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Tahina O. Ranaivoarisoa

Abstract

Pseudomonas syringae is a bacterial plant pathogen that infects a large variety of agricultural crops. Bacteria colonize leaf surfaces and enter plant mesophyll tissue through wounds or stomata. Once inside, *P. syringae* can alter plant cell signaling pathways and suppress plant defense responses enabling it to grow in the intercellular space in the mesophyll. *P. syringae* possesses at least two types of virulence factors that suppress plant defense responses: i) small phytotoxin molecules, and ii) effector proteins that are translocated through specialized secretion systems. Gram-negative bacteria possess at least six types of secretion systems. The *P. syringae* type II and type III secretion systems (T2SS and T3SS) are both involved in secreting proteins that are important for *P. syringae* pathogenesis. Functions of the other secretion systems have not been explored. This study investigates the role of the newly discovered type VI secretion system (T6SS) in *P. syringae* interaction with plants.

The results show that T6SS genes are expressed in three sequenced strains of *P. syringae*, *P. syringae* pv. tomato DC3000 (*Ps*t DC3000), *P. syringae* pv. phaseolicola 1448a (*Ps*p 1448a) and *P. syringae* pv. syringae B728a (*Ps*s B728a). The T6SSs of *Ps*p 1448a and *Ps*s B728a were also able to secrete the Hcp protein into culture supernatants, showing that they are active. *In planta* virulence and growth studies revealed that the T6SS may not be essential for *Ps*p 1448a and *Ps*s B728a to cause disease in host plants. However, the T6SS may be involved in regulating biofilm formation, since a mutant *Ps*p 1448a T6SS formed denser

biofilm than the wild-type bacteria. These results suggest that the T6SS may secrete factors important for controlling bacterial aggregation on leaves.

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Chapter I: Introduction

P. syringae, a model plant pathogen

The gram-negative bacterium *P. syringae* is a well-known cause of leaf spot disease in plants. The species is divided into more than 50 groups called pathovars (Hirano and Upper, 2002). A pathovar is defined as a group of bacteria that can infect only plants within a certain genus or species. For example, the *P. syringae* pathovar (pv.) tomato is a pathogen on tomato but not on beans, whereas *P. syringae* pv. phaseolicola causes halo blight disease on beans but not on tomato. The leaves and fruits of infected plants exhibit brown necrotic lesions that are often surrounded by yellowish haloes.

Because *P. syringae* (as a species) has a broad host range, it presents a threat to agriculture. The damage caused by halo blight in beans can reach 10 to 40% of crops (Asensio et *al* 2006). The decreases in both the yield and the quality of seeds and pods can lead to market devaluation. *P. syringae* is an attractive organism for laboratory studies on bacterial pathogenesis for several reasons. First, *P. syringae* is closely related to the opportunistic human pathogen *Pseudomonas aeruginosa*, which infects the lungs of cystic fibrosis patients (Potvin et *al*, 2003). Second, certain strains of *P. syringae* infect the model plant *Arabidopsis thaliana*. *A. thaliana* has a sequenced genome and many genetic tools are available for studying plant responses to bacterial infection. Third, the genome sequences of three pathovars of *P. syringae* are available. The genome sequence of *P. syringae* pathovar tomato DC3000 (here after *Ps*t DC3000) was available in 2003, followed by the *P. syringae* pv phaseolicola 1448a (here after *Ps*p 1448a) and *P. syringae* pv syringae B728a (here after *Ps*s B728a) in 2005 (Buell et al, 2003; Feil et *al*, 2005; Joardar et *al*, 2005). The availability of genomes from pathovars with different host ranges facilitates comparative genomic studies.

How does *P. syringae* invade plants?

Plant leaves are inhabited by millions of bacteria, which are transported between plants by natural agents such as wind, rain and even insects (Hirano and Upper, 2000). Bacteria that live on leaf surfaces are called epiphytes. *P*ss B782a is well known for its ability to survive epiphytically (Sabaratnam et al, 2003). After landing on the leaf surface, *P*ss B728a migrates to areas where carbon sources are available (Dulla et *al*, 2005). Once bacteria have reached areas rich in nutrients, they start to multiply and form large aggregates or biofilms. Studies have shown that aggregated bacteria are more resistant to environmental stresses such as moisture, drought, fluctuating temperatures, and UV light, and are even more resistant to antibiotics (Dulla et *al*, 2005). *P*ss B728a aggregation is regulated by a quorum sensing mechanism that involves acyl homoserine lactone (AHL) signaling molecules. At low population densities, bacteria are motile and produce AHLs that diffuse into the environment. As the bacterial population increases, the AHL signal secreted by one bacterium diffuses into neighboring bacteria. The AHL molecule then binds to a transcription factor that regulates gene expression. One outcome of this event is that bacteria become nonmotile and form a biofilm (Bodman et *al*, 2003).

Once *P. syringae* populations reach high levels on the leaf surface, bacteria begin to enter the leaf mesophyll through wounds or stomata, which are natural openings on the leaf that allow gas exchange. Inside the leaf, bacteria colonize the apoplast, or the space between plant cells. Unlike *P*ss B728a, *P*st DC3000 seems to be a poor epiphyte and finds the endophytic (inside the plant) area more convenient for growth (Li et *al*, 2002).

Virulence factors of *P. syringae*

So far, two types of virulence factors have been reported to be used by *P. syringae* to overcome plant defenses: phytotoxins and effectors. *P. syringae* pv. syringae secretes several

phytotoxins including syringomycin, which is a small lipopeptide that forms pores in the plasma membranes of plant cells. Pore formation destabilizes the influx of K^+, H^+, Ca^{2+}, which is deadly for plant cells (Bender et al, 1999). *P. syringae* pv. tomato also produces a phytotoxin called coronatine to overcome plant defenses. Coronatine is similar in structure to the phytohormone methyl jasmonate. Coronatine plays many roles during plant pathogenesis, including the induction of stomata opening (Melotto et *al*, 2008). It also helps *P. syringae* overcome the plant salicylic acid-dependant defense cascade (Uppalapati et *al*, 2007). Another important phytotoxin is phaseolotoxin (N-[N'-sulfo-diaminophosphinyl]-ornithyl-alanyl-homoarginine), which is secreted by *P. syringae* pv. phaseolicola and a few other pathovars. This toxin, which is a reversible inhibitor of the enzyme ornithine carbamoyltrasferase (OCTase), causes interruption of arginine biosynthesis in plants (Aguilera et *al*, 2007).

In addition to phytotoxins, *P. syringae* also secretes effectors, virulence proteins that vary in size from ~20 to 250 kD and are used by the bacteria to disrupt plant physiology and metabolism. *P. syringae* uses a dedicated secretion system termed the type III secretion system (T3SS) to translocate effector protein into plants. The type III secretion system of *Pst* DC3000 is one of the most studied secretion systems in bacteria. *Pst* DC3000 uses the T3SS to inject at least 33 proteins directly into plant cells (Schechter et *al*, 2006). These proteins collectively allow *P.syringae* to survive, grow and cause disease in plants. Many effectors manipulate plant signaling pathways that control the induction of defense responses. The function of two well-characterized effector proteins AvrPto and AvrPtoB are discussed below (Lin et *al*, 2006).

Plant defenses against *P. syringae*

Plants have two lines of defense against *P syringae*: basal defense and the hypersensitive response (HR). Basal defense is used by the plant to sense conserved molecules of microbes called PAMPs (Pathogen Associated Molecular Patterns). The most common molecules responsible for eliciting the basal defense mechanism are flagellin, lipopolysaccharide (LPS), peptidoglycan, and elongation factor EF-Tu. The basal defense response is nonspecific and occurs rapidly after recognition of bacteria. It triggers an increase in extracellular pH, increase of callose deposition in the cell wall, production of reactive oxygen species (ROS), and activation of mitogen-activated protein kinases (MAPKs) in plant cells (Abramovitch et al, 2006). Plant cells also form papillae, or areas where the cell wall is thickened, at sites of bacterial infection. Papillae contain several materials including the β-glucan polysaccharide callose. *P. syringae* uses a few effector proteins that alter plant signaling pathways to overcome elicitation of basal defense (Figure 1). For example, the effector AvrPto prevents the deposition of callose in plant cell walls following *P.syringae* infection (Hauck et al, 2003)

The (HR) is a rapid, localized programmed cell death of plant cells that contact invading pathogens. Resistance proteins (R proteins) inside plant cells recognize one or more cognate effectors delivered by bacteria (Figure 1). Upon their death, these plant cells release phenolic compounds and other factors that kill bacteria. The HR is triggered two to three hours after recognition of T3SS effectors (Abramovitch, 2006). However, some bacteria also possess effectors that block the HR caused by other effectors. For example, in tomato resistant to *P*st DC3000, AvrPto and AvrPtoB are both recognized by the resistance protein Pto, which triggers the HR. In tomato plants that lack Pto, which are susceptible to *P*st DC3000, AvrPtoB suppresses the HR caused by effectors recognized by other R proteins (Lin et *al*, 2006).

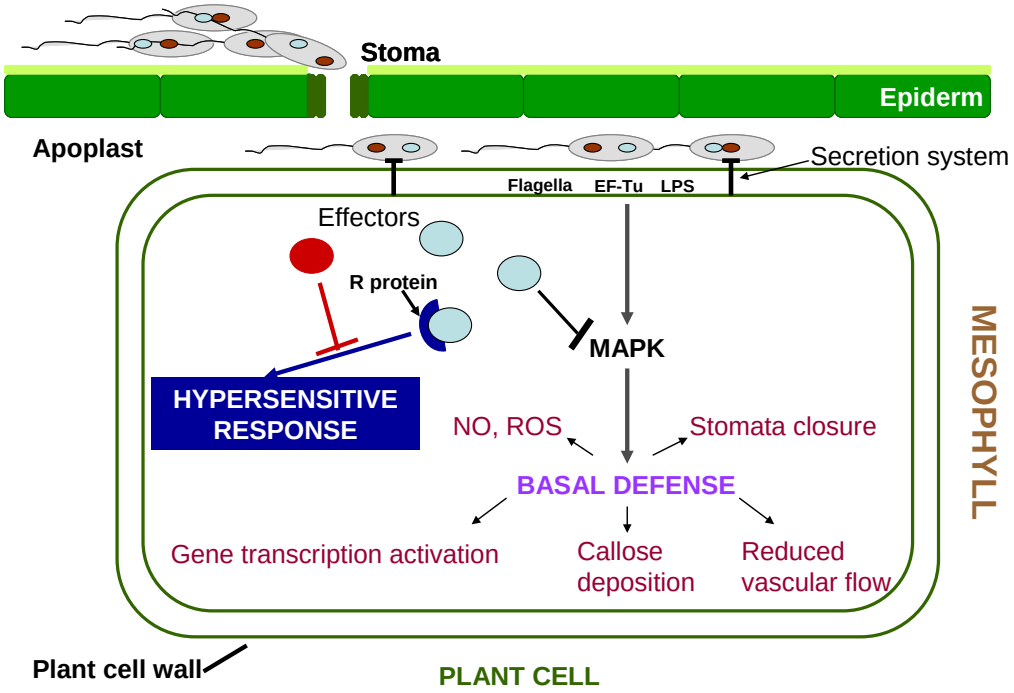


Figure 1 Model showing the plant-bacterial interaction. Bacteria swarm on the leaf surface and enter the leaf through stomata. Bacterial conserved components such as LPS and flagella trigger plant basal defenses such as stomata closure, increased callose deposition, reduced vascular flow, NO (Nitric Oxide), and ROS (Reactive Oxygen species) production. Effector proteins secreted by bacteria through the T3SS hamper MAPK pathways and suppress plant basal defenses. Effectors may also be recognized by plant resistance (R) proteins, which trigger the hypersensitive responses.

Secretion systems of *P. syringae*

In addition to the type III secretion system introduced earlier, five more secretion systems are known in Gram-negative bacteria, from type I to type VI (T1SS to T6SS).

The T1SS is related to the ATP-binding cassette (ABC) transporter family. ABC transporters are composed of three proteins that span the inner membrane, outer membrane, and periplasm. These transporters secrete various molecules including carbohydrates, lipids, and ions. The T1SS also secretes protein toxins such as haemolysin A of *Escherichia coli* (Beeckman and Vanrompay, 2009). The T2SS is used by bacteria to transport proteins outside of the cell in two steps. Proteins are first secreted across the inner membrane by the general secretory pathway (Sec system) or the Tat pathway and then transported through the outer membrane by the T2SS. *Erwinia carotovora* uses the T2SS to secrete plant cell wall degrading enzymes including cellulases and pectinases (Abramovitch et *al*, 2006). The T4SS is similar to bacterial conjugation systems. Bacteria use the T4SS to translocate DNA and/or proteins directly into host cells. For example, *Agrobacterium tumefaciens* delivers a tumour-inducing transfer DNA (tDNA) that gets integrated into the genomic DNA of the host plant, causing crown gall disease (Abramovitch et *al*, 2006). Some components of the T4SS share similarities with the T2SS. The T5SS secretes bacterial proteins into the extracellular milieu and is composed of only 1 or 2 proteins. This secretion system uses the Sec system to transport proteins from the cytoplasm into the periplasm. The secreted substrate contains: i) a C-terminal domain that forms a pore in the outer membrane, and ii) a passenger domain that is translocated through the pore. (In some cases, the two domains are in separate proteins). One of the well known proteins secreted through the T5SS is the immunoglobulin A1 (IgA1) protease secreted by *Neisseria gonorrhoeae* (Henderson et *al*, 2004). The T6SS is a newly discovered system and will be discussed in detail below.

P. syringae contains all of the secretion systems described above except for the T4SS. Deletion of the T2SS of *Ps*t DC3000 decreased its ability to grow and to cause chlorosis in *A. thaliana* and tomato (Bronstein et *al*, 2005). Thus, both the T2SS and T3SS are important for the pathogenicity of *P. syringae*. However, little is known about the roles of the other secretion systems in *P. syringae* virulence. This study aims to characterize the role of the T6SS in *P. syringae* interactions with plants.

Although the T6SS was first discovered in *R. leguminosarum*, its role in bacteria-host interactions was not fully appreciated until its discovery in the 037 strain of *Vibrio cholerae* (Bladergroen et *al* 2003; Pukatzki et *al*, 2006). Further analysis revealed that T6SSs are highly conserved in many bacteria. The T6SS is encoded by a cluster of several genes. The gene cluster contains 12 to 20 genes, depending on the bacterial species (Cascales, 2008). A survey of 400 bacterial genome sequences revealed that more than 100 bacteria have T6SS gene clusters (Bingle et *al*, 2008). Many bacteria contain only one T6SS gene cluster, such as *Ps*p 1448a and *Ps*s B728a. However, a few organisms possess more than one T6SS gene cluster. *Ps*t DC3000 encodes two and *Yersinia pestis* and *Photorhabus luminescens* each encode four (Bingle et *al*, 2008).

The Type VI Secretion System

Though little is known about the T6SS, some of the basic components have been characterized. The names of T6SS genes in *R. leguminosarum* and *P. aeruginosa* and their potential functions are listed in Table 1. The T6SS appears to form a channel spanning the inner and the outer membranes of Gram-negative bacteria (Fig 2). The inner membrane channel may be formed by ImpA/PA0082 family members and potentially other proteins (Table 1). The outer membrane channel is formed by a protein homolog of ImpL from *Rhizobium legumimosarum* (or IcmF of *P. aeruginosa*) (Table 1). The regulation of the T6SS

is controlled by several proteins located in the cytoplasm, including the phosphatase ImpM/PppA, the kinase, ImpN/PpkA, and ImpI/Fha. When Fha is dephosphorylated by PppA in *P. aeruginosa*, the T6SS is in its inactive state. The kinase PpkA counteracts PppA activity by phosphorylating Fha and turning on the T6SS system. PpkA is an inner membrane protein that may be activated by an unknown environmental cue in the bacterial periplasm (Figure 2). Secretion is also controlled by the ATPase ClpV, which is a member of the ClpB protein family. ClpV is a cytoplasmic protein located at the base of the apparatus that provides the force for assembling the system as well as energy for the secretion of proteins outside of the bacterium (Mougous et al, 2007) (Figure 2).

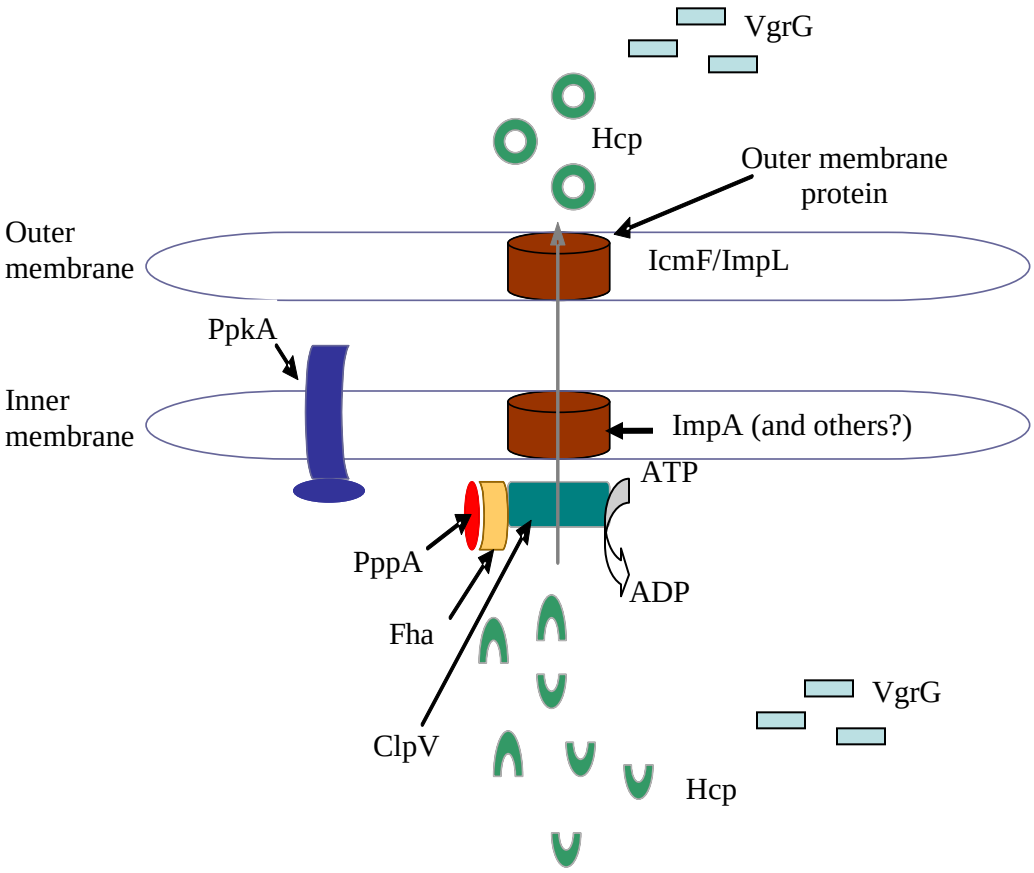


Figure 2 A model of the T6SS: The system is composed of inner and outer membrane channels. A protein kinase PpkA senses a signal in the environment, leading to phosphorylation of the activator protein Fha, which is kept in its inactive state when dephosphorylated by the protein phosphatase PppA. Phosphorylation of Fha activates the whole system, recruiting ClpV to provide energy by hydrolysis of ATP, leading to the secretion of Hcp and VgrG.

Table 1. The genes of the T6SS clusters in *Ps*p 1448a and *Ps*s B728a

R. l.[1] T6SS gene	*P. aeruginosa* T6SS (HSI-1) gene	*Ps*s B728a gene	% Id/sim B728a to *R. l* [2]	*Ps*p 1448a gene	% Id/sim 1448a to *R. l.*[3]	Other similar genes/ Potential function
impA	PA0082	4966	24/42	0121	25/40	Inner membrane protein
hcp	*hcp*	4965	27/47	0122	27/47	Secreted protein
impL	*icmF1*	4964	32/49	0123	31/50	Outer membrane protein
impM	*pppA*	4963	36/47	0124	35/48	Protein phosphatase
impL	*icmF1*	4962	27/45	0125	28/45	Outer membrane protein
impK	PA0078	4961	28/42	0126	28/42	Flagellar torque generating protein, Motb
impJ	PA0078	4960	30/49	0127	30/49	?
None	PA0080	4959	31/49	0128	32/48	Lipoprotein
clpV	*clpV*	4958	37/54	0129	37/55	AAA+ family ATPase
impH	PA0089	4957	33/48	0130	33/48	?
impG	PA0088	4956	37/53	0131	36/53	?
None	None	4955	28/55	0132	28/55	Lysozyme-related protein of *P. putida*
impC	PA0084	4954	54/71	0133	54/71	?
impB	PA0083	4953	50/65	0134	51/65	?
impN	*ppkA*	2101	34/51	2072	28/42	Protein kinase
impI	*Fha*	None	None	None	None	Fork-head domain

[1] *R. l. = Rhizobium leguminosarum*

[2] % Id/sim B728a to *R. l* = % identity/similarity of the *Ps*s B728a T6SS gene to the corresponding gene in *R. leguminosarum* bv. trifolii as determined by BLAST analysis.

[3] % Id/sim 1448a to *R. l.* = % identity/similarity of the *Ps*p 1448a T6SS gene to the corresponding gene in *R. leguminosarum* bv. trifolii as determined by BLAST analysis.

Two proteins have been shown to be secreted by the T6SS in *V. cholerae*: Hcp (haemolysin co-regulated protein) and VgrG (Valine-Glycine repeats) (Pukatzki et *al*, 2006). VgrG is translocated into host cells and has the ability to covalently crosslink actin (Pukatzki et *al*, 2006). Homologs of Hcp and VgrG are present in many bacteria. A recent study has also identified EvpP from *Edwardsiella tarda* as a secreted protein (Zheng and Leung, 2007). TssM from *Burkholderia mallei* is also a candidate substrate of T6SS because of the presence of an ubiquitin specific proteinase domain. Since bacteria lack ubiquitination systems, TssM may have a function inside eukaryotic cells. However, its secretion has yet to be demonstrated (Schell et *al*, 2007).

Many aspects of T6SS function remain to be characterized. One of the biggest debates centers on whether the T6SS is delivering secreted proteins into the extracellular milieu or into host cells. Even though Hcp and VgrG are secreted proteins, they may be also part of the secretion apparatus that delivers proteins into host cells. Deletion of either *hcp* or *vgrG* blocked the ability of *Edwardsiella tarda* to secrete both proteins into the supernatant. In addition, Hcp monomers associate into a ring-like structure that may act as a scaffold for the secretion system and also may be a channel for secretion of other proteins (Pukatzki et *al*, 2007, Zheng and Leung, 2007).

Role of the T6SS in bacterial pathogenesis

Studies have revealed a significant role of the T6SS in bacterial-host interactions. In *V. cholerae*, T6SS genes are necessary for its ability to kill the slime mold amoeba *Dictyostelium discoideum.* In addition, *V.cholerae* T6SS mutants lost the ability to cause cytotoxicity in J774 macrophages (Pukatzki et *al*, 2005).

Recently, a study on *Burkholderia mallei*, the bacterial cause of glanders disease in horses, mules, donkeys, and occasionally humans, has shown that this bacterium uses the T6SS to infect its hosts. A *B. mallei* mutant lacking the T6SS was unable to secrete Hcp into culture supernatants. Moreover, the mutant lost its virulence in the hamster model of infection (Schell et *al*, 2007).

In the opportunistic pathogen *P. aeruginosa*, the T6SS is encoded on the Hcp secretion island (HSI). Interestingly, *P. aeruginosa* has three HSI loci: HSI1, HSI2 and HSI3. To date, studies have mostly focused on the role of HSI1 in virulence. Analysis of sputum from patients with chronic fibrosis (CF) showed that they produce antibodies to Hcp1 which is encoded on HSI1. Hcp1 secretion by *P. aeruginosa* in culture was also shown to be dependent on the HSI1 T6SS. This allowed the authors to speculate that the HSI1 T6SS is required for the virulence of *P. aeruginosa* (Mougous et *al*, 2006). The T6SS is also required for the *P. aeruginosa* to cause chronic lung infection in rats (Potvin et al, 2003).

In a study conducted on *B. cenocepacia*, T6SS genes were reported to be controlled by a global virulence regulator, AtsR, which is a sensor kinase located in the inner membrane. Deletion of *atsR* both increased biofilm formation and altered T6SS function (Aubert et *al*, 2008).

The T6SS may also modulate bacterial interactions with plants. *Rhizobium leguminosarum*, a plant symbiont that forms nitrogen-fixing nodules on leguminous plant encodes a T6SS. A T6SS mutant strain of *R. leguminosarum* was able to form functional nodules on a plant that was not efficiently nodulated by a wild-type strain. Analysis of the proteins secreted by the two strains showed that the T6SS mutant was unable to secrete a protein of about 27 kDa (related to the size of Hcp). The genes encoding that secretion system were named *impA* to *impN* (<u>imp</u>aired in nodulation) (Bladergroen et *al.* 2002).

The T6SS in *P. syringae*

BLAST searches on the *Ps*t DC3000, *Ps*p 1448A and *Ps*s B728a genomes reveal the presence of a gene cluster encoding a T6SS. The cluster is composed of 14 genes in *Ps*p 1448a and *Ps*s B728a, and 24 genes in *Ps*t DC3000. Table 1 lists the genes found in the T6SS genes clusters of *Ps*p 1448a, as well as the percent identity and similarity to the T6SS genes in *R. leguminosarum*. In addition, the gene organization of the *Ps*s B728a, *Ps*p 1448a and *Ps*t DC3000 T6SS clusters is shown in Figure 3. The T6SS genes in *Ps*s B728a and *Ps*p 1448a are organized identically, while genetic rearrangements appear to have occurred in *Ps*t DC3000. Comparison of the three genomes also reveals that the T6SS gene clusters in *Ps*p 1448a and *Ps*s B728a lack homologs of *vgrG, ppkA,* and *fha* (Figure 3). These genes are all found in the T6SS gene cluster of *Ps*t DC3000. Although *vgrG* and *ppkA* are found elsewhere in the *Ps*p1448a and *Ps*s B728a chromosomes, *fha* appears to be completely absent from these strains. This may suggest that the T6SS of *Ps*p1448a and *Ps*s B728a are regulated differently or are nonfunctional. However, the T6SS of *V. cholerae* functions without Fha, suggesting that this component may not be essential (Pukatzki et *al*, 2006). Figure 3 also shows that the *Ps*t DC3000 T6SS gene cluster encodes a potential transcription factor (PSPTO5424) that is absent in *Ps*s B728a and *Ps*p1448a.

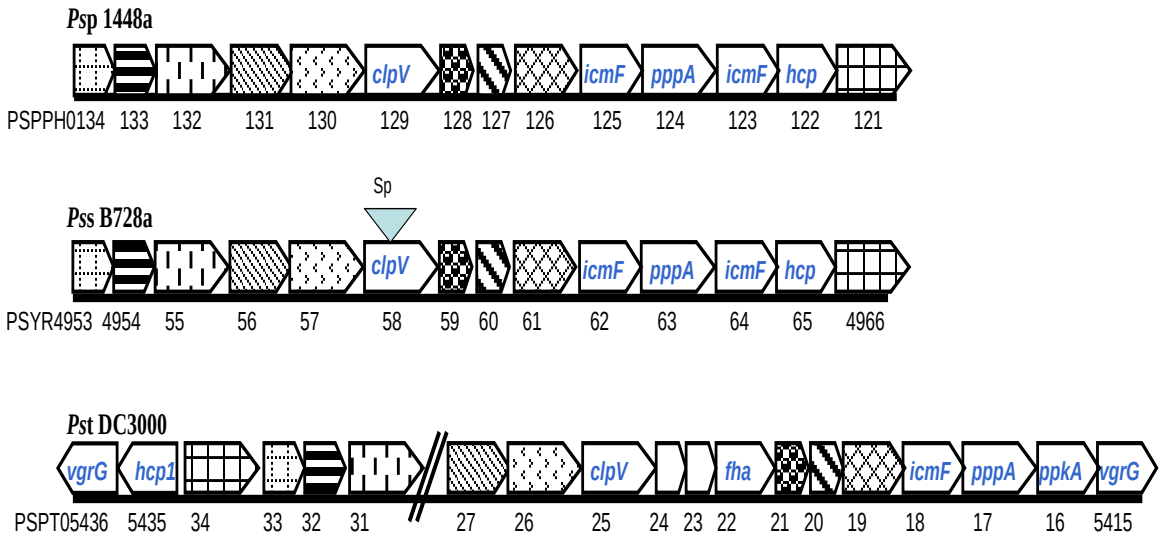


Figure 3 Comparison of T6SS genes in the three sequenced strains of *P. syringae*. The gene organization of the *Psp* 1448a *Pss* B728a and *Pst* DC3000 is shown. Homologous genes are colored and shaded identically. The genes with known functions in the T6SS (*clpV, pppA, pppkA, icmF, hcp, vgrG*) are indicated. Sp indicates the insertional mutation introduced into the *clpV* gene of *Pss* B728a. The region between PSPTO5131 and PSPT05435 in *Psp* DC3000, indicated by the slanted double bars, contains mobile genetic elements that are not conserved in other T6SS gene clusters.

Thesis overview

The sequenced strains of *P. syringae* all contain T6SS gene clusters. However, it is unclear whether the T6SS is produced or if it is functional in these strains. In this study, I verified that T6SS genes are expressed in *Pst* DC3000, *Psp* 1448a, and *Pss* B728a. I also found that the T6SSs in *Psp* 1448a and *Pss* B728a are functional, as they are able to secrete the Hcp protein.

In addition, I examined whether mutations in the *Psp* 1448a and *Pss* B728a T6SS gene clusters affect various *P. syringae* interactions with plants. My results show that the T6SS is not required for the virulence and the growth of *Pss* B728a and *Psp* 1448a in their host plants. However, the T6SS of *P. syringae* may modulate biofilm formation, since the *Psp* 1448a T6SS mutant showed altered biofilm formation. Thus the T6SS may play a role in regulating *P. syringae* aggregation on the leaf surfaces.

Chapter II: Materials and Methods

Strains and media

The stains of *P. syringae* used in this study are listed in Table 2 and the plasmids are listed in Table 4. Prior to growth and virulence assays, all strains of *P. syringae* were grown in King's B medium (King et *al,* 1954) at 30°C with the antibiotics listed in Table 2 at the concentration of 50 μg/ml. *E. coli* strains carrying the plasmids were grown in LB media at 37°C overnight with their respective antibiotics at the concentration of 50 μg/ml (Sambrook et *al*., 1989).

Gene expression assays

Bacteria were grown overnight in liquid media and washed three times with the respective media in which they were grown. Bacteria grown in KB (King *et al*, 1954) rich media were started at OD_{600}=0.1 as they grow faster in rich media, whereas for the Hrp minimal medium HrpMM, Hoitik-Siden medium amended with sucrose (HSS) (Peñaloza-Vásquez et *al*, 2000) and Mannitol-Glutamate (MG) minimal medium (Kaene et *al*, 1970), bacteria were started at OD_{600} = 0.05. Bacteria were grown up to ~OD_{600} = 0.5 at room temperature with shaking and pelleted for 20 min at 13000 rpm. RNA was extracted and cDNA was synthesized using random hexamers and Superscript III reverse transcriptase (Invitrogen). PCR was the performed on the cDNA using primers that hybridize to *hcp, clpV, hrpL,* or *gap1* (Table 5). PCR was also carried out on chromosomal DNA to insure the primers were functional and on DNA - free RNA to ensure that the RNA preparation was free of DNA contamination.

Construction of the T6SS gene cluster mutation in *Ps*p 1448a

Two fragments flanking the T6SS gene cluster of *Ps*p 1448a were PCR-amplified using ExTaq (Takara). The upstream flanking region of the T6SS gene cluster, including

ORF PSPPH0136 to PSPPH0133, was PCR amplified using primers p45 and p46 (Table5). p45 has an *EcoRI* restriction site attached at the 5'-end, and p46 has a *XhoI* at the 5'-end. The downstream region flanking the T6SS gene cluster containing the genes PSPPH0120 to PSPPH0115 was PCR amplified with p47and p48. A *XhoI* restriction site was attached at the 5'-end of p47 and a *Hind III* site was attached at the 5'-end of p48. pK18*mobsac* (Schäfer et *al*, 1994), a suicide vector was digested with *EcoRI* and *Hind III* restriction enzymes from New England Biolabs. The two digested PCR fragments (p45-46 and p47-48) were then ligated into pK18*mobsac*. This construct was named pTRI and was electroporated into *Ps*p 1448a. Recombination between the flanking regions of the T6SS gene cluster carried on pTR1 and the homologous sequences on the *Ps*s 1448a chromosome conferred kanamycin resistance and caused a duplication of sequences flanking the T6SS. Bacteria that underwent a second recombination event between the duplicated sequences were selected by plating on 5% sucrose. Since the *sacB* gene on pK18*mobsac* confers sucrose sensitivity, strains that lost the plasmid during the second recombination were not sensitive to 5% sucrose. Bacteria that contained the ΔT6SS mutation were confirmed by PCR using primers P144 and p145 (Table 5).

Construction of *Ps*s B728a *clpVΩ*

A truncated fragment of the *clpV* gene of *Ps*s B728a was PCR-amplified using p111 and p119. This fragment was ligated into the suicide vector pKnockoutΩ digested with *XcmI*, and the construct was named pTR8. pTR8 was electroporated into *Ps*s B728a. Bacteria that had pTR8 inserted into their genomes due to recombination between plasmid and chromosome *clpV* sequences were then selected on media containing spectinomycin. The *Ps*s B728a *clpVΩ* mutants were confirmed by PCR using two sets of primers: p134-p136 and p135-p137, as shown in Figure 6.

Protein preparation and Western analysis

A plasmid expressing the FLAG-tagged Hcp2 from *Pst* DC3000, called pHcp2-FLAG, was provided by K. Moore from the Schechter lab. pHcp2-FLAG was electroporated into both the wild-type and T6SS mutant *Psp* 1448a and *Pss* B728a along with pUFR034, a plasmid carrying the *nptII* cassette. Bacteria containing pHcp2-FLAG and pUFR034 were grown in KB media starting at OD_{600}=0.1 until they reached OD_{600}=0.4. Bacteria were then pelleted by centrifugation at 13000 rpm and the supernatant was filtered through a 0.2μM low protein binding filter. Proteins from the supernatant were precipitated with PRMM (Pyrogallol Red-molybdate-methanol) solution overnight as described by Randolph et *al* (2004), pelleted at 15000 rpm for 45 minutes at 4 °C, and dried for 30 minutes. Proteins from both supernatant and pellet were resolved by electrophoresis on 10% SDS-PAGE at 100V for ~4 hours. Proteins were then transferred onto PVDF and subjected to Western analysis by a standard protocol (Sambrook and Russel, 1989). Monoclonal antibodies against the FLAG tag were used to detect Hcp2-FLAG and polyclonal antibodies against NptII were used to detect NptII. Secondary antibodies conjugated to horseradish peroxidase were acquired from Sigma-Aldrich.

Bacterial growth *in vitro*

Bacteria were grown on KB plates for two days, scraped, and re-suspended to OD_{600} =0.1 in Hrp minimal or KB media. Bacteria were then grown with shaking at 24°C until sustained stationary phase was reached. Optical density measurements were taken with a spectrophotometer every hour for KB and every 3 hours for HrpMM.

Plant growth and care

Beans were grown in a growth chamber set at 16 hours light/8 hours dark and ~25-27 °C for 4 weeks. *Nicotiana. benthamiana* plants were grown in the greenhouse for 3-4

weeks at 23°C with natural lights and humidity of ~38%. *N. benthamiana* and *N. tobaccum* cv. Xhanti plants were transferred into the laboratory 12 hours prior to infection. *Arabidopsis thaliana* plants were grown in a growth chamber for six weeks at 20 °C with 12 hours light and 35-38% humidity.

Hypersensitive Response (HR) assay

Bacteria were suspended in 10mM $MgCl_2$ to OD_{600} = 0.3 (~$5x10^8$ CFU/ml). Serial dilutions of $5x10^7$, $5x10^6$, $5x10^5$, $5x10^4$ CFU/ml were then prepared. Leaves of *N. tobaccum* cultivar Xanthi, were poked with a needle and then infiltrated with the five different dilutions using a blunt ended syringe. Inoculated areas were circled with a permanent marker and the plants were kept at 24°C. Photographs were taken 24 hours post-inoculation.

Bacterial growth assay *in planta*

*P*sp 1448a and *P*ss B728a were grown for 2 days on KB plates, scraped, and suspended in 10 mM $MgCl_2$ to OD_{600} =0.3. Each bacterial suspension was then diluted 50 fold to ~10^8 CFU/ml and Silwet L-77 was added at 0.02%. Plants were dipped into the bacterial suspensions for 30 seconds. Infected plants were kept in a growth chamber at a relative humidity of ~80% and 28°C for 48 hours, and then transferred to 23°C and a relative humidity of ~43%. Photographs of tobacco infected with *P*ss B728a were taken after seven days to observe wilting symptoms and specks on leaves. To measure bacterial growth *in planta*, ten discs of 0.5 cm^2 were taken from four leaves of each plant. Discs were resuspended in 500 µl of 10 mM $MgCl_2$ and ground with an electrical Dremel. The grinder was also rinsed with 500 µl of $MgCl_2$ to recover the maximum number of bacteria. Ten-fold serial dilutions were prepared on 96-well plates and 2 µl samples were plated on KB containing the appropriate antibiotics and incubated at 30 °C. The plates also contained

cycloheximide at the concentration of 25µg/ml to prevent growth of fungi. Colonies were visually counted after ~48 hrs.

For the bacterial growth study in *A. thaliana*, six-week-old plants were infected by dipping in 1L of a 10^8 CFU/ml bacterial suspension. Three plants were collected per bacterial strain and placed into microfuge tubes after roots were removed. Bacteria were extracted by suspending leaves in 10 mM $MgCl_2$ + 0.02% silwet L-77 and shaking for an hour at 30˚C. Serial dilutions were prepared in 96-well plates, and 2 µl were plated on KB plates with appropriate antibiotics for 48 hours. Data was subjected to the Kruskal-Wallis test using the software SPSS.

Vascular flow assay

N. benthamiana leaves were poked with a needle in four spots and infiltrated with bacterial suspensions of *Pss* B728a in 10mM $MgCl_2$ at OD_{600}=0.1. Plants were incubated 6 hours at 24°C and leaves were detached using a razor blade. Petioles were dipped into 0.1% Neutral Red (pH 6.8) (Oh and Collmer, 2005) and photographs were taken 6-7 hours later. For quantification of the dye flow, three 0.5 cm-diameter leaf discs within the infected area were collected using a cork borer and suspended in 500µL 95% ethanol. Tubes were then shaken for an hour at 30°C to extract the Neutral Red dye. The extracted dye was diluted four times with 5mM MES (pH 5.5) and an optical density reading at 562 nm was taken using a spectrophotometer.

Biofilm formation assay

The biofilm assay was conducted according to the protocol of O'Toole *et al* (1998) with slight modification. Bacteria were grown in KB media overnight, then washed two times and resuspended in HrpMM to an OD_{600}=0.1. 100µl of each strain was transferred into a 96-

well PVC plate. Five replicates were performed for each strain. Plates were set at 24 °C for 48 hours to allow biofilm formation. The biofilm was then stained with 25 µl per well of 0.1% crystal violet (CV), shaken for 15minutes, and rinsed with distilled water to remove unbound cells and excess dye. The CV-stained biofilm was dissolved in 300 µl of 95% ethanol for 30 minutes at room temperature, and optical density readings were taken using a spectrophotometer set at 595nm. As a control, wells loaded with only 95% ethanol were used as blank.

Table 2 Bacterial strains used in this study

Strains	Antibiotic Resistance	References
*Ps*t DC3000	Rifampicin (Rif)	Cuppels, 1986
*Ps*p 1448A wild-type	Rifampicin (Rif)	Taylor et *al*, 1996
*Ps*p 1448A *hrpR::nptII*	Kanamycin (Km)	Provided by John Mansfield
*Ps*p 1448A ΔT6SS	Rifampicin (Rif)	This study
*Ps*s B728A wild-type	Rifampicin (Rif)	Willis et *al*, 1988
*Ps*s B728a *hrcC::nptII* (CUCPB5102)	Kanamycin (Km)	Hirano et *al*, 1999
*Ps*s B728A *clpV*Ω	Rif + Spectinomycin(Sp)	This study

Table 3 Genes examined by RT-PCR

Strains/Genes	*hcp*	*clpV*	*hrpL*	*Gap1*
*Ps*p1448a (Size in bp)	PSPPH0122 (437bp)	PSPPH0129 (380bp)	PSPPH01294 (522bp)	PSPPH01176 (445bp)
*Ps*t DC3000 (Size in bp)	PSPT05435 (388bp)	PSPT05424 (376bp)	PSPT01404 (328bp)	PSPT01287 (313bp)
*Ps*s B728a (Size in bp)	PSYR04965 (424bp)	PSYR04958 (519bp)	PSYR01217 (113bp)	PSYR01108 (430bp)

Table 4 List of plasmids used in this study

Plasmid	Antibiotic resistance	Description	Reference
pTR1	Kanamycin	Construction of ΔT6SS *Ps*p 1448a	This study
pTR8	Spectinomycin	Construction of *Ps*s B728a *clpVΩ*	This study
pKnockout Ω	Spectinomycin	Vector for creating chromosomal insertions	Windgassen et *al*, 2000
pHcp2-FLAG	Gentamycin	Encodes Hcp2 with C-terminal FLAG tag	Provided by Kristin Moore
pUFR034	Kanamycin	Cloning vector containing nptII gene	De Feyter, et *al*, 1990
pK18*mobsac*	Kanamycin	Creation of pTR1	Schäfer *et al*, 1994

Table 5: List of primers

Primers#	Sequences (5'-3')	Purpose
P33	CCAAGGCTACCGGCAGGACC	RT-PCR *gap1* DC3000
P34	GCTGGCCGGTGCTGAGATAATC	RT-PCR *gap1* DC3000
P45	GGGAATTCAAACAGCGGGCAAATAAGT	Construction of type VI 1448a mutant
P46	ATCTCGAGGGCGCGATTAGAGTCC	Construction of type VI 1448a mutant
P47	GCCTCGAGCAATTCGTAGGATCGGT	Construction of type VI 1448a mutant
P48	TAAAGCTTCCGCGCCCTGAGGATAAC	Construction of type VI 1448a mutant
P84	CCCAGCCACGCCAGGACTC	RT-PCR 1448a *hrpL*
P85	CACAGTGCCAATCGGAACGC	RT-PCR 1448a *hrpL*
P86	CGAGCATACGATTGGAGAACGC	RT-PCR 1448a *gap1*
P87	GCCTACACCAACGACCAGAACC	RT-PCR 1448a *gap1*
P96	ACCGAACCACGCCAACCATC	RT-PCR of B728a *hrpL*
P187	CCACATCATCAGCGTTCTTCACC	RT-PCR of B728a *hrpL*
P98	TGCCTACACCAACGACCAGAACC	RT-PCR of B728a *gap1*
P99	GTCGAGCATGCGATTGGAAAAC	RT-PCR of B728a *gap1*
P106	GCGTCGATCGAATTCGGTCAG	Knocking out *clpV* 1448A and RT-PCR
P107	GCTCGCGCTTCCACTGTTGC	Knocking out *clpV* 1448A
P108	AGGATCGGGTTGTTCTGACGG	RT-PCR *clpV* in 1448a
P109	CATACATCCAAATCGACGGCAT	RT PCR *hcp* 1448A
P110	ACGGCTCTGTTGGGTGTAGGT	RT PCR *hcp* 1448A
P111	TGATCCTGCGCCACTACGATG	RT PCR *clpV* B728a and knocking out *clpV*
P112	CGGTCAGGATCGGGTTGTTCT	RT PCR *clpV* B728a
P113	TGGCATTTGACGCATACATCC	RT PCR *hcp* B728A
P114	TTGATGCGAGCGAAGTTGAA	RT PCR *hcp* B728A
P115	ATGAGCAATGTAGACCTGCAACAAC	RT PCR *clpV* DC3000
P116	CGTAGCGCATCGGGTTGC	RT PCR *clpV* DC3000
P117	CGCCAGCGTACATGTCCATCA	RT PCR *hcp* DC3000
P118	TCTTGGCAATTGTGCATGTAGTCC	RT PCR *hcp* DC3000
P119	CGGTGATGTAGCGGTTGGACAG	Knocking out *clpV* B728a
P120	CCAGCCCCGTCAGCCATC	RT PCR *hrpL* DC3000
P121	ATGCCCCTCTACCTGATGACTGAC	RT PCR *hrpL* DC3000
P134	CTGTGCCTGTCGGAGAACCATTAC	Checking *clpV*' B728A insertion
P135	TTCCCAGTCACGACGTTGTAAAA	Checking *clpV*' B728A insertion
P136	CGCGCAATTAACCCTCACTA	Checking '*clpV* B728A insertion
P137	GATTTCCGCGTTCAGGTTGTC	Checking '*clpV* B728A insertion
P138	CGACAACAACGATAACGACCTG	Checking *clpV*' 1448A insertion
P139	CGATGGTGCCGCCGTCT	Checking *clpV*' 1448A insertion
P144	TTGTCGTCGAAGCCTGTAAAAC	Checking delta T6SS mutant 1448A
P145	AGAAATATCCGCTCACAAGACG	Checking delta T6SS mutant 1448A

Chapter III: Results

The T6SS is expressed in *Pst* DC3000, *Psp*1448A and *Pss* B728A

Even though T6SS genes are present on the chromosomes of the three sequenced *P. syringae* strains, it is not known if they are expressed. The T6SS gene clusters of the three stains of *P. syringae* used here are shown in Figure 3. *Psp* 1448a and *Pss* B728a both have a single copy of the T6SS gene cluster, and each contain 14 genes. The T6SS genes of *Psp*1448a span from the ORF PSPPH0121 to PSPPH0134. The T6SS genes of *Pss* B728a span from the ORF PSYR4953 to PSYR4966. *Pst* DC3000 has two copies of the T6SS gene cluster, one of which is interrupted by a transposon insertion and may not be functional. The other T6SS cluster of *Pst* DC3000 spans from the ORF PSPTO5413 to PSPTO5436 and contains 24 genes (Figure 3).

To determine whether T6SS genes are expressed, the genome sequences of *Psp* 1448a, *Pss* B728a and *Pst* DC3000, available in Genbank and at the PPI (*Pseudomonas* Plant- Interaction) web site (http://www.pseudomonas-syringae.org/psp_gen_analy.htm) were used to design primers for reverse transcription PCR (RT-PCR). The primers are listed in Table 5 in the Materials and Methods. Two key genes were selected for analysis: *clpV* and *hcp*. *clpV* encodes a protein in the AAA+ family ATPase proteins, which may provide essential energy for secretion via the hydrolysis of ATP. *hcp* encodes a protein previously shown to be secreted by the T6SS (Pukatzki et *al*, 2006). Expression of both *clpV* and *hcp* might indicate that *P. syringae* utilizes its T6SS. Primers that hybridize to the *hrpL* and *gap1* genes were also designed to use as controls. The *gap1* gene encodes glyceraldehyde-3-phosphate dehydrogenase, an enzyme involved in glycolysis. The *gap1* gene is constitutively expressed and has been used as a positive control in other RT-PCR experiments in *P. syringae* (Schechter et *al*, 2006a; Ferreira et *al*, 2006). The *hrpL* gene, which encodes a

regulator of T3SS gene expression, is expressed in minimal media but not rich media. The gene sizes for each RT-PCR product are given in Table 3 of Materials and Methods.

RNA was extracted from bacteria grown in four different media: Hrp minimal medium (HrpMM) (Huynh et al, 1989), Hoitink-Sinden medium amended with sucrose (HSS) (Peñaloza-Vázquez et *al*, 2000), Mannitol-Glutamate medium (MG) (Keane et *al*, 1970), and King's B medium (KB) (King et *al*, 1954). These four media were chosen because they support *P. syringae* growth and have been shown to induce different sets of genes. HrpMM enhances the expression of *hrpL* and T3SS genes and may mimic the nutritional environment in the plant leaf apoplast (Huynh et al, 1989). HSS is a minimal medium that optimally induces the expression of the coronatine biosynthesis genes in *Ps*t DC3000 (Peñaloza-Vázquez et *al*, 2000). MG is another minimum medium that is often used for *P. syringae* growth (Keane et *al*, 1970). KB is a rich medium that reduces T3SS gene expression. cDNA was synthesized and amplified with PCR. As a positive control, PCR was performed on chromosomal DNA. PCR was also conducted on DNA-free RNA to rule out chromosomal DNA contamination.

The results of the RT-PCR revealed that *hcp* is expressed at relatively high levels in *Ps*t DC3000, *Ps*p 1448a and *Ps*s B728a in all four media (Figure 4). The *clpV* gene was expressed in all four media in *Ps*t DC3000 and *Ps*s B728a. However, in *Ps*p 1448a *clpV* was not detected in HSS, and only barely detectable in HrpMM, MG and KB. As expected, *gap1* was constitutively expressed in all media, and *hrpL* was repressed in KB in the three strains. Although *hrpL* was more highly induced in HrpMM, in *Ps*p 1448a and *Ps*s B728a, it appears to be equally expressed in HrpMM, HSS and MG in the *Pst* DC3000. PCR on RNA that was not subjected to reverse transcription revealed that there was no DNA contamination in the

RNA preparation. Overall, these results indicate that *clpV* and *hcp* are expressed under several conditions in *P.syringae*.

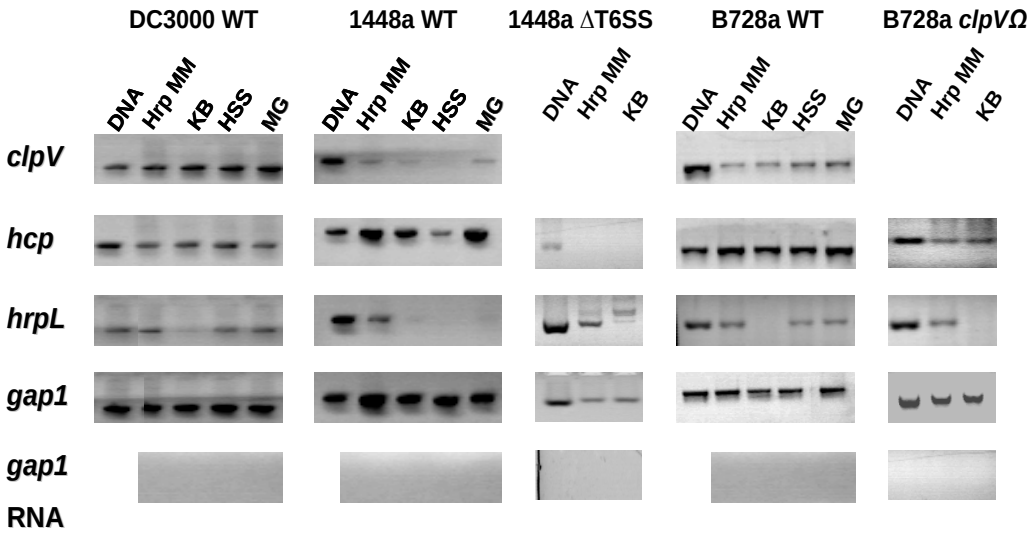


Figure 4 The T6SS is expressed in *Pst* DC3000, *Psp* 1448A and *Pss* B728A. *Pst* DC3000 WT, *Psp* 1448a WT, *Psp* 1448a ΔT6SS, *Pss* B728a WT, and *Pss* B728a*clpVΩ*, strains were grown in HrpMM, KB, HSS or MG media to OD_{600nm} =0.5. RNA was isolated and cDNA was synthesized as described in the Materials and Methods. The *clpV*, *hcp*, *hrpL* or *gap1* genes were then PCR amplified from cDNA samples using the primers in table 5. Reactions were also carried out on *P. syringae* DNA samples as positive controls and were run out in the lanes labeled DNA. The sizes of the PCR products are as follows: for *Pst* DC3000: *clpV*, 376bp; *hcp*, 388bp; *hrpL* 328bp; *gap1*, 313bp. For *Psp* 1448a: *clpV*, 380bp; *hcp*, 437bp; *hrpL*, 522bp; *gap1*, 445bp. For *Pss* B728a: *clpV*, 519bp; *hcp*, 424bp; *hrpL*, 113 bp; *gap1*,430 bp. The row labeled *gap1* RNA shows the PCR product of *gap1* primers using RNA samples that were not reverse transcribed into cDNA as the template.

Construction of a T6SS gene cluster mutations in *Ps*p 1448a and *P*ss B728a

Because T6SS genes are expressed in all three sequenced *P. syringae* strains, the next step was to assess whether the T6SS is functional. Two mutant strains were created: a *Ps*p 1448a strain that lacks the whole T6SS pathogenicity island, and a *Ps*s B728a strain containing an insertion in the *clpV* gene. The region encoding the T6SS of *Ps*p 1448a contains 14 genes (PSPPH0121-PSPPH0134), as described earlier. I created a *Ps*p 1448a mutant lacking the entire T6SS locus by PCR amplifying two ~1 kb regions flanking the T6SS cluster. These PCR products were cloned into the suicide vector pK18*mobsac* to create the plasmid pTR1 (Figure 5A). pTR1 does not replicate in *P. syringae* and contains a kanamycin resistance gene as well as the *sacB* gene, which confers sucrose sensitivity. These markers allow for selection of plasmid integration and excision from the *P. syringae* chromosome, as described in the Materials and Methods. Mutant candidates that underwent allele replacement were screened by PCR using the set of primers p144-p145. PCR amplification of the chromosome of the positive recombinants produced a ~2.4kb product as shown in Figure 5B. As a control, PCR was also performed on the wild-type genomic DNA. No product was produced, as the ~19 kb T6SS region is too large to amplify by PCR (Fig 5B). RT-PCR on the key gene *hcp* was also done on the *Ps*p 1448a ΔT6SS mutant. As shown in Figure 4, *hcp* was only expressed in the wild-type *Ps*p 1448a but not in the T6SS mutant, confirming that the mutation successfully knocked out the T6SS gene cluster. The faint band observed in the lane indicated DNA for the 1448a ΔT6SS mutant is slightly smaller than the expected size of the PCR product and may be a nonspecific product from the primer set. In addition, the *hrpL* gene was expressed in HrpMM but not in KB rich medium in the T6SS mutant. Thus, deleting the T6SS in *Ps*p 1448a does not appear to affect expression of *hrpL*.

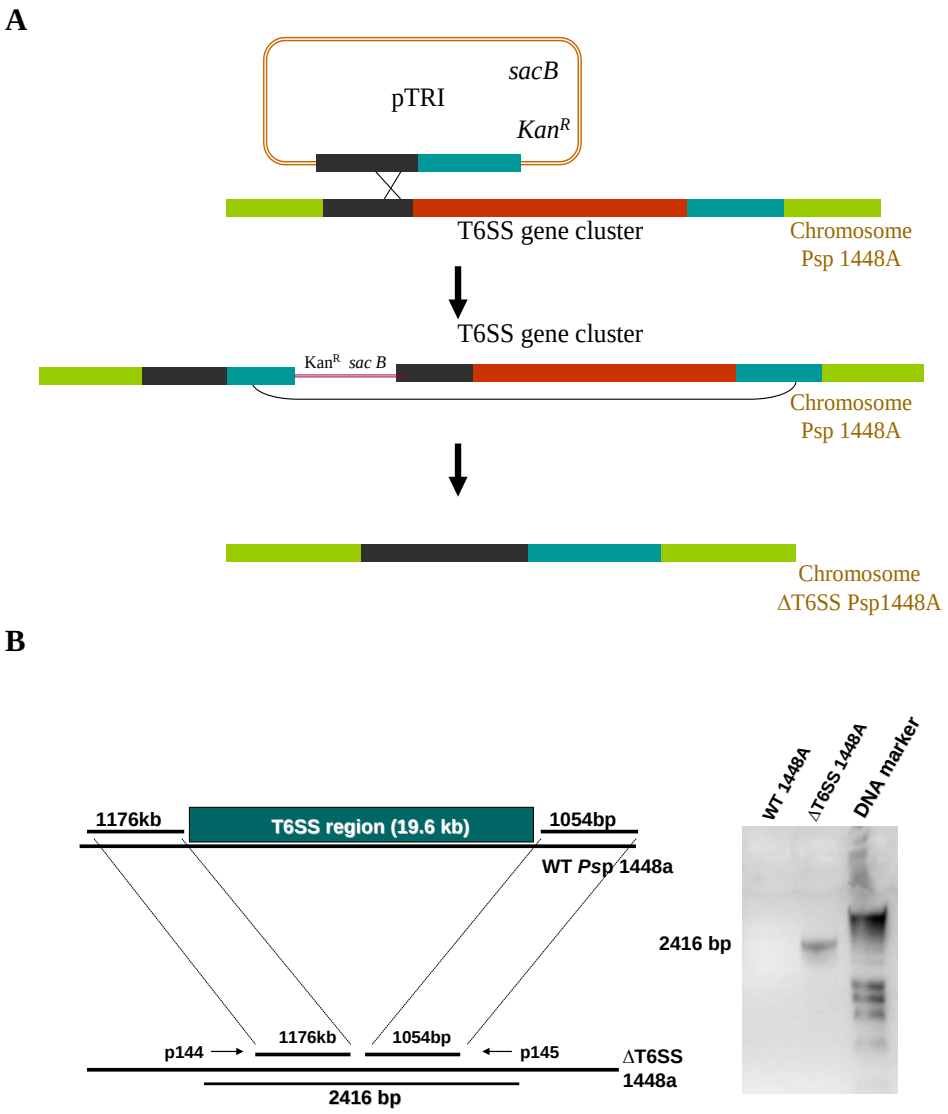


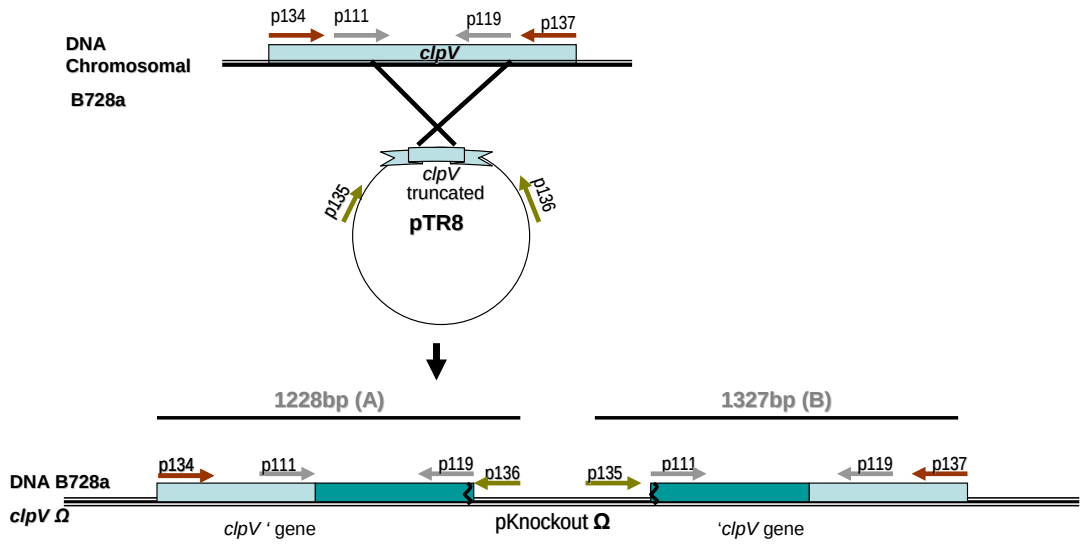
Figure 5 Creation of the ΔT6SS *Psp* 1448a mutant: ***A.*** Allele replacement was used to delete the T6SS gene cluster from *Ps*p 1448a.Two ~1 kb flanking regions upstream and downstream of the T6SS gene cluster were PCR amplified and ligated into the suicide vector pK18*mobsac* to obtain the plasmid pTR1. pTR1 was then electroporated into wild-type *Ps*p 1448a. After two crossover events the whole T6SS gene cluster was deleted from the genomic DNA. **B.** To screen for ΔT6SS mutants , chromosomal DNA was amplified with p144 and p145. These two primers bound outside of the two ~1 kb fragments in pTR1 in order to eliminate false positives. Products were electrophoresed on a 1% agarose gel. Presence of the 2146 bp band confirmed that the ΔT6SS mutant was obtained.

To inactivate the T6SS in *Pss* B728A, I created an insertion mutation in the homolog of *clpV* (PSYR4958). An internal fragment of the *clpV* gene was PCR amplified using primers p111 and p119 and cloned into pKnockout Ω (pKOΩ), a suicide vector carrying a spectinomycin resistance gene (Windgassen et al, 2002) (Figure 6A). The pKOΩ construct containing the truncated *clpV* gene was named pTR8. This plasmid was transformed into wild-type *Pss* B728a, and mutant bacteria containing pTR8 inserted in the chromosomal *clpV* gene were selected on spectinomycin. The insertion mutant, named *Pss* B728a *clpVΩ*, contains two non-functional, truncated copies of the *clpV* gene (Figure 6A). I verified that the *Pss* B728a *clpVΩ* mutant candidates contained the correct insertion by PCR using two sets of primers p134-p136 and p135-p137 (Figure 6A). Primers p134 and p137 anneal to *clpV* sequences in the chromosome that are absent from pTR8, whereas primers p135 and p136 anneal to vector sequences that are not present in wild-type bacteria. As shown in Figure 6B, PCR products of the expected sizes were only amplified when *Pss* B728a *clpVΩ* DNA was used as the template. No PCR products were generated with the primer sets p134-p136 or p135-p137 when wild-type was used as template, since p135 and p136 only anneal to vector sequences in pTR8 (Figure 6B).

The *clpVΩ* insertion mutation is assumed to disrupt the function of *clpV*. If the T6SS genes are organized in an operon driven by a single promoter, this insertion will also alter the expression of genes downstream from *clpV* (Figure 3). A study on the T6SS of the *Edwardsiella tarda* showed that the T6SS in this organism is controlled by a single promoter (Zheng et *al*, 2007). To determine whether the *clpVΩ* insertion affects expression of a downstream gene, RT-PCR was performed on the *hcp* gene of both wild type and *clpVΩ Pss* B728a. Since an RT-PCR product is detected for *hcp* in the *clpVΩ* mutant, the T6SS gene cluster (or at least the *hcp* gene) may be driven by more than one promoter (Figure 4). RT-PCR on the gene directly downstream from *hcp* (PSYR4966) also produced a product of

expected size (data not shown). In addition, the RT-PCR results show that the *hrpL* gene was expressed in HrpMM but not KB. Thus, as in *Ps*p 1448a, disruption of the T6SS does not appear to alter *hrpL* expression.

A



B

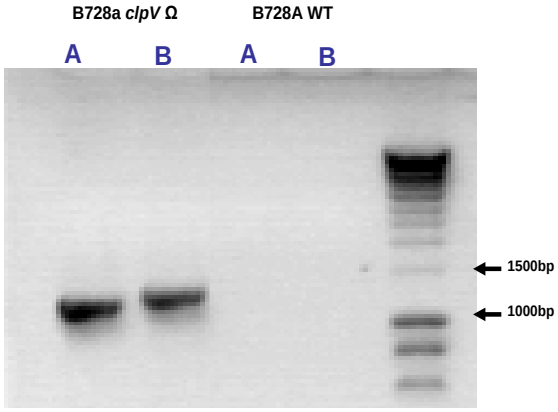


Figure 6 Construction of the *clpVΩ Pss* B728a: A. pTR8 was constructed by PCR-amplifying a portion of the *clpV* gene and inserting it into pKnockout Ω. Homologous recombination between pTR8 and the *P*ss B728a chromosome created *clpVΩ*, a mutant with a disrupted *clpV* gene. The *clpVΩ* mutant contains two truncated non-functional *clpV* genes: '*clpV* and *clpV*'. **B.** DNA from *clpVΩ* or wild-type *P*ss B728a were PCR-amplified using primers that hybridize to *clpV* and pKnockout Ω. Products were then run on a 1% agarose gel. DNA molecular weight markers were also run on the gel for size comparison. The sizes of the relevant PCR products are shown in part A. The positions of primers used to generate the truncated *clpV* fragment or to verify the insertion of pTR8 into the chromosome are indicated in part A.

The T6SS is required for the secretion of the Hcp protein in *P. syringae*

V. cholerae, P. aeruginosa and *E. tarda* have been shown to secrete the Hcp protein through the T6SS (Mougous et al, 2006, Pukatzki et al, 2006; Zheng and Leung, 2007). Therefore, I examined whether mutations in the *Ps*p1448a or *Ps*s B728a gene clusters reduce or even block Hcp secretion into the culture supernatant. To monitor Hcp secretion, a plasmid pHcp2-FLAG created by Kristin Moore from the Schechter lab was introduced into both wild-type and T6SS mutant *Ps*p 1448a and *Ps*s B728a strains. pHcp2-FLAG expresses a C-terminal epitope tagged version of the *Ps*t DC3000 Hcp2 protein (Table 4 of Materials and Methods). Bacteria were grown in KB and total cellular or secreted proteins were collected as described in the Materials and Methods. Proteins were then separated by SDS-PAGE and detected by Western analysis. As shown in Figure 7, the wild-type and T6SS mutant *Ps*p 1448a and *Ps*s B728a strains expressed Hcp2-FLAG as seen in the pellet fractions. Hcp2-FLAG was also detected in the culture supernatants of only the wild-type bacteria and not the mutants. To ensure that the Hcp2-FLAG in the supernatant was not due to cell lysis, the cytoplasmic protein Npt II was also examined in the cellular and secreted protein samples. The NptII protein was expressed from the pUFR034, a low copy plasmid that replicates in *P. syringae* (De Feyter et *al,* 1990). NptII was not detected in the secreted protein samples indicating that cell lysis did not occur. Taken together, these results show that the T6SSs of *P. syringae* are functional. Furthermore, the ΔT6SS and the *clpVΩ* mutations that I created disrupt the secretion of Hcp through the T6SS of *Ps*p 1448a and *Ps*s B728a, respectively.

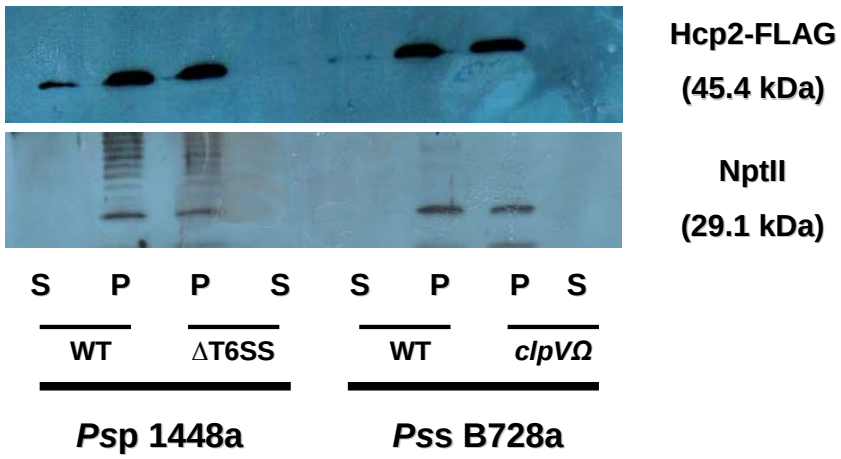


Figure 7 *Ps*p 1448a and *Ps*s B728a mutants lacking the T6SS do not secrete the Hcp protein.
ΔT6SS *P*sp 1448a and *clpVΩ* B728a or the corresponding wild-type (WT) strains carrying the plasmids pHcp2-FLAG and pUFR034 were grown in KB. Cell pellet (P) and culture supernatant (S) samples were then collected as described in the Material and Methods. Proteins were separated on a SDS-PAGE gel and Western analysis dwas performed using antibodies against the FLAG epitope tag or NptII.

Analysis of biofilm formation by *Ps*p1448a and *Ps*s B728a T6SS mutants

Biofilms are large aggregates of microorganisms that colonize solid surfaces. The formation of biofilms is important for many bacterial pathogens to infect their hosts. In fact, biofilm formation may be an important early step in *P. syringae* infection since survival on leaf surfaces is greatly enhanced if bacteria are located in large aggregates (Monier and Lindow, 2003). To determine whether the T6SS is involved in the modulation of biofilm formation, I assessed the ability of *P. syringae* to adhere to 96-well plates made of polyvinylchloride (PVC). This assay is based on the technique of O'Toole et *al.* (1998), who discovered that *P. fluorescens* adheres to PVC at the interface between the culture media in the well and the air.

As described in Materials and Methods, wild-type and T6SS mutant *P. syringae* strains were suspended in HrpMM and grown standing for 48 hours in 96-well plates. The biological stain crystal violet, which stains bacteria but not PVC, was then added to each well. The wells were rinsed and re-suspended in 95% ethanol. Finally, the amount of crystal violet in each well was quantitated with a spectrophotometer at OD_{595nm}.

As shown in Figure 8, *P*sp 1448a ΔT6SS showed an increase of more than 50% in biofilm formation compared to wild-type bacteria. Therefore, the T6SS may secrete factors that inhibit biofilm formation. In contrast, *Ps*s B728a formed a denser biofilm that was not altered by the *clpVΩ* mutation. These results may be explained by the fact that the *P*sp 1448a T6SS mutant lacks the entire T6SS gene cluster whereas the B728a T6SS mutant only has an insertion in the *clpV* gene. Alternatively, *Ps*s B728a may form biofilms independently of the T6SS.

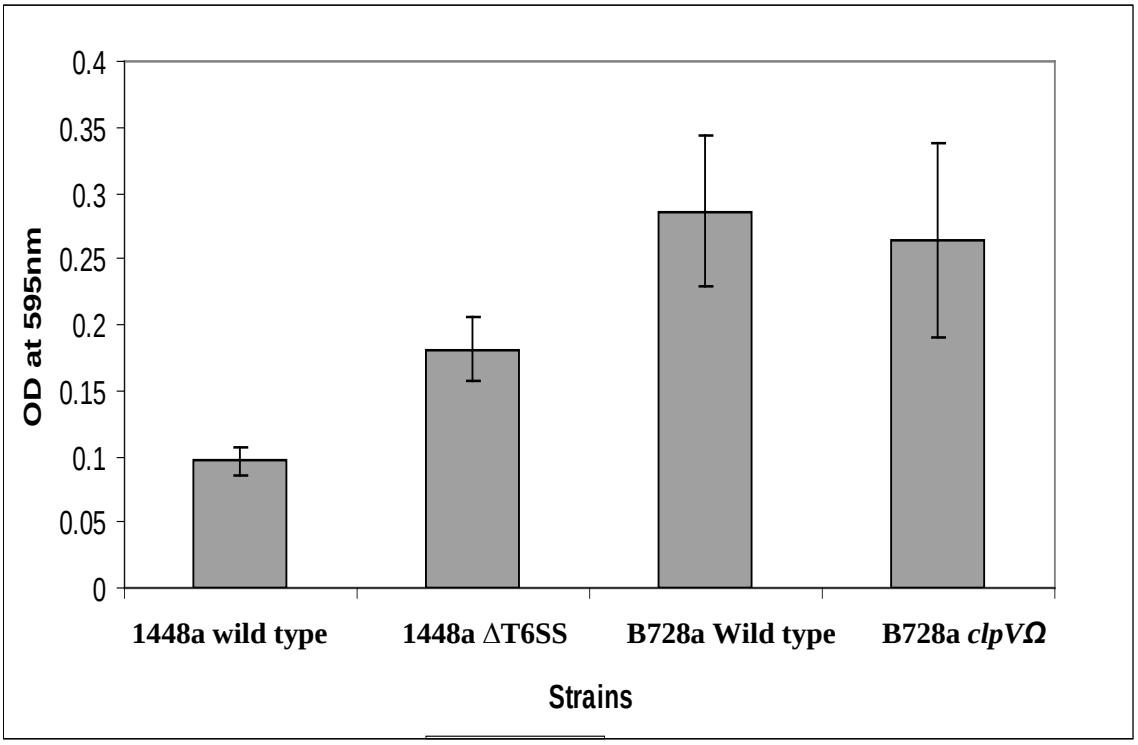


Figure 8 Quantitation of biofilm formation by *Ps*p 1448a and *Ps*s B728a T6SS mutants. Bacteria were suspended at OD_{600}=0.1 in HrpMM and incubated for 48 hrs in a 96-well plate to allow biofilm formation. Bacteria were then stained with crystal violet and the amount of stain in each well was determined at an optical density of 595 nm in a spectrophotometric plate reader. The values and error bars reported for each strain represent the average and standard deviation, respectively. Average values from five wells per strain were used.

Growth of *Ps*p 1448a and *P*ss B728a T6SS mutants in culture

The T6SS may be necessary for the growth of *P. syringae* in culture and/or *in planta*. To determine whether the ΔT6SS or *clpV*Ω mutations altered the growth of *Ps*p 1448a or *P*ss B728a in culture, wild-type and mutant bacteria were grown in HrpMM or KB until they reached the stationary phase. When grown in HrpMM, no significant difference was observed in the growth of the wild-type or T6SS mutant *Ps*p 1448a or *Ps*s B728a (Figure 9). The *hrpR::nptII* mutant of *Ps*p 1448a and the *hrcC::nptII* mutant of *Ps*s B728a were also included as controls. These mutants lack a functional T3SS and had growth rates similar to wild-type and T6SS mutant bacteria. In HrpMM, the *Ps*p 1448a strains reached a growth plateau after approximately 70 hrs, whereas *Ps*s B728a strains took only 50 hrs to reach stationary phase. When grown in the rich medium KB, bacterial growth was faster but still not significantly different between the wild-type and T3SS or T6SS mutant *Ps*p 1448a or *Ps*s B728a (Figure 9). Bacteria entered the exponential growth phase after about 6 hours and then plateaued after 10 to 12 hours. Overall these results show that the mutations in the T6SS gene cluster do not significantly alter *P. syringae* growth in culture media.

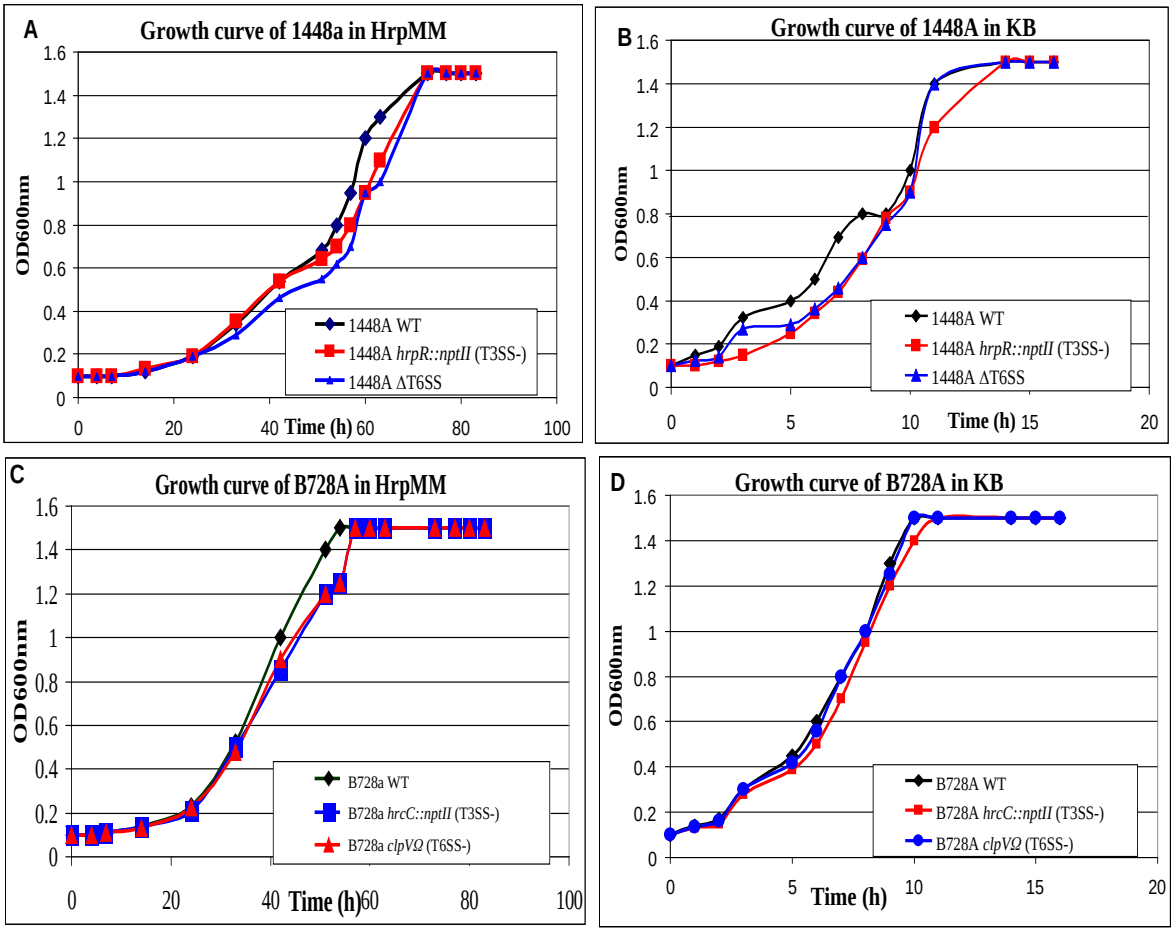


Figure 9 Mutations in the T6SSs of *Psp* 1448a and *Pss* B728a do not affect bacterial growth *in vitro*. The indicated bacteria were inoculated in 50 ml of HrpMM (A and C) or KB (B and D) and shaken at 24 °C until they reached sustained stationary phase. Bacterial growth was monitored by determining the optical density at 600nm (OD_{600nm}) in a Spectronic 20 Spectrophotometer. Each graph shows the result of one representative experiment. A repeated experiment yielded similar results.

Examination of *P*ss B728a *clpV*Ω growth and virulence *in planta*

N. benthamiana, a wild tobacco, is a host for *Ps*s B728a infection (Vinatzer et *al*, 2006). To determine whether the T6SS is required for *Ps*s B728a interactions with host plants, growth and virulence assays were performed in *N. benthamiana*. Plants were dipped into ~10^8 colony forming units (CFU) per ml suspensions of wild type or *clpV*Ω *Ps*s B728a. After a 7 day incubation period, leaf samples were collected and bacterial CFU per mg of leaf tissue were determined as described in the Materials and Methods. The results show that the *clpVΩ* mutant grew to between 10^7-10^8 cfu/mg in *N. benthamiana*, which was comparable to (or slightly better than) the wild-type *Ps*s B728a. Therefore, the T6SS is not required for optimal *Ps*s B728a growth *in planta*. As a control, plants were also inoculated with a *Ps*s B728a *hrcC::nptII* mutant, which lacks a functional T3SS. The *hrcC::nptII* mutant showed a reduced growth of about 34% when compared to wild-type *Ps*s B728a, which is consistent with previous findings (Vinatzer et *al*, 2006) (Figure 10).

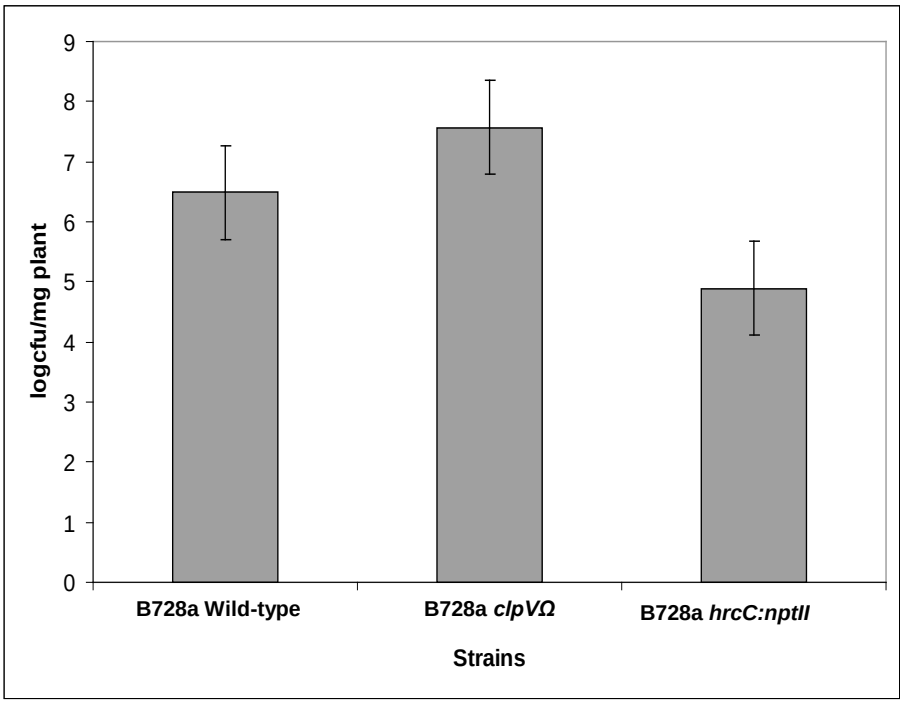


Figure 10 The *clpVΩ* mutation did not alter *P*ss B728a growth in *N. benthamiana*. Plants were infected with ~10^8 CFU/ml bacteria, incubated at 28 °C for 24 hours, and then transferred to 23 °C for 6 days. Bacteria were recovered from leaf samples and plated on selective media. The number of recovered bacteria is expressed as log cfu/mg leaf. Each bar represents the standard deviation of 4 samples of 10 leaf discs from 4 leaves of one plant. Repeated experiments yielded similar results.

Pss B728a causes wilt and speck lesions on *N. benthamiana*. To test the ability of the *clpVΩ* to cause disease in plants, *N. benthamiana* plants were dipped in 10^8 cfu/ml bacteria, incubated at 28°C for 24 hours and then transferred to 23°C for 6 days. As shown in Figure 11, wild-type and *clpVΩ* mutant bacteria caused similar wilting symptoms in *N. benthamiana*. Thus, the T6SS is not required for *Pss* B728a to cause disease in *N. benthamiana.* The *Pss* B728a *hrcC::nptII* mutant did not cause disease symptoms in *N. benthamiana*, in agreement with previous studies (Hirano et *al*, 1999, Vinatzer et *al*, 2006).

B728A *hrcC::nptII* (T3SS-) | B728A c*lpVΩ* (T6SS-) | B728A wild-type (WT) | Control (not dipped)

Figure 11 *Pss* B728a*clpVΩ* causes disease symptoms comparable to wild-type bacteria. *N. benthamiana* plants were dipped into ~ 10^8 cfu/ml bacterial suspensions and incubated at 28 °C for 24 hrs. Plants were then transferred to a growth chamber at 23 °C and 80% relative humidity. Photographs were taken 6 days after plants were transferred to 23°C.

Analysis of *Ps*p 1448a ΔT6SS growth and virulence in bean

*Ps*p 1448a causes halo blight on several varieties of beans, including Canadian Wonder and Tendergreen (Pitman et *al*, 2005). I tested the ability of the 1448a ΔT6SS mutant to infect and grow in these plants. Unlike in Tendergreen, the T3SS mutant *Ps*p 1448a showed decreased growth in Canadian Wonder in agreement with previous results (Rahme et *al*, 1992). Hence I chose to conduct the study in Canadian Wonder. Plants were dipped in bacterial suspensions of ~10^8 cfu/ml and bacteria were recovered from leaf tissue at various time points. At the beginning of the infection, 10^3 cfu per mg of leaf were extracted. Both wild-type and ΔT6SS mutant bacteria reached levels greater than ~10^6 cfu/mg leaf by 72 hours post-infection (Figure 12). The *Ps*p 1448a *hrpR::nptII* mutant, which does not express the T3SS, was used as negative control in this experiment. As expected, the *hrpR::nptII* mutant showed a significant growth defect in Canadian Wonder, which coincides with previous findings (Rahme et *al*, 1992).

When Canadian Wonder beans were infected with *Ps*p 1448a strains, the ΔT6SS mutant was able to cause water-soaked lesions on leaves and pods similar to that of wild type after seven days of infection (data not shown). This result is comparable to that obtained during *Ps*s B728a infection in *N. benthamiana.*

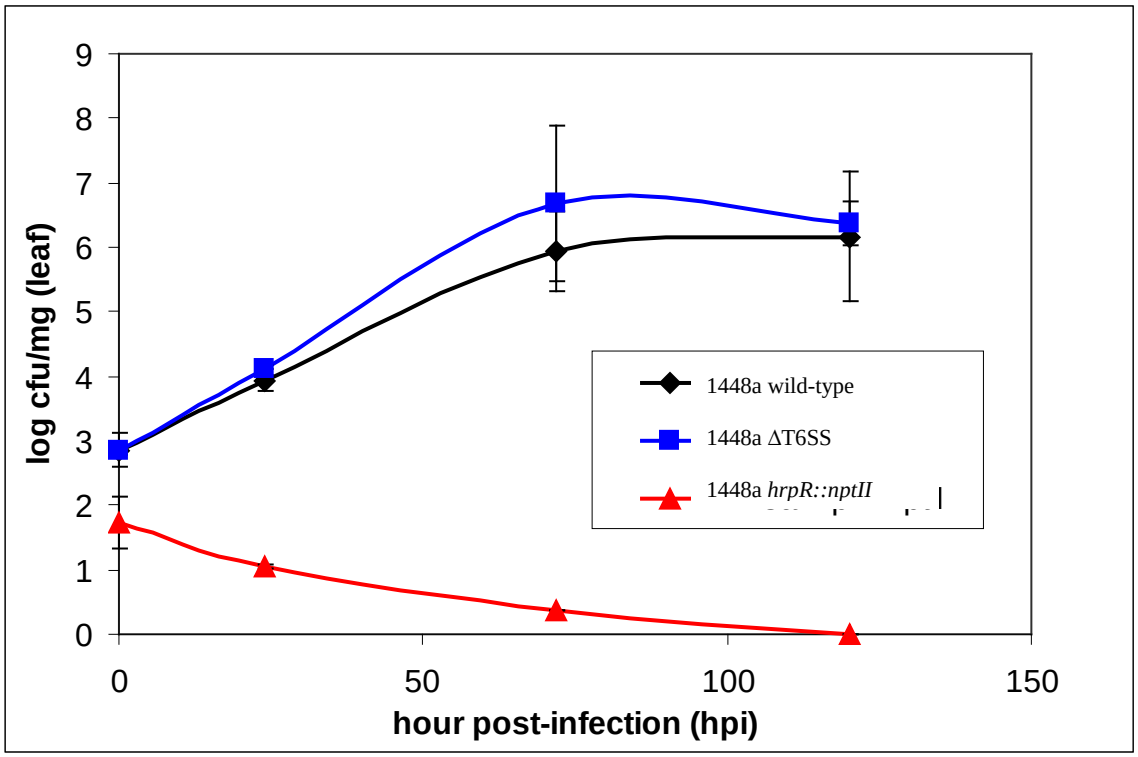


Figure 12 Growth of *Psp* 1448a ΔT6SS Plants were dipped in ~10^8cfu/ml bacterial suspension and incubated in growth chamber with a temperature of 23°C, relative humidity 43% and 12 hour photoperiod. Bacteria were recovered from leaf samples at 24 hours post infection (Hpi), 72 Hpi and 120 Hpi. Error bars represent the standard deviation of 4 samples.

Examination of the *Psp* 1448a ΔT6SS mutant growth in *Arabidopsis thaliana*

Even though *P. syringae* pv. phaseolicola does not cause disease in *A. thaliana*, it also does not cause a hypersensitive response. A recent study indicates that basal defenses are responsible for limiting *P. syringae* pv phaseolicola growth in *A. thaliana.* (Ham et *al* 2007). I tested whether the ΔT6SS mutation affects *Ps*p growth in *A. thaliana* after two days of infection. Wild-type *Ps*p 1448a was able to reach $10^{3.3}$ cfu/mg leaf, whereas the ΔT6SS mutant grew to $10^{4.3}$ cfu/mg leaf (Figure 13). This result indicates that the ΔT6SS *Ps*p 1448a mutant grows 10-fold greater in *A. thaliana* than its wild-type counterpart. To statistically validate the difference, a Kruskal-Wallis test was run using SPSS software. The p value obtained was 0.125, indicating that the difference was not significant.

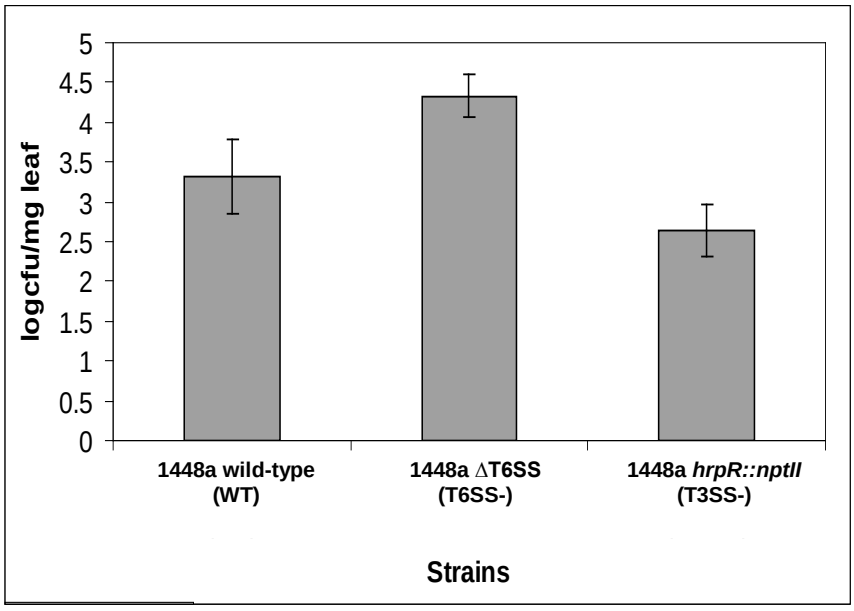


Figure 13 Growth of Psp 1448a ΔT6SS mutant in *A. thaliana*. *A. thaliana* was infected with ~10^8 cfu/ml bacteria by dipping and incubated in a growth chamber with a temperature of 23°C, and a 12 hour photoperiod. Bacteria were recovered from leaves after 48 hours post-infection. Bars represent the standard error of 4 samples. p = 0.125 for the three strains.

The *Ps*s B728a *clpVΩ* mutant suppressed vascular flow in *N. benthamiana*

Bacteria possess conserved features called PAMPs (Pathogen-Associated Molecular Patterns), which trigger basal plant defenses during infection. PAMPs known to trigger basal defenses include flagellin, LPS, and EF-Tu. Some responses to PAMPs are deposition of callose in the cell wall, production of reactive oxygen species, nitric oxide and reduction of vascular flow in minor veins (Abramovitch et al, 2006). To assess the function of the T6SS in modulating the basal defense responses of *N. benthamiana* against *Ps*s B728a, I performed a vascular flow assay. This technique was previously used successfully in *N. benthamiana* to show that the *Ps*t DC3000 effectors AvrPto1, AvrE and HopM1 suppress basal defenses (Oh and Collmer, 2005). In this experiment, *N. benthamiana* leaves were infiltrated with suspensions of wild-type *Ps*s B728A, the *clpVΩ* mutant, or the *hrcC::nptII* mutant. Following 6 hours of incubation, leaves were detached from plants and petioles were dipped into 0.1% neutral red, a dye that flows into and stains leaf veins. Wild-type *Ps*s B728a suppresses the reduction of vascular flow induced by the PAMPs in infected leaf tissue. As expected, the B728a *hrcC::nptII* mutant was not able to efficiently suppress the reduction of vascular flow in the infected area of the leaf (Figure 14). The area infected with the *hrcC::nptII* mutant accumulated about 33% less dye compared to the wild type bacteria, which is consistent with published results (Oh and Collmer, 2005)(Figure 14). In contrast to the T3SS mutant, the T6SS mutant does not appear to modulate vascular flow in infected leaves. B728a *clpVΩ* mutants suppressed reduction of vascular flow similar to wild-type bacteria (Fig 14). Thus the T6SS is not required for *Ps*s B728a to suppress basal defense.

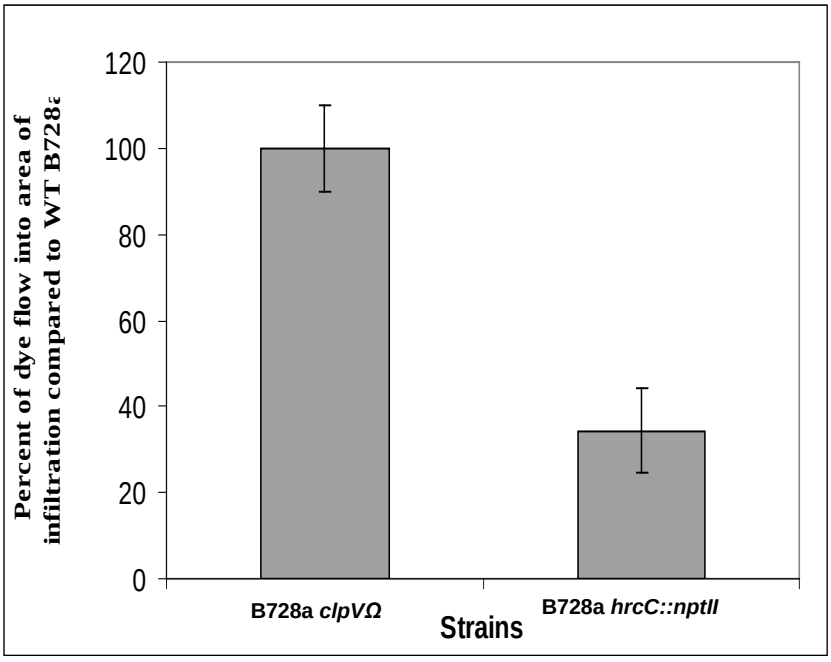
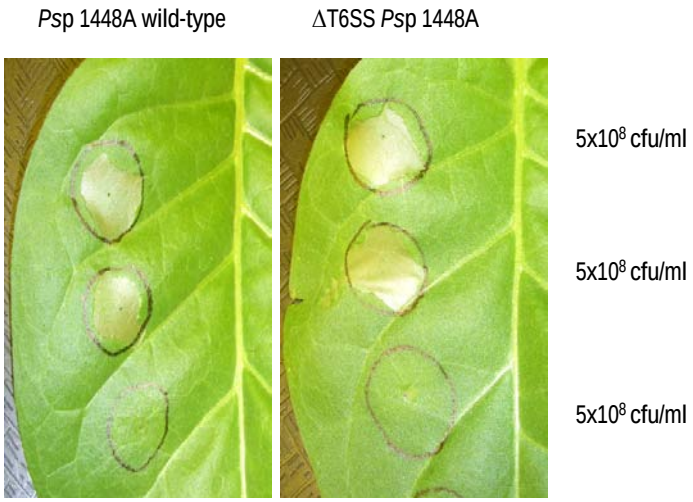


Figure 14 *Pss* B728a *clpVΩ* suppresses reduction of vascular flow in *N. benthamiana*. *N. benthamiana* leaves were infiltrated with bacterial suspensions of $OD_{600\ nm}$= 0.1 and incubated at 23 °C for 6 hours. Leaves were then detached and petioles were submerged in 0.1% neutral red for 6-7 hours. Neutral red accumulation in infiltrated leaf tissue was quantified after extraction of the dye from the three leaf discs in 500 µl of 95% EtOH, with shaking. The extracted dye solution was diluted four-fold with 5mM MES pH 5.5 and the OD at 562nm was determined in a spectrophotometer. The values of dye accumulation in B728a *clpVΩ* or *hrcC::nptII* infiltrated tissue are reported as a percentage of the dye accumulation in plant tissue infected with wild-type bacteria. Bars indicate the standard error of four samples.

Examination of the hypersensitive response caused by *Ps*p 1448aΔT6SS and *Ps*p B728a *clpVΩ* mutants

The HR is characterized by the rapid death of plant cells within an infected area resulting in necrosis. Plants initiate this response to bacteria or fungi to block them from spreading. The HR is triggered by plant resistance (R) proteins, which recognize bacterial T3SS secreted effectors that are delivered into plant cells. To assay whether *Ps*p 1448a or *Ps*s B728a T6SS mutants were able to cause the HR in a non-host plant, *Nicotiana tobacum* cultivar (cv.) Xanthi was infiltrated with bacterial suspensions at several cell densities (Figure 15A). The *Ps*p 1448a ΔT6SS mutant was able to cause the HR similar to wild-type bacteria at the cell densities of $5x10^8$ and $5x10^7$ cfu/ml. When the bacterial density was lower than $5x10^7$cfu/ml, neither strain was able to cause the HR (Figure 15A). The ability of the *Ps*s B728a *clpVΩ* mutant to cause the HR was also tested on *N. tobacum* cv. Xanthi (Figure 15B). Both wild-type and *clpVΩ Ps*s B728a strains caused the HR at $5x10^8$ to $5x10^5$ CFU/ml, however no HR was observed at the $5x10^4$ cfu/ml dilution. These results indicate that the T6SS is not required to secrete proteins that cause the HR in *N. tobacum* cv. Xanthi. As expected, control inoculations of B728a *hrcC::nptII* did not cause the HR even at the high bacterial density of $5x10^8$cfu/ml.

A



B

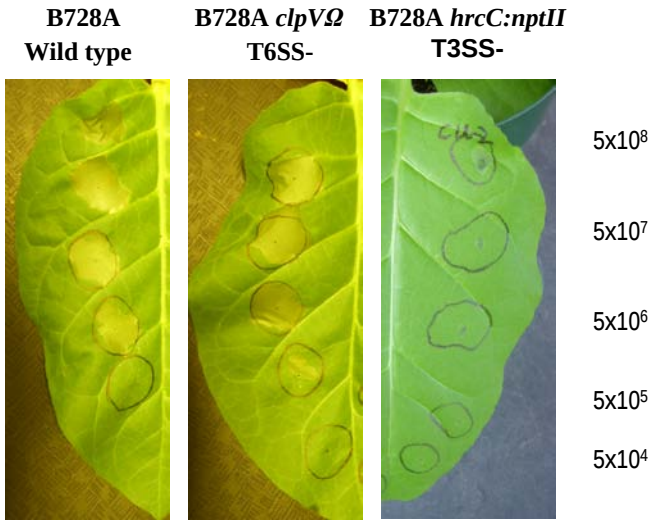


Figure 15 *Psp* 1448a and *Pss* B728a T6SS mutants trigger the hypersensitive response in a non-host plant. *N. tobaccum* cv. Xanthi leaves were infiltrated with bacterial suspensions at the cell density indicated to the right. Areas of infiltration were encircled and plants were incubated at room temperature for 24 hours, necroses were observed and photographs were taken of HR-associated cell death in the circled areas. A. Comparison of the HR caused by wild-type andΔT6SS *Psp* 1448a. B. Comparison of the HR caused by wild-type, *clpVΩ*, and *hrcC::nptII Pss* B728a.

Chapter IV: Discussion

In order to live and develop inside plants, bacteria must develop mechanisms to overcome plant defenses. Virulence factors secreted by bacteria alter plant signaling pathways and physiology. The type III secretion system of *P. syringae* has been shown to be the main virulence mechanism. However, a conserved T6SS gene cluster is present within three sequenced pathovars and it is important in the virulence of several other bacterial pathogens. Thus, I chose to investigate the role of the T6SS of *P. syringae* in its interaction with plants. Two pathovars are the main focus of this study: *P. syringae* pathovar phaseolicola 1448a and *P. syringae* pathovar syringae B728a

Expression of the T6SS occurs in a variety of growth media

Even though virulence factors are vital for bacterial survival in plants, their expression and secretion requires a significant amount of energy. Thus, expression of virulence genes is highly regulated. For example, the expression of T3SS genes is only initiated when the bacteria are in the plant apoplast or cultured in minimal media (Huynh et al, 1989, Rahme et *al*, 1992). When bacteria are grown in culture, T3SS genes are only highly expressed with in media with low osmolarity, low nutrient content, and low pH. These conditions may mimic the environment in the plant apoplast. My results from RT-PCR show that T6SS genes are expressed in *Ps*t DC3000, *Ps*p 1448a, and *Ps*s B728a when bacteria were grown in a variety of media. Unlike T3SS genes, which are repressed when bacteria are grown in rich media, T6SS genes are expressed in both minimal and rich media (Figure 4). This finding may infer that the T6SS is expressed in environments other than the apoplast or that it can be expressed in a broader range of environments.

In the future, studies could be performed to determine whether environmental conditions affect T6SS gene expression in *P. syringae*. For example, the effect of temperature on T6SS genes could be examined as it has been shown that *R. leguminosarum* T6SS gene expression is temperature dependent (Bladegroen et al, 2002). Carbon sources, osmolarity and pH are known to affect T3SS gene expression (Huyn et *a*l, 1992, Rahme et *al*, 1992). Therefore, the effect of these conditions on T6SS expression could also be assessed.

Since many virulence genes are induced upon host infection, *in planta* expression of T6SS should also be assayed. These studies could include examination of the expression of T6SS when bacteria are on the leaf surface or in the apoplast. Gene expression in apoplast bacteria can be specifically assayed by infiltrating mesophyll tissue and surface sterilizing leaves before taking the samples for RT-PCR. Gene expression in surface-localized bacteria could be measured by taking samples shortly after leaves are sprayed with bacteria.

The T6SSs in *Ps*p 1448a and *Ps*s B728a are functional

This study also revealed that the T6SSs in *Ps*p 1448a and *Ps*s B728a are able to secrete the Hcp protein when bacteria are grown in KB (Figure 7). Thus, the T6SS of *Ps*p 1448a and *Ps*s B728a are active *in vitro*. Besides Hcp, research on the T6SSs of *E. tarda*, and *B. mallei* reveals the existence of other secreted proteins including VgrG, EvpP or TssM. A BLAST search of those proteins against the genomes of *Ps*p 1448a and *Ps*s B728a revealed the presence of genes homologous to *vgrG*, but not *evpP* or *tssM*. In the future, it would be interesting to assess the secretion of the VgrG homologs in *Ps*p 1448a as well as in *Ps*s B728a using mass spectrometry. In addition, it would be helpful to identify other protein secreted through the T6SS in *P. syringae.* It is also important to determine whether VgrG and Hcp are translocated into plant cells.

The T6SS is not required for *P. syringae* growth or virulence or virulence in plants

To my surprise, bacterial growth and virulence assays revealed that the *Ps*s B728a *clpVΩ* mutant infects its host *N. benthamiana* as well as wild-type bacteria (Figure 10). In addition, the *Ps*s B728a *clpVΩ* mutant caused water-soaked lesions in both leaves and pods of Bush Blue Lake beans (data not shown). Furthermore, *Ps*p 1448a ΔT6SS grew as well as wild-type bacteria in Canadian Wonder beans (Figure 12).

Several explanations may account for the fact that no difference was seen in virulence and growth between the T6SS mutants and the wild-type bacteria. First, the T6SS may only make subtle contributions to *P. syringae* pathogenicity and its actions may be overshadowed by the T3SS. This could be resolved by creating a double mutant lacking both the T3SS and T6SS. Virulence and bacterial growth assays could be performed to see if a T3SS T6SS double mutant has a greater defect in plant infection than a single T3SS mutant. The contributions of the T6SS to *P. syringae* virulence may be more apparent in the absence of the T3SS. Second, the T6SS may only be important for *P. syringae* infection of specific host plants. In this study, the virulence of *P. syringae* T6SS mutants has been examined in only two different host plants, tobacco and beans. The T6SS may not be required for *P. syringae* to survive or overcome the defenses of these plant hosts. Third, it is possible that full activation of the T6SS might require specific environmental cues that were not present in my experiments. My studies were undertaken under laboratory conditions, which may not accurately simulate the natural environment. The T6SS may be required for optimal infection of plants when bacteria are exposed to stressful conditions such as temperature fluctuations, changes in humidity, or UV light. This could be resolved by performing virulence experiments in fields or under conditions mimicking the natural environment. Fourth, it could be possible that the T6SS is required for a late stage of infection that was not assessed here since I stopped all the experiments by day 7.

In some of my experiments, the T6SS mutants actually grew slightly better than the wild-type *in planta* (Figures 10 and 12). This result leads me to think that the T6SS may function opposite to the T3SS. Instead of suppressing basal defense, proteins secreted by the T6SS or structural proteins of the T6SS may be recognized by plant receptors and trigger basal defense. A further investigation of this idea could be assessed by examining the virulence and growth of T6SS T3SS double mutants in plants. If the T3SS secretes proteins that suppress basal defenses elicited by the T6SS, the double T6SS T3SS mutant may be able to grow better in plants than the single T3SS mutant. In addition, it would be interesting to study the induction of callose deposition by the *Ps*p 1448a ΔT6SS mutant in *A. thaliana*. If the *Ps*p 1448a ΔT6SS mutant causes less callose deposition than the wild-type bacteria, the T6SS may be secreting a factor that induces basal defense. It is unclear why *Ps*p 1448a has maintained the T6SS gene cluster even though it has a slight negative effect on pathogen virulence. *Ps*p 1448a may require the T6SS to increase its fitness in environments that were not explored in this study.

The T6SS regulates biofilm formation in *Ps*p 1448a but not *Ps*s B728a

In this study, I was unable to discover a role for the T6SS in the growth and virulence of *P. syringae* in plants. However, a biofilm formation assay revealed that the ΔT6SS *Ps*p 1448a mutant was able to form a biofilm on PVC that was 50% more dense than the wild-type bacteria (Figure 8). A similar result was obtained with a T6SS mutant in *Ps*t DC3000 (Joy Valenta, unpublished results). These two results lead me to hypothesize that the T6SS is involved in secretion of factor(s) that repress(es) biofilm formation. Because biofilm formation is upregulated in the *Ps*p 1448a T6SS mutant, it is possible that the T6SS is involved in dispersing bacteria aggregated on leaf surfaces before entry into the apoplast.

Screening of mutants with a biofilm formation defect in *P. fluorescens* WCS365 using transposon mutagenesis revealed that disruption of genes involved in flagellar synthesis

leads to a biofilm defect. This result is opposite of my findings, suggesting again that the T6SS might be involved in dispersing biofilm (O'Toole et *al*, 1998).

In a recent study on *B. cenocepacia*, T6SS and biofilm formation genes were shown to be regulated by the same regulator. AtsR is a hybrid two-component transcription factor that contains both sensor kinase and response regulator domains. Mutants lacking the regulator *atsR* over-expressed *hcp* and formed denser biofilms. Thus the T6SS in *B. cenocepacia* might be required for optimal biofilm formation, which is opposite to my results in *P. syringae*. This suggests that the T6SS may function differently according to species (Aubert et al, 2008).

In contrast to *Ps*p 1448a, the *Ps*s B728a *clpVΩ* mutant did not form denser biofilms than its wild-type counterpart (Figure 8). This finding may be explained by the fact that the *Ps*p 1448a mutant lacks the whole T6SS gene cluster, whereas the *Ps*s B728a mutant has only one disrupted *clpV* gene. The *Ps*s B728a *clpVΩ* mutant may therefore have a partially functional T6SS that can still repress biofilm formation. In the future, it would be helpful to perform more RT-PCR on gene on both the disrupted *clpV* gene downstream of the insertional mutagenesis of *Ps*s B728a as well as downstream genes of the *ClpV* including the ORF PSYR04959, PSYR04963 and PSYR04966. It would be interesting to create a *Ps*s B728a mutant that lacks the whole T6SS gene cluster. I hypothesize that this mutant, like the *Ps*p 1448a ΔT6SS mutant, will form denser biofilms. This could be done by dipping leaves with bacterial suspensions containing wild-type or T6SS mutant and wash leaves after a short time to recover bacteria on the surface. Bacterial suspension could be plated and colonies are counted.

To determine whether a factor secreted by the T6SS is involved in reducing biofilm formation, I could add supernatants from wild-type or ΔT6SS mutant cultures of *P. syringae*

to the biofilm assays. I would expect supernatants from wild-type cultures (but not T6SS mutants) to repress biofilm formation by *P. syringae* on PVC.

In conclusion, I have established that the T6SSs of *Ps*p 1448a and *P*ss B728a are expressed and active. Even though the role of the T6SS in *P. syringae* interaction with plants is still not understood, I have discovered that it may repress biofilm formation.

A role for the T6SS in *P. syringae* interactions with plants

A model for the role of the T6SS and T3SS in *P. syringae* interactions with plants is shown in Figure 16. The T6SS may regulate biofilm formation at the early stage of infection when the bacteria form large aggregates on the leaf surface. When bacteria move into the apoplast to initiate disease, the biofilm may be dispersed by the T6SS (Figure 16). The T6SS may not play a role in later stages of infection, since the T6SS did not contribute to bacterial growth *in planta* or the suppression of plant defenses (Figure 16). Once inside the apoplast, induction of the T3SS then allows *P. syringae* to suppress plant defenses and cause disease.

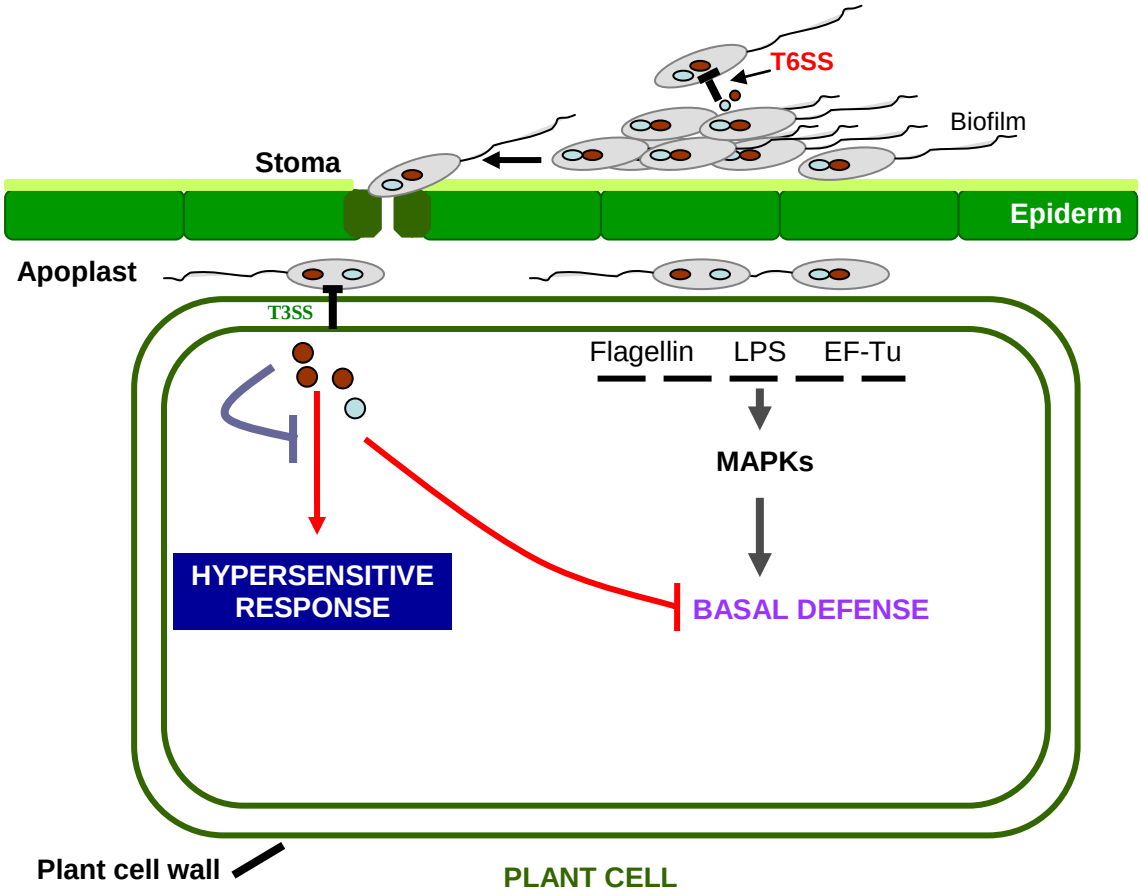


Figure 16 Model for the role of the T6SS and T3SS in *P. syringae* infection of plants. Bacteria land on the leaf surface and form biofilms to survive environmental stress. T6SS may be involved in dispersing biofilms to allow bacteria to enter the apoplast. Inside plant tissue, the T3SS translocates proteins into plant cells that suppress or activate the HR.

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