Environmental and density-dependent modulation of type III secretion system genes in Pseudomonas syringae pv. tomato DC3000

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Environmental and density-dependent modulation of type III secretion system genes in Pseudomonas syringae pv. tomato DC3000

Jennifer L. Stauber

A Thesis Submitted to the Graduate School at the University of Missouri – St. Louis
In partial fulfillment of the requirements for the degree
Master of Science in Cell and Molecular Biology

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Environmental and density-dependent modulation of type III secretion system genes in Pseudomonas syringae pv. tomato DC3000

Jennifer Stauber

Abstract

Pseudomonas syringae pathovar tomato strain DC3000 is a model bacterial pathogen that infects tomatoes and Arabidopsis. This bacterium utilizes a dedicated protein export apparatus, the type III secretion system (T3SS), to translocate virulence proteins called effectors directly into host cells. Because effectors suppress plant immune responses, activation of the T3SS is critical upon entry into the host. T3SS gene expression is controlled by a complex regulatory cascade. HrpL is an alternate sigma factor that regulates expression of genes that encode structural elements of the secretion apparatus, as well as secreted effectors, such as AvrPto. hrpL is activated by two members of the bacterial enhancer binding protein (bEBP) family, HrpR and HrpS, encoded by the hrpRS operon. The mechanisms regulating hrpRS activation of hrpL expression are unclear. Although previous studies have shown that T3SS genes are highly regulated by a variety of environmental signals, little is known about how these conditions control T3SS genes.

In this work, I examine how environmental stimuli modulate T3SS gene expression in P. syringae, and analyze how each variable modulates the hrpRS-hrpL regulatory cascade. Specifically, I show that hrpRS, hrpL, and avrPto in Pst DC3000 are regulated by pH and carbon sources in the growth media. Contrary to expectations, I report that several carbon sources, including sugars, a sugar alcohol, glycerol, and organic acids, initially induce Pst DC3000 T3SS gene expression. However, T3SS gene
expression decreases as the cell density increases in media with carbon sources that support faster bacterial growth rates. Furthermore, T3SS genes are lower when the bacteria are cultured at high cell densities regardless of carbon source, or at low cell densities in conditioned media. Therefore, I investigate the possibility that acyl homoserine lactone (AHL)-mediated quorum sensing regulates \textit{Pst} DC3000 T3SS gene expression. I confirm that \textit{psyRI} is responsible for production of 3-oxo-C6 AHL in \textit{Pst} DC3000, although neither addition of exogenous 3-oxo-C6 and C6 AHLs nor deletion of \textit{psyRI} has any effect on the density-dependent regulation of \textit{hrpL}. Therefore, I conclude that there is a T3SS-repressive signal secreted by \textit{Pst} DC3000 that accumulates at high cell densities, but the nature of the signal is still unknown. Lastly, I report that T3SS genes are repressed when the auxin IAA is added to cultures of \textit{Pst} DC3000, however the biological relevance of IAA as a T3SS repressing signal remains to be explored.
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Chapter I. Introduction
Pseudomonas syringae

*Pseudomonas syringae* is a gram-negative, flagellated, rod-shaped bacterium that causes leaf spot or blight in a variety of important agricultural and model plants (Whalen et al., 1991, Hirano & Upper, 2000). The taxonomic designation of *P. syringae* is based on the fact that it was first isolated from a diseased lilac (*Syringa vulgaris*) (Hirano & Upper, 2000). Since then, it has been found infecting many plants, including trees, ornamentals, fruits, vegetables, and grains. Although *P. syringae* has a broad host range, the species is broken down into ~50 subspecific pathovars (pvs) that each have narrower host ranges (Hirano & Upper, 2000). For example, *P. syringae* pathovar pv. tomato (hereafter *Pst*) infects tomatoes and not beans, whereas *P. syringae* pv. phaseolicola (hereafter *PsP*) infects beans and not tomatoes. Pathovar nomenclature refers to the diseased plant from which each strain was isolated, and does not reflect evolutionary phylogeny or host specificity (Sawada et al., 1999, Hwang et al., 2005). For instance, *P. syringae* pv. syringae (hereafter *Pss*) was named for its host lilac, but can also cause disease in as many as 80 different plants (Hirano & Upper, 2000). It is also important to note that strains within the same pathovar do not always share host specificity (Almeida et al., 2009). Different strains of *Pst* infect tomatoes, however *Pst* T1 infects only tomatoes, and *Pst* DC3000 can also infect *Arabidopsis thaliana* and plants in the genus *Brassica*, such as turnip, kale, collard, and cauliflower (Keith et al., 2003, Whalen et al., 1991).

*P. syringae* is a model pathogen

*P. syringae* has become a model plant pathogen because it is genetically tractable and there are several fully sequenced and annotated strains, *Pst* DC3000 (Buell et al., 2009).
2003), \( Psp \ 1448a \) (Joardar \textit{et al.}, 2005), and \( Pss \ B728a \) (Feil \textit{et al.}, 2005), representing three different pathovars. There is also now a draft sequences of \( Pst \ T1 \), allowing for genomic comparison between strains in pathovar tomato that have different host specificity (Almeida \textit{et al.}, 2009). In addition, draft sequences of pvs. oryzae 1-6 (Reinhardt \textit{et al.}, 2009) and tabaci 11528 (Studholme \textit{et al.}, 2009) have just been published, and the genomes of other pathovars are underway (Lindeberg, 2010).

In addition, \textit{P. syringae} utilizes a type III secretion system (T3SS) to infect plants (Höfte, 2006, Mohr \textit{et al.}, 2008). T3SSs are essential virulence factors for many gram-negative bacteria, including the plant pathogens \textit{Ralstonia}, \textit{Erwinia}, \textit{Pectobacterium}, \textit{Dickeya}, \textit{Pantoea}, and \textit{Xanthomonas}, as well as the animal pathogens \textit{Bordetella}, \textit{Vibrio}, \textit{Chlamydia}, \textit{Yersinia}, \textit{Salmonella}, \textit{Shigella}, \textit{Escherichia coli}, and \textit{P. aeruginosa} (Grant \textit{et al.}, 2006, Coburn \textit{et al.}, 2007). T3SSs are also conserved in symbiotic bacteria, such as \textit{Rhizobium} (Soto \textit{et al.}, 2006). Therefore, research on the T3SS in \textit{P. syringae} can have broad impacts on agriculture and human health.

Unlike some of the animal pathogens listed above, \textit{P. syringae} is an excellent candidate for studies on the T3SS because infections can be performed on its natural hosts, which are easy to infect in the laboratory. As mentioned previously, \textit{Pst DC3000} infects the model plant \textit{A. thaliana}. Other strains of \textit{P. syringae} can infect \textit{Nicotiana benthamiana}, which is widely used as a model plant for studying host-pathogen interactions (Goodin \textit{et al.}, 2008).
**P. syringae is not just a pathogen**

My research primarily focuses on the virulence of Pst DC3000. However, it is important to point out that *P. syringae* is not just a plant pathogen. Non-pathogenic strains of *P. syringae* have also been found colonizing plant leaves and roots, and some have even been shown to be beneficial as biocontrol agents for agriculture. For example, non-pathogenic *P. syringae* strain 508 has antimicrobial activity that is used to control the apple scab fungus (Burr *et al.*, 1996). Similarly, strains ESC-10 and ESC-11 (Bio-Save™) are used to protect crops from post-harvest fungal rot, and *P. syringae* strain TLP2 is even used to help protect tomato plants from Pst strains (Höfte, 2006, Wilson *et al.*, 2002, Mohr *et al.*, 2008).

Non-pathogenic strains of *P. syringae* can also damage crops via ice-nucleating activity, which raises the freezing temperature of water and increases the risk of frost damage (Lindow, 1983, Lindow *et al.*, 1982). Researchers were able to overcome this detrimental trait by removing the ice-nuclease gene from non-pathogenic strains of *P. syringae*. In 1989, ice-minus *P. syringae* became the first genetically modified organism deliberately released into the environment to outcompete the growth of ice-nucleating *P. syringae* (Drahos, 1991). Ice-minus *P. syringae* (Frostban™) is still commercially available to protect crops from frost damage.

**P. syringae is ubiquitous in the environment**

*P. syringae* is found everywhere linked to the water cycle, including clouds, pristine snow, and rivers (Morris *et al.*, 2007, Morris *et al.*, 2008, Hirano & Upper, 2000). Current research suggests that *P. syringae* aerosolizes from plants and water into clouds,
where its ice nucleating activity may play a significant role in precipitation (Christner et
al., 2008). Rain and snow deposit the bacteria onto undeveloped and agricultural
landscapes, where the bacteria can colonize plants (Figure 1) (Hirano & Upper, 2000,
Morris et al., 2008).
Figure 1. Life cycle of *P. syringae*.

*P. syringae* aerosolizes and precipitates with moisture, allowing it to move throughout the landscape. When the bacteria encounter a plant, they first colonize the leaf surface, and then sometimes the plant apoplast. Inside, they may trigger either a resistance response, such as the hypersensitive response (HR), or disease (necrosis and speck).
**P. syringae – plant interactions**

**P. syringae colonization of plants occurs in two stages**

*P. syringae* may be seed-borne and thereby present on emerging seedlings, or may arrive on leaves aeri ally, carried by wind, rain, or insects (Hirano & Upper, 2000, Morris et al., 2008). Once deposited on the leaf surface, *P. syringae* can survive as an epiphyte without causing disease (Hirano & Upper, 2000). Survival on leaf surfaces is not easy, as nutrients are scarce and environmental conditions are constantly fluctuating (Lindow & Brandl, 2003). Some strains of *P. syringae*, such as *Pss* B728a, are well adapted to an epiphytic lifestyle (Monier & Lindow, 2003). In contrast, research suggests that *Pst* DC3000 is a relatively poor epiphyte, preferring to enter into the nutrient-rich plant interior (Boureau et al., 2002). In fact, *P. syringae* infection of plants occurs in a two-step process: first in an epiphytic stage and then (if successful) in an endophytic stage (Figure 1).

**Epiphytic stage**

Once on a leaf, *P. syringae* are highly motile and search for areas rich in nutrients leaching from the plant interior (Dulla, 2005). These nutrients create a microhabitat, or “oasis”, that can support bacterial growth. Bacteria sense when their population has reached a certain size, or quorum, by detecting small diffusible molecules called acyl homoserine lactones (AHL) (Loh et al., 2002). These molecules are continuously produced by *P. syringae* and accumulate to high levels in dense populations, to facilitate the process known as quorum sensing (Dulla, 2005). AHLs signal *P. syringae* to down-regulate motility, aggregate into biofilms, and activate genes that provide protection from
environmental stresses (Dulla, 2005). Although these epiphytic biofilms do not cause disease, they are a source for infection for the leaf interior (Hirano & Upper, 2000).

**Endophytic stage**

Once established on leaf surfaces, *P. syringae* can invade the leaf mesophyll (or apoplast) by entering through wounds or natural gas exchange openings called stomata (Melotto *et al.*, 2008). Inside the apoplast, *P. syringae* can utilize many nutrients to support bacterial growth (Rico & Preston, 2008, Kamilova *et al.*, 2006). If plant defenses cannot impede *P. syringae* from manipulating plant physiology, the bacteria multiply and cause disease (Dulla, 2005). *P. syringae* is considered a hemibiotrophic pathogen because it does not kill plant tissue until later stages of infection (Rico & Preston, 2008).

Specific diseases caused by *P. syringae* are particular to each pathovar and host. In *Pst* DC3000, disease symptoms are characterized by brown necrotic lesions resulting from cell death on leaves and sometimes fruits, and chlorosis, which is yellowing of leaf tissue due to chloroplast disruption (Figure 2) (Bender *et al.*, 1999, Hirano & Upper, 2000).
Figure 2. *Pst DC3000* causes bacteria speck in tomato leaves. Tomato leaves dipped in *Pst* DC3000 bacterial suspensions develop necrotic specks surrounded by chlorotic halos.
**P. syringae uses a variety of virulence factors to cause disease in host plants**

The disease symptoms caused by *P. syringae* are the result of multiple overlapping virulence factors. Although the precise mechanisms of these virulence factors are still unclear, *P. syringae* orchestrates pathogenicity via both diffusible phytotoxins and secreted proteins known as effectors. The repertoire of phytotoxins and effectors varies between *P. syringae* pathovars and strains.

**Phytotoxins**

Phytotoxins produced by *P. syringae* include coronatine (a polyketide), tabtoxin (a β-lactam), phaseolotoxin (a sulfodiaminophosphinyl peptide), and syringomycin (a lipodepsinonapeptide) (Hwang et al., 2005). Coronatine acts as a phytohormone mimic to block salicylic acid-dependent plant defenses, open stomata, and cause chlorosis (Brooks et al., 2004, Bender et al., 1999, Boller & He, 2009). Tabtoxin and phaseolotoxin also contribute to chlorosis, while syringomycin forms holes in the plant plasma membrane, resulting in necrosis (Bender et al., 1999). A survey of 95 pathogenic strains of *P. syringae* reported that fewer than 50% of the strains produced any of these phytotoxins (Hwang et al., 2005). Thus, although phytotoxins contribute to severity of symptoms, they are not essential for disease development. *Pst* DC3000, the subject of this thesis, only produces the phytotoxin coronatine (Buell et al., 2003, Hwang et al., 2005).

**Effector proteins**

Effectors are virulence proteins that are delivered by bacterial secretion systems directly into plant cells. These proteins hijack plant signaling pathways to the benefit of
the invading bacteria (Gohre & Robatzek, 2008). *P. syringae* secretes numerous effectors to suppress plant defense responses and promote disease (Schechter et al., 2006, Lindeberg et al., 2006, Chang et al., 2005). Some effectors are also recognized by specific plants and trigger resistance. It is thought that evolutionary pressures to modulate plant responses while avoiding detection by defense systems have driven effector diversity and redundancy (Stavrinides et al., 2008).

**Plants have multiple layers of defenses against *P. syringae***

Most plants are resistant to most pathovars of *P. syringae*, due to two main types of pathogen detection and defense systems. PAMP-triggered immunity (PTI) occurs earlier (within 10 minutes) after bacterial infection and has features in common with immunity in animals (Ausubel, 2005, Abramovitch et al., 2006, Nurnberger et al., 2004). While PTI is successful against some pathovars of *P. syringae*, many plants have evolved another more specific level of resistance known as effector triggered immunity (ETI), or R-gene mediated resistance, which occurs a little later (2-3 hours) after bacterial infection (Figure 3) (Jones & Dangl, 2006).

**PAMP-triggered immunity***

Plant basal defenses are triggered in response to conserved bacterial molecules, collectively known as PAMPS (pathogen-associated molecular patterns) or MAMPS (microbe-associated molecular patterns) (Boller & He, 2009). Examples of PAMPS include lipopolysaccharide (LPS), peptidoglycan, flagellin, and the bacterial translation elongation factor EF-Tu (Zipfel, 2008). PTI is activated in response to both pathogenic and non-pathogenic microbes and is generally conserved among plants (Zipfel, 2008).
Figure 3. Activation and suppression of immune responses by *Pst DC3000*. Plants mount defense responses against *Pst DC3000* when PAMPs, such as flagella, are detected by PRRs. *Pst DC3000* secretes effectors, such as AvrPto, that can suppress PTI. However, plants with the R-protein Pto can recognize AvrPto and trigger ETI. Therefore, the presence of Pto determines whether *Pst DC3000* infection leads to bacterial pathogenicity or plant resistance.
PAMPs are detected by pattern recognition receptors (PRR) in the plant cell membrane, which then activate mitogen-activated protein kinase (MAPK) cascades that control defense gene expression (Boller & He, 2009, de Wit, 2007). For example, in A. thaliana, flagellin is detected by the PRR FLS2 (Gomez-Gomez & Boller, 2000). FLS2 then interacts with BAK1, a convergent signaling molecule for multiple PRRs (Chinchilla et al., 2007). Ultimately, flagellin perception leads to upregulation of nearly 1000 A. thaliana genes, and a variety of defense responses that limit bacterial invasion and spread (Chinchilla et al., 2007, Zipfel, 2008, Gomez-Gomez & Boller, 2002). These plant responses include closure of stomata, reinforcement of the cell-wall with β-1,3-glucan (callose), restriction of vascular flow, and increased production of reactive oxygen and nitrogen species (Abramovitch et al., 2006, de Wit, 2007, Chisholm et al., 2006, Kim et al., 2008).

Plants that utilize PTI to successfully defend against P. syringae infection are considered non-hosts. Many P. syringae effectors promote disease by counteracting PTI (Boller & He, 2009, Guo et al., 2009). For example, a Pst effector, AvrPto, inhibits expression of the PAMP-induced defense genes NHO1 and FRK1, suppresses callose deposition in the cell wall, reduces the production of reactive oxygen species, and reinstates vascular flow (Oh & Collmer, 2005, Hauck et al., 2003, Li et al., 2005, He et al., 2006a). Recent evidence suggests that AvrPto may suppress PTI by binding to BAK1, preventing its interaction with FLS2 (Shan et al., 2008).
**Effector-triggered immunity**

Some plants have evolved ETI as a second defense mechanism against effectors. ETI is mediated by plant resistance (R) proteins that detect the presence of a specific bacterial effector (Cui *et al.*, 2009). Each plant R protein works on a gene-for-gene basis, meaning that a particular R protein defends against one (or sometimes more) specific bacterial effector. If a plant has the resistance protein that corresponds to an effector encountered during infection, then ETI results in the hypersensitive response (HR), which is rapid, localized apoptosis (Boller & He, 2009). Plants that initiate ETI can successfully defend against *P. syringae* infection and are considered resistant. For example, the R-protein Pto recognizes the *P. syringae* effectors AvrPto and AvrPtoB (HopAB). Tomato plants that possess *Pto* are resistant to *Pst* DC3000, which delivers both AvrPto and AvrPtoB into plant cells (Figure 3)(Abramovitch & Martin, 2005). However, plants that lack *Pto* are susceptible to *Pst* DC3000 due to the virulence functions of AvrPto, AvrPtoB, in conjunction with other effectors.

Successful *P. syringae* pathovars also secrete effectors to overcome ETI. Recent analysis of *Pst* DC3000 effectors suggests that most can suppress ETI by blocking the ability of other effector proteins to trigger the HR (Guo *et al.*, 2009). There is also evidence for R-protein recognition of effectors that initially evolved to overcome ETI. For instance, although the N-terminus of AvrPtoB is recognized by Pto, the C-terminus of AvrPtoB has E3 ligase activity that targets Fen (a relative of Pto) for ubiquitination and degradation (Rosebrock *et al.*, 2007). When the E3 ligase activity of AvrPto is
inactivated, Fen recognizes the effector and triggers ETI in plants that lack Pto.

Therefore, AvrPtoB may have obtained a C-terminal E3 ligase domain to inactivate Fen and avoid ETI. Pto, on the other hand, may have evolved to evade the E3 ligase activity of AvrptoB and reinstate immunity (Rosebrock et al., 2007). Current evidence suggests that a new avrPto allele has evolved to evade Pto in Pst race 1 strains (Kunkeaw et al., 2010). Thus, the outcome of _P. syringae_ infection depends upon both the bacterial and host genotypes, and the success of the pathogen is linked to its ability to evolve new effectors and avoid detection.
The *P. syringae* T3SS

The T3SS injectisome and secreted effectors

T3SSs are dedicated protein export machines, structurally related to flagella, which inject effectors from the bacterial cytoplasm directly into the host cell cytoplasm (Figure 4) (Galán & Collmer, 1999). Components of the *P. syringae* T3SS apparatus are encoded by the *hrp/hrc* gene cluster, also known as the *hrp* pathogenicity island (Figure 4). This locus was named *hrp*, which stands for hypersensitive response and pathogenicity, because the *P. syringae* T3SS is responsible for causing both the HR in resistant plants and disease in susceptible plants (Alfano & Collmer, 1997). Some *hrp* genes were later renamed *hrc* (for *hrp* genes conserved across taxa) to denote the structural genes that are common to nearly all bacteria with T3SSs.

The *hrp/hrc* gene cluster is flanked by two genetic loci called the conserved effector locus (CEL) and exchangeable effector locus (EEL). As the names suggest, genes in these regions encode several (but not all) type III secreted effectors. Other T3SS effector genes, such as *avrPto*, are scattered throughout the genome. Bioinformatic and functional studies suggest that the *Pst* DC3000 genome encodes at least 33 T3SS effectors (Schechter et al., 2006).
Figure 4. The *P. syringae* T3SS.
The *hrp/hrc* pathogenicity island (shown above) contains T3SS regulatory genes (red) and genes encoding T3SS injectisome structural proteins (colors are coordinated between the genes and structural proteins). The injectisome is composed of intracellular and membrane-bound proteins in the bacterium, as well as the proteinaceous Hrp pilus and translocon, which functions to inject effectors directly from the bacterial cytoplasm into plant cells.
The *P. syringae* T3SS apparatus, also known as the injectisome, translocates effectors directly into plant cells, and is composed of many structural proteins that function together to penetrate two bacterial membranes and the plant cell wall and plasma membrane. Each component of the injectisome plays an important role in the translocation process and is thus essential for virulence.

Several proteins associate to form the base of the T3SS, which spans the bacterial inner and outer membranes. The bottom of the T3SS, in the bacterial cytoplasm, is composed of homo-oligomers of HrcN, an ATPase essential for secretion (Pozidis *et al.*, 2003). It has long been assumed that HrcN provides the energy for secretion of T3SS substrates. However, secretion of proteins through the base of the flagellum is driven by the proton motive force, not ATP hydrolysis (Minamino *et al.*, 2008). Thus, HrcN may instead play a role in loading effectors in the bacterial cytoplasm into the T3SS (Paul *et al.*, 2008, Buttner & He, 2009). The HrcQ and HrcQ_B proteins form a cytoplasmic ring, similar to that found at the base of flagellum (Fadouloglou *et al.*, 2004). HrcR, HrcS, HrcT, HrcU, and HrcV proteins form a channel through the bacterial inner membrane (He, 1998, He *et al.*, 2004). HrcC multimerizes to form a channel in the bacterial outer membrane, possibly with the help of the small HrpT protein (Yuan-Chuen Lin, 2006). HrcJ is a lipoprotein believed to connect the two channels in the periplasmic space, although other proteins may also be involved (Deng & Huang, 1998).

The base of the T3SS is connected to a hollow pilus (or proteinaceous extension) that radiates from the bacterial surface and serves as the conduit for translocation of effectors across the extracellular space into host cells (Jin & He, 2001). In animal
pathogens, the extracellular transport channel is a rigid, needle-like structure. In contrast, the *P. sryingae* T3SS pilus, which is composed of oligomers of the HrpA protein, is longer and more flexible (Buttner & He, 2009). These characteristics may allow the *P. syringae* T3SS to translocate effectors across the thick plant cell wall (Buttner & He, 2009). In fact, structural studies have revealed that the *Pst* DC3000 Hrp pilus extends 5 µm from the bacterial outer membrane and is ~6-8 nm in diameter, which is long enough to penetrate into plant cells but too narrow to hold many effectors in their folded form (Brown *et al.*, 2001). Therefore, it is hypothesized that effectors are secreted through the pilus in an unfolded state, and folding occurs after translocation into the host cytoplasm (Brown *et al.*, 2001). Recent research suggests that AvrPto folding is controlled by the environmental pH (Dawson *et al.*, 2009). At a mildly acidic pH, which exists in the bacterial cytoplasm, AvrPto unfolds and can be translocated. Refolding occurs after delivery into the plant cell cytoplasm, which has a neutral pH (Dawson *et al.*, 2009).

While the HrpA pilus may penetrate the plant cell wall and membrane, it is hypothesized that additional proteins at the tip of the pilus form a translocon that inserts into these eukaryotic barriers (Buttner & He, 2009). Although the composition of the *P. syringae* translocon has not been determined biochemically, genetic studies suggest that this channel may be composed of at least four different proteins: HrpK, HrpZ, HrpW, and HopAK1 (Kvitko *et al.*, 2007, Petnicki-Ocwieja *et al.*, 2005). The *hrpK* gene, located in the *hrp/hrc* cluster, encodes a protein that is similar to the putative translocator protein HrpF in *Xanthomonas campestris* pv. vasicatoria (Buttner *et al.*, 2002, Petnicki-Ocwieja *et al.*, 2005). Two lines of evidence support the idea that HrpK is a translocator. First, HrpK contains a transmembrane domain, which may allow it to associate with the
plant cell membrane. Second, Pst DC3000 *hrpK* mutants can secrete effectors into the extracellular milieu, but cannot efficiently translocate effectors into plant cells (Petnicki-Ocwieja et al., 2005). However, it is important to note that Pss B728a *hrpK* mutants cause the HR in resistant plants as well as wild-type bacteria (Collmer *et al.*, 2000).

HrpZ, HrpW, and HopAK1 are all in a class of proteins known as harpins. Harpins are T3SS-secreted glycine-rich proteins that lack cysteine residues and have a high isoelectric point. Curiously, purified preparations of harpin proteins also cause the HR when injected into the plant apoplast (Kvitko *et al.*, 2007, Charkowski *et al.*, 1998, He *et al.*, 1993). A role for harpins in translocation is supported by the finding that HrpZ binds to lipid bilayers and forms pores *in vitro* (Lee *et al.*, 2001). Although deletion of individual harpin genes does not affect the ability of Pst DC3000 to colonize host plants or cause the HR in non-hosts, a strain lacking all harpins caused the HR less efficiently (Charkowski *et al.*, 1998, Kvitko *et al.*, 2007).

A recent study suggests that HrpK, HrpZ, HrpW, and HopAK1 may function together in translocation. A mutant lacking *hrpK* and all harpin genes was significantly less efficient at translocating effectors than strains lacking just harpins or *hrpK* alone (Kvitko *et al.*, 2007). Ectopic expression of *hrpZ, hrpK, hrpW*, or *hopAK1* restored translocation in the polymutant (Kvitko *et al.*, 2007). Therefore, HrpK and harpin proteins may together compose a translocon that aids in translocation of effectors across the plant cell wall and membrane. The translocon may contain multiple harpins and other proteins that have redundant functions.
Type III secreted effectors are named Hops (Hrp outer proteins) or Avr (avirulence) proteins depending on how they were discovered (Collmer et al., 2000, Lindeberg et al., 2005). For example, AvrPto and AvrPtoB are named such because both are avirulence proteins recognized by Pto, although the two are functionally distinct effectors. Avr proteins were discovered based upon their ability to cause the HR, but may still be effective virulence factors in plants that lack cognate R proteins. On the other hand, Hops have been identified by their ability to be secreted by the T3SS (Lindeberg et al., 2005). However, the exact function of many Hop and Avr proteins is still unknown.

Pst DC3000 has over experimentally 30 confirmed T3SS effectors (Lindeberg et al., 2006, Schechter et al., 2006). Genomic comparison suggests that Pst DC3000 has considerably more effectors than other sequenced P. syringae pathovars, Psp 1448a or Pss B728a (Lindeberg et al., 2006). T3SS effector repertoires vary widely between these pathovars, which may be responsible for their distinct host specificities. Significant differences also occur in the repertoire of effectors in Pst DC3000 and of Pst T1, which may explain why Pst DC3000 is able to colonize plants other than tomato and Pst T1 does not (Almeida et al., 2009).

**Targeting effectors to the P. syringae T3SS**

Most P. syringae effectors contain a high percentage of polar amino acids within the first 50 residues and also lack negatively charged amino acids near the N-terminus (Petnicki-Ocwieja et al., 2002, Schechter et al., 2004). The N-terminal amino acid sequences appear to target most T3SS effectors for secretion. However, T3SS-secreted
effectors appear to lack a consensus targeting sequence, and the exact mechanism of effector recognition by the T3SS has remained elusive.

Chaperone proteins may also help guide effectors to the secretion system (Guttman et al., 2002). Chaperones are small, acidic proteins that are not secreted themselves, but assist in transporting type III secreted proteins to the injectisome (Buttner & He, 2009). T3SS chaperones also stabilize T3SS effectors in the bacterial cytoplasm (Losada & Hutcheson, 2005, Page & Parsot, 2002). Certain chaperones are dedicated to only one effector, while others can bind to several effectors (Wilharm et al., 2007, Page & Parsot, 2002). Often, dedicated chaperones are encoded by genes just upstream of their cognate effectors, suggesting a conserved evolutionary relationship (Cornelis, 2006).

T3SS injectisome proteins, such as HrpA and harpins, must be secreted prior to translocation of effectors. The order of secretion is dictated by substrate specificity switches, also known as T3S4 proteins (Buttner & He, 2009). In Xanthomonas campestris, HpaC controls the switch from pilus protein secretion to translocon and effector secretion (Lorenz et al., 2008). Although substrate specificity is not well understood in P. syringae, HrpP was recently identified as a T3SS substrate specificity switch in P. syringae (Morello & Collmer, 2009). In contrast to HpaC, HrpP is known to be a T3SS substrate. X. campestris also controls substrate specificity by means of a secreted protein, HpaA, and a global chaperone, HpaB. During assembly of the T3SS, HpaB is sequestered by HpaA inside the bacterial cytoplasm. When the T3SS is mature and HpaA is translocated, HpaB is released to chaperone other effectors (Lorenz et al.,
2008). However, it is unclear how *P. syringae* coordinates T3SS substrate specificity, because HrpP translocation is not necessary for its regulatory role (Morello & Collmer, 2009).
Regulation of *P. syringae* T3SS gene expression

In most bacteria, T3SS genes are highly regulated so that they are only expressed at the appropriate times during infection. *P. syringae* may need to regulate production of the T3SS because it is energetically expensive to synthesize and assemble the apparatus as well as to secrete effectors (Francis et al., 2002). In addition, since some effectors activate ETI defense responses, constitutive expression of the T3SS might prematurely alert the host. Activation of *P. syringae* T3SS gene expression occurs rapidly when bacteria are in plant tissue or media thought to mimic the plant apoplast (Hutcheson et al., 2001, Huynh et al., 1989, Rahme et al., 1992). Regulation of T3SSs is usually carried out by one or more transcription factors encoded within the T3SS gene cluster (Tang et al., 2006).

Multiple *P. syringae* T3SS regulators are encoded within the *hrp/hrc* island

In *P. syringae*, three transcriptional regulators, HrpR, HrpS, and HrpL, are encoded by genes within the *hrp/hrc* cluster and function in a cascade that activates all of the components of the *P. syringae* T3SS, including other *hrp/hrc* genes as well as effector genes (Figure 5). However, outstanding questions remain about how this cascade functions. And although several upstream components have been identified that modulate the *hrpRS-hrpL* cascade in response to environmental signals, little is known about how external cues are perceived by *P. syringae* or how they modulate T3SS regulatory elements.
Figure 5. Regulation of T3SS genes in *P. syringae*. T3SS genes include *hrp/hrc* genes (encoded within the *hrp* gene cluster) and effector genes (scattered throughout the genome), both of which are directly activated by regulators encoded by *hrpRS* and *hrpL*. T3SS genes and gene products (both shown in yellow) are modulated by environmental signals that transcriptionally and post-transcriptionally regulate the *hrpRS-hrpL* cascade, although the precise signals responsible for have not yet been characterized.
HrpL is an alternate sigma factor in the extracytoplasmic function (ECF) family, that binds directly to a consensus sequence (GGAACC-N_{16}-CCACNNA) found in T3SS gene promoters, known as the hrp box (Fouts et al., 2002, Xiao & Hutcheson, 1994, Ferreira et al., 2006). When HrpL binds to the hrp box, it recruits RNA polymerase to transcribe the downstream gene(s) (Xiao & Hutcheson, 1994). HrpL is highly conserved in plant pathogenic species of Erwinia, Dickeya, Pectobacterium, Pantoea, and Pseudomonas, and is responsible for transcription of most hrp/hrc genes and T3SS effectors in all pathovars of P. syringae (Figure 4 and 5) (Xiao & Hutcheson, 1994).

HrpL down-regulates flagellar genes and up-regulates genes for other virulence factors, such as those involved in coronatine production in Pst DC3000 (Ortiz-Martín et al., 2010, Ferreira et al., 2006, Fouts et al., 2002). HrpL positively controls expression of corR, which encodes another regulator of coronatine biosynthesis genes, and CorR has also been shown to regulate hrpL in a positive regulatory loop (Sreedharan et al., 2006). HrpL may regulate the hrpRS operon as well (Thwaites et al., 2004, Ortiz-Martín et al., 2010).

In P. syringae, hrpL expression is positively regulated by the products of the hrpRS operon and rpoN, which encodes an alternate sigma factor (σ^{54}) (Xiao et al., 1994). HrpR and HrpS are bacterial enhancer binding proteins (bEBP) that are ~60% identical (Xiao et al., 1994). Although HrpR is only found in P. syringae, HrpS is conserved in Pectobacterium, Dickeya, Erwinia and Pantoea species (Tang et al., 2006). The exact mechanism of HrpR and HrpS activation of hrpL is unclear. Other members of the bEBP family are known to bind to enhancer sequences located significantly upstream of the promoters they regulate (Rappas et al., 2007). They also interact with RNA
polymerase holoenzyme containing $\sigma^{54}$ (by DNA looping) and hydrolyze ATP to promote open complex formation (Rappas et al., 2007). Yeast two-hybrid and copurification experiments show that HrpR and HrpS interact, suggesting that they may activate *hrpL* expression by heterodimerization (Hutcheson et al., 2001). In *P. s* and *P. st*, the *hrpR* and *hrpS* genes are co-transcribed, and both proteins are required for optimal expression of a *hrpL-lacZ* reporter in *E. coli* (Hutcheson et al., 2001). Nevertheless, HrpS alone can also activate *hrpL-lacZ* expression in *E. coli*, albeit at much lower levels than when both HrpR and HrpS proteins are present (Hutcheson et al., 2001). In contrast, experiments in *P. p* suggest that HrpR activates *hrpS*, and that HrpS alone activates *hrpL* (Grimm et al., 1995). Further experiments will be required to resolve these contradictory findings.

Other *hrp* genes involved in regulation of the *hrpRS-hrpL* cascade include *hrpA*, *hrpV*, and *hrpG*. Mutating *hrpA*, which encodes the Hrp pilus protein, reduces the expression of *hrpRS* and downstream *hrp* genes by an unknown mechanism. HrpV is a negative regulator that binds to the HrpS protein and prevents it from activating *hrpL* (Ortiz-Martin et al., 2010, Preston et al., 1998). HrpG is a chaperone-like protein that may act as an anti-anti-activator by binding to HrpV and preventing its association with HrpS (Wei et al., 2005). Therefore, several *hrp* island genes play a crucial role in modulating T3SS gene expression. The finding that HrpV negatively regulates T3SS genes suggests a need to turn the secretion system off after it is activated.
Lon protease regulates \textit{P. syringae} T3SS genes

Lon is a cytoplasmic ATP-dependent protease with numerous substrates, many of which are involved in stress responses (Hori \textit{et al.}, 2002, Butler \textit{et al.}, 2006, Tsilibaris \textit{et al.}, 2006). The proteolytic activity of Lon affects regulation of the T3SS in several animal pathogens. Lon represses expression of T3SS genes in \textit{Salmonella} that are involved in promoting invasion of the intestinal epithelial cells (Takaya \textit{et al.}, 2002). More recently, Lon has also been implicated in environmental regulation of the \textit{Yersinia pestis} T3SS; although in this case, Lon degradation activates expression of the secretion system (Jackson \textit{et al.}, 2004)

Lon is also involved in regulating the \textit{Pst} DC3000 T3SS. Lon can target both effectors and transcriptional regulators for proteolysis. Lon degrades T3SS effectors when chaperones are not present (Losada & Hutcheson, 2005). In environmental conditions that repress T3SS genes, Lon inhibits transcription of \textit{hrpL} by rapidly degrading HrpR, but not HrpS (Figure 5) (Bretz \textit{et al.}, 2002). Thus \textit{Pst} DC3000 \textit{lon} mutants exhibit hypersecretion of T3SS effectors, and additionally cause an earlier HR response in non-host plants (Bretz \textit{et al.}, 2002). Surprisingly, Lon may also positively regulate T3SS gene expression, as \textit{lon} mutants in \textit{Pst} DC3000 and three different \textit{Psp} strains exhibit lower levels of \textit{hrpRS} and \textit{hrpL} expression in conditions that induce T3SS genes. These same \textit{lon} mutants also showed attenuated disease symptoms \textit{in planta} (Lan \textit{et al.}, 2007). Further studies will be required to resolve this conflicting data. Lon also may also be regulated by T3SS genes, as \textit{lon} transcripts are higher in both \textit{hrpRS} and \textit{hrpL} mutants of \textit{PstDC3000} under T3SS inducing conditions (Deng, 2009).
Two-component systems regulate *P. syringae* T3SS genes

Two component systems are generally composed of two proteins; a membrane-bound sensor kinase and a cytoplasmic response regulator (Beier & Gross, 2006). The sensor receives environmental signals and autophosphorylates itself on a conserved histidine residue. The phosphate is then transferred to a cognate response regulator, which binds to DNA and controls transcription of target genes (Beier & Gross, 2006). There are several two-component systems involved in regulating the *P. syringae* hrpRS-hrpL cascade. However, the mechanisms of gene regulation by these systems are not fully understood.

The Gac (Global regulator of antibiotic and cyanide production) two-component system is considered a master regulator of virulence in *P. syringae* (Chatterjee et al., 2003, Heeb & Haas, 2001, Mole et al., 2007). GacS is the sensor kinase that phosphorylates and activates the response regulator GacA (Rich et al., 1994). In *Pst* DC3000, GacA regulates T3SS gene expression, as well as swarming motility and the production of AHLs, phytotoxins, pigments, exopolysacharrides, and regulatory RNAs (Chatterjee et al., 2003, Mole et al., 2007, Tang et al., 2006). GacS/A modulates the T3SS regulatory cascade via transcriptional regulation of *hrpRS* and *rpoN*, which are both required for expression of *hrpL* (Figure 5) (Chatterjee et al., 2003). The exact signals perceived by GacS are unclear, although transcription of *gacA* may be regulated by Lon protease and environmental conditions such as stress responses and growth phase (Chatterjee et al., 2003, Lan et al., 2007).
The Rhp (regulator of hrp genes) two-component system was identified in Psp NPS3121 and is conserved in the sequenced strains Psp 1448a, Pss B728a, and Pst DC3000 (Xiao et al., 2007). Like the GacS/A system, environmental signals controlling the RhpR/S system are still unknown. In contrast to GacA, the hrpRS-hrpL regulatory cascade is repressed when the response regulator, RhpR, is phosphorylated. Under environmental conditions that induce T3SS genes, the sensor kinase RhpS reverses the phosphorylation of RhpR, inhibiting its ability to repress T3SS gene expression (Xiao et al., 2007). RhpR does not directly bind upstream of hrpRS, hrpL, or rpoN, and thus indirectly affects the T3SS regulatory cascade (Deng et al., 2010). In T3SS inducing conditions, RhpR may transcriptionally regulate AefR, which controls AHL production and T3SS gene expression in Psp NSP3121 (Deng et al., 2009). In addition, there is evidence that RhpR modulates T3SS gene expression by affecting Lon protease (Deng, 2009), although transcription of gacA is not affected (Xiao et al., 2007).

CorS and CorR are part of a modified two-component system responsible for production of the diffusible phytotoxin, coronatine (Rangaswamy & Bender, 2000). CorS is a sensor kinase and is modulated by temperature in vitro and in planta (Ullrich et al., 1995, Braun et al., 2008). Two response regulators, CorR and CorP, are associated with CorS; however, only CorR has a DNA binding domain (Ullrich et al., 1995, Penaloza-Vazquez & Bender, 1998, Smirnova & Ullrich, 2004). Coronatine production and the T3SS are co-regulated, as corR is directly transcriptionally regulated by HrpL, and hrpL is indirectly modulated by CorR (Sreedharan et al., 2006).
RNA-binding proteins and small RNAs regulate T3SS genes

RsmA (Regulator of secondary metabolism) is an RNA-binding protein that promotes degradation of target mRNAs, often involved in carbon storage and virulence. Untranslatable small RNAs (sRNA), such as rsmB or rsmX/Y/Z, can bind RsmA and thereby relieve translational repression (Lapouge et al., 2008). These sRNAs are often under the control of the Gac two-component system (Chatterjee et al., 2003). RsmA positively regulates T3SS genes in *P. aeruginosa*, and negatively regulates T3SS genes in *Erwinia carotovara* 71 (Mulcahy et al., 2006, O'Grady et al., 2006). In *E. carotovara*, rsmB positively regulates expression of *hrpL* by relieving repression by RsmA (Chatterjee et al., 2002). In *P. syringae*, overexpression of *rsmA* significantly reduces production of several phytotoxins (Kong, 2007). The *Pst* DC3000 genome contains several homologs of *rsmA*, including *rsmA1*, which negatively regulates expression of *hrpL* and other T3SS genes (Rife et al., 2005, Chatterjee et al., 2007). In addition, the sRNAs *rsmB* and *rsmZ* are expressed in *Pst* DC3000 (Heeb & Haas, 2001, Chatterjee et al., 2003).
Environmental conditions modulate the *P. syringae* T3SS

*P. syringae* T3SS genes are expressed and effectors are secreted in certain culture media, therefore, host-specific factors must not be essential for activation of the T3SS (Huynh et al., 1989, Rahme et al., 1992). However, studies performed on bacteria grown in culture may not provide the most accurate information about when T3SS genes are expressed during *P. syringae* infection of plants. Some studies have shown that *P. syringae* activates the T3SS more quickly upon entry into plant tissues than in culture (Rahme et al., 1992, Ortiz-Martín et al., 2010, Thwaites et al., 2004), while others suggest highest induction of T3SS genes in culture (Rico & Preston, 2008, Xiao et al., 1992). Regardless, manipulating conditions in *P. syringae* cultures has provided useful information about how the environment might affect expression of T3SS genes when the bacteria are *in planta*.

The list of variables suggested to modulate T3SS gene expression in *P. syringae* includes carbohydrates (Huynh et al., 1989, Rahme et al., 1992, Xiao et al., 1992), amino acids (Huynh et al., 1989, Xiao et al., 1992), organic acids (Huynh et al., 1989, Rahme et al., 1992), and fatty acids (Xiao et al., 2004), as well as factors such as pH (Rahme et al., 1992), temperature (Rahme et al., 1992), and osmolarity (Rahme et al., 1992). Thus far, however, how each of these factors influences T3SS genes in *Pst DC3000* has not thoroughly explained.

The *P. syringae* T3SS is repressed in complex media and induced in minimal media

When cultured in complex media, such as King’s B (KB) (King et al., 1954), *P. syringae* T3SS genes are repressed and effectors are not secreted. However, T3SS genes
are induced and effectors are secreted into culture when *P. syringae* is grown in a defined minimal medium, such as *hrp*-derepressing minimal medium (HDM), which consists of 50 mM potassium phosphate buffer, 7.6 mM (NH$_4$)$_2$SO$_4$, 1.7 mM MgCl$_2$, 1.7 mM NaCl, and 10 mM fructose (pH 6). HDM is thought to mimic the plant apoplast environment, although it is unclear what components of the medium signal *P. syringae* to express T3SS genes (Huynh et al., 1989, Rico & Preston, 2008).

Repression of T3SS genes in KB has been explained by the fact that the HrpR protein is degraded by Lon protease faster in KB than in HDM (Bretz et al., 2002). However, HrpS protein stability is similar in KB and HDM. Therefore, HrpR protein levels may be a limiting factor for expression of T3SS genes in KB. In addition, there is some debate as to whether *hrpRS* transcription is regulated by the composition of bacterial growth medium. Bretz et al. (2002) found that a *hrpRS::lacZ* reporter was constitutively active in both media in *Pss* 61, while Xiao et al. (2007) found that the *Pst DC3000 hrpR* mRNA levels were higher in HDM than in KB (Bretz et al., 2002, Xiao et al., 2007). These contradictory findings may be explained by the fact that the studies were carried out in different pathovars of *P. syringae*.

T3SS genes are also induced in another minimal medium, Hoitink-Sinden amended with sucrose (HSS) (50 mM potassium phosphate buffer, 18.6 mM NH$_4$Cl, 232.4 µM MgSO$_4$•7H$_2$O, 10 mM sucrose, 2 µM FeCl$_3$, pH 6.5) (Penaloza-Vazquez et al., 2000). However, *hrpL* expression levels are much lower in HSS than in HDM (Sreedharan et al., 2006). Several factors could be responsible for reduced T3SS gene expression in HSS, including the higher pH and/or the different carbon source.
The *P. syringae* T3SS is activated at a slightly acidic pH similar to that found in plants

Minimal media used to culture *P. syringae* for studies on the T3SS typically has a pH below 6.5. In fact, *P. syringae* T3SS genes are repressed at pHs higher than 6.5 in *Psp* NPS3121 (Rahme et al., 1992). The mechanism by which pH regulates T3SS genes in *Psp* NPS3121 is unclear since the effect of pH on the *hrpL* or *hrpRS* operon was not examined. In contrast, another study found that AvrPto, HopA1, and HrpZ protein levels were roughly equivalent at pH 6.0 and 7.0 in *Pst* DC3000 or *Pss* 61, suggesting that pH has little or no effect on transcriptional regulation of T3SS genes (van Dijk et al., 1999). However, both AvrPto and HopA1 were secreted into culture by *Pst* DC3000 and *Pss* 61, respectively, at pH 6.0 but not at pH 7.0 (van Dijk et al., 1999). More recently, AvrPto was found to have a pH-sensitive folding switch, a mechanism allowing the protein to be translocated prior to folding (Dawson et al., 2009). This mechanism supports a model in which pH plays a role in effector secretion without a strong regulatory effect at the transcriptional level.

*P. syringae* T3SS genes are differentially affected by various carbon sources

Different *P. syringae* pathovars infect distinct host plants, and the nutrients available within plants species may vary. Therefore, particular carbon sources available in the plant apoplast may provide signals for different pathovars to regulate the T3SS. *Pst* DC3000 can utilize many carbon sources within the tomato plant apoplast, including organic acids, such as citrate and succinate, as well as sugars, such as fructose and glucose (Kamilova et al., 2006, Rico & Preston, 2008). Also, *Pst* DC3000 regulates metabolism of specific carbon sources depending on environmental conditions, and in
some cases, in concert with T3SS genes. For instance, a fructose specific phosphotransferase system is induced in HDM and down-regulated in KB (Lan et al., 2006). In Pst DC3000, fructose and citric acid metabolism are both inducible by plant apoplast extracts, while glucose and succinic acid are utilized equally in various media and apoplast extracts (Rico & Preston, 2008).

Previous studies suggest that P. syringae T3SS genes are differentially induced in culture by various carbon sources. However, the effects of specific carbon sources on T3SS gene expression varies depending on the pathovar studied. T3SS genes in pathovar glycinea (hereafter PsG) race 0 were induced in HDM containing fructose, sucrose, or mannitol, but repressed when the carbon source was succinate or citrate (Huynh et al., 1989). The authors concluded that T3SS gene expression is inversely related with the growth supported by each carbon source and that preferred substrates, such as organic acids and other TCA cycle intermediates, repress T3SS genes, while carbohydrates that enter glycolysis prior to pyruvate induce T3SS genes (Huynh et al., 1989). On the other hand, T3SS genes in Psp NPS3121 were induced by fructose and citrate, but not by mannitol or succinate (Rahme et al., 1992, Xiao, 2005). In PsS 61, succinate induced T3SS gene expression equally as well as fructose, glucose, or glycerol (Xiao et al., 1992). Despite the conflicting results, much of the literature seems to favor the idea that sugars activate T3SS genes and organic acids repress T3SS genes (Tang et al., 2006, Huynh et al., 1989, Rahme et al., 1992).

Interestingly, HDM with either mannitol, fructose, or no carbon source induced similar levels of T3SS gene expression after 12 hours in both Pst DC3000 and P.
syringae pv. maculicola (hereafter \( P_{sm} \)) ES426 (Xiao, 2005). In \( P_{sm} \) ES426, T3SS genes continued to be induced over a longer period of time (up to 24 hrs) regardless of carbon source availability. However, at later time points, \( P_{st} \) DC3000 T3SS gene expression decreased in cultures with no carbon source (Xiao, 2005). In addition, fructose levels between 100 \( \mu \)M and 10 mM induced \( P_{sp} \) NPS3121 T3SS genes in a dosage-dependent manner, but the same genes were repressed in 500 mM fructose, possibly due to changes in osmolarity (Xiao, 2005). Together, these data suggest that carbon sources may not be required for activation of T3SS genes, but may still modulate their expression.
Small molecules could regulate *P. syringae* T3SS genes

Small diffusible molecules are involved in regulation of the T3SS in many plant-pathogenic bacteria. Autoinducers such as acyl homoserine lactones (AHLs), which are discussed in detail below, are involved in regulating some T3SSs via quorum sensing. However some bacteria use other autoinducers in a similar fashion. *Ralstonia solanacearum* utilizes an autoinducer 3-OH-plamitic acid methyl ester (3-OH-PAME) to down-regulate early stage virulence factors, such as T3SS genes, and up-regulate late stage virulence factors, such as cellulase (Mole et al., 2007). *Xanthomonas campestris* produces an autoinducer, cis-11-methyl-2-dodecenoic acid, called diffusible signal factor (DSF), which has also been implicated in down-regulation of T3SS genes (He et al., 2006b). Autoinducers have not been explored much in regulation of T3SS in *P. syringae*. However, there is some evidence in the literature that small signaling molecules produced by *P. syringae* can affect virulence.

**AHL-mediated quorum sensing may be involved in regulation of the T3SS**

As mentioned previously, AHLs are quorum sensing molecules that are produced by *P. syringae* to monitor population density on leaves (Dulla, 2005). AHl molecules contain a fatty acyl chain linked by an amide bond to a homoserine lactone ring. AHL was first described as the signal responsible for density-dependent regulation of luminescence (*lux*) genes in *Vibrio fischeri* (Fuqua & Greenberg, 2002). The Lux regulatory system consists of an AHL-synthase (LuxI), which produces AHLs, and a response regulator (LuxR), which detects AHLs and regulates density dependent genes (Ng & Bassler, 2009). Lux I and LuxR homologs have subsequently been found in many Gram-negative Proteobacteria, including plant and animal pathogens (Case et al., 2008).
AHL-mediated quorum sensing regulates expression of T3SS genes in *P. aeruginosa* (Hogardt et al., 2004, Bleves et al., 2005). Whether AHLs affect T3SS genes in *P. syringae* is unknown. Several different studies support the idea that AHLs regulate the virulence of *P. syringae*. First, transgenic tobacco plants producing 3-oxo-C6 AHL and C6 AHL exhibit decreased disease symptoms when infected with *P. syringae* pv. tabaci bacteria (Quinones et al., 2005). Second, deleting the *ahll* AHL synthase gene (a homolog of *luxI*) in *P. syringae* pv. *tabaci* B728a, increases virulence of the bacteria in beans (Quinones et al., 2005). Finally, mutation of *psrA* (*Pseudomonas* sigma regulator) in *Pst* DC3000 results in 8-fold higher AHL levels and reduced virulence *in planta* (Chatterjee et al., 2007). However, PsrA also positively regulates T3SS genes in *Pst* DC3000, possibly by its negative effect on RNA-binding proteins encoded by *rsmA1* (Chatterjee et al., 2007).

AHL molecules produced by different bacteria can vary: the number of carbons range from 4 to 14, and the third carbon can be bound to either hydrogen (H), oxygen (O), or hydroxyl group (OH) (Loh et al., 2002, Dumenyo, 1998, Elasri et al., 2001). In *V. fischeri*, LuxI synthesizes both N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6 AHL) and N-hexanoyl-homoserine lactone (C6 AHL) (Ng & Bassler, 2009, Kuo et al., 1994). *P. aeruginosa* has two Lux family quorum sensing circuits, LasRI and RhlR/I, which produce N-3-oxododecanoyl-homoserine lactone (3-oxo-C12 AHL) and N-butyryl-homoserine lactone (C4 AHL), respectively (Waters & Bassler, 2005). The *P. aeruginosa* T3SS is down-regulated by the Rhl quorum sensing system (Hogardt et al., 2004, Bleves et al., 2005).
The predominant AHL synthesized by *P. syringae* is 3-oxo-C6 AHL, although some pathovars may also produce C6 AHL and/or N-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8 AHL) (Marutani *et al.*, 2008, Elasri *et al.*, 2001, Cha *et al.*, 1998, Shaw *et al.*, 1997, Quiñones *et al.*, 2004, Taguchi *et al.*, 2006). *Pst* DC3000 contains a single LuxI-like protein, PsyI (PSPTO3864), which together with the LuxR homolog, PsyR (PSPTO3863), forms a putative AHL quorum sensing system (Chatterjee *et al.*, 2007). Case, Labbate, and Kjelleberg (2008) report a second LuxR homolog in *Pst* DC3000. Multiple LuxR proteins are not unusual in bacteria, and may be involved in sensing AHLs from other bacteria (Case *et al.*, 2008). It is also possible that *Pss* B728a and *Pst* DC3000 produce other AHL derivatives, since these bacteria contain homologs of another putative AHL synthase, HdtS (PSYR0009 and PSPTO0187, respectively) (Feil *et al.*, 2005). HdtS is not related to LuxI, but purportedly produces C6 AHLs as well as the longer chain AHLs, N-(3-hydroxy-7-cis-tetradecenoyl) homoserine lactone (3-hydroxy -C14 AHL) and N-decanoyl-homoserine lactone (C10 AHL) (Laue *et al.*, 2000). However, thin layer chromatography analysis of *Pst* B728a and several strains of *Pst* (although not DC3000) detected primarily 3-oxo-C6 AHL and only trace amounts of C6 AHL or 3-oxo-C8 AHL (Elasri *et al.*, 2001, Quiñones *et al.*, 2004, Cha *et al.*, 1998). Therefore, 3-oxo-C6 AHL is likely the main AHL signal produced by *Pst* DC3000. Further studies will be required to determine whether HdtS is active and produces physiologically relevant levels of AHL.

**Auxin may be involved in regulation of T3SS genes**

Auxins are an essential group of plant hormones that control plant growth and development (Vanneste & Friml, 2009). The most important and abundant plant auxin,
indole-3-acetic acid (IAA), is also produced endogenously by diverse plant-associated bacteria, including *P. syringae* (Glickmann *et al.*, 1998, Spaepen *et al.*, 2007). Most studies to date have focused on the effects of bacterially-produced IAA on plant physiology. However, recent research suggests that IAA may also function as a regulatory molecule in bacteria (Charkowski, 2009, Spaepen *et al.*, 2007). For example, in *Agrobacterium tumefaciens*, IAA represses expression of *vir* genes, which are required for transfer of T-DNA into plant cells (Liu & Nester, 2006). IAA may inhibit *vir* gene expression by interacting with the two-component sensor kinase, VirA (Liu & Nester, 2006).

Bacteria produce IAA via several tryptophan-dependent pathways, including the indole-3-acetamide (IAM) and indole-3-pyruvate (IPyA), indole-3-acetonitrile (IAN), and tryptamine (TAM) pathways (Spaepen *et al.*, 2007). There may also be tryptophan-independent pathways, however they are less well characterized (Spaepen *et al.*, 2007). The IAM pathway requires *iaaM*, which encodes tryptophan-2-monoxygenase, and *iaaH*, which encodes indole-3-acetamide hydrolase, and both genes have been found in many plant pathogenic bacteria (Mazzola & White, 1994, Glickmann *et al.*, 1998, Buell *et al.*, 2003, Spaepen *et al.*, 2007, Joardar *et al.*, 2005).

Several lines of evidence suggest that IAA regulates T3SS gene expression in various bacteria. For example, a mutation in *iaaM* in *Erwinia chrysanthemi* reduces expression of T3SS genes (Yang *et al.*, 2007). In contrast, an *iaaH* mutant of *Pantoea agglomerans* pv. gypsophilae (*D. Dadantii*) has higher T3SS gene expression (Chalupowicz *et al.*, 2009). In addition, a signal that inhibits T3SS gene expression in *P.*
aeruginosa was identified in the culture medium of mutants that did not produce AHLs (Shen et al., 2008). This novel quorum sensing molecule was dependent on trpA (a tryptophan biosynthesis gene) for its synthesis, and addition of IAA to P. aeruginosa cultures had a similar inhibitory effect on T3SS genes (Shen et al., 2008). Whether IAA (or an IAA-derivative) can function as a quorum sensing molecule in P. aeruginosa remains to be determined.

Many pathovars of P. syringae produce IAA (Glickmann et al., 1998). The three sequenced and annotated strains of P. syringae (Pss B728a, Psp 1448a, and Pst DC3000) all contain homologs of iaaM and iaaH (Joardar et al., 2005, Buell et al., 2003). In addition, Pss B728a encodes a nitrilase enzyme that can hydrolyze indole-3-acetonitrile and produce IAA via the IAN pathway (Feil et al., 2005, Howden et al., 2009). Although Pst DC3000 has a homologous nitrilase gene, it does not appear to be active (Howden et al., 2009). Pst DC3000 also contains iaaL, which encodes an IAA-lysine ligase that inactivates IAA (Glass & Kosuge, 1988, Romano et al., 1991).

The idea that bacterially-produced IAA could regulate P. syringae genes expression has not been explored to date. However, Pss Y30 mutants that are deficient in IAA biosynthesis produce disease symptoms in bean plants quicker and at lower inoculum levels than the wild-type parental strain (Mazzola & White, 1994). Furthermore, disruption of iaaL in P. savastanoi (previously P. syringae pv. savastanoi) increases IAA accumulation in bacterial culture and decreases virulence in oleander plants (Glass & Kosuge, 1988). Taken together, these results suggest that IAA or IAA-lysine could regulate virulence in plant pathogenic Pseudomonads. Intriguingly, iaaL in
*Pst* DC3000 is regulated by *hrpL* (Buell et al., 2003). Therefore, inactivation of auxin could play a role in virulence or T3SS regulation in *Pst* DC3000.
Thesis Overview

The T3SS is essential for *P. syringae* to inactivate host defenses and successfully colonize plants. The *hrpRS-hrpL* regulatory cascade is central to control of T3SS genes, and is turned on quickly under very specific conditions. Previous studies have defined several environmental factors that modulate T3SS gene expression, including growth media components, such as carbon sources, and pH, however few have examined how these variables affect *hrpRS* or *hrpL*. In addition, studies in different pathovars of *P. syringae* do not always reach the same conclusions as to how each specific condition regulates T3SS genes. The goal of my thesis research is to identify environmental variables that modulate expression of T3SS genes in the sequenced strain *Pst* DC3000 and to determine whether conditions that regulate *hrpRS* or *hrpL* act at the transcriptional or post-transcriptional level. To this aim, I created *T3SS::gusA* transcriptional reporter strains by fusing a promoterless *gusA* reporter to three T3SS genes: *hrpRS*, *hrpL*, and *avrPto*. These reporters can be used for *in vitro* and *in planta* studies, as neither *P. syringae* nor plants have naturally occurring beta-glucuronidase (GUS). I analyzed GUS specific activity of each *T3SS::gusA* reporter strain (*hrpRS::gusA, hrpL::gusA, & avrPto::gusA*) under variable culture conditions reported to activate or repress T3SS gene expression. I verified that repression of *Pst* DC3000 T3SS genes in KB acts upstream of the *hrpRS* operon, and demonstrate that this repression can be relieved by overexpression of either *hrpR* or *hrpS*. In addition, I show that pH has a small but measurable effect on *Pst* DC3000 T3SS genes. Results of several different assays suggest that quorum sensing may be involved in regulation of the T3SS in *Pst* DC3000. First, T3SS genes are optimally expressed in the presence of carbon sources that promote
slower growth rates, and when bacteria are at low cell densities. In addition, I show that a T3SS repressive signal is released into Pst DC3000 cultures and accumulates at high cell densities. However, density-dependent repression of T3SS genes is independent of psyRI, which mediates quorum sensing by acyl homoserine lactones (AHLs). T3SS gene expression was unaffected by exogenous application of 3-oxo-C6 or C6 AHLs. In contrast, another small molecule produced by Pst DC3000, IAA, had a strong inhibitory effect on T3SS gene expression when added to the growth medium.
Chapter II. Materials and Methods
**Materials and Methods**

**Bacterial strains, plasmids, and culture conditions**

Bacterial strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. *Escherichia coli* was maintained in Luria-Bertani (LB) or LM supplemented with 8.6 mM phosphate buffer (Hanahan, 1983, Sambrook *et al.*, 1989). *P. syringae* strains were maintained in King’s Broth (KB) or LM (King *et al.*, 1954). For GUS assays, *P. syringae* strains were cultured in *hrp*-depressing medium (HDM) at pH6 with 10 mM fructose, except where otherwise noted (Huynh *et al.*, 1989) and in HSS (Sreedharan *et al.*, 2006). Antibiotics and other additives were used at the following concentrations: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 20 μg/ml; kanamycin (Km), 50 μg/ml; rifampicin (Rf), 50 μg/ml; tetracycline (Tc), 10 μg/ml; bromo-chloro-indolyl-galactopyranoside (X-gal), 20 μg/ml; and 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc), 20 μg/ml. Synthetic AHLs, N-(3-Oxohexanoyl)-L-homoserine lactone (3-oxo-C6 AHL) and N-hexanoyl-L-homoserine lactone (C6 AHL) (Cayman Chemicals), were dissolved in DMSO to make 140 mM stock solutions, and 60 mM aqueous dilutions were made fresh before each assay. Final concentrations (10 μM) of AHLs were obtained by adding aqueous dilutions of AHLs to KB for the cross-streak assay or HDM for the GUS assay. Optical density (OD$_{600}$) of bacterial suspensions was determined using a Spectronic 20+ spectrophotometer (Thermo Electron Corporation).
DNA manipulations

PCR for cloning was performed with Vent DNA polymerase (New England Biolabs), while diagnostic PCR was performed with Taq polymerase purified from *E. coli*. All primers were obtained from Integrated DNA Technologies. Plasmids were isolated using standard methods (Sambrook et al., 1989) or Wizard Plus Miniprep DNA purification kits (Promega). Ligations were performed using T4 DNA Ligase (TaKaRa). All restriction digestions were performed using enzymes from New England Biolabs. DNA sequencing was performed at the University of Missouri – Columbia DNA Core Facility using the 3730 DNA Analyzer (Applied Biosystems).

Construction of T3SS::gusA transcriptional fusions

To create the *Pst* DC3000 T3SS::gusA fusions, approximately 1 kb upstream and downstream of the *hrpRS, hrpL, and avrPto* translation stop sites were PCR-amplified using the appropriate primer pairs listed in Table 3 (Figure 6). PCR products were then digested with the relevant restriction enzymes and products were ligated into pUC18, which was then transformed into *E. coli* DH5αmcr. The resulting plasmids, containing the *hrpRS, hrpL, or avrPto* region, were sequenced to ensure that no mutations were introduced into the T3SS genes. A promoterless β-glucuronidase gene (*gusA*) was then inserted after the translation stop codon of *hrpS, hrpL, or avrPto* using the engineered XhoI restriction site to create the plasmids, pJS8, pJS7, and pJS9, respectively. All three T3SS::gusA fusions were then subcloned into the broad host range suicide vector, pK18mobsac (Schafer et al., 1994). The resulting plasmids, pJS3, pJS1, and pJS6 were electroporated into *Pst* DC3000 and allele replacement was performed as described in Schäfer et al (1994) and diagramed in Figure 21 (Schafer et al., 1994). Briefly, *P.*
syringae transformants were plated on LM + kanamycin to select for those colonies in which the suicide vector has integrated via a single homologous recombination event. Several colonies were tested for sucrose sensitivity on LM + kanamycin +5% sucrose, and then one or more of the most sensitive colonies were plated on KB + 10% sucrose with X-gluc to force a second recombination event. The resulting colonies were screened for kanamycin sensitivity and rifampicin resistance on LM and blue color on HDM + X-gluc, confirming excision of the plasmid and chromosomal insertion of the T3SS::gusA gene fusion. The T3SS::gusA reporter strains were then confirmed by colony PCR using primers to gusA and Pst DC3000 chromosomal sequences (Table 3). Three independently constructed biological replicates were maintained for each T3SS::gusA strain.
Figure 6. Creation of T3SS::gusA reporter strains.

(I.) Creation of pJS3, a suicide vector containing the hrpRS gene region with insertion of gusA at the end of the hrpRS operon. This vector allows positive selection (Km$^R$) and negative selection (SacB, which confers sucrose sensitivity). (II. And III.) Homologous recombination of pJS3 into WT Pst DC3000 replaced the endogenous allele and resulted in (IV.) the hrpRS::gusA reporter strain. Similar strategies were used to create both the hrpL::gusA and avrPto::gusA reporter strains.
Construction of *psyRI* mutants

Regions flanking *psyRI* were PCR amplified with the following primer pairs: p166-p167 (flanking region #1) and p168-p172 (flanking region #2). PCR products were approximately 1 kb each and were engineered to contain the restriction sites *Xba*I and *Bam*HI (flanking region #1) or *Bam*HI and *Eco*RI (flanking region #2). These fragments were digested and cloned into the vector, pUC18. A gel-purified chloramphenicol resistance cassette from pH45ΩCm (Fellay *et al.*, 1987) was then ligated into the *Bam*HI site. The resulting plasmid, pJS12, was confirmed by restriction digest analysis and the insert (*psyRI* flanking regions with chloramphenicol resistance cassette) was ligated into pK18mobsac (Schafer *et al.*, 1994) to create pJS16. This plasmid was then electroporated into *Pst* DC3000 and derivative T3SS::gusA reporter strains. Chromosomal integration of pJS16 was selected on LM + kanamycin + chloramphenicol, and replacement of *psyRI* was achieved by streaking individual colonies onto LM + chloramphenicol + 10% sucrose. Sucrose-resistant colonies were patched onto LM + chloramphenicol and onto LM + chloramphenicol + kanamycin to confirm excision of pK18mobsac, leaving only the chloramphenicol resistance cassette in place of *psyRI*. All *psyRI* deletion strains were confirmed by colony PCR using primers that hybridized to the chloramphenicol cassette and chromosomal sequences flanking *psyRI*. Two independently constructed biological replicates were maintained for each Δ*psyRI* deletion strain.

Overexpression of *hrpR* or *hrpS*

For overexpression, I cloned *hrpR* or *hrpS* under control of the *lac* promoter (*P_{lac})*. Briefly, *hrpR* and *hrpS* were subcloned from pS308 and pS284 into the broad host
range vector, pCPP46, resulting in the plasmids, pJS14 (P_{lac}::hrpR) and pJS15 (P_{lac}::hrpS), respectively. pJS14, pJS15, and the vector control, pCPP46, were then electroporated into Pst DC3000 WT and the hrpL::gusA and avrPto::gusA reporter strains. Plasmids were selected for and maintained using tetracycline.

**Hypersensitive Response (HR) Assay**

Pst DC3000 strains were harvested from plates and suspended in 5 ml MES [2-(N-morpholino)ethanesulfonic acid] buffer to an optical density at 600 nm (OD$_{600}$) of 0.3 (~1x10$^8$ CFU/ml) and then serially diluted to create suspensions of approximately 2x10$^7$, 4x10$^6$, and 8x10$^5$ CFU/ml. These dilutions were then infiltrated into 4-6 week old Nicotiana tabacum cultivar Xanthi leaves by pricking the leaves with a sterile needle and inoculating bacteria suspensions using a blunt syringe. The area of infiltration was circled. Leaves were observed and photographed approximately 48 hrs later.

**GUS Reporter Assays**

Bacterial strains were inoculated from plates into a liquid KB preculture and shaken at 30 °C shaker for 1-2 days. Bacterial cells were then harvested from the preculture by centrifugation, washed 3-4 times in 10 mM MgCl$_2$ buffer, and inoculated into 5-6 ml of fresh media at an OD$_{600}$ of approximately 0.05 unless otherwise noted. Specific media and conditions for individual assays are provided in the figure legends. All assay cultures were incubated at room temperature (~25°C) with shaking. At specified time intervals, noted as hours post inoculation (hpi), optical densities of each culture were determined and 50-100 µl samples were collected in 96 well plates and frozen at -80 °C until assayed for β-glucuronidase (GUS) specific activity. To dilute
samples and lyse bacteria, GUS extraction buffer (50mM NaHPO$_4$ pH 7.0, 10mM Na$_2$EDTA, 0.1% sodium laryl sarcosine, 0.1% Triton-X 100, 10 mM β-mercaptoethanol) was added to frozen samples in the 96 well plates. Cells were lysed as samples thawed to limit changes in gene expression. Sample/extraction buffer dilution ratios varied by assay (depending upon final optical density of cultures) and were used to calculate culture volume (ml) for GUS specific activity. Diluted samples (8-10 µl) were transferred to opaque 96-well plates and brought up to 37°C in a water bath. 50 µl of GUS reaction buffer [extraction buffer plus 1 mg/ml 4-methylumbelliferyl β-D-glucuronide (MUG) (Gold Biotechnology, Inc.)] preheated to 37°C was then added to each sample well. Reactions were incubated at 37°C for 10-15 minutes, and terminated by adding 200 µl of 0.2 M Na$_2$CO$_3$. Preliminary studies tested several incubation times (5-20 minutes) to verify that the assay results were linear over time. Results within the linear range were consistently achieved within 10-15 minutes of incubation. The fluorescent product, 4-methylumbelliferone (MU), was quantified in a fluorometer (VICTOR$^2$ Perkin-Elmer) and fluorescent units (counts) were compared to an MU (Sigma-Aldrich) standard curve to determine pmol MU produced. Only samples within the linear range of the MU standard curve (<10,000,000 counts) were used in the final analysis. GUS specific activity was then calculated as follows: (pmol MU)/(ml culture)(min assay)(OD$_{600}$).

**Cross-streak Assay to Screen for AHL Production**

*Pst* DC3000 strains to be tested for AHL production were cultured in KB without antibiotics with shaking at 30°C for two days. *E.coli* JLD271 harboring biosensor or control plasmids (Table 2) were cultured overnight with shaking in LB + tetracycline at
37°C. JLD271 does not produce AHLs and has a deletion in the native LuxR homolog, sdiA, to reduce interference with AHL detection.

Each *Pst* DC3000 strain was cross-streaked with each biosensor pair as described by Ahmer et al. (2007). Briefly, 20 µl of the culture to be tested was rolled horizontally onto KB agar plates without antibiotics, followed by two separate cross-streaks with 10 µl each of the *E. coli* biosensor or control strain (Ahmer *et al.*, 2007). DMSO was used as a negative controls, and 3-oxo-C6 AHL (10 µM) was used as a positive control for strains with pAL103 and pAL104. Plates were incubated for 24 hours at room temperature (~25°C) and photographed in the dark with a CCD camera (Canon Powershot A610) set to ISO 400 and a 15 second exposure time.
Table 1. List of bacterial strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Abbreviation</th>
<th>Antibiotic Resistance</th>
<th>Reference</th>
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<tr>
<td><em>E. coli</em></td>
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<td>DH5α mer</td>
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<td>Stratagene (Lindsay &amp; Ahmer, 2005)</td>
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<td>JLD271 ΔsdiA</td>
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<td><em>P. syringae pv. tomato</em></td>
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<td>DC3000 Wild-type</td>
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<td>Rf</td>
<td>(Cuppels, 1986)</td>
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<td><em>Pst</em> DC3000 ΔT3SS</td>
<td>ΔT3SS</td>
<td>Cm</td>
<td>(Fouts <em>et al.</em>, 2003)</td>
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<td>SCH776 <em>Pst</em> DC3000 <em>hrpRS::GUSA Δ</em>psyRI* (1)</td>
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<td>RSA1</td>
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<td>Cm</td>
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### Table 2. List of plasmids

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<tr>
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<th>Reference</th>
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<tr>
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<tr>
<td>pK18mobsac</td>
<td>mob, Suc&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Km</td>
<td>(Schafer et al., 1994)</td>
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<td>chloramphenicol resistance cassette</td>
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<td>(Fellay et al., 1987)</td>
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<td>pCPP46</td>
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<td>pS308</td>
<td>hrpS with S-tag and his-tag at 5' end</td>
<td>Km</td>
<td>Gift of Thota and Schechter</td>
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<td>rhlII':luxCDABE, rhlR control for pAL101</td>
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<td>(Lindsay &amp; Ahmer, 2005)</td>
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<td>avrPto::gusA</td>
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<td>pJS14</td>
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Table 3. List of primers.

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<td>P10</td>
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<td>Cloning gusA</td>
</tr>
<tr>
<td>P13</td>
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</tr>
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<td>P17</td>
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<td>P176</td>
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* Engineered restriction digest sites are underlined
Chapter III: Results
Construction of \textit{Pst DC3000 T3SS::gusA} reporter strains

In order to monitor gene expression at multiple levels of the T3SS regulatory cascade, I inserted a promoterless \textit{gusA} gene downstream of the translation stop site of three T3SS genes, without interrupting upstream or downstream genes, resulting in the \textit{gusA} transcriptional fusion (\textit{T3SS::gusA}) strains SCH788-796 listed in Table 1 [hereafter referred to as \textit{hrpRS::gusA} (RS1-3), \textit{hrpL::gusA} (L1-3), and \textit{avrPto::gusA} (A1-3)]. All of the \textit{T3SS::gusA} reporter strains formed blue colonies when plated on HDM with 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc) and were confirmed by PCR using primers that hybridize to each T3SS gene region and \textit{gusA} (Figure 7). Primers for confirmation of the \textit{T3SS::gusA} strains were either upstream or downstream of primers used to construct the plasmids for allele replacement, to ensure that amplification was not from plasmids used for allele replacement. When chromosomal DNA from the \textit{hrpRS::gusA} strains was used as template, PCR products of the expected size were amplified (~1.9 kb product from p25 - p126, and a ~1.1 kb product from p126 - p128). Similarly, PCR of chromosomal DNA from \textit{hrpL::gusA} and \textit{avrPto::gusA} strains specifically amplified products of the expected sizes (~1.2 kb product from p123 - p126, a ~1.1 kb product from p127 - p129, ~1.1 kb products from p130 and p126, and 127 and 131). These products were not amplified when the WT \textit{Pst DC3000} chromosomal DNA was used as the template, although non-specific bands were seen in reaction products from primer pairs p127 - p129 and p123 - p126.
Figure 7. PCR Confirmation of \textit{T3SS::gusA} reporter strains.

Diagrams of the \textit{Pst} DC3000 (A) \textit{hrpRS::gusA}, (B) \textit{hrpL::gusA}, and (C) \textit{avrPto::gusA} chromosomal regions are shown, including the positions of PCR primers. The expected sizes of PCR products for all three \textit{T3SS::gusA} reporters are shown below each diagram. (D) Electrophoresis of PCR products confirming \textit{hrpRS} (lanes 2-5), \textit{hrpL} (lanes 7-10), and \textit{avrPto} (lanes 12-15) gene regions. Lanes 1, 6, and 11 are DNA markers. PCR reaction contained chromosomal DNA from either wild-type (W), \textit{hrpRS::gusA} (R), \textit{hrpL::gusA} (L), or \textit{avrPto::gusA} (A) \textit{PstDC3000} strains. The primers used in each reaction are: lanes 2-3, p25-p126; lanes 4-5, p127-p128; lanes 7-8, p123-p126; lanes 9-10, p127-p129; lanes 12-13, p130-p126; lanes 14-15, p127-p131.
**gusA fusion does not disrupt the function of hrpRS and hrpL**

Previous studies have shown that disrupting the function of the T3SS abolishes the ability of *P. syringae* to cause the HR in resistant plants (Deng *et al.*, 1998, Huang *et al.*, 1991, Lindgren *et al.*, 1986). Therefore, disruption of *hrpRS* and *hrpL* should also abolish the ability of *Pst* DC3000 to cause the HR in resistant plants. To confirm that insertion of *gusA* did not disrupt *hrpRS* and *hrpL* function, I inoculated *N. tabacum* cultivar Xanthi with the *hrpRS::gusA*, *hrpL::gusA*, and *avrPto::gusA*, as well as wild-type (WT) *Pst* DC3000 and a mutant lacking the entire T3SS gene cluster (ΔT3SS) at various concentrations of inoculum (1x10^8, 2x10^7, and 4x10^6 CFU/ml) (Fouts *et al.*, 2003).

As indicated by visible necrosis within the area of infiltration, *Pst* DC3000 WT bacteria caused the HR at concentrations of 2x10^7 CFU/ml or above, while the ΔT3SS mutant did not cause the HR at any concentration (Figure 8). The three reporter strains, *hrpRS::gusA*, *hrpL::gusA*, and *avrPto::gusA*, all caused the HR at the same concentrations as WT, confirming that the *hrpRS* and *hrpL* function normally in the *gusA* reporter strains. *Pst* DC3000 lacking *avrPto* still causes the HR in *N. tabacum* cultivar Xanthi, suggesting that AvrPto is not the sole determinant of ETI in the plant, nor does it impact function of the T3SS (Lin & Martin, 2005). Therefore, the experiment in Figure 8 does not discern whether the *gusA* insertion downstream from *avrPto* alters the function of the *avrPto* gene. However, since my research is concerned with the regulatory cascade upstream of *avrPto*, the *avrPto::gusA* strain is primarily used to confirm results observed in the *hrpRS::gusA* and *hrpL::gusA* reporter strains.
**Figure 8.** Induction of the HR by *Pst* DC3000 strains in *N. tabacum* cv. Xanthi.

WT *Pst* DC3000, ΔT3SS, and T3SS::gusA reporter strains were infiltrated into 4-6 week old leaves at the inoculum densities indicated to the left and the areas of infiltration were circled. After 24 hours, leaves were inspected for HR (indicated by darker, necrotic tissue within the area of infiltration) and photographed.
**T3SS::gusA reporters are differentially regulated in various growth media**

Previous studies have shown that T3SS genes are repressed when *P. syringae* is grown in rich medium, such as King’s B (KB) or Luria-Bertani (LB), and induced when *P. syringae* is grown in defined minimal media, such as *hrp*-derepressing minimal medium (HDM), Mb3M and M9-sucrose (Huynh et al., 1989, Rahme et al., 1992, Rico & Preston, 2008, Xiao et al., 1992). T3SS genes are induced to a lesser degree in another minimal medium, Hoitink-Sinden medium with sucrose (HSS) (Sreedharan et al., 2006). To confirm that my T3SS::gusA reporters are regulated in a manner consistent with previously published results, I assayed three independently constructed biological replicates of *hrpRS::gusA* (RS1, RS2, RS3), *hrpL::gusA* (L1, L2, L3), and *avrPto::gusA* (A1, A2, A3) for GUS specific activity after culturing the bacteria in KB, HSS, and HDM.

*T3SS::gusA* reporter strains were routinely grown overnight in KB liquid cultures prior to each assay. Because KB represses T3SS gene expression, bacterial cells harvested from these precultures were washed in 10 mM MgCl₂ buffer before resuspending in different assay media (Rico & Preston, 2008). If 10 mM MgCl₂ induces T3SS genes, it is not a suitable wash buffer for assaying T3SS gene expression in potentially repressive conditions. Thus, I tested whether the buffer altered T3SS gene expression by comparing GUS activity of *T3SS::gusA* reporter strains cultured in KB and in 10 mM MgCl₂. Reporter strains exhibited low basal levels of GUS activity over a 22 hour period in KB, confirming repression of T3SS gene expression. Comparable levels of GUS activity were observed when reporter strains were assayed in 10 mM MgCl₂, suggesting that GUS activity in 10 mM MgCl₂ reflects basal activity from KB pre-
cultures and T3SS genes are not induced in the wash buffer (Figure 9).
Figure 9. Analysis of GUS activity in *PstDC3000* after growth in various media or buffer.

WT and *T3SS::gusA* *PstDC3000* reporter strains were grown overnight in KB broth precultures and washed in 10mM MgCl₂. Cells were then inoculated into KB, HSS, HDM, or 10 mM MgCl₂ to OD₆₀₀ ~0.05. (A-C) Comparison of three independently constructed biological replicates of (A) *hrpRS::gusA*, (B) *hrpL::gusA*, and (C) *avrPto::gusA* *PstDC3000* reporter strains. Graphs show the average GUS specific activity (pmol MU)/(ml culture)/(min assay)/(OD₆₀₀) for two samples taken from each biological replicate, at 0.5, 4, 6, and 22 hours post inoculation (hpi), and error bars represent the standard deviations. Similar results were obtained in two independent assays. (D) Growth of *PstDC3000* WT and one biological replicate of each *T3SS::gusA* reporter strain in HDM, HSS, KB, and 10 mM MgCl₂.
As expected, T3SS::gusA reporter strains are highly induced in HDM and somewhat induced in HSS (Figure 9). Induction of hrpRS::gusA, hrpL::gusA, and avrPto::gusA expression was observed in HDM within 6 hours post inoculation (hpi) and continued to increase over the entire 22 hours. Expression of T3SS genes also increased in HSS, but GUS activity levels were much lower than in HDM. These results are in agreement with previous studies of T3SS gene expression. Therefore, the hrpRS::gusA, hrpL::gusA, and avrPto::gusA reporter strains can be used to accurately measure T3SS gene regulation under different environmental conditions.

My data also shows that KB repressed the T3SS regulatory cascade by acting upstream of the hrpRS operon. These results are in agreement with Xiao et al. (2007), who showed that the hrpRS operon is expressed more in HDM than in KB (Xiao et al., 2007). However, hrpL and avrPto expression were more induced by HDM than hrpRS. By 22 hpi, hrpRS::gusA expression was approximately 5-fold higher in HDM than in KB, while hrpL::gusA and avrPto::gusA were each more than 20-fold higher in HDM (Figure 9).

There are no published studies showing induction of hrpRS in HSS. My results show that the average expression level of hrpRS::gusA is slightly higher in HSS than KB, although the difference is less than a 2-fold difference (Figure 9). By 22 hpi, there was no significant difference between hrpRS expression in HSS and KB. Nevertheless, hrpL and avrPto expression were clearly induced in HSS at 6 hpi, with approximately 4-fold higher expression in HDM than in KB by 22 hpi. Together, these data suggest that HSS
only minimally induces *hrpRS*, but at levels that may be sufficient to induce downstream T3SS genes.

Growth rates of *PstDC3000* also varied significantly when cultured in KB, HDM, or HSS (Figure 9D). Within 22 hpi, a *Pst DC3000* culture in KB was saturated (OD$_{600}$ >1.4), while bacteria in HDM were still at the beginning of exponential phase (OD$_{600}$ ~ 0.2). HSS supported faster growth than HDM, although not as fast as KB. The *T3SS::gusA* reporter strains had comparable growth rates to WT *PstDC3000* in all media tested. None of the bacterial cultures grew in 10 mM MgCl$_2$. Several of the variable components of KB, HSS, and HDM, such as carbon sources and pH, have been previously suggested to modulate T3SS genes. While these variables may affect growth rates and T3SS genes independantly, it is also possible that the rate of growth regulates expression of T3SS genes.
Overexpression of \( hrpR \) or \( hrpS \) can overcome repression of T3SS genes in KB

My results suggest that KB represses expression of the T3SS regulatory cascade by reducing transcription of \( hrpRS \). Therefore, I hypothesized that expression of \( hrpR \) or \( hrpS \) from a heterologous promoter might overcome repression of T3SS genes by KB. To explore whether over-expression of either \( hrpR \) or \( hrpS \) would relieve repression of \( hrpL \) in KB, I transformed plasmids pJS14 (\( P_{lac}::hrpR \)) and pJS15 (\( P_{lac}::hrpS \)) into the \( hrpL::gusA \) and \( avrPto::gusA \) reporter strains. These plasmids ectopically express \( hrpR \) or \( hrpS \) from the \( lac \) promoter in the broad host range vector pCPP46.

Both \( hrpL::gusA \) and \( avrPto::gusA \) show significantly higher expression levels in KB when either \( hrpR \) or \( hrpS \) are constitutively expressed (Figure 10). These results confirm that repression of the T3SS in KB via transcriptional regulation of \( hrpRS \) can be overcome by increasing expression of either \( hrpR \) or \( hrpS \). Interestingly, \( hrpS \) overexpression induced higher levels of \( hrpL::gusA \) than \( hrpR \) overexpression, despite the fact that each gene should be similarly expressed from the \( lac \) promoter in pCPP46. It is possible that HrpS is more stable than HrpR, or that HrpS is a better activator of the \( hrpL \) promoter.
Figure 10. The effect of overexpressing hrpR or hrpS on hrpL and avrPto expression after culturing in T3SS-repressive conditions.

(A) hrpL::gusA and (B.) avrPto::gusA Pst DC3000 reporter strains containing no plasmid or either pJS14 (P_{lac}::hrpR), pJS15 (P_{lac}::hrpS), or pCPP46 (empty vector) were grown overnight in KB, and then washed and resuspended in 10mM MgCl₂ to standardize the bacteria to OD₆₀₀ ~0.05, and immediately sampled for GUS activity. GUS specific activity is shown as the average of two biological replicates for each strain with error bars representing the standard deviations.
Pst DC3000 T3SS genes are transcriptionally regulated by pH

The effect of pH on P. syringae T3SS gene expression has not been examined in detail. The plant apoplast has a pH below 6.5 (Felle, 2001). Rahme et al (1992) found that expression of several T3SS genes significantly decreased in minimal media as the pH was raised above 5.5 (Rahme et al., 1992). However, this study did not examine the effect of pH on the hrpRS operon. On the other hand, van Dijk et al (1999) found that pH does not alter AvrPto protein levels, suggesting that pH does not significantly affect regulatory elements upstream of avrPto (van Dijk et al., 1999). To determine if Pst DC3000 T3SS gene expression is regulated by pH, I assayed expression of hrpRS::gusA, hrpL::gusA, and avrPto::gusA in HDM with variable pHs, ranging from 5.4 to 7.6.

My results show that hrpRS, hrpL, and avrPto are regulated by pH and suggest that the regulatory effect is upstream of the hrpRS operon (Figure 11). Maximum expression of hrpRS::gusA, hrpL::gusA, and avrPto::gusA was seen at pH 5.9 and minimal expression at 7.6. However, there was only an ~2-fold difference in GUS activity between pH 5.9 and pH 7.6 in all three reporter strains. The pHs of KB, HSS, and HDM are normally 7.5, 6.5, and 6.0, respectively. Therefore, although pH is one factor that varies between these media, pH alone is not enough to account for the large differences in T3SS gene expression seen in KB, HSS, and HDM (Figure 9). The growth of Pst DC3000 in HDM at different pHs was similar, although by 21.5 hpi, the bacteria grew slightly faster at the higher pHs (>7) (Figure 11D).
Figure 11. The effect of pH on expression of *Pst DC3000* T3SS genes. *PstDC3000* (A) *hrpRS::gusA*, (B) *hrpL::gusA*, and (C) *avrPto::gusA* strains were suspended to an OD$_{600}$ of ~0.05 in 6 ml HDM with final pHs of 5.4, 5.9, 6.3, 6.8, 7.2, and 7.6. Each reporter strain preculture was washed in each of the 6 different pHs of HDM prior to inoculation into culture tubes containing the corresponding pH medium. Average GUS specific activity is shown for two samples per culture for each T3SS::gusA reporter strain at 21.5 hpi, with error bars showing standard deviations. Similar results were observed in two independent assays. (D) Growth curves of WT *PstDC3000* in HDM with various pHs.
**Carbon sources differentially affect Pst DC3000 T3SS genes**

Various carbon sources have been reported to differentially regulate T3SS genes in diverse *P. syringae* pathovars. Minimal media with fructose has been suggested to provide the optimal conditions for T3SS gene expression, although other sugars or sugar alcohols still induce T3SS genes (Huynh et al., 1989, Rahme et al., 1992, Xiao et al., 1992). HSS, which contains sucrose as the carbon source, induces *hrpL* expression in *Pst* DC3000, albeit at a lower level than in HDM (Figure 9) (Sreedharan et al., 2006). To determine if carbon sources differentially regulate the *Pst* DC3000 T3SS, expression of *hrpRS::gusA*, *hrpL::gusA*, and *avrPto::gusA* was assayed in HDM containing the sugars fructose, glucose, or sucrose, or the sugar alcohol mannitol.

All three *T3SS::gusA* reporters were induced by fructose, glucose, sucrose, and mannitol by 9 hpi (Figure 12). Induction of *hrpRS::gusA*, *hrpL::gusA*, and *avrPto::gusA* expression in the first few hours was similar regardless of which carbon source was used in the culture medium, suggesting that none of these carbon sources repress T3SS genes. However, by 26.5 hpi, expression of all three *T3SS::gusA* reporters leveled off in HDM containing sucrose, glucose, or mannitol. Only bacteria growing in HDM with fructose showed increasing T3SS gene expression levels over the entire assay. By 26.5 hpi, *hrpRS::gusA*, *hrpL::gusA*, and *avrPto::gusA* expression was much higher in HDM containing fructose than with any of the other carbon sources. Interestingly, expression patterns in HDM with sucrose were similar to those previously observed in HSS (Figures 9 and 12), suggesting that carbon source variation may be sufficient to explain differences in T3SS gene expression between HDM and HSS, despite other dissimilarities between the two media.
Figure 12. The effect of sugars or sugar alcohols on expression of *Pst* DC3000 T3SS genes. WT and *T3SS::gusA* reporter strain precultures were washed in 10mM MgCl$_2$ and inoculated to OD$_{600}$ ~0.5 into HDM containing 10mM glucose, 10mM sucrose, 10 mM mannitol, or 10 mM fructose as the carbon source. Expression patterns for (A) *hrpRS::gusA*, (B) *hrpL::gusA*, and (C) *avrPto::gusA* are shown as average GUS specific activity for two biological replicates of each reporter strain, with error bars representing the standard deviation. (D) Growth curves of *hrpRS::gusA* in the same media are shown as average optical density (OD$_{600}$) for two biological replicates, with error bars representing the standard deviation. Samples for both expression and growth were taken at 2, 5, 9, and 26.5 hours post inoculation (hpi). Similar GUS activities and growth rates were observed in two independent experiments.
Tricarboxylic acid (TCA) cycle intermediates, such as the organic acids citrate and succinate, have been shown to repress *P. syringae* T3SS genes (Huynh et al., 1989, Rahme et al., 1992). Glycerol enters glycolysis before the TCA cycle and does not inhibit T3SS genes (Xiao et al., 1992, Huynh et al., 1989, Xiao, 2005). Therefore, I hypothesized that expression of *Pst* DC3000 T3SS genes would be higher in HDM containing sugars or glycerol than in HDM containing organic acids, and I assayed expression of the *Pst* DC3000 *hrpRS::gusA*, *hrpL::gusA*, and *avrPto::gusA* reporters in HDM containing fructose, glycerol, citrate, or succinate.

By 8 hpi, the *hrpL::gusA* and *avrPto::gusA* fusions were similarly induced by fructose, glycerol, citrate, or succinate (Figure 13). Therefore, at early time points after inoculation into HDM, organic acids do not repress T3SS genes. In contrast, by 23.5 hpi, expression of T3SS genes in HDM containing succinate was approximately 4-5-fold lower compared to HDM with fructose. In addition, bacteria grown in HDM containing glycerol or citrate expressed intermediate levels of GUS.
Figure 13. Organic acids and glycerol do not repress Pst DC3000 T3SS gene expression. WT and T3SS::gusA reporter strain precultures were washed in 10mM MgCl2 and inoculated to OD_{600} ~0.5 into HDM containing 20 mM glycerol, 10 mM succinate, 10 mM citrate, or 10 mM fructose as the carbon source. The pH of HDM containing succinate and citrate was adjusted to be the same as the media containing fructose or glycerol (pH6). Expression patterns for (A) hrpRS::gusA (B) hrpL::gusA (C), and avrPto::GUS are shown as average GUS specific activity for two biological replicates of each reporter strain, with error bars representing the standard deviation. (D) Growth curves of hrpRS::gusA in the same media are shown as average optical density (OD_{600}) for two biological replicates, with error bars representing the standard deviation. Samples for both expression and growth were taken at 0.5, 2, 5.5, 8, 10, 20, and 23.5 hours post inoculation (hpi). Similar GUS activities and growth curves were observed in two independent experiments.
None of the previously published studies tested the effect of varying carbon sources on transcription of the hrpRS operon. hrpRS expression was induced by fructose, glycerol, citrate, and succinate, although it was only ~2-fold higher in HDM containing succinate than the basal expression in MgCl$_2$ wash buffer controls (data not shown).

Small differences (<2-fold) in expression of hrpRS between the different carbon source treatments were observed at earlier time points than in hrpL and avrPto. Still, by 24 hpi, hrpRS expression was approximately 3-fold lower in HDM when fructose was replaced with succinate, and an intermediate level (~2-fold lower) of hrpRS expression was observed when the carbon source was glycerol or citrate (Figure 13). Because expression of hrpRS in HDM containing fructose, glucose, sucrose, and mannitol, glycerol, citrate, or succinate fit the same pattern as observed in the downstream T3SS genes (Figures 12 and 13), I conclude that carbon source related conditions regulate T3SS upstream of the hrpRS operon. However, there may also be post-transcriptional regulation of hrpRS, as there is a larger effect seen at the level of hrpL and avrPto.

**Pst DC3000 T3SS gene expression patterns are correlated to growth rate**

Growth curves for hrpRS::gusA are shown in Figures 12D and 13D, and the growth rates were similar for wild-type Pst DC3000, hrpL::GUS, and avrPto::GUS in HDM with each carbon source tested (data not shown). However, various carbon sources differentially affected the growth rate of Pst DC3000 (Figures 12D and 13D). Furthermore, the growth rate supported by each carbon source was inversely related to the level of T3SS::gusA expression. For example, Pst DC3000 strains grew slowest in HDM containing fructose, the medium that induced the highest expression of T3SS::gusA
fusions. The cell density of *Pst* DC3000 cultures in HDM with fructose barely rose above OD\textsubscript{600} 0.2 by the end of the assay. In contrast, *Pst* DC3000 cultures grew fastest in HDM containing succinate; yet *T3SS::gusA* expression peaked within 6 hpi, and then decreased, as the OD\textsubscript{600} of the culture surpassed 0.2. In fact, *T3SS::gusA* expression leveled off or decreased in HDM with all the other carbon sources once the OD\textsubscript{600} rose above 0.2. Therefore, I hypothesized that growth rate or culture density, rather than the particular carbon sources, may be affecting T3SS gene expression.

**Pst DC3000 T3SS genes are regulated by cell density**

Many pathogens regulate virulence gene expression in response to population density. For instance, *P. aeruginosa* down-regulates T3SS genes in response to high cell density (Bleves et al., 2005, Hogardt et al., 2004). To test whether cell density affects T3SS gene expression in *Pst* DC3000, I assayed the GUS activity of *PstDC3000 T3SS::gusA* reporter strains after the bacteria were suspended in HDM at different cell densities.

A ~5 fold decrease in *hrpL* expression was observed in bacterial cultures that were inoculated at high cell densities (OD\textsubscript{600} ~0.5) compared to low cell densities (OD\textsubscript{600} ~ 0.02) (Figure14). The difference in expression of *hrpRS* in high and low cell density cultures was only ~2-fold (Figure 13), however other assays in this study have shown that very slight increases in *hrpRS* expression can be amplified into much larger increases in *hrpL* expression (Figures 9, 11, 12, and 13). Therefore, I conclude that cell density may affect *hrpRS* transcription at a subtle level, which could account for increased transcriptional regulation observed in downstream T3SS genes.
Figure 14. The effect of cell density on hrpRS and hrpL expression in PstDC3000. WT and T3SS::gusA reporter strain KB precultures were washed in HDM and inoculated to a low (~0.02), medium (~0.5), and high cell density (~1.5). Samples for both expression and cell density were taken at 5.5 hours post inoculation (hpi). Expression of hrpRS::gusA and hrpL::gusA at various cell densities is shown as average GUS specific activity for two samples from each T3SS::gusA reporter strain culture, with error bars representing standard deviations. Similar results were obtained in multiple independent experiments.
**Pst DC3000 T3SS genes are repressed by high cell density conditioned media**

Regulation of gene expression by cell density usually involves small diffusible molecules that accumulate extracellularly as bacteria grow and divide (Waters & Bassler, 2005). To explore whether *Pst* DC3000 releases a small molecule that causes repression of T3SS genes in high cell density cultures, I assayed expression of *T3SS::gusA* reporters in conditioned media. Conditioned media was made by inoculating *Pst* DC3000 into HDM at either a lower cell density (OD$_{600} = 0.04$) or a higher cell density (OD$_{600} = 0.6$), growing the bacteria for 16 hours, and removing the cells by filtration. These conditioned media were then inoculated with *T3SS::gusA* reporter strains. If small signaling molecules accumulating at high cell density are responsible for repressing T3SS gene expression, *hrpL::gusA* should be repressed when bacteria are inoculated at low cell density into the high cell density conditioned medium. In contrast, *hrpL::gusA* expression should be induced normally when bacteria are inoculated into the low cell density conditioned medium or into fresh HDM.

After a six hour incubation, the *hrpL::gusA* strain expressed comparable amounts of GUS in fresh or low density conditioned media. However, *hrpL::gusA* expression was approximately 3-fold lower in the high-cell-density conditioned media (Figure 15A). There was no observable effect of conditioned media on *hrpRS* expression.

To rule out the possibility that repression of T3SS gene expression by high cell density conditioned HDM could be due to depletion of nutrients, I examined the growth of *Pst* DC3000 in fresh or conditioned HDM supplemented with an additional 50 or 100% of the standard HDM nutrients. Analysis of growth showed that bacteria grew
similarly in fresh HDM and unsupplemented conditioned media prepared from either low (starting OD<sub>600</sub> = 0.04) or higher (starting OD<sub>600</sub> = 0.6) cell density cultures (Figure 14). In addition, supplementing fresh HDM or either conditioned medium (low or high-cell density) allowed the bacteria to grow faster in a similar manner (Figure 15B). Therefore, I concluded that neither conditioned medium was nutritionally depleted.

Supplementing the fresh and conditioned media also confirmed that changes in nutrient levels did not alter T3SS::gusA expression data, as I assayed the hrpRS::gusA and hrpL::gusA reporter strains in fresh or conditioned HDM, with or without supplementation. My results show that extra nutrients did not significantly alter T3SS gene induction (Figure 15A). Therefore, I conclude that the repression of hrpL expression observed at higher cell densities is due to a small molecule that is released by Pst DC3000 into the culture medium.
Figure 15. The effect of conditioned HDM on the expression of \textit{hrpL}.
Conditioned HDM was prepared by inoculating fresh HDM with WT \textit{Pst DC3000} at a lower density (OD$_{600} = 0.04$) or higher density (OD$_{600} = 0.6$), followed by shaking overnight (16 hrs) at 23 °C. Bacteria were removed by filtration and the conditioned media (supernatant) were tested for sterility by plating on KB. Supplemented conditioned media were made by adding either 50% (+1/2X) or 100% (+1X) of the standard concentrations of HDM nutrients (HDM salts, fructose, and MgCl$_2$). WT and \textit{T3SS::gusA} reporter strain precultures were washed in 10 mM MgCl$_2$ buffer and inoculated into fresh HDM or conditioned media (high or low density), with or without supplementation. (A) Expression of \textit{hrpL::gusA} (green) and \textit{hrpRS::gusA} (blue) was measured at 6 hpi. (B) Growth of \textit{hrpL::gusA} in the various media treatments over an extended period of time. GUS specific activity is shown as the average from 2 biological replicates for each reporter strain, with error bars representing the standard deviation. Similar results were observed in two independent assays.
Ps\textsubscript{t} DC3000 produces AHLs that activate LuxR

Results from the cell density and conditioned media assays prompted me to explore whether \textit{Ps}\textsubscript{t} DC3000 T3SS gene expression is regulated by AHL-mediated quorum sensing. Surveys of various pathovars suggest that the predominant AHL synthesized by \textit{P. syringae} is 3-oxo-C6 AHL, although some pathovars also produce C6 AHL and/or 3-oxo-C8 AHL (Marutani et al., 2008, Elasri et al., 2001, Cha et al., 1998, Shaw et al., 1997, Quiñones et al., 2004, Taguchi et al., 2006). To examine AHL production by \textit{Ps}\textsubscript{t} DC3000, I used three different biosensor strains of \textit{E. coli} designed to produce bioluminescence in response to various AHL species (Table 2). Each biosensor strain contains a plasmid encoding a different LuxR homolog (\textit{rhlR}, \textit{luxR}, or \textit{lasR}), which activates a \textit{luxCDABE} operon regulated by the promoter of the corresponding LuxI homolog (\textit{rhlI'}, \textit{lux I'}, or \textit{las I'}). \textit{E. coli}/pAL101 utilizes RhlRI' to detect C4 AHLs, while \textit{E. coli}/pAL103 contains LuxRI', and responds best to 3-oxo-C6 AHL, but can also detect C6, C8, and 3-oxo-C8 AHLs. \textit{E. coli}/pAL105 contains LasRI', and is designed to detect 3-oxo-C12 AHL, but also responds to C10, C12, and 3-oxo-C10 AHLs. Each biosensor strain glows in the dark when the bacteria are exposed to an AHL detected by the specific LuxR homolog, while control strains (\textit{E. coli}/pAL102, \textit{E. coli}/pAL104, \textit{E. coli}/pAL106) contain a plasmid lacking the LuxR homolog, and thus cannot respond to AHLs (Lindsay & Ahmer, 2005).

In order to identify AHLs produced by \textit{Ps}\textsubscript{t} DC3000, bacterial suspensions were cross-streaked on agar plates with the \textit{E. coli} biosensor strains containing pAL101, pAL102, pAL103, pAL104, pAL105, or pAL106. The \textit{E. coli}/pAL103 biosensor strain
produced luminescence that intensified in the vicinity of the WT Pst DC3000 or 10 µM 3-oxo-C6 AHLs cross-streak, while the E. coli/pAL104 control strain did not show differential luminescence (Figure 16). Luminescence was not detected when either E. coli strain was cross-streaked with a solvent control, DMSO (data not shown). Based on previous reports of AHLs produced by P. syringae, I conclude that the AHLs detected are most likely 3-oxoC6 AHL, although they could also be C6 and/or 3-oxo-C8 AHLs. Pst DC3000 did not induce luminescence in other E. coli strains containing pAL101 or pAL105 (data not shown), consistent with the fact that shorter and longer chain AHLs have not been detected in P. syringae.

Although it is unknown how much AHL is produced by Pst DC3000, saturated cultures of Pss B728a generate nearly 500 µM AHL (Quiñones et al., 2004). Previously published results suggest that Pst DC3000 produces less AHL than Pss B728a (Cha et al., 1998). In Pss B728a, the luxI homolog ahlI is autoregulated by its LuxR homolog, AhlR (Quiñones et al., 2004) Exogenous application of 10 µM 3-oxo-C6 AHL rescued AHL-deficient mutant strains (ΔaefR and ΔgacA) of Pss B728a, by inducing ahlI levels above that of WT (Quiñones et al., 2004). In addition, I observed that WT Pst DC3000 or 10 µM 3-oxo-C6 AHL both induced similar qualitative levels of luminescence in the E. coli/pAL103 biosensor (Figure 16). Therefore, I hypothesized that adding 10 µM exogenous AHLs to low-cell-density Pst DC3000 cultures would be sufficient to mimic high cell-density conditions and might thereby repress hrpL expression.
Figure 16. Production of AHLs by Pst DC3000.
A suspension of PstDC3000 or 10 µM 3-oxo-C6 AHL (Caymen Chemical) was rolled horizontally across a KB agar plate. *E. coli* strains containing pAL103 (*luxR luxI’::luxCDABE*) or pAL104 (*luxI’::luxCDABE*) were cross-streaked by rolling bacterial suspensions vertically down the same plate. Plates were incubated at room temperature (~25°C) for ~24 hrs and then photographed in the dark. The *E. coli*/pAL103 biosensor strain emits light strongest in the vicinity of AHLs, while the *E. coli*/pAL104 control strain produces basal levels of luminescence, which do not increase near the cross-streak. Non-luminescent bacteria are barely detectable in the photograph. Similar results were obtained from three independent assays.
Neither 3-oxo-C6 AHL nor C6 AHL affect T3SS gene expression

Results from my previous assay suggest that *Pst* DC3000 may produce 3-oxo-C6 AHL or C6 AHL. Also, transgenic tobacco expressing both 3-oxo-C6 AHL and C6 AHL are more resistant to infection by *P. syringae* pv tabaci than wild-type plants (Quinones et al., 2005). Therefore, to determine if expression of *hrpL* is repressed by exogenous AHLs, I assayed GUS activity in low cell density cultures of the *hrpL::gusA* reporter strain in HDM with and without the addition of 3-oxo-C6 AHL, C6 AHL, or DMSO (solvent control). Unexpectedly, my results show that expression of *hrpL::gusA* was not affected by addition of either exogenous AHL (Figure 17).
Figure 17. The effect of exogenous AHLs on hrpL::gusA expression. 
*P. stutzeri* DC3000 hrpL::gusA reporter strain KB precultures were washed in 10 mM MgCl$_2$ buffer, and resuspended to OD$_{600}$ ~0.05 in HDM without AHLs (white), or with 10 µM concentrations of either 3-oxo-C6-HSL (horizontal stripes) C6-HSL (vertical stripes), or DMSO (grey). GUS specific activity is shown for each media treatment at both 4.25 and 23.25 hpi as the average of 2 samples per culture with error bars representing the standard deviation. Similar results were observed in two independent assays.
Construction of Pst DC3000 psyRI deletion Strains

Exogenous AHLs may not adequately mimic in vivo quorum sensing signals, and it is possible that Pst DC3000 produces other AHLs than 3-oxo-C6 and C6 AHLs. Therefore, I also created an AHL-deficient mutant strain of Pst DC3000. There are two genes in Pst DC3000 that could encode AHL-synthases: an hdtS homolog (PSPTO0187) and a luxI homolog, psyI (PSPTO3864) (Feil et al., 2005). PsyI in P. syringae pv tabaci reportedly produces oxo-C6 AHL and C6 AHL, and the PsyI homolog in Pss B728a, AhI, produces 3-oxo-C6 AHL (Taguchi et al., 2006, Quiñones et al., 2004). HdtS is not related to LuxI and may synthesize multiple AHLs, including C6, C10, and 3-OH-C14 AHLs in P. fluorescens, although it is unknown if homologs in P. syringae encode functional AHL synthases (Laue et al., 2000, Feil et al., 2005). I chose to focus on PsyI for several reasons. First, prior studies suggest that PsyRI is actively involved in quorum sensing in Pst DC3000 (Chatterjee et al., 2007). In addition, results from my previous assay show that Pst DC3000 produces AHLs consistent with those synthesized by PsyI homologs. Furthermore, Pss B728a ∆ahlI mutant bacteria caused increased disease symptoms in host plants compared to WT bacteria (Quinones et al., 2005).

Two LuxR homologs have been reported in Pst DC3000 (Case et al., 2008). The luxR homolog, psyR, is adjacent to and in an opposite orientation from psyI (Figure 18A), and both psyR and psyI are expressed at low levels when Pst DC3000 is grown in KB (Chatterjee et al., 2007). To determine whether psyRI is responsible for repression of hrpL at high cell densities, I deleted psyRI in the Pst DC3000 WT and hrpL::gusA reporter strain. I hypothesized that expression of hrpL::gusA could be derepressed at high cell densities in the psyRI mutant.
Figure 18. Creation and confirmation of psyRI deletion strains.
(A) Organization of the psyRI locus in Pst DC3000. The psyR gene encodes a homolog of LuxR, an AHL-responsive transcription regulator, and psyI encodes a homolog of LuxI, an AHL synthase. The genes are convergently transcribed and overlap at their 3’ ends. Primers p166, p167, p168, and p172, which were used to construct the allele replacement vector, pJS16 (described in the Materials and Methods), are indicated by arrows below the diagram. Primers p170 and p171, which hybridize to psyR and psyI, respectively, were used to detect the presence of the psyRI sequences in Pst DC3000 WT and hrpL::gusA reporter strains, and ΔpsyRI mutant derivatives. The gel to the right of the diagram shows the results of colony PCR with p170 and p171 on the following Pst DC3000 strains: (1) WT, (2) hrpL::gusA, and (3 and 4) two biological replicates of hrpL::gusA ΔpsyRI. The last lane (5) is DNA marker. (B) Replacement of psyRI with a Cm\textsuperscript{r} cassette. The Cm\textsuperscript{r} cassette, derived from pHP45ΩCm (Fellay et al., 1987), contains transcription terminators at both ends. Primers p173 and p175, shown as arrows below the diagram, hybridize to sequences upstream of psyRI (and p166) and within the Cm\textsuperscript{r} cassette, respectively. The results of colony PCR on the Pst DC3000 strains described in (A) are shown on the gel to the right of the diagram.
I made psiRI deletion strains by replacing psiRI in the Pst DC3000 WT and hrpL::gusA reporter strains with a chloramphenicol resistance cassette as described in the Materials and Methods. The absence of psiRI in the Pst DC3000 psiRI deletion strains was confirmed by PCR with p170 and p171, which amplified a 2.3 kb product from WT Pst DC3000, but not from the psiRI deletion strains (Figure 18A). To confirm that the Cm\textsuperscript{r} cassette replaced psiRI in the \(\Delta\text{psiRI}\) mutants, a PCR reaction was performed with p173 and p175, which hybridize to the Cm\textsuperscript{R} cassette and upstream of psiRI, respectively. A ~1.3 Kb product was present in the \(\Delta\text{psiRI}\) mutants and not in the WT Pst DC3000 (Figure 18B).

**Pst DC3000 psiRI deletion strains are deficient in AHL activity**

To confirm that deletion of psiRI affects Pst DC3000 AHLs, I assayed AHL activity in the Pst DC3000 WT, hrpL::gusA, and derivative psiRI deletion strains. The E. coli/pAL103 biosensor produced strong luminescence in the vicinity of the cross-streak with WT Pst DC3000 and the hrpL::gusA reporter strain, but not with either of the psiRI deletion strains (Figure19). The control strain (E. coli/pAL104) produced little or no luminescence, and intensity did not increase close to any of the cross-streaked strains. Therefore, I conclude that deletion of psiRI resulted in decreased production of AHLs by Pst DC3000.
Figure 19. Detection of 3-oxo-C6-HSL production by psyRI deletion strains. Pst DC3000 strains to be tested (A) WT, (B) hrpL::gusA, and (C and D) their respective ΔpsyRI mutant derivatives were horizontally streaked on KB agar plates. The 3-oxo-C6 AHL biosensor (E. coli/pAL103) and the control strain that lacks luxR (E. coli/pAL104) were then streaked vertically across each plate. Plates were incubated at room temperature (~25°C) for approximately 24 hrs and then photographed with a CCD camera in the dark. Basal luminescence is higher in pAL104 than in pAL103, but does not increase near the cross-streak, as noted by Lindsay and Ahmer (2005). Similar results were observed in three independent assays.
**psyRI does not regulate Pst DC3000 T3SS gene expression**

To determine if expression of *hrpL* is derepressed in the *psyRI* mutant, I assayed *Pst* DC3000 *hrpL::gusA* and its Δ*psyRI* derivative for GUS activity in low and high cell density cultures. My results show that deletion of *psyRI* has no effect on expression of *hrpL::gusA* (Figure 20). Because I did not delete the *hdtS* homolog, which may also synthesize AHLs in *Pst* DC3000, I cannot rule out the possibility that an AHL molecule involved in quorum sensing mediated regulation of T3SS genes. However, PsyI appears to be the primary enzyme responsible for AHL production in *Pst* DC3000 (Figures 16 and 19). Therefore, I conclude that T3SS genes are not regulated by the PsyRI AHL-mediated quorum sensing system.
Figure 20. The effect of psyRI on T3SS gene expression. *P*. *s.* DC3000 *T3SS::gusA* reporter strains (A) *hrpRS::gusA* and (B) *hrpL::gusA* and their ΔpsyRI derivatives were suspended in HDM at low cell density (OD600 ~ 0.1) or high cell density (OD600~ 0.7) and shaken at 23°C for 6 hours. Values are the average GUS specific activity for 2 samples per culture for each strain with error bars representing the standard deviation. Similar results were seen in multiple independent assays.
Exogenous IAA inhibits Pst DC3000 T3SS gene expression

Since psyRI and exogenous AHLs do not appear to regulate Pst DC3000 T3SS genes, a non-AHL quorum sensing molecule could be responsible for the observed repression of hrpL and avrPto at high cell densities. A tryptophan-dependent stationary-phase T3SS-repressing signal was recently reported in supernatants from P. aeruginosa mutants unable to produce known quorum sensing signals (Shen et al., 2008). Shen et al. (2008) found that the auxin indole-3-acetic acid (IAA) represses P. aeruginosa T3SS genes (Shen et al., 2008). IAA is primarily known as a plant hormone, but bacteria also produce IAA and use it as a signaling molecule (Charkowski, 2009, Lambrecht et al., 2000, Spaepen et al., 2007). Most pathovars of P. syringae produce IAA, especially when supplemented with tryptophan, however little is known about why (Glickmann et al., 1998, Fett et al., 1987). To ascertain whether IAA similarly represses T3SS genes in P. syringae, I assayed expression of hrpRS and hrpL in the presence or absence of exogenous IAA using concentrations ranging from 10µM to 1 mM.

Indeed, hrpL::gusA was repressed in a concentration dependent manner by IAA (Figure 21A). My results show that after 9 hour incubation in the presence of 1 mM exogenous IAA, hrpL expression was reduced 20-fold. There was a 3-4-fold difference in expression in the presence of 500 µM IAA, but the reduction in hrpL expression was less than 2-fold with 100 µM IAA. There was a slight reduction in hrpRS in high concentrations of exogenous IAA, although the effect was less than 2-fold. Therefore, both IAA and high cell density repress T3SS genes in a similar manner.
Figure 21. Exogenous auxin has a dosage-dependent effect on *hrpL::gusA* expression and growth of *Pst* DC3000.

WT and *T3SS::gusA* *Pst* DC3000 KB precultures were washed in 10 mM MgCl\(_2\) buffer and inoculated to OD\(_{600}\) =0.05 into HDM containing indole-3-acetic acid sodium salt (Sambrook *et al.*)(Sigma-Aldrich) at the indicated concentrations. The pH of HDM was not altered at any of the concentrations of IAA tested. (A) Expression of *hrpRS::gusA* and *hrpL::gusA* at 9 hpi shown as average GUS specific activity of 3 biological replicates for each reporter strain, with error bars representing standard deviation. (B) Growth of WT *Pst* DC3000 under the same conditions. Optical densities were taken at 9 and 21 hpi. Similar growth was observed in the *T3SS::gusA* reporter strains. This assay was repeated twice with comparable results.
IAA also had a significant effect on *Pst* DC3000 growth (Figure 21B). At 9 hpi, the growth *Pst* DC3000 was reduced in a concentration dependent manner by IAA, although the effect was small. However, by 21 hpi, growth of *Pst* DC3000 was ~50% lower in HDM with 1 mM exogenous IAA. When 10 µM IAA was added to the culture medium, growth was not inhibited, but there was no observable effect on T3SS gene expression. Previously, exogenous IAA was reported to affect both virulence gene expression and growth of *A. tumefaciens* cultures (Liu & Nester, 2006). My results also show *Pst*DC3000 responds similarly to increased IAA levels.
Chapter IV: Discussion
Although it is well known that environmental conditions affect the expression of *P. syringae* T3SS genes, the molecular mechanisms underlying this regulation are not well understood. Many of the previous studies on this subject have yielded conflicting results. In addition, few studies have tested whether environmental signals affect the expression or activity of HrpR, HrpS, and HrpL, which are key components of the T3SS regulatory cascade. In this work, I explored how various environmental conditions modulate expression of T3SS genes in *Pst* DC3000. To do this, I constructed chromosomal *gusA* fusions to T3SS genes that encode regulatory factors or secreted proteins. Importantly, these fusions did not alter the function of the T3SS (Figure 8). These reporter strains were then utilized to address some areas of disagreement in the literature, such as which environmental factors encountered in plants alter *P. syringae* T3SS genes expression in culture, and how each condition affects the *hrpRS*-*hrpL* regulatory cascade. While many of my results confirm previous reports, the breadth of conditions tested and the techniques employed allowed me to define regulatory patterns that may have gone unnoticed in previous studies. In addition, I identified new environmental variables that modulate expression of *Pst* DC3000 T3SS genes in culture, which could also potentially regulate the T3SS *in planta*.

KB (a rich growth medium) represses *P. syringae* T3SS genes, while HDM (a defined minimal medium) may mimic the plant apoplast because it induces the same genes. One way that KB might inhibit T3SS genes is by repressing the expression of *hrpRS*. In fact, Xiao et al (2007) found that *hrpR* transcript levels in *Psp* 3121 were significantly lower when bacteria were cultured in KB (Xiao et al., 2007). My results confirm that *hrpRS* expression is also repressed by KB in *Pst* DC3000 (Figure 9).
Another way that KB might repress T3SS gene expression is by modulating T3SS regulators at the post-transcriptional level. For example, one study found that the \textit{hrpR} gene is similarly expressed in KB and HDM, but that in both \textit{Pss} 61 and \textit{Pst} DC3000, HrpR protein is preferentially degraded by the Lon protease in KB (Bretz et al., 2002). My results show that both \textit{hrpL} and \textit{avrPto} expression were significantly higher in KB when \textit{hrpR} was overexpressed, suggesting that activation of \textit{hrpL} by HrpR is not affected by Lon when \textit{hrpR} is expressed at sufficient levels (Figure 10). In contrast to HrpR, the HrpS protein was not degraded by Lon and was equally stable when \textit{Pss} 61 and \textit{Pst} DC3000 were grown in KB or HDM (Bretz et al., 2002). I also found that \textit{hrpL} expression was higher in KB when \textit{hrpS} was overexpressed compared to when \textit{hrpR} was overexpressed (Figure 9), which could be explained by greater HrpS stability. However, Hutcheson et al. (2001) observed that HrpS alone activated the \textit{hrpL} promoter in \textit{E. coli}, while HrpR alone did not (Hutcheson et al., 2001). These results might indicate the: (i) HrpS is also more stable in \textit{E. coli}, or (ii) HrpS is a more effective activator of the \textit{hrpL} promoter than HrpR. Further experiments will be required to distinguish between these two possibilities. One way to answer this question would be to overexpress tagged \textit{hrpR} and/or \textit{hrpS} proteins in a \textit{ΔhrpRS} mutant derivative of the \textit{hrpL::gusA} \textit{Pst} DC3000 reporter strain. The relative expression of HrpR and/or HrpS could then be correlated to the expression of \textit{hrpL}.

My results also confirm the finding by Sreedharan et al. (2006) that \textit{hrpL} expression in \textit{Pst} DC3000 was higher when the bacteria were cultured in HDM compared to HSS (Sreedharan et al., 2006). I additionally found that \textit{hrpRS} and \textit{avrPto} are lower in HSS than in HDM (Figure 9). The differences between expression of T3SS genes in HSS
and HDM may be attributed to the different carbon sources in the two media (sucrose and fructose, respectively). This hypothesis is supported by the finding that expression levels of \textit{hrpRS}, \textit{hrpL}, and \textit{avrPto} were similar in HSS and HDM containing sucrose in place of fructose (Figure 9 and 12).

Because the pH in the plant apoplast is slightly acidic, pH could be an important environmental signal controlling expression of the T3SS. However, my results indicate that pH by itself is not entirely responsible for the T3SS expression differences between KB and HDM. I found that \textit{hrpL} and \textit{avrPto} were maximally expressed at pH ~5.9, and that expression decreases by only ~2.5-fold at pH 7.6 (Figure 11). The effect of pH on \textit{hrpRS} was relatively small (<2-fold lower at pH 5.9 compared to 7.6). However, the small changes in \textit{hrpRS} at various pHs could lead to the larger effect of pH on \textit{hrpL} and \textit{avrPto}. In contrast to my results, van Dijk et al. (1999) demonstrated that AvrPto protein levels were comparable in \textit{Pst DC3000} grown at pH 6 or 7, suggesting that pH does not affect transcriptional regulation of \textit{avrPto} (van Dijk et al., 1999). However, the difference in expression of \textit{avrPto} between pH 5.9 and 7.2 was less than 2-fold in my experiment. Thus, my conclusions may not contradict the results of van Dijk et al (1999).

The other study that examined the effect of pH on transcriptional regulation of \textit{P. syringae} T3SS genes was performed in \textit{Psp NPS3121} (Rahme et al., 1992). Rahme et al. (1992) reported maximum expression of several T3SS genes at pH 5.5, with a significant decrease (several log units) at pH 7.5. Because neither van Dijk et al. (1999) nor I found such a large effect of pH on \textit{Pst DC3000} T3SS genes, it is possible that pH differentially regulates T3SS genes in \textit{Pst DC3000} and \textit{Psp NPS3121}. However, Rahme et al. (1992)
tested five T3SS apparatus genes, and curiously, one of the genes was only slightly affected by the increase in pH. Therefore, another possibility is that pH regulates hrpRS in both pathovars, and in addition, independently and differentially regulates downstream T3SS genes.

*P. syringae* is exposed to various carbon sources during the process of colonizing plants (Kamilova et al., 2006, Rico & Preston, 2008). Because pathovars may encounter different levels and types of carbon sources in their plant hosts, various carbon sources may signal *P. syringae* to activate the T3SS. Overall, my results show that initial induction of T3SS gene expression is similar in HDM regardless of whether sugars, sugar alcohols, organic acids, or glycerol were used as the sole carbon sources (Figures 12 and 13). At later time points, induction of T3SS genes continued when the carbon source was fructose, but not other preferred growth substrates, such as citrate and succinate. These findings may explain some of the conflicting observation reported in other studies. For example, the organic acid succinate was reported to repress T3SS genes in *Ps* race 0 (Huynh et al., 1989) and *Ps* NPS3121 (Rahme et al., 1992), but not in *Ps* 61 (Xiao et al., 1992). However, Xiao et al. (1992) analyzed T3SS expression at 6 hpi, while Huynh et al. (1989) and Rahme et al. (1992) analyzed samples at 12 hpi and 10 hpi, respectively. I found that succinate induced T3SS genes at 6 hpi, but repressed them by 10 hpi (Figure 13). Therefore, my results do not actually differ much from previous studies. My conclusions are different, however, because I analyzed the effect of various carbon sources on changes in T3SS expression over time rather than at any individual time point. Particularly in *Pst* DC3000, it makes sense that T3SS expression is not inhibited by succinate. Succinate is one of the most abundant organic acids in tomato plants, where
organic acids are a more available carbon source than sugars (Kamilova et al., 2006). To successfully colonize tomato plants, Pst DC3000 must be able to activate the T3SS in the presence of succinate.

I also found that sugars and sugar alcohols affected expression patterns of T3SS genes in Pst DC3000 in ways that differed from previous studies. For instance, both mannitol and fructose were comparable in inducing T3SS genes in Psg race 0 (Huynh et al., 1989) and in Pss 61 (Xiao et al., 1992), yet I detected variation in expression patterns over time using these two carbon sources. Although fructose and mannitol had similar effects on avrPto in Pst DC3000 at 12 hpi, avrPto expression was ~3-fold lower in mannitol compared to fructose at 24 hpi (Figure 12). My results are similar to those reported in the Ph.D. thesis of Yan Mei Xiao (2005), who also found that fructose and mannitol differentially affected T3SS genes in P. syringae pv. tabaci 11528 and Pst DC3000 (Xiao, 2005). However, Psp NPS3121 T3SS genes were not induced at all by mannitol (Xiao, 2005). Therefore, particular carbon sources may not uniformly affect T3SS genes in diverse pathovars of P. syringae.

Similar to Huynh et al. (1989), I observed that the level of T3SS gene expression in P. syringae varies inversely with the growth rate supported by various carbon sources. However, Huynh et al. (1989) concluded that preferred growth substrates (such as the TCA cycle intermediates citrate and succinate) repress T3SS genes, while those that enter glycolysis before pyruvate (such as sugars and sugar alcohols) do not. My data suggest that the carbon source itself may not repress T3SS genes. Instead, I propose that T3SS gene expression declines when cultures reach higher cell densities.
Rahme et al. (1992) additionally noted that growth rates of *Psp* NPS3121 were variable when different carbon sources were added to the inducing media. The authors attempted to minimize the variation by pre-adapting the bacteria to each specific carbon source in the KB pre-culture before inoculating bacteria into inducing media (Rahme et al., 1992). I did not find a significant change in growth rates in HDM after pre-adaptation to each carbon source. *Pst* DC3000 grew slower in HDM containing fructose, whether fructose was included in the KB pre-culture or not (data not shown).

The effect of carbon sources on growth rate and expression of T3SS genes led me to investigate the effect of cell density on T3SS expression. I report here that *hrpL* expression is inversely related to cell density and is highest at low cell densities (<0.1) (Figure 14). This is surprising for a couple of reasons. First, many bacterial pathogens up-regulate their virulence genes at high population densities (Antunes et al., 2010, Mole et al., 2007). Second, T3SS genes are positively regulated by GacA, and *gacA* transcripts increase with growth phase (Chatterjee et al., 2003). GacA also positively regulates AHL production, as well as *rpoS*, which encodes a stationary-phase sigma factor (σS) (Chatterjee et al., 2003). Thus one might expect GacA to mediate increased expression of T3SS genes at high cell densities. Since T3SS genes are actually repressed at high cell densities, other regulatory systems may counteract the positive effects of GacA in this condition.

Cell density dependent gene regulation is usually mediated by small extracellular signaling molecules. I found that when *Pst* DC3000 was inoculated to a low cell density in conditioned medium from a high cell density culture, T3SS gene expression decreased.
(Figure 15). This result suggested that \textit{Pst} DC3000 secretes a T3SS-inhibiting molecule into the culture medium that accumulates as bacteria reach high-cell densities. AHLs were a strong candidate for this signaling molecule, since they are known to be produced by \textit{P. syringae} (Quiñones et al., 2004, Dumenyo, 1998), and I confirmed that \textit{Pst} DC3000 produces AHLs (Figure 16). Furthermore, \textit{Pss} B728a mutants that do not synthesize 3-oxo-C6 AHL are more virulent in beans (Quinones et al., 2005). Unexpectedly, I found that exogenous application of 3-oxo-C6 AHL or C6 AHL did not alter expression of T3SS genes (Figure 17). In addition, deletion of \textit{psyRI} reduced production of AHLs by \textit{Pst} DC3000 (Figure 19), but did not alter density dependent repression of \textit{hrpL} (Figure 20). It is possible that another AHL is responsible for quorum sensing regulation of T3SS genes, since the \textit{Pst} DC3000 genome encodes an HdtS-family AHL synthase. Nevertheless, the only species of AHL reportedly produced by HdtS which may not have been detected by the \textit{E. coli} biosensors in this study is 3-OH-C14 AHL. Still, it is possible that \textit{Pst} DC3000 produces \textit{psyRI}-independent AHLs below the threshold of detection of these biosensors. However, it seems likely that a non-AHL signaling molecule may be involved in quorum sensing regulation of the T3SS in \textit{Pst} DC3000.

Many other types of AHL-independent quorum sensing signals have been identified in bacteria. Examples mentioned earlier, 3OH PAME and DSF, are diffusible molecules responsible for density-dependent regulation of virulence in \textit{R. solanacearum} and \textit{X. campestris}, respectively. \textit{P. aeruginosa} coordinates virulence genes using another quorum sensing molecule, Pseudomonas quinolone signal [(PQS) (3,4-dihydroxy-2-heptylquinoline)], which links together two AHL-mediated quorum sensing. \textit{V. cholerae} utilizes several quorum sensing signals in conjunction with AHLs to orchestrate virulence.
gene expression, including autoinducer-2 [(AI-2), which is a furanosyl borate diester (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate, as well as cholerae autoinducer-1 (CAI-1), which is (S)-3-hydroxytridecan-4-one] (Miller et al., 2002, Higgins et al., 2007). A homolog of the CAI-1 synthase, CqsA, may be present in Pst DC3000, but there are no obvious homologs of the other known quorum sensing molecule synthases (Schechter personal communication). Therefore, quorum sensing regulation of Pst DC3000 T3SS genes expression may occur by a novel mechanism.

Shen et al. (2008) recently suggested that the tryptophan catabolite IAA or a related molecule might serve as a non-AHL quorum sensing signal that represses T3SS genes in P. aeruginosa (Shen et al., 2008). Since IAA is produced by P. syringae (Glickmann et al., 1998, Spaepen et al., 2007), I tested whether IAA could also repress T3SS genes in Pst DC3000. Although I found that IAA reduced hrpL expression in a concentration dependent manner, relatively high levels of IAA were required to the effect. Exposure of Pst DC3000 to 1 mM IAA reduced hrpL expression by 20 fold, while 0.1 - 0.5 mM IAA reduced hrpL expression by ~2-3-fold (Figure 21). In contrast, Liu et al. (2006) found that only 32 µM IAA repressed vir genes by 50% in A. tumefaciens (Liu & Nester, 2006). However, Shen et al. (2008) also found that high levels of IAA were required to decrease T3SS gene expression in P. aeruginosa, with 1 mM IAA decreasing T3SS gen expression by 2-3-fold. IAA had a much more modest effect on hrpRS expression, as 1 mM IAA reduced hrpRS::gusA by less than 2-fold. Again, it is possible that this small effect on transcription of hrpRS is magnified downstream in expression of hrpL. However, it is also possible that IAA may act on hrpRS at the post-transcriptional level or may affect hrpL in a hrpRS-independent manner.
The correlation between IAA and cell-density dependent repression of hrpL raises an intriguing question: is IAA a secreted cell to cell signaling molecule in \textit{P. syringae}? To answer this question, I attempted to quantitate the levels of IAA in the supernatant from a high cell density culture of \textit{Ps} \textit{t DC3000}. Unfortunately, I was unable to detect extracellular IAA using Salkowski’s reagent (data not shown). Therefore, more sensitive tests may be required to quantitate IAA or IAA may not be the density-dependent T3SS repressing signal. Future biochemical and genetic studies will be required to determine if IAA or a related molecule functions as a quorum sensing molecule in \textit{P. syringae} cultures at high cell densities.

In addition to synthesizing IAA, \textit{P. syringae} is exposed to plant-produced auxins during colonization of its hosts. Curiously, infection of \textit{A. thaliana} with \textit{Pst DC3000} causes an increase in free IAA levels in plants, although it is not clear whether the auxin is derived from the plant or bacteria (Schmeltz, 2003). It remains to be determined whether \textit{Pst DC3000} encounters high enough levels of plant or bacterial derived IAA for the auxin to be a biologically relevant T3SS repressor during infection. HDM does not contain tryptophan and \textit{Pst DC3000} reportedly produces relatively low levels of IAA unless the culture media is supplemented with tryptophan (Glickmann et al., 1998, Fett et al., 1987) (Kunkel personal communication). It will be interesting to see if IAA also inhibits T3SS gene expression in other pathovars, such as \textit{P. syringae pv. syringae}, which synthesizes high levels of IAA with or without tryptophan supplementation (Glickmann et al., 1998, Fett et al., 1987).
It is also possible that IAA is converted to another molecule that is involved in repression of T3SS genes. Indole and derivatives of indole have been implicated in quorum sensing in several bacteria (Ryan & Dow, 2008). For example, besides AHLs and DSF, *R. solanacearum*, may utilize (3S)-3-hydroxy-indolin-2-one as a quorum sensing molecule (Delaspre *et al.*, 2007). This indole is unusual in that it appears to interact with a LuxR homolog, which normally only responds to AHLs (Delaspre *et al.*, 2007). PQS is another indole derivative that functions as a signaling molecule in *P. aeruginosa* (Mole *et al.*, 2007). However, as mentioned above, *P. syringae* appears to lack the enzymes that synthesize this molecule. Finally, the IaaL enzyme may convert IAA into IAA-lysine in *Pst DC3000*, which could repress T3SS genes. Alternatively, conjugating lysine to IAA might inactivate the ability of IAA to repress T3SS genes. In support of this theory, mutations of *iaaL* in *P. savastanoi* increase IAA accumulation in culture and decrease virulence *in planta* (Glass & Kosuge, 1988). Further studies are needed to ascertain whether IaaL inactivates auxin or affects virulence in *Pst DC3000*. Interestingly, HrpL activates *iaaL* expression, suggesting that production of IAA-lysine may be important under T3SS-inducing conditions.

Although my experiments were performed on bacterial cultures, cell-density dependent regulation of T3SS gene expression in *Pst DC3000* may also be important for efficient infection of plants. Considering that the primary function of *P. syringae* type III secreted effectors is to disarm plant defense responses, the T3SS would be essential for survival of the first bacteria entering the apoplast, which would be at low population densities. My data suggest a model in which a quorum sensing signaling molecule would enable *P. syringae* to transition from early stage to late stage virulence factors. T3SS
genes may be repressed in biofilms on the leaf surface due to a variety of environmental cues, including an extracellular density dependent signaling molecule (Figure 22). Upon entry into the apoplast, *P. syringae* would initially be at low cell densities. Therefore, repression of T3SS genes would be relieved, allowing the secretion system to be employed to translocate effectors and disarm plant defenses. Once the bacteria overcome barriers to multiplication in the apoplast, the density dependent signal could again accumulate and down-regulate T3SS genes, allowing *P. syringae* to conserve energy. The signal involved may reduce T3SS expression by modulating the levels or activity of HrpV, a known repressor of HrpS activity (Ortiz-Martin et al., 2010, Preston et al., 1998). Alternatively (or additionally), the repressing signal might increase Lon-mediated degradation of HrpR. However, it remains to be seen exactly how quorum sensing might be involved in density dependent regulation of *P. syringae* T3SS genes during infection.
Figure 22. Model for cell-density dependent regulation of *P. syringae* T3SS gene expression. (I.) T3SS genes are repressed by high cell density conditions, such as encountered in biofilms on the leaf surface. (II.) The repressive signaling molecule diffuses away from the first bacteria entering into the plant apoplast, and repression of the *hrpRS-hrpL* regulatory cascade is relieved. HrpL then activates production of the T3SS injectisome and type III secreted effectors. (III.) *P. syringae* utilizes the T3SS to disarm plant defense responses, allowing the bacteria to multiply. (IV.) Multiplication of *P. syringae* within the apoplast leads to high-cell density conditions and T3SS genes are once again repressed.
References


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