQLMSGGSSSERVDEDVNADIHIPLLL
HopPtoD1   231 QAASIAGVQLEMRPRRDIEKALEEFKGAFTVVKAQLMSGANSSERVDEDVNADIHIPLLL
HopPtoD3   237 QAASIDGVQLEMRPTRDIEQALKKFKDAFTVEKAQLMSVTNSSERADEDINADIHIPLLL
HopPtoD2       ------------------------------------------------------------

AvrPphD    291 KAIERGAGAFGPGALIEIADGGQISAKAFLASCAPTITSNDDVLSEFINQKLKGDDDLQV
HopPtoD1   291 KAIERGAAAFGP-----NASIGQNSAKAFLASCAPKITSNDDVLSEFINQKLKGDDDLQV
HopPtoD3   297 KAIEQGAAAFGP-----NASIGQNSAKAFLASCAPTITSNDDVLSAFINQKLKGDDDLKV
HopPtoD2       ------------------------------------------------------------

AvrPphD    351 RLGAQELLHVATKKEFQLGGLAGSIGVSSILGSAWELGASELLKNAIFGKNFSPSQYALQ
HopPtoD1   346 RLGAQELLHVATKKEFQLGGLAGSIGVSSILGSAWELGASELLKNAIFGKNFSPSQYALQ
HopPtoD3   352 RLGAQELLHVATKKEFQLGGLAGSIGVSSILGSAWELGASELLKNAIFGKNFSPSQYALQ
HopPtoD2       ------------------------------------------------------------

AvrPphD    411 LAGIDSVPPFIIEAMDSFCVLVIIKGMKGELWSMKDLLPKALKAGAISSAMSFPNNVLQY
HopPtoD1   406 LAGIDSVPPLIIESMDTMCVLAIIKGMKGEEWSMSDLLPKALKAGAISSVVSFPNNVLQY
HopPtoD3   412 LAGIDSVPPLMIESMDTMCVLAIIKGMKGEEWSMSDLLPKALKAGAISSVVSFPNNVLQY
HopPtoD2       ------------------------------------------------------------

AvrPphD    471 AGFKSRVADLAANSITTEAAIFGAASGIPPEVKESEELMRAGLFQSMKDGVMAHPGEGMD
HopPtoD1   466 AGFKSRVGDLAANSVTTEAAIFGAASGIPPEVKESEELMRAGLFQSMKDGVMAHSGEGVD
HopPtoD3   472 AGFKSRVGDLAANSVTTEAAIFGAASGIPPEVKESEELMRAGLFQSMKDGVMAHPGEGVD
HopPtoD2       ------------------------------------------------------------

AvrPphD    531 TKETIERMTRHALDIAPGESTAVKSMGLAAIVGMIPLIASSKATGLVSEQILRIFRNAVF
HopPtoD1   526 TKKTIERMTRHALDIAPGESTAVKSMGLASIVGMIPLIASNKATGLLSEQVLRIFRSAVF
HopPtoD3   532 TKETIERMTRHALDIAPGESTAVKSMGLASIVGMIPLIASNKATGLLSEQVLRIFRSAVF
HopPtoD2       ------------------------------------------------------------

AvrPphD    591 NPIEAIALNALALGGRVNVPGLFDSDNAKHARVAQTILARASQHMEAGDREISAEELHQM
HopPtoD1   586 NPIEAIALNALALGGRVNVPGLFDSDNAKHARVVQTILARASQHMEAGDRDISAEELHQM
HopPtoD3   592 NPIEAIALNALALGGRVNVPGLFDSDNAKHARVVQTILARASQQMEAGDRNISAEELHQM
HopPtoD2       ------------------------------------------------------------

AvrPphD    651 LAPRSEFLRHVGSAIVNGMNASFEAIPALVRKLGYGEAPLAERIPYQDLAVPDTSRQPAP
HopPtoD1   646 LAPRSEFLRHVGSAIVNGMNASFEAIPALVRKLGYGEAPLAERIPYQDLAVPDTSRQPAP
HopPtoD3   652 LAPRSEFLRHVGSAIVNGMNASFEAIPALARKLGYGEAPLAERIPYQDLAVRDSPRQPAP
HopPtoD2       ------------------------------------------------------------
```

Figure 5-3: The IS52-like element of *hopPtoD3.* The nucleotide sequence of the IS52-like insertion element from *hopPtoD3* was aligned against an IS52 insertion element from *P. syringae* pv. *savastanoi* and the JR1 insertion element from *Pseudomonas* sp. JR1 using CLUSTALW (43). The largest ORF in the IS52-like element from *hopPtoD3* encodes a 330 residue putative transposase that is 91% identical to the JR1 transposase. Identical residues are marked in black. The direct repeat sequences from the insertion site in the DC3000 genome are marked by asterisks.

```
                    *****
hopPtoD3      1 --------CTAGGGAGGCGCTGCAAAAATAGCCAACTGTCCCCACCCTTGGCACACTAAG
JR1           1 TCTTTTCACTAGGGAGACGCTGCAAAAATAGCCAACCGTCCCCACCCTTGGCACACTGAG
IS52          1 ----TTCTTTAGGGAGCCGCTGCAAAAATAGCCAACAGCCCGTAGCCTTGGCACACTGAG

hopPtoD3     53 TTCCTTCAACCAGCCTCCCTCTCCGTGAGC--GTTACGCGCGTGCAGAAAACCTTCTCCG
JR1          61 TACCTTCAACCAGCCACCCTCTCCGTGAGC--GTTACGCGCGTGCAGAAGACCTTCTCCG
IS52         57 CACCTTCAACCACCC--CCTCTCCGTGAGCCTGTTACGCGCGTGCAGAAGACCTTCTCCG

hopPtoD3    111 AACTCGAATATACCGGCAAGAAAAAGCAGACTCGCCGAGATCGCTTCCTGGCTGACCTTG
JR1         119 AACTCGAATACACCAGCAAAAAAAAGCAGACTCGCCGAGATCGCTTCCTGGCTGACCTTG
IS52        115 AACTCGAATACACCGGCAAGAAAAAGCAGACTCGTCGCGACCGCTTCTTGGCCGACCTTG

hopPtoD3    171 AACAGTTGGTGCCCTGGGCCCAGCTGGAGGCGCAAGTGGCGCCGTTTTATAGCAACACCG
JR1         179 AACAGCTGGTGCCCTGGGCACTGCTGGAGGCGCAAGTGGCGCCGTTCTATAGCGACACCA
IS52        175 AACAGTTGGTGCCCTGGGCGCAACTCGAAGCGCAAGTGGCTCCTTTCTATAGCGACACCA

hopPtoD3    231 CAGGCAAGCGCGGACGCCCTGCGATAGGGGTGTCGCGCATGTTGCGCATGTACGTCGTGC
JR1         239 CAGGCAAGCGCGGACGCCCTGCGATTAGGTTGTCACGCATGCTGCGCATGTATGTTGTGC
IS52        235 CAGGCAA-CGCGGGCGCCCGGCGATCGGGTTGTCGCGCATGCTGCGCATGTATGTCGTGC

hopPtoD3    291 AGCAGTGTTTCGGTTTCTCCGATGAAGGTTGCGAAGATGCCGTCTACGACAGCCAGGCCA
JR1         299 AGCAGTGTTTCGGTTTCTCCGATGAAGGTTGCGAAGATGCCGTCTACGACAGCCAGGCCA
IS52        294 AGCAGTGTTTTGGTTTCTCCGATGAAGGTACCGAAGATGCCGTCTACGACAGCCAGGCCA

hopPtoD3    351 TCCGCGGTTTTATGGGTATCGACCTGGGTCGCGAGTCGGCACCGGATGCCACCACCTTGC
JR1         359 TCCGTGGTTTTATGGGCATCGACCTGGGCCGCGAGTCGGCACCGGATGCCACTACCTTAC
IS52        354 TTCGTGGTTTTATCGGCATCGATCTGGGCCGTGAGTCGGCACCGGATGCCACCACCCTGC

hopPtoD3    411 TGCGTTTTCGCCGCTTGCTGGAAACCCATCAGCTAACGCGGCTGCTGTTTGAAACGATTA
JR1         419 TGCGTTTTCGCCGCTTGCTGGAAACCCATCGGCTAACGCGGGTGCTGTTTGAAACGATTA
IS52        414 TGCGATTTCGTCGCTTGCTGGAAACCCATCAGCTGACACGAGTGCTGTTTGAAACGATTA

hopPtoD3    471 ACCAGCATCTGGCCAGCCGGGGGCTGCTGCTCAAGGAAGGCACTATCGTCGACGCTACCC
JR1         479 ACCTGCATCTGGCCGGCCGGGGTCTGCTGTTCAAGGAAGGCACTATCGTCGACGCTACCC
IS52        474 ACCAGCATCTGGCCAGCCGAGGTCTGCTGCTCAAGGAAGGCACTATCGTCGACGCTACCC

hopPtoD3    531 TGATCGCCGCGCCGCCCTCGGTCAAGAACCGAGAAGGCAAGCGTGATCCTGAGATGCATC
JR1         539 TGATCGCCGCGCCGCCCTCGGTCAAGAACCGAGAAGGCAAGCGTGATCCTGAGATGCATC
IS52        534 TGATCGCCGCGCCGCCCTCGGTCAAGAATCGAGAAGGCAAGCGTGATCCCGAGATGCATC

hopPtoD3    591 AGGCCAGGAAAGGCAATCAATGGCACTTCGGGATGAAGGCCCACATTGGTGTAGACGCCA
JR1         599 AAGCCAAGAAAGGCAATCAATGGCACTTCGGGATGAAGGCTCACATTGGTGTCGACGCCA
IS52        594 AGGCTAAAAAAGGTAATCAGTGGCACTTTGGGATGAAGGCCCACATTGGTGTCGACGCCA

hopPtoD3    651 CGTCGGGGCTGGTGCACAGCGTAGTAGGGACGGCCGCTAACGTGGCGGATGTCACCCAGG
JR1         659 CGTCGGGGCTGGTGCACAGCGTGGTAGGGACGGCCGCCAACGTGGCGGATGTCACCCAGG
IS52        654 CGTCGGGGCTGGTGCACAGCGTAATAGGCACGGCTGCTAACGTGGCGGATGTCACCCAGG

hopPtoD3    711 TTGGCCAGTTGCTGCACGGTGACGAAACCTATGTTTCGGGTGACGCTGGATACACCGGTG
JR1         719 TCGACCAGTTGCTGCACGGTGCCGAAACCTATGTTTCGGGTGACGCTGGATACACCGGTA
IS52        714 TTGATCAGTTGCTGCACGGTGACGAAACCTATGTTTCGGGTGACGCTGGATACACCGGTG

hopPtoD3    771 CGGCCAAGCGACCGGAGCATGCTGAACGGGACGTTATCTGGTCGATTGCAGCACGGCCAA
JR1         779 CGGCCAAGCGACCGGAGCATGCTGAACGGGACGTTATCTGGTCGATTGCAGCCCGGCCAA
IS52        774 CGGCCAAGCGACCGGAGCATGCTGAACGGGACGTTATCTGGTCGATTGCAGCACGGCCAA
```

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hopPtoD3  831 GCAGTTACAAGCAGCACGGCGAAGGCAGCGTGCTGTATCGGGTCAAGCGTAAAATTGAAT
JR1       839 GCAGTTACAAGCAGCACGGCGAAGGCAGTGTGCTGTACAGGGTCAAGCGCAAGATCGAAT
IS52      834 GCAGTTACAAGCAGCACGGCGAAGGCAGCGTGCTCTATCGGGTCAAGCGCAAAATTGAAT

hopPtoD3  891 ATGCCAAGGCGCAACTGCGCGCCAAGGTCGAGCACCCCTTCCAGGTAATCAAGGTGCGCT
JR1       899 ACGCCAAGGCGCAACTGCGTGCCAAGGTCGAGCACCCATTTCAGGTAATCAAGGTGCGCT
IS52      894 ATGCCAAGGCGCAACTGCGTGCCAAGGTCGAGCACCCCTTCCAGGTAATCAAGGTGCGCT

hopPtoD3  951 TCAATCATCGCAAGGTTCGCTACCGTGGGCTGGAAAAGAATACAGCGCAGTTGTTC-AGT
JR1       959 TCAATCATCGCAAGGTTCTCTACCGTGGGCTGGAAAAGAATACGGCGCAGTTGTTCCAGT
IS52      954 TCAATCATCGCAAGGTTCGCTACCGTGGGCTGGAAAAGAATACGGCGCAGTTGTTC-AGT

hopPtoD3 1010 TTGTTTGGGTTGGCCAATCTGATGCTGGCCAAGCGGTATTTGCAACAGGCGGCAGGATAA
JR1      1019 CTGTTTGGGTTGGCCAATCTGATGCTGGCCAAGCGGTATTTGCAACGGGCGGCGGGATAA
IS52     1013 TTGTTTGGGTTGGCCAATCTGATGCTGGCCAAGCGGTATTTGCAACGGGCGGCGGGATAA

hopPtoD3 1070 ATCCGTCTGAAAGGCGGGACTGGCCCGCCAATCAGCAAAAGGAGGGCAGAAATCTGCCCG
JR1      1079 ATCCGTCTGAAAGGCGGGT-TGACCCGCAAATCAGCAAAATGAGGGCGAAAATCGGCCCT
IS52     1073 ATCCGTCTGAAAGACGGGACTGGCCCGTAAATCAGCAAAATGAGGGGAGAAACCTGCCCC

hopPtoD3 1130 AGAAACGCAAAGCAAGGCCGGCAGGTTGAAAAAAACCGGCTTGGAAATGAAGACGGTGCG
JR1      1138 GGAAATGCAAAT-AAGGCCGGTAGGTTGAAAAAAACCGGCTTGGAAATGGGGGCGGTGCG
IS52     1133 CGAAACGTAAAACAAGGTCGGCAGGGCGAAAAAAAC-GACTTGGAAATGGGGGCGGTGCG

                                    *****
hopPtoD3 1190 AACGG-GTTAATTTTTCAGCGTCTCCCTAGG-------
JR1      1197 AACGG-GTTAATTGTTCAGCATCTCCCTAGAGGGT---
IS52     1192 AACGGAGTTAATTGTTCAGCGTCTCCTTAGTTGCCCCG
```

Identification of a candidate protein tyrosine phosphatase. The identification of an insertionally inactivated ortholog of the widely-distributed AvrPphD (8) was unusual. Using the amino terminus of *hopPtoD3* as a probe, the genome of DC3000 was found to carry two other homologs of *avrPphD*. The first, *hopPtoD1,* was an apparent wild-type allele of *avrPphD* (705 aa) that was 89% I/ 91% S to the *Pph* AvrPphD (70)(Figure 5-2). The second homolog was equivalent to *hopPtoD2* (177) and was located 1577 bp 5' to *hopPtoD3* (Figure 5-4). The amino terminal 142 aa of this homolog retained 61% I / 70% S to the amino terminal 142 aa of AvrPphD (Figure 5-2), whereas the remaining 326 aa carboxyl terminal domain exhibited no detectable similarity to AvrPphD. However, a protein tyrosine phosphatase (PTP) domain was identified in the carboxyl terminal domain by the conserved domain finder of the BLAST algorithm. The PTP active site signature sequence [LIVMF]HCxAGxxR[STC][STAG] (68) at position 376-386 contained the critically spaced cysteine and arginine residues that are essential for PTP activity (Figure 5-5). In addition, the general acid motif typical of other PTPs was also detected at position 351.

HopPtoD2 has protein tyrosine phosphatase activity. While translocated PTPs have been identified as TTSS-dependent effectors of *Yersinia* and *Salmonella* (22, 88, 121, 165), similar effectors had not been reported previously for a plant pathogenic bacterium. Due to difficulties in purifying HopPtoD2, culture filtrates carrying secreted proteins from wild-type DC3000 and JB4, a Δ*hopPtoD2* mutant of DC3000,

Figure 5-4: *P. syringae* pv. *tomato* DC3000 genomic region containing unique AvrPphD alleles. Solid lines above arrows indicate regions represented by constructs indicated on the left. Dashed lines indicate unsequenced portions of pJB015. Filled arrows indicate open reading frames. Bent arrows indicate HrpL-dependent promoters. Black boxes represent areas similar to *avrPphD* from *P. syringae* pv. *phaseolicola*. Hashed box indicates 1210 bp IS52 insertion element in HopPtoD3. Carboxy-terminal tyrosine phosphatase domain of HopPtoD2 is represented by the gray arrow.

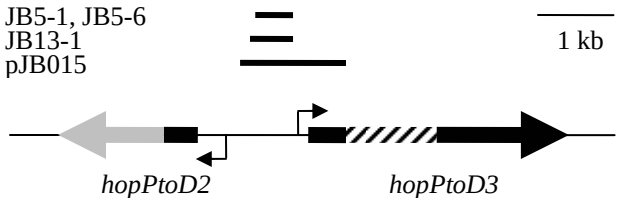
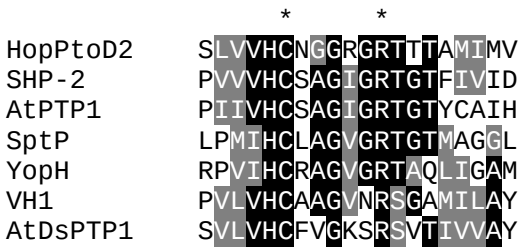

JB5-1, JB5-6
JB13-1
pJB015
1 kb
hopPtoD2
hopPtoD3

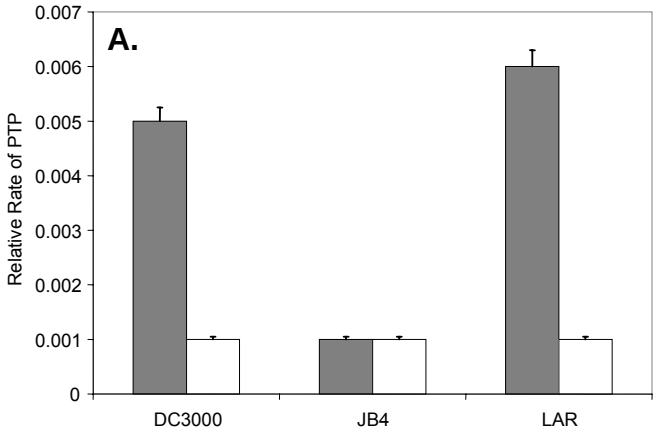
Figure 5-5: Conservation of the protein tyrosine phosphatase signature sequence of HopPtoD2. The HopPtoD2 apparent active site was aligned with the active site of other PTPs (68) using CLUSTALW (43). The catalytically required cysteine and arginine residues are marked by *. Identical residues are framed in black while similar residues are marked in gray.


*
*
HopPtoD2 SLVVHCNGGRGRTTTAMIMV
SHP-2 PVVVHCSAGIGRTGTFIVID
AtPTP1 PIIVHCSAGIGRTGTYCAIH
SptP LPMIHCLAGVGRTGTMAGGL
YopH RPVIHCRAGVGRTAQLIGAM
VH1 PVLVHCAAGVNRSGAMILAY
AtDsPTP1 SVLVHCFVGKSRSVTIVVAY

were used to determine if HopPtoD2 had PTP activity. These filtrates were analyzed for their ability to hydrolyze an artificial PTP substrate, *para*nitrophenyl phosphate (*p*NPP) (249). Culture filtrates from DC3000 were able to hydrolyze *p*NPP at a rate similar to the activity of LAR, a known PTP (215). Culture filtrates from JB4 hydrolyzed *p*NPP at a much lower rate (Figure 5-6A). Addition of a PTP inhibitor, sodium orthovanadate (74), eliminated detectable phosphatase activity in culture filtrates of DC3000. To confirm that HopPtoD2 was capable of hydrolyzing a phosphorylated protein substrate, extracts of *E. coli* transformants expressing HopPtoD2 were surveyed for activity against a commercially available phosphotyrosine peptide derived from the insulin receptor. Extracts of cells expressing HopPtoD2 were capable of hydrolyzing the insulin receptor substrate at rates 2-3 fold faster than the control extracts (Table 5-4). The gain of PTP activity by *E. coli* transformants specifically expressing *hopPtoD2* combined with the strong conservation of the critical PTP active site motifs indicate that HopPtoD2 is responsible for the PTP activity detected in DC3000 culture filtrates.

HopPtoD2 is translocated into host cells by the *hrp* TTSS. The high similarity of the amino terminus of HopPtoD2 to the amino terminus of AvrPphD is consistent with translocation of HopPtoD2 into plant cells via the *hrp* TTSS. Because secreted effectors are labile and not readily detected in planta, an alternative approach to detect secretion is necessary. In *P. syringae*, the secretion signal required for *hrp*-dependent secretion is carried by the first 50 amino acids of the secreted effector, whereas the effector activity localizes to the carboxyl terminal domain (89, 163). The carboxyl

Figure 5-6: Protein tyrosine phosphatase activity of DC3000 and Psy61 culture filtrates. The indicated strains were incubated under *hrp*-inducing conditions to an OD_{600} of approximately 1.2. Culture supernatant was collected, concentrated 50-fold and normalized for protein content. Phosphatase activity was assayed using pNPP as described in the Experimental Procedures. Product accumulation was monitored spectrophotometrically and the relative rate of PTPase activity calculated from the slope of the reaction (ΔA_{410}/min) averaged over the 75 min assay period. The reactions were near linear during the entirety of the assay period. Gray bars represent activity of the enzyme alone. White bars represent the activity observed in the presence of 1 mM sodium orthovanadate, a PTP inhibitor. Error bars represent the standard deviation obtained in at least three separate experiments. **A.** DC3000 (WT, HopPtoD2$^+$) and JB4 (*ΔhopPtoD2*) culture filtrates were analyzed for PTPase activity. One unit of purified LAR (leukocyte antigen-related protein), a known PTP, was used as a positive control for the assay. **B.** Culture filtrates from Psy61 (HopPtoD2$^-$) carrying the following plasmids were analyzed for PTPase activity: pDSK600 (empty vector); pJBHopPtoD2 (full length HopPtoD2); or pJBHopPtoD2ΔC (carboxy terminal truncation of HopPtoD2 lacking the PTP domain).


A.
Relative Rate of PTP
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
DC3000
JB4
LAR

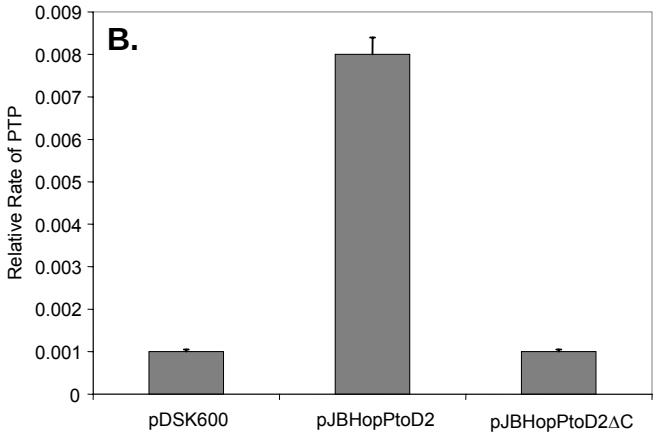

B.
Relative Rate of PTP
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
pDSK600
pJBHopPtoD2
pJBHopPtoD2ΔC

Table 5-4: Protein tyrosine phosphatase activity detected in lysates of *E. coli* expressing *hopPtoD2*.

Expressed gene[1]	PTP activity[2]
None	88±13
HopPtoD2	259±4
LAR[3]	210±11

[1] None, *E. coli* DH5α (pDSK600); HopPtoD2, *E. coli* DH5α (pJBHopPtoD2); LAR, purified leukocyte antigen related protein, a known PTP.

[2] whole cell extracts of the indicated strains were monitored for PTP activity using residues 1142-1153 of the insulin receptor as the phosphotyrosine substrate. PTP activity measured as the amount of phosphate released per minute (pmole PO_4^{2-}/min). The value reported represents the mean of duplicate samples and the experiment was repeated three times with similar results.

[3] LAR, leukocyte antigen-related protein. One unit of purified LAR, a known PTP, was used as a positive control for the assay.

terminal effector domain of AvrRpt2 has been shown to function independently as a reporter for translocation into host cells (90, 163). Fusion proteins carrying the effector domain of AvrRpt2 elicit the HR in the *RPS2* ecotypes of *A. thaliana* after translocation into plant cells via the *hrp* TTSS. When DC3000 expressing a fusion between the amino terminal 171 aa of HopPtoD2 and the 'AvrRpt2 effector domain was tested using this assay, the strain carrying the HopPtoD2':'AvrRpt2 fusion elicited the HR in the reactive *RPS2* line of *A. thaliana* but not in the nonresponsive *rps2* derivative (Table 5-5). Expression of the HopPtoD2':'AvrRpt2 fusion in A9, a Δ*hrpA* mutant of DC3000 that lacks a functional TTSS (231), produced a null phenotype. These results indicate that HopPtoD2 is translocated into plant cells via the *hrp* TTSS.

DC3000 Δ*hopPtoD2* mutant exhibits reduced virulence in *A. thaliana*. To evaluate whether HopPtoD2 functions during pathogenesis, virulence of DC3000 and JB4 were compared in the susceptible *A. thaliana* ecotype Columbia (234). Although typical disease symptoms appeared 24-48 hours post-infection for both strains, populations of JB4 were reduced relative to the wild-type DC3000. In experiments in which initial populations were indistinguishable, populations of the Δ*hopPtoD2* mutant were nearly two orders of magnitude lower than those of DC3000 by 72 h after inoculation (Figure 5-7) and populations of JB4 remained below those of DC3000 for the subsequent 48 hr.

Table 5-5: Translocation of HopPtoD2':'AvrRpt2 fusions into plant cells.

Strain	Expressed Gene[2]	Response[1]	
		RPS2[3]	*rps2*[4]
DC3000	None[5]	D[6]	D
	avrRpt2	HR[7]	D
	hopPtoD2	D	D
	hopPtoD2':'avrRpt2	HR	D
A9[8]	None	null	null
	hopPtoD2':'avrRpt2	null	null

[1] each strain was inoculated into individual leaves of the same plants by syringe infiltration and monitored for induction of the HR or development of disease. A total of 9 leaves were infiltrated with each strain. All inoculated leaves responded in the indicated manner.

[2] indicated gene or construct expressed from *lacUV5* promoter of pDSK600

[3] *A. thaliana* ecotype Columbia; recognizes the 'AvrRpt2 peptide and is resistant to *P. syringae* strains expressing AvrRpt2.

[4] susceptible *A. thaliana* derivative of ecotype Columbia that does not recognize 'AvrRpt2 peptide

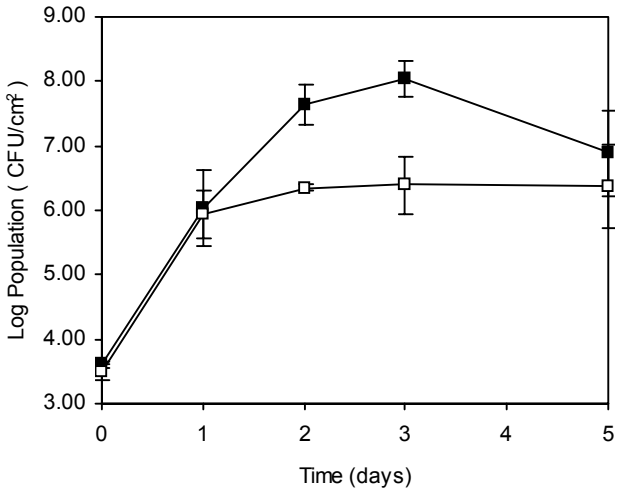
[5] the unmodified vector pDSK600 alone

[6] classic disease (D) symptoms for DC3000 in *A. thaliana* developed after 48 hrs

[7] tissue collapse and subsequent necrosis typical of the HR observed 12-24 hrs post-inoculation

[8] DC3000 Δ*hrpA* mutant lacking a functional TTSS

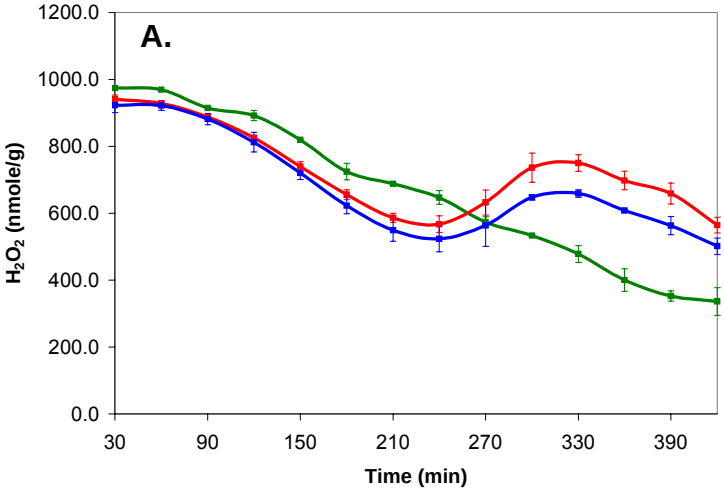
Figure 5-7: HopPtoD2 facilitates *P. syringae* DC3000 growth during pathogenesis in *Arabidopsis thaliana.* *A. thaliana* ecotype Columbia (Col-0) leaves were infiltrated with 10^5 CFU/ml DC3000 (solid squares) or JB4 (*ΔhopPtoD2*)(open squares). Bacterial populations were monitored over 5 days.

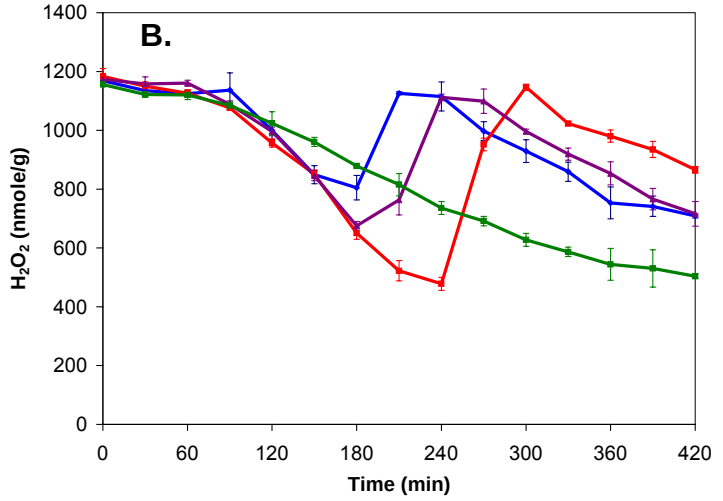

9.00
8.00
7.00
6.00
5.00
4.00
3.00
Log Population (CFU/cm2)
0
1
2
3
4
5
Time (days)

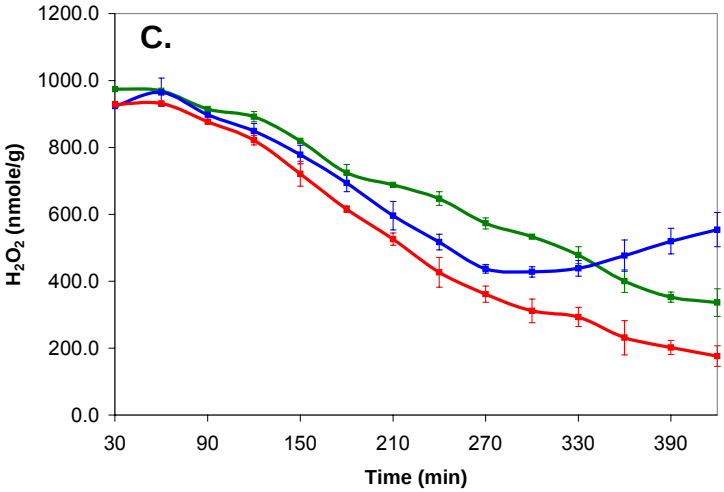
HopPtoD2 modulates host defense responses to infection. The role of HopPtoD2 in suppressing host defense responses was examined. The HR elicited by the Δ*hopPtoD2* mutant JB4 in *Nicotiana tabacum* L. leaves was indistinguishable from that of DC3000 under all conditions tested. In contrast, a phenotype was detected when HopPtoD2 was ectopically expressed in Psy61, which does not appear to carry a native allele of HopPtoD2. When *N. tabacum* leaves were inoculated with the lowest concentration of bacteria capable of producing a visible HR (10^6 CFU/ml), the HR elicited by Psy61 (pJBHopPtoD2) was observed 2-3 hrs after the response elicited by the control Psy61 strain or a Psy61 transformant (Psy61 (pJBHopPtoD2ΔC)) expressing the amino terminal 321 aa of HopPtoD2 from the same promoter as the full length construct. The HopPtoD2ΔC fragment carries the secretion signals for TTSS as shown above but lacks PTP activity (Figure 5-6B). Because ectopic expression of the truncated HopPtoD2ΔC construct did not cause a delay in the plant response, it is unlikely that the expression system for HopPtoD2 was impeding translocation of other effectors.

An increase in active oxygen production commonly accompanies the HR of tobacco cells and is one of the earliest known responses of responding plant cells (15). In collaboration with Norti Mock and Jacyn Baker at the USDA, the active oxygen response of cultured tobacco cells inoculated with *P. syringae* expressing HopPtoD2 was monitored. Consistent with its HR phenotype in leaves, inactivation of *hopPtoD2* in DC3000 had little affect on active oxygen production from inoculated *N. tabacum* cells (Figure 5-8A). A delayed active oxygen response, however, was

Figure 5-8: The effect of HopPtoD2 on the active oxygen response of cultured tobacco cells. Suspensions of cultured tobacco cells two days (A and B) or four days (C) after transfer were inoculated with 5 x 10^7 CFU/ml of the indicated strains and production of H_2O_2 was monitored using luminol. Data reported are the mean of four concurrent assays. **A.** Inactivation of HopPtoD2 in DC3000 had little effect on active oxygen production. Active oxygen production from *N. tabacum* cells inoculated with either DC3000 (red) or the *ΔhopPtoD2* mutant JB4 (blue) could be detected after approximately 210 minutes. **B.** Ectopic expression of HopPtoD2 in Psy61 delayed the active oxygen response. The active oxygen response of the cultured tobacco cells could be detected between 180 and 210 minutes after inoculation with either Psy61 (pDSK600) (blue) or Psy61 (pJBHopPtoD2ΔC) (purple). Active oxygen production from cells incubated with Psy61 (pJBHopPtoD2) (red) could only be detected after 240 minutes. **C.** The age of cultured tobacco cells affects active oxygen production in response to Psy61 infection. Active oxygen production from 4 day old *N. tabacum* suspension cells inoculated with Psy61 (pDSK600) (blue) occurred after approximately 300 minutes. A definitive active oxygen response was not detected in 4 day old cells inoculated with Psy61 (pJBHopPtoD2) (red) during the period tested. The cells only control is represented by the green line in each experiment.


A.
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
H_2O_2 (nmole/g)
30
90
150
210
270
330
390
Time (min)


B.
1400
1200
1000
800
600
400
200
0
H_2O_2 (nmole/g)
0
60
120
180
240
300
360
420
Time (min)


C.
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
H_2O_2 (nmole/g)
30
90
150
210
270
330
390
Time (min)

detected when cells were inoculated with Psy61 (pJBHopPtoD2) (Figure 5-8B). Plant cells ordinarily express several peroxidases and other enzymes that degrade hydrogen peroxide (13) (Cells only; Figure 5-8); thereby requiring the addition of basal levels of hydrogen peroxide in the assay medium to overcome this antioxidant activity. Hydrogen peroxide production from *N. tabacum* cells inoculated with Psy61 (pDSK600) could be detected between 180 and 240 min after inoculation of the culture as indicated by the increase in hydrogen peroxide levels in the medium relative to the "cells only" control. Thereafter, the basal rate of hydrogen peroxide degradation was restored. Hydrogen peroxide production from *N. tabacum* cells inoculated with Psy61 (pJBHopPtoD2ΔC) initiated at 210 min but was also complete by 240 min. The hydrogen peroxide production elicited by Psy61 (pJBHopPtoD2), however, was first detected at 240 min and continued until 300 min. Thus, the active oxygen response elicited by Psy61 (pJBHopPtoD2) occurred at least one hour later than that elicited by Psy61 transformants not expressing HopPtoD2. At least a three hour delay was observed in older cultures of *N. tabacum* cells (Figure 5-8C). Thus, translocation of HopPtoD2 delays the active oxygen response of tobacco cells, consistent with the observed delay in HR-associated PCD.

To determine if HopPtoD2 modulates other plant responses, expression of PR1 was monitored during DC3000 pathogenesis and during elicitation of the HR by Psy61. PR1 is a pathogenesis-related gene associated with multiple defense responses (225) and is induced during pathogen infection through a MAPK-linked pathway (241). To estimate PR1 activity, expression of a PR1-GUS fusion created in *A. thaliana* (202)

was monitored in tissue inoculated with DC3000, JB4, Psy61 (pDSK600), Psy61 (JBHopPtoD2) and Psy61 (pJBHopPtoD2ΔC). Modest levels of PR1-driven GUS expression were observed during pathogenesis by DC3000 in *A. thaliana* (Figure 5-9). The *ΔhopPtoD2* mutant JB4 elicited a 2-fold higher level of GUS expression. Complementary responses were observed in the tissue inoculated with the Psy61 transformants ectopically expressing *hopPtoD2*. PR1 expression was induced by the control Psy61 strain, but expression of HopPtoD2 caused a 65% reduction in expression of the PR1-GUS fusion. Psy61 derivatives expressing HopPtoD2ΔC elicited a response indistinguishable from that elicited by Psy61. Thus, during interactions with both resistant (*N. tabacum*) and susceptible plants (*A. thaliana*), HopPtoD2 is associated with suppression of plant defense responses.

Distribution of HopPtoD2 among *P. syringae* strains. To determine which *hopPtoD* alleles are carried by other *P. syringae* strains, *P. syringae* strains of diverse host ranges were surveyed for the presence of *hopPtoD* alleles. Primer pairs were designed to amplify regions of DNA specific to each *hopPtoD* allele. Similar to a previous report (8), a fragment indicative of the presence of *hopPtoD1* could be amplified from 32 of the 44 strains surveyed (71%)(Table 5-6). Indicative fragments for *hopPtoD2* or *hopPtoD3* could only be amplified from DC3000, *P. syringae* pv. *maculicola* 10 and *P. syringae* pv. *tomato* 2844. Similar results were obtained by hybridization using a probe unique to the PTP domain of *hopPtoD2*. Only the three strains that the PCR studies indicated carried the *hopPtoD2* and *hopPtoD3* alleles

Figure 5-9: Expression of HopPtoD2 suppresses PR1 expression. The indicated strains were infiltrated into leaves of *A. thaliana* Col0/PR1-GUS. GUS activity was assayed two days post-inoculation. The bars represent the mean units of GUS activity detected in five leaf discs corrected for the activity detected in water-inoculated tissue. GUS acitivity for water-inoculated leaves was less than 5% of the amount measured for *P. syringae* inoculated leaves. Two DC3000 derivatives were assayed: DC3000 (WT, HopPtoD2^{+}) and JB4 (*ΔhopPtoD2*). Psy61 (HopPtoD2^{-}) strains expressing the following plasmids were also assayed: pDSK600 (empty vector), pJBHopPtoD2 (full length HopPtoD2), and pJBHopPtoD2ΔC (carboxy terminal truncation of HopPtoD2 lacking the PTP domain).

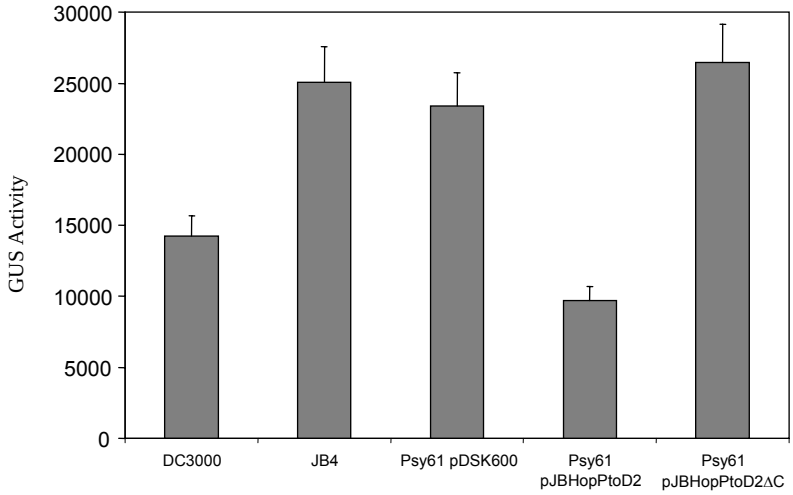

30000
25000
20000
15000
10000
5000
0
GUS Activity
DC3000
JB4
Psy61 pDSK600
Psy61 pJBHopPtoD2
Psy61 pJBHopPtoD2ΔC

Table 5-6: Distribution of *avrPphD* homologs among *Pseudomonas syringae* strains.

Strain	Detection of		
	hopPtoD1	*hopPtoD2*	*hopPtoD3*
P.s. pv. *tomato* strains			
DC3000	P[1]	P[2],H[3]	P[4]
T1	P	-	-
B368	-	-	-
PDDCC 4355, 3523, 3375[5]	P	-	-
B76,	-	-	-
B67, 88, 117, 118, 120, 121, 122, 125	P	-	-
T4B1	P	-	-
2424	P	-	-
Denny 125	P	-	-
JL1060, 1075, 1105, 1120	P	-	-
RG-4	P	-	-
NCPPB 880	P	-	-
CNBP1323	P	-	-
PT14, 21, 30	P	-	-
DAR 30555, 31861	P	-	-
832F	-	-	-
2844	-	P,H	P
84-15	-	-	-
85-274	-	-	-
DAR 26742	-	-	-

Strain	Detection of		
	hopPtoD1	*hopPtoD2*	*hopPtoD3*
PSP 343	P	-	-
P.s. pv. *phaseolicola*			
NPS 3121	P	-	-
NK343	P	-	-
B130	P	-	-
BK378	-	-	-
#1	-	-	-
#5	P	-	-
#10	P	P,H	P
1083-3	-	-	-
P.s. pv. *syringae*			
Psy61	-	-	-

[1] Colony PCR employing primers DC1183 and DC2050R (from C-terminal region) was used to screen the indicated strains for *hopPtoD1* alleles. The presence of an 867 bp fragment was scored as positive.

[2] To identify the *hopPtoD2* allele, primers DC285 and DC1048R were used in colony PCR to amplify a 763 bp fragment unique to *hopPtoD2*. The presence of this fragment was scored as positive.

[3] To verify the presence of the *hopPtoD2* allele, the PCR fragment amplified using primers DC285 and DC1048R was labeled with [α^{32}-P] dCTP and used as a probe in colony hybridization to survey the indicated strains.

[4] To specifically identify *hopPtoD3*, primers DC971 and DC929R were used in colony PCR to amplify a 630 bp fragment spanning the IS*52*/*hopPtoD3* border. The presence of this fragment was scored as positive.

[5] Strains with the same prefix.

hybridized to the probe (Table 5-6). Thus, although *hopPtoD2* has a significant role in the virulence of DC3000, it appears that only a few strains carry an ortholog to *hopPtoD2*.

Discussion

Pathogenesis by *P. syringae* strains is dependent upon translocated effectors that facilitate parasitism of the host cells and suppress innate immunity responses (49, 54). Using a regulated HrpL-dependent promoter trap assay to survey the *P. syringae* pv. *tomato* DC3000 genome for effector genes, several homologs of *avrPphD* were identified. One of the homologs, HopPtoD2, was shown to be a TTSS-dependent effector with a carboxyl terminal PTP domain. Like other TTSS-dependent effectors of *P. syringae* (89, 163, 177) and several mammalian pathogens (e.g. (121, 208)), HopPtoD2 was modular. The amino terminus of HopPtoD2 retained structural features typical of TTSS-dependent effectors, and consistent with the detection of HopPtoD2 in culture filtrates of DC3000 (177), HopPtoD2 was demonstrated to be translocated into plant cells by the *hrp* TTSS. The carboxyl-terminus of HopPtoD2, in contrast, included characteristic motifs indicative of PTP activity. This region included a general acid motif positioned 24 residues from a consensus PTP active site domain in which the critical residues were conserved. Because the 321 aa amino terminal domain was ineffective in eliciting or affecting plant defense responses, the carboxyl terminal domain of HopPtoD2 appears to be responsible for the observed activities of this effector.

Consistent with the retention of diagnostic PTP structural motifs, HopPtoD2 was shown to be a PTP. Culture filtrates of DC3000 expressing HopPtoD2 dephosphorylated three PTP-specific substrates. This phosphatase activity was sensitive to orthovanadate and was substantially reduced in culture filtrates obtained from a DC3000 Δ*hopPtoD2* mutant. *E. coli* and Psy61 strains expressing HopPtoD2 acquired the ability to degrade PTP-specific substrates. The presence of diagnostic structural motifs, the ability to dephosphorylate *p*NPP, and the sensitivity of the phosphatase activity to orthovanadate are considered to be defining features of a PTP (68). Among the four general classes of PTPs that have been characterized, HopPtoD2 seems to be most similar to the non-receptor-like PTP family that includes the *Yersinia* YopH. The PTP active site domain was nearly identical to that of other non-receptor-like PTPs and HopPtoD2 contained no obvious P-loop typical of dual-specificity PTPs, such as the VH1 PTP.

Several TTSS-dependent effectors have been shown to contribute to the pathogenic fitness of *P. syringae* strains. For example, the effectors AvrE and AvrRpm1 are required for full virulence of their source *P. syringae* strain (146, 184, 185). AvrPphC, AvrPphF and VirPphA enhanced the virulence of a *P. syringae* pv. *phaseolicola* strain by suppressing R-gene-dependent PCD (111, 224). Similarly, the broadly conserved AvrPtoB, a homolog of VirPphA (111), inhibited the Pto-dependent PCD elicited by AvrPto in *N. benthamiana* (1). Likewise, HopPtoD2 was also found to contribute to pathogenic fitness of DC3000 in susceptible hosts.

Populations of a DC3000 Δ*hopPtoD2* mutant were typically reduced by 97% relative to the parent strain after three days growth. This is among the largest reported reductions in virulence of a *P. syringae* strain that can be attributed to inactivation of a single TTSS-dependent effector. Thus, a translocated protein tyrosine phosphatase can be added to the growing list of TTSS-dependent effectors that are shared in common between plant and mammalian pathogens and are required for virulence (211). Interestingly, HopPtoD2 has very little overall sequence similarity with other TTSS-secreted PTPs and therefore appears to be an example of convergent evolution. Analogs, and in some cases, partial homologs of the *Yersinia* YopJ (171), YopT (201), and YopH (22) have now been detected in plant pathogenic bacteria.

Unlike several *P. syringae* effectors that have been shown to be epistatic to other effectors (1, 111), HopPtoD2 appears to act more subtly. Ectopic expression of *hopPtoD2* delayed plant defense responses but did not fully suppress these responses. The RPS2-dependent recognition of AvrRpt2 did not appear to be altered by the co-expression of *hopPtoD2* resident in the wild-type DC3000 genome. Virulence enhancement caused by the effectors translocated by the *hrp*-encoded TTSS has been attributed to interference with effector-receptor interactions, reduced detection by host receptors, or suppression of the signal transduction pathway(s) leading to a defense response (224). HopPtoD2 most likely falls into the third category.

Plant cells have multiple signal transduction pathways that can be differentially activated in response to distinct elicitors and may function differently in distinct plant

species (33). For several mammalian pathogens that utilize TTSS in pathogenesis, translocated PTPs , such as the *Yersinia* YopH (22) and the *Salmonella* SptP (121), affect signal transduction pathways (21, 50, 60). One possible target for the PTP activity of HopPtoD2 is one or more of the MAPK-dependent signal transduction pathways controlling defense responses (170, 247). Greater than 20 distinct MAPKs have been identified in the *Arabidopsis* genome (118) and their roles in signal transduction are only beginning to be established (108, 170). For example, HR-associated programmed cell death (242), active oxygen production (183), and induction of secondary metabolism and pathogenesis-related proteins, such as PR1 (241), have been linked to MAPK signal cascades. Thus, the apparent HopPtoD2-mediated suppression of the HR-associated programmed cell death, active oxygen production, and PR1 expression are consistent with interference of one or more signal transduction pathways, perhaps linked to MAPKs. Experiments are underway to test this hypothesis, but other mechanisms are possible as well. Irrespective of the mechanism by which HopPtoD2 acts, the inability to suppress some host defense responses might account for the reduced virulence of the Δ*hopPtoD2* mutant.

While HopPtoD2 is important for DC3000 virulence, not all strains of *P. syringae* carry an ortholog of HopPtoD2. Although many strains appear to have alleles of *hopPtoD1* (8), a survey of 44 *P. syringae* strains of diverse host ranges revealed that only two other strains carried an allele of DC3000 *hopPtoD2*. The strains that lack an apparent *hopPtoD2* ortholog are fully virulent on their respective hosts, indicating either that PTP activity is not essential to the virulence of all *P. syringae* strains or

that other effectors have a similar activity. Interestingly, all three strains shown to carry the *hopPtoD2* allele also appear to contain the *hopPtoD3* allele as well. This suggests that these genes may be part of a mobile gene cassette like those reported for some effector genes of other *P. syringae* strains (38, 112).

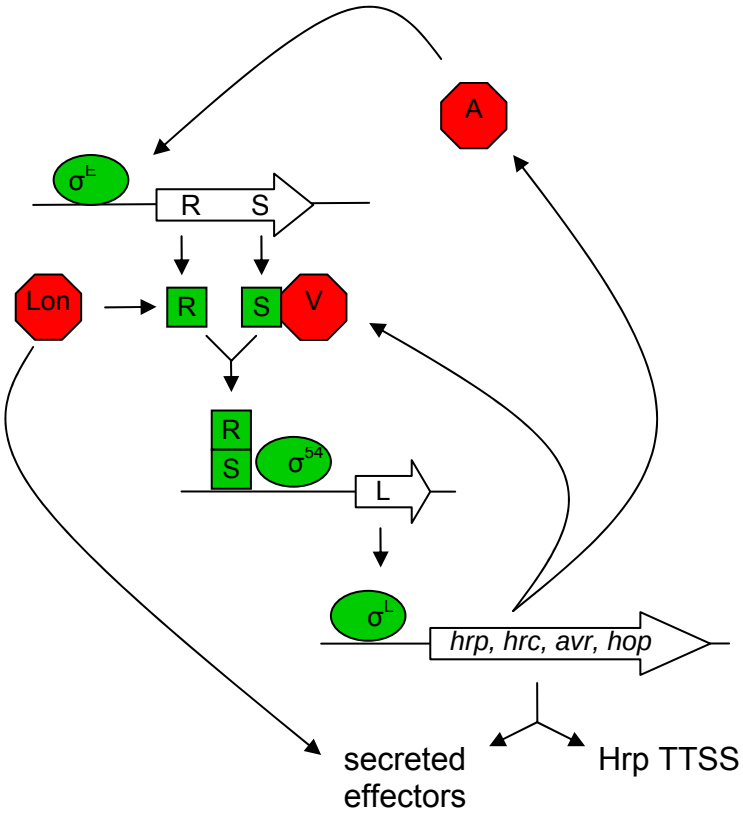
In summary, this report and a report from Espinosa *et al* (67) demonstrate that HopPtoD2 is a translocated PTP that suppresses host defense responses. The observed delays in programmed cell death, active oxygen production, and PR1 expression coupled with reduced virulence of the Δ*hopPtoD2* mutant are consistent with this hypothesis. Thus, HopPtoD2 can be added to a growing list of virulence-enhancing effectors produced by plant pathogenic bacteria. Virulence-enhancing effectors provide a molecular explanation for the original observation that *hrp* genes are required for pathogenicity of *P. syringae* strains (142). These virulence-enhancing effectors are likely to be strain-specific by affecting the activity of one or a few effectors but could also be host species-specific like other effectors, such as *avr* genes. With the identification of biochemical activities associated with specific effectors, such as HopPtoD2, the mechanism by which these effectors function to suppress at least a portion of the innate immune response of susceptible plants can now be explored.

Chapter 6: Conclusions and future perspectives

When this research began, only some of the factors involved in *P. syringae hrp* TTSS regulation had been identified. Out of those factors identified, only the role of HrpL was clearly defined. Now, the mechanism by which HrpR and HrpS activate *hrpL* expression has been established. In addition, Lon protease has been added to the list of *hrp* regulatory proteins. Although other factors are surely involved, the interplay of these four factors appears to provide a mechanism for environmental regulation of the *hrp* TTSS and its associated secreted effectors.

Using the data from this dissertation as well as from other sources, it is possible to describe a regulatory system that is dedicated to the expression of the *hrp* regulon (Figure 6-1). In this model, HrpR and HrpS are constitutively expressed from the *hrpRS* operon. Under non-inductive conditions, HrpR is actively degraded by Lon protease. As a result, HrpR does not interact with HrpS and full induction of *hrpL* expression does not occur. However, under *hrp*-inductive conditions, HrpR is stabilized via an unknown mechanism. HrpR interacts with HrpS to form a heteromeric complex. This complex, in conjunction with σ^{54}, activates expression of *hrpL*. HrpL, an alternative sigma factor, activates expression of the *hrp* regulon (the *hrp*-encoded TTSS and *hrp*-secreted effectors). The combination of the *hrp* TTSS and secreted effectors allows *P. syringae* to successfully parasitize a susceptible host. The combination of TTSS and effectors also allows resistant plants to recognize and quickly repel *P. syringae* infection.

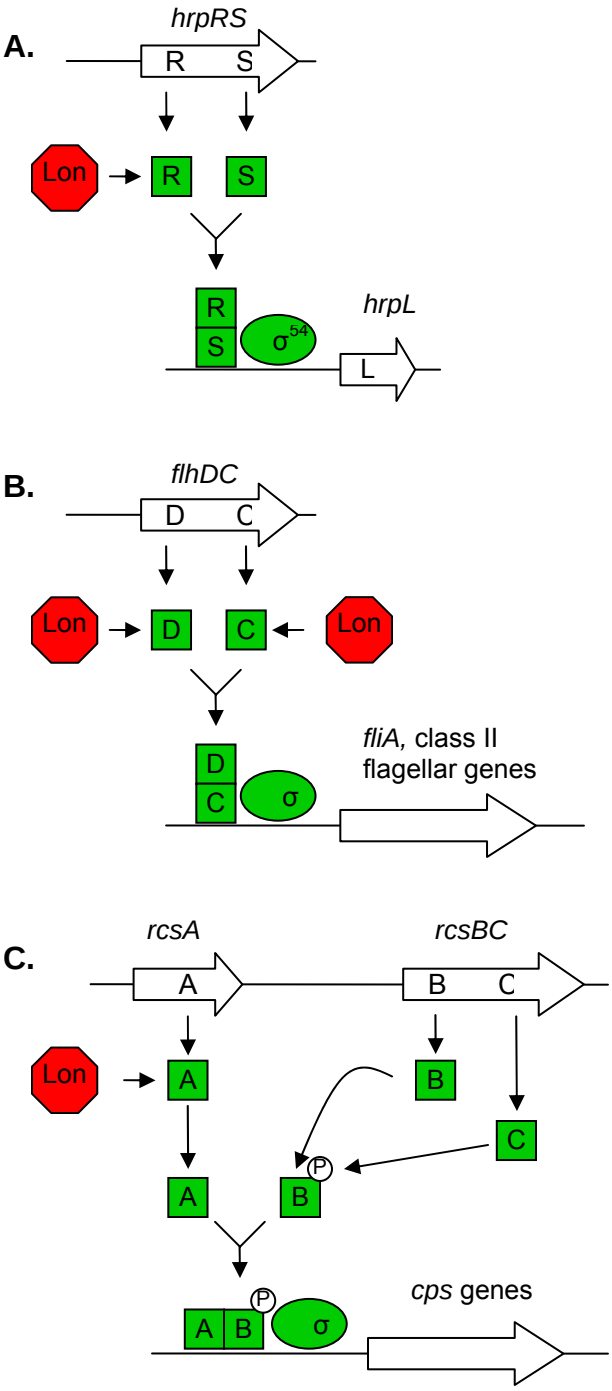
Figure 6-1: Current model of the *hrp* regulatory system. Several factors play a key role in both positively and negatively regulating *hrp* expression. At the top of the regulatory cascade, the *hrpRS* promoter is constitutively expressed from a putative σ^E promoter. The enhancer binding proteins HrpR and HrpS physically interact to drive expression of the σ^{54}-dependent *hrpL* promoter. *hrpL* is only expressed in planta or during growth in an acidic minimal salt media. HrpL is an alternative sigma factor that activates expression of the *hrp*-encoded TTSS and its associated secreted effectors. Lon protease negatively regulates *hrp* regulon expression by degrading HrpR. Under inductive conditions, HrpR is stabilized via an unknown mechanism to allow full activation of *hrp* expression. Lon protease also regulates secretion by degrading secreted effectors (147). HrpV negatively regulates *hrp* expression via an unknown mechanism that may involve a direct physical interaction with HrpS (179). The Hrp pilus structural protein, HrpA, appears to be a negative regulator of *hrp* expression at the level of *hrpRS* expression (231). Positive acting factors are green. Negative acting factors are red. Genes are represented by the open arrows.


A
σE
R
S
Lon
R
S
V
R
S
σ54
L
σL
hrp, hrc, avr, hop
secreted
effectors
Hrp TTSS

In many aspects, the *P. syringae hrp* regulatory cascade closely resembles flagellar regulation in *E. coli* and *Proteus mirabilis* (Figure 6-2). Control of flagellar regulation is governed by the *flhDC* operon (152). FlhD and FlhC encode DNA binding proteins that act as transcriptional activators. Similar to HrpR and HrpS, FlhD and FlhC form a heteromeric complex that activates expression of class II flagellar genes (144). The class II operons consist of FliA and many of the genes that encode the components of the flagellar machinery. FliA, an alternative sigma factor closely related to HrpL, induces expression of the third level of flagellar genes (152). Although the environmental signals that activate flagellar gene expression are different than those that activate *hrp* expression, Lon protease has also been implicated in flagellar regulation. In *P. mirabilis,* Lon degrades FlhD and FlhC (47). Interestingly, it appears that increased expression of the *flhDC* operon occurs during differentiation into swarming cells (65, 71). It is thought that this transient increase in expression allows for the accumulation of FlhD and FlhC and the subsequent activation of class II flagellar genes (47). A similar phenomenon may occur in *P. syringae* as a modest increase in *hrpRS* expression is seen during growth in *hrp*-inducing media (Chapter 2).

The HrpR/HrpS system also shares significant similarity with the RcsB/RcsA system that regulates capsular biosynthesis in *E. coli* (81)(Figure 6-2). In this system, RcsB interacts with RcsA to regulate *cps* expression. RcsB is a typical response regulator found in a two component system. RcsB is phosphorylated by RcsC. RcsB and RcsC can activate low level *cps* expression by themselves. For full expression, RcsA must

Figure 6-2: Comparison of the *hrp* regulatory cascade to other systems. The HrpR/HrpS regulatory system closely resembles two other systems from *E. coli*. In flagellar biosynthesis, FlhD and FlhC are transcriptional activators that are expressed as an operon. Similar to HrpR and HrpS, FlhD and FlhC form a complex that is required for the expression of class II flagellar genes. Both FlhD and FlhC are negatively regulated via Lon-mediated degradation. In capsular biosynthesis, *cps* gene expression is dependent upon three proteins. RcsB and RcsC are expressed as an operon. RcsB is response regulator that is activated by the protein kinase RcsC. Full *cps* gene expression requires the auxiliary protein RcsA. Intracellular levels of RcsA are regulated by Lon protease. Positive acting factors are green. Negative acting factors are red. Genes are represented by the open arrows.


A.
hrpRS
R
S
Lon
R
S
R
S
σ54
hrpL
L
B.
flhDC
D
C
Lon
D
C
Lon
fliA, class II
flagellar genes
D
C
σ
C.
rcsA
rcsBC
A
B
C
Lon
A
B
C
A
B
P
P
A
B
σ
cps genes

also be present. RcsA is an auxiliary protein whose intracellular levels are regulated by Lon protease. Similar to flagellar biosynthesis, Lon degrades RcsA in response to environmental signals distinct from the signals that activate *hrp* expression. In *P. syringae,* HrpR appears to play the role of the auxiliary protein. Expression of HrpS is sufficient for low level expression of the *hrp* regulon (85, 105). However, HrpR is needed for maximal expression. Therefore, it appears that Lon protease degrades unstable regulatory proteins in both *E. coli* and *P. syringae* in response to different environmental stimuli.

Two other *hrp*-encoded factors also play a role in regulation. As mentioned previously, HrpV is a negative regulator of *hrp* expression (Figure 6-1). Research in our lab indicates that HrpV negatively regulates *hrpL* expression. Yeast two hybrid data indicated that HrpS and HrpV physically interact (S. Hutcheson and T. Sussan, unpublished data). Because HrpV is a member of the *hrp* regulon, it appears that HrpV provides a form of feedback regulation during pathogenesis. The precise mechanism by which HrpV negatively regulates *hrp* expression is unknown. It's possible that HrpV acts as the missing modulatory domain for HrpS or simply interferes with the HrpR/HrpS heteromeric complex to block *hrpL* expression.

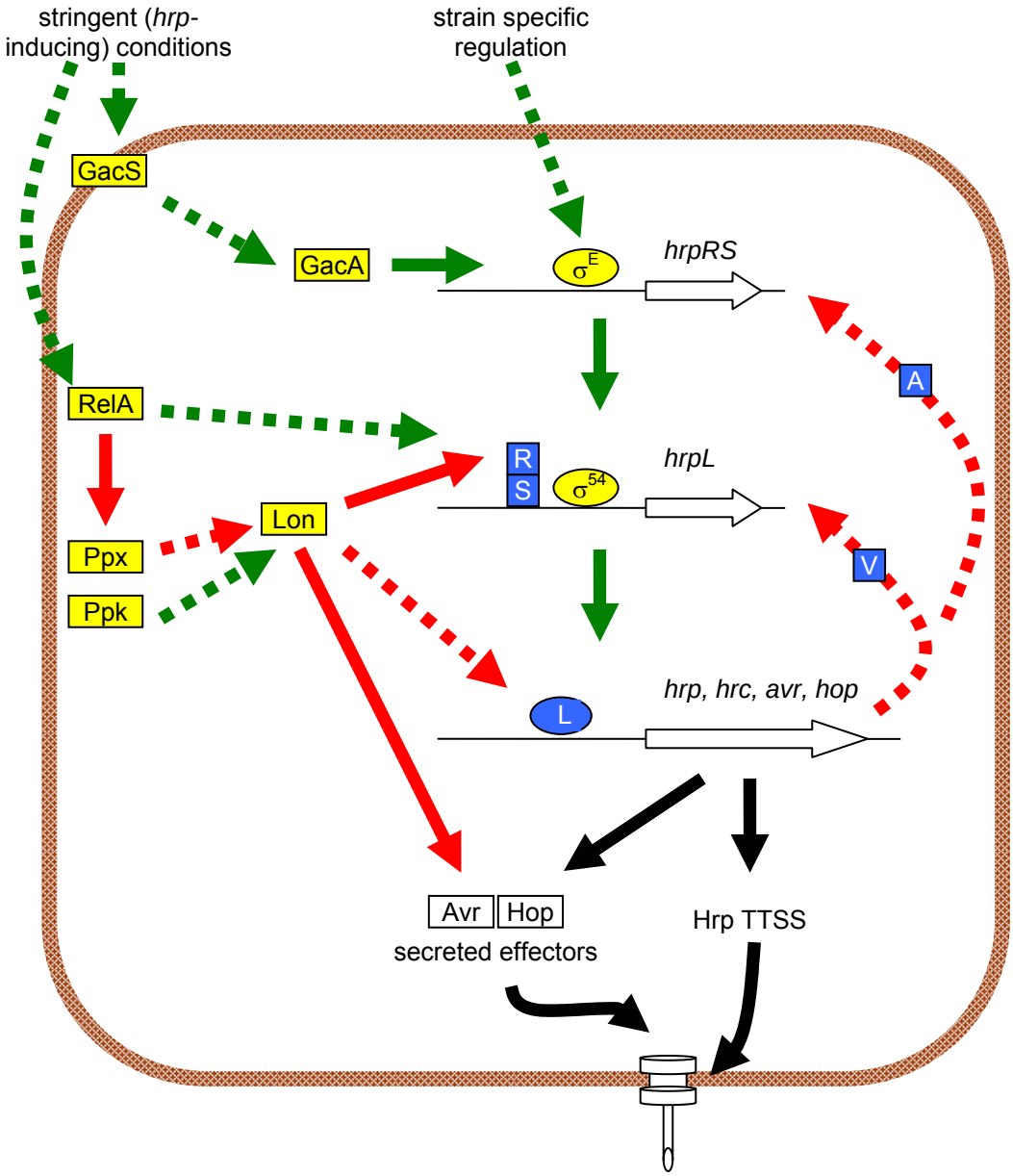
The other *hrp*-encoded factor involved in regulation is HrpA (Figure 6-1). HrpA is the *hrp* pilus structural protein and is required for disease development and PCD induction (186). Similar to HrpV, HrpA seems to be involved in feedback regulation of *hrp* expression. In a *ΔhrpA* mutant, *hrpRS* mRNA levels are depressed (231). This

decrease in *hrpRS* expression leads to decreased expression of the rest of the *hrp* regulon. This regulatory activity has been localized to the carboxy terminal end of the HrpA protein (231). HrpA has also been shown to affect effector stability. In a *ΔhrpA* mutant, effectors were considerably less stable than in the wild-type strain (147). This decrease in effector stability may be due to the decrease in *hrp* expression observed by Wei *et al* (231) for *ΔhrpA* mutants. Regardless of the mechanism, HrpA plays an essential role in *hrp* regulation by preventing expression and production of the Hrp TTSS and associated effectors when the HrpA pilus is not functional.

With the exception of Lon protease, the factors mentioned above form a dedicated *hrp* regulatory system. Recent efforts have been focused on identifying the more elusive global factors that help to regulate the *hrp* regulon. It has long been postulated that *hrp* expression is linked to global regulatory pathways (Figure 6-3). The identification of Lon protease as a negative regulator of the *hrp* regulon provided the first bridge between the dedicated *hrp* regulatory system and global regulatory pathways.

As mentioned elsewhere, Lon protease is a general ATP-dependent protease responsible for the degradation of abnormally folded proteins and other non-functional proteins (80). Lon also plays an important role in the regulation of a variety of processes by degrading unstable regulatory proteins. In *P. syringae*, Lon has been shown to degrade HrpR and several secreted effectors. In addition to these two targets, Lon may also target HrpL. While overexpression of *hrpRS* leads to

Figure 6-3: Current model for global regulation of the *hrp* regulon. In addition to the factors that are dedicated to regulating *hrp* expression (see Figure 6-1), many other global regulatory networks have been implicated in *hrp* regulation. GacS and GacA are members of a two component regulatory system that has been identified in numerous bacteria. Similar to the *hrp* regulon, expression of the response regulator GacA increases in acidic minimal salts media. GacA has been shown to positively regulate expression of both the *hrpRS* and *hrpL* operons (40). Lon protease has been implicated in numerous regulatory networks (see Figure 6-2). In *P. syringae*, Lon protease negatively regulates *hrp* expression by rapidly degrading HrpR under *hrp* repressive conditions. If *P. syringae* responds to stringent conditions like *E. coli* does, RelA, Ppx, and Ppk may affect *hrp* expression by affecting how Lon protease identifies targets. Under stringent conditions, polyphosphate would direct Lon away from HrpR and towards ribosomal proteins and other non-essential proteins. Green arrows represent positive regulation of *hrp* expression while red arrows indicate negative regulation of *hrp* expression. Direct links between regulatory factor and target are marked with solid arrows. Dashed arrows represent postulated or indirect links between regulatory factor and target. Yellow shaded areas indicate factors involved in global environmental regulation of the *hrp* regulon. Blue shaded factors represent members of the dedicated *hrp* regulatory system (see Figure 6-1).


stringent (*hrp*-inducing) conditions
strain specific regulation
GacS
GacA
σ^E
hrpRS
RelA
R
S
σ^{54}
hrpL
A
V
Lon
Ppx
Ppk
L
hrp, hrc, avr, hop
Avr
Hop
secreted effectors
Hrp TTSS

expression of *hrpL*, this does not lead to increased expression of HrpL-dependent genes. This discrepancy could be explained if Lon also degrades HrpL. Lon-mediated degradation of sigma factors has been demonstrated before. In *Bacillus subtilis*, Lon has been shown to degrade the sporulation sigma factor σ^s (194). In addition to helping to regulate flagellar and capsular biosynthesis, Lon has been implicated in many other regulatory pathways. For instance, in several bacterial species, intracellular levels of the cell division inhibitor SulA are regulated by Lon. During cell division Lon rapidly degrades SulA. However, during the SOS response, SulA production increases dramatically and overcomes Lon-mediated degradation. SulA accumulates and prevents septation of the cell (195). Lon also targets the CcdA protein involved in plasmid addiction (228). HrpR is simply the latest in the list of regulatory factors that are targeted by Lon protease.

Lon protease involvement in regulating TTSS has also been demonstrated in *Salmonella.* In *S. enterica* serovar Typhimurium, Lon protease appears to be a negative regulator of the SPI-1 TTSS that is involved in epithelial cell invasion (221). Similar to what occurs in *P. syringae,* the *Salmonella lon* mutant showed increased transcription of genes involved in the positive regulation of SPI-1 and increased secretion of effectors. As a result, the *lon* mutant was highly invasive (221). Interestingly, Lon was found to be essential to systemic infection (220). Recently, Boddicker and Jones (24) reported that Lon down regulated SPI-1 expression after epithelial cell invasion possibly by targeting the SPI-1 positive regulator HilD.

These studies have also implicated two other global regulatory factors that may be involved in regulating the *P. syringae hrp* regulon. RelA and Ppx are both involved in the stringent response. As mentioned previously, Lon substrate targeting in *E. coli* is affected by the stringent response. RelA produces (p)ppGpp when it detects idling ribosomes during stringent conditions in *E. coli* (36). This increase in (p)ppGpp leads to an increase in polyphosphate levels because (p)ppGpp inhibits the exopolyphosphatase Ppx (131). In turn, polyphosphate directs Lon to degrade the ribosomal proteins S2, L9, and L13 and other expendable proteins (130). If the same process were occurring in *P. syringae,* polyphosphate would guide Lon protease to other substrates and thereby allow for the accumulation of HrpR and expression of the *hrp* regulon. In this model, Ppx would be a negative regulator of *hrp* expression because it would decrease the intracellular levels of polyphosphate (Figure 6-3). Without polyphosphate to guide Lon to ribosomal proteins, Lon would continue to degrade HrpR and repress *hrp* expression. A *ppx* mutant would mimic stringent conditions because it would allow for the accumulation of polyphosphate and the reciprocal stabilization of HrpR.

Incorporating RelA into the global model of *hrp* regulation is more problematic. The Tnp screen in Chapter 4 identified RelA as a negative regulator of *hrp* expression. However, if the stringent response in *P. syringae* is similar to the stringent response in *E. coli,* RelA would be predicted to be a positive regulator of *hrp* expression via its effects on polyphosphate accumulation. While it is possible that (p)ppGpp negatively regulates the expression of genes that encode proteins that negatively regulate *hrp*

expression, all data in the literature seem to indicate that (p)ppGpp would be a positive regulator of *hrp* expression. For example, in other Pseudomonads, (p)ppGpp is required for the expression of at least two σ^{54}-dependent promoters (34, 218). The *hrpL* promoter is also σ^{54}-dependent and therefore may also be a target for positive regulation by (p)ppGpp. In addition, SpoT-mediated accumulation of (p)ppGpp has been shown to be a positive regulator of SPI-1 in *Salmonella* (207).

A third global factor involved in *hrp* regulation was identified by another group. GacS and GacA form a two component regulatory system that has been identified in several Pseudomonads as well as numerous other bacteria (95). GacS is the sensor kinase which activates the response regulator GacA. In both plant and animal pathogens, both GacS and GacA are important for virulence (23). In *Pseudomonas* spp., this two-component system is involved in the regulation of several extracellular products including toxins and antibiotics (95). In most species, the signal that activates this phosphorelay is unknown. Recent work by Chatterjee *et al* (40) has shown that transcript levels of GacA in DC3000 are higher when the cultures are grown under *hrp*-inducing media than when grown in rich media. They also show that GacA positively regulates expression of *hrpRS, hrpL, rpoN*, and several HrpL-dependent genes (Figure 6-3). In a *ΔgacA* mutant, not only is expression of these genes repressed, but both disease and the HR are either eliminated or dramatically reduced (40). Through its effects on both *hrpRS* and *rpoN* expression, GacA has a major effect on *hrpL* and downstream *hrp* regulon expression. GacA (and by

conjecture GacS) seem to lie at or near the top of the *hrp* regulatory cascade (Figure 6-3).

The factors presented above certainly provide a more comprehensive picture of *P. syringae hrp* regulation than was previously available. However, many questions still remain to be answered. The following is a list of relevant questions that need to be answered to provide a more comprehensive model of *P. syringae hrp* regulation.

1. **What other factors are involved in *P. syringae hrp* regulation?** The search for other factors involved in *hrp* regulation should begin with the *P. syringae* transposome (Tnp) mutants identified in Chapter 4. Work is already under way with four of these mutants. Other members of our lab are using mutants JB102, JB201, JB202, and JB203 to investigate the roles of RelA and Ppx in *hrp* regulation. Tnp mutant TK04 may also be of interest. TK04 contains an insertion in the gene that encodes for the putative heat shock protein HtpG. In many cases, Lon-mediated degradation of abnormally folded proteins involves chaperones encoded by heat shock genes (80, 125, 214). Like other heat shock proteins, HtpG may also play a role in Lon-mediated degradation of HrpR.

2. **How are environmental signals transduced into the *hrp* regulon?** Another way of looking at this question is by asking how *hrp* regulation is integrated into global *P. syringae* biology. In addition to using the Tnp

mutants as described above, other factors involved in *P. syringae hrp* regulation may be identified by looking at the regulation of type III secretion in other bacteria. The mechanisms by which other bacteria recognize their environment and induce TTSS expression are known. For example, in *Ralstonia solanacearum,* plant cell contact initiates expression of the *hrp*-encoded TTSS. A non-diffusible signal from the plant cell wall is recognized by the bacterial outer membrane receptor protein PrhA (3, 30). Similarly, target cell contact is known to affect TTSS expression in *Yersinia* spp. (188), but a bacterial encoded receptor has not been identified. Instead, several different environmental signals are recognized. Expression of the *Yersinia* TTSS and of the Yop secreted effectors is induced by temperature and extracellular glutamate. Secretion of effectors into the media is triggered by serum albumin while secretion into epithelial cells is triggered by Ca^{2+} (135). Similarly, *Salmonella enterica* serovar Typhimurium TTSS is induced by contact and culture conditions. *Salmonella* encodes two TTSS (SPI-1 and SPI-2) that function at different times during the infection process. SPI-1 is involved in invasion of epithelial cells and is induced by contact and alkaline pH (53, 251). SPI-2 is required for *Salmonella* growth in macrophages and is induced by low pH, low Mg^{2+} levels, and phosphate starvation (19, 55). Because *P. syringae hrp* expression is induced by low pH and amino acid starvation (181, 240), mechanisms similar to both *Yersinia* and *Salmonella* may be important. Plant cell contact may also play a role in *hrp* regulation. In

general, only a few Hrp-secreted proteins can be found in culture media (227, 244). The other Hrp-secreted effectors cannot be found in the culture media but as evidenced by their in planta effects are certainly translocated into the plant cell. It is possible that *P. syringae* assembles the TTSS when it encounters an acidic, amino acid-depleted environment but does not secreted effectors until it makes contact with the plant cell wall.

3. **What is the mechanism that modulates HrpR stability?** As mentioned previously, HrpR stability may be modulated by the stringent response. In this scenario, Lon protease activity does not actually change during growth under stringent (*hrp*-inducing) conditions. Rather, Lon protease itself is directed by its affinity for polyphosphate to degrade ribosomal proteins. This "targeting" change by Lon leads to an accumulation of HrpR and subsequent *hrp* regulon expression. An alternative is that HrpR is modified under *hrp*-inducing conditions. This modification can be covalent or could be simply a conformational change caused by HrpR interacting with another unidentified protein. Both of these scenarios are currently being investigated in our lab.

4. **Why is HrpR and not HrpS targeted by Lon protease in *P. syringae?*** The answer to the previous question may provide the answer to this question. In *E. coli*, both HrpR and HrpS are degraded by Lon protease.

In *P. syringae,* only HrpR is targeted for degradation by Lon and only during growth in rich media such as KB. The reasons for this discrepancy are not clear. Lon does not seem to target specific substrates. Rather, the relative affinity of a substrate for Lon is the deciding factor in substrate specificity (80). Specific regions of substrate proteins seem to be important in substrate recognition. For example, the amino terminus of UmuD is required for Lon recognition (78) while the carboxy terminus of SulA is required (110). In *P. syringae*, the carboxy terminus of the effector HopPtoM is important for Lon recognition (147). Unlike other proteases, Lon does not appear to cleave specific sequences (167, 168, 229). In both Psy61 and DC3000, the amino acid sequence of HrpR and HrpS are approximately 60% identical and 70% similar. Conformational changes in the structures of the two proteins may account for the difference in Lon specificity. Post-translation modification such as glycosylation or phosphorylation could affect the stability of either protein. In addition, an interaction with another protein, or with HrpS, could stabilize HrpR. In *Proteus,* FlhC and FlhD are stabilized by their interaction with each other (47). Likewise, in *E. coli,* the plasmid addiction protein CcdA is stabilized by its interaction with CcdB (229).

5. **Does *hrp* regulation vary between *P. syringae* strains?** For all their similarities, upon closer examination, there are many differences in type III secretion between *P. syringae* strains. While the core *hrc*-encoded

components of the TTSS are highly conserved, other components are not so closely related. For example, the pilin structural protein, HrpA is essential for pathogenesis (186) but is quite diverse between strains. In DC3000, this critically important structural protein only shares 28% identity and 45% similarity with its counterparts in other *P. syringae* strains (180). The array of effectors secreted by the Hrp TTSS of each strain is also a source of variation between strains. DC3000 appears to encode for 58 probable effectors, while B728a encodes only 29 (49, 84). In addition, some effectors are found in almost all strains while other effectors have been identified in only a few strains. Regulation may also vary between strains. Data presented by Chatterjee *et al* (40) indicate that while *hrpRS* expression is regulated by GacA in DC3000, GacA seems to have no effect on *hrp* expression in B728a. The analysis of the *hrpRS* promoter sequence presented in Chapter 2 showed significant variation between strains. Differences in *hrp* regulation between strains could be a way for individual *P. syringae* strains adapt to their particular host plant.

Study of *P. syringae hrp* regulation has also led to advances in understanding the inter-connection of the various processes that occur during *P. syringae* pathogenesis. For example, the regulation of the genes that control production of the phytotoxin coronatine has been linked to RpoN, HrpL, and GacA (2, 40, 70, 175). Production of another phytotoxin, syringomycin, has also been linked to GacA and possibly HrpL (40, 70, 149). Perhaps the area in which the most progress has been made is in the

identification of secreted effectors. Numerous studies, including the study presented in Chapter 5, have used the regulatory machinery to identify Hrp-secreted effectors (38, 49, 84, 147).

The best example of how the *hrp* regulatory system has been used to further the understanding of *P. syringae* pathogenesis was in the identification of HopPtoD2 (Chapter 5). Here, a HrpL-dependent promoter trap assay was used to identify an insertionally inactivated DC3000 homolog of AvrPphD. Using this allele, HopPtoD3, to screen the DC3000 genomic sequence available at TIGR, HopPtoD2 was identified. This newly discovered secreted protein turned out to have profound effects on *P. syringae* pathogenesis.

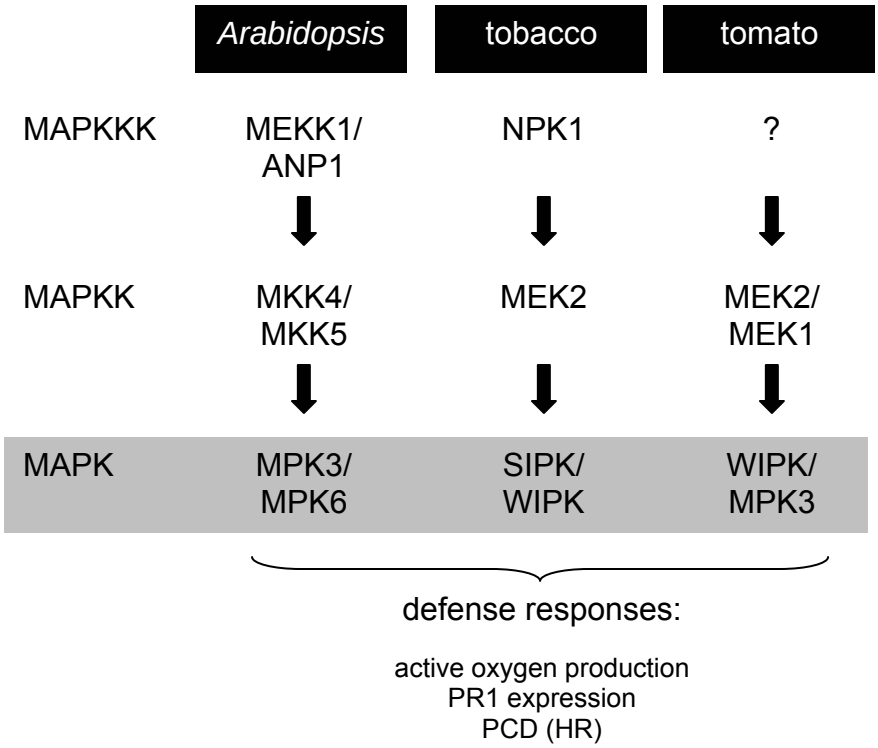
Unlike many other secreted effectors whose enzymatic activity remains a mystery, HopPtoD2 was quickly identified as a protein tyrosine phosphatase (PTP). Because HopPtoD2 was a secreted PTP, all signs indicated that HopPtoD2 would interfere with host cell defense signaling. Unfortunately, the intracellular target of HopPtoD2 has yet to be determined. Plant cells utilize multiple signaling pathways to respond to distinct environmental stimuli (33). The important point of future research will be to establish a link between HopPtoD2's target and each of the observed phenotypes. It will also be important to identify and compare the signaling components involved in each plant species tested (*Arabidopsis*, tobacco, and tomato). The data presented in Chapter 5 and by Espinosa *et al* (67) indicate that HopPtoD2 interferes with signaling during disease, the HR, active oxygen production, and induction of pathogenesis

related genes. In fact, each of these phenotypes has been linked to MAP kinase (MAPK) signal cascades (183, 241, 242).

MAPK cascades consist of three signaling molecules that create a phosphorelay. At the top of the cascade is the MAPK kinase kinase (MAPKKK) which is activated in response to environmental stimuli. The MAPKKK activates a MAPK kinase (MAPKK) by phosphorylation of serine/threonine residues at a conserved motif. MAPKK's are dual specificity threonine/tyrosine kinases that activate MAPK's. MAPK's are serine/threonine kinases that phosphorylate other substrates including transcription factors. Several different MAPKKK's can activate the same MAPK thereby allowing different environmental stimuli can converge at the same point to activate the same response (118).

While multiple MAPK cascade components have been identified in *Arabidopsis* and other plant species (118), very few of these have been organized into discrete pathways. The best studied pathway related to plant defense involves the MAPK's MPK3 and MPK6 from *Arabidopsis.* These MAPK's are activated by the MAPKK's MEK4 or MEK5. The MAPKKK's MEKK1 and ANP1 can initiate this cascade by activating MEK4 or MEK5 (Figure 6-4). MPK6 has been linked to disease resistance in *Arabidopsis* (157, 169). Homologues of this MAPK are also associated with plant defense in tobacco and tomato (66, 155, 242, 248). The MAPKK's that activate these MAPK's also have been implicated in pathogen defense in all three plant species

Figure 6-4: Potential host cell targets for HopPtoD2. The studies on HopPtoD2 revealed multiple phenotypes were affected by HopPtoD2 in at least three distinct host plants. Because it is a protein tyrosine phosphatase (PTP), it seems likely that HopPtoD2 interferes with host cell signaling. In plant cells, MAPK cascades are involved in signaling the response to multiple environmental stimuli, including pathogen attack. MAPKs are phosphorylated on threonine and tyrosine residues and are thus ideal targets for a PTP. Espinosa *et al* (67) indicated that HopPtoD2 exerted its effect downstream of MEK2 in tobacco. Therefore, SIPK and WIPK are candidate targets of HopPtoD2. Their counterparts in *Arabidopsis* and tomato are also potential targets for the HopPtoD2 PTP. Gray box indicates potential targets for HopPtoD2. The information for this figure was complied from multiple sources (66, 118, 155, 183, 187, 223, 241, 242, 247).


Arabidopsis
tobacco
tomato
MAPKKK
MEKK1/
ANP1
NPK1
?
MAPKK
MKK4/
MKK5
MEK2
MEK2/
MEK1
MAPK
MPK3/
MPK6
SIPK/
WIPK
WIPK/
MPK3
defense responses:
active oxygen production
PR1 expression
PCD (HR)

(183, 241, 242). Therefore, it appears likely that this pathway may be a target for HopPtoD2 in these plant species. To further this point, preliminary data presented by Espinosa *et al* (67), indicated that HopPtoD2 exerted its affects downstream of MEK2 in tobacco. Unfortunately, Espinosa did not identify a specific MAPK target. While the data presented above seem to indicate that HopPtoD2 targets one of the MAPK's downstream of MEK2, it's worth noting that *Arabidopsis* encodes at least 20 MAPK's (118), and the MAPK's mentioned here are by no means the only potential targets for HopPtoD2.

What emerges from studies of *P. syringae* effectors is that the interaction of *P. syringae* strains with their plant hosts is highly complex, involving multiple pathogenicity and virulence factors to enable the successful colonization of the host. *P. syringae* strains express a variety of effectors that are translocated into the cytosol of host cells by their TTSS and are required for pathogenicity in the susceptible host. While most mammalian pathogens do not appear to secrete such a large number of effectors, this appears to be a common feature of many plant pathogenic bacteria, such as *Erwinia* (73), *Xanthomonas* (32), and *Ralstonia* spp. (189). This diversity of effectors could be due to the complexity of the host cell itself and the diversity of hosts that a strain interacts with.

While some *P. syringae* effectors are at least partially conserved among strains, many are only found in a few strains. For example, of the effectors with known or predicted biochemical activity, orthologs of the cysteine protease AvrPphB are

broadly conserved (201), whereas the PTP HopPtoD2 appears to have a limited distribution among *P. syringae* strains (29, 67). This is consistent with the observation that each strain appears to be specialized to parasitize a specific plant host. Each effector is thought to have a specific cellular target to aid in the parasitism of that susceptible host. Because these hosts can be from divergent taxonomic families of plants, distinct effectors are likely to be necessary to facilitate parasitism in these different hosts. In addition, deletion of individual effectors usually has little effect on virulence yet loss of the TTSS invariably abolishes pathogenicity (100, 142). Therefore, *P. syringae* strains also appear to express functionally redundant effectors.

The plant hosts, in turn, are not passive recipients to these pathogens and have evolved complicated defense mechanisms that respond to effectors. Plants apparently express a large array of R genes in all living cells as part of a surveillance system to detect the presence of pathogens (be they viral, bacterial, fungal, protist or animal). For those that respond to translocated bacterial effectors, the sensors do not appear to be receptors for effectors, but rather appear to monitor the cellular targets of the *P. syringae* effectors (54, 99). The loss or modification of the cellular target is the eliciting signal for the defense response in these cases. In addition, more than one sensor may monitor a single cellular target to detect different types of modifications. For example, RPS2 monitors proteolytic degradation of RIN4 whereas RPM1 seems to detect phosphorylation of RIN4 (12, 150, 151).

It is also important to recognize that translocated effectors are not the only bacterial signals to which plant cells respond. In addition to translocated effectors, flagellin can elicit defense responses and its cognate host cell surface receptor has been identified (77). Acyl lactone elicitors of defense responses (syringolides) and lipopolysaccharides appear to elicit distinct sets of defense responses in some plants (62, 76, 91). Thus, plants have evolved multifaceted "immune" systems to prevent disease that must be evaded or suppressed for disease to occur.

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