CHARACTERIZING THE TYPE VI SECRETION SYSTEMS OF PLANT BENEFICIAL BACTERIA *PSEUDOMONAS CHLORORAPHIS* 30-84 AND THEIR ROLES IN RHIZOSPHERE DYNAMICS AND ECOLOGY

A Dissertation

By

EMILY BOAK

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Chair of Committee,	Elizabeth A. Pierson
Committee Members,	Leland S. Pierson
	Patricia Klein
	Terry Gentry
Head of Department,	Amit Dhingra

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ABSTRACT

Host-associated bacterial species have evolved numerous mechanisms to enable them to interact and compete with other microorganisms and with their hosts. One such mechanism is the bacterial Type VI Secretion System (T6SS). Many plant-beneficial bacteria have one or moreT6SS, but their functions in plant-associated niches are poorly understood. T6SS are bacterial machines studied predominantly in human and plant pathogens as a mechanism for bacterial competition, killing, and the transmission of effector proteins critical for virulence into prokaryotic and eukaryotic cells. T6SS may respond to environmental stimuli differently and may have different spectra of influence on community members depending on how firing is regulated, and the types of effectors delivered, suggesting that in strains with multiple T6SS, each may have non-redundant functions. The present work focuses on *Pseudomonas chlororaphis* 30-84, a well-studied wheat rhizosphere-colonizing plant-beneficial bacterial strain with two distinct T6SS that may perform non-redundant functions. The role of each T6SS (T6SS-1, T6SS-2) in microbial competition and persistence in the rhizosphere and anti-predation was examined. Mutants deficient in T6SS function were less persistent in the rhizosphere in natural field soil in repeat-harvest assays. Mutants deficient in T6SS function were also less competitive than the wild type against other bacteria in competition assays *in vitro* and on wheat roots, with T6SS-2 playing an important role against competing strains lacking their own T6SS. Finally, mutants lacking both T6SS lost the ability to effectively protect themselves from resist predation by several eukaryotic bacterivores. These data indicate important roles for the T6SS of plant-beneficial bacteria in rhizosphere dynamics, interactions with eukaryotes, and overall soil ecology.

DEDICATION

To my parents, Bob and Kelly Boak and Rachel and Dan Nyberg, for all their love and support for my education and willingness to listen.

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Contributors

This work was supervised by a dissertation committee consisting of Dr. Elizabeth A. Pierson and Dr. Patricia Klein of the Department of Horticultural Sciences, Dr. Leland S. Pierson of the Department of Plant Pathology and Microbiology, and Dr. Terry Gentry of the Department of Soil and Crop Sciences at Texas A&M University

The research was designed by Dr. Elizabeth A. Pierson and Dr. Leland S. Pierson. All other work conducted for the dissertation was completed by Emily Boak independently.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1.General Introduction

A long-standing goal of agricultural research is to ensure that we continue to feed the world and provide sufficient renewable sources of fiber and fuel. To accomplish this, we need to continuously seek ways to make agriculture more efficient, including improvements in crop productivity and sustainability. Many believe that the next advances in agriculture, on parallel with the Green Revolution, will come from a focus on soil health and the health of soil microbial communities that positively contribute to plant health and productivity (Chaparro et al. 2012; Dubey et al. 2019; Ratnakar and Shikha 2019). A recent center of research related to soil and plant health has been on the plant microbiome, the community of microorganisms that actively associate with plants (Berendsen, Pieterse, and Bakker 2012). The microbiome can be thought of as a second plant genome providing the plant with services and functionalities it does not encode in its own genome (Berendsen, Pieterse, and Bakker 2012; Lebeis 2014; Riva et al. 2019; Rout 2014).

The plant rhizosphere, first defined in 1904, is the area surrounding the plant roots that is influenced by exudates and secondary metabolites from the plant, is an important source of plant-beneficial microorganisms (Bais et al., 2006; Hartmann et al., 2008). This zone of influence allows the plant to actively recruit plant-beneficial microorganisms, known as plant growth promoting bacteria or PGPB (Bakker et al. 2012; Berendsen, Pieterse, and Bakker 2012; Pascale et al. 2020). These microorganisms are recruited from the surrounding bulk soil, an area rich in diversity but not in population density (Bais et al., 2006; Hartmann et al., 2008). Studies

have shown that the rhizosphere microbiome is highly diverse, though not as diverse as bulk soil, in terms of microbial populations, species present, and multitrophic interactions (Berendsen, Pieterse, and Bakker 2012; Rossmann et al. 2020). Opportunities for utilizing well-characterized PGPB from the rhizosphere for plant health applications depend on understanding how PGPB effectively integrate into the rhizosphere niche to reliably provide the desired plant growth promoting activities.

The ability of individual species to employ a spectrum of competitive mechanisms and responses to challenges may be essential to their survival in diverse communities such as the rhizosphere, where competitive stress and predation may take many forms (Stubbendieck and Straight, 2016). One of the premier microbial defenses is the formation of a biofilm community wherein the cells may be buffered and protected from biotic and abiotic stresses by a self-produced matrix (Costerton 1995; Yin et al. 2019). This lifestyle results in coordinated multicellular behavior among sister cells and offers greater protection from competition with other microorganisms and grazing by predators (Hibbing et al. 2010; Matz and Kjelleberg 2005; Matz et al. 2005). Many bacteria also possess an arsenal of diffusible weapons including antibiotics, toxins, bacteriocins and extracellular enzymes enabling them to defend their "space" (Dorosky et al. 2017; Ghoul and Mitri 2016; Granato, Meiller-Legrand, and Foster 2019; Hibbing et al. 2010). Bacteria also employ short-range defensive mechanisms for the delivery of weapons when contact with other bacterial cells is made. The Type VI Secretion System (T6SS) delivery of toxins and other antimicrobials is one of the best examples of a short-range delivery system used by bacteria (Basler 2015; Cianfanelli, Monlezun, and Coulthurst 2016; Smith et al. 2020).

T6SS are common among Gram-negative bacteria and have been studied extensively in pathogenic strains (plant and animal) for their roles in virulence (Asolkar and Ramesh 2020; Chen et al. 2011; Kim et al. 2020; Leung et al. 2012; Ma and Mekalanos 2010; Pukatzki et al. 2006; Tian et al. 2017). Many pathogenic strains have more than one T6SS (Basler, Ho, and Mekalanos 2013; Schwarz et al. 2010). Moreover T6SS have been shown to serve functions other than in virulence and pathogenicity, such as influencing competition and interactions with both prokaryotes and eukaryotes in the environment (Bernal et al. 2017; Gallique et al. 2017; Pukatzki et al. 2006). Interestingly, T6SS are also common in PGPB strains, but little is known regarding how they contribute to plant beneficial activities. A few previous studies of T6SS in PGPB have demonstrated roles of T6SS in interactions with prokaryotes and eukaryotes, but further research must be performed to elucidate their roles in the rhizosphere environment (Bernal et al. 2017; Marchi et al. 2013). I believe a more thorough understanding of the ecological benefits of T6SS to PGPB is important for improved understanding of what makes a PGPB strain effective. Specific knowledge may be useful for the selection of better PGPB-based applications.

Essential for any mechanistic study is having the right tools. In my dissertation, I focus on a well-characterized PGPB strain, *Pseudomonas chlororaphis* 30-84. Previous work (described in the literature review below) demonstrates its effectiveness as a rhizosphere colonist and its contributions to mediating disease and stress tolerance in wheat. Additionally, previous studies demonstrate that *P. chlororaphis* 30-84 possesses a diverse arsenal of highly effective defense weapons that contribute to rhizosphere competence, including two T6SS (Wang et al. 2013). As a consequence, essential tools for focusing specifically on the contribution of T6SS to rhizosphere interactions exist including mutants deficient in secondary metabolite production

(but not T6SS) or regulatory mutants reduced in the expression of both. I was particularly intrigued that *P. chlororaphis* 30-84 had two T6SS, leading me to hypothesize that if both T6SS are functional, they may serve non-redundant functions. In this study, I created mutants defective in either or both of the T6SS and used these derivatives to characterize the role of each T6SS in rhizosphere competence, bacterial competition, and protection from bacterivores.

In the following literature review, I discuss previous research on *P. chlororaphis* 30-84, describe in detail the structure and functions of known T6SS, and provide more discussion of the rhizosphere environment and the potential role of T6SS. Below I provide a summary of the rationale, central hypothesis, specific objectives, and long-range goals of my research.

The rationale for my research is that although T6SS have been shown to contribute to virulence and pathogenicity of plant pathogens, T6SS in PGPB likely play other roles important for survival in a diverse and dynamic rhizosphere environment. Many of the best studied PGPB have a wealth of defensive mechanisms involved in exploitation and interference competition including but not limited to the production of siderophores, antibiotics, bacteriocins and extracellular enzymes, yet also have one or more T6SS. Why? I hypothesize these must perform other functions related to rhizosphere competence or modify the host to possibly provide plant beneficial functions or improve rhizosphere conditions beyond the previously established repertoire of mechanisms known to be conferred by T6SS. In my dissertation I focus on a well-characterized PGPB, *P. chlororaphis 30-84*, which has two distinct T6SS-encoding gene clusters.

The central hypothesis is that the two T6SS-encoding gene clusters produce two separate functional T6SS that are important for PGPB activities. Moreover, given the differences

in the composition and organization of the two T6SS-encoding gene clusters, each system may confer different, non-redundant ecological benefits.

The specific objectives of my dissertation study aim to characterize the two systems in *P. chlororaphis* 30-84 and elucidate their ecological roles. Given some of the most important drivers of community dynamics are competition and predation, these were the focus of my research.

The long-term goal of my research is to improve knowledge of how T6SS contribute to interactions between a PGPB and other rhizosphere residents and potentially use this information for more effective application of PGPB and greater improvement in soil and plant health.

1.2. Literature Review

1.2.1. The biological system: *Pseudomonas chlororaphis* 30-84 on wheat

My dissertation research focuses on elucidating the role of T6SS in interactions performed by PGPB and how T6SS affect rhizosphere dynamics and soil ecology. A specific phenazine-producing PGPB strain, *Pseudomonas chlororaphis* subsp. *aureofaciens* 30-84, is utilized as the experimental system in this study. *P. chlororaphis* 30-84 is a well-characterized PGPB that is an effective wheat rhizosphere colonizer (Pierson and Pierson 1996). This strain was selected as a biocontrol to protect against wheat-take all disease caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (*Ggt*). The production of phenazines by *P. chlororaphis* 30-84 (a redox active secondary metabolite) is required for *Ggt* inhibition, disease suppression, and rhizosphere competence, and subsequent work showed that phenazines are inhibitory to other fungal pathogens (Pierson and Pierson 1996; Yu et al. 2018). Phenazines are important for biofilm production and the formation of surface attached biofilm communities (Zhang and Pierson 2001, Maddula et al. 2006, Wang et al. 2016). Phenazine production also has been shown to be involved in ameliorating drought and salt stress in wheat plants (Mahmoudi et al.

2019; Yuan et al. 2020). Another class of diffusible defense compounds produced by *P. chlororaphis* 30-84 are bacteriocins (antibacterial proteins that resemble bacteriophage tails), which improve the competitive ability of *P. chlororaphis* 30-84 against a certain other *Pseudomonas* competitors (Dorosky et al. 2017). With a diverse spectrum of protective mechanisms already at its disposal, the question must be asked, why would *P. chlororaphis* 30-84 need two T6SS? Could they provide protection from a different spectrum of antagonists, or do they serve some other purpose?

In the present study, *P. chlororaphis* 30-84 mutants deficient in the function of either or both T6SS were generated. I also employed a number of existing mutants as controls in my experiments. These include *P. chlororaphis* 30-84 GacA, a strain with a specific mutation in the GacS/GacA/RsmZ signal transduction pathway (Chancey et al., 1999). Previous studies showed that the GacS/GacA signal transduction pathway regulates both T6SS in *P. chlororaphis* 30-84 (Wang et al., 2013), and GacS/GacA regulation of T6SS has been noted previously in other systems (Hassan et al., 2010, Records and Gross 2010, Chen et al., 2015). This same pathway also regulates the production of phenazines, so *P. chlororaphis* 30-84 GacA mutants are deficient in the production of phenazines and T6SS (Wang et al., 2013). In addition, I employed mutants deficient in phenazine production, but not T6SS activity. These included *P. chlororaphis* 30-84 ZN (containing a mutation in the phenazine biosynthetic pathway, Pierson et al., 1994) and *P. chlororaphis* 30-84 I/I2 (containing mutations in phenazine regulatory genes, Zhang and Pierson 2001). In the absence of exogenously produced signal *P. chlororaphis* 30-84 I/I2 does not produce phenazines (Zhang and Pierson 2001). These mutants provide necessary treatments to evaluate the specific role of T6SS in rhizosphere interactions (e.g., enable me to consider the roles of phenazines and each T6SS independently).

1.2.2. General Overview of Bacterial T6SS

Type VI Secretion Systems (T6SS) genes are found in the genomes of 25% of Gram-negative bacteria and many strains have genes encoding more than one T6SS. These systems closely resemble bacteriophage-tails, having a needle-like apparatus that has been shown to inject effector proteins directly into nearby bacterial or eukaryotic cells (Abby et al., 2016; Barret et al., 2011; Basler et al., 2013; Bernal et al., 2017; Ho et al., 2014; Records, 2011), and, based on the similarities of the T6SS to phage-tails, it is hypothesized that bacteria likely acquired T6SS from phages through horizontal gene transfer with some duplication events occurring after acquisition (Abby et al., 2016; Basler et al., 2013; Bernal et al., 2017; Ho et al., 2014). This is further supported by the fact that some T6SS proteins can still interact with phage proteins (Barret et al. 2011). Among plant-associated bacteria, the T6SS has been studied predominantly in plant pathogenic bacteria as a mechanism for increased pathogenicity and the transmission of effector proteins critical for virulence (Chen et al. 2015; Jiang et al. 2016; Tian et al. 2017). However, T6SS are also present in non-pathogenic, plant-associated strains. For example, genes encoding T6SS are present in most *Pseudomonas* species, which includes species with pathogenic (plant and animal), commensal, and host-beneficial lifestyles (Bernal et al. 2017; Bernal, Llamas, and Filloux, 2017; Mougous et al. 2006). For example, of the 34 recognized *Pseudomonas* species, 33 have genes encoding at least one T6SS (with *P. stutzeri* being the only exception); 27 of these have genes encoding more than one system (Barret et al. 2011; Chen et al. 2015). These observations suggest that T6SS are not solely virulence mechanisms but may play other roles important for bacterial fitness in a plant-associated environment. In support of this idea, there is evidence suggesting that the T6SS of non-pathogenic, rhizosphere-colonizing strains are upregulated on plant roots (Barret et al. 2011; Lutz et al. 2013; Marchi et al. 2013). It has been

hypothesized that these systems play roles in mediating microbial community dynamics or interactions with plants and/or other eukaryotes such as bacterial predators or fungal competitors (including plant pathogens). Evidence also suggests that T6SS may contribute to biofilm formation and intercellular communication as observed for *P. aeruginosa* and *P. fluorescens*, respectively (Chen et al. 2015; Gallique et al. 2017), both of which are important functions for rhizosphere colonizers.

Below, I discuss how T6SS function and are controlled, including differences in regulation, and will provide an overview on some of the types of effectors found associated with T6SS. I will then discuss how each of these contribute to T6SS diversity and organization into distinct genetic clades.

1.2.2.1. T6SS Complex and the assembly, firing, and disassembly cycle

1.2.2.1.1. Assembly (Figure 1.1a)

The T6SS is composed of three distinct parts: the needle-like structure, the baseplate, and the intermembrane structure which keeps the complex anchored to the cell membrane (Ho, Dong, and Mekalanos 2014). These parts are referred to collectively as the T6SS complex and are made up of 12 to 15 structural proteins, most of which are designated “Tss” consistent with the nomenclature of the system (Barret et al., 2011; Bernal et al., 2017). The final size of the assembled complex is determined by the width of the cell, ending only when it runs into the other side of the cell, meaning the system could reach up to 1μm in length (Santin et al. 2019). This large complex can attack an area up to half of its total length and therefore could penetrate neighboring cells as deeply as 500 nm (Ho, Dong, and Mekalanos 2014). This provides sufficient length to extend from the cytoplasm across the outer membrane of the cell and beyond with

sufficient extracellular length to penetrate entirely through a neighboring bacterial cell or through the cell wall of plant cells (Basler et al. 2012; Basler and Mekalanos 2012).

The formation of the Type VI structure begins with the localization of the membrane spanning proteins TssJ, TssL, and TssM (Zoued et al. 2017). TssJ is the first to position and anchors the system in the outer membrane of the forming cell (Aschtgen et al. 2008; Zoued et al. 2017). Once TssJ is in position it interacts with TssM through a periplasmic domain on TssM (Zoued et al. 2017). TssM spans both membranes and connects to the inner membrane through TssL which anchors the complex with an OmpA-like extension (Marek Basler 2015). There are ten copies of each protein in the transmembrane complex which form a pore in the membranes (Zoued et al. 2017).

The baseplate is recruited next. TssA, TssE, TssF, TssG, and TssK are all known baseplate components but very little is known about the interactions involved and order of formation. It is known that TssA forms a dodecamer, ring-shaped complex with a central hole measuring around 10 nm, suggesting that TssA coordinates the Hcp-TssB/C assembly using an interaction with Hcp via the central hole and an interaction with TssB/C with the outside diameter (Planamente et al. 2016; Zoued et al. 2017). This structure connects to the membrane complex through TssK interacting with TssL (Basler 2015). TssK also stably interacts with TssF and TssG, however the function of these two components is unknown (Marek Basler 2015). There is some homology between TssF and the N-terminal region of a baseplate component of phage gp6, and with TssA showing homology to the C-terminal region which suggests that in the early evolutionary history of T6SS, these two proteins were fused to create a stable baseplate structure, and that TssF could be a remnant of this structure (Planamente et al. 2016). TssG could possibly be stabilizing the interaction between TssK, the membrane complex, and TssE as it

interacts with each of these components (Zoued et al. 2017). TssE is suggested to be binding directly to the sheath structure and could be providing further stabilization (Basler 2015; Planamente et al. 2016). It is thought that TssE, TssF, TssG, and TssK are first to attach to the membrane complex, though whether they combine in the cytoplasm or individually at the membrane is unknown.

Finally, the TssA, Hcp, and TssB/C needle/sheath structure is attached (Zoued et al. 2017). The needle structure is formed by a rigid, hollow tube of haemolysin co-regulated protein (Hcp or sometimes TssD) rings roughly 4 nm wide surrounded by a sheath of interlocking TssB and TssC proteins that interact with Hcp using a bacterial two hybrid system (Basler, 2015; Bernal et al., 2017). TssB and TssC are found free-floating in the cytosol as assembled into a 12-tooth cog wheel-like shape prior to localizing to the point of formation (Basler et al. 2012; Ho, Dong, and Mekalanos 2014). A 1 μm long Type VI structure is composed of roughly 1,500 TssB/C sheath subunits (Basler 2015), the exact number of Hcp proteins involved is unknown. This tube is topped with a valine-glycine repeat protein G (VgrG) trimer which is in turn topped by one proline-alanine-alanine-arginine (PAAR) repeat protein using hydrogen bonds which then acts as a sharpener enabling the end to penetrate neighboring cells (Basler 2015; Bernal, Llamas, and Filloux, 2017; Ho, Dong, and Mekalanos 2014; Shneider et al. 2013). Full assembly of the T6SS takes 20-30 sec. and once it is formed it can stay in its extended form for anywhere between a few seconds to several minutes and the signal that determines this has yet to be identified (Basler et al. 2012).

1.2.2.1.2. Firing (Figure 1.1b)

After assembly, the system is ready to fire. When firing, the extended TssB/C sheath contracts to about half of its original size by using a twisting motion that brings the TssB and TssC subunits closer together (Basler 2015). This twisting motion makes the puncturing of the neighboring cell analogous to drilling a hole, and the contraction of the system powers the secretion and "drilling" (Basler 2015; Basler et al. 2012). In total the contraction of the sheath takes about 5 milliseconds (Basler et al. 2012). To determine how much energy is produced by the system, its contraction was compared to that of pyocins, a bacteriocin found in *P. aeruginosa*, which is very similar to the Type VI structure. Pyocins gain around 12 kcal/mol per subunit in energy, a roughly comparable amount to Type VI subunits. As mentioned previously, a 1μm long Type VI structure is composed of roughly 1,500 TssB/C sheath subunits, so upon contraction the total energy released would be roughly 18,000 kcal/mol, which equates to around 1,600 molecules of ATP. This level of energy propels the system's payload forward at a speed of at least 100 μm/s (Basler 2015). Once through the membranes of the neighboring cell the Hcp and associated proteins break off inside of the neighbor (Basler 2015; Basler et al. 2012).

1.2.2.1.3. Disassembly (Figure 1.1c)

The contraction of the TssB/C sheath immediately triggers disassembly by recruiting the protein ClpV, an ATPase used for recycling components of the system (Zoued et al. 2014). ClpV disassembles the structure using ATP and by binding to the TssB/C sheath (Basler and Mekalanos 2012). ClpV binds weakly to TssB and acts mostly on TssC. The N-terminus of TssC exposed on the sheath is threaded through the ClpV ring while ATP is hydrolyzed (Basler 2015). It is likely that rather than threading the entire structure through, that the slight pulling on some

of the TssC proteins is enough to destabilize the structure and release the sheath components back into the cytosol (Basler 2015). This mechanism also ensures that recycling is not triggered until the contraction and firing event occurs as, while extended, the N-terminus of TssC is not exposed (Basler 2015). The baseplate components of the system are also recycled using ClpV and the status of the membrane complex after firing is not known (Basler et al. 2012; Basler and Mekalanos 2012). While the components released into the neighboring cell (Hcp, VgrG, PAAR, and effectors) cannot be recycled, if the system is fired on a sister cell that cell could potentially reuse the components (Gallique et al. 2017). In total disassembly takes 22-46s (Basler and Mekalanos 2012).

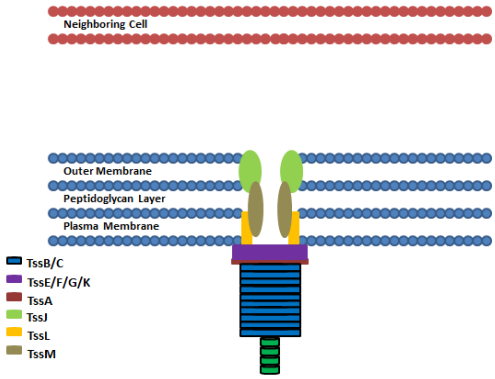

Figure 1A: Assembly of the T6SS

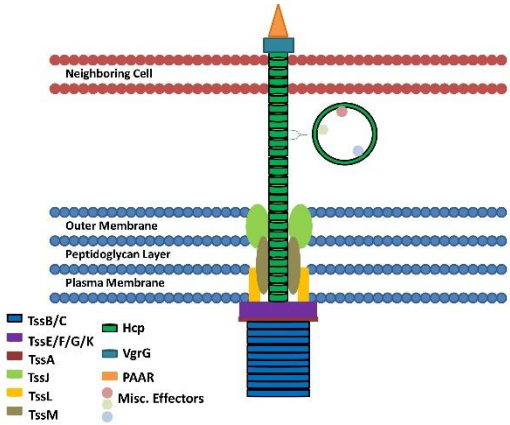

Figure 1B: Firing of the T6SS

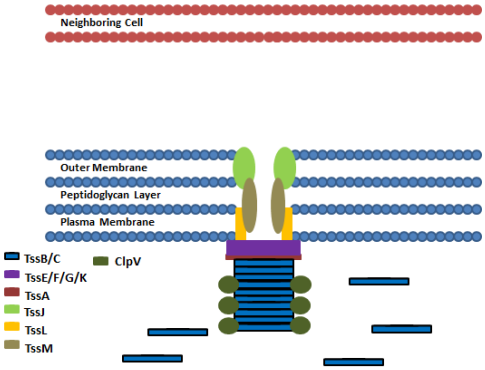

Figure 1C: Disassembly of the T6SS sheath

Figure 1.1: Representation of the assembly, firing, and disassembly cycle of the T6SS, adapted from Cascales and Cambillau, 2012 and Cianfanelli et al. 2016. A) This shows the initial steps in assembly of the T6SS where the Hcp tube is fed through the sheath made by TssB and TssC proteins. TssJ, TssL, and TssM anchor the system to the cell membrane and TssA, TssE, TssF, TssG, and TssK act as the glue holding the sheath to the anchor. **B)** When assembled and ready for firing, the VgrG and PAAR proteins are attached to the Hcp tube. This image also shows how various effectors can be attached to VgrG and PAAR proteins or attached to the inside of the Hcp tube. **C)** The system is recycled by ClpV (Basler and Mekalanos 2012)**.**

1.2.2.2. Regulation of the System

Much of what is known regarding the regulation of T6SS in *Pseudomonas* comes from studies of on *P. aeruginosa*. In *P. aeruginosa,* T6SS are transcriptionally regulated by the quorum sensing regulators LasR and MvfR (Chen et al. 2015). These differentially regulate the three T6SS in *P. aeruginosa,* with T6-1 being negatively regulated and T6-2 and T6-3 being positively regulated. The production of non-recyclable components of T6SS (Hcp, VgrG, PAAR, effectors) are more tightly regulated given the energy expenditure and have been found to be regulated by RpoN and VasH (Ho, Dong, and Mekalanos 2014). RetS and LadS are regulators that mediate the switch between expression of genes for acute infection (RetS) and long-term colonization (LadS) (Records and Gross 2010a). These directly interact with the GacS/GacA/RsmZ signal transduction pathway by influencing the levels of RsmZ and RsmY which antagonize the protein RsmA. RsmA is an RNA binding protein which both posttranscriptionally and posttranslationally regulates T6SS formation (Records and Gross 2010; Chen et al. 2015).

Posttranslationally, T6SS are further regulated by phosphorylation. The serine/threonine kinase, PpkA, is autophosphorylated by an unknown environmental signal and then binds to Fha1 (forkhead associated-1) which is required for T6SS action and Hcp secretion. Fha1 localizes to a locus in the cell and recruits the components of T6SS (Kulasekara and Miller 2007). The serine/threonine phosphatase (PppA) antagonizes PpkA, preventing its phosphorylation, and posttranslationally repressing T6SS formation (Chen et al. 2015).

Previous work identified contact dependent firing, a dueling T6SS activity unique to pseudomonads (Basler, Ho, and Mekalanos 2013). This dueling response is regulated by the signaling cascade TagQRST. This cascade was shown to signal the phosphorylation of PpkA.

TagQ and TagR are outer membrane associated proteins that sense perturbations to the cell membrane and signal TagS and TagT which are related to ABC transporters. This triggers the autophosphorylation of PpkA (Ho, Dong, and Mekalanos 2014). The absence of this cascade leads to a "random firing" phenotype, that is, the T6SS forms and fires in any direction with a currently unknown trigger.

1.2.2.3. Effector Biology

It is believed that a primary function of T6SS is to secrete bacterial proteins known as effectors. Among pathogenic bacteria, T6SS are used to introduce effectors into the cells of their host and are required for virulence and pathogenicity. Although bacterial effectors involved in virulence are typically secreted into their host using the Type III secretion system, Type IV and T6SS may also be employed. To be successfully delivered, effectors of the T6SS must be attached to parts of the system that are broken off inside of the target cell **(Fig. 1.1)**. To accomplish this, the effectors bind to the Hcp proteins that make up the tube itself or the VgrG or PAAR proteins associated with the tube using adaptor or chaperone proteins like Tap/Tec and EagR (Bernal et al. 2017, 2018; Liang et al., 2015). There are five different mechanisms for effector delivery: 1) attach directly to the PAAR protein, 2) attach to extensions attached to the PAAR protein such as Rhs cages, 3) attach to a VgrG protein, 4) attached through evolved VgrG domains which are hybrids of the N-terminus of the VgrG protein and the C-terminus of an effector domain, and 5) attached to the inside of the hollow Hcp tube (Ho et al., 2013). Because the VgrG and PAAR proteins can bind to the Hcp tube with varying combinations, it is thought that several different effectors can be delivered by a single T6SS (Bernal et al. 2017, 2018; Cianfanelli et al., 2016). This prevents the need for the cell to assemble, disassemble, and

reassemble with different effectors or have several T6SS assembled at once, conserving valuable energy.

Several superfamilies of T6SS effectors exist, such as the Type VI secretion amidase effector (Tae) and Type VI secretion glycoside hydrolase effector (Tge) families. The Tae family includes Tse1, a novel effector from *Pseudomonas aeruginosa* that hydrolyzes the murein in the periplasm of cells (Ding et al. 2012) and Ssp1 and Ssp2 from *Serratia marcescens*. The Tge family includes Tse3 muramidase from *P. aeruginosa,* which also acts as a hydrolyzer, and Tge2 and Tge3 from *Pseudomonas protegens. Vibrio cholerae* also utilizes another effector family called VgrG3, which has muramidase activity unrelated to Tge (Ma et al. 2014). A third superfamily of effectors includes the phospholipase Tle, which degrades bacterial membranes changing the integrity of the target cells (Ma et al. 2014). In a more recent discovery, it was found that *Agrobacterium tumefaciens* produces a family of Type VI DNase effectors (Tde) that inhibit antibacterial DNase activity (Ma et al. 2014). It has also been shown that Hcp itself can cause damage to the cell and even cause apoptosis (Abby et al. 2016). Although potential *Pseudomonas chlororaphis* 30-84 effectors are mostly unannotated, my study identified several hypothetical genes having high similarity to genes encoding several different types of effectors. These effector types include Tae, Tle, Tpl, and Pld from the lipase effector superfamily (this study).

T6SS effector genes are typically found to be paired with genes encoding partner immunity proteins that protect the producing cells from the effector's activity (Ding et al. 2012). These immunity proteins serve mainly to protect the cells from accidental attacks by their sister cells (Dong et al. 2013). The immunity proteins encoded by the T6SS gene clusters seem to be highly specific, having no homology to other immunity proteins. This suggests that specific

pairing between a secreted effector and an immunity protein occurs, with no immunity proteins doubling up on the effectors they can counteract (Dong et al. 2013). Similar to how certain effector genes are closely linked with the *hcp* genes in the T6SS gene cluster, the genes of immunity proteins have been found in close proximity to the genes encoding their effector counterpart. The conserved genetic linkages between genes encoding effectors and immunity proteins and *hcp* or *vgrG* have provided clues for the identification of effector-immunity proteins among the hypothetical genes encoded within T6SS gene clusters (English et al. 2012; Spiewak et al. 2019).

1.2.2.4. T6SS Gene Clusters: Organization and Diversity

It has been shown that bacterial species differ genetically in the T6SS they possess and that many species have more than one T6SS. For example, *P. fluorescens* Q287 has three systems, *P. fluorescens* SBW25 has one system (Loper et al. 2012), and *P. chlororaphis* 30-84 has two T6SS encoded within two separate gene clusters (**Fig. 1.2**). As stated previously, each T6SS is composed of 12 to 15 protein components that are typically encoded within the same gene cluster (Bernal et al., 2017). These gene clusters also encode regulatory and associated proteins to help with the construction and disassembly of the system (Bernal et al., 2017). Some proteins, such as Hcp and VgrG, have several different genes involved in their production, some of which are scattered throughout the bacterial genome. The genes encoding the proteins that make up the tube, sheath, and baseplate are relatively well conserved and vary only in linkages to other genes and their relative order within clusters (Barret et al. 2011; Bernal et al. 2017). Several important conserved linkages have been found among these genes. For example, *tssB* and *tssC*, the genes that encode the proteins responsible for sheath formation, have been found to be linked, indicating the interdependent relationship of these proteins (Bernal et al. 2017). Genes

that encode effectors are also linked with the *vgrG* and *hcp* genes, suggesting they bind to the Hcp tube or VgrG trimer for delivery, which will be discussed later (Bernal et al. 2017).

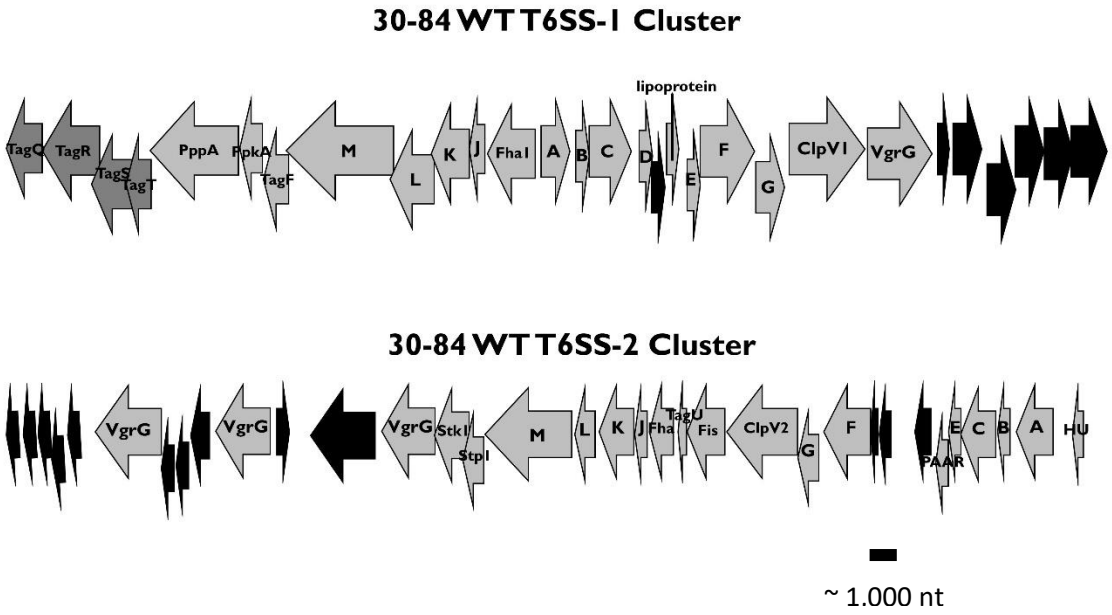

Figure 1.2: *P. chlororaphis* 30-84 T6SS gene clusters. A) The cluster of genes encoding proteins involved in T6SS-1. **B)** The cluster of genes encoding proteins involved in T6SS-2. Light grey arrows that are labeled with individual letters designate genes encoding the proteins TssA-TssM associated with each T6SS. Black arrows designate genes encoding hypothetical proteins. The dark gray arrows designate genes encoding proteins associated with the TagQRST system in T6SS1.

Based on phylogenetic analysis of sequenced T6SS gene clusters, T6SS are assigned to different clades (Boyer et al. 2009). Recently, these clades were parsed into further subgroups (Barret et al. 2011). Clade 1, subgroup 1.1 includes T6SS from many different pseudomonads such as *P. aeruginosa* and *P. fluorescens* strains. Subgroup 1.2 has two branches: branch "A"

includes T6SS from *P. putida* and branch "B" includes T6SS from *Dickeya* and *Pectobacterium*. Clade 2 includes T6SS mostly from *P. putida*, but also some from *P. fluorescens* and *Erwinia*. Clade 3 includes T6SS from many genera. Clade 4, subgroup 4A includes T6SS from *P. putida*, *P. aeruginosa*, and *P. fluorescens*. Clade 4, subgroup 4B-B1 includes T6SS mostly from *P. putida* and *P. syringae*, and B2 includes T6SS from *Burkholderia* and *Xanthomonas*. Finally, clade 5 includes T6SS from *Agrobacterium* and *Rhizobium* (Bernal et al., 2018). Characterization of the T6SS using this clade system illustrates both the diversity of species having T6SS as well as the diversity of T6SS found within a single species. For example, *Pseudomonas* species have T6SS belonging to clades 1.1, 1.2-A, 2, 3, 4A, and 4B-1 and a single species, *P. putida*, has T6SS belonging to all of these clades. In contrast, *P. syringae* appears to have T6SS belonging to only two clades (Bernal, Llamas, and Filloux, 2017).

In species with more than one T6SS, it is not uncommon for the systems to cluster with different clades. The two T6SS in *P. chlororaphis* 30-84, T6SS-1 and T6SS-2, cluster in clades 3 and 1.1, respectively **(Fig. 1.3)**. This diversity in T6SS suggests each system is potentially derived from different ancestral origins and is cited as supporting the theory that T6SS originated from separate horizontal gene transfer events with few subsequent duplication events occurring after (Bernal et al., 2018; Chen et al., 2011). These observations also support the intriguing hypothesis that different T6SS may serve different, non-redundant functions for the producing host, though this has not been established (Abby et al., 2016; Bernal et al., 2018). Differing functions in relation to clade has not been established, though there are two general camps: those that believe the function:clade relationship exists and those that believe function is related mainly to what effectors are present within the cluster (Bernal et al., 2018).

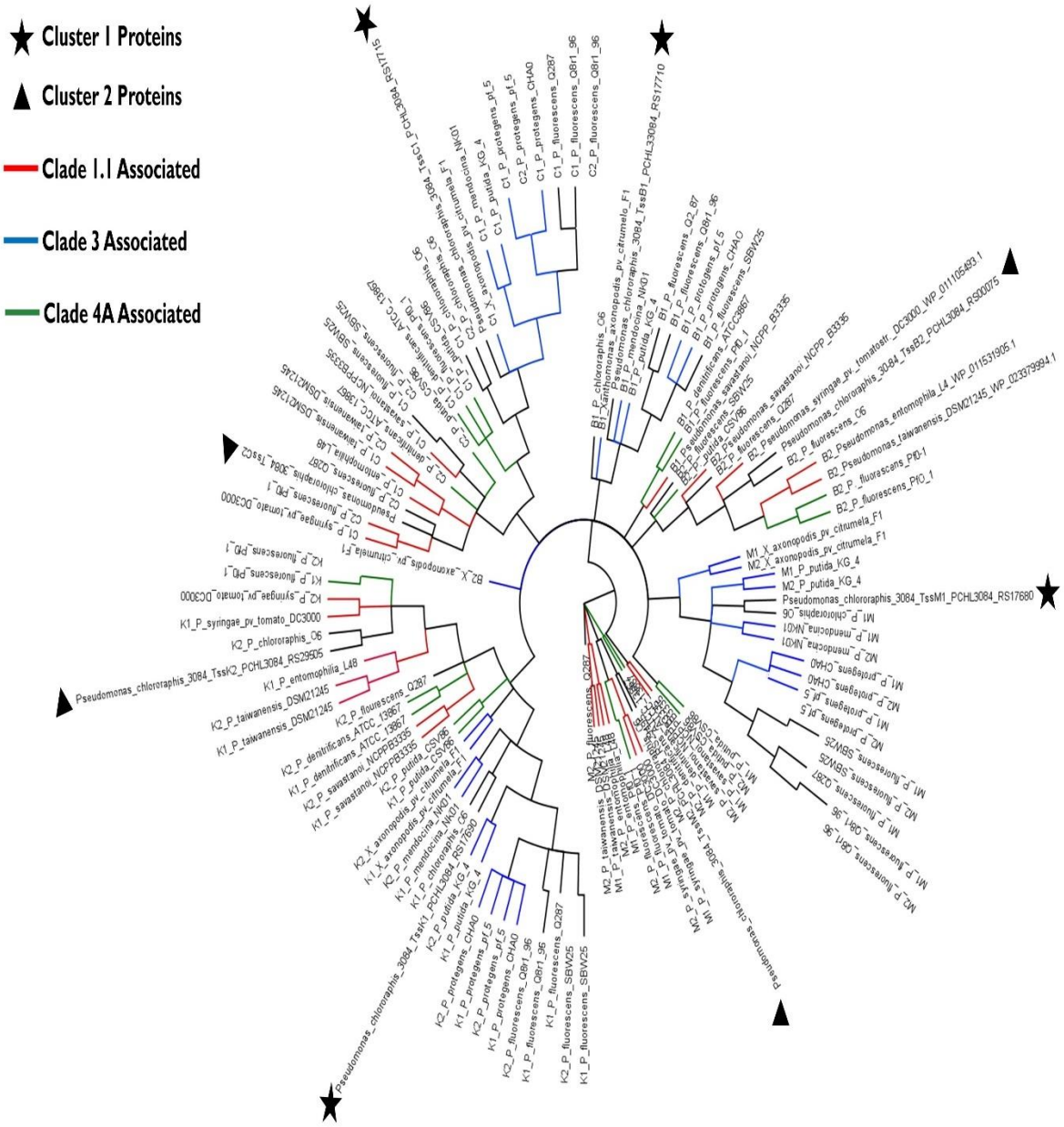

Figure 1.3: Phylogenetic tree comparing the sequences of four T6SS proteins from each of the two T6SS clusters to known homologs from organisms. The amino acid sequences for four conserved T6SS proteins, TssB, TssC, TssK, and TssM from both of the two *P. chlororaphis* 30-84 T6SS clusters were compared to the corresponding sequences from individuals within each established clade referenced in Bernal et al. 2017 using NCBI BLASTp. The *P. chlororaphis* 30-84 sequences and representative sequences from these clades were then aligned and a maximum likelihood tree was constructed using MEGA7. The program FigTree was used to convert the MEGA7 tree into figure format. Proteins in *P. chlororaphis* 30-84 cluster 1 (indicated by stars) was most similar to clade 3 whereas proteins from *P. chlororaphis* 30-84 cluster 2 (indicated by triangles) were most similar to clade 1.1.

1.2.3. T6SS Roles in Rhizosphere Ecology

In this section I discuss interactions that occur in the rhizosphere and how T6SS affect these interactions through their roles in contact-dependent antagonistic behavior, in eukaryotic interactions, and other functions.

1.2.3.1. Interactions in the Rhizosphere

The specific mechanisms for the interaction of bacteria with other rhizosphere dwelling organisms fall into two categories, exploitative or interference interactions (Birch 1957). Exploitative interactions are a passive mechanism in which an organism uses up the limited resources in their surroundings, preventing other organisms from using them (Hibbing et al. 2010). This mechanism encompasses diffusible compounds such as siderophores which are used to capture iron from the surrounding environment (Hider and Kong 2010). Interference competition refers to an organism directly antagonizing another and impeding their growth and survival (Hibbing et al. 2010). This mechanism includes both diffusibles as well as contact dependent methods for competition.

There are a wide variety of diffusible compounds utilized by bacteria for interference competition in the rhizosphere, these include volatile organic compounds and anti-microbials, including phenazines and bacteriocins. Volatile compounds produced by bacteria have been shown to be involved in interactions with other bacteria, plants, animals, fungi, and other eukaryotes (Kai et al. 2009). Furthermore, these interactions involve promoting plant growth, communication and signaling with bacteria, and pathogen inhibition (Kai et al. 2009; Syed-Ab-Rahman et al. 2019; Ryu et al. 2003; Yuan et al. 2012). Production of the antibiotic phenazine was implicated in pathogen inhibition as well as biofilm formation and stress tolerance in plants (Maddula, Pierson, and Pierson 2008; Mahmoudi et al. 2019; Yuan et al. 2020), and bacteriocins

contribute to rhizosphere persistence and bacterial competition (Dorosky et al. 2017; Dorosky, Pierson, and Pierson 2018). When organisms are in close contact in the rhizosphere, the utilization of a close range, contact-dependent mechanism for interference competition is vital. T6SS are such mechanisms.

Interestingly, T6SS have been found to have functions that would fall into both exploitative and interference competition. Beyond the previously mentioned roles in contact-dependent competition, virulence and killing, and biofilm formation and communication, T6SS have also been shown to be involved in nutrient acquisition (Bernal et al. 2017; Chen et al. 2015; Chen et al. 2016; DeShazer 2019; Gallique et al. 2017). Previous work has shown that many species have more than one T6SS and suggests that, in the case of multiple systems, each may provide a different function (Chen et al. 2011; Schwarz et al. 2010). This suggests the possibility of T6SS performing both exploitative and interference competition within the same organism. With roles in both methods of competition and interaction, the T6SS is a dynamic mechanism that can have great influence in the rhizosphere environment and on overall soil ecology.

1.2.3.2. T6SS function in contact-dependent, antagonistic behavior

The T6SS assembly and firing is the most widely used example of contact-dependent antagonistic behavior (Ho, Basler, and Mekalanos 2013; Pukatzki et al. 2006; Russell et al. 2014; Sana et al. 2016). As previously mentioned, T6SS contact-dependent firing is regulated by signals (TagQRST) that indicate the location of the point of attack by the T6SS of neighboring cells. This location-dependent signaling causes the T6SS to form at the site of attack and fire back (Marek Basler, Ho, and Mekalanos 2013). When the genes from this signaling cascade are absent in the T6SS gene cluster, the system randomly fires (Marek Basler, Ho, and Mekalanos 2013). The T6SS is not continually active, and completion of three different stages, extension,

contraction, and disassembly, collectively referred to as "T6SS activity" **(Fig. 1.1)** must occur each time the T6SS is fired (Basler, Ho, and Mekalanos 2013; Ho, Basler, and Mekalanos 2013). When fired at other cells, the T6SS can cause significant morphological changes in the targeted cell such as cell rounding, blebbing, plasmolysis, and lysis. Although these changes are usually linked to the injection of effectors, the physical damage caused by the needle puncturing the cell cannot be ruled out. All of these morphological changes may affect the function and survival of the targeted cells (Basler, Ho, and Mekalanos 2013). Research into T6SS activity as a defensive competitive mechanism has focused on bacterial behavior in the presence of competitors with intact or mutation defective T6SS. For example, *P. aeruginosa* was found to only attack *V. cholerae* cells that had a fully functional T6SS and did not efficiently kill *V. cholerae* cells that had a non-functioning T6SS (Basler, Ho, and Mekalanos 2013). These observations support a theory termed "T6SS dueling" in which cells will respond to an attack using the T6SS as a defense mechanism (Basler, Ho, and Mekalanos 2013). Moreover, a role for T6SS in bacterial competition is supported by the close association between effector and immunity proteins within T6SS clusters. Indeed, Ma et al. (2014) determined that the plant pathogen *Agrobacterium tumefaciens* used its T6SS to inject competing bacteria with effectors rather than actively using the system for virulence. It should be noted that the T6SS does not only form in response to an attack by another T6SS but has been found to respond to the invasive components of the T4 conjugation machinery as well (Ho, Basler, and Mekalanos 2013).

1.2.3.3. T6SS role in eukaryotic interactions

The main point of focus in T6SS research has been on plant and animal pathogens that utilize their T6SS for virulence. Among plant pathogens, direct correlation between function of their T6SS and disease symptom presence in the infected plant has been generally observed

(Asolkar and Ramesh 2020; Kim et al. 2020; Tian et al. 2017). Among animal pathogens, T6SS were shown to be necessary for virulence and killing using animal model systems such as *Caenorhabditis elegans* and *Dictyostelium discoideum* (Lin et al. 2015; Sana et al. 2013; Miyata et al. 2011). However, even in research with higher animals, such as mice and fish, T6SS function and virulence were linked (Ma and Mekalanos 2010; Leung et al. 2012; Wang et al. 2009). Beyond action as a virulence mechanism, bacterial T6SS were also shown to protect them from predation by eukaryotic organisms (Pukatzki et al. 2006; MacIntyre et al. 2010). This presents an interesting focal point for research on the T6SS of plant-associated beneficial bacteria, as a beneficial bacterium is unlikely to conserve effectors for virulence. In their environment, bacteria could encounter many different predators and a variety of feeding styles they would need to combat. Several of these potential predators including *Dictyostelium discoideum, Tetrahymena thermophila,* and *Caenorhabditis elegans* will be discussed in future chapters. A brief background of each is provided below.

Dictyostelium discoideum is a soil dwelling amoeba that preys on bacteria at the soil surface. It moves through its environment through the use of a pseudopod (Eidi 2017) and consumes bacteria via phagocytosis (Dunn et al. 2018; Williams and Kay 2018). Phagocytosis is the process by which the amoeba engulfs the bacteria and forms a vacuole or "phagocytic cup" which becomes highly acidic and can degrade the contents within (Dunn et al. 2018). These amoeba typically live as a single-celled organism that reproduces via fission until it encounters an environmental stress, at which point cells begin to aggregate together (Brock and Gomer 1999). This aggregate forms a migrating slug which then forms a fruiting body dedicated to spreading spores (Brock and Gomer 1999). *D. discoideum* has been observed in experiments with many different bacterial strains as they are considered to be one of the simplest systems to

measure bacterial interactions and virulence (Froquet et al. 2009). These traits plus the conservation of phagocytosis make it a model organism for studying predation on plant beneficials.

Like *D. discoideum, Tetrahymena thermophila* utilizes phagocytosis to prey on bacteria and other particles in its environment (Luan et al. 2012), however, *T. thermophila* is a ciliate filter feeder, meaning that it can swim through its aquatic environment and use its cilia to sweep food into its oral structure (Gavin 1980; Dürichen et al. 2016). *T. thermophila* has shown observable feeding preferences and an ability to overcome bacterial biofilms in order to feed (Dopheide et al. 2011). *T. thermophila* also undergoes observable mating when cells of different germlines are exposed to each other (Cervantes et al. 2013). Similar to *D. discoideum,* this mating behavior can be used as a measurement of stress. Despite its lifecycle as a purely aquatic living organism, *T. thermophila* represents a good model organism for studies with soil-dwelling, plant-associated microbes due to its impacts on biofilms. By utilizing an organism that can overcome the production of biofilms, it eliminates the biofilm as a potential mechanism of protection from this predator, allowing the effects of T6SS function to be determined in this system.

Finally, the soil-dwelling nematode *C. elegans* is a more complex organism than the others considered here. This organism possesses a rudimentary digestive system, complete with intestines, and so digests its prey through a combination of contractions and griding (Avery and You 2018). Nematodes that have been starved display significantly retarded growth, providing a quantifiable trait to use in experiments (Avery and You 2018). Previous work on the predation of bacteria by this nematode has shown that the production of biofilms by the bacteria disrupt the motility and grazing behavior of *C. elegans* (Chan et al. 2021). Furthermore, in studies with *P.*

aeruginosa PAO1, the T3SS has been indicated as the primary anti-grazing mechanism (Lewenza et al. 2014). This makes *C. elegans* an interesting predator model for this research, as *P. chlororaphis* 30-84 does not encode a T3SS, but does form biofilms, allowing the level of importance of the T6SS to be determined.

1.2.3.4. Other known functions of T6SS

As mentioned previously, the function of T6SS were first studied in human and plant pathogens due to their role in the delivery of effectors critical for virulence. Thus, it was thought that the primary role of the T6SS to serve as a virulence and pathogenicity mechanism. The observation that many strains have multiple, functional T6SS and the discovery that T6SS are found in non-pathogenic bacteria have been cited as the basis for the theory that different T6SS may provide cells with different types of functionalities, and that not all these functions are related to virulence. In support of this theory, it was shown that distinct T6SS of *Burkholderia thailandensis*, which has five T6SS, are used in different ways. The T6SS-1 was found to play a role in biofilm formation and competition, whereas T6SS-5 was found to play a role in virulence (Schwarz et al. 2010). Similarly, different roles for separate T6SS in *Aeromonas hydrophila* were observed (Schwarz et al. 2010; Suarez et al. 2008).

It was shown that the presence of a T6SS may confer greater fitness to microbes compared to those without a T6SS in natural environments (Bernal et al. 2018). This has been attributed to the potential role of T6SS in dynamics between bacterial competitors as well as interactions with eukaryotic competitors. For example, Haapalainen et al. (2012) found that bacteria may use the T6SS to obtain a growth advantage over bacteria and yeast in the soil. Some bacteria are also able to use the T6SS to obtain zinc, manganese, and iron giving them a

competitive edge over other bacteria (Bernal, Llamas, and Filloux, 2017; Chen et al. 2016; DeShazer 2019; Lin et al. 2017). Using the T6SS to obtain a growth advantage over pathogenic fungi, may also be involved in applications related to biocontrol. For example, the *P. fluorescens* T6SS genes *vgrG* and *clpV* were more highly expressed on wheat roots infected with the fungal pathogen *Gaeumannomyces graminis* var. *tritici* compared to healthy roots. These observations suggest a possible role for the T6SS in disease suppression, although this has yet to be proven (Barret et al. 2009; Marchi et al. 2013). Another experiment using *P. putida* performed by Bernal et al. (2017) showed that when in direct contact with various plant pathogens in plant leaves, such as *P. syringae, P. putida* was able to use its T6SS to outcompete the pathogen, suggesting a direct mechanism for pathogen suppression.

As mentioned previously, the T6SS has been shown to play a role in the formation and maintenance of biofilms (Chen et al. 2015; Gallique et al. 2017; Mikkelsen, Sivaneson, and Filloux 2011). Experiments have shown that mutations in certain components of the T6SS can negatively affect biofilm formation, including disruption in the secretion of compounds necessary to form the biofilm matrix (Aschtgen et al. 2008; Bernal et al. 2018). An important compound for biofilm matrix formation is thought to be an Hcp-like protein secreted by the T6SS and upregulation in the T6SS lead to an increase in biofilm formation (Aubert, Flannagan, and Valvano 2008; Khajanchi et al. 2009). It is also possible that, within biofilms, the T6SS plays a role in communication, conveying signals among biofilm cells and thus orchestrating biofilm development and architecture and influencing rhizosphere dynamics (Gallique et al. 2017).

1.3. Experimental Design

In my dissertation, I examine potential ecological benefits of having two distinctly different T6SS for a PGPB using the well-characterized PGPB strain, *P. chlororaphis* 30-84 as the biological system. In my study, I created mutants defective in either or both T6SS and used these derivatives to characterize the role of each T6SS in rhizosphere competence, bacterial competition, and protection from bacterivores. My research was facilitated by previous research resulting in the availability of additional genetic tools as well as information regarding competitors/predators that were not targeted by other mechanisms (e.g., phenazines or bacteriocins). Bioinformatic analysis as part of my dissertation provided insights into important differences between the two T6SS-encoding clusters in terms of genetic organization, potential firing regulation, and toxic cargo. It is interesting to consider whether ecological function can be predicted by relatedness (clade) to other well-characterized systems, firing regulation (dueling vs random firing), stockpile of effector and immunity proteins or some combination. Based on the overall differences between the two T6SS-encoding operons, I predicted that they were likely to serve non-redundant functions, important for life in the rhizosphere niche. Using these tools, I addressed the following questions:

1) What does bioinformatic analysis of the two T6SS-encoding gene clusters reveal about potential differences in their structure or regulation?

2) Are the two T6SS-encoding gene clusters simply duplications? Are they sufficiently similar that they fall into the same genetic clades, or are they sufficiently different to indicate that they are structurally, functionally, or evolutionarily different?

3) Are each of these systems functional? If so, do the two T6SS function in a non-redundant manner? Do they each play roles in rhizosphere competence, competition, or predation. Do they target the same spectrum of organisms?

4) Does gene organization (and clade in which they are members) or potential regulation of the two T6SS-encolding gene clusters yield any insights into differences or similarities in their ecological roles?

5) What does genomic analysis reveal about the existence of potential effector proteins and are these paired with immunity proteins? Does bioinformatic analysis of effectors and immunity proteins shed any light on why they target certain organisms?

Chapter II provides all experimental results and is written in manuscript format. I describe the genetic organization of the two *P. chlororaphis* 30-84 T6SS and form and test hypotheses related to their potential functions. I discuss methods to obtain single and double mutants and how I used these tools to test the importance of each T6SS in rhizosphere persistence, bacterial competition, and predation. I provide evidence that both T6SS are functional. I also conduct bioinformatic analyses to identify potential effector proteins that may contribute to the functionalities I observe. I develop hypotheses on how T6SS in PGPB may have direct and indirect impacts on the rhizosphere environment and community dynamics.

Chapter III provides an executive summary and possible future directions as well as potential methods in which to address future directions.

Appendix A includes supplementary material to chapter II and Appendix B includes research on other potential roles of T6SS in PGPB.

CHAPTER II

THE TYPE VI SECRETION SYSTEM FUNCTIONS IN RHIZOSPHERE PROKARYOTIC AND EUKARYOTIC INTERACTIONS

2.1. Introduction

The Type VI Secretion System (T6SS) comprises a needle-like structure that is found in roughly 25% of Gram-negative bacteria (Basler, Ho, and Mekalanos 2013). T6SS have been shown to be important for the delivery of effector proteins, signaling molecules involved in plant-pathogen interactions in prokaryotic and eukaryotic interactions (Abby et al. 2016; Barret et al. 2011; Basler, Ho, and Mekalanos 2013; Bernal et al. 2017). The primary focus of research on these secretion systems is their role in virulence and pathogenesis. A role for T6SS in virulence was first demonstrated using *Vibrio cholerae* and the model eukaryote *Dictyostelium discoideum* (Pukatzki et al. 2006). T6SS have been implicated in virulence and killing of other eukaryotes such as the animal model *Caenorhabditis elegans* (Vaitkevicius et al. 2006). T6SS have been shown to be important virulence factors among human pathogens such as *Burkholderia pseudomallei*, *Pseudomonas aeruginosa, Vibrio cholerae*, and *Salmonella typhimurium* and their T6SS have been linked directly to their virulence (Pukatzki et al. 2006; Sana et al. 2016; Aubert et al. 2016; Basler, Ho, and Mekalanos 2013). T6SS are also important virulence factors among plant pathogens such as *Ralstonia solanacearum* and *Erwinia amylovora* (Asolkar and Ramesh 2020; Tian et al. 2017). Although, T6SS have been well studied in pathogenic systems, much less information exists for their importance in the lifestyle of non-pathogenic organisms. Interestingly, many plant growth promoting bacteria (PGPB) also possess

one or more T6SS (Bernal et al. 2017; Marchi et al. 2013; Loper et al. 2012). Thus, it is likely that T6SS in PGPB may be involved in other functions related to their host-associated niche or plant beneficial activities.

Generally, T6SS are composed of 13-15 structural proteins divided into three interlocking structures: the intermembrane anchor, the baseplate, and the needle/sheath. The length of the entire structure has been shown to be determined by the width of the cell, which can measure up to 1 μm in length, allowing it to interact with both prokaryotes and eukaryotes (Santin et al. 2019; Basler, Ho, and Mekalanos 2013; Abby et al. 2016; Bernal et al. 2017). This needle is topped with a valine-glycine repeat protein G (VgrG) trimer which is in turn topped by one proline-alanine-alanine-arginine (PAAR) repeat protein using hydrogen bonds (Shneider et al. 2013) which then acts as a sharpener enabling the end to penetrate neighboring cells (Basler 2015; Ho, Dong, and Mekalanos 2014). ClpV "recycles" the system by detaching the proteins and allowing them to reform elsewhere (Kapitein et al. 2013; Zoued et al. 2014). Other genes, such as the serine/threonine kinase and phosphatase (*ppkA* and *pppA*) are associated with those genes encoding structural proteins and are heavily involved in the regulation of firing of the system (Chen et al. 2015; Kulasekara and Miller 2007).

A great deal of genetic diversity has been found among the operons encoding T6SS and efforts to characterize this diversity has led to T6SS-encoding operons being partitioned into five clades based on their genetic organization and the presence or absence of specific genes (Boyer et al. 2009; Bernal, Llamas, and Filloux, 2017). Characterization of T6SS using this clade system illustrates both the diversity in bacterial taxa having T6SS as well as the diversity of systems found within a single species. For example, *Pseudomonas* species have T6SS belonging to clades 1.1, 1.2-A, 2, 3, 4A, and 4B-1 and a single species, *P. putida*, has T6SS belonging to all these

clades. In contrast, *P. syringae* appears to have T6SS belonging to only two clades (Bernal, Llamas, and Filloux, 2017).

Many species that have T6SS have operons that encode more than one system (Chen et al. 2015; Chen et al. 2011; Loper et al. 2012; Spiewak et al. 2019). When more than one system is present, it is possible that the systems perform different, non-redundant functions, increasing the repertoire of functionalities provided. For example, *Burkholderia thailandensis* possesses five different T6SS and these systems have been shown to perform different roles (Schwarz et al. 2010). The different T6SS in a single strain can differ in terms of their genetic organization, the presence or absence of certain effectors, and their regulation. In *Pseudomonas*, T6SS can differ further in terms of the type of stimuli that causes firing, e.g., contact-dependent firing or random firing. Contact-dependent firing is regulated by the signaling cascade TagQRST. This signaling cascade alerts the cell when damage has been caused to its membrane and triggers the formation of the T6SS in what is known as dueling behavior (Basler, Ho, and Mekalanos 2013). To fire randomly, the TagQRST signaling cascade is not needed. Within the same *Pseudomonas,* there can be T6SS with both dueling and random firing behavior, such as *P. fluorescens* Q287, which contains three T6SS clusters, one of which contains the TagQRST signaling cascade (Basler, Ho, and Mekalanos 2013; Loper et al. 2012, this study). A direct method to determine T6SS function has not been established based purely on sequence analysis, but the genetic organization, effector types, and firing regulation are all possible determinants of functionality. Some groups hypothesize that function is determined by genetic organization and where T6SS fall in the genetic clades, whereas other groups believe function is simply determined by which effectors are linked to the system (Bernal, Llamas, and Filloux, 2017).

To gain a better understanding of the importance of T6SS for the rhizosphere lifestyle, I focus on *Pseudomonas chlororaphis* 30-84, a well-characterized, plant growth promoting bacteria (PGPB) that is an effective rhizosphere colonizer. This strain was previously shown to utilize the diffusible, anti-microbial compound phenazine to protect wheat plants against take-all disease and mediate water and salt stress (Pierson and Weller 1994; Mahmoudi et al. 2019; Yuan et al. 2020). The genome of *P. chlororaphis* 30-84 encodes two genetically distinct T6SS (Loper et al. 2012, this study). As observed in other *Pseudomonas* sp., the genes of T6SS are notably down regulated in Gac mutants (Hassan et al. 2010; Wang et al. 2013; Records and Gross 2010; Chen et al. 2015). The GacS/GacA two component system also controls the production of secondary metabolites (including phenazines) and extracellular enzymes involved in pathogenicity, biocontrol, ecological fitness, or stress tolerance, providing an important control to determine specific T6SS functions (Heeb and Das 2001). I hypothesize that if both T6SS are functional, they may serve non-redundant functions. In this study, I created mutants defective in one or both T6SS and used these derivatives to characterize the role of each T6SS in rhizosphere competence, bacterial competition, and protection from bacterivores. I also took advantage of an existing collection of mutants deficient in the production of phenazines (but not T6SS activity) or reduced in the expression of both phenazines and T6SS (*P. chlororaphis* 30-84 GacA) to characterize the importance of T6SS relative to other mechanisms of interference.

2.2. Materials and Methods

2.2.1. Bacterial strains and media

The bacterial strains and plasmids used in this study are described in **Table 2.1**. A spontaneous rifampin-resistant derivative of *P. chlororaphis* 30-84 was used in all studies, hereafter referred to as wild type (30-84 WT). *P. chlororaphis* and wheat rhizosphere test strains

was grown at 28°C in the following media: Luria-Bertani (LB) (Fisher BioReagents, Hampton, NH), AB minimal (2% glucose) (Chilton et al. 1974) amended with 2% casamino acids (AB+CAA) (CAA is from BD Bacto, San Jose, CA) or King's medium B (KMB) (King, Ward, and Raney 1954). *Escherichia coli* was grown at 37°C in LB medium, unless otherwise noted. *E. coli* and *Pseudomonas* strains were grown in liquid culture with agitation (200 rotations/min) or on solid medium (amended with agar at 15 g/l). Antibiotics were used in the following concentrations for *E. coli:* kanamycin (Km), gentamicin (Gm), carbenicillin (Cb), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 50, 15, 100, and 40 μg/ml, respectively; and for *P. chlororaphis:* Km, Gm, Cb, rifampicin (Rif), and Cycloheximide (Cyclohex) at 50, 50, 100, 100, and 100 μg/ml, respectively.

Table 2.1: Bacteria strains and plasmids used in this study.

Strain	Description	Reference
Pseudomonas		
30-84 WT	*P. chlororaphis* 30-84, Rif^r, "wild type"	W.W. Bockus
30-84 ZN	Phz^-, Rif^r, *phzB::lacZ* genomic fusion	Pierson et al. 1994
30-84 GacA	Phz^- Rif^r spontaneous *gacA* mutant	Chancey, Wood, and Pierson 1999
30-84 I/I2	*phzI::npt* and *csaI::uidA-Gm* genomic fusion, Gm^r	Zhang and Pierson 2001
ΔTssA1	T6SS-1 mutant: Pchl3084_RS17705 replaced with Km^r cassette	This Study
ΔTssA2	T6SS-2 mutant: Pchl3084_RS00080 replaced with Km^r cassette	This Study
ΔTssA1/2	T6SS-1/2 mutant with Pchl3084_RS17705 replaced with Km^r and Pchl3084_RS00080 replaced with Gm^r cassette	This Study

Table 2.1 continued.

Strain	Description	Reference
Rhizosphere colonizing, biocontrol strains		
P. protegens Pf-5	Rhizosphere associated (formerly *P. fluorescens* Pf-5)	Howell and Stipanovic 1979
P. synxantha 2-79	Rhizosphere associated (formerly *P. fluorescens* 2-79)	Weller and Cook 1983
P. fluorescens Q2-87	Rhizosphere associated	Weller and Pierson 1994
Plant Pathogenic Strains		
P. putida F1	Environmental isolate	TAMU Teaching Collection
P. syringae pv. *tomato* DC3000	Plant pathogen with Type III Secretion System	Petnicki-Ocwieja et al. 2002
Agrobacterium tumefaciens C58	Gall-forming plant pathogen	TAMU Teaching Collection
Pectobacterium carotovorum subsp. *carotovorum*	Plant pathogen	TAMU Teaching Collection
Escherichia		
E. coli DH5α	*F⁻recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(argF-lacZYA) Iq69 Φ80lacZΔ*M15λ	GIBCO-BRL
E. coli HB101	F⁻ *hsds20*($r_B^-m_B^-$)*supE44recA1 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-5* λ^-	GIBCO-BRL
E. coli ΔB	*D. discoideum* food source	*Dictyostelium* Stock Center
E. coli OP50	*C. elegans* food source	Brenner 1974
Plasmids	**Description**	**References**
pEX18Ap	Ap^r	Hoang et al. 1998
pUC4K	Km^r, Ap^r	Grindley and Joyce 1981
pUCP20Gm	Gm^r, pUCp20 derivative containing constitutive promoter P_{lac} with *Sma*I-flanked Gm^r cassette inserted into the unique *Sca*I site within *bla*	Chiang and Burrows 2003
pEX18A+TSSA2	pEX18A containing *tssA*-2 upstream and downstream sequences separated by a KpnI restriction site	This study
pEX18A+TSSA2/KMR	pEX18A containing *tssA*-2 upstream and downstream sequences separated by a Km resistance cassette	This study

Table 2.1 continued.

Plasmids	Description	References
pEX18A+TSSA1/KMR	pEX18A containing *tssA*-1 upstream and downstream sequences separated by a Km resistance cassette	This study
pEX18A+TSSA2/GMR	pEX18A containing *tssA*-2 upstream and downstream sequences separated by a Gm resistance cassette	This study

Ap^r, Km^r, Gm^r, Rif^r indicate ampicillin, kanamycin, gentamicin, and rifampin, respectively.

2.2.2. Phylogenetic analysis of chromosomal regions containing two putative T6SS

P. chlororaphis 30-84 genes annotated as encoding two T6SS are shown in **Fig. 1.1**. We determined how these two T6SS compared to clades established by Boyer et al. (2009) and expanded by Bernal et al. (2017). The amino acid sequences encoded by four highly conserved genes, *tssB*, *tssC*, *tssK*, and *tssM*, from each of the two *P. chlororaphis* 30-84 T6SS and the corresponding sequences from five species representing each clade were retrieved from the National Center for Biotechnology Information (NCBI) database and compared using BLASTp (Altschul et al., 1990). Based on levels of amino acid sequence identity, the *P. chlororaphis* 30-84 proteins were most similar to proteins in clades 3, 1.1 and 4A. The same *P. chlororaphis* 30-84 amino acid sequences were then compared to the corresponding amino acid sequences of 12 other plant-associated species belonging to clade 1.1, 3, or 4A. Also included were corresponding amino acid sequences from several biological control strains (Loper et al. 2012). The sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) through the program MEGA7 (Kumar, Stecher, and Tamura 2016). Once aligned, the Jones, Taylor, and Thorton (JTT) model in MEGA7 and bootstrap analysis with 1000 bootstrap replicates was used to build a maximum likelihood (ML) tree for each of the conserved

genes. The program FigTree v1.4.4 (Rambaut 2009; Gardner and Hall 2013) was used to visually represent the ML tree.

2.2.3. Generation of single and double T6SS mutants

A derivative of *P. chlororaphis* 30-84 containing a *tssA-2* deletion mutation (ΔTssA2) was generated using the suicide vector pEX18Ap and using methods described previously (Hmelo et al. 2015). Briefly, DNA sequences (1,000 nucleotides [nt]) flanking the gene *tssA-2* were amplified by two-step PCR using the primer pairs TssA2KO-UP-F-EcoRI and TssA2KO-UP-R-KpnI, and TssA2KO-DWN-F-KpnI and TssA2KO-DWN-R-HindIII, respectively **(Supplementary Table 2.1)**. Amplification using primers TssA2KO-UP-F-EcoRI and TssA2KO-DWN-R-HindIII and using the product of the previous PCRs as a template resulted in a construct that contained the upstream fragment separated from the downstream fragment by a *Kpn*I restriction site. This fragment was ligated into the *Eco*RI and *Hin*dIII restriction enzyme sites in the multiple-cloning region of pEX18a to create plasmid pEX18A+TSSA2. A kanamycin resistance cassette with its promoter was obtained via PCR amplification using pUC4K as the template and the primers TssA2KO-UP-R-KpnI and TssA2KO-DWN-F-KpnI and ligated between the upstream and downstream fragments at the *Kpn*I site in pEX18Ap. The final construct (pEX18A+TSSA2/KMR) was transformed into *E. coli* DH5α, and transformants were selected on LB amended with Km and Xgal. After conjugation, double-crossover mutants into *P. chlororaphis* were obtained by counterselection on LB amended with Rif, Km, and 6% sucrose and confirmed using PCR primers specific to the internal regions of *tssA-2*. PCR was performed using GoTaq® Green Master Mix (Promega, Madison, WI) according to manufacturer recommendations. *E. coli* transformation and *P. chlororaphis* conjugation were performed as described previously (Pierson and Thomashow 1992; Wang et al. 2012).

A derivative of *P. chlororaphis* 30-84 containing a *tssA-1* deletion mutation (ΔTssA1) was generated using the suicide vector pEX18Ap. Briefly, DNA sequences upstream (~1200 nt) and downstream (~1,100 nt) flanking *tssA-1* were amplified via PCR using the primer pairs TssA1KO-repliQa-UP-F and TssA1KO-repliQa-UP-R, and TssA1KO-repliQa-DWN-F and TssA1KO-repliQa-DWN-R, respectively **(Supplementary Table 2.1)**. The kanamycin resistance cassette with its promoter was obtained via PCR amplified using pUC4K as the template and primers TssA1KO-repliQa-KmR-F and TssA1KO-repliQa-KmR-R. The final construct (pEX18A+TSSA1/KMR) was obtained using repliQa HiFi Assembly according to manufacturer recommendations ("RepliQa HiFi ToughMix | Superior Speed and Inhibitor Tolerance | Quantabio"). DH5α transformants were selected on LB amended with Km and Xgal. *P. chlororaphis* double-crossover mutants were obtained by counterselection on LB amended with Rif, Km and 6% sucrose and confirmed using PCR primers specific to the internal regions of *tssA-1*.

To generate the double mutant ΔTssA1/2, a gentamicin resistant cassette with its promoter was amplified using the plasmid pUCP20Gm as the template and the primer pairs DoubleKO-GnR+A2UP-R and DoubleKO-GnR+A2DWN-F. The plasmid pEX18A+TSSA2 was digested with *Kpn*I. The final construct containing the gentamicin resistance cassette (pEX18A+TSSA2/GMR) inserted at the *Kpn*I site was obtained using repliQa HiFi Assembly. The plasmid pEX18A+TSSA2/GMR was transformed into *E. coli* DH5α and transformants were selected on LB amended with Gm and Xgal. *P. chlororaphis* double-crossover mutants in ΔTssA1 were obtained by counter selection on LB amended with Rif, Km, Gm and 6% sucrose and confirmed using PCR primers specific to the internal regions of *tssA-1* and *tssA-2.*

2.2.4. Growth in planktonic culture and surface attached biofilms

The strains 30-84 WT, ΔTssA1, ΔTssA2, and ΔTssA1/2 were grown in LB medium at 28°C with agitation and the optical density (OD_{620}) was measured at one-hour intervals until 8 h. and then at two-hour intervals between 24 h. and 30 h. The experiment was performed with two biological replicates and repeated three times.

Surface attached biofilm formation was quantified via 96-well microtiter assay routinely used in our lab (Maddula et al. 2006) with slight modifications. Briefly, pre-cultures were grown in LB medium (28°C, with agitation, 16 h). These cultures were resuspended in fresh LB medium and grown to a final cell density of OD_{620} = 0.8. Each strain (1.2 µL) was inoculated into 120 µL AB + CAA in separate wells of a 96 well polystyrene cell culture plate (Corning Inc., Corning, NY, USA). The plate was incubated at 28°C for 72 h without agitation in a sealed container to minimize evaporation. Unattached cells were removed by inversion of the plate with vigorous tapping. The adherent bacteria were fixed to the plate (20 min, 50°C) and stained (1 min, 150 µL of 0.1% crystal violet). Excess stain was removed by inversion of the plate followed by two washings with sterile distilled water. The adherent cells were decolorized (to release the dye) with a 20% acetone/80% ethanol solution (200 µL, 5 min, room temperature). A sample (100 µL) of each well was transferred to a new 96-well plate and the amount of dye (proportional to the density of adherent cells) was quantified (OD_{540}). The experiment was performed with two biological replicates (started from separate colonies) and five technical replicates and repeated three times.

2.2.5. Rhizosphere colonization and persistence assay

We used repeated planting/harvest cycles to evaluate rhizosphere persistence as described previously (Mazzola et al. 1992). The assay was performed using methods described previously (Dorosky et al. 2017) with minor modifications. Soil used for rhizosphere experiments was a Pullman clay loam collected from the USDA-ARS, Bushland, TX dryland wheat plots at a depth of 1 to 15 cm. Prior to use in experiments, it was necessary to sieve (2 mm mesh) and mix the soil with sand (soil:sand, 2:1, v:v) to facilitate drainage as described previously (Mahmoudi et al. 2019). The soil-sand mix is hereafter referred to as soil. The hard red winter wheat cultivar TAM 304 developed by Texas A&M AgriLife Research was used for all rhizosphere studies (Rudd et al. 2015).

Bacteria were grown overnight with antibiotic selection, washed twice with sterile water, and resuspended in sterile water at a final concentration of $1x10^9$ CFU/mL. Inoculum was added to either steam-sterilized soil (autoclaved twice: 121°C, 15 psi, 45 min, 24 h pause between cycles) or untreated (field) soil. Final bacterial concentrations were adjusted to 10^6 CFU/g by dilution using sterile water, adding the diluted suspension to soil (20 mL solution per 500 g), and mixing thoroughly daily for four days. Soil was then added to clean conical plastic growth tubes (Ray Leach Cone-tainers, 4 cm diameter, 21 cm height).

Wheat seeds (TAM 304) were surface disinfested by incubation in 10% bleach (10 min.) and then washed with sterile water (5 times for 1 min. each). Disinfested seeds were pregerminated on germination paper for 48 h. The seedlings were planted in the growth tubes four days after the soil was inoculated with bacteria. A total of 50 plants were sown at the start of the experiment. Plants were grown on a light bench (8 h.:16 h. dark/light cycle, room temperature) and given sterile water (10 mL) every five days. After 20 days of growth, 10 of the

50 plants from each treatment were randomly selected and harvested and rhizosphere populations determined. The unharvested plants (remaining 40 of the 50 plants/treatment) were removed individually from their containers, the shoot system was excised and discarded, and the soil and root system were transferred to a clean paper cup, mixed by shaking, and returned to the conical growth tube from which they were obtained. This soil was then replanted with disinfested, pregerminated wheat seeds to initiate the second 20-day planting/harvest cycle. At each harvest, ten of the remaining plants from each treatment were harvested and rhizosphere populations determined. The planting to harvest cycle was repeated for a total of 5 cycles. The entire experiment was repeated twice.

2.2.6. *In vitro* and rhizosphere competition assays

Competitive fitness assays compared the populations of competitors grown separately and in 50:50 mixed cultures *in vitro*. Briefly, bacterial strains were grown overnight in LB at 28°C with agitation (200 rpm), harvested, washed, and resuspended in fresh medium (cell densities were adjusted to OD_{620} = 0.5) before creating the single strain or mixed starting cultures. Mixed cultures were prepared using equal volumes of competitors. A total of 10 µl per treatment was placed onto ~ 1 cm^2 pieces of nitrocellulose filter paper on LB plates, and plates were incubated at 28°C for 5 h. Nitrocellulose papers then were transferred separately to sterile tubes containing 1 mL sterile water (sufficient to cover filter paper), and cells were collected by vortexing for 30 sec. twice with a 5 min. rest in between. Bacterial populations were enumerated via serial dilution plating on LB after 48 h. The experiment was performed with two biological replicates and repeated at least three times.

Competitive fitness assays comparing the growth of competitors grown separately and in 50:50 mixed cultures in the wheat root rhizosphere were performed similar to previous methods

(Dorosky et al. 2017). The inoculum was prepared as described for the *in vitro* assay, but the final total cell density used to inoculate seeds was 10^9 CFU/ml (OD_{620} = ~1.0). Wheat seeds were surface disinfested and pregerminated (as described above) and then suspended in bacterial inoculum for 10 min. The seeds were sown into steam-sterilized soil (prepared as above). Plants were grown and maintained as above. After 28 days, the entire root system and loosely adhering soil was transferred to a sterile plastic tube (15 mL), immersed in 5 mL of sterile water and sonicated and vortexed three times (10 sec. each). Serial dilutions were plated onto LB amended with cycloheximide and bacterial populations quantified after 48 h. The roots were dried for 48 h. at 65°C and populations were standardized to root dry weight. The experiment was performed with 8-10 replicates/treatment and repeated three times.

2.2.7. Predator-Prey studies

2.2.7.1. *Dictyostelium discoideum*

D. discoideum strain AX2 was purchased from the *Dictyostelium* stock center (Fey et al. 2013). *D. discoideum* cells were grown in SIH medium (Formedium, Hunstanton, England) with agitation as described previously (Brock and Gomer 1999; Rijal et al. 2019). *D. discoideum* cells were collected by centrifugation (500 x g, 3 min.), resuspended/washed in fresh SIH twice, and resuspended at a final concentration of 1 x 10^6 cells/mL in SIH (determined via direct counts using a hemocytometer). *D. discoideum* cells (1 mL/well) were added to 24-well plates (Cat. #353047, Corning, NY), allowed to adhere for 30 min., and then the liquid medium was replaced with low nutrition PBM or high nutrition HL5 (Phillips and Gomer 2012).

Bacterial cultures were grown in LB media for 16 h., and bacterial cells were collected by centrifugation (2,000 x g, 1 min.) and resuspended in PBM. Bacterial cultures were then

standardized to a low cell density (OD_{600} = 0.1) and added to the wells containing *D. discoideum* cells. Bacteria used as prey in the feeding assay included 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, or 30-84 ZN. *D. discoideum* cells growing without bacteria as a food source or with *E. coli* ΔB (used in lab as a preferred prey source) were used as controls. After 24 h., the mixed cultures were slowly resuspended with a pipettor to detach the *D. discoideum* cells from the wells and 200 μL from each well was transferred into a 96-well microtiter-plate suitable for microscopy (#160822/1, ibidi, Martinsried, Germany). The *D. discoideum* cells were left to adhere for 30 min. Differential Interference Contrast (DIC) images were obtained using a Nikon Ti2 Eclipse Microscope (40X, 100X oil). Another 200 μL from each well was used for serial dilutions to determine bacterial populations.

To measure Contact Site A protein (CsA) and Discoidin I levels in *D. discoideum* cells, the phagocytosis assay was performed as above. After 24 h., the supernatant was removed from each well and 200 μL of 2X SDS was added to each well. The lysates were then heated to 95 °C for 5 min. Samples were electrophoresed and blotted as described previously (Bakthavatsalam et al. 2008) with the exception that the blots were blocked in 5% non-fat skim milk (Difco, Franklin Lakes, NJ) in PBST (Phosphate Buffered Saline (pH 7.4) + 0.1% Tween-20) for 1 h. and stained as previously described (Rijal et al. 2019) with either 1:500 #20-121-1-s anti-CsA (Developmental Studies Hybridoma Bank, Iowa City, IA) or 1:500 #80-52-13-s anti-discoidin I (Developmental Studies Hybridoma Bank, Iowa City, IA) and 1:2500 #715-036-150 peroxidase-conjugated donkey anti-Mouse IgG (Jackson ImmunoResearch, West Grove, PA). Staining was detected with Supersignal West Pico PLUS Chemiluminescent Substrate for 10 min. (Cat # 34087, Thermo, Waltham, MA). Images of the membrane were taken using a BioRad ChemiDoc XRS system and quantified using the Image Lab software (BioRad, Hercules, CA).

To determine the effect of predation on bacterial fitness, a bacterial clearing assay was performed as described previously (Phillips and Gomer 2010). Briefly, bacterial cultures and *D. discoideum* cells were grown and collected as described above with the exception that the final concentration of *D. discoideum* was 500,000 cells/mL. 100 µL of each bacterial culture were spread onto SM/5 (pH = 6.5) medium plates and 10 µL of *D. discoideum* was then transferred to the center of the plate. Plates were incubated at 22°C for 5 days and the diameter of the zone of clearing was measured after 2 and 5 days. This experiment was repeated using four biological replicates.

2.2.7.2. *Tetrahymena thermophila*

For the feeding assay, *T. thermophila* CU427 (*Tetrahymena* Stock Center, Cornell University, Ithaca, NY) were grown according to a previous protocol with minor modifications (Wilson et al. 1999). Briefly, *T. thermophila* was grown in PPYS (2% proteose peptone, 90 µM sequestrene, 0.2% yeast extract) liquid medium overnight with agitation (200 rpm, 30°C) and then transferred to 200 mL fresh media. Populations were adjusted to $3x10^5$ cells/20 mL via direct population counts using a hemocytometer and Leitz (Epivert) microscope (100X). Bacterial cultures were grown in LB for 16 h., and bacterial cells were collected by centrifugation (3,000 x g, 15 min.) and washed with an equal amount of sterile water. Bacterial cultures were then standardized to a low cell density (OD_{620} = 0.1). Bacteria used as prey in the feeding assay included 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, or 30-84 ZN. *T. thermophila* without prey bacteria was used as a control. Bacterial and *T. thermophila* cultures were mixed (5 mL, 20 mL, respectively) and grown in 50 mL tubes with agitation (200 rpm, 27 °C). After 4 h. and 24 h., *Tetrahymena* populations in mixed cultures were enumerated

via direct counts using a hemocytometer and bacterial populations were enumerated via serial dilution plating. This experiment was repeated six times.

For the mating assay, *T. thermophila* with different germlines, CU427 and CU330, were selected. These were selected as they are non-self-cells of different germlines and will reproduce together (Cervantes et al. 2013). CU427 and CU330 (*Tetrahymena* Stock Center, Cornell University) were grown in PPYS liquid media overnight (200 rpm, 30 °C) and collected via centrifugation (3,000 x g, 10 min.) and washed in an equal amount of 10 mM Tris Buffer (pH = 7.4) twice. *T. thermophila* were grown overnight (200 rpm, 30 °C) in 10 mM Tris Buffer to induce starvation and then populations were standardized to a cell density of 1.5-2 x 10^5/10 mL (via direct counts). Bacterial cultures were prepared as described above and standardized to OD_{620} = 0.1. *T. thermophila* CU427 and CU330 cultures were mixed in equal amounts (10 mL each) with 1 mL of the bacterial culture. After 4 h. the treatments were viewed under the Leitz (Epivert) microscope, and the frequency of mated cell pairs (number of mated pairs/total number of observations) determined. Mating is defined by the joining to two *T. thermophila* cells vs. cells that remain single. This experiment was repeated three times.

2.2.7.3. *Caenorhabditis elegans*

C. elegans N2 hermaphrodites (*Caenorhabditis* Genetics Center, University of Minnesota, Minneapolis, MN) were partially synchronized by allowing the nematodes to crowd a Nematode Growth Media (NGM) plate (Brenner 1974) and consume all of their food source. Once the eggs produced on this plate hatched to stage L1 they were removed to a fresh NGM plate with *E. coli* OP50 for food and allowed to grow (20 °C) to stage L4. Once mature, five L4 adult nematodes were selected and placed onto new NGM plates inoculated with one of the different prey bacteria: *E coli* OP50 (control), 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84

GacA, 30-84 I/I2, or 30-84 ZN. Nematodes were allowed to lay eggs for 1 h. (at room temperature) and then transferred to a new prey-containing plate. This transfer protocol was repeated until there were four plates per treatment, with the original nematodes being removed from the fourth plate. The plates were observed every 24 h. for 72 h. The numbers of immature and mature nematodes were enumerated, and the percentage of adult nematodes calculated. Images of the plates were taken after 72 h. using a Zeiss Stemi SV11 scope (26X magnification) and a Hamamatsu ImagEM EM-CCD camera. Bacterial clearing was estimated from total space occupied by *C. elegans* at the end of 72 h. Experiments were replicated three times.

2.2.8. Statistical Analyses

All data presented are the mean ± the standard error from at least two experiments. Multiple comparisons were analyzed ($\alpha = 0.05$) using ANOVA and either Tukey HSD or Student's t tests and significant differences ($P < 0.05$) are indicated by lowercase letters unless otherwise stated. All data were analyzed using JMP Version 16 Software (SAS Institute In., Cary, NC).

2.3. Results

2.3.1. *P. chlororaphis* 30-84 has two putative T6SS

P. chlororaphis 30-84 contains two separate T6SS gene clusters, T6SS-1 and T6SS-2 **(Fig. 1.1)**, both of which contain genes encoding at least 12 of the 13 conserved T6SS proteins (*tssA*-*tssM*). These proteins combine into subunits that make the three structures necessary for T6SS formation: the intermembrane structure (composed of the proteins TssJ, TssL, and TssM); the baseplate structure (composed of TssE, TssF, TssG, and TssK); and the sheath and needle-like structure (TssB, TssC and Hcp/TssD) with TssA coordinating the assembly of the final

structure (Planamente et al. 2016; Zoued et al. 2017). The gene encoding Hcp is not present in the T6SS-2 cluster, but an additional putative *hcp* gene is found elsewhere in the genome. The needle-like structure is topped with a valine-glycine repeat protein G (VgrG/TssI) trimer that in most cases is associated with effector proteins (Ho, Dong, and Mekalanos 2014). The proline-alanine-alanine-arginine (PAAR) repeat protein sits atop VgrG and acts as a sharpener enabling the end to penetrate neighboring cells (Basler 2015; Ho, Dong, and Mekalanos 2014; Shneider et al. 2013). Both T6SS-1 and T6SS-2 have genes encoding putative VgrG proteins associated with their clusters (one and two genes, respectively), and an additional five putative VgrG-encoding genes are found elsewhere in the genome. Only the T6SS-2 contains a gene encoding PAAR in its cluster, with nine more putative PAAR-encoding genes occurring elsewhere in the genome. ClpV (TssH) is involved in recycling the system (Kapitein et al. 2013; Zoued et al. 2014) and is found in both T6SS clusters. Hcp, VgrG, and PAAR proteins are associated with the delivery of effectors (Ho, Dong, and Mekalanos 2014) and effectors are frequently identified based on their proximity to the encoding genes in the genome (Spiewak et al. 2019). The locations of these genes in the genome were used to search for putative effectors associated with both T6SS.

The two T6SS differ in organization. In T6SS-1, the structural genes are divergently transcribed whereas in T6SS-2 the genes are transcribed in the same direction. The two gene clusters also differ in that the genes encoding the regulation cascade system TagQRST, shown previously to be responsible for contact-dependent firing used in T6SS dueling (Basler, Ho, and Mekalanos 2013). TagQRST are found only within T6SS-1 gene cluster, suggesting this system may be fired in a contact-dependent manner. A serine/threonine kinase (PpkA) must be present and able to phosphorylate the protein Fha for the dueling signal to be received and induce T6SS formation, and a serine/threonine phosphatase (PppA) is required to dephosphorylate Fha

allowing the T6SS to be dismantled and recycled by ClpV (Ho, Dong, and Mekalanos 2014; Casabona et al. 2013). Although the T6SS-2 cluster lacks the Tag regulatory cascade, it contains genes encoding a serine/threonine kinase (Stk1), Fha, and a serine/threonine phosphatase (Stp1), suggesting random firing as seen in other systems (Basler and Mekalanos 2012). Based on the differences in the organization and regulation of these systems, we hypothesized that they respond to different stimuli and potentially serve non-redundant functions.

We compared the two *P. chlororaphis* 30-84 T6SS clusters to the genetic clades established by Boyer et al. (2009) and expanded by Bernal et al. (2017). The predicted amino acid sequences of four highly conserved structural genes, *tssB, tssC, tssK*, and *tssM* from T6SS-1 and T6SS-2 and the corresponding sequences from five species representing each clade were compared using BLASTp. Based on the levels of amino acid sequence identity, the proteins in T6SS-1 were most similar to clade 3, whereas the amino acid sequences in T6SS-2 were similar to both clades 1.1 and 4A. The predicted protein sequences of these genes in T6SS-1 and T6SS-2 were then compared to corresponding sequences from 12 other plant-associated species belonging to clade 1.1, 3, or 4A. A maximum likelihood tree **(Fig. 1.2)** was constructed and confirmed that the amino acid sequences in T6SS-1 group aligned optimally with clade 3. Clade 3 includes T6SS from a wide variety of genera, including the *P. aeruginosa* T6SS involved in contact-dependent dueling (Basler, Ho, and Mekalanos 2013). The sequences in T6SS-2 predominately align with clade 1.1, which includes T6SS from many different pseudomonads such as *P. fluorescens* strains that appear to be random firing systems (Bernal, Llamas, and Filloux, 2017).

2.3.2. Growth of *P. chlororaphis* T6SS mutants

To study the function of each T6SS, mutants were generated to disrupt T6SS assembly via deletion of *tssA* in each system and double mutants generated in both systems. In planktonic culture, there was no difference in the growth rates of wild type (30-84 WT) or the single T6SS mutants (ΔTssA1 and ΔTssA2), although the double mutant (ΔTssA1/2) consistently grew somewhat slower and reached a slightly, but significantly lower cell density after 30 h. **(Fig. A1A)**. However, in surface-attached biofilms, population levels of 30-84 WT and the single or double T6SS mutants were no different after 72 h **(Fig. A1B)**.

We also looked at the ability of 30-84 WT, ΔTssA1, ΔTssA2, and ΔTssA1/2 to colonize and persist in the wheat rhizosphere after multiple plant/harvest cycles in steam-sterilized and untreated field soil. No differences in the rhizosphere populations of strains were observed at the end of one harvest cycle in either sterile or field soil **(Fig. 2.1)**, indicating no loss in colonizing ability by the mutants. In the steam-sterilized soil, although the rhizosphere populations were slightly smaller at the end of five plant harvest cycles compared to the first harvest, there were still no statistical differences among treatments. In contrast, in natural soil the rhizosphere populations of ΔTssA1/2 were significantly reduced compared to 30-84 WT whereas populations of ΔTssA1, ΔTssA2 were intermediate **(Fig. 2.1)**. These data suggested that having at least one T6SS is not necessary for rhizosphere colonization but *is* important for competitive persistence.

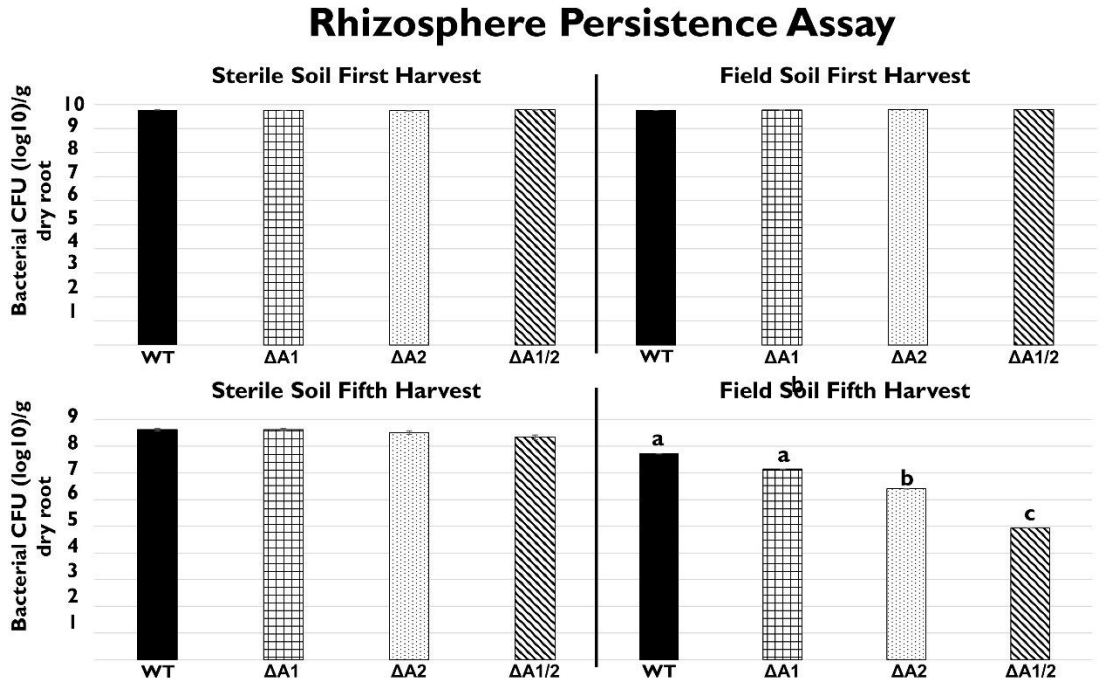

Figure 2.1: Rhizosphere persistence over repeat harvests. Bacterial populations (log10 CFU/g dry root weight) in sterile and field soil after the first and fifth harvest. Strains tested included 30-84 WT, the single T6SS mutants, ΔTssA1 and ΔTssA2, and the double mutant, ΔTssA1/2. Data are the mean and standard errors (bars too small to see) of three replicates (n=30/treatment). Lettering indicates level of significant difference. Data were analyzed using a one-way ANOVA and Tukey's tests and significant differences are indicated, $p<0.05$.

2.3.3. Bacterial competition assays

We explored the hypothesis that disruption of either or both T6SS alters competitive fitness using single strain competition assays *in vitro*. For this assay, 30-84 WT and each of the T6SS mutants were grown separately or in 50:50 mixtures with other well-characterized rhizosphere colonizing *Pseudomonas* biological control strains. These included rhizosphere colonizing strains that were not affected by phenazines and for which there was information about their T6SS and the sequence similarity of each to the *P. chlororaphis* 30-84 T6SS clusters.

Rhizosphere competitors included *P. synxantha* 2-79 (having no T6SS), *P. protegens* Pf-5 (having a T6SS-1 homolog), and *P. fluorescens* Q2-87 (having three T6SS, including T6SS-1 and T6SS-2 homologs) (Loper et al. 2012, and **Fig. 1.2**). For mixed strain treatments, strains were grown independently then mixed and applied to filter paper on solid LB medium, whereas for single strain treatments the same total volume of the one strain was applied. The population of each strain in mixture were compared the populations of their single strain counterparts to observe any effect on growth. Competition with 30-84 WT, ΔTssA1, ΔTssA2, or ΔTssA1/2 are shown as separate analyses **(Fig. 2.2)**. All strains grew well when cultured separately on filters. In competition with *P. synxantha* 2-79, 30-84 WT and ΔTssA1 reduced *P. synxantha* 2-79 populations, whereas ΔTssA2 and ΔTssA1/2 permitted substantial growth of *P. synxantha* 2-79 in mixed cultures. These results suggest, T6SS-2 (which may be randomly firing), but not TSS6-1 (which may be contact dependent) confers a competitive advantage to *P. chlororaphis* 30-84 over *P. synxantha* 2-79 (which lacks a T6SS). In contrast, neither of the T6SS had an appreciable effect on the growth of *P. protegens* Pf-5, whereas *P. protegens* was able to reduce the growth ΔTssA1/2 in mixed culture. In competition with *P. fluorescens* Q2-87, 30-84 WT and both single T6SS mutants virtually eliminated *P. fluorescens* Q2-87, whereas ΔTssA1/2 permitted growth of *P. fluorescens* Q2-87 in mixed culture, indicating having at least one T6SS conferred a competitive advantage to 30-84 WT **(Fig. 2.2)**.

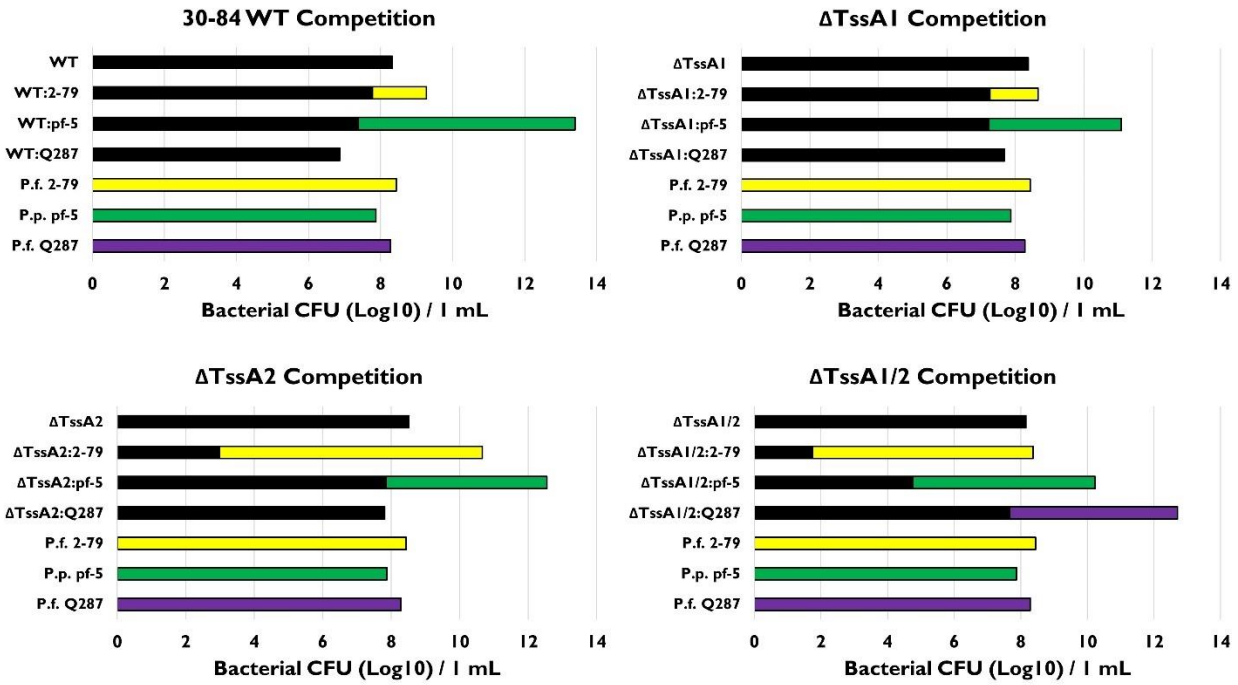

Figure 2.2: *In vitro* competition assays. The competitive fitness of 30-84 WT and T6SS mutants (black color) were evaluated by comparing their populations when grown separately or in 50:50 mixtures with other *Pseudomonas* rhizosphere colonizing bacteria in liquid media, including *P. fluorescens* 2-79 (yellow color, having no T6SS), *P. protegens* Pf-5 (green color, having a T6SS-1 homolog), and *P. fluorescens* Q2-87 (purple color, having three T6SS, including T6SS-1 and T6SS-2 homologs). Individual bacterial cultures or mixture cultures were spotted onto nitrocellulose filters on LB plates and incubated at 28 °C, 5 h. Bacterial cells were washed from filters, collected via centrifugation, and populations were enumerated after 48 h. via serial dilution plating. Data are the means (log10 CFU/1 mL) of 5 replicates/treatment pooled across three experiments (n = 15/treatment).

In the rhizosphere, in mixed treatments with 30-84 WT or either of the single T6SS mutants and one of the biological control treatments, none of the strains (*P. synxantha* 2-79, *P. protegens* Pf-5, or *P. fluorescens* Q2-87) had an obvious competitive advantage **(Fig. 2.3)**. However, ΔTssA1/2 populations were reduced in the presence of *P. protegens* Pf-5 and *P. fluorescens* Q2-87, but not *P. synxantha* 2-79, suggesting that at least one T6SS is needed for competitive fitness in mixtures with certain biological control agents having at least one T6SS.

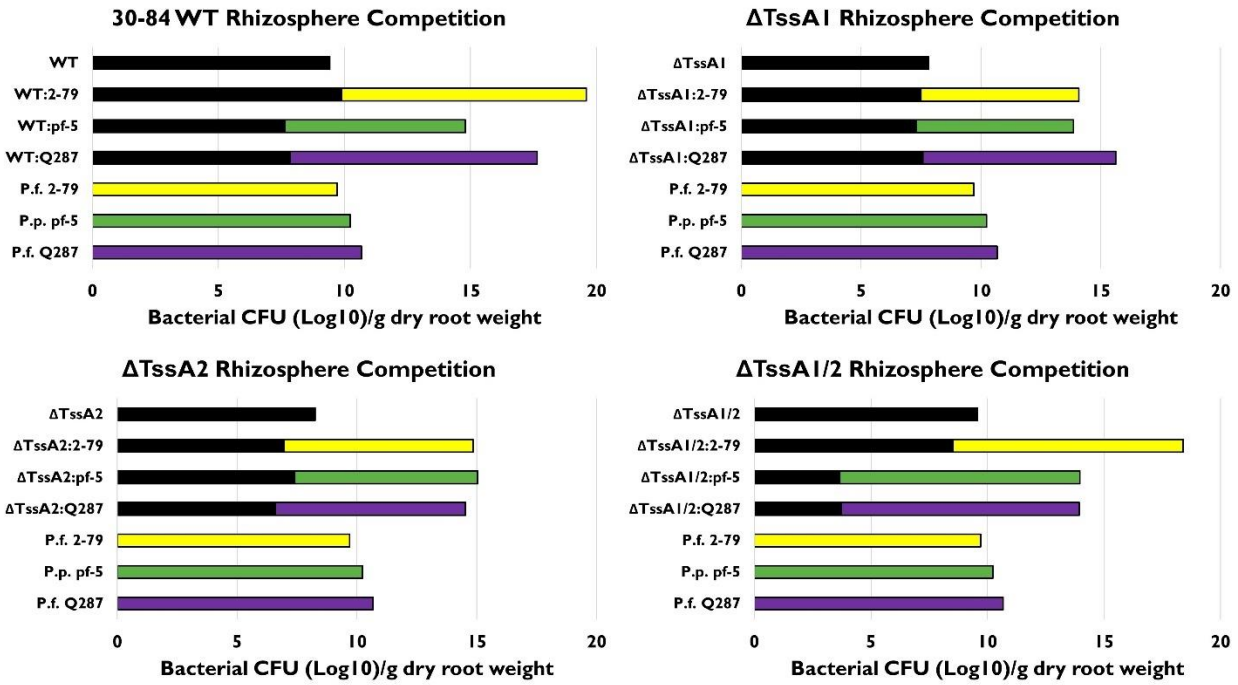

Figure 2.3: Rhizosphere competition assays. The competitive fitness of 30-84 WT and T6SS mutants (black color) were evaluated by comparing their populations when grown separately or in 50:50 mixtures with other *Pseudomonas* rhizosphere colonizing bacteria in the rhizosphere, including *P. fluorescens* 2-79 (yellow color, having no T6SS), *P. protegens* Pf-5 (green color, having a T6SS-1 homolog), and *P. fluorescens* Q2-87 (purple color, having three T6SS, including T6SS-1 and T6SS-2 homologs). After 28 days, bacterial populations from the entire root system and loosely adhering soil were collected and enumerated via serial dilution plating. Populations were standardized to root dry weight. Data are the means (log10 CFU/g dry root weight) of at least 8 replicates/treatment pooled across three experiments (n = 24/treatment).

We also examined the importance of one or both T6SS using the wild type and T6SS mutants in competition assays with several environmental isolates or plant pathogens **(Fig. A2)**. We found that the environmental isolate *P. putida* F1, having no T6SS performed similarly to *P. synxantha* 2-79 in mixed culture being inhibited by 30-84 WT and ΔTssA1, whereas ΔTssA2 and ΔTssA1/2 permitted substantial growth. *Pseudomonas syringae* pv. tomato DC3000, and *Agrobacterium tumefaciens* C58 populations were substantially reduced when grown with 30-84 WT and both single mutants but were able to maintain higher populations than the double

mutant. *Pectobacterium carotovorum* subsp. *carotovorum* inhibited the double mutant but was able to coexist with wild type and the single mutants **(Fig. A2)**. This indicates that having at least one T6SS is important for competition against plant pathogens, with T6SS-2 being important for competition against strains lacking their own T6SS.

2.3.4. T6SS as an anti-predation mechanism against different bacterivores

2.3.4.1. *Dictyostelium discoideum*

To test our hypothesis that one or both of the T6SS function in anti-predation defense against *D. discoideum* amoeba cells, we observed the behavior of *D. discoideum* amoebae when the only food source was offered prey choices consisting of 30-84WT and derivatives deficient in the production of phenazines (Phz), quorum sensing (QS) signal production, or one or both T6SS system, e.g., 30-84 WT (Phz^+, QS^+, $T6SS^+$), 30-84 ZN (Phz^-, QS^+, $T6SS^+$), 30-84 I/I2 (Phz^-, QS^-, $T6SS^+$), ΔTssA1, ΔTssA2, ΔTssA1/2 (Phz^+, QS^+), and 30-84 GacA (Phz^-, QS^-, $T6SS^{reduced}$). We performed the assay in two different types of medium: low nutrition (PBM) and high nutrition (HL5) medium. Because starvation stress in this amoeba results in aggregation and formation of multicellular fruiting bodies, we assessed aggregation behavior (Kessin 2001). In low nutrition medium, we observed that after 24 h., *D. discoideum* cells growing without a bacterium food source (control) began to thin and stream and formed extensive aggregates **(Fig. 2.4)**. Similarly, when offered 30-84 WT or derivatives 30-84 ZN, 30-84 I/I2, ΔTssA1, or ΔTssA2, all having at least one intact T6SS, extensive aggregation resulted. In contrast, *D. discoideum* cells growing with *E. coli* ΔB (used in lab as a preferred prey source) showed no aggregation. *D. discoideum* growing with ΔTssA1/2 and 30-84 GacA treatments showed no aggregation, similar to cells grown on *E. coli* ΔB, indicating that the *D. discoideum* was able to gain adequate nutrition by using these strains as a food source **(Fig. 2.4)**. In HL5 media, little to

no aggregation behavior was seen, consistent with *D. discoideum* having adequate nutrition **(Fig. A3)**. These results suggest that having at least one functional T6SS causes *D. discoideum* stress behavior typically associated with starvation, whereas neither the absence of phenazine or quorum sensing signal production reduced the aggregation behavior, indicating neither phenazine nor QS signals play a significant role in promoting the stress response.

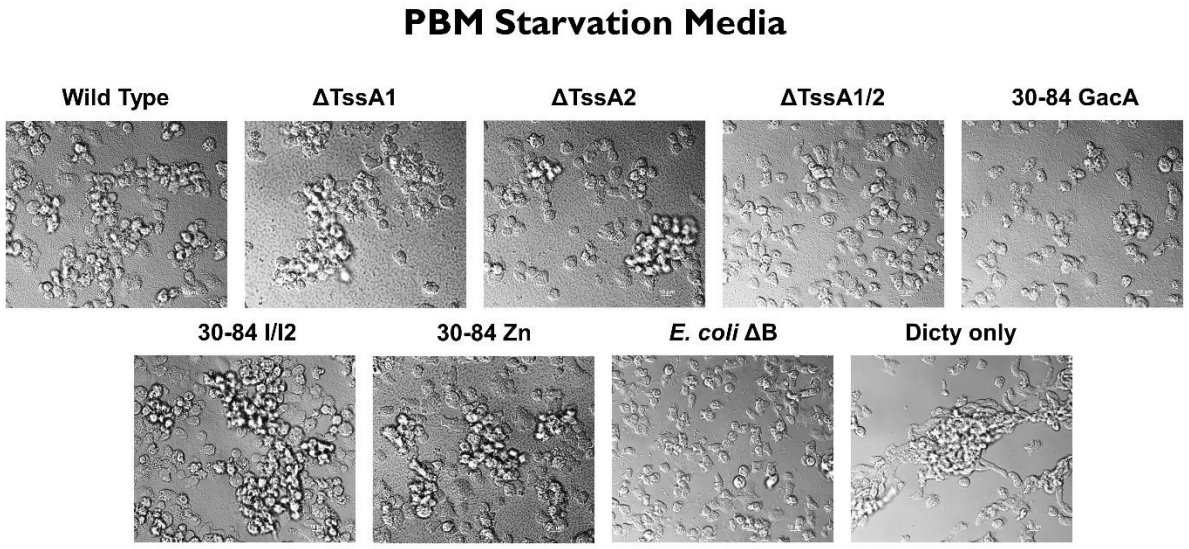

Figure 2.4: Aggregation behavior of *Dictyostelium discoideum* grown with different bacterial strains. Bacteria used as prey in the feeding assay included 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, or 30-84 ZN and *E. coli* ΔB (used as a preferred prey in the lab). *D. discoideum* without prey bacteria was used as a negative control. *D. discoideum* cells were grown in 24-well plates in low nutrient PBM media for 24 h. and aggregation behavior was observed using DIC microscopy (100X oil). The *D. discoideum* control (no prey) showed high levels of aggregation caused by stress due to the lack of nutrition in the media. *D. discoideum* growing with 30-84 WT, ΔTssA1, ΔTssA2, 30-84 I/I2, and 30-84 Zn displayed a similar level of aggregation, indicating that *D. discoideum* cannot eat these strains. *D. discoideum* grown with *E. coli* ΔB showed little to no aggregation. *D. discoideum* growing with ΔTssA1/2 and 30-84 GacA treatments showed similarly low levels of aggregation. Two replicate experiments were performed, and representative images from the same replicate are presented.

To confirm that bacterial T6SS induce stress during feeding, we measured the level of the production of *D. discoideum* development markers Discoidin I and Contact site A (CsA). Discoidin I is a protein involved in adhesion that is detectable at low levels in vegetative amoeba cells but is expressed at high levels with aggregation (Springer, Cooper, and Barondes 1984). CsA is a glycoprotein involved in cell-cell binding during development (Harloff, Gerisch, and Noegel 1989). We found that when grown individually (negative control) in low nutrition media, *D. discoideum* produced high levels of both Discoidin I and CsA **(Fig. 2.5)**. When grown in mixture with *E. coli* ΔB (positive control) the production of both proteins was significantly lower compared to the negative control. Production levels when 30-84 WT, 30-84 I/I2, or 30-84 ZN was the prey were all comparable to the negative control indicating that phenazines were not the driving mechanism behind this response. *D. discoideum* also produced high levels of both development markers when grown with ΔTssA1 and ΔTssA2. When *D. discoideum* was grown with ΔTssA1/2 and 30-84 GacA, levels of both proteins were comparable to the *E. coli* ΔB control **(Fig. 2.5)**, indicating that the presence of one or both T6SS causes stress, which deters *D. discoideum* predation.

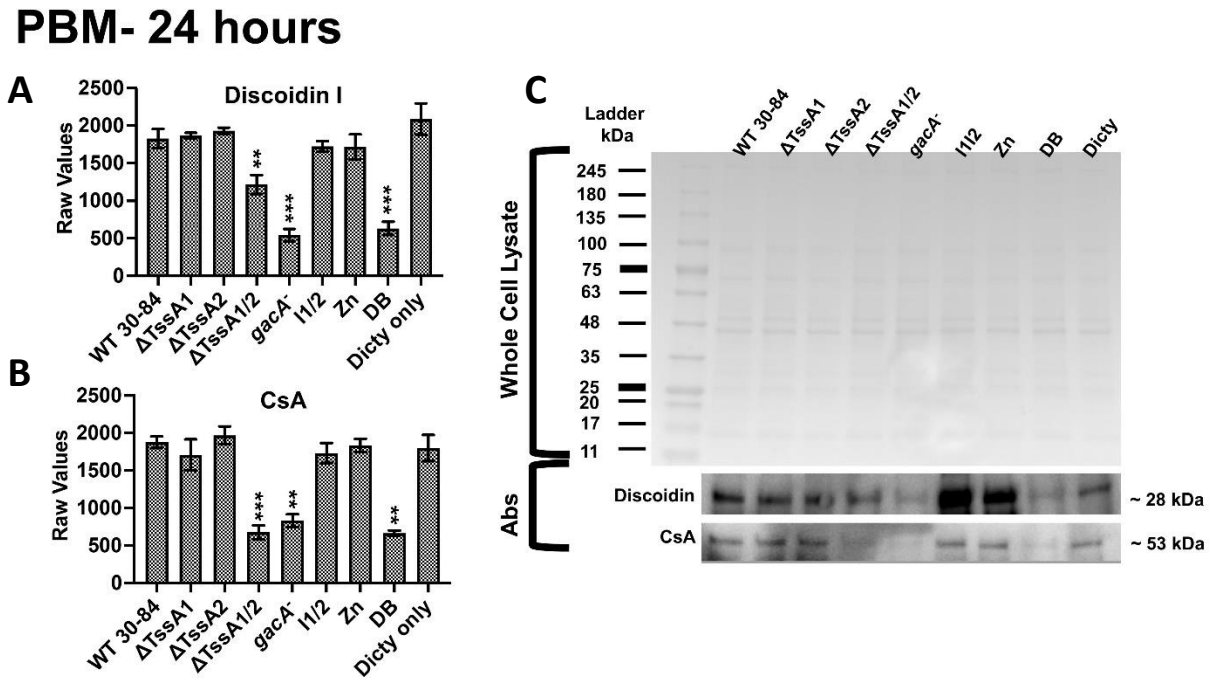

Figure 2.5: Western Blot observing two starvation markers in *Dictyostelium discoideum*. Starving *Dictyostelium* cells express higher levels of Discoidin and CSA. *D. discoideum* AX2 cells were incubated in PBM with different bacterium strains (30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, or 30-84 ZN and *E. coli* ΔB (DB)) for 24 h. Graphs show the levels of **A)** discoidin or **B)** CSA normalized to the AX2 control. Values are mean and standard error of 2 independent experiments. ** indicates $p < 0.01$, *** $p < 0.001$ compared to *D. dictyostelium* AX2 control (Unpaired t-tests, Welch's correction). **C)** At 4 and 24 h., an aliquot of the samples was used for a Coomassie-stained gel. These were electrophoresed on SDS-polyacrylamide gels, and western blots were stained with an anti-discoidin or an anti-CSA antibody. Band intensities were normalized to the corresponding Coomassie gel scans.

To determine how *D. discoideum* predation effects bacterial fitness, a plate clearing assay was performed in which the size of the zone of clearing (and *D. discoideum* spread) after 5 days was used as a measure of bacterial cell death due to predation. On all plates, the initial colony of *D. discoideum* resulted in a clear zone ~1 cm in diameter after 2 days. After 5 days, the clearing

zone on plates containing 30-84 WT, ΔTssA1, ΔTssA2, 30-84 I/I2, and 30-84 ZN did not increase significantly i.e., the zones were within 0.05 cm of the clearing diameter measured at day 2 **(Fig. 2.6)**. The clearing zone on plates containing the positive control ΔB and 30-84 GacA grew to 3.0 $\pm$ 0.2 and 3.8 $\pm$ 0.02 cm, respectively, and the colony grown on ΔTssA1/2 grew to 1.7 $\pm$ 0.06 cm. These results indicate that 30-84 GacA and ΔTssA1/2 populations suffered greater losses due to *D. discoideum* feeding **(Fig. 2.6)**.

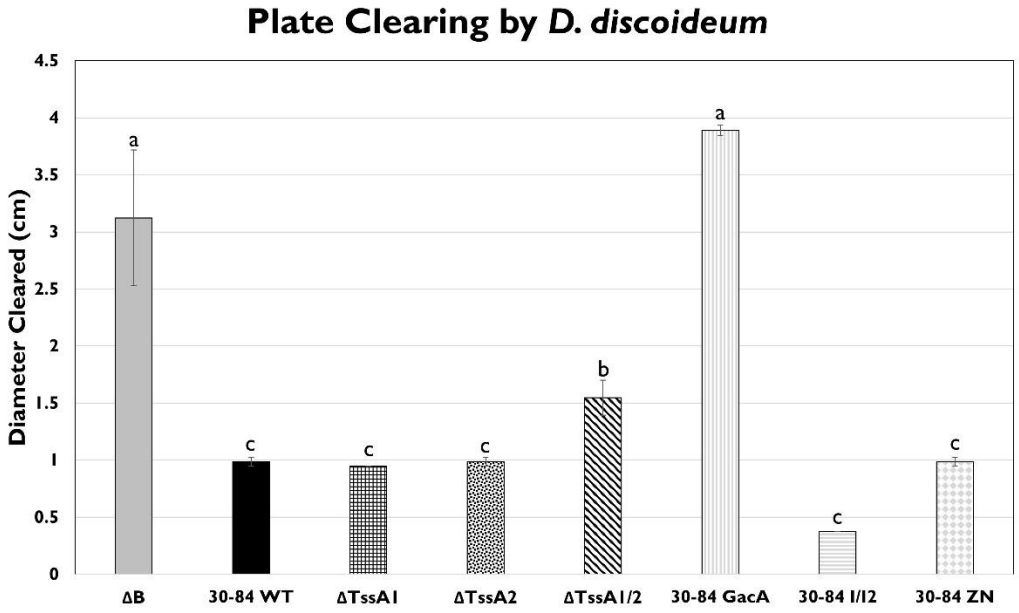

Figure 2.6: Bacterial plate clearing by *Dictyostelium discoideum.* Bacteria used as prey in the feeding assay included 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, or 30-84 ZN and *E. coli* ΔB (used as a preferred prey in the lab). The diameter of bacterial lawn cleared by *D. discoideum* was measured in cm after 72 h. Lettering indicates level of significant difference. *D. discoideum* colonies grown on 30-84 WT, both single mutants, 30-84 I/I2, and 30-84 ZN showed little to no clearing, indicating low to no bacterial cell death. *D. discoideum* growing on *E. coli* ΔB showed significant levels of clearing. When grown on 30-84 GacA, levels of clearing similar to the control were observed. When grown on ΔTssA1/2, less clearing was observed than on the control, but levels were still significantly higher than clearing on 30-84 WT and the single mutants, indicating a decrease in bacterial fitness when lacking at least one functional T6SS. Data are the means and standard error of four biological replicate experiments (n = 4/treatment). Data were analyzed using one-way ANOVA and Student t tests, $p<0.05$.

2.3.4.2. *Tetrahymena thermophila*

In the feeding assay, the predator was the model ciliate *Tetrahymena thermophila* CU427. As above, predators were offered the same single prey choice strains (30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, 30-84 ZN), and population densities of

both predator and prey in mixed culture were measured after 4 and 24 h. No significant differences in *T. thermophila* population densities were found even after 24 h **(Fig. A4)**. However, we also assessed stress related behaviors of *T. thermophila* when offered the different prey choices via a mating assay. Two *T. thermophila* of different mating types, CU427 and CU330, were selected for this assay as they are recognized as "non-self" cells with different germlines, and will therefore reproduce (Cervantes et al. 2013). In the mating assay, *T. thermophila* were starved prior to their exposure to the different prey. If *T. thermophila* continued to experience starvation stress, this would increase in instances of mating (Cole 2013). After 4 h growth, the frequency of mating was ~55% for the control (no food source) and the frequency of mating for 30-84 WT, 30-84 I/I2, or 30-84 ZN as the food source was similar (62%, 53%, and 62%, respectively; **Fig. 2.7**). Since all three of these derivatives have wild type T6SS expression, but differ in their ability to produce phenazines, these data demonstrate that the production of phenazines did not enhance mating. In contrast, the frequency of mating was significantly lower for *T. thermophila* growing with ΔTssA1/2 or 30-84 GacA (19% and 36%, respectively, **Fig. 2.7**). These observations support the hypothesis that having an intact T6SS causes predator stress, and this results in reduced levels of bacterivory.

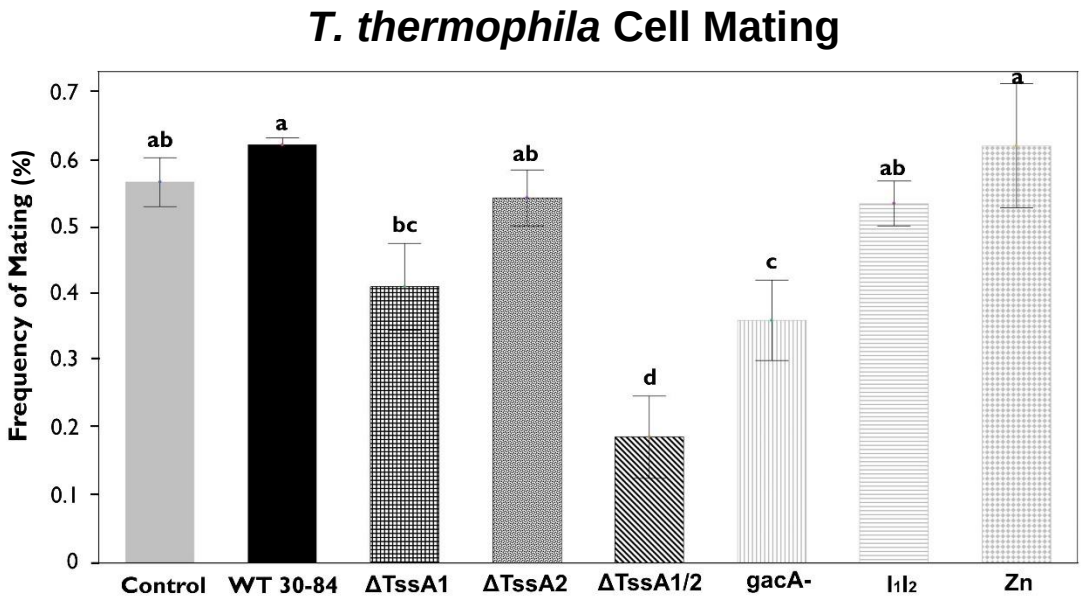

Figure 2.7: *Tetrahymena* Mating Assay. *T. thermophila* CU427 and CU330 were grown separately overnight in Tris Buffer (pH=7.4) to induce starvation. Populations were standardized to a cell density of 1.5 - 2 x 10^5/10 mL (via direct counts) in fresh Tris Buffer and mixed together with only the bacterial strain as a food source. After 4 h, the treatments were viewed using a Leitz (Epivert) microscope (100X magnification), and the frequency of mated cell pairs (number of mated pairs/total number of observations) determined. This experiment was repeated three times (n = 3/treatment). Letters denote significant differences. Data were analyzed using one-way ANOVA and Student t tests, $p<0.05$.

No differences in prey populations were observed at 4 h, but by 24 h ΔTssA1/2, and 30-84 GacA populations were significantly lower than 30-84 WT populations **(Fig. 2.8)**. All strains grew to the same density in the absence of *T. thermophila* (data not shown), confirming that predation was affecting bacterial density. These observations suggest that having at least one T6SS protects *P. chlororaphis* 30-84 from *T. thermophila* bacterivory.

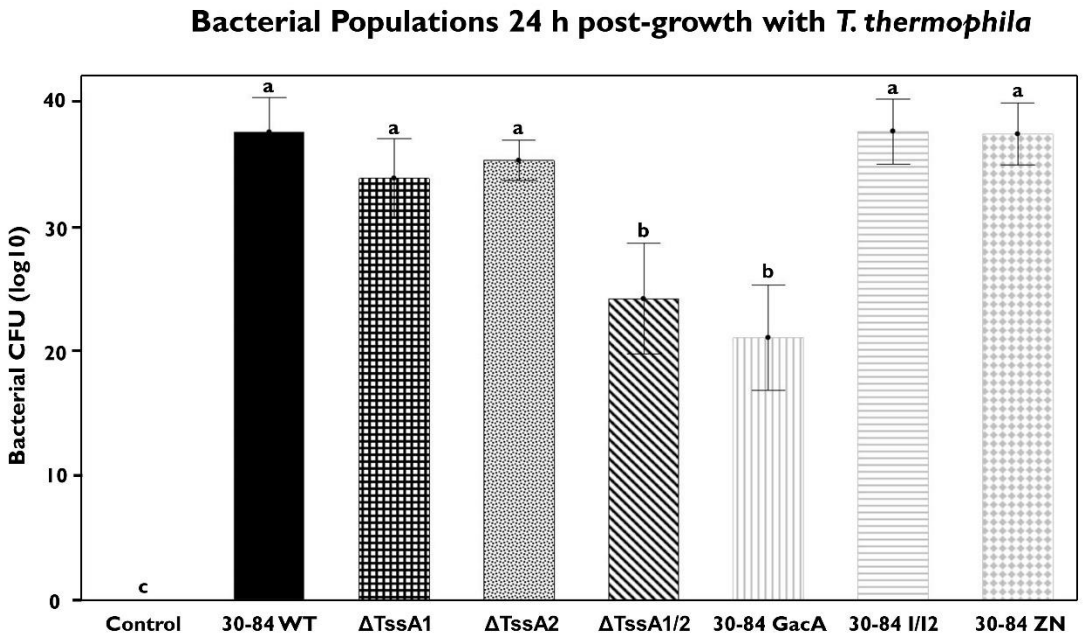

Figure 2.8: Bacterial populations after 24-h *Tetrahymena* feeding assays.
Bacteria used as prey in the feeding assay included 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, or 30-84 ZN. *T. thermophila* without prey bacteria was used as a negative control. Bacterial and *T. thermophila* cultures were mixed (5 mL, 20 mL, respectively) and grown in 50 ml tubes with agitation (200 rpm, 27 °C). After 24 h., bacterial populations were enumerated via serial dilution plating. Data are the means (log10 CFU) and standard errors of 6 replicate experiments (n = 6/treatment). Data were analyzed using a one-way ANOVA and Student t tests and letters indicate significant differences, $p<0.05$.

2.3.4.3. *Caenorhabditis elegans*

As in the previous experiments, the bacterivorous nematode *C. elegans* was offered different prey choices. In this experiment, 5 *C. elegans* adults were allowed to graze for 1 h. on a lawn of each bacterial strain and then moved successively to a fresh prey-containing plate every 1 h. three more times, enabling nematodes to lay eggs on each of the plates. Plates were then

observed for 72 h. at 24 h. intervals and adult and juvenile nematodes maturing from eggs were counted. Well-fed nematodes will mature from eggs within 72 h.

After 72 h., a large proportion (~45%) of nematodes grown on *E. coli* OP50, the *C. elegans* normal laboratory food source, were mature **(Fig. 2.9)**. The percentages of adult nematodes observed on plates having ΔTssA1/2 and 30-84 GacA as food sources were also high (~55%), whereas the percentages were lower when *C. elegans* was grown on plates having 30-84 WT, ΔTssA1, ΔTssA2, 30-84 I/I2, or 30-84 ZN as the food source (2%, 2%, 0%, 9.5%, and 0.03% adults, respectively) **(Fig. 2.9)**. Moreover, for plates containing 30-84 WT, 30-84 I/I2, or 30-84 ZN as the food source, *C. elegans* avoided the center of the plates where the bacterial density was greatest, instead moving to the edges of the plate **(Fig. 2.10)**. On plates containing these prey sources, less than 25% of the plate was cleared (where clearing indicates bacterial consumption, **Table 2.2**). Since all three of these derivatives have wild type T6SS expression, but differ in their production of phenazines, these data demonstrate that the production of phenazines was not the primary feeding deterrent. Similarly, the nematodes moved to the outside of the plate and generally cleared less than 25% of the plate when grown on plates containing ΔTssA1 or ΔTssA2 as the food source. On ΔTssA1/2 and 30-84 GacA, nematodes were found in the center of the plates and often cleared greater than 90% of the plate **(Fig. 2.10, Table 2.2)**. These data indicate that having either T6SS is a deterrent to *C. elegans* feeding, and that *C. elegans* prefer prey lacking expression of both systems.

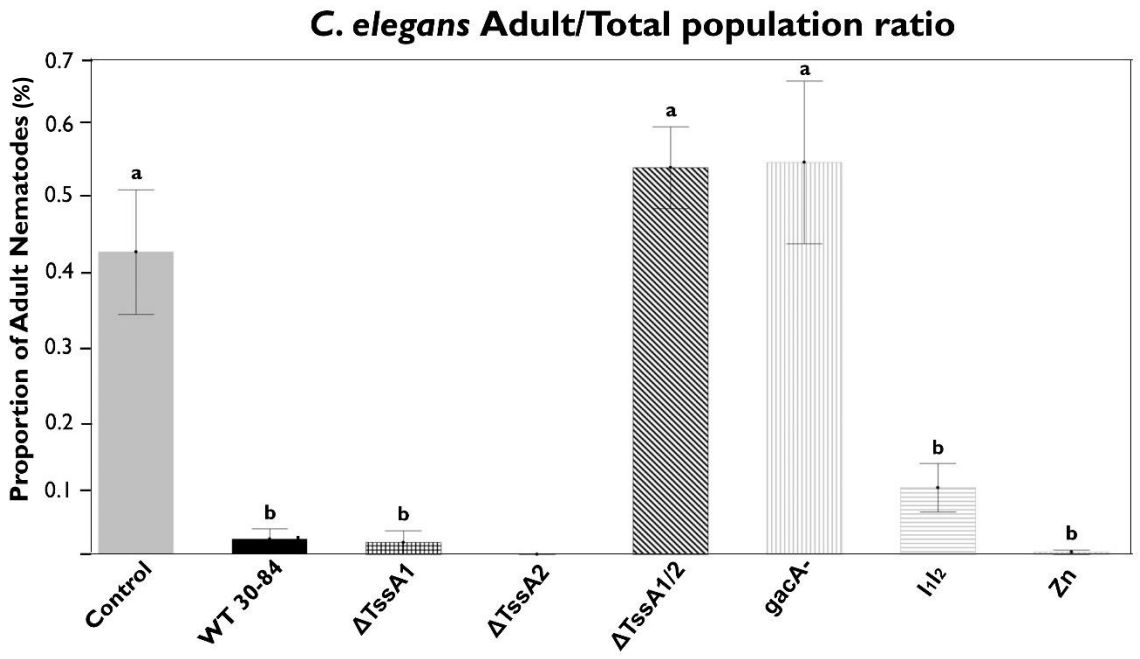

Figure 2.9: Proportion of *C. elegans* that reach maturity after 72 h. Five adult *C. elegans* were transferred at 1-hour intervals to new prey-containing plates to facilitate egg laying (a total of 4 successive transfers), and then the percentage of nematodes maturing to adults were measured every 24 h over a 72-h period. Plates contained either 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, 30-84 ZN, or *E. coli* OP50 (control) as a food source. Data are the mean and standard errors of 3 replicates (pooled across the four plates)/treatment (n = 12/treatment). Data were analyzed using one-way ANOVA and Tukey tests. Letters indicate significant differences, $p<0.01$.

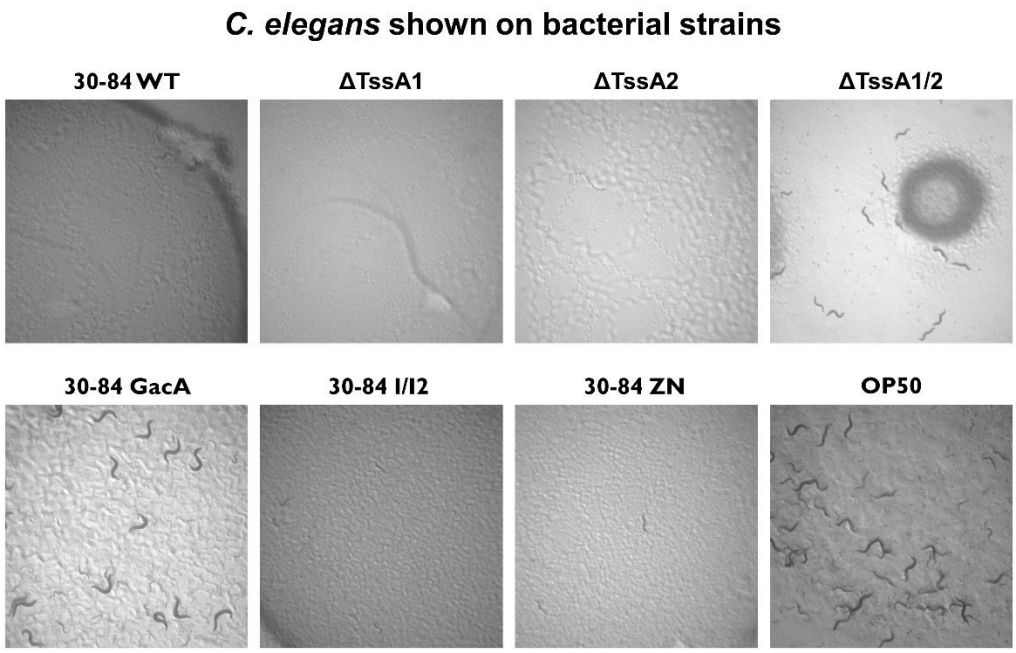

Figure 2.10: Images of *C. elegans* on plates containing different prey after 72 h. Five adult *C. elegans* were transferred at 1-hour intervals to new prey-containing plates to facilitate egg laying (a total of 4 successive transfers), and then images were taken at 72 h. Images are of the center of the plate where bacterial cultures were applied. Plates contained either 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, 30-84 ZN, or *E. coli* OP50 (control) as a food source. Images were obtained using a Zeiss Stemi SV11 scope (26X magnification) and a Hamamatsu ImagEM EM-CCD camera. Adult nematodes can be seen on the plates containing ΔTssA1/2, GacA or *E. coli* OP50 (ΔTssA1/2 image contains air bubble), whereas no or only a few, immature nematodes can be seen on the other prey sources. The experiment and imaging were repeated 3 times with 4 plates/treatment/replicate (n = 12/treatment) and representative images from the same replicate are shown.

Table 2.2: Predation on Bacteria by *C. elegans* after 72 h. Estimates of % clearing of the bacterial lawn on each plate are made. This experiment was repeated 3 times with 4 plates/treatment/experiment. Final n = 12 per treatment.

Prey	**>90% clearing**	**90-25% clearing**	**<25% clearing**
OP50	8	4	0
30-84 WT	0	0	12
ΔTssA1	0	2	10
ΔTssA2	0	1	11
ΔTssA1/2	7	5	0
30-84 GacA	10	2	0
30-84 I/I2	0	0	12
30-84 ZN	0	0	12

2.3.5. In silico identification of putative T6SS-dependent effectors and immunity proteins

Neither of the T6SS clusters in *P. chlororaphis* 30-84 contain genes previously annotated as encoding T6SS-dependent effectors. However, since T6SS-dependent effector genes are often located in close proximity to genes encoding VgrG, Hcp, or PAAR proteins, we analyzed the

predicted protein sequences of genes closely associated with all copies of these genes using BLASTp (Lien and Lai 2017; Spiewak et al. 2019). Putative effectors and immunity proteins were identified based on predicted functional domains and homology to proteins within established T6SS effector superfamilies. A diversity of putative T6SS effectors and associated proteins were identified using this approach, with all VgrG or Hcp encoding genes being associated with at least one putative effector **(Table 2.3)**. Within the T6SS-1 cluster, adjacent to an Hcp encoding gene are genes encoding a Tae4-like protein and a hypothetical protein (RS17720, 25, and 30, respectively). Tae (type VI amidase effectors) family effectors have peptidoglycan amidase activity and are typically located adjacent to a cognate immunity protein (H. Zhang et al. 2013). However, the hypothetical protein (RS17730) bears no amino acid sequence homology to the known Tae4 immunity protein Tai4 and has no well characterized conserved domains. These two genes (encoding Tae4 and the hypothetical protein) are found in the *P. chlororaphis* 30-84 T6SS-1 gene cluster but are absent from the gene cluster of the T6SS-1 homolog in *P. protegens* Pf-5. Also located within the T6SS-1 cluster and adjacent to a *vgrG* gene are two genes coding for hypothetical proteins (RS17760, RS17765) with DUF6484 and DUF2169 domains. Although DUF6484 domains are not well characterized, in *A. tumefaciens*, adapter proteins with the DUF2169 domain have been shown to be necessary for the efficient secretion of T6SS effectors (Bondage et al. 2016). Located nearby the T6SS-1 gene cluster and associated with a *vgrG* gene (RS17845) are genes encoding three hypothetical proteins (RS17830, RS17835, and RS17840) predicted to have lipase (class 3) activity, be a cytoplasm-localized lipoprotein, or contain a DUF4123 domain, respectively. Proteins having a DUF4123 domain were shown to serve as T6SS effector chaperones (Liang et al. 2015). Two other VgrG-encoding proteins (RS15635 and RS20265) are adjacent to hypothetical proteins predicted to

have lipase (class 3) activity (RS15625 and RS20275). A protein predicted to have a DUF4123 domain (RS20270) is found with one of these lipases. Located between two VgrG encoding genes in the T6SS-2 cluster (RS29465 and RS29480) is a hypothetical protein (RS29475) having a lysozyme-like domain similar to the C-terminal domain of pesticin. Pesticin is an antibacterial toxin involved in hydrolysis of the peptidoglycan in the periplasm. Proteins with a pesticin C-terminal domain are typically involved in the hydrolysis of beta-1,4- linked polysaccharides (Patzer et al. 2012). Also adjacent to one of the *vgrG* genes (RS29465) are three hypothetical proteins, one with a DUF4123 domain, one encoding a Tli1-like immunity protein with a DUF3304 domain, previously shown to be associated with immunity proteins (Crisan et al. 2019), and one encoding a Tle1-like phospholipase protein with a DUF2235 domain (Ma et al. 2017) (RS29560, RS29555, and RS29550). Including the Tle-like effector associated with T6SS-2, there were three putative effectors predicted to have phospholipase activity, but belonging to different phospholipase effector families: Tle, Tpl, and Pld. A gene encoding a TplE-like protein is located adjacent to a *vgrG* gene (RS20065 and RS20070, respectively). Putative immunity protein pairs, which are paralogs, and a PAAR encoding gene are located adjacent to the putative *tplE* gene (RS20060, RS20055, and RS20050). A gene encoding a putative type VI secretion phospholipase D effector (Pld) in the same ortholog group as *tle5B* in *P. aeruginosa* PAO1 was located adjacent to a *vgrG* gene (RS20580 and 85, respectively). In *P. aeruginosa* PAO1 three immunity proteins were found adjacent to *tle5B* (Wen et al. 2020). No similar immunity proteins were found adjacent to the putative *pld* in *P. chlororaphis* 30-84. However, similar to the *P. aeruginosa* immunity proteins, the hypothetical protein adjacent to *pld* in *P. chlororaphis* 30-84 contains multiple (3) SLR repeats. The three *P. aeruginosa* PAO1 proteins each contain 4, 3, or 2 SLR repeats. Proteins from Sel1-like repeat (SLR) family share similar alpha-helical

conformations but typically differ in consensus sequence length and topologies. First described in *C. elegans*, Sel1-like proteins have been shown to play roles in signal transduction pathways in both eukaryotes and prokaryotes (Mittl and Schneider-Brachert 2007). While my organism does not contain a Sel1-like protein, the presence of the SLR motifs is interesting and should be further researched. Elsewhere in the genome, a hypothetical protein with a peptidoglycan-binding LysM binding motif is associated with another hypothetical protein with a DUF4123 binding domain and the VgrG-encoding gene (RS14860, 65, and 70, respectively). Additionally, a gene encoding a RhsA- like protein (RS01885) is found within a four gene cluster containing genes encoding a TAP (T6 adaptor protein) with unknown function, VgrG and Hcp (RS01890, RS01895, and RS01900, respectively). Rhs (rearrangement hotspot) proteins, typically have multiple repeats of RHS sequences and have been associated with toxicity and bacterial competition (Koskiniemi et al. 2013). The arrangement of the four genes is the same as the RhsP2-containing cluster in *P. aeruginosa* PA14 previously shown to be involved in T6SS secretion (Jones et al. 2014).

Table 2.3: Putative effectors of *Pseudomonas chlororaphis* 30-84. Bolded words indicate structural proteins of the T6SS needle complex. Red text indicates proteins found within the T6SS-1 cluster. Green text indicates proteins found within the T6SS-2 cluster.

Locus Tag	Putative Effector	Predicted Activity	Localization
PCHL3084_RS01885	RhsP2	Unknown function; General Antibiotic activity	Cytoplasmic Membrane
PCHL3084_RS01890	Tap (T6 Adaptor Protein)	T6 related but currently unknown; DUF4123 containing	Cytoplasmic
PCHL3084_RS01895	**VgrG**	Needle Subunit	Cytoplasmic
PCHL3084_RS01900	**Hcp**	Needle Subunit	Extracellular
PCHL3084_RS04425	**PAAR**	Needle Subunit	
PCHL3084_RS04675	**PAAR**	Needle Subunit	
PCHL3084_RS11710	**PAAR**	Needle Subunit	
PCHL3084_RS15945	Putative effector	Homology to several different effectors	
PCHL3084_RS17720	**Hcp**	Needle Subunit	**Extracellular**
PCHL3084_RS17725	Tae4	Amidase activity	
PCHL3084_RS17730	Hypothetical protein	Unknown function	
PCHL3084_RS17755	**VgrG**	Needle Subunit	**Cytoplasmic**
PCHL3084_RS17760	Hypothetical protein	Unknown function; DUF6484 domain containing	**Cytoplasmic**
PCHL3084_RS17830	Hypothetical protein	lipase	
PCHL3084_RS17835	Hypothetical protein	Putative lipoprotein	**Cytoplasmic**
PCHL3084_RS17840	Hypothetical protein	DUF4123 containing	
PCHL3084_RS17845	**VgrG**	Needle Subunit	Cytoplasmic

Table 2.3 Continued.

Locus Tag	Putative Effector	Predicted Activity	Localization
PCHL3084_RS18065	**PAAR**	Needle Subunit	
PCHL3084_RS18305	**PAAR**	Needle Subunit	
PCHL3084_RS20050	**PAAR**	Needle Subunit	
PCHL3084_RS20055	Putative TplEi	TplEi Immunity protein; inparalog	
PCHL3084_RS20060	Putative TplEi	TplEi Immunity protein; inaralog	
PCHL3084_RS20065	Putative TplE	Phospholipase	
PCHL3084_RS20070	**VgrG**	Needle Subunit	Cytoplasmic
PCHL3084_RS20265	**VgrG**	Needle Subunit	Cytoplasmic
PCHL3084_RS20580	Phospholipase D family protein	Phospholipase; Homology to T6SS effector Tle5b	
PCHL3084_RS20585	**VgrG**	Needle Subunit	Cytoplasmic
PCHL3084_RS21180	**PAAR**	Needle Subunit	
PCHL3084_RS24445	**PAAR**	Needle Subunit	
PCHL3084_RS27685	**PAAR**	Needle Subunit	
PCHL3084_RS29450	Tle1; DUF2235 containing	Phospholipase	
PCHL3084_RS29455	Tli1	Tle1 Immunity protein	
PCHL3084_RS29465	**VgrG**	Needle Subunit	Cytoplasmic
PCHL3084_RS29475	Lysozyme-like domain similar to pesticin	Hydrolysis of peptidoglycan	
PCHL3084_RS29480	**VgrG**	Needle Subunit	Cytoplasmic
PCHL3084_RS00060	**PAAR**	Needle Subunit	

2.4. Discussion

Type VI Secretion Systems (T6SS) are known to be involved in many different types of bacterial interactions with prokaryotes and eukaryotes, and previous studies showed that in comparison to bacterial strains lacking T6SS, the presence of a T6SS confers greater fitness to bacteria in their environments (Bernal et al. 2017; Haapalainen et al. 2012). It was also observed that many species possess one to several T6SS and that the different T6SS can be involved in different types of interactions (Chen et al. 2011), and thus possessing more than one T6SS can increase the repertoire of potential benefits to the strain (Schwarz et al. 2010). Plant growth promoting pseudomonads have been found to either lack T6SS or possess one to several different T6SS, but their importance in activities related to their plant-associated habitat have not been well studied. In the present study, we describe the structure and organization of two separate T6SS gene clusters in the PGPR strain *P. chlororaphis* 30-84 and provide evidence to support a role for both in competition against bacterial species, including other PGPR and phytopathogens, and protection against different types of bacterivorous predators.

Bioinformatic analysis revealed that *P. chlororaphis* 30-84 has two separate T6SS clusters, each of which contain at least 12 of the 13 conserved T6SS structural proteins, but that the two clusters are quite different in terms of their organization and regulation. Comparative genomic analyses suggested that the two systems are not redundant and differ in their potential for responding to environmental stimuli. The T6SS-1 gene cluster contains putative Tag protein-encoding genes, which are involved in contact dependent firing, and was organizationally similar to the gene cluster encoding the *P. aeruginosa* T6SS previously shown to display dueling behavior (Marek Basler, Ho, and Mekalanos 2013). Together, these findings led us to hypothesize that T6SS-1 is fired in a contact dependent manner and thus may not fire, or may

fire at a lower level, unless fired upon. The absence of the Tag-encoding genes in the T6SS-2 cluster led us to hypothesize random firing of this T6SS (i.e., not contact-dependent). Consistent with these hypotheses, *P. chlororaphis* 30-84 derivatives lacking T6SS-2 (ΔTssA2 and ΔTssA1/2), and thus lacking random firing, permitted substantial growth of *P. synxantha* 2-79 (which lacks a T6SS) in mixed cultures, whereas derivatives having the T6SS-2 cluster (30-84 WT and ΔTssA1) competitively reduced *P. synxantha* 2-79 *in vitro*. We saw the same pattern of behavior in competition assays using *P. putida* F1, which also lacks a T6SS.

Previous studies have shown that T6SS are important for the inhibition of competitors. For example, Bernal et al. (2017) showed the biological control strain *P. putida* KT2440 possesses three different T6SS and, using mutagenesis to disrupt all of them, demonstrated that these were integral for the control of certain plant pathogens. In the present study, we examined competitive fitness both in terms of the ability of the mutants to limit the growth of challengers and also to resist the impact of challenges on their own growth. We found that wild type was significantly more effective than the double mutant in inhibiting the growth of several different plant pathogens and even different biological control strains in mixed cultures *in vitro*. Moreover, in competition with certain strains (*P. synxantha* 2-79, *P. protegens* Pf-5, *Pectobacterium carotovorum*), the double mutant population was significantly reduced, indicating a lack of ability to protect itself. Competition assays utilizing the single mutants clearly showed that, in most cases, having either system was sufficient for competitor inhibition, although as noted above there were some exceptions demonstrating that T6SS-2 was effective against a broader spectrum of competitors (including those having no T6SS). It was interesting that derivatives having either T6SS system significantly inhibited *P. fluorescence* Q2-87 (having three T6SS, including T6SS-1 and T6SS-2 homologs) in mixed cultures, whereas *P. fluorescence*

Q2-87 was able to grow in the presence of the double mutant. These observations are consistent with the hypothesis that despite having multiple T6SS systems, Q2-87 may not have immunity to *P. chlororaphis* 30-84 T6SS effectors. In some cases, pathogens (*Pseudomonas marginalis*) or biological control agents (*Pseudomonas putida* KT2440) that were chosen for competition assays were highly sensitive to phenazine or bacteriocin production and would not grow in the competition assay even with ΔTssA1/2. In other cases, strains (*P. putida* F1, *P. syringae* DC3000) that were shown previously to be sensitive to bacteriocins produced by *P. chlororaphis* 30-84 (Dorosky et al. 2017) were able to sustain somewhat higher populations in mixed culture with the double mutant compared to wild type. However, the majority of biological control strains tested (*P. syxantha* 2-79, *P. protegens* Pf-5, *P. fluorescens* Q2-87) were neither sensitive to phenazines nor bacteriocins, highlighting the importance of contact-dependent mechanisms in a rhizosphere where the "resistome", the population of bacteria that contain highly efficient antibiotic resistant genes, may be already well established (O'Brien and Wright 2011).

Interestingly, the rhizosphere habitat offered some protection from competition, presumably because strains could escape interaction spatially. In general, ΔTssA1/2 was less competitive than wild type. This deficit is unlikely to be the result of insufficiency in colonizing ability, because ΔTssA1/2 colonized the rhizosphere as well as 30-84 WT in sterile and field soil in our persistence assay **(Fig. 2.1)**. However, after 5 harvest cycles ΔTssA1/2 populations were significantly smaller than 30-84 WT populations, but only in field soil. The populations of the single mutants were also reduced under these conditions, suggesting that both T6SS contribute to competitive persistence in the rhizosphere. In this study, we purposely looked at competition between *P. chlororaphis* 30-84 and other well-characterized PGPR strains because these are also

known to be good rhizosphere colonists. These results highlight the importance of considering T6SS compatibility when considering multi-strain mixtures of PGPR.

We also examined the importance of T6SS for protecting our rhizosphere colonizing biological control strain from predators with different feeding styles including, *Dictyostelium discoideum, Tetrahymena thermophila,* and *Caenorhabditis elegans.* Previous studies demonstrated that bacterial T6SS play a vital role in virulence against two of these three systems and survival against the third (Pukatzki et al. 2006; Sana et al. 2013; Wang et al. 2018). Our study is somewhat unique in that rather than focusing on virulence and killing, we examine the effect of each T6SS on the behavior and feeding of the predators *and* the impact of predation on bacterial populations. This perspective allows us to consider the changes in soil dynamics and overall ecology. For all three predators, we found that being grown with a bacterial food source having a T6SS increased predator stress, altered predator behavior, and reduced predation. For example, when grown with 30-84 WT or either of the single mutants as the primary food source, *D. discoideum* formed extensive aggregates, a common stress response (Kessin et al. 2001). Levels of developmental proteins related to aggregation behavior (Discoidin I and CsA) were also high in *D. discoideum* populations grown on 30-84 WT or either of the single mutants as the primary food source. In contrast, when grown with the double mutant or 30-84 GacA as the primary food source, no aggregation was observed, and development protein levels were reduced. When grown together in high nutritional medium there were no differences in predator behavior or stress levels, demonstrating that there were no limitations to co-existence when there was sufficient food for the predator. In assays using pre-starved *T. thermophila,* mating, a stress related behavior, was observed at high levels only when *T. thermophila* was grown with 30-84 WT or either of the single mutants as the primary food source. *C. elegans* also displayed

behavior indicating its avoidance of 30-84 WT or both single mutants, moving to the edges of the plates, away from the bacterium. Having a T6SS also had important consequences on bacterial fitness. In all cases, having a functional T6SS resulted in greater bacterial populations than for those without a functional T6SS as determined from direct counts or clearing zones. However, we did not see any differences in the behavior of any of the predators when grown with either ΔTssA1 or ΔTssA2, indicating both systems were effective predator deterrents.

Previous work demonstrated a role for T6SS in the delivery of effectors important for competition and virulence (Chen et al. 2015; Russell et al. 2011; Bernal et al. 2017). There are five different mechanisms for effector delivery: 1) attach directly to the PAAR protein, 2) attach to extensions attached to the PAAR protein such as Rhs cages, 3) attach to a VgrG protein, 4) through evolved VgrG domains which are hybrids of the N-terminus of the VgrG protein and the C-terminus of an effector domain, and 5) attached to the inside of the hollow Hcp tube (Ho, Dong, and Mekalanos 2014). Based on the linkages between effectors and the structural components of the needle, we identified a number of potential effectors that could be delivered via T6SS based on their proximity to genes known to shuttle effectors (VgrG or Hcp), a method commonly used for effector discovery (English et al. 2012; Spiewak et al. 2019). Notably these included several proteins having potential lipase or phospholipase activity, although the functionality of these was not specifically addressed in this study. Previous studies have shown that predicted lipases are often encoded adjacent to *vgrG* homologs and those associated with T6SS may function to degrade bacterial membrane phospholipids (Russell et al. 2013). Previous studies also showed that antibacterial effectors are often in small gene clusters with genes encoding cognate immunity proteins and VgrG. With the exception of *pld*, the phospholipase-like effectors were all found with genes encoding VgrG and putative immunity proteins. T6SS

secreted effectors have been shown to increase virulence against amoebas (MacIntyre et al. 2010; Pukatzki et al. 2006), and some studies have attributed this to effectors specifically targeting lipids in the membrane of eukaryotes (Miyata et al. 2011; Durand et al. 2014; Jiang et al. 2014). The presence of potential phospholipase effectors in *P. chlororaphis* 30-84 suggests a potential mechanism for the interaction observed with eukaryotic bacterivores. Furthermore, the gene encoding a putative Pld effector is adjacent to a gene with Sel1-like repeats (SLR) which are important in signal transduction in both prokaryotes and eukaryotes (Mittl and Schneider-Brachert 2007). Notably, these Sel1-like proteins were first described in *C. elegans*, a bacterivore observed in this study. The presence of this Sel1-like protein could indicate another potential mechanism by which *P. chlororaphis* 30-84 interacts with potential eukaryotic predators. In addition, we also found effectors with potential amidase, lysozyme, and peptidoglycan degrading activity as well as an Rhsp2 homolog. Rhs effectors have been shown to have antibacterial properties and are involved in bacterial competition (Hachani et al. 2014; Whitney et al. 2014; Alcoforado Diniz and Coulthurst 2015). An interesting finding was the repeated presence of genes containing DUF4123 domains, shown to be utilized in effector chaperoning (Liang et al. 2015), adjacent to VgrG encoding genes, suggesting this domain as being important for chaperoning and cargo loading in *P. chlororaphis* 30-84 T6SS. We also identified proteins having a DUF2169 motif, previously shown to be involved in efficient secretion of effectors, with some VgrG encoding genes (Bondage et al. 2016). The diversity of effectors found and their activities, portrays likely mechanisms for targeting both prokaryotic and eukaryotic systems.

Somewhat contrary to our hypothesis, despite their genetic differences, T6SS-1 and TSS-2 do not have non-redundant functions and, generally, have overlapping target ranges. While this

may be due to the limited number of strains tested, both systems were found to be important in bacterial competition (against other beneficials as well as pathogens and environmental isolates), with T6SS-2 being more important for competition against strains lacking their own T6SS. These systems were also found to be vital to anti-predator behavior, preventing the consumption of the bacteria as well as modulating the behavior of the predators. It is interesting to speculate that this direct effect on predators could also have an indirect, beneficial effect on the plant being colonized. Apart from eukaryotes that pose a threat to soil bacteria, there are also eukaryotes that threaten plants (Bergeson 1972; Haldar et al. 2006; Sasser 1980). Given the modulation of predator behavior in the presence of T6SS, it could be hypothesized that T6SS will interact with, and modulate the behavior of, eukaryotes intent on harming the plant. These possible interactions provide an interesting path of further research that could be pursued.

CHAPTER III

SUMMARY AND CONCLUSIONS

3.1. Rationale

Type VI Secretion Systems (T6SS) play an important role in bacterial interactions with other prokaryotes and eukaryotes. Previous work on T6SS characterized their roles to range from virulence, competition, biofilm formation, anti-predation, and nutrient acquisition (Bernal et al. 2017; Chen et al. 2015; DeShazer 2019; Gallique et al. 2017; Ma et al. 2014; MacIntyre et al. 2010; Pukatzki et al. 2006). While much work has gone into studying T6SS, the majority of the research has considered the roles in plant or animal pathogenic bacteria, and information of the roles of T6SS in rhizosphere dwelling, plant beneficial bacteria is limited.

Given that rhizosphere communities are heavily driven by an organism's ability to persist in the environment, a better understanding of how T6SS affect a bacterium's interactive behavior and fitness is vital. Furthermore, by understanding how these systems drive interactions between a plant beneficial bacterium, other microbes, and its environment may lead to more effective application of beneficial microbes and an improvement of overall soil and plant health. Here in this dissertation, the T6SS of the model rhizosphere colonizing plant growth promoting bacteria (PGPB) *Pseudomonas chlororaphis* 30-84 were studied. Through this, we elucidate the roles T6SS play in bacterial competition with both plant pathogens and other rhizosphere colonizing species, as well as their role in anti-grazing activity against several eukaryotic predators.

3.2. Summary and Conclusions

In this dissertation, we identified two genetically distinct T6SS (T6SS-1 and T6SS-2) in the biological control species *P. chlororaphis* 30-84. Bioinformatic analysis of these systems highlighted unique differences between them and suggested that T6SS-1 fires in a contact dependent manner, better known as T6SS dueling (Basler, Ho, and Mekalanos 2013), whereas T6SS-2 may be firing in a random manner. Phylogenetic analysis allowed these two systems to be classified is separate genetic clades (Bernal, Llamas, and Filloux, 2017; Boyer et al. 2009), further suggesting that they may be functioning in different manners. To determine specific functions of each system, mutants deficient in one or both systems were generated and tested.

Using a set of repeat harvest assays, it was first established that deficiency in either or both systems did not affect *P. chlororaphis* 30-84's ability to colonize and survive in the wheat root rhizosphere in sterilized soil. Through the same assay it was found that mutants deficient in both T6SS had a deficit in competitive ability in the microbiome of a wheat root rhizosphere grown in natural field soil. This competitive disability was further confirmed through single strain competition assays both in the soil and *in vitro*. In the soil, it was found that mutants deficient in both systems suffered a competitive disadvantage against two other rhizosphere dwellers, *P. fluorescens* Q287 and *P. protegens* Pf-5. It was found that, *in vitro*, against strains lacking their own T6SS, *P. synxantha* 2-79 and *P. putida* F1, deficiency in T6SS-2 function resulted in decreased competitive ability. This supports the hypothesis that T6SS-1 is involved in contact-dependent firing and T6SS-2 in involved in random firing. *In vitro* competition against five further strains, both other rhizosphere dwellers and pathogens, revealed that lacking function in both systems resulted in decreased competitive ability. Together, these results indicate that having at least one T6SS is needed for competitive fitness in mixtures with bacterial strains

containing their own T6SS, and T6SS-2 is of slightly more importance as it serves to compete against strains that do not have T6SS.

We next aimed to determine the role of T6SS in anti-predation activity. Three eukaryotic predators with different lifestyles were selected to test the efficacy of T6SS against different types of predators. These predators were: *Dictyostelium discoideum,* a free-living, soil-dwelling amoeba that utilizes pseudopodia and phagocytosis to move towards and consume its prey (Dunn et al. 2018; Eidi 2017), *Tetrahymena thermophila,* a ciliate that utilizes phagocytosis, and *Caenorhabditis elegans,* a more complex organism that has a rudimentary digestive system complete with intestines. Feeding assays revealed that having at least one functional T6SS provides adequate protection from all three of these predators. Furthermore, observing predator behavior revealed that the presence of functional T6SS resulted in modulated predator behavior, such as aggregate forming by *D. discoideum,* mating by *T. thermophila,* and avoidance by *C. elegans*.

Finally, once it was determined that both T6SS genetic clusters formed functional T6SS, we searched for putative effectors and immunity proteins associated with the systems. The genes associated with the T6SS gene clusters in *P. chlororaphis* 30-84 were relatively unannotated so bioinformatic analysis was used to identify genes with potential to be effectors or immunity proteins. This analysis revealed four different effector family types: Tle, Tpl, Pld, and Tae. Furthermore, most putative effectors were encoded with a putative immunity protein and a VgrG (part of the T6SS needle). These effector types have been previously shown to interact with both prokaryotic and eukaryotic species (MacIntyre et al. 2010; Durand et al. 2014; Jiang et al. 2014; Ma et al. 2014; Whitney et al. 2014; Alcoforado Diniz and Coulthurst 2015; Mittl and Schneider-

Brachert, 2007), suggesting a potential function in both bacterial competition and anti-predatory behavior.

3.3. Future Directions

In conclusion, we characterized two genetically distinct T6SS that fire in different manners. Both systems are involved in bacterial competition and anti-grazing behavior, and several putative effectors were identified with possible relation to the performed functions of the systems. Further work should focus on interactions with eukaryotes in the rhizosphere and how this could affect the plant host and confirming the associated effectors and determining their direct effects on interactions performed by *P. chlororaphis* 30-84. This work should be completed through several methods. Initially, confirmation that bacterial T6SS continue to interact with predators in the rhizosphere is needed and could be determined through a modified rhizosphere persistence assay. In this assay, the populations of WT and the single and double mutants could be compared to each other and to the populations obtained in the *in vitro* experiments. It would also be important to obtain population counts of the predators in the area immediately adjacent to the plant root rhizosphere to indicate whether they are excluded from the environment and if this varies based on T6SS function. To confirm that the putative effectors uncovered in this study are in fact secreted by T6SS, assays similar to those performed in Pukatzki et al. (2006) should be carried out. Briefly, this involves the isolation of the supernatant of the cell culture and isolation of the secreted proteins, indicating what has been secreted by the T6SS. This methodology could aid in effector discovery and classification of random firing systems, but for contact-dependent firing systems further functionality of these effectors can be confirmed through systematic mutagenesis in which the genes for the putative effectors of

interest are knocked out and their abilities to carry out the established competitive and anti-grazing activities measured.

APPENDIX A: SUPPLEMENTARY MATERIAL FOR CHAPTER II

This section contains research materials and results that supplement the findings of Chapter II.

Table A1: Primers used in this study

Primers	
TssA1KO-repliQa-UP-F	TTTGAGACACAACGTGGCTTTCCCCAGCTGTAGCCCTTGCACCA
TssA1KO-repliQa-UP-R	GACCATGATTACGAATTCGAGCTCGCAGGCGGGCGAAAG
TssA1KO-repliQa-DWN-F	GCAGGTCGACTCTAGAGGATCCCCGGGTACAGGGTCTTGTGCAGGTC
TssA1KO-repliQa-DWN-R	GAGCATTACGCTGACTTGACGGGACACGAGCCCTCAAGCCC
TssA1KO-repliQa-KmR-F	ACCGGCACGGGGCTTGAGGGCTCGTGTCCCGTCAAGTCAGCGTAAT
TssA1KO-repliQa-KmR-R	CCGGCCTGGTGCAAGGGCTACAGCTGGGGAAAGCCACGTTGTGTC
TssA2KO-UP-F-EcoRI	GAATTCCCCGTCTGCCGCAGAGTAGAGCCTG
TssA2KO-UP-R-KpnI	CCTATTCGAGGGTACCGTAGGACATCCATGGCCTTTTTTG
TssA2KO-DWN-F-KpnI	GATGTCCTACGGTACCCTCGAATAGGCCCCAGGGCCAACA
TssA2KO-DWN-R-HindIII	CCCAAGCTTGCGGTGTAGATGTGCTTGTACAGGCC
KanR-F-BamHI	GGATCCTAGAAAAACTCATCGAGCATCAAATGAAACTG
KanR-R-BamHI	CCTAGGATGAGCCATATTCAACGGGAAACG

Table A1 Continued.

Primers	
DoubleKO-GnR+A2UP-R	AAGGCCATGGATGTCCTACCGGTACTCTTACTGTCATGCCATCC
DoubleKO-GnR+A2DWN-F	TGGGGCCTATTCGAGGGTACGCAACTCGGTCGCCGCATACA

Red coloration indicates restriction enzyme sites.

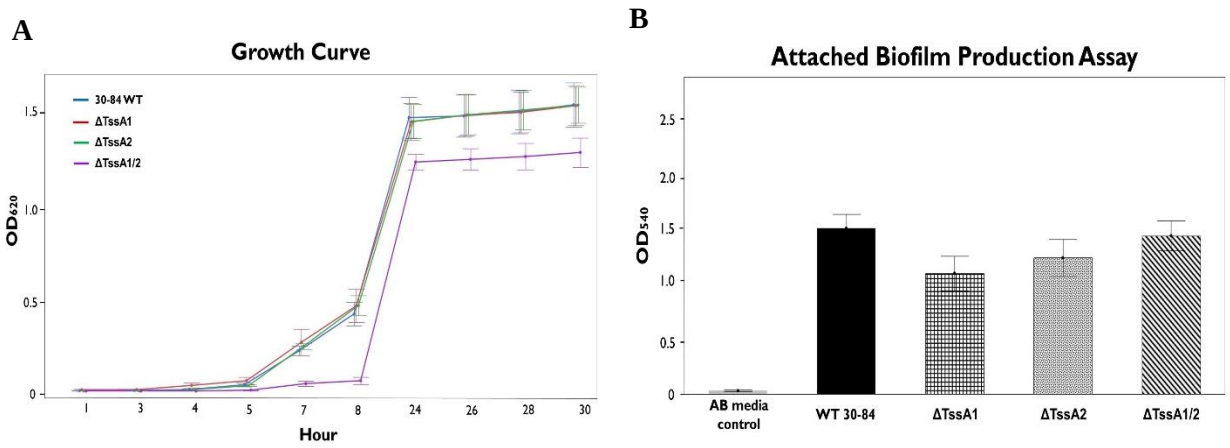

Figure A1: Planktonic Growth Curve and Attached Biofilm Production. A) Planktonic growth: bacteria were grown in LB medium at 28°C with agitation and populations were measured (OD_{620}) over a period of 30 hours. Data are the mean and standard error of 6 replicates/treatment pooled across experiments (n = 6/treatment, $p<0.05$). **B)** Attached biofilms: separate wells of 96-well plates were inoculated with bacteria and grown at 28°C without agitation for 72 h. After removal of non-adhering cells, surface-attached biofilms were stained with crystal violet. The optical density (540 nm) of crystal violet released from the biofilms was used as a relative measure of biofilm population density. Data are the means and standard errors of two biological replicates per strain (started from separate colonies) and five technical replicates performed three times (n = 30/treatment). Strains tested included 30-84 WT, the single T6SS mutants ΔTssA1 and ΔTssA2, and the double mutant ΔTssA1/2. Data were analyzed using a one-way ANOVA and Tukey's tests. No significant differences were found, $p< 0.05$.

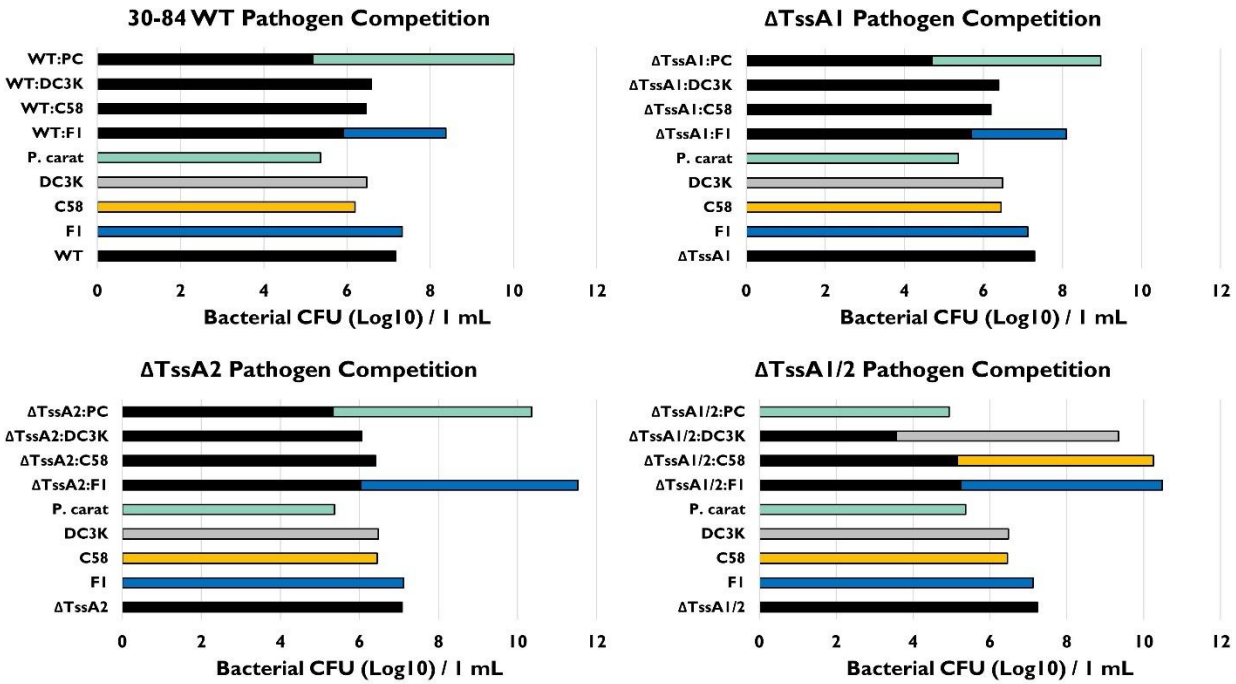

Figure A2: *In vitro* Competition Assays against Environmental Isolates and Plant Pathogens. The competitive fitness of 30-84 WT and T6SS mutants (black color) against environmental isolates and pathogenic strains were evaluated by comparing their populations when grown separately or in 50:50 mixtures in liquid medium. Strains used include *P. putida* F1 (blue color), *Agrobacterium tumefaciens* C58 (orange color), *P. syringae* DC3000 (gray color), and *Pectobacterium carotovorum* (light green color). Individual bacterial cultures or mixed cultures were spotted onto nitrocellulose filters on LB plates and incubated at 28°C, 5 h. Bacterial cells were washed from filters, collected via centrifugation, and populations were enumerated after 48 h. via serial dilution plating. Data are the means (log10 CFU/1 mL) of at least 5 biological replicates/treatment pooled across experiments (n = 5/treatment).

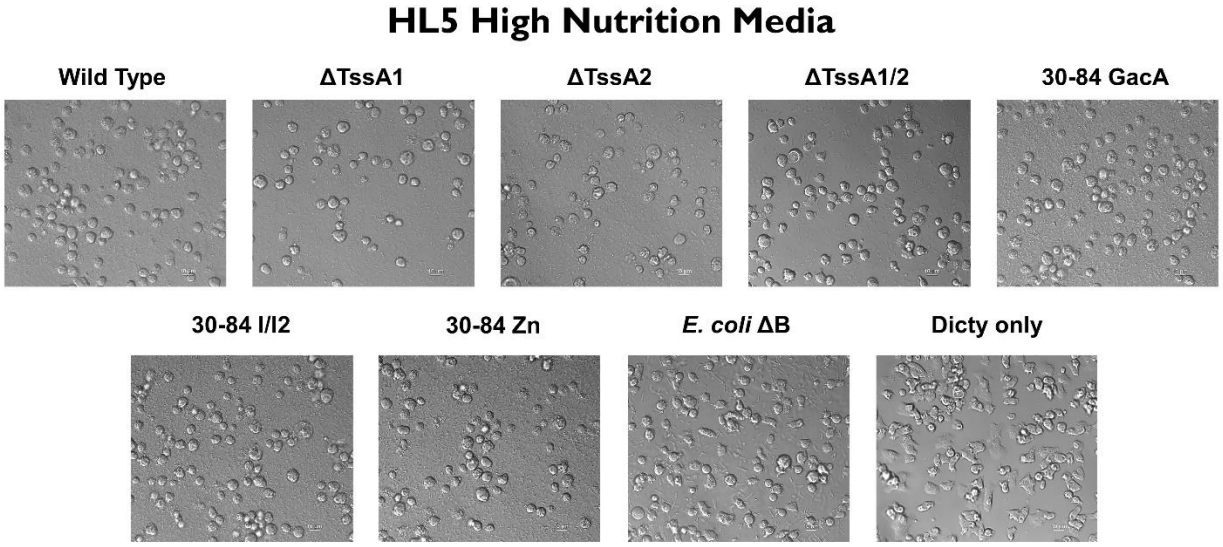

Figure A3: Aggregation behavior of *Dictyostelium discoideum* grown with different bacterial strains in high nutrition HL5 medium. Bacteria used as prey in the feeding assay included 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, or 30-84 ZN and *E. coli* ΔB (used as a preferred prey in the lab). *D. discoideum* without prey bacteria was used as a negative control. *D. discoideum* cells were grown in 24 well plates in high nutrient HL5 media for 24 h and aggregation behavior was observed using DIC microscopy (100X oil). All treatments showed no aggregation behavior, indicating no stress. Two replicate experiments were performed, and representative images from the same replicate are presented.

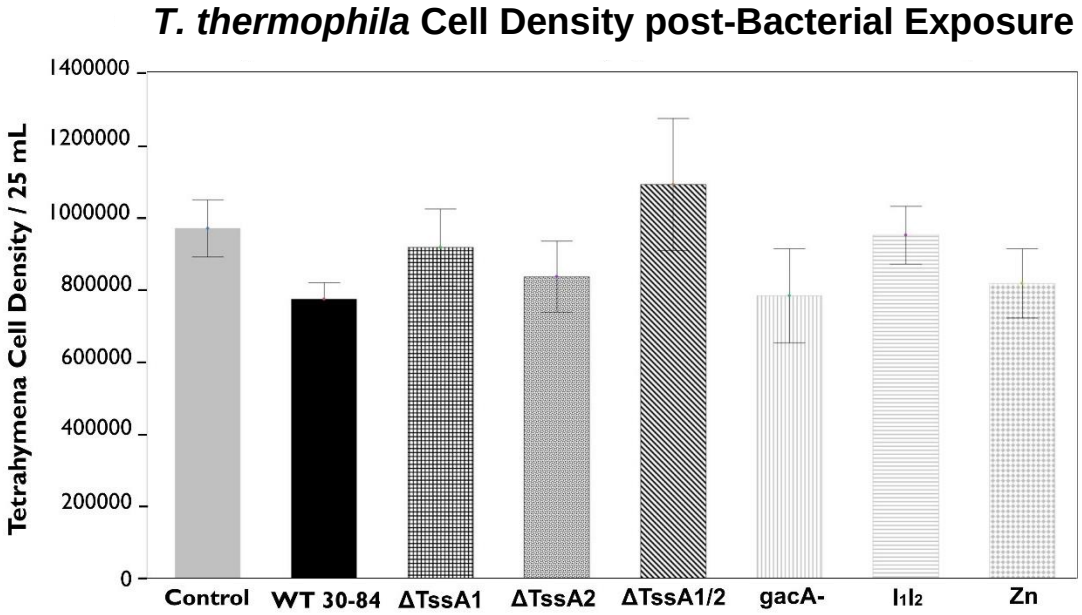

Figure A4: *T. thermophila* populations after 24 h feeding assay. Bacteria used as prey in the feeding assay included 30-84 WT, ΔTssA1, ΔTssA2, ΔTssA1/2, 30-84 GacA, 30-84 I/I2, or 30-84 ZN. *T. thermophila* without prey bacteria was used as a negative control. Bacterial and *T. thermophila* cultures were mixed (5 mL, 20 mL, respectively) and grown in 50 mL tubes with agitation (200 rpm, 27 °C). After 24 h., *T. thermophila* populations in mixed cultures were enumerated via direct counts using a hemocytometer. Data are the means and standard errors of 6 replicate experiments (n = 6/treatment). Data were analyzed using a one-way ANOVA and Student t tests. No significant differences were found, $p<0.05$.

APPENDIX B: SOME FURTHER RESEARCH

A.1. Introduction

Previous research has shown that Type VI Secretion Systems (T6SS) are used in interactions with plant cells, primarily for virulence, and integral for biofilm formation (Asolkar and Ramesh 2020; Tian et al. 2017; Chen et al. 2015). Thus, it was hypothesized that plant growth promoting bacteria (PGPB) may use their T6SS for modified interactions with plant cells and/or be involved in the production of biofilm communities. To test these effects, we performed growth curve analysis, attached biofilm production and extracellular matrix production assays, and plant immune response assays.

Mutants in each or both T6SS were created in 30-84 ZN to further observe the specific effects of T6SS vs. phenazine production. These mutants were not used in this research project but may provide a useful tool for future T6SS research.

A.2. Materials and Methods

A.2.1. Generation of single and double mutants in *P. chlororaphis* 30-84 ZN

Single mutants, ZN-ΔTssA1 and ZN-ΔTssA2, were created in the phenazine deficient mutant *P. chlororaphis* 30-84 ZN using plasmids pEX18A+TSSA2/KMR and pEX18A+TSSA1/KMR, respectively (Table A1). Double-crossover mutants in *P. chlororaphis* 30-84 ZN were obtained by counterselection on LB amended with Rif, Km, and 6% sucrose and confirmed using PCR primers specific to the internal regions of *tssA-1* or *tssA-2*. PCR was performed using GoTaq® Green Master Mix (Promega, Madison, WI) according to manufacturer recommendations. *E. coli* transformation and *P. chlororaphis* 30-84 ZN

conjugation were performed as described previously (Pierson and Thomashow 1992; Wang et al. 2012).

The *P. chlororaphis* 30-84 double mutant ZN-ΔTssA1/2 was created using ZN-ΔTssA1 as the starting material and plasmid pEX18A+TSSA2/GMR for the *tssA-2* disruption (Table A1). Double-crossover mutants in *P. chlororaphis* 30-84 ZN-ΔTssA1 were obtained by counter selection on LB amended with Rif, Km, Gm and 6% sucrose and confirmed using PCR primers specific to the internal regions of *tssA-1* and *tssA-2*.

A.2.2. Growth in planktonic culture and surface attached biofilms

The strains 30-84 WT, ΔTssA1, ΔTssA2, and ΔTssA1/2 were grown in LB medium at 28°C with agitation and the optical density (OD_{620}) was measured at one-hour intervals until 8 hours and then at two-hour intervals between 24 hours and 30 hours. The experiment was performed with two biological replicates and repeated three times.

Surface attached biofilm formation was quantified via a 96 well microtiter assay routinely used in our lab (Maddula et al. 2006) with slight modification. Briefly, pre-cultures were grown in LB medium (28°C, with agitation, 16 h). These cultures were resuspended in fresh LB medium and grown to a final cell density of OD_{620}=0.8. Each strain (1.2 µL) was inoculated into 120 µL AB + CAA in separate wells of a 96 well polystyrene cell culture plate (Corning Inc., Corning, NY, USA). The plate was incubated at 28°C for 72 h without agitation in a sealed container to minimize evaporation. Unattached cells were removed by inversion of the plate with vigorous tapping. The adherent bacteria were fixed to the plate (20 min, 50°C) and stained (1 min, 150 µL of 0.1% crystal violet). Excess stain was removed by inversion of the plate followed by two washings with sterile distilled water. The adherent cells were decolorized (to release the

dye) with a 20% acetone/80% ethanol solution (200 µL, 5 min, room temperature). A sample (100 µL) of each well was transferred to a new 96-well plate and the amount of dye (proportional to the density of adherent cells) was quantified (OD_{540}). The experiment was performed with two biological replicates (started from separate colonies) and five technical replicates and repeated three times.

A.2.3. Extracellular Matrix Production

Extracellular matrix was quantified via assays routinely used in our lab (Wang et al. 2016). Briefly, pre-cultures were grown overnight in AB + CAA (28°C, 200 rpm agitation, 16 h). The pre-cultures then were diluted to an OD_{620}=0.1 in fresh AB + CAA and 1.5 ml per well were added to wells of 24-well polystyrene cell culture plates (Corning Inc.). The plates were maintained at 28°C without agitation in a sealed container to minimize evaporation. At 48 h and 72 h the entire non-adhering contents of each well was transferred to separate Eppendorf tubes, and cells were collected by centrifugation (16,000 x g for 5 min). The supernatants were discarded, and the mass of the cells and hydrated matrix were measured. The experiment used two biological replicates and four technical replicates and was performed three times.

A.2.4. FRK1 and WRKY46 Luminescence Assays

A luciferase assay was used to determine whether the T6SS were involved in plant immune response. 30-84 WT or mutant derivates, *P. syringae* pv. *tomato* DC3000, a DC3000 mutant deficient in the T3SS (ΔHrcC), and another DC3000 mutant which causes a hypersensitive immune response (avrpt2) were grown in 3 mL LB (or LB+Km^{50} for mutants) or KB medium (DC3000 strains) at 28°C and 200 rpm for 16 hours. These were then subcultured into 50 mL of fresh LB or KB medium and grown until they reached an optical density of 0.8.

The cells were then centrifuged at 4,000 rpm for 15 minutes and the pellet resuspended in ddH_2O. The cultures were then normalized to an optical density of 0.5. Each culture was then syringe infiltrated into the leaves of four-week-old *Arabidopsis thaliana* plants. The *Arabidopsis* plants used were previously generated to either contain the FRK1 promoter linked to a luciferase reporter gene (*pFRK1::Luc*) (Li et al. 2014) or the WRKY46 promoter linked the a luciferase reporter gene (*WRKY46::Luc*) (He et al. 2006). The plants were grown according to Li et al. (2014). Briefly, the plants were grown in pots containing Metro Mix 360 soil in a growth room at 23°C, 60% relative humidity, and a 12-hour photoperiod. Each strain was infiltrated into leaves of 12 *Arabidopsis* plants of each type. These infiltrated plants were then left at room temperature for 16 hours. Leaf discs were taken from the infiltrated leaves and added to a 96-well plate with the bottom of the leaf facing upwards. The plate was sprayed with 0.2 mM luciferin and the bioluminescence signal was read using a luminometer (Promega Glomax Multi Detection System Model E7031). These experiments were repeated two times each.

A.3. Results

A.3.1. Growth of *P. chlororaphis* T6SS mutants

To study the function of each T6SS, mutants were generated to disrupt T6SS assembly via deletion of *tssA* in one or both systems. In planktonic culture, there was no difference in the growth rates of wild type (30-84 WT) or the single T6SS mutants (ΔTssA1 and ΔTssA2), although the double mutant (ΔTssA1/2) consistently grew somewhat slower and reached a slightly, but significantly lower cell density after 30 h **(Fig. A.1A)**. However, in surface attached biofilms, population levels of 30-84 WT and the single or double T6SS mutants were no different after 72 h, indicating that neither T6SS is involved in the production of attached biofilms **(Fig. A.1B)**. This further indicates that the difference in planktonic growth, while

statistically significant, is not biologically significant as there is no growth deficit in biofilm or in the rhizosphere **(chapter II)**.

A.3.2. Extracellular Matrix Production

To determine whether either T6SS was involved in the production of extracellular matrix in biofilms, rather than the attached biofilm itself, a matrix was performed to measure the total weight of the produced matrix in grams. This assay showed a slight, but significant, increase in matrix produced by ΔTssA1/2 **(Fig. A5)**. This shows that neither T6SS is involved in matrix production, and further concludes that neither system is involved in the aspects of biofilm production tested here.

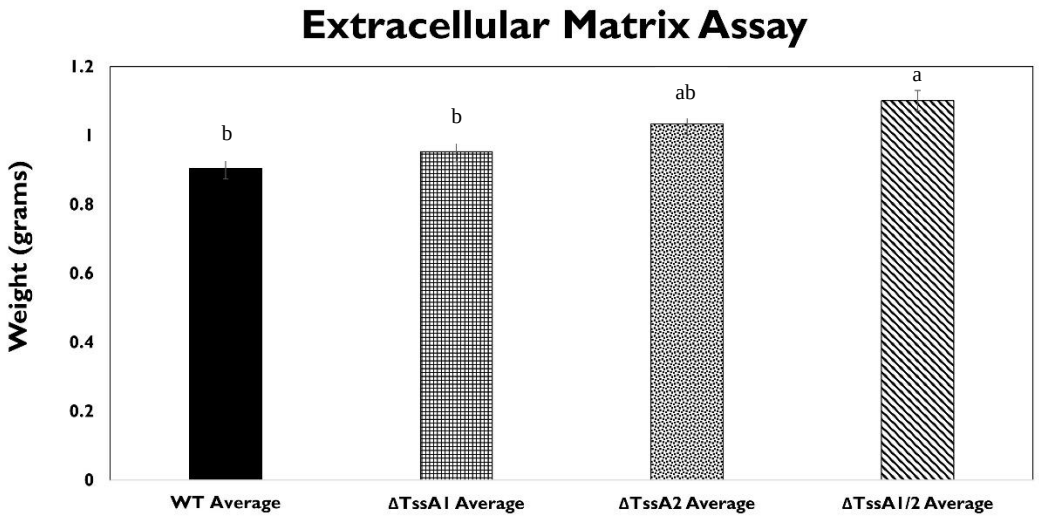

Figure A5: Comparison of extracellular matrix production. Bacteria were grown for 72 h in AB + CAA at 28°C, without agitation**.** Matrix was collected and weighed. Strains tested included 30-84 WT, the single T6SS mutants ΔTssA1 and ΔTssA2, and the double mutant ΔTssA1/2. Data are the means and standard errors of 12 replicates/treatment pooled across experiments (n = 12/treatment). Data were analyzed using a one-way ANOVA and Tukey HSD tests. Letters denote groups of treatments that are significantly different, $p<0.05$.

A.3.3. T6SS and their roles in plant recognition and immune response

T6SS have been shown to be heavily involved in interactions with plant cells. To determine if the T6SS of *P. chlororaphis* 30-84 perform similar interactions, plant immune response assays were performed. An FRK1 Luciferase assay was done to see the role of *P. chlororaphis* 30-84 in triggering host plant Pattern Triggered Immune response (PTI). 30-84 WT was shown to trigger a high level of luciferase activity, indicating a high level of PTI response, higher even than the positive control, ΔHrcC. The mutants, ΔTssA1, ΔTssA2, and ΔTssA1/2 showed a significant decrease in this activity, around 30-35% less. This suggest that both

mutants are recognized by the plant host and are involved in triggering host immunity, but there is no additive reduction in recognition correlated with loss of both T6SS **(Fig. A6)**.

A WRKY46 Luciferase assay was performed to determine the role of *P. chlororaphis* 30-84 in downstream, late-stage host immune response which is typically triggered by effectors. 30-84 WT was shown to cause a high level of luciferase activity. Although there was a slight increase in triggering by ΔTssA2 and ΔTssA1/2, it does not appear to be biologically significant **(Fig. A7)**.

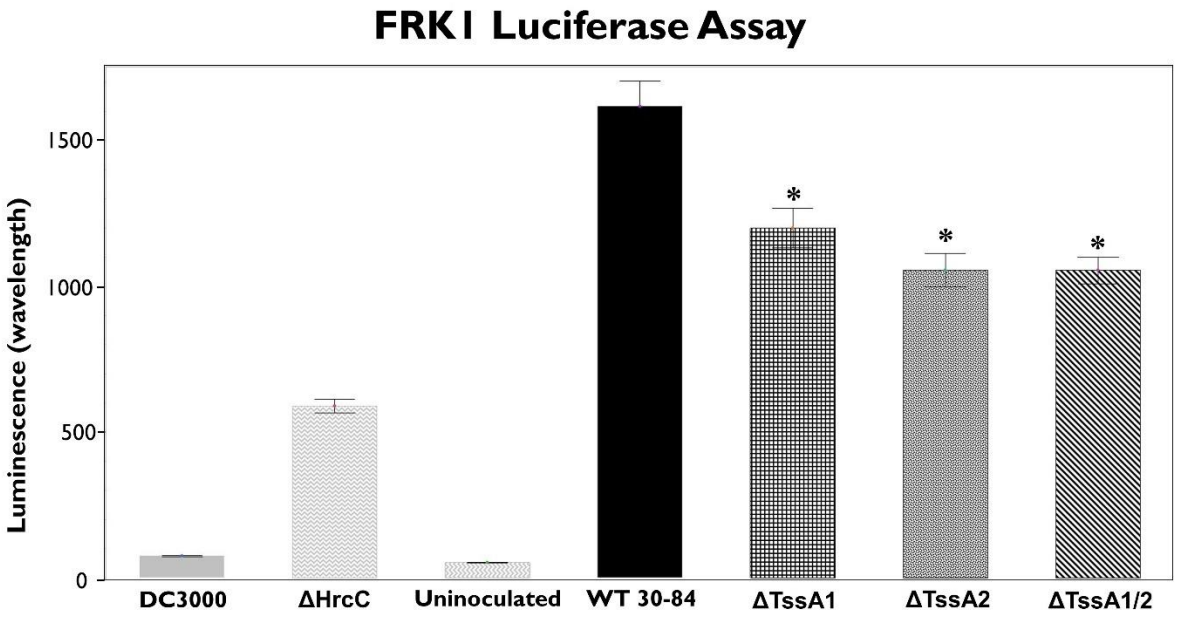

Figure A6: Comparison of wild type and mutants using the FRK1 luciferase assay. 12 samples/treatment from each of 2 experiments were observed for wild type and the mutants with statistically determined outliers removed. These were then compared to uninoculated plants, *P. syringae* DC3000, which utilizes a T3SS and shuts down PTI response, and ΔHrcC, which is deficient in the T3SS; these served as the negative and positive controls, respectively. This was repeated four times and revealed significant differences between wild type and all mutants, indicating that the T6SS are involved in recognition by the plant. The double mutant, however, is not significantly different than either single mutant, suggesting that there is not an additive effect of the T6SS on plant recognition. Data are means and standard error of at least 8 replicates per treatment from two experiments (n = 16/treatment). Data were analyzed using one-way ANOVA and Tukey HSD tests. Significant treatments are indicated, $p<0.05$.

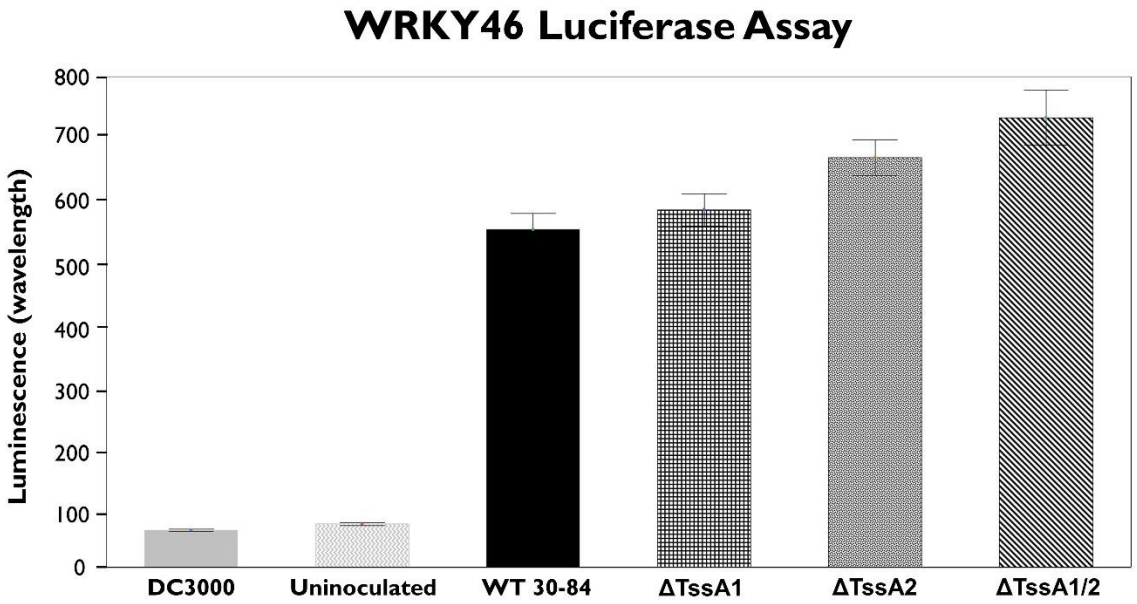

Figure A7: Comparison of wild type and mutants using the WRKY46-luciferase assay. Samples were compared to uninoculated plants, *P. syringae* DC3000 which utilizes a T3SS and shuts down PTI response and Δavrpt2, which causes a hypersensitive response in plant leaves, which served as the positive and negative controls, respectively. Leaves from avrpt2 were unable to be scanned due to their hypersensitive response. Results show that mutants deficient in T6SS-2 function have slightly higher levels of immune response triggering, though we do not believe this is biologically relevant. This indicates that neither T6SS is involved in WRKY46 immune response, suggesting that neither system delivers effectors to plant cells. Data are the means and standard error of at least 8 replicates per treatment from two experiments (n = 16/treatment). Data were analyzed using one-way ANOVA and Tukey HSD tests, $p<0.05$.

A.4. Conclusion

Methods and results presented here are extra experiments performed concurrently with the research in Chapter II. Given the need for further research and expansion on T6SS roles in rhizosphere dynamics, these data and materials could be used to further future experiments.

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