Characterisation of the type VI secretion system in *Pantoea ananatis*

BY

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Submitted in partial fulfilment of the requirements for the degree

Doctor of Philosophy

In the Faculty of Natural and Agricultural Sciences, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa

February 2014

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DECLARATION

I declare that the thesis "Characterisation of the type VI secretion system in *Pantoea ananatis*", which I hereby submit for the Doctor of Philosophy degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:…….. DATE:.....……..

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ACKNOWLEDGEMENTS

I am deeply grateful to God for having taken me this far in my life.

I would want to acknowledge the invaluable supervision of Prof. TA Coutinho, Dr. Lucy Moleleki, Prof. SN Venter and Dr. Ian Toth who guided me professionally.

Many thanks also to Prof. Jacques Theron for your invaluable technical assistance, most of all thank you for helping me think outside the box.

Many thanks to my siblings and parents for being there for me all these years.

Thanks also to Mr John Menjo and his wife Ruth Menjo for providing me an escape from the rigors of academic work during the weekends and holidays. Special thanks to Ms Vimbai Siziba for all her support, you stood by me, listened and supported me through what I consider the hardest part of my PhD studies.

I will also like to acknowledge the funding bodies: National Research Foundation (NRF), Tree Protection Cooperative Programme (TPCP), Centre of Excellence in Tree Health Biotechnology (CTHB), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

PREFACE

Pantoea ananatis is a Gram-negative, facultative, non-spore forming bacterium that belongs to the family *Enterobacteriaceae*. Pathogenic strains of *P. ananatis* are known to cause disease in several economically important plants including onion, maize, melon, cotton, cantaloupe fruit, Sudan grass, rice and *Eucalyptus* spp. Despite the extended host and geographical range of *P. ananatis*, there is limited information on how this pathogen is able to colonize and cause disease symptoms in different susceptible host plants. Therefore, the overall objective of this study was to determine what role the newly described type six secretion system (T6SS) plays in the biology of *P. ananatis*.

The first chapter of this thesis is a review of the literature dealing with the different secretion systems found in bacterial genomes, with a specific emphasis on the T6SS. This review highlights the role of the different secretion systems found in Gram-negative bacteria. This chapter also addresses different aspects of the T6SS such as structure, genetic organization, phylogeny, regulation and the different phenotypes that have been associated with a functional T6SS found in other bacterial strains and species. It also highlights some of the effectors that have been shown to be secreted by T6SS.

The second chapter provides an in-depth comparative analysis of the different T6SS gene clusters found in the genome sequences of eight strains of *P. ananatis*. PCR and probes were used to determine the distribution of the different T6SS gene clusters in environmental and pathogenic strains of *P. ananatis* from different ecological niches. This chapter, therefore, addresses issues such as the gene content, operon structure, evolutionary history and differential distribution of T6SS-1, -2 and -3 gene clusters in different strains of *P. ananatis*.

In Chapter 3 the objective was to experimentally determine what role (s) T6SS-1, T6SS-2 and T6SS-3 play in the biology of *P. ananatis*. This involves systematic deletion of individual T6SS gene clusters found in the genome sequences of *P. ananatis* strains LMG 20103 and LMG 2665^{T}, which are pathogens of *Eucalyptus* spp. and pineapple, respectively. We functionally characterized each T6SS mutant based on virulence in susceptible onion plants and their ability to inhibit growth of other Gram-negative bacteria following co-culture on LB agar.

In Chapter 4 the objective was to determine if the observed phenotypes described in chapter 3 were dependent on proteins encoded by genes found within the T6SS gene cluster. In other

words, since the T6SS is a multi-component secretion system consisting of 15-30 different genes, we wanted to disrupt some of these conserved genes to determine if they encode proteins which are essential for biosynthesis of a functional T6SS.

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LIST OF ABBREVIATIONS

%:	Percentage
Ω:	Omega
τ:	Time constant
^{o}C:	Degree Celsius
x g:	Centrifugal force
~:	Approximately
λ:	Lambda
Δ:	Delta
&:	And
μM:	Micromole
μl:	Microlitre
A:	Absorbance
Ap:	Ampicillin
BCC:	Bacterial Culture Collection
BLAST:	Basic Local Alignment Search Tool
bp:	Base pair
CDD:	Conserved Domain Database
CFU:	Colony Forming Units
Cm:	Choloramphenicol
COG:	Classification of Orthologous Groups
Cv:	Cultivar

ddH_2O:	Double distilled water
DNA:	Deoxyribonucleic Acid
dNTP:	Deoxynucleoside triphosphate
dUTP:	Deoxyuridine triphosphate
E:	Electric field strength
e.g.:	For example
EDTA:	Ethylenediaminetetra-acetic acid
FABI:	Forestry and Agricultural Biotechnology Institute
Fig:	Figure
Gm:	Gentamycin
Hcp:	Hemolycin coregulated protein
Hr:	Hour
HSI:	Hcp Secretion Island
IPTG:	Isopropyl-β-D-thiogalactopyranoside
Kb:	Kilobase
Kb:	Kilobase pairs
kDa:	Kilodalton
Km:	Kanamycin
LB:	Luria-Bertani
Log:	Logarithm
LPP-1:	Large Universal Pantoea Plamid-1
M:	Molar
MEGA:	Molecular Evolutionary Genetics Analysis

mg:	Milligram
ml:	Millilitre
mm:	Millimetre
mM:	Millimole
NCBI:	National Center for Biotechnology Institute
ng:	Nanogram
nt:	Nucleotide
OD:	Optical density
PA:	*Pantoea ananatis*
PG:	Peptidoglycan
pmol:	Picomole
Rap:	Resistance associated protein
RSA:	Republic of South Africa
RtxA:	Repeats-in-toxin A
s:	Seconds
SPI:	Salmonella Pathogenicity Island
spp:	Specie
Ssp:	Small secreted proteins
Subsp:	Subspecies
T1SS:	Type I secretion system
T2SS:	Type II secretion system
T3SS:	Type III secretion system
T4SS:	Type IV secretion system

T5SS:	Type V secretion system
T6SS:	Type VI secretion system
T7SS:	Type VII secretion system
Tag:	*tss* - associated genes
Tc/Tet:	Tetracycline
Tm:	Melting temperature
Tris-HCL:	Tris (hydroxymethyl) aminomethane hydrochloride
Tse:	Type six effector
Tsi:	Type six immunity
Tss:	Type six secretion
U:	Unit
USA:	United States of America
UV:	Ultraviolet
v.	Version
v/v:	Volume per volume
VgrG:	Valine-glycine repeat G

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CHAPTER 1

SECRETION SYSTEMS IN BACTERIA WITH A FOCUS ON THE TYPE VI SECRETION SYSTEM

1.1 GENERAL INTRODUCTION

Pantoea ananatis (synonyms: *Erwinia uredovora, Erwinia ananas, Pantoea ananas*) is a Gram-negative, facultative anaerobic bacterium belonging to the family *Enterobacteriacae* (1, 2). *Pantoea ananatis* represents a wide collection of pathogenic and non-pathogenic bacteria. The cells are rod-shaped, approximately 1.0 μm to 2.0 μm in length and 0.5 μm to 0.75 μm wide (3). The colonies are mostly yellow due to the production of carotenoids (4) encoded by the carotenoid gene cluster found on the Large *Pantoea* Plasmid-1 (LPP-1) (5). They are usually glucose fermenters, nitrate reducers and are oxidase negative (6). They are motile with peritrichous flagella. *P. ananatis* can be transmitted by insect vectors such as the cotton fleahoppers (*Pseudatomoscelis seriatus* Reuter) and the tobacco thrip (*Frankliniella fusca*) (7, 8). In addition, virulent strains of *P. ananatis* have been isolated from asymptomatic onion seeds, which were shown to produce central rot symptoms in onion plants and by the introduction of contaminated onion seeds (9, 10, 11, 12). They can also gain entry into host plants through flowers, mechanical injury and plant-to-plant contact during high winds (1, 13).

Pathogenic strains of *P. ananatis* have been shown to cause disease symptoms on several economically important plants. They infect pineapple (1), cotton (7), onion (8, 9, 11), sudangrass and sorghum (13), maize (15), rice (16), cantaloupe fruit (18), honeydew melons (17) and netted melon (19). In South Africa, the pathogen causes diseases in maize, onion and *Eucalyptus* (3, 10, 21). In *Eucalyptus, P. ananatis* causes blight and die-back in a number of clones, hybrids, and species including *E. grandis*, *E. saligna*, *E. dunnii, E. nitens, E. smithii, E. grandis X E. camadulensis,* and *E. grandis X E. urophylla* (3). During outbreaks large economic impacts have been noted. *P. ananatis* was identified in Georgia (USA) in 1997 as the causal agent of central rot in onion (8) and accounted for 100% losses in some fields. Goszcynska *et al*. (2006) isolated the pathogen from onion seeds in South Africa and it is the causal agent of brown stalk-rot of maize in this country (21). The pathogen infected over 70% of the maize crops in some fields and was associated with underdeveloped seed cobs in South Africa (21). To date, the genome sequences of eight strains of *P. ananatis* are currently available. These genomes are providing valuable information on the genetic diversity, pathogenicity and environmental adaptation strategies of *P.*

ananatis. Sequenced strains include *P. ananatis* LMG 20103 isolated from an infected *Eucalyptus grandis* seedling (22), the soil saprophyte AJ13355 (23), the biocontrol agent PA13 (24), the clinical isolate LMG 5342 (25), the onion epiphyte B1-9 (26), the pineapple isolate LMG 2665^{T}, the maize isolate BD442 and the onion isolate PA-4. The genomes of strains LMG 2665^{T}, BD442 and PA-4 were recently sequenced by our laboratory and have not been published at the time of this study. All sequenced strains have a similar size (4.7 to 5.1 million base pairs), GC content (~ 53%), protein coding capacity (4038 to 4645) and harbour a 280-352 kb megaplamid called the Large *Pantoea* Plasmid-1 (LPP-1) containing 238-320 putative genes (5).

Genome mining revealed that *P. ananatis* contains several genes encoding adhesins, fimbria, type IV pili, chemotaxis genes, different transport systems such as several RND multidrug transporters, ABC transport and iron uptake systems (siderophores) as well as a wide range of response regulators (22, 23, 24, 25, 26). It has been suggested that these proteins enhance fitness of bacteria in different ecological niches (27). Most of the genes encoding pathogenicity factors of *P. ananatis* remain to be identified and experimentally validated. Genes encoding phytotoxins and plant cell wall degradation enzymes are not found in the genome of sequenced strains of *P. ananatis*. Of interest to this review is the finding that the genome sequence of *P. ananatis* strain LMG 20103 contains gene clusters which could encode the type VI secretion system (T6SS) (28). This secretion system has been shown to play a role in pathogenicity and fitness of different bacterial species (29, 30). This review, therefore, focuses on advances made towards our understanding of the structure, function and regulation of the T6SS of *P. ananatis*.

1.2 Secretion systems

Secretion systems are dedicated protein channels found in both Gram-negative and Gram-positive bacteria (31). These channels are used to translocate effector molecules from the cytoplasm to the exterior of bacterial cells or in some cases directly into eukaryotic host cells (32, 33). Secretion systems have been classified (Type I to VII) based on their organization and the nature of the effectors released by each system (31, 32, 33, 34). These different secretion systems have been implicated in virulence and fitness of different bacteria (Figure 1.1). The Type VII secretion system will not

be discussed in this review because it is not well characterized and is restricted to Gram-positive bacteria such as the mycobacteria.

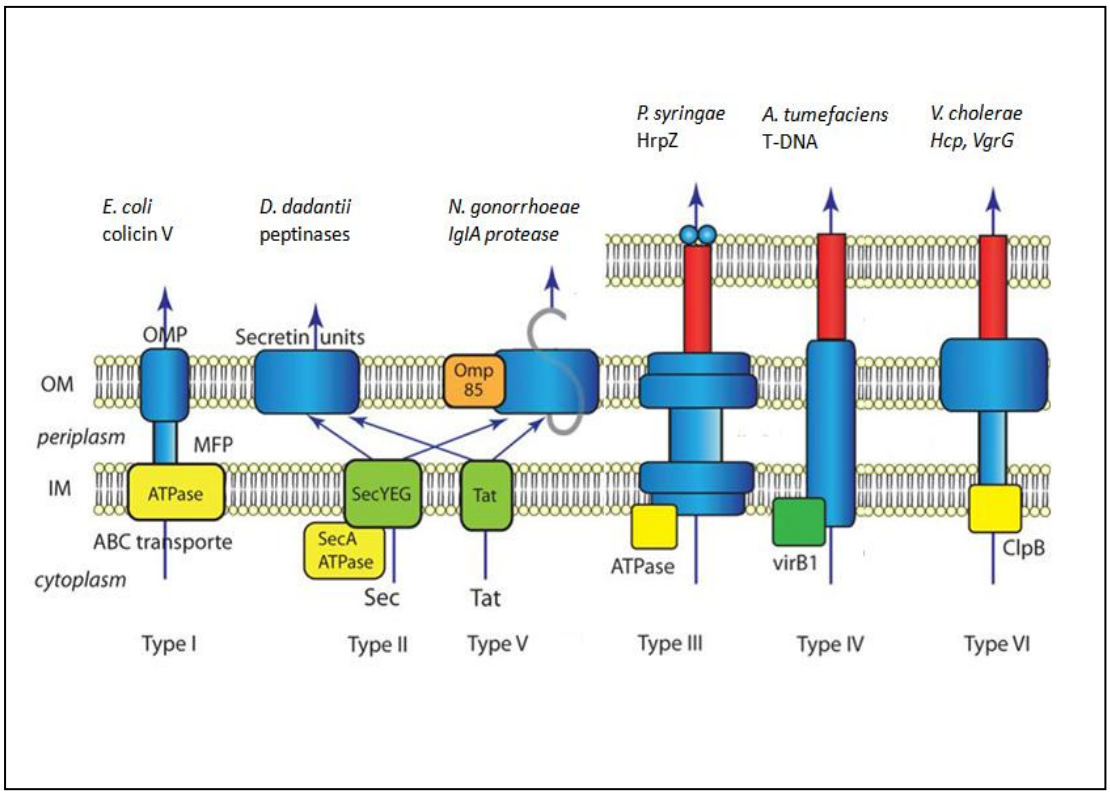

Figure 1.1: Schematic representation of type I-VI secretion systems. HM: Host membrane; OM: outer membrane; IM: inner membrane; OMP: outer membrane protein; MFP: membrane fusion protein. ATPases are shown in yellow. *E. coli* = *Escherichia coli*; *K. oxytoca* = *Klebsiella oxytoca*; *N. gonorrhoeae* = *Neisseria gonorrhoeae*; *D. dadantii* = *Dickeya dadantii*; *A. tumefaciens* = *Agrobacterium tumefaciens*; *V. cholerae* = *Vibrio cholerae*. (Modified from Tseng *et al*., 2009) (33).

The type I secretion system (T1SS) has been associated with secretion of proteins and peptides such as hemolycin, cyclolysin, alpha hemolysin, colicin V, proteases and antimicrobial peptides (bacteriocins and microcins) (34, 35, 36). These effectors inhibit growth of recipient bacteria through processes related to DNA damage, membrane disruption or inhibition of protein synthesis (34). Bacteria secreting these toxic effectors are protected from autoinhibition by producing the cognate immunity factor (35, 36). In addition, gall-forming *Agrobacterium tumefaciens* uses the T1SS to secrete β-1,2-Glucans, which are required for attachment and virulence on several plants (37). The T1SS can, therefore, be considered as a versatile secretion system

that can play different roles related to virulence, symbiosis, survival and ecological fitness of different bacterial species.

The type II, III and IV secretion systems are multicomponent secretion systems made up of 12-25 different proteins (30, 31). These secretion systems are major virulence factors restricted to a few bacterial species. For example, the T2SS is involved in secretion of virulence factors such as the ADP-ribosylation toxin by *E. coli*, cholera toxin produced by *Vibrio cholera* and cell wall degrading enzymes such as pectinase and pectic lyase produced by *Dickeya dadantii* and *Erwinia amylovora* (38, 39). The T3SS and T4SS differ from the T2SS in two important aspects: a) secretion by both systems is independent of the Sec and Tat pathway and b) both systems form an injectisome-like structure which spans both the inner and outer membrane, allowing secretion of effectors from the cytoplasm directly to the exterior of the cell or directly into the host cytosol. The Hrp secretion system (T3SS) of *Pseudomonas syringae* has been shown to secrete HrpZ, HrpW and Avr proteins (such as AvrPto, AvrRpt2 and AvrB) (40). These proteins are involved in different host pathogen interactions, which can lead to induction of host cell immunity and hypersensitivity reactions thus limiting the host range of the pathogen. The type IV secretion system (T4SS) is evolutionary related to the bacterial conjugation system and is capable of trans-kingdom transfer of virulence factors and DNA-protein complexes into bacteria, plants and yeasts (41). *A. tumefaciens* uses the T4SS encoded by the *virB2- virB11* genes to transfer oncogenic T-DNA and effectors into plants (42).

The type V secretion system (T5SS) is subdivided into the type Va to type Ve system based on the structural diversity of the exported protein (43, 44). Only the type Vb secretion system will be discussed in this review because it encodes toxin/immunity systems associated with bacterial contact-dependent growth inhibition (CDI) (45). CDI systems mediate inter-bacterial competition by secreting toxins which cause growth inhibition of neighbouring cells (45, 46). Genes involved in CDI are found in an operon that comprises *cdiA*/*cdiB*/*cdiI* and encode the toxin, transporter and the immunity proteins, respectively (Figure 1.2).

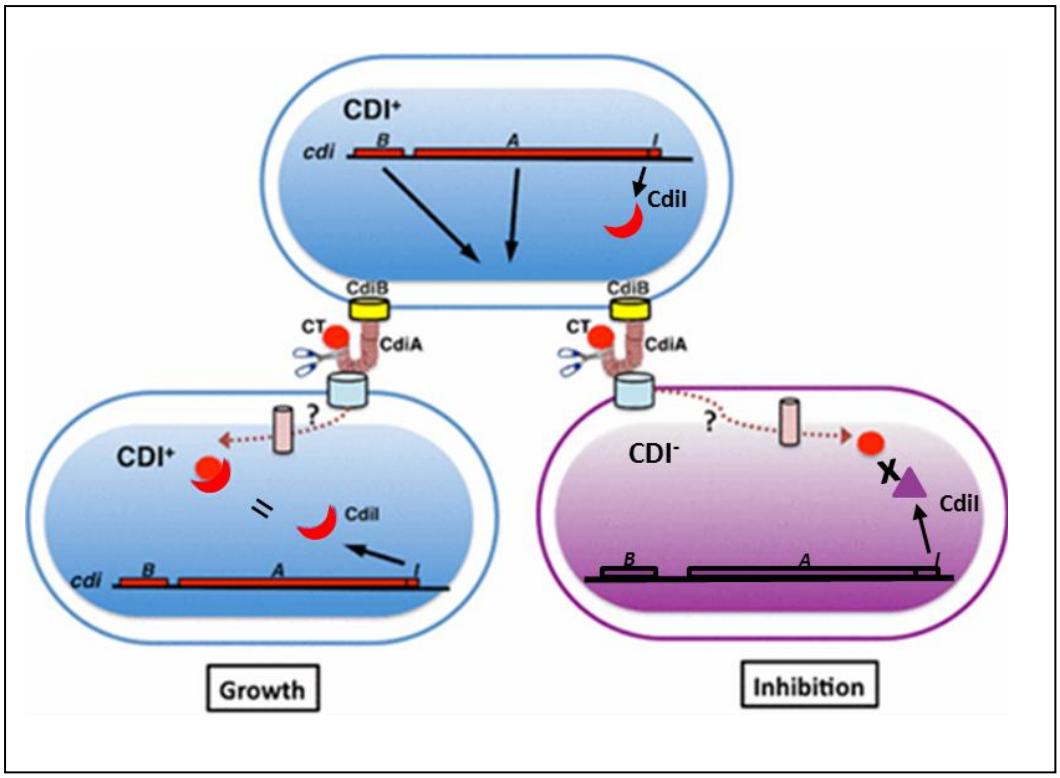

Figure 1.2: Mechanism of contact-dependent growth inhibition (CDI) associated with the type Vb secretion system. CDI^+ cells containing the *cdiBAI* gene cluster express CdiB and CdiA at the cell surface. Cells carrying the identical CDI system (depicted in blue cells) are protected from growth inhibition by the CdiI immunity protein. Non-immune cells (CDI^-) and cells expressing a different CDI system are indicated in purple. Figure was modified from Morse *et al*. (2012) (51).

CdiA toxins have a highly conserved N-terminal domain of over 3000 amino acids and a highly variable C-terminal domain of 200-300 amino acids (45). This variable C-terminal domain constitutes the functional domain of the toxin and is referred to as CdiA-CT (50). CdiA-CT is demarcated from the N-terminal domain by conserved amino acids called the VENN peptide motif. Most CdiA toxins are usually RNA and DNA nucleases, although other effector domains such as deaminases, ADP-ribosyl transferase and peptidases have been identified in a few CdiA toxins (50, 51). Following expression of the *cdi* locus, CdiA and CdiB proteins are translocated to the periplasm by the Sec transport system. The CdiB protein assembles to form a β-barrel channel in the outer membrane, through which CdiA toxin is transported out of the cell (47, 48). Extracellular CdiA binds to BamA protein on target cells and is internalised through unknown mechanisms, where it causes growth inhibition in target

cells that do not express the cognate immunity protein CdiI (49). Bacteria cells expressing the CdiA toxin are protected from auto-inhibition by expressing CdiI immunity proteins. Several bacteria have been shown to encode different CDI systems with up to 10 genetically distinct CDI gene clusters identified in *Burkholderia pseudomallei* (52). In addition, bacterial Rhs/YD repeat proteins have been shown to encode toxin/immunity protein pairs, which play a role in contact-dependent growth inhibition (53, 54). Similar to CdiA proteins, Rhs proteins have a conserved N-terminal domain and variable C-terminal domain (Rhs-CT) (55). This variable Rhs-CT domain represents the functional part of the toxin. Bacteria expressing Rhs-CT also express the cognate immunity protein (Rhs-I) from the *rhsI* gene, which is located immediately downstream of the *rhsD* gene (53). Despite the close similarity between the Rhs and CDI modules, the secretion mechanism of Rhs-CT is unknown. The proximity of the Rhs element to VgrG proteins has led to the speculation that Rhs-CT toxins may be secreted by the type VI secretion system (T6SS) (53).

The type VI secretion system (T6SS) is evolutionarily related to contractile phage particles and is made up of 15-23 different proteins, which assemble to form an injectisome structure (56, 57). Similar to the T3SS, the T6SS delivers effectors in a single step, from the cytoplasm to either the exterior of the cell or directly into bacterial and eukaryotic host cells. The T6SS has been associated with several processes related to fitness, virulence and inter-bacterial competition in different bacteria (28, 29, 58). Despite the widespread distribution of the T6SS in different bacteria, the role of this secretion system remains largely unknown. This review will focus on the structure, function and regulation of this secretion system and its possible role in plant-associated bacteria.

1.3 The type VI secretion system

1.3.1 Discovery of the type VI secretion system (T6SS)

The concept of a previously undescribed T6SS existing in bacteria arose from different lines of research related to the symbiotic relationship between nitrogen-fixing bacteria and leguminous plants. This interaction is usually specific and restricted to certain plant cultivars (59), e.g., *Rhizobium fredii* strain USDA275 nodulates cowpea, and several soybean genotypes but not the McCall cultivar, while *R. leguminosarum* bv. *trifolii* nodulates most clovers except cv. Woogenellup (60, 61,

62). Transposon mutants of *R. fredii* were identified with improved nodulation to include McCall and other soybean cultivars (61). The locus responsible for impairment in nodulation (*imp*) in *R. trifolii* was mapped to an 11kb chromosomal region (63). The homologous *imp* locus in *R. leguminosarum* strain RBL5523 was sequenced and the genes designated *imp*A-N (64). Blast analysis showed that *impL* and *impK* were highly similar to *icmF* and *dotU*, respectively (64). In *Legionella pneumophilia*, *icmF* and *dotU* genes are located next to the *icm/dot* genes that encode the type IVb secretion system (Figure 1.3) (65, 66). The *icmF* and *dotU* genes of *L. pneumophila* have been functionally characterized and shown to provide support to the T4bSS (67). This finding led the authors to investigate the possible association of the *imp* locus with protein secretion. Interestingly, the authors showed that the *imp* locus was responsible for the secretion of an approximately 27kDa protein. This protein was similar to a ribose-binding protein (RbsB) of *E. coli* and contained a cleavable amino terminal Sec signal sequence (64).

Continued interest in the *dot/icm* genes of *L. pneumophila* led to the identification of an *icmF*-like gene cluster in 17 sequenced bacterial genomes (68). Unlike *L. pneumophilia*, homologs of *icmF* and *dotU* genes found in other bacteria were not associated with the T4SS but were linked to a unique cluster called the IcmF-associated homologous protein cluster (IAHP). This IAHP cluster contained genes encoding several hypothetical proteins of unknown function. In 2006, Pukatzki *et al.* characterized the IAHP cluster in *V. cholera* 037 and showed that this cluster was responsible for contact-dependent killing of amoeba cells (28). This IAHP or VAS (virulence-associated secretion) cluster was also shown to secrete Hcp, VgrG-1, VgrG-2 and VgrG-3 without a cleavable N-terminal Sec signal sequence (28). The authors proposed the name - type VI secretion system (T6SS) - to represent this unique system.

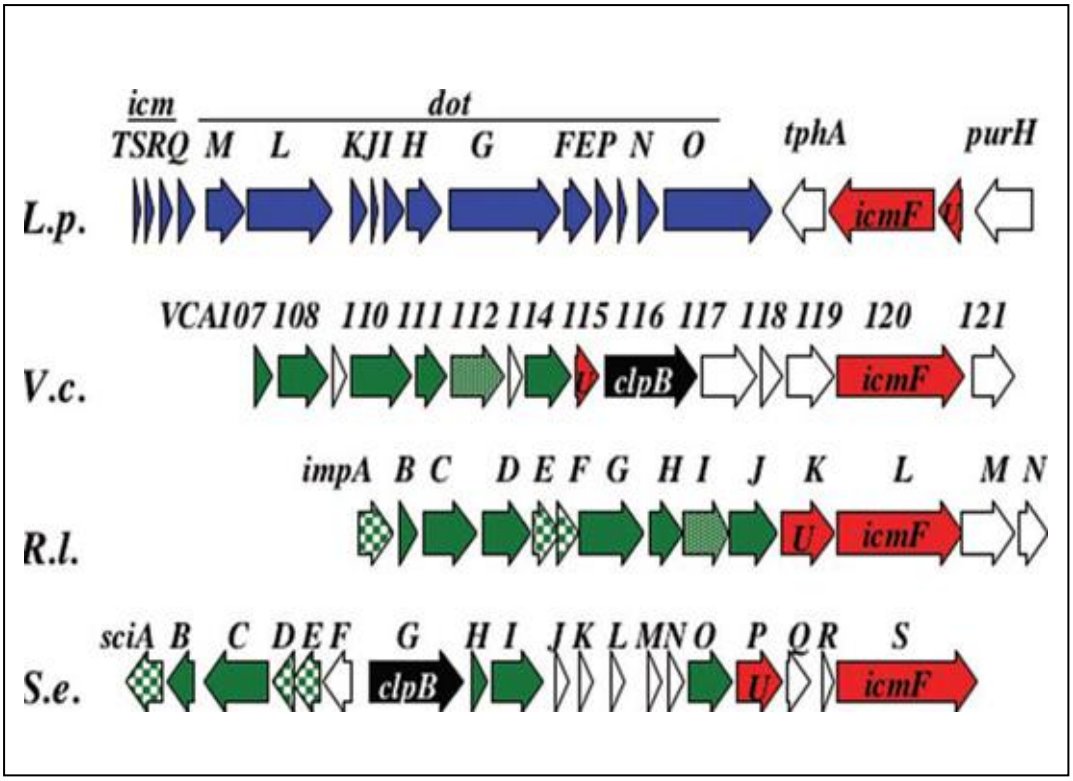

Figure 1.3: Genetic organization of the IcmF-associated homologous protein cluster. The *dot/icm* genes of *Legionella pneumophila* (L.p.) encode the type VIb secretion system. The IcmF-like cluster of *Vibrio cholerae* (V.c.), *Rhizobium leguminosarum* (R.l.) and *Salmonella enterica* (S.e.). Arrows with the same colour and shading represents conserved genes encoded by different bacteria. The figure was modified from Sexton *et al.* (2004) (67).

Genome mining identified homologous T6SS gene clusters in the genomes of several pathogenic and non-pathogenic bacteria mostly belonging to the family *Proteobacteriaceae* (69, 70, 71, 72, 73, 74, 75, 76). *In silico* analysis revealed that a single bacterial genome can contain more than one T6SS gene cluster, with up to six identified in *Yersinia pestis* and *Burkholderia pseudomallei* (69, 70). Phylogenetic analysis using conserved T6SS genes showed that bacterial T6SS gene clusters can be clustered into distinct groups, namely Group A to D (Figure 1.4) with the *Francisella tularensis* T6SS forming an outgroup (69). However, these phylogenetic groups cannot delineate the T6SS gene clusters from pathogenic and non-pathogenic bacteria, human, plant pathogens, symbionts or the ecological niche of the bacterial species.

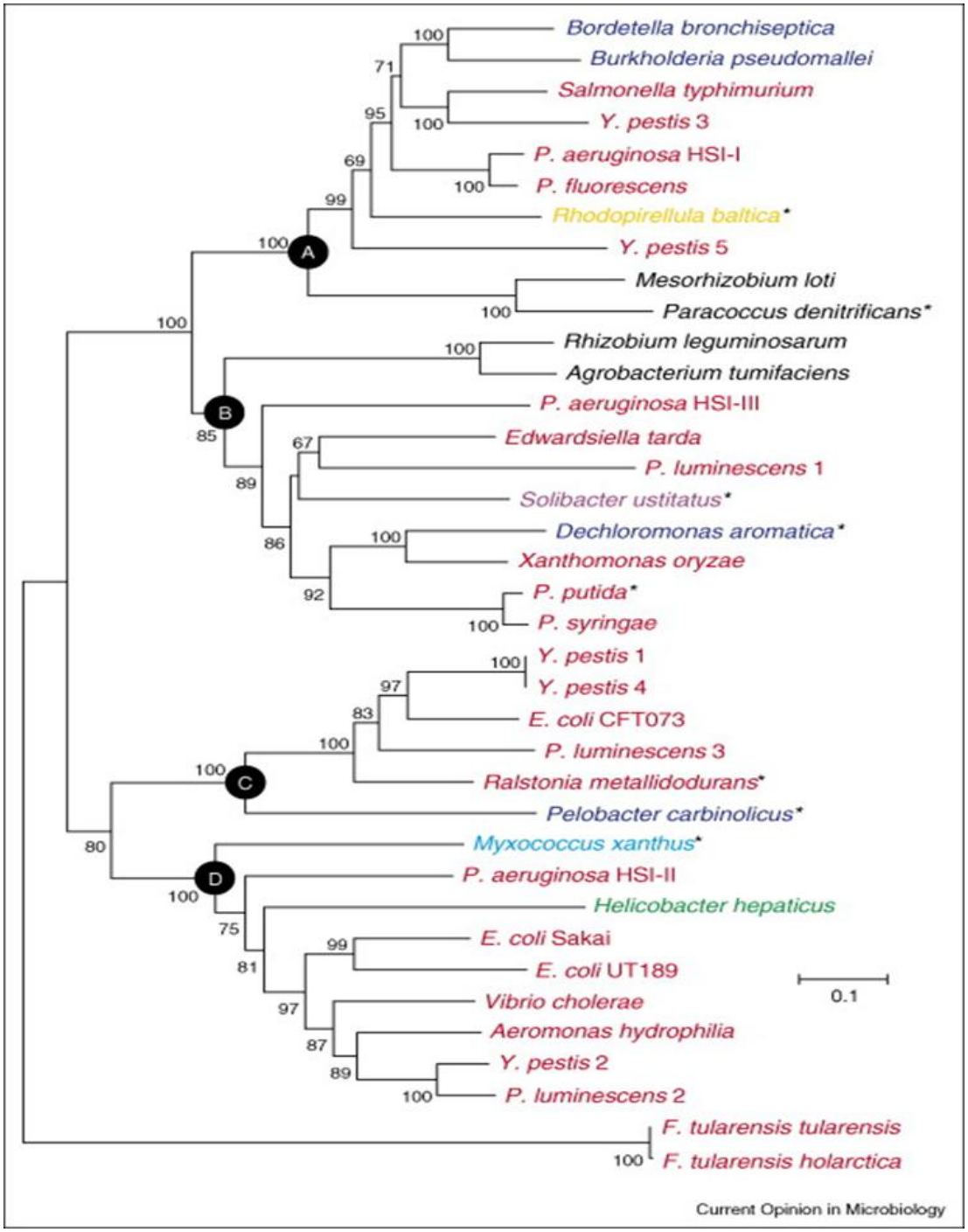

Figure 1.4: Evolutionary relationships of bacterial type VI secretion systems. Tree based on concatenated protein motifs DUF770 and DUF877. Bacterial taxons are indicated by font colour: Alphaproteobacteria, black; Betaproteobacteria, blue Gammaproteobacteria, red; Deltaproteobacteria, turquoise; Epsilonproteobacteria, light green; Acidobacteria, purple; Planctomycetales, yellow. Asterisk indicates species that are not considered to be pathogens or symbionts. The scale bar indicates 0.1 substitutions per site. Figure from Bingle *et al.* (2008) (69).

The identification of T6SS gene clusters in several different bacterial species has led to problems related to T6SS gene nomenclature. For example the T6SS *icmF* gene homolog is also known as *vasK* (*V. cholerae*), *evpO (E. tarda)* or *impL* (*R. leguminosarum*). In a bid to resolve this problem Shalom *et al.* (2007) proposed that the conserved core T6SS genes be named *tss* (type six secretion) while the accessory genes be called *tag* (*tss*-associated genes) (Table1.1) (78).

Table 1.1: Standardized nomenclature for the type VI secretion system genes

T6SS genes	*Rl*	*V. cholera*	*E. tarda*	Domain or putative function of the protein
tssA	*impA*	*vasJ/L*	*evpK*	
tssB	*impB*		*evpA*	Similar to bacteriophage T4 tail sheath protein
tssC	*impC/D*		*evpB*	Similar to bacteriophage T4 tail sheath protein
tssD (*hcp*)		*vasD*	*evpC*	Secreted/ homolog of bacteriophage T4 tail tube
tssE			*evpE*	
tssF		*vasA*	*evpF*	
tssG	*impH*	*vasB*	*evpG*	
tssH (*clpV*)		*vasG*	*evpG*	AAA+ ATPase
tssI (*vgrG*)		*vgrG*	*evpI*	Secreted/ homolog of bacteriophage T4 tail spike
tssJ		*vasD*	*evpL*	Outer membrane lipoprotein
tssK	*impJ*	*vase*	*evpM*	
tssL (*dotU*)	*impK*	*vasF*	*evpN*	Anchor T6SS to the cell wall (DotU homolog)
tssM (*icmF*)	*impL*	*vasK*	*evpO*	Anchor the T6SS to the cell wall (IcmF homolog)
tagA				
tagB				Pentapeptide repeat protein; unknown function
tagAB				TagA-TagB chimaeric protein
tagC				
tagD				
tagE				Ser/Thr kinase; post translational regulation
tagF				SciT domain protein
tagG	*impM*			Ser/Thr phosphatase; post translational regulation
tagH	*impI*	*vasC*		FHA; post translation regulation of the T6SS
tagI				Sec-dependent OM lipoprotein
tagJ	*impE*			
tagK				
tagL				IM protein with MotB/OmpA motif
tagM				OM lipoprotein; contains TPR domain
tagN				Periplasmic protein with MotB/OmpA motif
tagO		*vasI*		
tagP				IM protein; IcmF-MotB chimaera

The type VI secretion gene nomenclature proposed by Shalom *et al*. (2007) (78). *Tss* (type six secretion) = conserved T6SS genes while *tag* (*tss* associated genes) = non-conserved genes found in the T6SS gene clusters of limited bacterial species. IM (inner membrane), OM (outer membrane), Rl (*Rhizobium leguminosarium*), *V. cholerae* (*Vibrio cholerae)*, *E. tarda* (*Edwardsiella tarda*). Ser/Thr (Serine/threonine).

1.3.2 Structure of the T6SS

Systematic mutagenesis of the *evp* gene cluster (T6SS) of *Edwardsiella tarda* showed that 15 genes were required for the secretion of TssD (79). *In silico* analysis showed that 13 of these genes were conserved in the T6SS gene cluster of several different bacteria. These 13 genes are often referred to as "core" type VI secretion system genes and are believed to represent the minimal set of genes needed for biosynthesis of a functional type VI secretion system (69, 70). The core genes are interspersed by a variable number of non-conserved genes encoding mostly hypothetical proteins.

One of the first T6SS genes to be characterized was *tssD*, which encodes a ~17kDa TssD protein (Hcp). Electron microscopy and X-ray crystalographic analysis showed that TssD of *Pseudomonas aeruginosa* formed hexameric ring-shaped structures with an inner diameter of 4 nm (Figure 1.5A) (58). Ballister *et al*. (2007) later demonstrated that the hexameric TssD monomers polymerize *in vitro* to form tube-like structures up to 100 nm in length (Figure 1.5B) (80). In this regard, TssD is considered evolutionary related to the Gp19 protein of T4 bacteriophages, which polymerizes to form the phage tube (81).

Another hallmark T6SS gene is *tssI* that encodes the TssI (VgrG) protein. All characterized TssI proteins have a conserved N-terminal domain, an internal Gp5-like domain and a variable C-terminal domain (Figure 1.5C) (82). This domain architecture is highly similar to the trimeric Gp27-Gp5 tail spike complex of T4 bacteriophage (83). In contractile bacteriophages, the tail spike complex assembles on the tip of the phage tail tube and is required to hydrolyse the cell wall of bacterial cells (83). It is hypothesized that TssI proteins assemble on the tip of polymerized TssD rings to form the piercing structure (82). In addition to being essential structural components, TssD and TssI proteins are also effector proteins that are actively secreted by the T6SS of most bacteria (28, 58, 82).

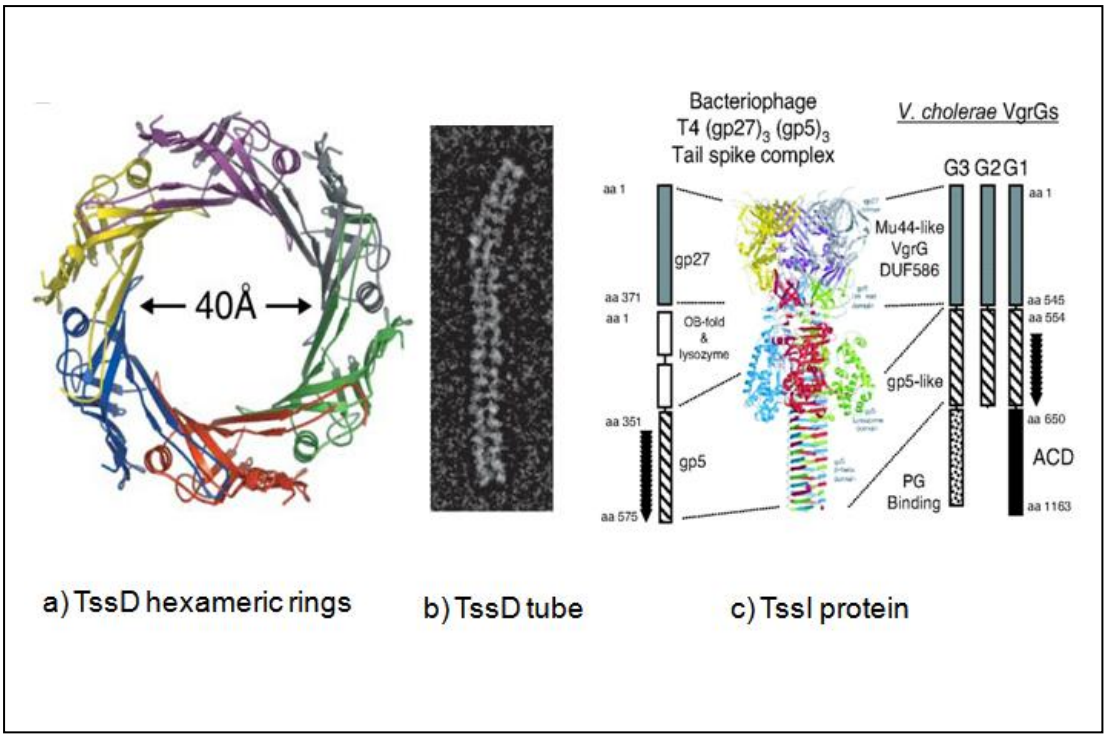

Figure 1.5: Schematic representation of TssD and TssI proteins. (A) Top view of a ribbon representation of the crystallographic TssD hexamer; (B) Electron micrographs of TssD nanotube prepared *in vitro* from completely reduced subunits; (C) Structural relationship between TssI proteins and T4 spike complex. PG = peptidoglycan, ACD = actin cross-linking domain. The images were obtained from: A) = Mougous *et al*., 2006 (58), B) = Ballister *et al*., 2007 (80) and C) from Pukatzki *et al*., 2007 (82).

The T6SS genes *tssB* and *tssC* encode the cytoplasmic proteins TssB and TssC, respectively. TssB and TssC proteins interact with each other to form a cogwheel tubular structure, up to 500 nm in lenth with an internal diameter of 100 Å (84). This structure is analogous to the bacteriophage tail sheath, which is composed of Gp18 proteins arranged in 23 hexameric rings around the tail tube (85). Using a combination of time-lapse microscopy and electron cryotomographic imaging, Basler *et al*. (2012) observed the contracted and relaxed conformations of the TssB/C cogwheel structures (86). TssB/C proteins are believed to polymerize and surround the TssD tube. Analogous to the bacteriophage tail sheath, contraction of the TssB/TssC sheath leads to propulsion of the TssD/TssI complex out of the cell (Figure 1.6) (57).

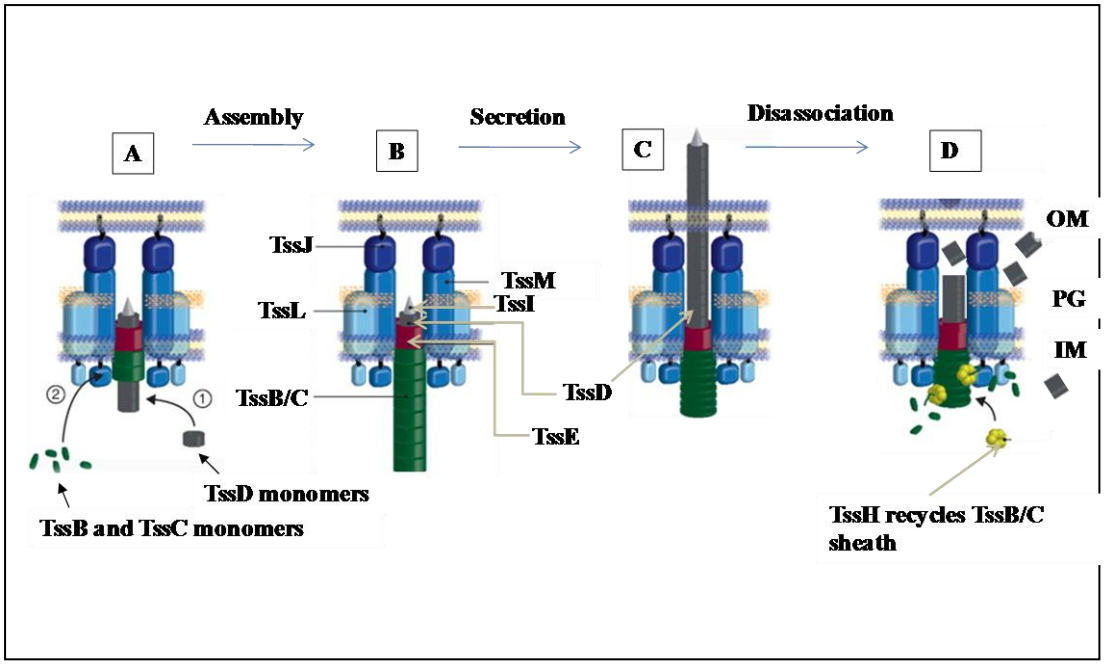

Figure 1.6: Schematic representation of assembly, secretion and dissociation of the T6SS. (A) Assembly of the T6SS; (B) Fully assembled T6SS; (C) Contraction of TssB/C sheath proteins and ejection of TssD/TssI out of the cell; (D) Relaxation of TssB/C sheath proteins leads to retraction the TssD/TssI complex into the cell followed by disassembly of the T6SS (Modified from Kapitein and Mogk, 2013) (87).

TssH encodes TssH, which is an ATPase that belongs to a family of functionally diverse proteins called AAA^+ (ATPases associated with diverse cellular activities) (88, 89). Proteins within this family have been extensively studied because of their role in protein quality control. For example, ClpA, ClpC and ClpX recognize, unfold and translocate proteins to the ClpP peptidase for degradation, while ClpB cooperates with the DnaK chaperone systems to disaggregate and refold large protein aggregates allowing bacteria to survive extreme temperatures (88, 90). TssH has a domain organization similar to ClpB consisting of an N-terminal domain, two AAA^+ domains (ATP binding and hydrolysis or Walker A/B motifs), which are separated by a middle domain (M-domain) (Figure 1.7) (84, 91). Substrate binding specificity of AAA^+ proteins is determined by the N-terminal domain and cognate adaptor proteins, which bind different substrates under different physiological conditions (92).

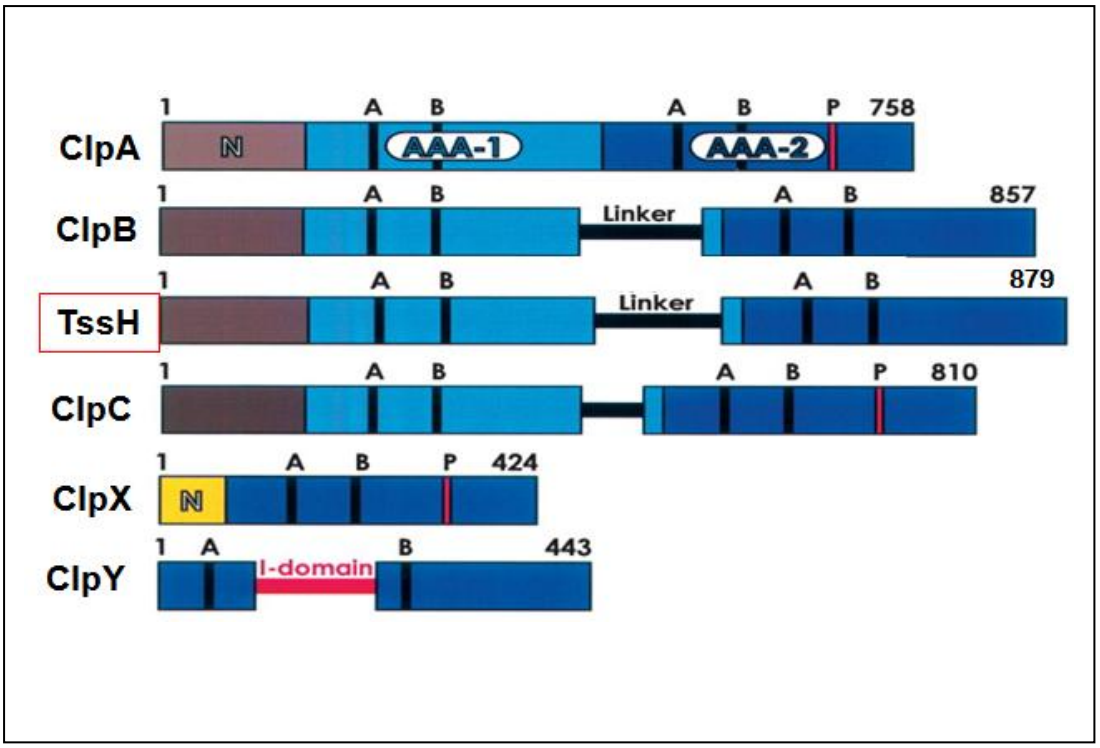

Fig 1.7: Schematic representation of Clp AAA$^+$ proteins. N = N-terminal domain associated with substrate binding specificity; A and B represent Walker A/B motifs found within the ATPase domain (AAA) region; P = ClpP recognition motif ([LIV]-G-[FL]). Figure modified from Dougan *et al.*(2002) (92).

TssH is different from other Clp AAA$^+$ proteins in three important aspects; 1) TssH does not play a role in thermo-tolerance and fails to solubilize aggregated proteins *in vitro*, 2) TssH lacks the P motif required for recognition and interaction with peptidase ClpP and 3) TssH is the only Clp AAA$^+$ protein that has been associated with protein secretion (93). Genes encoding different ATPases have been associated with all secretion systems, suggesting a conserved requirement of these proteins in protein secretion (94). In *Salmonella enterica*, the type III secretion system ATPase (InvC) plays a dual role in a) releasing chaperones from chaperone-effector protein complexes and b) unfolds effectors destined for secretion by the T3SS (95). However, TssH does not interact with known T6SS effectors like TssD and TssI but instead interacts with TssB, a constituent of the T6SS sheath protein (84). This interaction is mediated by the N-terminal of TssH that binds the α-helix of TssB proteins in the

contracted cogwheel structure, leading to depolymerization of the TssB/C sheath (84, 91). The role of TssH therefore seems to be the recycling of the TssB/C sheath following contraction of the T6SS (96).

The *tssE* gene encodes TssE, which is a 15kDa protein that belongs to the PF04965 family (GPW_gp25) (57). This family also includes the T4 bacteriophage Gp25 protein, which is an important structural component of the phage baseplate (79). In T4 bacteriophages, this protein is responsible for connecting the baseplate wedges thereby providing support to the baseplate (79). It is hypothesized that the Gp25 orthologs associated with the T6SS could play a role in assembly and stability of the T6SS baseplate structure (56, 57).

Further stability of the T6SS is provided by the gene products of *tssL*, *tssM* and *tssJ*, which encode the membrane proteins TssL and TssM, and the lipoprotein TssJ, respectively (98, 99, 100). TssL and TssM are inner membrane proteins with coiled-coil structures similar to IcmH (DotU) and IcmF, respectively. In *Legionella pneumophila,* IcmF and DotU proteins provide support to the type IVb secretion system (66, 67). In the T6SS, TssL and TssM are believed to anchor the T6SS to the cell wall using one or more of their transmembrane helices or the peptidoglycan binding domain found in the C-terminal of most TssL homologs (99). TssJ is a lipoprotein anchored to the outer membrane and exposed to the periplasm (100). This lipoprotein contains a protruding L1-2 loop, which interacts with the periplasmic domain of TssM and deletion of this loop abrogates TssD secretion (101). Multiple alignment of TssJ homologs from different bacteria revealed that the L1-2 loop is not conserved. The variable L1-2 loop has been suggested to determine the specificity of interaction between the cognate TssJ and TssM proteins when multiple T6SS gene clusters are encoded in a single bacterial (101).

The TssM homologs associated with the T6SS of some bacterial species such as *E. tarda*, *V. cholerae*, *Y. pestis* and *Salmonella* Typhimurium contain an N-terminal Walker A/B motif whose role in ATP binding and hydrolysis is controversial (79, 102). Systematic deletion of conserved amino acid residues within the Walker A domain in the *E. tarda* TssM protein did not affect secretion of the T6SS effector proteins, TssD and TssI (79). However, similar experiments involving deletion or substitution of the Walker A motif in the TssM homologs of *A. tumefaciens* abrogated

TssD secretion (102). In addition, the TssM Walker A/B domains of *A. tumefaciens* have been shown to bind and hydrolyse ATP (103). Co-precipitation assays showed that TssD interacts with the inner membrane protein, TssL. It is speculated that the ATPase activity of TssM is required to mobilize TssD to the TssM-TssL complex (103).

1.3.3 Role of the T6SS

The T6SS has been extensively studied in the context of host-pathogen interactions. Pukatzki *et al.* (2006) first demonstrated a link between the T6SS of *V. cholerae* and virulence where the VAS cluster (T6SS) was associated with a contact-dependent killing of amoebae and macrophages (29). Since then, the T6SS has been shown to be a virulence determinant of several different pathogenic bacteria such as *Edwardsiella tarda, Aeromonas hydrophila, Burkholderia mallei, Pseudomonas aeruginosa, Salmonella enterica* Typhimurium and the plant pathogen *Pectobacterium atrosepticum* (79, 104, 104, 105, 106, 107, 108) (Table 2). However, for some pathogenic bacteria, the contribution of the T6SS to virulence remains largely unknown (109). For example, experimental evidence demonstrated that T6SS mutants of enteroaggregative *E. coli,* uropathogenic *E. coli, Yersinia pestis, Vibrio anguillarum* and *Pseudomonas syringae* produced disease symptoms similar to the wild-type strains, when inoculated into susceptible hosts (73, 100, 110, 111, 112, 113, 114). These findings suggest that the T6SS of some pathogenic bacteria could play other roles not related to virulence (69). This is demonstrated in *V. anguillarum,* a fish pathogen, which uses its T6SS to modulate quorum sensing and the stress response by positively regulating RpoS and the quorum sensing regulator VanT (112). In addition, the T6SS of *Vibrio parahaemolyticus* and enteroaggregative *E. coli* have been implicated in biofilm formation (98, 100, 116). Confocal laser scanning electron microscopy revealed that *V. parahaemolyticus tssD* mutants were defective in submerged biofilm formation, a phenotype that was associated with single cell attachment and defects in later stages of biofilm development (116).

Table 1.2. T6SS gene clusters reported to have a role in virulence and/or interaction with eukaryotic cells

Organism	T6SS-dependent phenotype	References
Vibrio cholerae	Virulence towards amoeba and macrophages	29
R. leguminosarum	Nodulation of pea plants	64
Francisella tularensis	Virulence in mouse	74
Edwardsiella tarda	Virulence in fish	79
Aeromonas hydrophila	Virulence in mouse	104
B. mallei	Virulence in hamsters	105
Pseudomonas aeruginosa	Virulence in mouse	106
P. atrosepticum	Virulence in potato stems and tubers	108
Helicobacter hepaticus	Anti-inflammatory response	120
B. thailandensis	Virulence in mouse	124
Avian *Escherichia coli*	Virulence in chicks	127
B. cenocepacia	Resistance to amoeba predation	128
B. pseudomallei	Virulence in hamsters	129
Campylobacter jejuni	Colonization of mouse	130
Escherichia coli K1	Invasion of human brain endothelial cells	131
SE serovar Gallinarum	Colonization of chicken organs	132
SE serovar Typhimurium	Virulence and intracellular replication in mouse	133
Vibrio parahaemolyticus	Adhesion to cultured cell monolayers	134

The above table shows the contribution of the T6SS in virulence and interaction with different eukaryotic host models. *P = Pectobacterium*; *R = Rhizobium*; *SE = Salmonella enterica*; *B = Burkholderia*. Table was modified from Coulthurst (2013) (115).

The T6SS of some bacteria has been shown to limit disease development, thereby favouring a symbiotic or mutualistic interaction with the host (116). This is exemplified in *Rhizobium leguminosarum*, which forms nitrogen-fixing nodules in leguminous plants belonging to the clover group (60, 61, 62). *R. leguminosarum* mutants with a transposon insertion in the *imp* gene cluster (T6SS) were identified that could nodulate pea plants (61, 63). This finding suggested that the T6SS of *R.*

leguminosarum releases effector molecules that limit symbiosis and nodulation of specific legumes. Another example involves the enteric pathogen *Salmonella enterica* serovar Typhimurium, the causal agent of gastroenteritis in humans. This pathogen is disseminated in macrophages and eventually kills the macrophages 18 hours post-infection (118). The *S.* Typhimurium *tssM* mutant was identified that grew to high cell numbers and persisted in macrophages 24 hours post-infection without causing host cell lysis (119). This *tssM* mutant was hyper-virulent, showing an eight-fold lethality on infected mice compared to the wild-type strain. In addition, transcriptome analysis of the *Helicobacter hepaticus* Δ*tssM* mutant revealed elevated levels of proinflammatory cytokines such as interleukin-17, -21, -23, tumor necrosis factor-α and nitric oxide synthase (120). In addition, *H. hepaticus* Δ*tssM* and Δ*tssD* mutants displayed increased attachment, internalization and colonization of mouse intestinal epithelial cells compared to the wild-type strain (120). These findings suggest that *H. hepaticus* uses the T6SS to limit intestinal colonization and inflammatory responses, thereby maintaining a non-pathogenic relationship with the host.

Recently, the T6SS has been shown to play a role in inter-bacterial competition. This was first observed in a secretome analysis of the Δ*tagG* and Δ*tssH* mutants of *P. aeruginosa* H1-T6SS (29). This study demonstrated that the H1-T6SS of *P. aeruginosa* actively secreted TssI, TssD and three previously uncharacterized proteins, Tse 1-3 (type six exported 1-3) (29). Tse2 is a toxin, while Tse1 and Tse3 are lytic antimicrobial enzymes (121). Together, these effectors cause growth stasis (Tse2) and peptidoglycan degradation (Tse1 and Tse3) in recipient cells, thus giving *P. aeruginosa* a fitness advantage when co-cultured with strains missing the immunity proteins Tsi1-3 (type six immunity 1-3) (29, 121). This fitness advantage requires a functional T6SS and direct cell-to-cell interaction. Effector-producing strains of *P. aeruginosa* are protected from auto-inhibition by expressing the cognate Tsi immunity proteins (121). The contribution of the T6SS to inter-bacterial competition has also been reported in *P. syringae* DC3000 (114) *V. cholerae* (122), *Serratia marcescens* (123), *B. thailandensis* (124), *Acinetobacter baumannii* (125) and *V. parahaemolyticus* (126).

1.3.4 Effectors released by the T6SS

V. cholera V52 secretes TssD (Hcp), VgrG1, VgrG2 and VgrG3 proteins into the culture supernatant (29). These proteins lack an N-terminal signal sequence and their secretion requires a functional T6SS (28). VgrG1 of *V. cholerae* contains a C-terminal extension with structural homology to the actin cross-linking domain of the RTX toxin (29, 82). The T6SS of *V. cholerae* secretes VgrG1 into eukaryotic cells where it cross-links actin, resulting in cytotoxicity and virulence against macrophages and amoeba cells (82). VgrG3 (VCA0123) contains a C-terminal peptidoglycan binding domain (29). This protein is a bactericidal effector that causes apoptosis of *E. coli* cells not expressing the cognate immunity protein TsiV3 (VCA0124) (126, 135). The TssD and VgrG2 proteins of *V. cholera* V52 contain no C-terminal extensions and their exact role as effectors is unknown. Like *V. cholerae*, the T6SS of different bacteria actively secrete TssD and/or TssI homologs with no C-terminal effector domains (73, 74, 102, 104, 105, 114, 122, 136). A subset of *tssI* genes with C-terminal effector domains have been identified in a limited number of bacterial genomes (82). Some of these domains include S-pyosin domain (*S. enterica*), tropomyosin-like domain (*Y. pestis*), zinc-metaloprotease domain (*P. aeruginosa*), fibronectin-like domain (*P. entomophila*) and pertactin-like domain (*Y. pestis*) (82). While the function of these proteins has been predicted solely on the basis of structural homology to known proteins, VgrG1 of *Aeromonas hydrophila* has been functionally characterized. This protein contains a C-terminal VIP2 (vegetative insecticidal protein) domain with actin ADP-ribosyltransferase activity (137). The T6SS of *A. hydrophila* actively secretes VgrG1 into eukaryotic cells, where it causes actin reorganization and apoptosis (137). VgrG proteins with additional C-terminal effector domains are now called “evolved” VgrG and constitute a subset of putative T6SS effectors (82).

In addition to TssD and VgrG1-3, the T6SS of *V. cholerae* strain V52 also secretes VasX (VCA0020) and TseL (VC1418) proteins with no N-terminal Sec signal sequences (135, 138). VasX and TseL are anti-bacterial effectors that provide a fitness advantage to *V. cholerae* when co-cultured with strains lacking the cognate immunity proteins TsiV1 (VC1419) and TsiV2 (VCA0021) (135). In addition to their role as

antibacterial effectors, TseL and VasX are anti-eukaryotic effectors required for killing amoeba, and deletion of *vasX* and/or *tseL* abolished this virulence (135, 138). Other T6SS effectors such as Tle1-5 (type VI lipase effectors 1-5) are lipase enzymes that hydrolyze bacterial membrane phospholipids, leading to increased cellular permeability and cell death (139). The T6SS of several different bacteria have been shown to secrete two broad families of cell wall degrading enzymes, namely type VI amidase 1-4 (Tae 1-4) and type VI secretion glycoside hydrolase effectors (Tge1-3) (140, 141). These proteins are bacteriocidal effectors that usually co-occur with the immunity proteins. Other T6SS effectors such as AaiC of enteroaggressive *Escherichia coli* and EvpP of *Edwardsiella tarda* showed limited homology to TssD and TssI, and their exact function is unknown (73, 79).

Mass spectrophotometry has been used to identify putative T6SS effectors of *B. thailandensis* and *S. marcescens* (140, 142). *B. thailandensis* T6SS-1 secretes 11 effectors, which include TssI homologs, hypothetical proteins, a filamentous hemagglutinin (BTH_I1830), a putative bacteriocin LlpA (BTH_II0310), a putative colicin Ia (BTH_I2691) and the Tae2 peptidoglycan amidase (BTH_I0068) (140). With the exception of Tae2 these effectors have not been functionally characterized, and it remains to be determined whether or not they represent true substrates that are secreted by T6SS-1 of *B. thailandensis*. Similarly, the T6SS of *Serratia marcescens* DB10 secretes 14 putative substrates (142). Two of these proteins, Ssp1 and Ssp2 (Small secreted protein), were previously shown to be antimicrobial effectors that caused growth stasis in the absence of Rap1a and Rap2a immunity proteins (Resistance associated protein) (143). Other putative substrates secreted by the T6SS of *Serratia marcescens* include three TssD homologs, two TssI homologs, TssH (SMA2274), Rhs family protein (SMA2278), Penicillin-binding protein 2 (SMA0444) and four Small secreted proteins (Ssp3 to Ssp6). Ssp3-6 were shown to be anti-bacterial effectors associated with *S. marcescens* intra-strain competition, while the remaining effectors still need to be characterized (142).

1.3.5 Induction and regulation of the T6SS

The observation that *V. cholerae* N16961 does not secrete detectable TssD proteins into the culture supernatant while the T6SS of *V. cholerae* V52 and *S. marcescens* DB10 are constitutively expressed, suggests different *in vitro* expression and *in vivo*

induction conditions (29, 122, 143). The T6SS of several bacteria is induced by host and plant extracts, necrotic roots and copper (78, 135, 144, 145, 146, 147). The mechanism of activation by these compounds is unclear. It is also unclear whether or not these compounds activate the T6SS directly or indirectly through modulation of other transcriptional regulators. Transcriptional regulation of the T6SS by the ferric uptake response regulator (Fur) and the alternate sigma factor sigma54 encoded by *rpoN* has been documented in a few bacteria (148, 149, 150).

Quorum sensing regulates the T6SS gene clusters of several different bacteria (139, 149, 150). In *Pectobacterium atrosepticum*, the diffusible N-(3-oxohexanol)-L-homoserine lactone signal molecule is encoded by *expI* (108). Whole-genome microarray analysis of an *expI* mutant of *P. atrosepticum* revealed that 11 of the 18 T6SS gene homologs were down-regulated compared to the wild-type (108). These results showed for the first time in any pathogen that the T6SS is regulated by quorum sensing. In *Aeromonas hydrophila* SSU, the quorum sensing mutant Δ*ahyRI* lost the ability to secrete both the TssD and TssI proteins into the culture supernatant (151). Secretion of these proteins was restored in the Δ*ahyRI* mutant by trans complementation of *ahyRI* or addition of exogenous C4-homoserine lactone (151).

In *P. aeruginosa*, another level of control of the T6SS is via the sensor kinases, RetS /LadS (152). These sensor kinases are usually associated with the GacS/GacA two-component sensor kinase system with RetS activating and LadS repressing the GacS/GacA system (58, 152, 153). Post-translational regulation of the T6SS-1 of *P. aeruginosa* is mediated by the concerted action of proteins encoded by *tagR*, *pknA* (serine/threonine kinase), *pppA* (serine/threonine phosphatase) and *tagH* (which encodes TagH) (154, 155). The signalling cascade begins with the perception of unknown environmental cues by the periplasmic TagR protein. This signal is then transmitted to PknA, which autophorylates its threonine residue. Phosphorylated PknA protein transfers a phosphate group from its threonine residue to the cytoplasmic TagH protein. Phosphorylated TagH then interacts with TssH and the TagH-TssH complex is recruited to the base of the assembled T6SS, leading to activation of the T6SS (156). This phosphorelay activation of the T6SS is counteracted by PppA (serine/threonine phosphatase), which dephosphorylates TagH and PknA leading to inactivation and disassociation of the T6SS.

1.4 The T6SS in plant-associated bacteria

Genome mining has identified T6SS gene clusters in the genome of several plant-associated bacteria including plant epiphytes (*Pantoea vagans, Erwinia billingiae* and *Erwinia tasmaniensis*) and plant symbionts (*Rhizobium etli* strain CIAT652, *Bradyrhizobium japonicum*, *Mesorrhizobium loti* and *Cupriavidus taiwanensis*) (28, 157, 149). In addition, T6SS gene clusters have also been identified in several plant pathogens such *Pseudomonas syringae, Agrobacterium tumefaciens, P. atrosepticum* and other phytopathogens belonging to the genus *Pantoea*, *Xanthomonas, Erwinia*, *Pseudomonas* and *Dickeya* (28, 102, 108, 113, 157, 158). Variable numbers of T6SS gene clusters have been identified in some but not all plant-associated bacteria. For example, *Xanthomonas campestris* pv. *vesicatoria*, which infects pepper and tomato, contains two genetically distinct T6SS gene clusters that are absent from all three sequenced strains of *X. campestris* pv. *campestris*, the etiological agent of black rot disease of brassicas (157). However, the exact role of the T6SS in most plant-associated bacteria remains unknown since detailed functional characterization has been limited to *Pectobacterium atrosepticum*, *Pseudomonas syringae* and *Agrobacterium tumefaciens* (108, 113, 102).

Pseudomonas syringae pv. *syringae* B728a encodes a functional T6SS that secretes TssD into the culture supernatant. However, the contribution of this secretion system to virulence is unknown (113). Functional studies showed that the Δ*tssH* mutant of strain B728a produced biofilms and colonized leaf surfaces similar to the wild-type strain (113). This Δ*tssH* mutant also multiplied *in planta* and produced disease symptoms on bean plants similar to the wild-type (113). Similar results were obtained using the tomato pathogen *Ps.* pv. *tomato* DC3000 (114). This pathovar encodes two T6SS, called Hcp secretion islands I and II (HSI-I and HSI-II). Deletion of *tssD*1 (*hcp*1) and *tssD*2 (*hcp*2) did not affect virulence and colonization efficiency of the T6SS mutants on tomato and *Arabidopsis* plants (114). Inter-bacterial competition assays showed that wild-type DC3000 was able to inhibit growth of *E. coli*, *Proteus vulgaris*, *Pectobacterium carotovorum* subsp. *carotovorum* (SCC1) and *Pectobacterium wasabiae* (SCC3193). These results, for the first time, showed that the T6SS of a phytopathogen played a role in inter-bacterial competition.

Pectobacterium atrosepticum is a phytopathogen that causes soft rot of potato tubers and blackleg disease on potato stems. The genome of *P. atrosepticum* encodes a single T6SS, which is induced by potato extracts and regulated by quorum sensing (108, 136). Secretome analysis confirmed secretion of a TssI (ECA3427) and four TssD (Hcp) proteins encoded by ECA4275 (Hcp-1), ECA3428 (Hcp-2), ECA2866 (Hcp-3) and ECA0456 (Hcp-4) (136). Deletion of ECA4275 (*hcp*-1), ECA3438 (*tssK*) or ECA3444 (*tssC*) resulted in reduced virulence in potato tubers, while over-expression of Hcp1 led to increased virulence (108, 136). Interestingly, deletion of *tssM* led to hyper-virulence in potato tubers (145). This Δ*tssM* mutant also grew to a higher cell density and produced more pectolytic enzymes in LB and minimal media compared to the wild-type strain (145). The significance of these finding are currently unknown.

Agrobacterium tumefaciens is the causal agent of crown gall in several different plants. This pathogen is known to use the VirB/VirD4 T4SS to transfer oncogenic DNA and effector proteins from the bacteria into host plant cells (156, 160, 161). The T6SS of *A. tumefaciens* is able to secrete TssD (Atu4345) into the culture supernatant and secretion of TssD is dependent on an intact T6SS (162). Deletion of the *A. tumefaciens tssD* gene led to reduced tumorogenic efficiency of the Δ*tssD* mutant compared to the wild-type, while deletion of the entire T6SS or *tssM* (Atu4332) did not produce similar results (162). These finding led the authors to conclude that a) the T6SS of *A. tumefaciens* is not absolutely required for virulence and b) intracellular TssD, and not secreted TssD, could have enhanced the tumerogenic efficiency of the wild-type strain (162).

1.5 The T6SS of *Pantoea ananatis* strain LMG 20103

P. ananatis strain LMG 20103 was initially isolated from *Eucalyptus grandis* seedlings and displayed typical symptoms of blight and dieback (3). Genome mining identified three genetically distinct T6SS gene clusters in LMG 20103 (28). These clusters were designated T6SS-1, T6SS-2 and T6SS-3. T6SS-1 corresponds to a region of 35.2 kb containing 26 genes, which are located between PANA_2348 to PANA_2373. Representatives of all 13 conversed T6SS genes were present in this cluster (28, 157). T6SS-2 corresponds to a 19 kb region found on the LPP-1 megaplasmid and contains 22 genes (PANA_4130-PANA_4153) (5, 28). This cluster

contains genes encoding all 13 core gene components of the T6SS. The third T6SS cluster, T6SS-3 corresponds to a region ~ 8.4 kb and contains 8 genes located between PANA_1650 to PANA_1657. Only 2 of the 13 core gene components of the T6SS (i.e. *icmF* and *dotU* homologs) were found to be present in the T6SS-3 gene cluster of *P. ananatis* strain LMG 20103 (28). The absence of these conserved gene components of the T6SS has led to the speculation that T6SS-3 of *P. ananatis* represents an incomplete and probably a non-functional T6SS (157).

Phylogenetic analysis revealed that the T6SS-1 and T6SS-2 gene clusters of *P. ananatis* strain LMG 20103 were phylogenetically close to the T6SS gene clusters of *Pantoea agglomerans* and *Pantoea* sp aB-*valens*, respectively, while the T6SS-3 gene cluster of *P. ananatis* strain LMG 20103 was phylogenetically closest to *Pantoea* sp. At-9B (28). A second analysis by Sarris *et al.* (2011), which included several important plant-associated bacteria showed that the T6SS-1 and T6SS-2 gene clusters of *P. ananatis* strain LMG 20103 were phylogenetically close to the T6SS gene clusters of *E. amylovora* (157). Taken together, these results suggest that the different T6SSs of *P. ananatis* strain LMG 20103 (T6SS1-3) could play different roles related to ecological fitness or virulence of this species. However, none of the T6SS gene clusters of *P. ananatis* LMG 20103 have been functionally characterized.

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CHAPTER TWO

Comparative genomics of type VI secretion systems in strains of *Pantoea ananatis* from different environments

2.1 Abstract

Background: The type VI secretion system (T6SS) has been identified in several different bacteria, including the plant pathogen *Pantoea ananatis*. Previous *in silico* analyses described three different T6SS loci present in the pathogenic strain of *P. ananatis* LMG 20103. This initial investigation has been extended to include an additional seven sequenced strains of *P. ananatis*, together with 39 strains from different ecological niches. Comparative and phylogenetic analyses were used to investigate the distribution, evolution, intra-strain variability and operon structure of the T6SS in the sequenced strains.

Results: Three different T6SS loci were identified in *P. ananatis* strain LMG 20103 and designated PA T6SS 1-3. PA T6SS-1 was present in all sequenced strains of *P. ananatis* and in all 39 additional strains examined in this study. In addition, PA T6SS-1 included all 13 core T6SS genes required for synthesis of a functional T6SS. The plasmid-borne PA T6SS-2 also included all 13 core T6SS genes, but was restricted to only 33% (15/46) of the strains examined. In addition, PA T6SS-2 was restricted to strains of *P. ananatis* isolated from symptomatic plant material. This finding raises the possibility of an association between PA T6SS-2 and either pathogenicity or host specificity. The third cluster PA T6SS-3 was present in all strains analyzed in this study but lacked 11 of the 13 core T6SS genes, suggesting it may not encode a functional T6SS. Inter-strain variability was also associated with *hcp* and *vgrG* islands, which are associated with the T6SS and encode a variable number of proteins of unknown function. These proteins may play a role in the fitness of different strains in a variety of ecological niches or as candidate T6SS effectors. Phylogenetic analysis indicated that PA T6SS-1 and PA T6SS-2 are evolutionarily distinct.

Conclusion: Our analysis indicates that the three T6SSs of *P. ananatis* appear to have been independently acquired, and may play different roles relating to pathogenicity, host range determination and/or niche adaptation. Future work will be directed toward understanding the roles that these T6SSs play in the biology of *P. ananatis*.

2.2 Background

Pantoea ananatis is a Gram-negative, motile, facultative anaerobe belonging to the gamma Proteobacteria. This bacterium can survive and multiply in a variety of ecological niches as a saprophyte, endophyte, epiphyte and pathogen (1). In its latter role, *P. ananatis* infects a wide range of economically important plants. In South Africa the pathogen causes disease on maize, onion and *Eucalyptus* spp. (2, 3, 4, 5), but has also been reported to infect pineapple (6), rice (7), melon (8), Sudangrass and sorghum (9, 10). The mechanism of spread of *P. ananatis* between host plants is largely unknown. However, Walcot *et al*. (2002) isolated virulent strains of *P. ananatis* from onion seeds, which went on to produce disease symptoms on susceptible onion plants (11). In addition, Gitaitis *et al*. (2003) demonstrated that tobacco thrips (*Frankliniella fusca*) were able to transmit *P. ananatis* into 52% of onion plants analyzed (12). These studies show that seeds and insect vectors are important sources of inoculation and could serve as vehicles for the spread of *P. ananatis* to different geographical regions. Current control measures are limited to cultivation of resistant plant cultivars, eradication of infected plant material and/or the use of biocontrol in the form of lytic phages (1, 13, 14). Despite the wide geographical spread and host range of *P. ananatis*, there is limited information on the genetic determinants of virulence and ecological fitness of the species.

To date, seven different secretion systems have been described in bacteria; namely type I-VII (15, 16). These secretion systems release factors that modulate the host environment to favour bacterial fitness and, in some cases, virulence. The type VI secretion system (T6SS) was first described by Pukatzki *et al*. (2006) in *Vibrio cholerae* and was shown to be required for virulence against amoeba and macrophages (17). This secretion system consists of 15-23 different proteins, which assemble to form an injectisome-like structure similar to an inverted contractile phage particle (18, 19, 20). The T6SS has since been identified in the genome of several pathogenic but also non-pathogenic Gram-negative bacteria, suggesting that it may be involved in functions unrelated to pathogenicity (21, 22, 23, 24). The role of the T6SS in virulence, symbiosis, biofilm formation and stress has been documented in several bacteria (25, 26, 27, 28, 29, 30). In addition, the T6SSs of *Pseudomonas aeruginosa*,

Vibrio cholerae, *Pseudomonas fluorescens*, *Pseudomonas protegens*, *Burkholderia thailandensis* and *Serratia marcescens* have been shown to secrete bactericidal effectors, which inhibit growth of bacterial species that lack the cognate immunity protein (31, 32, 33, 34, 35, 36, 37, 38, 39, 40). Similarly, the T6SSs of *Pseudomonas syringae* pv. *tomato* DC3000, *Acinetobacter baumannii*, *Acinetobacter baylyi*, *Vibrio parahaemolyticus* and *Citrobacter rodentium* have also been shown to play a role in inter-bacterial competition (41, 42, 43, 44). Some bacteria encode more than one evolutionarily distinct T6SS in their genome (23). Multiple T6SS gene clusters found in a given bacterial genome are believed to have been acquired by independent horizontal gene transfer events, possibly to play different roles in the biology of different bacteria (23, 24, 45).

This study focused on the T6SS of the pathogen *Pantoea ananatis*. To date, the genome sequences for eight strains of *P. ananatis* are available, representing both pathogenic and non-pathogenic strains. Type II, type III and type IV secretion systems are well documented virulence determinants of several human and plant pathogens (15, 16), although genome mining showed that they were all absent from the genome of *P. ananatis* strain LMG 20103 (1, 46). These findings raise the possibility that the T6SS of *P. ananatis* could play a role in either virulence or ecological fitness of the species. Previous comparative studies identified three distinct T6SS loci on the genome of a single *Eucalyptus* pathogenic *P. ananatis* strain LMG 20103 (46, 47, 48). Thus, to better understand the distribution of the T6SS in this versatile pathogen we carried out an in-depth comparative analysis of the T6SS in eight sequenced strains, of which three were recently sequenced as part of this study. To this end, we analyzed the gene content, sequence similarity, synteny, operon structure, and possible evolutionary history of each T6SS locus. PCR and dot blot hybridizations were used to study the distribution of the T6SS in 39 additional strains from a variety of ecological niches. This study represents the first detailed intra-species comparative analysis of the T6SS in *P. ananatis*.

2.3 Results and Discussion

2.3.1 *In silico* identification of T6SS gene clusters in *P. ananatis*

The 13 conserved gene components of the T6SS from *P. ananatis* strain LMG 20103 (48) were used as a bait to identify the T6SS in all sequenced strains of *P. ananatis.* BLASTN and BLASTP searches were done using all eight currently available genomes of *P. ananatis*, which included the LPP-1 megaplasmid found in all sequenced strains (49). In this chapter we have used the standard T6SS gene nomenclature proposed by Shalom *et al*. (2007). Based on this nomenclature, the conserved T6SS genes were designated *tss A-M* (Type Six Secretion A-M), while the accessory or non-conserved T6SS genes were designated *tag A-P* (Type Six Associated Genes A-P) (50).

Homologs of the T6SS genes were clustered in two distinct genomic regions in all sequenced strains of *P. ananatis*. These regions were designated PA T6SS-1 and PA T6SS-2 (*Pantoea ananatis* T6SS 1, 2). The PA T6SS-1 gene cluster was located on the genome of all eight sequenced strains of *P. ananatis*, while the PA T6SS-2 gene cluster was located on a ~30 kb region of the LPP-1 megaplasmid of strains AJ13355, LMG 20103 and PA-4. This 30 kb region was missing from the LPP-1 plasmid of *P. ananatis* strains LMG 2665^{T}, LMG 5342, BD442, B1-9 and PA-13. We also found that the PA T6SS-1 and PA T6SS-2 gene clusters contained all 13 core gene components of the T6SS (23, 24). In addition, we also identified a 9 kb region containing homologs of *icmF* and *dotU* in all eight sequenced strains of *P. ananatis*. This gene cluster was designated T6SS-3 and was found to be missing the remaining 11 core gene components of the T6SS. Whether or not the PA T6SS-3 gene cluster encodes a functional T6SS or represents a truncated T4SS is currently unknown. However, this putative PA T6SS-3 gene cluster was included in this study for purposes of comparative analysis. The overall genetic organization of each T6SS of *P. ananatis* is presented in Figure 2.1. The list of all core and accessory T6SS gene components found in the T6SS gene clusters of *P. ananatis*, including their putative functions and COG classification is presented in Table 2.1.

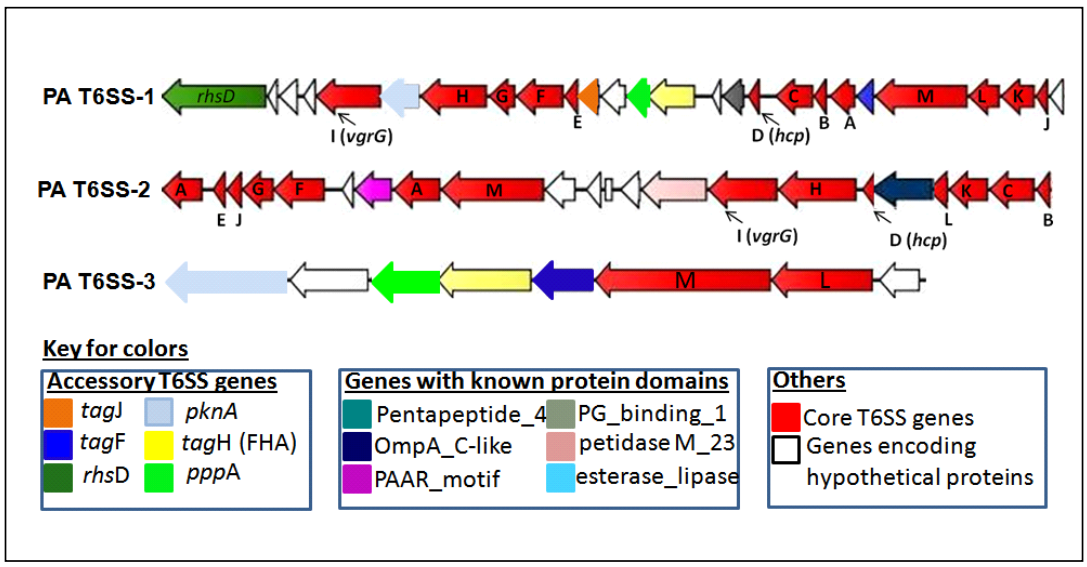

Figure 2.1: Genetic organization of the different type VI secretion gene clusters (T6SS) in *Pantoea ananatis* (PA). Genes are indicated by arrows and the direction of the arrows represents the direction of transcription of the gene related to the rest of the genome. We used the type VI secretion system gene nomenclature of Shalom *et al.* (2008). Conserved core gene components of the T6SS (*tssA-M*) are indicated in red, while non-conserved genes associated with the T6SS of limited bacteria (*tagA-P*) are indicated in different colors. The *tag* genes found in the T6SS gene clusters of other bacteria are indicated in the key. The figure is not drawn to scale, PA T6SS-1 and PA T6SS-2 are both over 30 kb and contain up to 20 genes each, while PA T6SS-3 (9 kb) contains eight genes in all sequenced strains.

Table 2.1. List of core and accessory proteins associated with the type VI secretion system gene clusters of *Pantoea ananatis*

Gene	Homologues	COG classification	Putative function
tssI	*vgrG*	COG3501	Effector/structure: Forms the T6SS spike
tssD	*Hcp*	COG3157	Effector/structure: Homologous to T4 phage tube
tssC	*impC, vipB*	COG3517	Homologous to T4 phage tail sheath proteins
tssB	*impB, vipA*	COG3516	Homologous to T4 phage sheath proteins
tssH	*clpV, vasG*	COG0542	ATPase /effector chaperon/recycling TssB/C
tssM	*vasK, icmF*	COG3523	Anchoring T6SS to cell wall
tssL	*ompA/dotU*	COG3455	Anchoring T6SS to cell wall
tssJ	*vasD, lip*	COG3521	Anchoring T6SS to cell wall
tssE	*impF,vasS*	COG3518	Homolog of phage Gp25 proteins
tssG	*impH, vasB*	COG3520	Unknown function
tssF	*impG, vasA*	COG3519	Unknown function
tssA	*impA/vasJ*	COG3515	Unknown function
tssK	*impJ, vase*	COG3522	Unknown function
tagB	*BB0796*	COG1357	Pentapeptide_4 protein, unknown function
tagAB	*BB0795*	COG1357	Pentapeptide_4 protein, unknown function
tagE	*pknA/ppkA*	COG0515	post-translational regulation
tagF	*impM, sciT*	COG3913	Unknown function
tagG	*pppA*	COG0631	post-translational regulation
tagH	*impI*	COG3456	FHA protein post-translational regulation
tagJ	*impE*	COG4455	Unknown function
	c3389	COG2885/COG1360	OmpA_C-like protein, unknown function
	VCA0105		PAAR protein associated with the VgrG spike
		COG3409	Protein with a peptidoglycan binding domain, putative effector
	L376_02862		Protein with a peptidase M_23 domain, putative endopeptidase effector
	Ebc_4130		Protein with an esterase_lipase domain, unknown function

Tss (type VI secretion) genes refers to the T6SS gene nomenclature proposed by Shalom *et al*. (2007). These genes have been shown as essential for secretion of at least two proteins, Hcp and VgrG, and are conserved in the genome sequence of over 100 different bacteria encoding a T6SS similar to the prototype described by Pukatzki *et al*. (2006).

2.3.2 Operon structure of the T6SS

The T6SS encoded by most bacteria is organized in discreet transcriptional units or operons (51, 52), suggesting coordinated expression (23, 24). We therefore, investigated the organization of conserved genes in the *P. ananatis* T6SS. The core genes of PA T6SS-1 were clustered in three highly conserved operons; group 1 (*tssJ-tssK-dotU-icmF*) group 2 (*tssB-tssC-hcp*) and group 3 (*tssE-tssF-tssG-tssH*). PA T6SS-2 showed a considerable level of gene shuffling compared to PA T6SS-1, with the gene order being highly variable between each of the different groupings. The consensus grouping in PA T6SS-2 included *tssB-tssC-tssK-dotU* and *tssF-tssG-tssJ-tssE*, while *tssH-vgrG* and *icmF-tssA* were stand-alone operons linked to non-conserved T6SS genes. The start and stop codons of all nine genes located in PA T6SS-3 gene cluster overlapped with each other, suggesting that the PA T6SS-3 represents a single transcriptional unit. These different operon structures suggested the independent acquisition of the T6SS clusters, each of which may play a different role in the biology of *P. ananatis*.

2.3.3 Distribution of the PA T6SS in other strains of *P. ananatis*

2.3.3.1 PCR and dot blot analysis

To determine the prevalence of the three PA T6SS clusters (1, 2 and 3) among *P. ananatis* strains, we analyzed the distribution of each T6SS cluster in 46 different strains of the pathogen. BLASTP analysis showed that the gene products of PA T6SS-1 and -2 shared less than 50% amino acid similarity (Table S1). This low sequence similarity allowed specific PCR primers to be designed within conserved regions located on the DNA sequence of the targeted genes. For primer design, the targeted T6SS gene homologs from eight sequenced strains of *P. ananatis* were aligned in BioEdit using the ClustalW multiple alignment program and PCR primers were designed within the conserved DNA regions located on these genes. BLASTN analysis showed that the conserved regions used for primer design were not present on the DNA sequences of T6SS gene homologs found in other clusters. We, therefore, saw no cross-reactivity during the PCR amplification. The list of primers and strains of *P. ananatis* used for PCR analysis is provided in Tables 2.2 and 2.3, respectively.

Table 2.2: List of primers used for PCR amplification of type VI secretion system genes from different strains of *Pantoea ananatis*

Genes	Sequences 5' to 3'	Amplicon (base pairs)	Reference
	PA T6SS-1 primers		
tssH (PANA_2354)	clpVF – CTAATTGGTGGGATTAATCAGC	2577	This study
	clpVR – CTGATCACAGCTGACCTGCAC		This study
hcp (PANA_2364)	hcpF – GATATGTATTTGAAGGTAGACG	448	This study
	hcpR – TCCAGCCAGCGCTGCTTTCTG		This study
tagF (PANA_2368)	tagFF – AGTGGTCTAACTGGTTTCAGG	533	This study
	tagFR – TAAGTTTCCACTGTGGACATG		This study
tssK (PANA_2371)	tssKF – AGCAGGCAGAAAACTTTCTGC	1278	This study
	tssKR – ATTCTGACTGACTGCGAATGG		This study
	PA T6SS-2 primers		
tssA (PANA_4130)	tssA1F – TCAGGTGCATTTCCAGTTGAG	1234	This study
	tssA1R - CCTGATGTGAACTGGCTCTATG		This study
tssE (PANA_4131)	lysF – ACAGACGGGTTAAGGTATCAAG	407	This study
	lysR – CAATTTCTCCGGTGGTCTGAGC		This study
tssJ (PANA_4132)	tssJF – CAGATAATGACTCCCGGCTTC	503	This study
	tssJR – TGACTAAATCTGCACGCTGGC		This study
tssA (PANA_4137)	tssA2F – ACTGTCGAGCTCTTCCAACAG	1407	This study
	tssA2R – ATGAGCACTCTACAGAATCTG		This study
ompA (PANA_4147)	ompF – ATCTGACAGGCATTCGCCTGAG	1165	This study
	ompF – TGATTCTGAATTGTCAGATCGG		This study
tssK (PANA_4149)	tssKF – TCAAGTTCAGGCTCACCGAGC	1260	This study
	tssKR – TCCAGCAACAGGTCGCGTATG		This study
	Plasmid specific primers		
parA (PANA_4187)	parAF - ATGGCTAACGATGATAGTCAGG	1118	This study
	parAR –TCACTGCTACCTACATAGGTTG		This study
	PA-T6SS-3 flanking primers		
pebP (PANA_4129)	pebF – TAGCGCCAGATTGCCGGTAGC	1350	This study
clcA (PANA_4151)	clcR – GTATCATCTTGAAGCGGCAAC		This study
	PA T6SS-3 primers		
pknA (PANA_1650)	c1aF – CTAATTGGTGGGATTAATCAGC	805	This study
	c1aR – TGGCAACCTGTCCGACGAAACC		This study
pppA (PANA_1652)	c1cF – CTGAGTCAGCCAGCGTGTAGAG	700	This study
	c1cR – CAGGATCGAACCGGTGTGATAC		This study
tagH (PANA_1653)	c1bF – GGAGTCTTTATACTGATTGAC	878	This study
	c1bR – TGAAGCGTGGCGTGCAGGTGC		This study
tssL (PANA_1656)	c1dF – CTCAGTGTTTCAGGTGCGGCG	437	This study
	c1dR – CTGGATTTACGCCAGCGGCTC		This study

PA T6SS = *Pantoea ananatis* type VI secretion system. PA-T6SS-2 primers were used for PCR and DNA dot blot experiments. The *ompA*-like gene found in PA T6SS-2 cluster belongs to COG2885 and COG1360 and is different from the *tssL*/*icmF*/*dotU* gene homologs which belongs to COG3455. * indicates the two *tssA* homologs found in T6SS-2. The *parA* gene encodes the plasmid partition protein.

Table 2.3: Strains of *Pantoea ananatis* tested for the presence of T6SS gene clusters

Bacteria strain	Local reference numbers	Relevant characteristics or host of isolation	Location	Collector (a) or Reference (b)
Mmir 9	BCC0053	*Mirridae* sp.	RSA	Roux
Mmir 2	BCC0054	*Mirridae* sp.	RSA	Roux
AJ13355*	BCC1049	Soil	Japan	66
LMG 5342*	BCC0192	Human	USA	Brenner
LMG 5256	BCC0157	White mutant	N/A	Thiry (1983)
LMG 2807	BCC0155	*Cattleya* sp.	Brazil	Robbs (1965)
LMG 2628	BCC0644	*Musa* sp.	Germany	Mannheim (1981)
ICMP 10132	BCC0601	*Saccharum officinarum*	Brazil	Almeida (1991)
Yomogi-101	N/A	*Artemisia* sp.	Japan	Kobayashi (1997)
SUPP2582	N/A	*Cucumis melo*	Japan	67
SUPP1128	N/A	*Cucumis melo*	Japan	Makino (1986)
SUPP1791	N/A	*Cucumis melo*	Japan	67
ATCC 35400	BCC0151	*Cucumis melo*	USA	Wells (1981)
LMG 2678	BCC0195	*Puccinia graminis*	Zimbabwe	Hayward (1961)
LMG 2675	BCC0149	*Puccinia graminis*	Hungary	Klement (1957)
DAR76142	BCC0371	*Oryza sativa*	Australia	DAAR (2004
RAMI7971	BCC0376	*Oryza sativa*	Australia	N/A
DAR76144	BCC0373	*Oryza sativa*	Australia	DAAR (2004
DAR76143	BCC0372	*Oryza sativa*	Australia	DAAR (2004
SUPP 2113	N/A	*Oryza sativa*	Japan	Takikawa (2004)
SUPP2219	BCC0147	*Oryza sativa*	Japan	67
LMG 2101	BCC0158	*Oryza sativa*	India	Fujii, Urano (2001)
BD 442*	BCC0635	*Zea mays*	RSA	Goszczynska (2004)
BD 602	BCC0641	*Zea mays*	RSA	Goszczynska (2004)
BD 622	BCC0640	*Zea mays*	RSA	Goszczynska (2004)
BD 588	BCC0639	*Zea mays*	RSA	Goszczynska (2004)
BD 647	BCC0643	*Zea mays*	RSA	Goszczynska (2004)
BD 640	BCC0642	*Zea mays*	RSA	Goszczynska (2004)
BD 336	BCC0630	*Allium cepa*	RSA	Goszczynska (2004)
BD 310	BCC0628	*Allium cepa*	RSA	Goszczynska (2004)
N/A	BCC0156	*Allium cepa*	RSA	Coutinho
PA-4*	BCC0633	*Allium cepa*	RSA	Goszczynska (2004)
BD 301	BCC0626	*Allium cepa*	RSA	Goszczynska (2004)
BD 315	BCC0416	*Allium cepa*	N/A	Goszczynska
LMG 20103*	BCC0127	*Eucalyptus grandis*	RSA	Coutinho (1998)
N/A	BCC0112	*Eucalyptus* sp.	RSA	Len van Zyl
Uruguay 37	BCC0381	*Eucalyptus* sp.	Uruguay	Wingfield (2004)
Uruguay 40	BCC0382	*Eucalyptus* sp.	Uruguay	Wingfield (2004)
LMG 20104	BCC0091	*Eucalyptus grandis*	RSA	Coutinho (1998)
LMG 20105	BCC0084	*Eucalyptus grandis*	RSA	Coutinho (1998)
LMG 2665^{T}*	BCC0132	*Ananas comosus*	Brazil	Robbs (1965)
LMG 2664	BCC0154	*Ananas comosus*	Hawaii	Spiegelberg (1958)
LMG 2669	BCC0142	*Ananas comosus*	Hawaii	Spiegelberg (1958)
LMG 2668	BCC0181	*Ananas comosus*	Hawaii	Spiegelberg (1958)
LMG 2666	BCC0128	*Ananas comosus*	Hawaii	Spiegelberg (1958)
LMG 2667	BCC0143	*Ananas comosus*	Hawaii	Spiegelberg (1958)
ATCC 14028^{T}	BCC0433	*Salmonella* Typhimurium	N/A	N/A

* = Genome sequence available. N/A = data not available. BCC = Bacterial Culture Collection, Department of Microbiology and Plant Pathology, University of Pretoria, Republic of South Africa (RSA). USA = United States of America.

PCR amplification showed that the T6SS-1 and T6SS-3 homologs were present in all tested strains of *P. ananatis* (Table S2). PCR amplification using PA T6SS-2 gene-specific primers identified homologs in only 15 of the 46 strains of *P. ananatis* (33%) tested (Table 2.4). The distribution of PA T6SS-2 was further confirmed by dot blot hybridization using probes for *tssA*, *tssE*, *tssJ* and *tssK* (results not shown). Our results showed that PA T6SS-2 was present in strains of *P. ananatis* isolated from symptomatic maize, onion, pineapple fruit and *Eucalyptus* spp. However, not all strains of *P. ananatis* isolated from the same host plant contained PA T6SS-2. The cluster was only found in three of the six strains of *P. ananatis* (50%) isolated from either maize, onion, pineapple and *Eucalyptus* spp., while the remaining three strains, isolated from the same host but different plants, did not have the cluster. In addition, PA T6SS-2 was absent in all strains of *P. ananatis* isolated from symptomatic wheat (two isolates), honeydew melon (four isolates), rice (seven isolates) and sugarcane (one isolate). These results suggest that PA T6SS-2 could be a host range or virulence determinant of *P. ananatis*. Future studies will undertake pathogenicity and cross-inoculation trials to validate this correlation. In addition, the cluster was also found in *P. ananatis* strains AJ13355 and Yomogi-101, which have been shown not to cause disease on a range of host plants (53, 54, and data not shown), suggesting that PA T6SS-2 could be involved in other processes related to the ecological fitness of the species.

2.3.4 Comparative analysis of T6SS gene clusters from different strains of *P. ananatis*

Homologous PA T6SSs encoded by different strains of *P. ananatis* were highly conserved in terms of sequence similarity, gene content and operon structure (Figure 2.2 and Figure S1). A detailed description of the genes found in individual T6SSs encoded by all sequenced strains of *P. ananatis* analyzed in this study are provided in supplementary data (Tables S3 to S13).

Table 2.4: The distribution of type VI secretion gene clusters-2 (T6SS-2) in 46 strains *Pantoea ananatis* clusters

Bacteria strain	Location	Homologs of T6SS-2 genes targeted for PCR amplification							
		tssA1	*tssE*	*tssJ*	*tssA2*	*ompA* -like	*tssK*	*pepF /chlR*	*parA*
Mmir 9 (*Mirridae* sp.)	RSA	-	-	-	-	-	-	+	+
Mmir 2 (*Mirridae* sp.)	RSA	-	-	-	-	-	-	+	+
AJ13355* (Soil isolate)	Japan	+	+	+	+	+	+	-	+
LMG 5342* (Clinical isolate)	USA	-	-	-	-	-	-	+	+
LMG 5256 (white mutant)	N/A	+	+	-	+	+	+	-	+
LMG 2807 (*Cattleya* sp.)	Brazil	-	-	-	-	-	-	+	+
LMG 2628 (*Musa* sp.)	Germany	-	-	-	-	-	-	+	+
ICMP 10132 (*S. officinarum*)	Brazil	-	-	-	-	-	-	+	+
Yomogi-101 (*Artemisia* sp.)	Japan	+	+	+	+	+	+	-	+
SUPP2582 (*Cucumis melo*)	Japan	-	-	-	-	-	-	+	+
SUPP1128 (*Cucumis melo*)	Japan	-	-	-	-	-	-	+	+
SUPP1791 (*Cucumis melo*)	Japan	-	-	-	-	-	-	+	+
ATCC 35400 (*Cucumis melo*)	USA	-	-	-	-	-	-	+	+
LMG 2678 (*Puccinia graminis*)	Zimbabwe	-	-	-	-	-	-	+	+
LMG 2675 (*Puccinia graminis*)	Hungary	-	-	-	-	-	-	+	+
DAR76142 (*Oryza sativa*)	Australia	-	-	-	-	-	-	+	+
RAMI7971 (*Oryza sativa*)	Australia	-	-	-	-	-	-	+	+
DAR76144 (*Oryza sativa*)	Australia	-	-	-	-	-	-	+	+
DAR76143 (*Oryza sativa*)	Australia	-	-	-	-	-	-	+	+
SUPP 2113 (*Oryza sativa*)	Japan	-	-	-	-	-	-	+	+
SUPP2219 (*Oryza sativa*)	Japan	-	-	-	-	-	-	+	+
LMG 2101 (*Oryza sativa*)	India	-	-	-	-	-	-	+	+
BD 442* (*Zea mays*)	RSA	-	-	-	-	-	-	+	+
BD 602 (*Zea mays*)	RSA	+	+	+	+	-	+	-	+
BD 622 (*Zea mays*)	RSA	+	+	-	+	-	-	-	+
BD 588 (*Zea mays*)	RSA	-	-	-	-	-	-	+	+
BD 647 (*Zea mays*)	RSA	+	+	+	+	+	+	-	+
BD 640 (*Zea mays*)	RSA	-	-	-	-	-	-	+	+
BD 336 (*Allium cepa*)	RSA	-	-	-	-	-	-	-	-
BD 310 (*Allium cepa*)	RSA	-	-	-	-	-	-	+	+
N/A (*Allium cepa*)	RSA	-	-	-	-	-	-	-	-
PA-4* (*Allium cepa*)	RSA	+	+	+	+	+	+	-	+
BD 301 (*Allium cepa*)	RSA	+	+	+	+	+	-	-	+
BD 315 (*Allium cepa*)	N/A	+	+	-	+	+	+	-	+
LMG 20103* (*E. grandis*)	RSA	+	+	+	+	+	+	-	+
N/A (*Eucalyptus* sp.)	RSA	+	+	+	+	-	+	-	+
Uruguay 37 (*Eucalyptus* sp.)	Uruguay	-	-	-	-	-	-	+	+
Uruguay 40 (*Eucalyptus* sp.)	Uruguay	-	-	-	-	-	-	+	+
LMG 20104 (*E. grandis*)	RSA	+	+	+	+	+	+	-	+
LMG 20105 (*E. grandis*)	RSA								
LMG 2665^{T}* (*Ananas comosus*)	Brazil								
LMG 2664 (*Ananas comosus*)	Hawaii	+	+	-	+	+	+	-	+
LMG 2669 (*Ananas comosus*)	Hawaii	+	+	-	+	+	+	-	+
LMG 2668 (*Ananas comosus*)	Hawaii	+	+	-	-	-	+	-	+
LMG 2666 (*Ananas comosus*)	Hawaii	-	-	-	-	-	-	+	+
LMG 2667 (*Ananas comosus*)	Hawaii	-	-	-	-	-	-	+	+
ATCC 14028T (*S.* Typhimurium)	N/A	-	-	-	-	-	-	-	-

Homologs of T6SS-3 genes were restricted to strains of *P. ananatis* shaded in blue. *S. officinarum = Saccharum officinarum, E. grandis = Eucalyptus grandis, S.* Typhimurium = *Salmonella* Typhimurium.

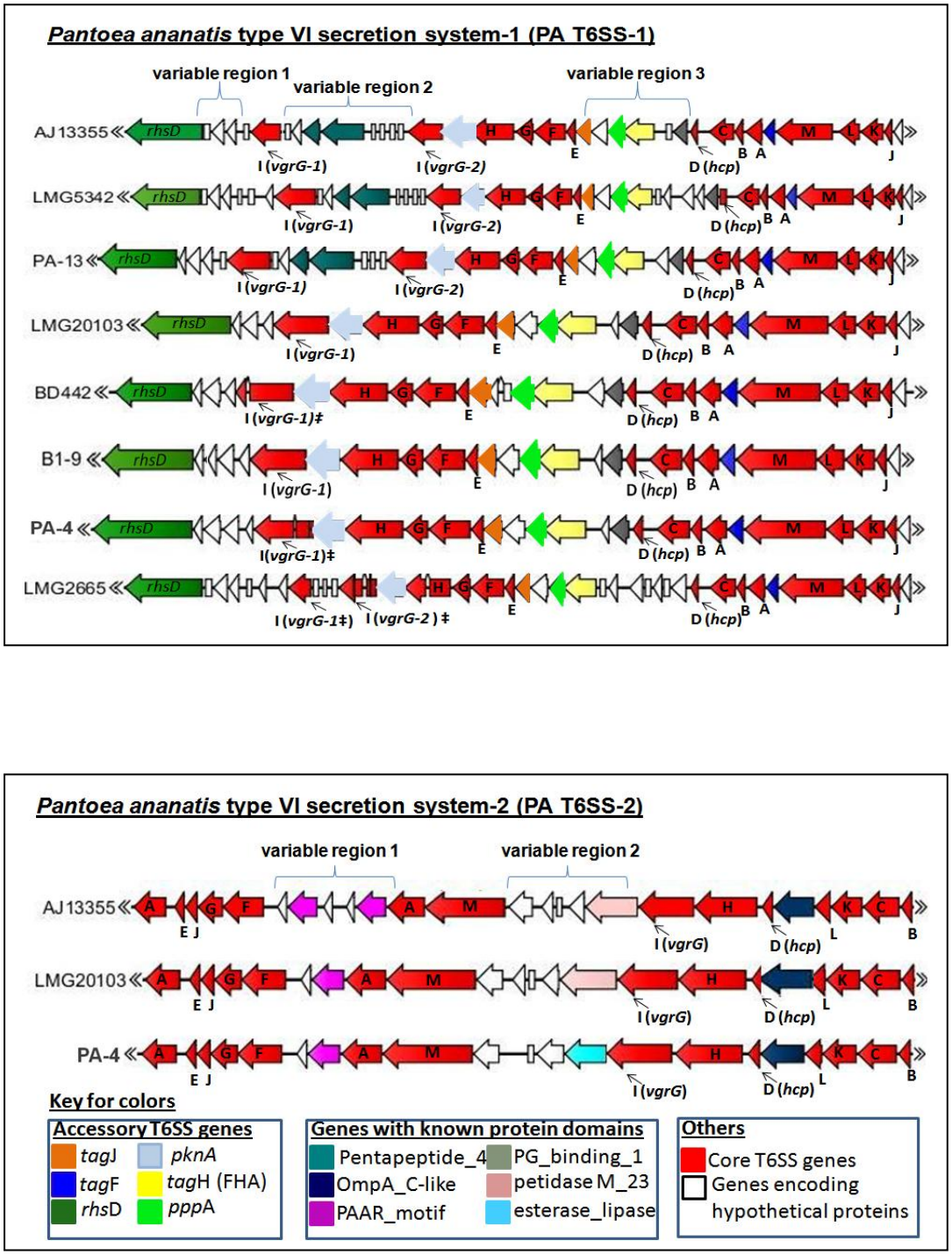

Figure 2.2: Comparison of the *Pantoea ananatis* type VI secretion systems 1 and 2 (T6SS-1 and T6SS-2). All 13 conserved core gene components of the T6SS are indicated in red, while non-conserved genes associated with the T6SS of limited bacteria are indicated in different colors. The letters in the figure represent conserved T6SS genes based on the nomenclature of Shalom *et al*. (2007).

2.3.4.1 PA T6SS-1

The genetic architecture of PA T6SS-1 was shown to be conserved amongst all sequenced strains of *P. ananatis*. The *tssD* (*hcp*) and *tssI* (*vgrG*) genes found in this cluster encoded Hcp and VgrG proteins that do not have C-terminal extensions as found in "evolved" VgrG and Hcp proteins (45, 55, 56). The C-terminal extension of some evolved VgrG proteins, such as VgrG1 of *V. cholerae* and *Aeromonas hydrophila*, have been associated with actin cross-linking and actin ADP ribosylation activity in mammalian host cells, respectively (55, 56). *P. ananatis* strains PA-4, BD442, B1-9 and LMG 20103 had a single *vgrG* gene, while strains AJ13355, PA-13, LMG 5342 and LMG 2665^{T} had an additional copy of *vgrG*. These *vgrG* genes appear to encode VgrG proteins with different domain architectures, characterized by the presence or absence of a C-terminal Beta-N-acetylglucosaminidase domain (Figure 2.3). This C-terminal domain is associated with lysozymes belonging to the glycoside hydrolase family 73 (PF01832) (57, 58). It is possible, therefore, that the different VgrG proteins encoded by each *vgrG* gene are mobilized to the T6SS baseplate under different physiological conditions or play different roles either as effectors, structural elements or both.

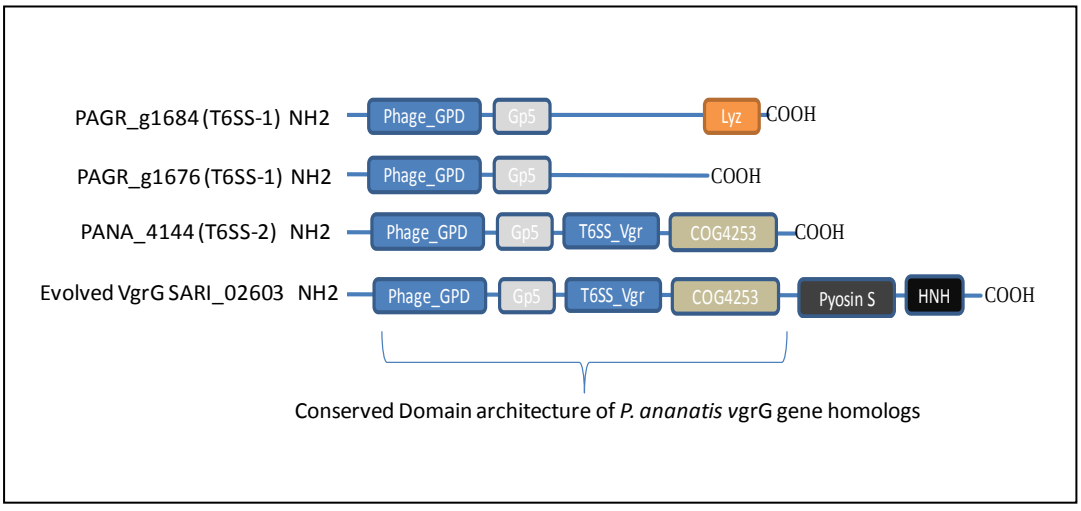

Figure 2.3: **Homolog of *vgrG* genes found in the type VI secretion system gene clusters of sequenced strains of *Pantoea ananatis***. Domains are represented in different colors. Lyz = lysozyme / Beta-N-acetylglucosaminidase domain is found in PAGR_g1684, which is missing from the *vgrG* homolog (PAGR_g1676). PANA_4144 found in T6SS-2 of strain LMG 20103 has a domain architecture similar to part of SARI_02603 of *Salmonella enterica* subspecies *arizonae*.

Regions associated with *hcp* and *vgrG* contain genes that encode a variable number of accessory and hypothetical proteins that account for strain-specific differences. The first variable region in PA T6SS-1 is located between the major *rhsD* element and *vgrG*. Genes found in this region encode mostly hypothetical proteins and proteins with either a PAAR (proline-alanine-alanine-arginine) repeat or pentapeptide_4 domains. PAAR repeat proteins of bacteria have been categorized into different classes (Class 1-7) based on their domain architectures (58). PAAR repeat proteins of several bacteria have effector domains on the N- or C-terminal (58). Some of these effector domains include: transthyretin, lipase, nuclease, deaminase, and ADP-ribosyl transferase (58). The gene products of PA-13 PAGR_g1683 and LMG 5342 PANA5342_1748 belong to the Class 1 PAAR domain architecture proteins and share 100% sequence similarity (Figure 2.4). In addition, PAGR_g1683 and PANA5342_1748 have no N- or C-terminal extensions and showed structural homology to the PAAR repeat protein of *V. cholerae* (4jiv_D – Hhpred score 105.4). A recent study showed that the PAAR repeat proteins of *E. coli* and *V. cholerae* bind to the Gp5-VgrG complex by means of non-covalent interactions (59). In addition, PAAR repeat proteins of *V. cholerae* and *Acinetobacter baylyi* were shown to be bactericidal effectors associated with T6SS-mediated killing of *E. coli* (59). These findings have led to the speculation that PAAR repeat proteins, carrying different effector domains located on either their N- or C-terminal extensions, may also bind to the VgrG spike and mediate secretion of these effectors by the T6SS (59, 60). It is also speculated that PAAR repeat proteins may form non-covalent interactions with different effectors, thereby recruiting them to the T6SS spike complex (59). It is, therefore, possible that the PAAR repeat proteins encoded by genes located in the *vgrG* island of the *P. ananatis* T6SS-1 gene cluster may either be T6SS effectors associated with inter-bacterial competition or may mediate secretion of other effectors.

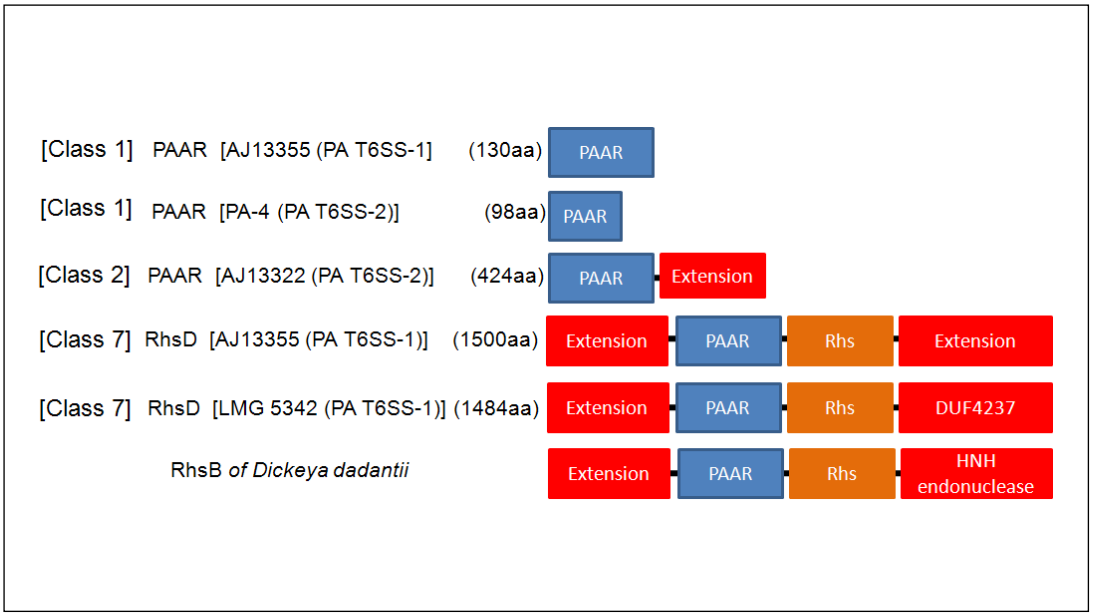

Figure 2.4: Domain architecture of PAAR repeat proteins associated with *Pantoea ananatis* type VI secretion system 1 and 2 (T6SS-1 and T6SS-2). We used the nomenclature of Shneider *et al*. (2013) (59), which categorized PAAR proteins into different classes (1-7) based on domain architecture. The different domains and extensions associated with *P. ananatis* PAAR proteins are indicated by different colors. No putative effector domains were associated with the N- or C-terminal extensions located in most PAAR repeat proteins of *P. ananatis*. However, the RhsD protein of LMG 5342, LMG 2665^{T} and B1-9 showed extensive structural homology to the insecticidal YenC2 toxins of *Yersinia entomophaga*. The domain architecture of the RhsD protein of *P. ananatis* is similar to the RhsB endonuclease toxin of *Dickeya dadantii*.

PAAR-repeat domains are also found in the *rhsD* gene of PA T6SS-1. A recent study, aimed at identifying polymorphic toxins in bacterial genomes using comparative analysis, sequence and structural analysis, identified RhsD as putative T6SS effectors of *P. ananatis*, based on the presence of PAAR repeats on this protein (59). Similarly, the RhsD protein of *Serratia marcescens* was recently identified in a T6SS secretome analysis, suggesting that this protein is secreted by the T6SS (40). Bacterial Rhs proteins have been associated with different phenotypes such as social motility, inflammasome-mediated cell death, virulence in mice, insecticidal toxin production, polysaccharide transport and bacteriocin production (61, 62, 63, 64, 65, 66, 67, 68, 69). In addition, the *rhsA* and *rhsB* genes of *Dickeya dadantii* encode endonuclease toxins that have been associated with contact-dependent killing of other bacterial species (70, 71). Toxin-producing strains of *D. dadantii* also express the cognate immunity factors from the *rhsI* gene located downstream of the *rhsA* and *rhsB* genes (70, 71). The *rhsD* locus of *P. ananatis* has a genetic organization similar to that of *D. dadantii* consisting of the *rhsD* gene that is followed by the *rhsI* homolog (Figure 2.5). In addition, *P. ananatis rhsD* genes have a conserved N-terminal domain and a variable C-terminal domain, which is characteristic of several bacterial Rhs toxins (Figure S2) (72, 73). We identified a DUF4237 domain of unknown function on the C-terminal extensions of RhsD proteins of *P. ananatis* strains B1-9, LMG 2665^{T} and LMG 5342, which is missing from the remaining strains. Furthermore, the RhsD proteins of strains B1-9, LMG 2665^{T} and LMG 5342 showed extensive structural homology to the insecticidal YenC2 toxin of *Yersinia entomophaga* (4igl_B – Hhpred score 574, 559.5 and 573.4, respectively). These findings suggest that the *rhsD* loci associated with the T6SS-1 gene clusters of *P. ananatis* may encode different toxin/immunity factors that play different roles as either bactericidal or insecticidal toxins. Alternatively, RhsD proteins of *P. ananatis* may mediate secretion of other T6SS effectors, which are non-covalently associated with either their N- or C-terminal extension, as predicted for other PAAR proteins (59).

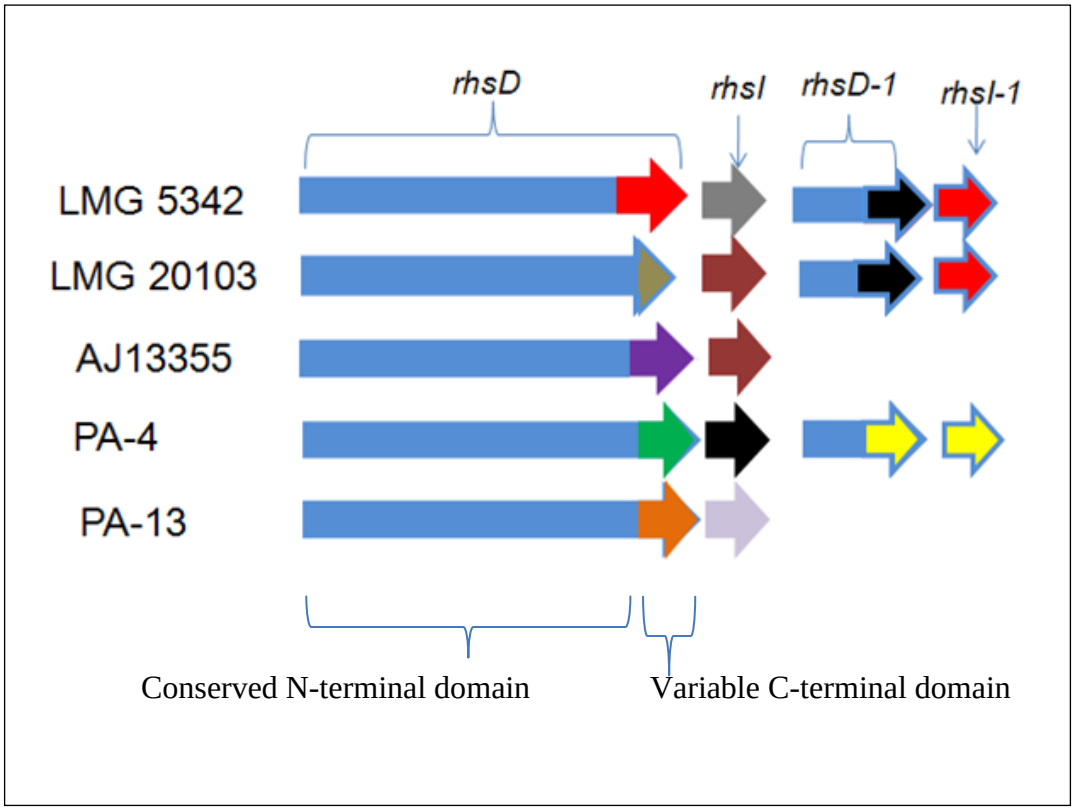

Figure 2.5: Genetic organization of the different *rhs* loci associated with the type VI secretion system 1 gene cluster (T6SS-1) of *Pantoea ananatis*. Representative strains of *P. ananatis* with distinct *rhs* loci are indicated in the figure. Strains LMG 5342, B1-9 and LMG 2665^{T} have identical *rhsD*/*rhsI* gene homologs. Similarly, strains PA-4 and BD442 also have identical *rhsD*/*rhI* gene homologs. Conserved and variable regions associated with *rhsD* genes are indicated in the figure. The *rhsI* gene is located downstream of the *rhsD* gene and the colors indicate the different *rhI* genes encoded by different strains of *P. ananatis*. The figure is not drawn to scale.

Homologs of *tagA* and *tagB,* which encode pentapeptide repeat proteins, were also identified in the *vgrG* islands of *P. ananatis* strains PA-13, AJ13355 and LMG 5342. In strain PA-13, the pentapeptide repeat proteins are encoded by PAGR_g1680 and PAGR_g1681 which correspond to *tagA* and *tagB* genes, respectively. Sequence analysis performed on PAGR_g1680 and PAGR_g1681 predicted no signal sequence and no transmembrane helices (75, 76). PsortB analysis predicted the gene product of PAGR_g1680 to be a secreted effector, while the sub-cellular localization of the PAGR_g1681 protein is unknown (77). Homologs of *tagA* and *tagB* genes have also been identified in the T6SS gene clusters of *Serratia marcescens, Azotobacter vinelandii, Burkholderia bronchiseptica,* including the T6SS-5 gene clusters of *B. mallei, B. pseudomallei* and *B. thailandensis*. However, these genes have not been functionally characterized and the biological function of their gene products is currently unknown (30, 38, 50).

The second variable region found in the T6SS-1 gene cluster of *P. ananatis* is located between *hcp* and *tagH*, and contains a gene encoding a protein with a peptidoglycan-binding domain (PG_binding). In strain LMG 5342, the protein is encoded by PANA5342_1731 and showed structural homology to the lytic transglycosylase enzyme of *Pseudomonas aeruginosa* bacteriophage phiKZ (3bkh_A Hhpred - score 146), while the corresponding homologs found in strains LMG 20103, PA-4, B1-9 and AJ13355 showed structural homology to the pesticin toxin of *Yersinia pestis* (4aqm_A Hhpred – score 290.2, 283.4, 290.2 and 283.4, respectively). Pesticin and lytic transglycosylase enzymes are bactericidal effectors which degrade peptidoglycan by cleaving the β-1,4 glycosidic bond between *N*-acetylmuramic acid and the *N*-acetylglucosamine moieties (78, 79, 80, 81, 82). These findings suggest that genes found in the *hcp* island of the *P. ananatis* T6SS-1 cluster may encode bactericidal effectors that are functionally related to the type VI secretion glycoside hydrolase effectors 1-3 (Tge1-3) (37, 83). Genes encoding proteins with the PG_binding domain were not identified in the T6SS-1 *hcp* island of *P. ananatis* strain LMG 2665^{T}. However, the gene product of LMG 2665^{T} N454_00628 showed weak structural homology to Colicin S4 of *Escherichia coli* (3few_X - Hhpred score 55.8). Colicin S4 is a pore-forming bacteriocin that kills bacterial species not expressing the immunity factor (84, 85). It is, therefore, possible that the gene product of LMG 2665^{T} N454_00628 may be a bacteriocidal effector, which plays a role in inter-bacterial

competition. We did not identify homologs of type VI amidase 1-4 (Tae 1-4), type VI lipase effectors 1-5 (Tle 1-5), small secreted proteins 1-6 (Ssp 1-6) and type VI secretion exported 1, 2 (Tse 1, 2) within the T6SS-1 gene cluster of *P. ananatis*, based on sequence and structural analysis (34, 36, 39, 40).

2.3.4.2 PA T6SS-2

The genetic architecture of PA T6SS-2 is highly conserved in all sequenced of *P. ananatis* that harbor the cluster. PA T6SS-2 was found to contain a single *vgrG* gene that encodes a VgrG protein with an additional C-terminal DUF2345 (COG4253) domain of unknown function. Conserved Domain architecture analysis showed that this domain was conserved in the *vgrG* genes of several different bacteria. All strains of *P. ananatis* that harbor this cluster encode two copies of the *tssA* gene within the cluster, showing 22% amino acid similarity. Duplication of the *tssA* gene has also been reported in the T6SS gene clusters of *Vibrio cholerae*, *Escherichia coli* O157:H7, *Yersinia pseudotuberculosis* and *Salmonella enterica* serotype Gallinarum (21, 45, 50). The reason for this duplication is unknown, as is the exact role that TssA plays as an essential T6SS structural protein.

Two variable regions were identified in PA T6SS-2, which contained genes encoding non-conserved T6SS components and hypothetical proteins. The first variable region is located between *tssA* and *tssF*, and encodes several strain-specific hypothetical proteins including proteins with a PAAR_motif. The genes products of LMG 20103 PANA_4136, AJ13355 PAJ_p0154 and PA-4 N455_00706 contain 424 amino acids, sharing 99% amino acid similarity and belong to the Class 2 PAAR domain architecture proteins. Furthermore, these PAAR proteins all contained identical C-terminal extensions with no putative effector domains. The gene products of PANA_4136, PAJ_p0154 and PA-4 N455_00706 may either be T6SS effectors or mediate secretion of other effectors bound to their C-terminal extensions (59, 60). The second variable region in the PA T6SS-2 gene cluster is located between *icmF* and *vgrG*. This region in LMG 20103 contains four genes (PANA_4140 to 4143) encoding hypothetical proteins with homologs present in strain AJ13355, but is missing from strain PA-4. The *PANA_4143* gene encodes a protein with a

peptidase_M23 domain. This protein is a putative endopeptidase and is predicted to be a T6SS effector based on its high structural homology to the secreted chitinase G of *Streptomyces coelicolor* (48, 86). The corresponding variable region in *P. ananatis* strain PA-4 contains a gene with an esterase_lipase domain and belongs to the family lecithin: cholesterol acyltransferase (PF02450). This protein is predicted to be involved in extracellular metabolism of plasma lipoproteins, including cholesterol (87). Genes with an esterase_lipase domain have been found in the vicinity of *vgrG* genes outside the major T6SS of *Pseudomonas* spp. In *P. aeruginosa*, these genes form part of the "cargo" genes predicted to have been acquired by recent horizontal gene transfer (88). In addition, the N455_00710 gene, which is located in the *vgrG* island of strain PA-4 T6SS-2 cluster, encodes a Class 1 PAAR domain architecture protein containing 98 amino acids with no N- or C-terminal extensions. This PAAR protein may play a role as a T6SS effector associated with inter-bacterial competition (59, 60). Homologs of functionally characterized T6SS effectors such as type VI lipase effectors 1-5, type VI amidase 1-4, type VI secretion glycoside hydrolase effectors, Small secreted proteins 1-6 and Type VI secretion exported 1-2, were not identified in *P. ananatis* T6SS-2 gene cluster, using sequence analysis and structural homology search tools.

2.3.4.3 PA T6SS-3

Comparative analysis of PA T6SS-3 showed that there was no variability of this cluster between the different strains of *P. ananatis*. The genetic architecture, gene order and gene content of PA T6SS-3 was conserved in all sequenced strains of *P. ananatis*. Interestingly, all genes found in PA T6SS-3 showed high sequence similarity to genes found in *Pantoea* sp. At-9b, *Pantoea* sp. aB-valens, *P. vagans* C9-1, *P. agglomerans* E325 and *Erwinia billingiae* Eb661. These *Pantoea* and *Erwinia* spp. have a homologous cluster highly similar to PA T6SS-3 in terms of gene content and operon structure (48). The high conservation of this cluster suggests a strong selective pressure to maintain the gene content and order, although its specific role is unknown. The fact that the cluster is missing 11 core gene components of the T6SS suggests that this system does not encode a functional T6SS, although this is yet to be confirmed.

2.3.5 "Orphan" Hcp and VgrG proteins

When the T6SS genes *hcp* and *vgrG* are present outside the T6SS they are often referred to as "orphan" *vgrG* and *hcp* genes. Our analysis did not identify "orphan" *vgrG* genes in any of the sequenced genomes of *P. ananatis*. However, we identified three different "orphan" *hcp* genes in *P. ananatis* strain PA-13 (PAGR_g1583, PAGR_g1584 and PAGR_g3636). These "orphan" *hcp* genes were designated *hcp*-1, *hcp*-2 and *hcp*-3 to correspond to PAGR_g1583, PAGR_g1584 and PAGR_g3636, respectively. Genes *hcp*-1 and *hcp*-2 are adjacent to each other and have homologs present in all sequenced strains of *P. ananatis*, while *hcp*-3 is restricted to *P. ananatis* PA-13. Multiple alignments of the amino acid sequences of representative *P. ananatis* Hcp proteins showed that the Hcp-3 protein is highly divergent from Hcp-1, Hcp-2 and T6SS-associated Hcp proteins (Figure S3). All "orphan" *hcp* genes in *P. ananatis* are found in the vicinity of genes encoding hypothetical proteins and a putative endoribonuclease SymE, which is part of an SOS inducible toxin/antitoxin system (89). It remains to be determined whether "orphan" *hcp* genes of *P. ananatis* are associated with the major T6SSs, as either T6SS effectors or structural proteins, or whether the conserved association between *symE* and "orphan" *hcp* genes is important in other aspects of the biology of *P. ananatis*.

2.3.6 Phylogenetic analysis of the T6SS

Phylogenetic analysis was used to infer the evolutionary history of the T6SS using the Maximum Likelihood Method based on the Le and Gascuel (LG) + G + F amino acid substitution model, as determined by ProtTest (101, 102). Representative bacteria from the different T6SS phylogenetic groups A-D were included in the analysis (23, 45) (Table S14). The analysis showed that PA T6SS-1 belonged to phylogenetic Group A, while PA T6SS-2 belonged to Group C (Figure 2.6). PA T6SS-1 was phylogenetically closest to T6SS loci 1 of *Pantoea* sp. At-9b (an insect endophyte), while PA T6SS-2 was closest to T6SS loci 2 of *Erwinia amylovora* CFBP1430 (a plant pathogen). Our phylogenetic analysis resembled the analysis performed by Bingle *et al*. (2008), which showed that phylogenetic Group A to D contained pathogenic and non-pathogenic bacteria associated with different ecological niches

(23). Similarly, functionally characterized T6SSs with a known role in virulence or inter-bacterial competition were represented in the different Groups. For example, the H1-T6SS of *P. aeruginosa*, which is known to secrete Tse1 to 3 antimicrobial effectors, was found in Group A, while the T6SSs of *Vibrio cholerae* and *Pseudomonas syringae* pv. *tomato*, with a similar role in inter-bacterial competition, belonged to Group D (31, 32, 41). In addition, all four phylogenetic Groups contained bacterial T6SSs that have been associated with virulence. Together, these findings suggest that bacterial T6SSs found in phylogenetic Group A to D are evolutionarily distinct and play different roles in pathogenic and non-pathogenic bacteria (23, 24).

Our phylogenetic tree also showed that *P. ananatis* T6SS-1 and T6SS-2 clustered away from the T6SS of several important plant pathogens such as *Xanthomonas citri*, *X. campestris, X. oryzae, Pseudomonas syringae* pv. *syringae, Pseudomonas syringae* pv. *phaseolicola, Pectobacterium atrosepticum* SCRI1043, *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium carotovorum* subsp. *brasiliensis*. Functional studies have shown that the T6SS gene clusters of *P. atrosepticum* and *P. syringae* are only partially required for disease development. The T6SS mutants of these bacteria were either only slightly reduced in the ability to cause disease or caused disease symptoms on susceptible host plants similar to the wild-type strains (41, 90, 91, 92, 93). These findings, together with our T6SS phylogenetic groupings, suggests that 1) the T6SS of different plant pathogens were acquired from unrelated bacteria or distantly-related ancestors, 2) the T6SS clusters found in different phytopathogens may play different roles depending on the host plant or ecological niche, and 3) T6SS-1 and T6SS-2 may play an important role in the virulence of *P. ananatis* in susceptible host plants.

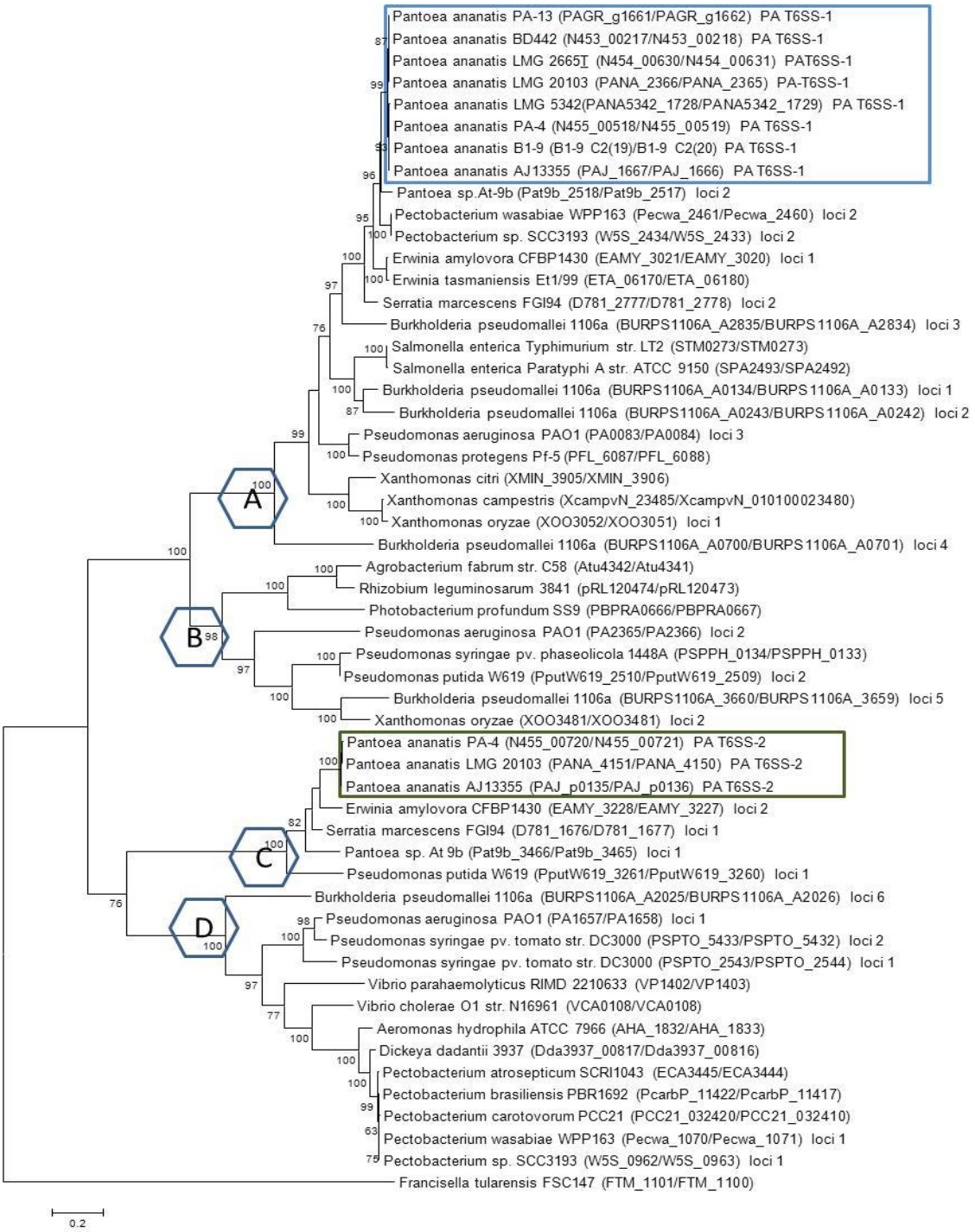

Figure 2.6: Evolutionary relationships of the different type VI secretion systems of *Pantoea ananatis* using concatenated TssB and TssC amino acid sequences. Phylogenetic analysis were conducted in MEGA6 (100). The amino acid substitution model was determined by ProtTest (101). The evolutionary history was inferred using the Maximum Likelihood method based on the Le and Gascuel (LG) + G + F model (102). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The TssB/C homologs used in this study are indicated next to the name of the bacteria. Blue octagons represent T6SS phylogenetic Groups A-D proposed by Bingle *et al*. (2008). Representative position of PA T6SS-1 and PA T6SS-2 are shown in blue and green rectangles, respectively. Details of all bacteria used in are given in supplementary data (Additional file S14).

To infer the evolutionary history of "orphan" *hcp* genes (*hcp*-1, 2 and 3) in *P. ananatis*, we constructed a second Maximum Likelihood tree using the General Reverse Transcriptase (rtRev) + I + G + F amino acid substitution models, as determined by ProtTest (101, 103). Our phylogenetic analysis also included representative T6SS-associated *hcp* genes and "orphan" *hcp* genes from other bacteria (Table S15). The resulting phylogeny revealed four distinct clusters, which we have designated as *hcp* Cluster 1-4 to distinguish them from T6SS phylogenetic Group A-D (23) and Group I-V (24). Our results indicate that *hcp*-1 and *hcp*-2 belong to Cluster 3, together with the T6SS-associated *hcp* genes found in PA-T6SS-1 (Figure 2.7). Hcp-3 (PAGR_g3636) belongs to Cluster 2, together with T6SS-associated *hcp* genes present in PA-T6SS-2. Hcp-3, however, forms part of a subset of Cluster 2, and was phylogenetically close to orphan Hcp proteins from *Klebsiella varriicola* At-22 and *Erwinia amylovora* ATCC BAA-2158. These *hcp* groupings suggest either independent acquisition of "orphan" *hcp* genes from different ancestors or gene duplication and rearrangement of T6SS-associated *hcp* genes.

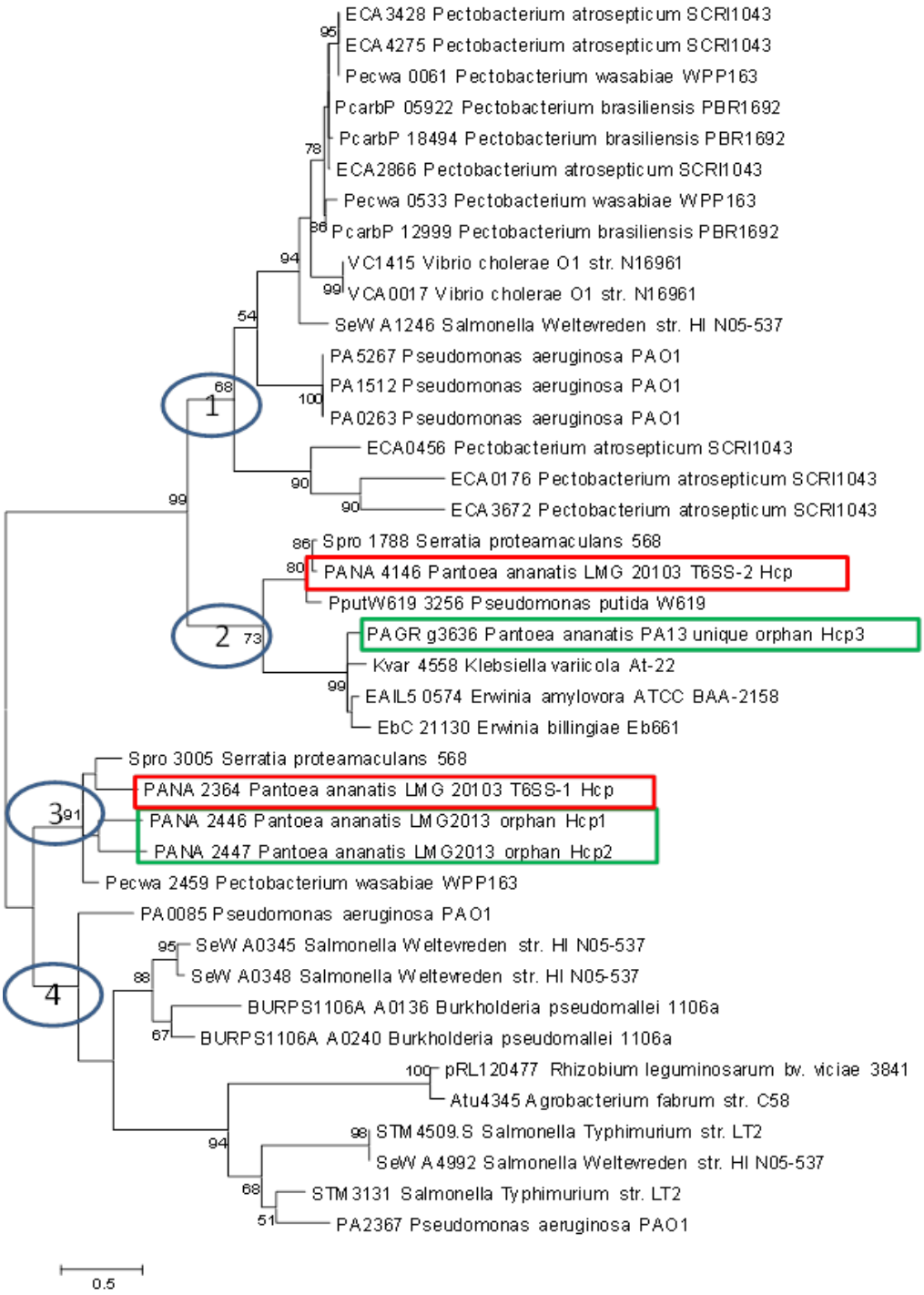

Figure 2.7: Evolutionary relationships of type VI secretion system - linked Hcp and "orphan" Hcp proteins in *Pantoea ananatis.* Phylogenetic analysis were conducted in MEGA6 (1000). The evolutionary history was inferred using the Maximum Likelihood method based on the General Reverse Transcriptase (rtRev) + I + G + F amino acid substitution models, as determined by ProtTest (101, 103). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The different Hcp Groups (1 to 4) are shown in rectangles. The green and blue rectangles correspond to orphan Hcp proteins and type VI secretion system-associated Hcp proteins of *P. ananatis*, respectively. P = *Pantoea*

2.4 Conclusion

Comparative analyses of the T6SS in the genomes of sequenced strains of *P. ananatis* identified three putative gene clusters PA T6SS-1, PA T6SS-2 and PA T6SS-3. The former two of these were potentially functional, as they contained the 13 core genes necessary for synthesis of a functional T6SS (23, 24, 30). PA T6SS-1 was widespread in the genome of all sequenced strains including environmental isolates, while PA T6SS-2 was plasmid-borne and restricted mostly to pathogenic strains of *P. ananatis* isolated from certain classes of plants. This finding suggests a potential association of PA T6SS-2 with host range determination. However, the finding that PA T6SS-1 and PA T6SS-2 were present in both pathogenic and non-pathogenic strains of *P. ananatis* supports the concept that the T6SS may evolve to play different roles unrelated to pathogenicity, e.g., competition against other microbes, fitness and/or niche adaptation (18, 23, 27, 39). The genetic organization and phylogenetic groupings of PA T6SS-1 and -2 further suggests that these clusters were independently acquired to play differing roles in the different strains of *P. ananatis*. Furthermore, the variable regions associated with *hcp* and *vgrG* genes could account for specialization of each T6SS based on the needs of the specific strain. In the future, key questions that need to be addressed include determining: 1) whether the T6SSs of *P. ananatis* are functionally active and what roles they play in host-pathogen interactions and fitness; 2) which *in vitro* and *in vivo* conditions activate the T6SSs; 3) which effectors are secreted by the T6SSs of *P. ananatis* and their physiological relevance to fitness and host-pathogen interactions; and 4) how T6SSs are regulated in these strains.

2.5 Materials and Methods

2.5.1 *P. ananatis* T6SS data acquisition

The annotated genome sequences of different *P. ananatis* strains were downloaded from NCBI with the accession numbers LMG 5342 (chromosome HE617160.1, plasmid pPANA10 HE617161.1); AJ13355 (chromosome AP012032.1, plasmid AP012033.1); PA-13 (chromosome CP003085.1, plasmid PAGR_pCP003086.1); LMG 20103 (chromosome CP001875.2) and the draft genome of B1-9 (CAEJ00000000.1). The draft genomes of *P. ananatis* BD 442, PA-4 and LMG 2665^{T} were sequenced and partially assembled in our laboratory. The sequences and fully annotated contigs representing the different type VI secretion system gene clusters

have been deposited in GenBank for strain BD 442 (KF552073, KF552074), PA-4 (KF590029, KF590030, KF590028) and LMG 2665^{T} (KF590026, KF590027). All eight sequenced strains of *P. ananatis* were obtained from different geographical regions and were isolated from different diseased plants or other environmental samples. Detailed information on the strains is presented in Table 2.3.

2.5.2 *In silico* identification of T6SS clusters

Genes associated with the T6SS, including flanking regions identified in *P. ananatis* strain LMG 20103 by De Maayer *et al*. (2010), were used as bait to search for T6SS homologs in all sequenced *P. ananatis* genomes and plasmids using BLASTN and BLASTP (94, 95). Nucleotide sequences representing entire T6SS regions, including flanking regions, were extracted from each genome and used for *de novo* gene prediction using F-GenesB (www.Softberry.com). All predicted genes were searched against non-redundant protein databases at NCBI to identify homologs. SignalP 4.0 and TMHMM Server v.2.0 were used to predict signal peptides and trans-membrane helices (75, 76). Protein localization and functional classifications were done using PSORTb (77), InterProScan (96), Conserved domains and CDD domain architecture-Search tool on NCBI (97). Protein structural homology analyses were performed using using the HHpred (98). Proteins were clustered based on their COG groups (99).

2.5.3 PCR and dot blot hybridizations

A minimum of four primer pairs were designed per T6SS cluster to amplify locus-specific genes. For primer design, the nucleotide sequences for each gene of interest were extracted from all eight sequenced strains of *P. ananatis*. BLASTP analysis showed that T6SS gene products from homologous T6SS clusters found in all sequenced strains of *P. ananatis* were over 98% similar (Table S7 to S10). In addition, BLASTP analysis showed that there was less than 70% similarity between gene products found in the different T6SS clusters (Table S1). The low sequence similarity between gene homologs in each T6SS cluster, therefore, allowed specific primers to be designed within conserved T6SS gene regions. Importantly, PCR analysis showed that the primers were specific and did not amplify T6SS homologs found in other T6SS gene clusters found in the genome sequence of *P. ananatis*.

Genomic DNA was isolated from 46 different *P. ananatis* strains using the Quick-gDNA^{TM} MiniPrepkit (Zymo Research, USA) following the manufacturer's instructions. PCR amplification using SuperTherm DNA polymerase (Southern Cross Biotechnology, RSA) was performed with a Veriti^{R} Thermal Cycler (Applied Biosystems, USA). DNA sequencing was done using the ABI PRISM3100 Genetic Analyzer (Applied Biosystems) at the DNA Sequencing Facility (University of Pretoria, RSA). Colony hybridization was used to validate the PCR results. Probes for hybridisation were labeled with Digoxigenin-11-dUTP using the DIG PCR labeling Kit (Roche Applied Science, USA) according to the manufacturer's instructions. Membrane hybridization, washing and detection were done using the DIG DNA labeling and detection kit (Roche Applied Science, USA) as directed by the manufacturer.

2.5.4 Phylogenetic analyses

Two phylogenetic analyses were carried out, one using concatenated amino acid sequences of TssB and TssC, and the second using amino acid sequences of Hcp and "orphan" Hcp. The TssBC analysis showed the phylogenetic grouping of the different T6SSs of *P. ananatis*, while a further phylogenetic grouping examined the relationship between T6SS-associated Hcp and "orphan" Hcp proteins. Amino acid sequences of TssB, TssC, Hcp and "orphan" Hcp proteins representing bacteria from T6SS phylogenetic Groups A-D were downloaded from NCBI (23, 24). Amino acid sequences were aligned by ClustalW and phylogenetic analysis conducted in MEGA6 (100). The amino acid substitution models were determined by ProtTest and applied to this study (101). The evolutionary history of TssB/C and Hcp proteins were inferred by using the Maximum Likelihood method based on 1) the Le and Gascuel (LG) + G + F amino acid substitution model (102) for TssB/C proteins and 2) the General Reverse Transcriptase (rtRev) + I + G + F amino acid substitution model (103) for Hcp proteins.

2.6 Availability of supporting data

The data sets supporting the results of this study are included within the supplementary files. Alignments and Phylogenetic trees that support the findings

presented in this study are available online in the Dryad Digital Repository (doi:10.5061/dryad.vd7k7).

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Table S1: BLASTP analysis of type VI secretion system (T6SS) homologs found in *Pantoea ananatis* strain LMG 20103

T6SS-1 genes	T6SS-2 homologs	T3SS-3 homologs
	% identity; E-value	% identity; E-value
PANA_2348 (*rhsD*)	-	-
PANA_2352 (*vgrG*)	137/512 (26%);3e-26	-
PANA_2353 (*tagE*)	-	-283/499 (56%); e-146
PANA_2354 (*tssH*)	400/881 (45%);0.0	-
PANA_2355 (*tssG*)	43/176 (24%);2e-012	-
PANA_2356 (*tssF*)	175/631 (27%); 8e-55	-
PANA_2357 (*tssE*)	-	-
PANA_2358 (*tagJ*)	-	-
PANA_2360 (*tagG*)	-	-126/260 (48%); 1e-67
PANA_2361 (*tagH*)	-	-163/259 (62%); 7e-84
PANA_2364 (*hcp*)	-	-
PANA_2365 (*tssC*)	192/427 (44%); e-108	-
PANA_2366 (*tssB*)	44/139 (31%); 9e-017	-
PANA_2367 (*tssA*)	33/108 (30%); 5e-005*	-
	24/66 (36%); 2e-004**	-
PANA_2368 (*tagF*)	-	-
PANA_2369 (*icmF*)	224/1070 (20%); 2e-015	361/674 (53%); 0.0
PANA_2370 (*dotU*)	-	196/382 (51%); e-113
PANA_2371 (*tssK*)	120/450 (26%); 6e-32	-
PANA_2372 (*tssJ)*	30/145 (20%); 0.13	-

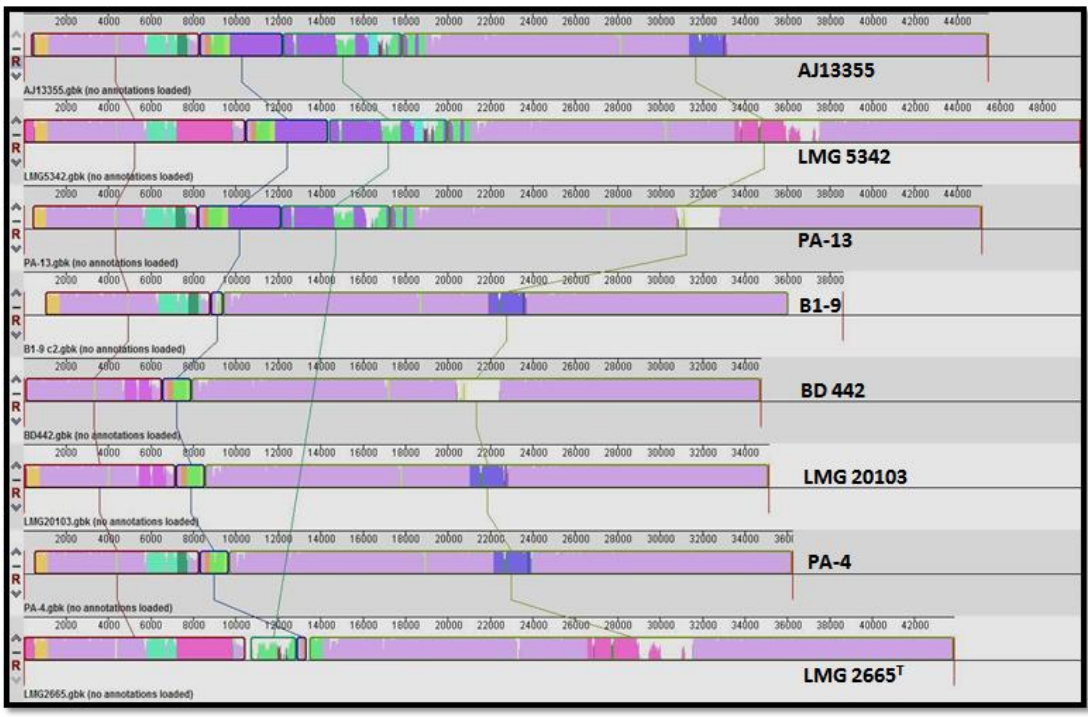
PANA_ = gene locus tags for *Pantoea ananatis* strain LMG 20103. The corresponding T6SS genes are indicated in indicated in brackets next to the locus tags. We used the T6SS gene nomenclature (*tss*/*tag*) proposed by Shalom *et al.* (2007), including some more commonly used gene names such *vgrG* (*tssI*), *hcp* (*tssD*), *icmF* (*tssM*) and *dotU* (*tssL*). The T6SS-1 genes of *Pantoea ananatis* strain LMG 20103 were used as bait for BLASTP analysis. The amino acid identity of T6SS gene homologs found in T6SS-1 and T6SS-3 gene cluster are indicated in columns 2 and 3, respectively. The two *tssA* gene homologs found in the T6SS-2 gene cluster of LMG 20103 are indicated by * = PANA_4137 and ** = PANA_4130.

Table S2: Distribution of the type VI secretion-1 and -3 gene clusters in 48 strains of *P. ananatis*

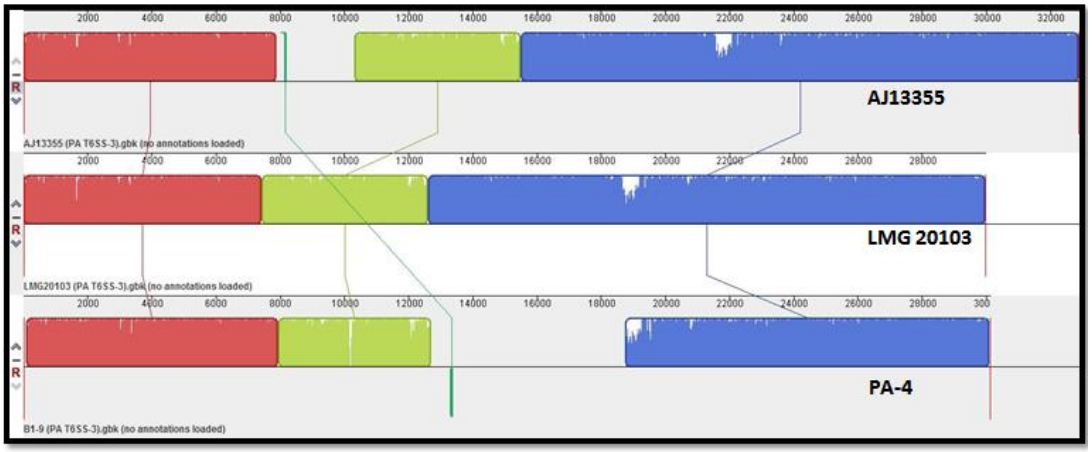
Bacteria strain	T6SS-1				PA T6SS-3			
	tssH	*hcp*	*tagF*	*tssK*	*pknA*	*pppA*	*tagH*	*dotU*
Mmir 9 (*Mirridae* sp.)	+	+	+	+	+	+	+	+
Mmir 2 (*Mirridae* sp.)	+	+	+	+	+	+	+	+
AJ13355* (soil)	+	+	+	+	+	+	+	+
LMG 5342* (clinical)	+	+	+	+	+	+	+	+
LMG 5256 (white mutant)	+	+	+	+	+	+	+	+
LMG 2807 (*Cattleya* sp.)	+	+	+	+	+	+	+	+
LMG 2628 (*Musa* sp.)	+	+	+	+	+	+	+	+
ICMP 10132 (*S. officinarum*)	+	+	+	+	+	+	+	+
Yomogi-101 (*Artemisia* sp.)	+	+	+	+	+	+	+	+
SUPP2582 (*Cucumis melo*)	+	+	+	+	+	+	+	+
SUPP1128 (*Cucumis melo*)	+	+	+	+	+	+	+	+
SUPP1791 (*Cucumis melo*)	+	+	+	+	+	+	+	+
ATCC 35400 (*Cucumis melo*)	+	+	+	+	+	+	+	+
LMG 2678 (*Puccinia graminis*)	+	+	+	+	+	+	+	+
LMG 2675 (*Puccinia graminis*)	+	+	+	+	+	+	+	+
DAR76142 (*Oryza sativa*)	+	+	+	+	+	+	+	+
RAMI7971 (*Oryza sativa*)	+	+	+	+	+	+	+	+
DAR76144 (*Oryza sativa*)	+	+	+	+	+	+	+	+
DAR76143 (*Oryza sativa*)	+	+	+	+	+	+	+	+
SUPP 2113 (*Oryza sativa*)	+	+	+	+	+	+	+	+
SUPP2219 (*Oryza sativa*)	+	+	+	+	+	+	+	+
LMG 2101 (*Oryza sativa*)	+	+	+	+	+	+	+	+
BD 442* (*Zea mays*)	+	+	+	+	+	+	+	+
BD 602 (*Zea mays*)	+	+	+	+	+	+	+	+
BD 622 (*Zea mays*)	+	+	+	+	+	+	+	+
BD 588 (*Zea mays*)	+	+	+	+	+	+	+	+
BD 647 (*Zea mays*)	+	+	+	+	+	+	+	+
BD 640 (*Zea mays*)	+	+	+	+	+	+	+	+
BD 336 (*Allium cepa*)	+	+	+	+	+	+	+	+
BD 310 (*Allium cepa*)	+	+	+	+	+	+	+	+
N/A (*Allium cepa*)	+	+	+	+	+	+	+	+
PA-4* (*Allium cepa*)	+	+	+	+	+	+	+	+
BD 301 (*Allium cepa*)	+	+	+	+	+	+	+	+
BD 315 (*Allium cepa*)	+	+	+	+	+	+	+	+
LMG 20103* (*E. grandis*)	+	+	+	+	+	+	+	+
N/A (*Eucalyptus* sp.)	+	+	+	+	+	+	+	+
Uruguay 37 (*Eucalyptus* sp.)	+	+	+	+	+	+	+	+
Uruguay 40 (*Eucalyptus* sp.)	+	+	+	+	+	+	+	+
LMG 20104 (*E. grandis*)	+	+	+	+	+	+	+	+
LMG 20105 (*E. grandis*)	+	+	+	+	+	+	+	+
LMG 2665^{T}* (*Ananas comosus*)	+	+	+	+	+	+	+	+
LMG 2664 (*Ananas comosus*)	+	+	+	+	+	+	+	+
LMG 2669 (*Ananas comosus*)	+	+	+	+	+	+	+	+
LMG 2668 (*Ananas comosus*)	+	+	+	+	+	+	+	+
LMG 2666 (*Ananas comosus*)	+	+	+	+	+	+	+	+
LMG 2667 (*Ananas comosus*)	+	+	+	+	+	+	+	+
ATCC 14028^{T} (*S.* Typhimurium)	-	-	-	-	-	-	-	-

S. officinarum = Saccharum officinarum, E. grandis = Eucalyptus grandis, S. typhimurium = Salmonella typhimurium. * = Genome sequence available. N/A = data not available.

A: Alignment of the type VI secretion system-1 (T6SS-1) found in sequenced strains of *Pantoea ananatis*



B: Alignment of type VI secretion system-2 (T6SS-2) found in sequenced strains of *Pantoea ananatis*



C: Alignment of the type VI secretion system-3 (T6SS-3) found in sequenced strains of *Pantoea ananatis*

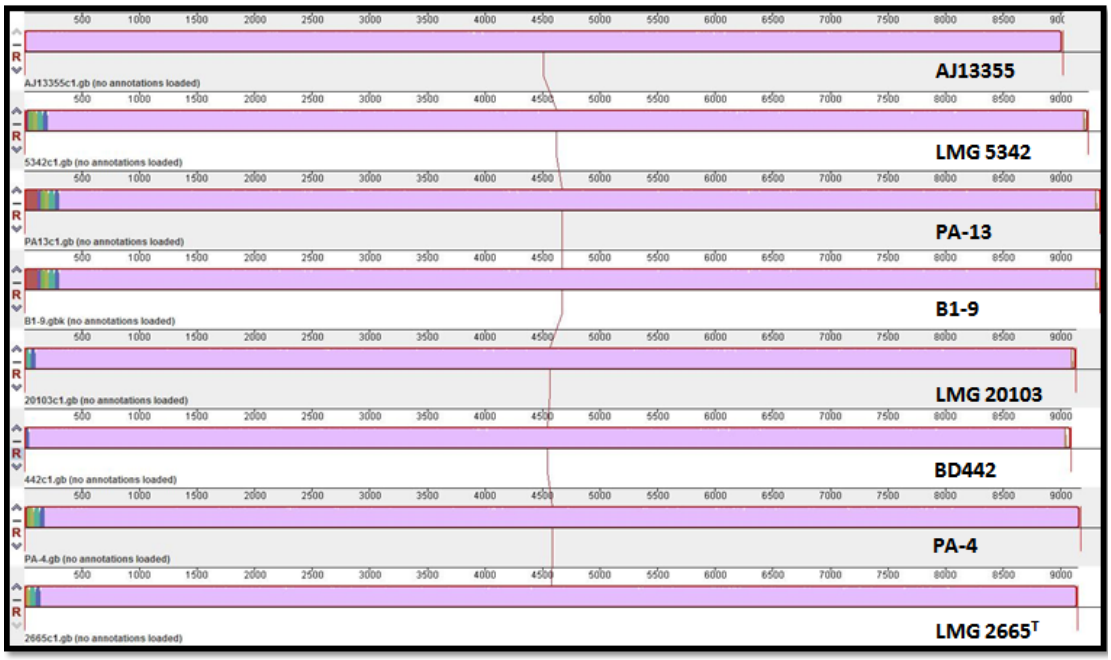


Figure S1: Synteny between the different type VI secretion system clusters encoded by strains of *Pantoea ananatis*. Figure S1C shows that the T6SS-3 gene cluster is highly syntenic in all strains of *P. ananatis* analyzed. Figures S1A and S1B show that the varible and conserved regions associated with PA T6SS-1 and PA T6SS-2 gene clusters, respectively. These variable regions are usually clustered around *hcp* and *vgrG* genes and encode mostly hypothetical proteins. The alignments were generated using Mauve v.2.3.1

Table S3: Gene content of type VI secretion system cluster 1 (T6SS-1) found in *Pantoea ananatis* strain LMG 20103

T6SS genes	Homologues	CD-Search	COGnitor	Amino acids	PSORTb	Locus_tag
rhsD	*rhsD*	PAAR/RHS	COG3209	1367	IM	PANA_2348
-	-	DUF1795	-	146	-	PANA_2349
-	-	-	-	255	-	PANA_2350
-	-	-	-	176	Extracellular	PANA_2351
tssI	*vgrG*	VI_Rhs_Vgr	COG3501	854	Cytoplasmic	PANA_2352
tagE	*pknA/ppkA*	PKc_like	COG0515	487	IM	PANA_2353
tssH	*clpV, vasG*	P-loop_NTPase	COG0542	869	Cytoplasmic	PANA_2354
tssG	*impH, vasB*	DUF1305	COG3520	349	-	PANA_2355
tssF	*impG, vasA*	DUF879	COG3519	625	-	PANA_2356
tssE	*impF*	GPW_gp25	COG3518	191	-	PANA_2357
tagJ	*impE*	ImpE	COG4455	275	-	PANA_2358
-	-	-	-	337	Periplasmic	PANA_2359
tagG	*pppA*	PP2Cc	COG0631	263	-	PANA_2360
tagH	*fha*	FHA	COG3456	631	Cytoplasmic	PANA_2361
-	-	DUF1311	-	133	-	PANA_2362
-	-	PG_binding_1	COG3409	291	Cytoplasmic	PANA_2363
tssD	*hcp*	DUF796	COG3157	160	Extracellular	PANA_2364
tssC	*impC, vipB*	DUF877	COG3517	499	Cytoplasmic	PANA_2365
tssB	*impB, vipA*	DUF770	COG3516	176	Cytoplasmic	PANA_2366
tssA	*impA*	ImpA-rel_N	COG3515	340	Cytoplasmic	PANA_2367
tagF	*impM*	DUF2094	COG3913	237	Periplasmic	PANA_2368
tssM	*vasK, icmF*	IcmF-related	COG3523	1208	IM	PANA_2369
tssL	*ompA/dotU*	OmpA_C-like	COG3455	412	IM	PANA_2370
tssK	*imp, vase*	DUF876	COG3522	448	Cytoplasmic	PANA_2371
tssJ	*vasD, lip*	T6SS-SciN	COG3521	167	-	PANA_2372
-	-	-	-	195	Periplasmic	PANA_2373
-	*matE*	MatE	COG0534	479	IM	PANA_2374

The contigous region representing T6SS-1 was identified from the published genome sequence and used for *ab initio* gene predictions as detailed in Materials and Methods. T6SS-1 is flanked by *rhsD* and *matE* genes, which are not T6SS genes. We used the T6SS gene nomenclature proposed by Shalom *et al.* (2007). Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. IM = inner membrane.

Table S4: Gene content of type VI secretion system cluster 1 (T6SS-1) found in *Pantoea ananatis* strain PA-13

T6SS genes	Homologues	CD-Search	COGnitor	Amino acids	PSORTb	Locus_tag
-	*rhsD*	PAAR/RHS	COG3209	1462	IM	PAGR_g1689
-	-	DUF1795	-	146	-	PAGR_g1688
-	-	-	-	266	-	PAGR_g1687
-	-	DUF 1311	-	210	-	PAGR_g1686
-	-	-	-	126	-	PAGR_g1685
tssI	*vgrG*	VI_Rhs_Vgr	COG3501	829	Cytoplasmic	PAGR_g1684
-	-	PAAR_motif	-	130	-	PAGR_g1683
-	-	DUF3540	-	201	-	PAGR_g1682
-	-	pentapeptide_4	COG1357	356	-	PAGR_g1681
-	-	pentapeptide_4	COG1357	846	Extracellular	PAGR_g1680
-	-	-	-	128	-	PAGR_g1679
-	-	-	-	124	-	PAGR_g1678
-	-	-	-	126	IM	PAGR_g1677
tssI	*vgrG*	VI_Rhs_Vgr	COG3501	747	cytoplasmic	PAGR_g1676
tagE	*pknA/ppkA*	PKc_like	COG0515	487	IM	PAGR_g1675
tssH	*clpV, vasG*	P-loop_NTPase	COG0542	869	Cytoplasmic	PAGR_g1674
tssG	*impH, vasB*	DUF1305	COG3520	349	-	PAGR_g1673
tssF	*impG, vasA*	DUF879	COG3519	625	-	PAGR_g1672
tssE	*impF*	GPW_gp25	COG3518	191	-	PAGR_g1671
tagJ	*impE*	ImpE	COG4455	275	-	PAGR_g1670
-	-	-	-	332	Periplasmic	PAGR_g1669
tagG	*pppA*	PP2Cc	COG0631	263	-	PAGR_g1668
tagH	*fha*	FHA	COG3456	631	Cytoplasmic	PAGR_g1667
-	-	DUF1311	-	271	Periplasmic	PAGR_g1666
-	-	PG_binding_1	COG3409	276	-	PAGR_g1665
tssD	*hcp*	DUF796	COG3157	160	Extracellualr	PAGR_g1664
tssC	*impC, vipB*	DUF877	COG3517	499	Cytoplasmic	PAGR_g1663
tssB	*impB, vipA*	DUF770	COG3516	176	Cytoplasmic	PAGR_g1662
tssA	*impA*	ImpA-rel_N	COG3515	340	Cytoplasmic	PAGR_g1661
tagF	*impM*	DUF2094	COG3913	237	Periplasmic	PAGR_g1660
tssM	*vasK, icmF*	IcmF-related	COG3523	1208	IM	PAGR_g1659
tssL	*ompA/dotU*	OmpA_C-like	COG3455	412	IM	PAGR_g1658
tssK	*impJ, vase*	DUF876	COG3522	448	Cytoplasmic	PAGR_g1657
tssJ	*vasD, lip*	T6SS-SciN	COG3521	167	-	PAGR_g1656
-	-	-	-	195	Periplasmic	PAGR_g1655
-	*matE*	MatE	COG0534	472	IM	PAGR_g1654

PAGR_g1692 (150 nucleotides) was annotated in the genome as a putative Rhs YD repeat-containing protein. We did not predict this gene during our gene prediction and as such was eliminated from all analysis. Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. IM = inner membrane.

Table S5: Gene content of type VI secretion system cluster 1 (T6SS-1) found in *Pantoea ananatis* strain LMG 5342

T6SS genes	Homologues	CD-search	COGnitor	Amino acids	PSORTb	Locus_tag
-	*rhsD*	PAAR/RHS/DUF4237	COG3209	1484	IM	PANA5342_1754
-	-	DUF1795	-	146	-	PANA5342_1753
-	-	-	-	266	-	PANA5342_1752
-	-	DUF1311	-	210	-	PANA5342_1751
-	-	-	-	116	-	ORF1
-	-	-	-	160	-	ORF2
-	-	-	-	186	-	PANA5342_1750
tssI	*vgrG*	VI_Rhs_Vgr	COG3501	903	-	PANA5342_1749
-	-	PAAR_motif	-	130	-	PANA5342_1748
-	-	DUF3540	-	201	-	PANA5342_1747
-	-	pentapeptide_4	COG1357	356	-	PANA5342_1746
-	-	pentapepdide_4	COG1357	846	Extracellular	PANA5342_1745
-	-	-	-	99	-	ORF3
-	-	-	-	115	-	ORF4
-	-	-	-	126	IM	ORF5
-	-	-	-	125	-	ORF6
tssI	*vgrG*	VI_Rhs_Vgr	COG3501	741	Cytoplasmic	PANA5342_1744
tagE	*pknA/ppkA*	Pkc_like	COG0515	487	IM	PANA5342_1743
tssH	*clpV, vasG*	AAA+ ATPase	COG0542	869	Cytoplasmic	PANA5342_1742
tssG	*impH, vasB*	DUF1305	COG3520	349	-	PANA5342_1741
tssF	*impG, vasA*	DUF879	COG3519	625	-	PANA5342_1740
tssE	*impF*	GPW_gp25	COG3518	191	-	PANA5342_1739
tagJ	*impE*	ImpE	COG4455	275	-	PANA5342_1738
-	-	-	-	337	Periplasmic	PANA5342_1737
tagG	*pppA*	PP2Cc	COG0631	263	-	PANA5342_1736
tagH	*fha*	FHA	COG3456	631	Cytoplasmic	PANA5342_1735
-	-	DUF1311 #	-	133		PANA5342_1734
-	-	-	-	183		PANA5342_1733
-	-	DUF1311 #	-	286	-	PANA5342_1732
-	-	-	-	183	-	ORF7
-	-	PG_binding_1	-	285	-	PANA5342_1731
tssD	*hcp*	DUF796	COG3157	160	Extracellular	PANA5342_1730
tssC	*impC, vipB*	DUF877	COG3517	499	Cytoplasmic	PANA5342_1729
tssB	*impB, vipA*	DUF770	COG3516	176	Cytoplasmic	PANA5342_1728
tssA	*impA*	ImpA-rel_N	COG3515	340	Cytoplasmic	PANA5342_1727
tagF	*impM*	DUF2094	COG3913	237	Periplasmic	PANA5342_1726
tssM	*vasK, icmF*	IcmF-related	COG3523	1208	IM	PANA5342_1725
tssL	*ompA/dotU*	OmpA_C-like	COG3455	412	IM	PANA5342_1724
tssK	*impJ, vasE*	DUF876	COG3522	448	Cytoplasmic	PANA5342_1723
tssJ	*vasD, lip*	T6SS-SciN	COG3521	167	-	PANA5342_1722
-	-	-	-	195	Periplasmic	PANA5342_1721
-	*matE*	MatE	COG0534	472	IM	PANA5342_1720

= genes contain the same DUF1311 domain with low amino acid similarity 59/183(32%). ORF1-7 corresponds to intergenic regions not annotated as genes in the published genome sequence but showed strong homology to genes encoding hypothetical proteins annotated in other sequenced strains of *P. ananatis*. Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. IM = inner membrane

Table S6: Gene content of type VI secretion system cluster 1 (T6SS-1) found in *Pantoea ananatis* strain AJ13355

T6SS genes	Homologues	CD-search	COGnitor	Amino acids	PSORTb	Locus_tag
-	*rhsD*	PAAR/RHS/Rhs_ass	COG3209	1500	IM	PAJ_1646
-	-	DUF1795	-	146	-	ORF1
-	-	-	-	266	-	PAJ_1647
-	-	DUF1311	-	209	-	PAJ_1648
-	-	-	-	126	-	ORF2
tssI‡	*vgrG*	VI_Rhs_Vgr	COG3501	542	Cytoplasmic	PAJ_1649
tssI‡	-	phage_GPD	COG3501	285	Cytoplasmic	PAJ_1650
-	-	PAAR	-	130	-	ORF3
-	-	DUF3540 (pfam12059)	-	201	-	PAJ_1651
-	-	Pentapeptide_4	COG1357	356	-	PAJ_1652
-	-	Dpentapeptide_4	COG1357	846	Extracellular	PAJ_1653
-	-	-	-	99	-	ORF4
-	-	-	-	115	-	ORF5
-	-	-	-	126	IM	ORF6
-	-	-	-	125	-	ORF7
tssI	*vgrG*	VI_Rhs_Vgr	COG3501	685	Cytoplasmic	PAJ_1654
tagE	*pknA/ppkA*	Pkc_like	COG0515	487	IM	PAJ_1655
tssH	*clpV, vasG*	AAA+ ATPase	COG0542	869	Cytoplasmic	PAJ_1656
tssG	*impH, vasB*	DUF1305	COG3520	349	-	PAJ_1657
tssF	*impG, vasA*	DUF879	COG3519	625	-	PAJ_1658
tssE	*impF*	GPW_gp25	COG3518	191	-	PAJ_1659
tagJ	*impE*	ImpE	COG4455	275	-	PAJ_1660
-	-	-	-	337	Periplasmic	PAJ_1661
tag	*pppA*	PP2Cc	COG0631	263	-	PAJ_1662
tagH	*fha*	FHA	COG3456	631	Cytoplasmic	PAJ_1663
-	-	DUF1311	-	133	-	ORF8
-	-	PG_binding_1	COG3409	291	Cytoplasmic	PAJ_1664
tssD	*hcp*	DUF796	COG3157	160	Extracellular	PAJ_1665
tssC	*impC, vipB*	DUF877	COG3517	499	Cytoplasmic	PAJ_1666
tssB	*impB, vipA*	DUF770	COG3516	176	Cytoplasmic	PAJ_1667
tssA	*impA*	ImpA-rel_N	COG3515	340	Cytoplasmic	PAJ_1668
tagF	*impM*	DUF2094	COG3913	237	Periplasmic	PAJ_1669
tssM	*vasK, icmF*	IcmF-related/	COG3523	1107	IM	PAJ_1670
tssL	*ompA/dotU*	OmpA_C-like	COG3455	412	IM	PAJ_1671
tssK	*impJ, vase*	DUF876	COG3522	448	Cytoplasmic	PAJ_1672
tssJ	*vasD, lip*	T6SS-SciN	COG3521	167	-	PAJ_1673
-	-	-	-	195	Periplasmic	PAJ_1674
-	*matE*	Multi-drug efflux pump	COG0534	431	Inner membrane	PAJ_1675

‡ = interrupted by a stop codon generating two genes representing the N-terminal and the C-terminal of full-length *tssI* gene. ORF1-8 corresponds to intergenic regions not annotated as genes in the published genome sequence of strain AJ13355, but showed strong homology to genes encoding hypothetical proteins in other sequenced strains of *Pantoea ananatis*. Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. IM = inner membrane.

Table S7: Gene content of type VI secretion system cluster 1 (T6SS-1) found in *Pantoea ananatis* strain PA-4

T6SS genes	Homologues	CDD search	COGnitor	Amino acids	Psortb	Locus_tag	% amino acid identity to sequenced *P. ananatis* strains
-	*rhsD*	PAAR	COG3209	1479	IM	N455_00500	PAGR_g1689 (98)
-	-	DUF1795	-	146	-	N455_00501	PANA5342_1753 (99)
-	-	-	-	266	-	N455_00502	PAJ_1647 (99)
-	-	DUF1311	-	247	-	N455_00503	PAJ_1648 (99)
-	-	-	-	126	-	N455_00504	PAGR_g1685 (100)
*tssI**	*vgrG*	VI_Rhs_Vgr	COG3501	827	Cytoplasmic	N455_00505	PAGR_g1684 (99)
*tagE**	*pknA/ppkA*	Pkc_like	COG0515	451	IM	N455_00506	PAJ_1655 (92)
tssH	*clpV, vasG*	AAA+ ATPase	COG0542	869	Cytoplasmic	N455_00507	PANA5342_1742 (100)
tssG	*impH, vasB*	DUF1305	COG3520	349	-	N455_00508	PANA_2355 (100)
tssF	*impG, vasA*	DUF879	COG3519	625	-	N455_00509	PANA_2356 (100)
tssE	*impF*	GPW_gp25	COG3518	191	-	N455_00510	PANA_2357 (100)
tagJ	*impE*	ImpE	COG4455	275	-	N455_00511	PANA_2358 (99)
-	-	-	-	337	Periplasmic	N455_00512	PANA_2359 (99)
tagG	*pppA*	PP2Cc	COG0631	263	-	N455_00513	PANA_2360 (100)
tagH	*fha*	FHA	COG3456	631	Cytoplasmic	N455_00514	PANA_2361 (99)
-	-	DUF1311	-	133	-	N455_00515	PANA_2362 (99)
-	-	PG_binding_1	COG3409	291	Cytoplasmic	N455_00516	PANA_2363 (99)
tssD	*hcp*	DUF796	COG3157	160	Extracellular	N455_00517	PANA_2364 (100)
tssC	*impC, vipB*	DUF877	COG3517	499	Cytoplasmic	N455_00518	PANA_2365 (99)
tssB	*impB, vipA*	DUF770	COG3516	176	Cytoplasmic	N455_00519	PANA_2366 (100)
tssA	*impA*	ImpA-rel_N	COG3515	340	Cytoplasmic	N455_00520	PANA_2367 (100)
tagF	*impM*	DUF2094	COG3913	237	periplasmic	N455_00521	PANA_2368 (99)
tssM	*vasK, icmF*	IcmF	COG3523	1208	IM	N455_00522	PANA_2369 (99)
tssL	*ompA/dotU*	OmpA_C-like	COG3455	412	IM	N455_00523	PANA_2370 (100)
tssK	*impJ, vasE*	DUF876	COG3522	448	IM	N455_00524	PANA_2371 (100)
tssJ	*vasD, lip*	T6SS-SciN	COG3521	167	-	N455_00525	PANA_2372 (100)
-	-	-	-	195	Periplasmic	N455_00526	PANA_2373 (99)
-	*matE*	MatE	COG0534	479	IM	N455_00527	PANA_2374 (99)

Putative genes were given the provisional locus tags N455_00500 to N455_00527. BLASTP analysis were done and the percentage identity to homologs found in sequenced strains of *P. ananatis* recorded. * = the N- and C-terminal of the full-length gene are found in two unassembled contigs. Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. IM = inner membrane.

Table S8: Gene content of type VI secretion system cluster 1 (T6SS-1) found in *Pantoea ananatis* strain BD442

T6SS genes	Homologues	CDD search	COGnitor	Amino acids	Psortb	Locus_tag	% identity to sequenced *P. ananatis* strains
-	*rhsD*	-	COG3209	1461	-	N453_00200	PANA_2348 (97)
-	-	DUF1795	-	146	-	N453_00201	PANA_2349 (99)
-	-	-	-	255	-	N453_00202	PAGR_g1687 (98)
-	-	-	-	176	-	N453_00203	PANA_2351(100)
*tssI**	*vgrG*	VI_Rhs_Vgr	COG3501	823	Cytoplasmic	N453_00204	PANA_2352 (96)
tagE	*pknA/ppkA*	Pkc_like	COG0515	488	IM	N453_00205	PANA_2353 (100)
tssH	*clpV, vasG*	AAA+ ATPase	COG0542	869	Cytoplasmic	N453_00206	PANA_2354 (99)
tssG	*impH, vasB*	DUF1305	COG3520	350	-	N453_00207	PANA_2355 (100)
tssF	*impG, vasA*	DUF879	COG3519	626	-	N453_00208	PANA_2356 (100)
tssE	*impF*	GPW_gp25	COG3518	191	-	N453_00209	PANA_2357 (100)
tagJ	*impE*	ImpE	COG4455	275	-	N453_00210	PANA_2358 (99)
-	-	-	-	282	-	N453_00211	PANA_2359 (81)*
tagG	*pppA*	PP2Cc	COG0631	263	-	N453_00212	PANA_2360 (100)
tagH	*fha*	FHA	COG3456	631	Cytoplasmic	N453_00213	PANA_2361 (99)
-	-	DUF1311	-	271	Periplasmic	N453_00214	PAGR_g1666(100)
-	-	lysozyme_like	COG3409	276	-	N453_00215	PAGR_g1665(100)
tssD	*hcp*	DUF796	COG3157	160	Extracellular	N453_00216	PANA_2364 (100)
tssC	*impC, vipB*	DUF877	COG3517	499	Cytoplasmic	N453_00217	PANA_2365 (100)
tssB	*impB, vipA*	DUF770	COG3516	176	Cytoplasmic	N453_00218	PANA_2366 (100)
tssA	*impA*	ImpA-rel_N	COG3515	340	Cytoplasmic	N453_00219	PANA_2367 (100)
tagF	*impM*	DUF2094	COG3913	237	Periplasmic	N453_00220	PANA_2368 (100)
tssM	*vasK, icmF*	IcmF-related	COG3523	1208	IM	N453_00221	PANA_2369 (100)
tssL	*ompA/dotU*	OmpA_C-like	COG3455	412	IM	N453_00222	PANA_2370 (100)
tssK	*impJ, vasE*	DUF876	COG3522	448	cytoplasmic	N453_00223	PANA_2371 (100)
tssJ	*vasD, lip*	T6SS-SciN	COG3521	167	IM	N453_00224	PANA_2372 (100)
-	-	-	-	195	Periplasmic	N453_00225	PANA_2373 (99)
-	*matE*	MatE	COG0534	479	IM	N453_00226	PANA_2374 (99)

* = the N- and C-terminal of full-length gene are found in two unassembled contigs. Putative genes were given the provisional locus tags N453_00200 to N453_00226. BLASTP analysis were done and the percentage identity to homologs found in sequenced strains of *P. ananatis* recorded. Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. IM = inner membrane.

Table S9: Gene content of type VI secretion system cluster 1 (T6SS-1) found in *Pantoea ananatis* strain LMG 2665^{T}

T6SS genes	Homologues	CD search	COGnitor	Amino acids	PSORTb	Locus_tag	% amino acid identity to sequenced *P. ananatis* strains
-	*rhsD*	PAAR/RHS	COG3209	1455	IM	N454_00601	PANA5342_1754 (99)
-	-	DUF1795	-	146	-	N454_00602	PANA5342_1753 (99)
-	-	-	-	282	-	N454_00603	PANA5342_1752 (92)
-	-	DUF1311	-	209	-	N454_00604	PANA5342_1751 (93)
-	-	-	-	116	-	N454_00605	PAGR_g1685 (88)
-	-	-	-	160	-	N454_00606	PANA_2351 (54)
-	-	-	-	186	-	N454_00607	PANA5342_1750 (100)
tssI	*vgrG*	LYSOZYME	COG3501	435	Extracellular	N454_00608	PANA5342_1749 (100)
-	-	DUF3592	-	125	-	N454_00609	Pecwa_2444 (46)
-	-	-	-	93	-	N454_00610	PAGR_g1679 (59)
-	-	-	-	125	-	N454_00611	PAGR_g1678 (59)
-	*vgrG*	VI_Rhs_Vgr	COG3501	741	-	N454_00612	PANA_2352 (74/44)
tagE	*pknA/ppkA*	Pkc_like	COG0515	487	IM	N454_00613	PANA_2353 (98)
*tssH**	*clpV, vasG*	AAA+ ATPase	COG0542	824	Cytoplasmic	N454_00614	PANA_2354(94)
tssG	*impH, vasB*	DUF1305	COG3520	349	-	N454_00615	PANA_2355 (99)
tssF	*impG, vasA*	DUF879	COG3519	625	-	N454_00616	PANA_2356 (99)
tssE	*impF*	GPW_gp25	COG3518	191	-	N454_00617	PANA_2357 (100)
tagJ	*impE*	ImpE	COG4455	275	-	N454_00618	PANA_2358 (100)
-	-	-	-	344	-	N454_00619	PANA_2359 (90)*
tagG	*pppA*	PP2Cc	COG0631	263	-	N454_00620	PANA_2360 (100)
tagH	*fha*	FHA	COG3456	631	Cytoplasmic	N454_00621	PANA_2361 (99)
-	-	DUF1311	COG3755	133	-	N454_00622	PANA5342_1734 (100)
-	-	-	-	183	-	N454_00623	PANA5342_1733 (100)
-	-	DUF1311	-	269	-	N454_00624	PANA5342_1732 (96)
-	-	DUF1311	-	132	-	N454_00625	PANA_2362 (47)
-	-	-	-	187	-	N454_00626	PANA5342_1733 (29)
-	-	-	-	137	IM	N454_00627	PanABDRAFT_2447 (42)
-	-	-	-	306	-	N454_00628	Pat9b_2515 (73)
tssD	*hcp*	DUF796	COG3157	160	Extracellular	N454_00629	PANA_2364 (100)
tssC	*impC, vipB*	DUF877	COG3517	499	Cytoplasmic	N454_00630	PANA_2365 (99)
tssB	*impB, vipA*	DUF770	COG3516	176	Cytoplasmic	N454_00631	PANA_2366 (99)
tssA	*impA*	ImpA-rel_N	COG3515	340	Cytoplasmic	N454_00632	PANA_2367 (99)
tagF	*impM*	DUF2094	COG3913	237	Periplasmic	N454_00633	PANA_2368 (100)
tssM	*vasK, icmF*	IcmF-related	COG3523	1208	IM	N454_00634	PANA_2369 (99)
tssL	*ompA/dotU*	OmpA_C-like	COG3455	412	IM	N454_00635	PANA_2370 (100)
tssK	*impJ, vasE*	DUF876	COG3522	448	Cytoplasmic	N454_00636	PANA_2371 (100)
tssJ	*vasD, lip*	T6SS-SciN	COG3521	167	IM	N454_00637	PANA_2372 (100)
-	-	-	-	195	Periplasmic	N454_00638	PANA_2373 (99)
-	*matE*	MatE	COG0534	497	IM	N454_00639	PANA_2374 (100)

* = the N- and C-terminal of full-length gene are found in two unassembled contigs. Putative genes were given the provisional locus tags N454_00601 to N454_00639. BLASTP analysis were performed and the percentage identity to homologs found in sequenced strains of *P. ananatis* recorded.

Table S10: Gene content of type VI secretion system cluster 1 (T6SS-1) found in *Pantoea ananatis* strain B1-9

T6SS genes	Homologues	CD search	COGnitor	Amino acids	PSORTb	Locus_tag	% amino acid identity to sequenced *P. ananatis* strains
-	*rhsD*	-	-	1485	IM	B1-9 C2(1)	PANA5342_1754(99)
-	-	DUF1795	-	150	-	B1-9 C2(2)	PANA5342_1753 (99)
-	-	-	-	300	Unknown	B1-9 C2(3)	PANA5342_1753(99)
-	-	DUF1311	-	271	-	B1-9 C2(4)	PANA5342_1751(97)
-	-	-	-	129	Unknown	B1-9 C2(5)	PAGR_g1685 (100)
tssI	*vgrG*	VI_Rhs_Vgr	COG3501	574	Cytoplasmic	B1-9 C2(6)	PAJ_1649(99)
tagE	*pknA/ppkA*	Pkc_like	COG0515	508	IM	B1-9 C2(7)	PANA_2353(98)
tssH	*clpV, vasG*	AAA+ ATPase	COG0542	892	Cytoplasmic	B1-9 C2(8)	PANA_2354(100)
tssG	*impH, vasB*	DUF1305	COG3520	377	-	B1-9 C2(9)	PAGR_g1673(100)
tssF	*impG, vasA*	DUF879	COG3519	632	-	B1-9 C2(10)	PAGR_g1673(100)
tssE	*impF*	GPW_gp25	COG3518	204	-	B1-9 C2(11)	PANA_2357(100)
tagJ	*impE*	ImpE	COG4455	275	-	B1-9 C2(12)	PANA_2357(99)
-	-	-	-	364	Periplasmic	B1-9 C2(13)	PANA_2359(99)
tagG	*pppA*	PP2Cc	COG0631	264	-	B1-9 C2(14)	PANA_2360(100)
tagH	*fha*	FHA	COG3456	636	Cytoplasmic	B1-9 C2(15)	PAJ_1663(100)
-	-	DUF1311	-	137	-	B1-9 C2(16)	PANA_2362(99)
-	-	PG_binding_1	-	291	Cytoplasmic	B1-9 C2(17)	PANA_2363(99)
tssD	*hcp*	DUF796	-	165	Extracellular	B1-9 C2(18)	PANA_2364(100)
tssC	*impC, vipB*	DUF877	COG3517	503	Cytoplasmic	B1-9 C2(19)	PANA_2365(100)
tssB	*impB, vipA*	DUF770	COG3516	201	Cytoplasmic	B1-9 C2(20)	PANA_2366(99)
tssA	*impA*	ImpA-rel_N	COG3515	373	Cytoplasmic	B1-9 C2(21)	PANA_2367(100)
tagF	*impM*	DUF2094	COG3913	245	Periplasmic	B1-9 C2(22)	PANA_2368(100
tssM	*vasK, icmF*	IcmF-related	COG3523	1209	IM	B1-9 C2(23)	PANA_2369(99)
tssL	*ompA/dotU*	OmpA_C-like	COG3455	415	IM	B1-9 C2(24)	PANA_2370(100)
tssK	*impJ, vasE*	DUF876	COG3522	453	Cytoplasmic	B1-9 C2(25)	PANA_2371(100)
tssJ	*vasD, lip*	T6SS-SciN	COG3521	176	-	B1-9 C2(26)	PANA_2372(100)
-	-	-	-	246	Periplasmic	B1-9 C2(27)	PANA_2373(99)
-	*matE*	MatE	COG0534	491	IM	B1-9 C2(28)	PANA_2374(99)

Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. Putative genes were given the provisional locus tags B1-9 C2 (1 to 28). BLASTP analysis were done and the percentage identity to homologs found in sequenced strains of *P. ananatis* recorded. IM = inner membrane.

Table S11: Gene content of type VI secretion system cluster 2 (T6SS-2) found in *Pantoea ananatis* strain 20103

T6SS genes	Homologues	CD-Search	COGnitor	Amino acids	PSORTb	Locus_tag
-	*ybhB/ybcL*	PEBP	COG1881	178	-	PANA_4128
-	*ydcX*	DUF2566	-	57	IM	PANA_4129
tssA	*vasJ/L, impA*	-	COG3515	463	-	PANA_4130
tssE	*gp25*	VI_zyme	COG3518	151	-	PANA_4131
tssJ	*vasD, lip*	T6SS-SciN	COG3521	181	-	PANA_4132
tssG	*impH, vasB*	DUF1305	COG3520	361	-	PANA_4133
tssF	*impG, vasA*	DUF879	COG3519	584	Cytoplasmic	PANA_4134
-	-	-	-	144	IM	PANA_4135
-		PAAR_motif	-	424	-	PANA_4136
tssA	*vasJ/L, impA*	*impA*-rel_	COG3515	533	Cytoplasmic	PANA_4137
tssM	*impL, icmF*	IcmF-related	COG3523	1161	IM	PANA_4138
-	-	-	-	351	IM	PANA_4139
-	-	-	-	162	IM	PANA_4140
-	-	-	-	93	-	PANA_4141
-	-	-	-	234	-	PANA_4142
-	-	petidase M_23	-	743	Cytoplasmic	PANA_4143
tssI	*vgrG*	VI_Rhs_Vgr	COG3501	783	Cytoplasmic	PANA_4144
tssH	*clpV, vasG*	AAA+ ATPase	COG0542	884	Cytoplasmic	PANA_4145
tssD	*hcp*	DUF796	COG3157	163	Extracellular	PANA_4146
tagL	*c3389*	OmpA_C-like	COG2885/COG1360	574		PANA_4147
tssL	*impK, ompA*	DUF2077	COG3455	229	Cytoplasmic	PANA_4148
tssK	*impJ, vase*	DUF876	COG3522	446	Cytoplasmic	PANA_4149
tssC	*impC*	DUF877	COG3517	513	Cytoplasmic	PANA_4150
tssB	*impB*	DUF770	COG3516	241	Cytoplasmic	PANA_4151
-	*clcA*	Voltage_CLC	COG0038	346	IM	PANA_4152

Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. We used the T6SS gene nomenclature proposed by Shalom *et al.* (2007), *tss* (type VI secretion gene) represents conserved T6SS genes, while *tag* (type VI associated gene) represents accessory T6SS genes found in the T6SS gene clusters of a few bacteria.

Table S12: Gene content of type VI secretion system cluster 2 (T6SS-2) found in *Pantoea ananatis* strain AJ13355

T6SS genes	Homologues	CD-Search	COGnitor	Amino acids	PSORTb	Locus_tag
-	*ybhB/ybcL*	PEBP	COG1881	178	-	PAJ_p0162
-	*ydcX*	DUF2566	-	57	IM	PAJ_p0161
tssA	*impA*	-	COG3515	463	-	PAJ_p0160
tssE	*gp25*	VI_zyme	COG3518	151	-	PAJ_p0159
tssJ	*vasD, lip*	T6SS-SciN	COG3521	181	-	PAJ_p0158
tssG	*impH, vasB*	DUF1305	COG3520	361	-	PAJ_p0157
tssF	*impG, vasA*	DUF879	COG3519	584	Cytoplasmic	PAJ_p0156
-	-	-	-	144	IM	PAJ_p0155
-	-	PAAR_motif	-	424	-	PAJ_p0154
-	-	-	-	142	IM	PAJ_p0153
-	-	-	-	127	IM	PAJ_p0152
-	-	PAAR_motif	-	425	Cytoplasmic	PAJ_p0151
tssA	*vasJ/L, impA*	*impA*-rel_	COG3515	533	Cytoplasmic	PAJ_p0150
*tssM**	*impL, icmF*	IcmF-related	COG3522	515	-	PAJ_p0149*
*tssM**	*impL, icmF*	IcmF-related	COG3522	591	IM	PAJ_p0148*
-	-	-	-	351	IM	PAJ_p0147
-	-	-	-	162	IM	PAJ_p0146
-	-	-	-	93	-	PAJ_p0145
-	-	-	-	234	-	PAJ_p0144
-	-	petidase M_23	-	743	Cytoplasmic	PAJ_p0143
tssI	*vgrG*	VI_Rhs_Vgr	COG3501	783	Cytoplasmic	PAJ_p0142
tssH	*clpV, vasG*	AAA+ ATPase	COG542	886	Cytoplasmic	PAJ_p0141
tssD	*Hcp*	DUF796	COG3157	163	Extracellular	PAJ_p0140
tagL	*c3389*	OmpA_C-like	COG2885/ COG1360	574	IM	PAJ_p0139
tssL	*impK, ompA, vasF*	DUF2077	COG3455	229	Cytoplasmic	PAJ_p0138
tssK	*impJ, vase*	DUF876	COG3522	446	Cytoplasmic	PAJ_p0137
tssC	*impC*	DUF877	COG3517	513	Cytoplasmic	PAJ_p0136
tssB	*impB*	DUF770	COG3516	165	Cytoplasmic	PAJ_p0135
-	*clcA*	Voltage_CLC	COG0038	225	IM	PAJ_p0134*
-	*clcA*	Voltage_CLC	COG0038	178	IM	PAJ_p0133*

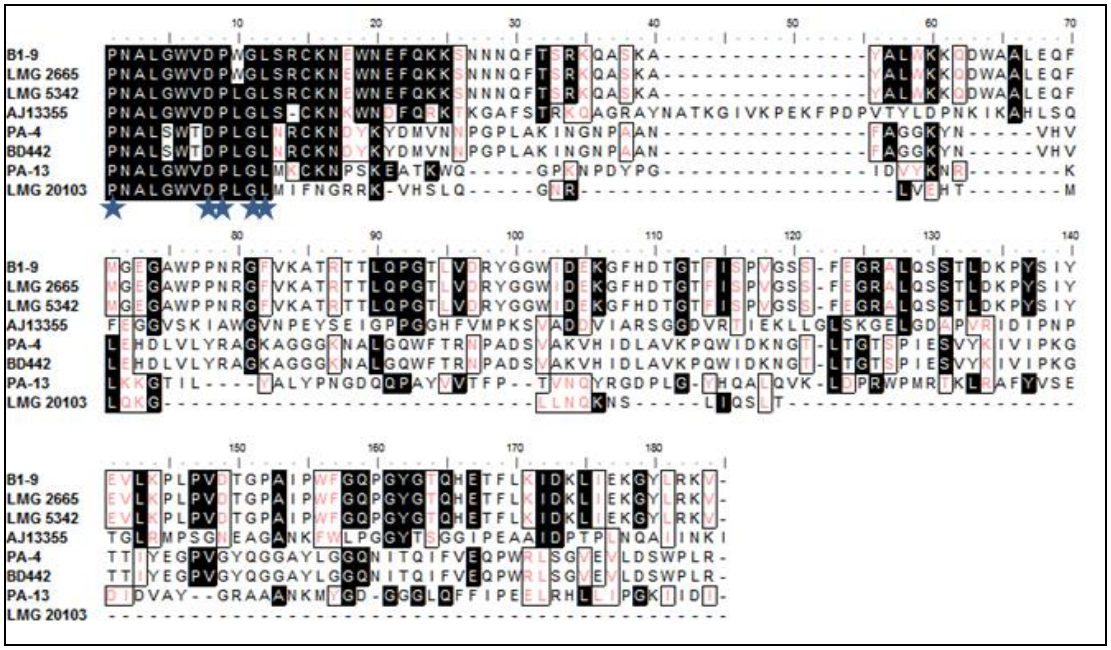
We used the T6SS gene nomenclature proposed by Shalom *et al.* (2007). The *tss* genes (type VI secretion gene) represents conserved T6SS genes, while the *tag* genes (type VI associated gene) represents accessory T6SS genes found in the T6SS gene clusters of a few bacteria. * = truncated *tssM* gene, interrupted by a stop codon generating two ORFs corresponding to the C- and N-terminals of full-length *tssM* gene. Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. IM = inner membrane.

Table S13: Gene content of type VI secretion system cluster 2 (T6SS-2) found in *Pantoea ananatis* strain PA-4

T6SS genes	Homologues	CD-Search	COGnitor	Amino acids	PSORTb	Locus_tag	% amino acid identity to sequenced *P. ananatis* strains
-	*ybhB/ybcL*	PEBP	COG1881	178	-	N455_00698	PANA_4128 (99)
-	*ydcX*	DUF2566	-	57	IM	N455_00699	PANA_4129 (98)
tssA	*vasJ/L, impA*	-	COG3515	463	-	N455_00700	PANA_4130 (98)
tssE	*gp25*	VI_zyme	COG3518	151	-	N455_00701	PANA_4131 (99)
tssJ	*vasD, lip*	T6SS-SciN	COG3521	181	-	N455_00702	PANA_4132 (94)
tssG	*impH, vasB*	DUF1305	COG3520	361	-	N455_00703	PANA_4133 (99)
tssF	*impG, vasA*	DUF879	COG3519	584	Cytoplasmic	N455_00704	PANA_4134 (100)
-	-	-	-	144	IM	N455_00705	PANA_4135 (100)
-	-	PAAR_motif	-	424	-	N455_00706	PANA_4136 (100)
tssA	*vasJ/L, impA*	*impA*-rel_	COG3515	533	Cytoplasmic	N455_00707	PANA_4137 (100)
tssM	*impL, icmF*	IcmF-related	COG3523	1172	IM	N455_00708	PANA_4138 (89)
-	-	-	-	346	IM	N455_00709	unique protein
-	-	PAAR_motif	-	92	-	N455_00710	unique protein ‡‡
-	-	-	-	363	-	N455_00711	unique protein
-	-	Esterase_Lipase	-	556	-	N455_00712	unique protein ‡
*tssI**	*vgrG*	VI_Rhs_Vgr	COG3501	408	Cytoplasmic	N455_00713	PANA_4144 (66) C-terminal
tssI	*vgrG*	Phage_GPD	COG3501	527	Cytoplasmic	N455_00714	PANA_4144 (99) N-terminal
tssH	*clpV, vasG*	P-loop_NTPase	COG0542	884	Unknown	N455_00715	PANA_4145 (99)
tssD	*hcp*	DUF796	COG3157	163	Extracellular	N455_00716	PANA_4146 (100)
tagL	-	OmpA_C-like	COG2885/ COG1360	574	IM	N455_00717	PANA_4147 (99)
tssL	*impK, ompA*	DUF2077	COG3455	229	Cytoplasmic	N455_00718	PANA_4148 (99)
tssK	*impJ, vasE*	DUF876	COG3522	446	Cytoplasmic	N455_00719	PANA_4149 (99)
tssC	*impC*	DUF877	COG3517	513	Cytoplasmic	N455_00720	PANA_4150 (99)
tssB	*impB*	DUF770	COG3516	165	Cytoplasmic	N455_00721	PANA_4151 (99)
-	*clcA*	Voltage_CLC	COG0038	341	IM	N455_00722	PANA_4152 (100)

‡ = similar to PMI17_02526 PGAP1-like protein [*Pantoea* sp. GM01] (esterase lipase) identity 212/536 (40%). We assigned the provisional gene locus tags N455_00698 to N455_00672 genes found in PA-4 T6SS-2 gene cluster. ‡‡ = N455_00710 encodes a PAAR protein with 49% (44/90) identity to the PAAR protein encoded by LMG 20103 PANA_4136. * = *tssI* gene occurs in two unassembled contigs, consistent with the amino and carboxyl terminals of the full-length *tssI* homolog found in *P. ananatis* LMG 20103 T6SS-2. Genes represented by a dash (-) either encode hypothetical proteins with no conserved domains, domains of unknown function or have not been functionally characterized. IM = inner membrane.

A



B

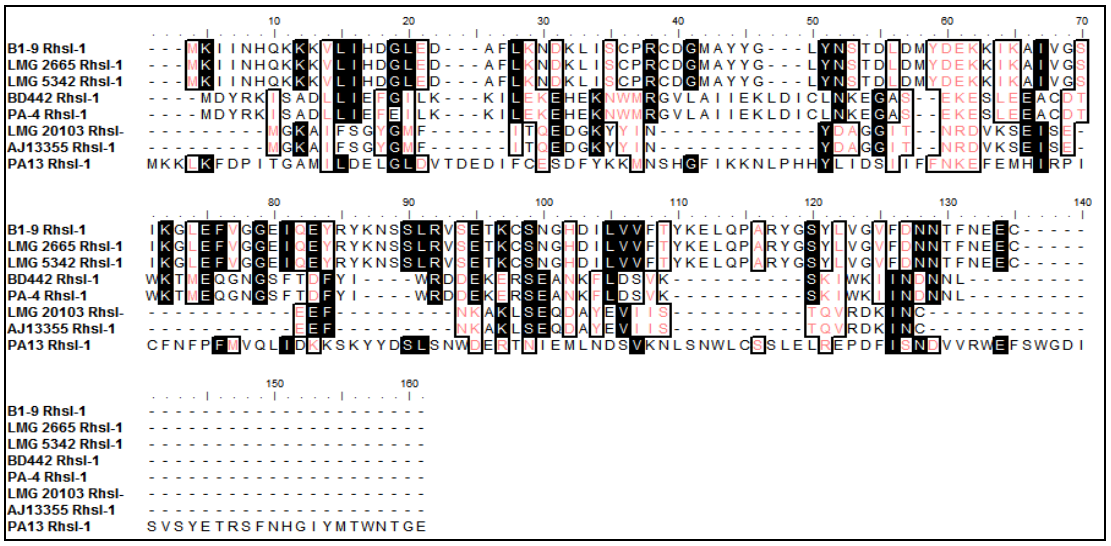


Figure S2: Alignment of the RhsD and RhsI proteins associated with *Pantoea ananatis* type VI secretion system 1. A) Shows alignment of the variable C-terminal domain of RhsD proteins from eight different strains *of P. ananatis*. The variable C-terminal domain is demarcated from the conserved N-terminal domain by a PxxxxxxDPxGL peptide motif indicated in the figure by blue stars. B) Shows alignment of the full-length RhI proteins encoded by the *rhsI* gene located downstream of the *rhsD* gene. Conserved residues are indicated by the different shadings. *P. ananatis* strains B1-9, LMG 2665^{T} and LMG 5342 have identical C-terminal extensions and encoded identical RhsI homologs.

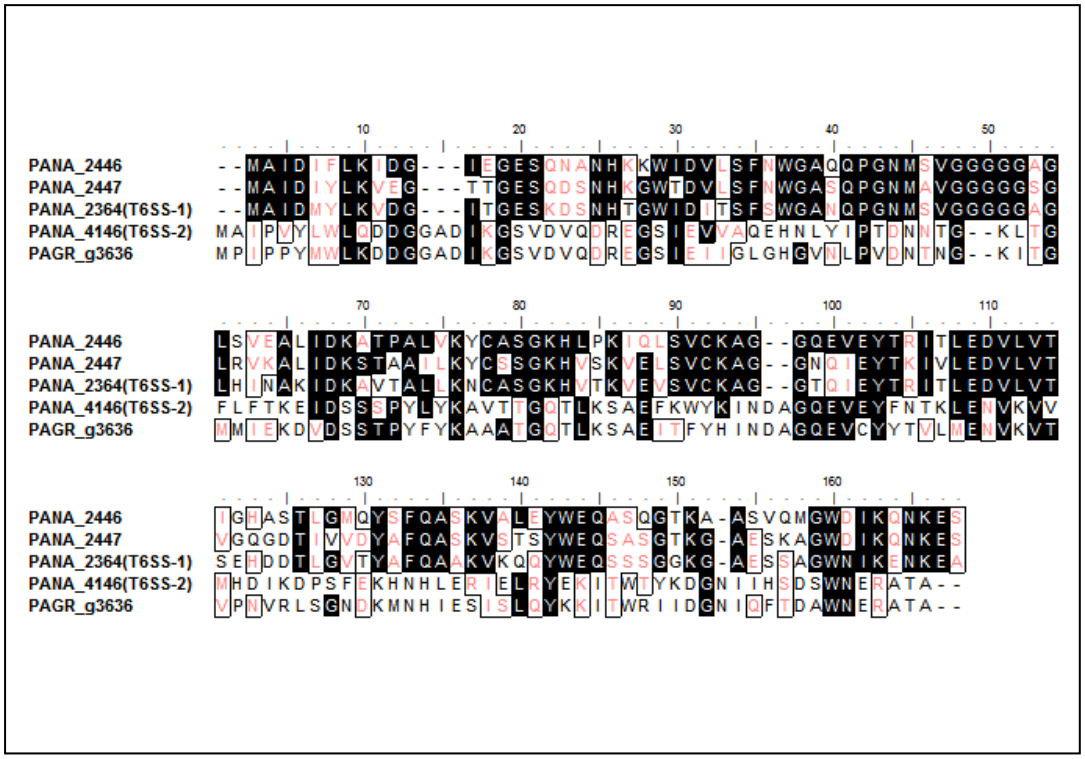


Figure S3: Alignment of all representative Hcp proteins encoded by strains of *Pantoea ananatis.* Representative Hcp amino acid sequences were aligned in BioEdit using ClustalW2 with default settings. The orphan Hcp proteins of *P. ananatis* strain LMG 20103 encoded by PANA_2446 and PANA_2447 are highly similar to the T6SS-associated Hcp protein encoded by PANA_2364. *P. ananatis* strain PA-13 encodes an additional orphan Hcp protein (PAGR_g3636) which is unique to this strain and is highly divergent from all other Hcp proteins.

Table S14: List of bacterial strains used in TssB/C phylogenetic tree

Bacterial strains	Accession number	TssB/TssC locus tags	
Aeromonas hydrophila ATCC 7966	NC_008570.1	AHA_1832/AHA_1833	
Agrobacterium fabrum str. C58	NC_003063.2	Atu4342/Atu4341	
Burkholderia pseudomallei 1106a	NC_009076.1	BURPS1106A_A0134/BURPS1106A_A0133	BURPS1106A_A0243/BURPS1106A_A0242
		BURPS1106A_A0700/ BURPS1106A_A0701	BURPS1106A_A2025/BURPS1106A_A2026
		BURPS1106A_A2835/BURPS1106A_A2834	BURPS1106A_3660/BURPS1106A_3659
Dechloromonas aromatica RCB	NC_007298.1	Daro_2177/Daro_2176	
Erwinia amylovora CFBP1430	NC_013961.1	EAMY_3228/EAMY_3227	EAMY_3021/EAMY_3020
Erwinia tasmaniensis Et1/99	NC_010694.1	ETA_06170/ETA_06180	
Francisella tularensis subsp. *mediasiatica* FSC147	NC_010677.1	FTM_1100/FTM_1101	
Pantoea ananatis strain LMG 5342	NC_016816.1	PANA5342_1728/PANA5342_1728	
Pantoea ananatis strain AJ13355	NC_017533.1	PAJ_1667/PAJ_1666	PAJ_p0135/PAJ_p0136
Pantoea ananatis strain LMG 20103	NC_013956.2	PANA_2365/PANA_2366	PANA_4151/PANA_4150
Pantoea ananatis strain PA-13	NC_017554.1	PAGR_g1662/PAGR_g1663	
Pantoea ananatis strain B1-9	CAEJ00000000.1	B1-9 C2(20)/ B1-9 C2(19)	
Pantoea ananatis strain BD442	PRJNA213372	N453_00218/ N453_00217	
Pantoea ananatis strain PA-4	PRJNA213375 *	N455_00519/ N455_00518	
Pantoea ananatis strain LMG 2665^{T}	PRJNA213373 *	N454_00631/ N454_00630	
Pantoea sp. At-9b	NC_014837.1	Pat9b_2518/Pat9b_2517	Pat9b_3466/Pat9b_3465
Pectobacterium atrosepticum SCRI1043	NC_004547.2	ECA3445/ECA3444	
Pectobacterium carotovorum subsp. *brasiliensis* PBR1692	NZ_ABVX01000021.1	PcarbP_11422/PcarbP_11417	
Pectobacterium carotovorum subsp. *carotovorum* PCC21	NC_018525.1	PCC21_032420/PCC21_032410	
Pectobacterium sp. SCC3193	CP003415.1	W5S_0962/W5S_0963	W5S_2434/W5S_2433
Pectobacterium wasabiae WPP163	NC_013421.1	Pecwa_1070/Pecwa_1071	Pecwa_2461/Pecwa_2460
Pseudomonas aeruginosa PAO1	NC_002516.2	PA0083/PA0084	PA1657/PA1658
		PA2365/PA2366	
Pseudomonas syringae pv. *phaseolicola* 1448A	NC_005773.3	PSPPH_0134/PSPPH_0133	
Pseudomonas syringae pv. *tomato* str. DC3000	NC_004578.1	PSPTO_2543/PSPTO_2544	PSPTO_5433/PSPTO_5432
Rhizobium leguminosarum bv. *viciae* 3841	NC_008378.1	pRL120474/pRL120473	
Salmonella Paratyphi A str. ATCC 9150	NC_006511.1	SPA2493/SPA2492	
Salmonella Typhimurium str. LT2	NC_003197.1	STM0273/STM0273	
Serratia marcescens FGI94	NC_020064.1	D781_1676/D781_1677	D781_2777/D781_2778
Vibrio cholerae N16961	NC_002506.1	VCA0108/VCA0108	
Yersinia pestis KIM10+	NC_004088.1	y0037/y0038	y1545/y1546
		y2706/y2705	y2706/y2705
Xanthomons citri LMG 941	NZ_CAHO01000067.1	XMIN_3905/XMIN_3906	
Xanthomonas campestris NCPPB 702	NZ_ACHS00000000.1	XcampvN_010100023480/XcampvN_010100023485	
Xanthomonas oryzae KACC 10331	NC_006834.1	XOO3052/XOO3051	XOO3481/XOO3481
Dickeya dadantii 3937	NC_014500.1	Dda3937_00817	

The table contains the list of TssB and TssC homologs from different bacterial species which were used for phylogenetic analysis. The accession numbers of all bacterial strains used in this study including the *tssB* and *tssC* gene locus tags are provided.

Table S15: List of bacterial strains used in Hcp phylogenetic tree

Bacterial strains	Accession number	locus tags(Hcp)
Agrobacterium fabrum str. C58	NC_003063.2	Atu4345
Burkholderia pseudomallei 1106a	NC_009076.1	BURPS1106A_A0136
Erwinia amylovora ATCC BAA-2158	FR719186	EAIL5_0574
Erwinia billingiae Eb661	NC_014306.1	EbC_21130
Pantoea ananatis strain LMG 20103	NC_013956.2	PANA_4146/ PANA_2364
		PANA2446/PANA_2447
Pantoea ananatis strain PA-13	NC_017554.1	PAGR_g3636
Pectobacterium atrosepticum SCRI1043	NC_004547.2	ECA4276/ECA3428/ECA3672
		ECA0176/ECA0456
Pectobacterium carotovorum subsp. *brasiliensis* PBR1692	NZ_ABVX01000021.1	PcarbP_12999
		PcarbP_05922/PcarbP_18494/
Pectobacterium wasabiae WPP163	NC_013421.1	Pecwa_0061
		Pecwa_0533/Pecwa_2459/
Pseudomonas aeruginosa PAO1	NC_002516.2	PA0263/PA1512/PA5267
		PA0085/PA2367/
Pseudomonas putida W619	CP000949.1	PputW619_3256
Rhizobium leguminosarum bv. *viciae* 3841	NC_008378.1	pRL120477
Salmonella enterica subsp. *enterica serovar* Typhimurium str. LT2	NC_003197.1	STM4509.S/STM3131
Salmonella enterica subsp. *enterica* serovar str. HI_N05-537	NZ_ABFF01000003.1	SeW_A0348/SeW_A0345
		SeW_A4992/SeW_A1246/
Serratia proteamaculans 568	NC_009832.1	Spro_1788/Spro_3005
Vibrio cholerae O1 biovar eltor str. N16961	NC_002506.1	VC1415/VCA0017
Klebsiella variicola At-22	NC_013850.1	Kvar_4558

Amino acid sequences of the orphan Hcp and T6SS-associated Hcp proteins were identified by BLASTP using sequence from *Pantoea ananatis* strain LMG 20103. Homologs of PANA_4146, PANA2446, PANA_2447 and PANA_2364 are found in all eight sequenced strains of *Pantoea ananatis* with over 98% amino acid similarity and as such they were not included in our analysis. PAGR_g3636 (Hcp-3) was only found in *Pantoea ananatis* strain PA-13.

Chapter 3

Functional characterization of the different type VI secretion system gene clusters of *Pantoea ananatis*

3.1 ABSTRACT

Pantoea ananatis is a pathogen of several economically important plants. It is currently unknown how this pathogen is able to colonize and cause disease on susceptible host plants. The type VI secretion system (T6SS) has been associated with virulence, fitness and inter-bacterial interactions in a few bacterial species. In a previous *in silico* analysis, we identified three genetically distinct T6SS gene clusters namely, T6SS-1, -2 and -3 in the genome sequence of eight strains of *P. ananatis*. To date, none of these T6SS gene clusters have been functionally characterized. In this study, we deleted the different T6SS gene clusters of *P. ananatis* strains LMG 20103 and LMG 2665^{T} and investigated their contribution to pathogenicity and bacterial competition. Results from pathogenicity assays in onion plants demonstrated that the T6SS-1 of LMG 2665^{T} was required for onion pathogenicity. Remarkably, the ΔT6SS-1 mutant of LMG 20103 was as pathogenic as the wild-type strain, when inoculated into susceptible onion plants. In addition, the T6SS null mutant [ΔT6SS-(1, 2 &3)] of LMG 20103 was as pathogenic as the wild-type strain, suggesting that there was no functional complementation following deletion of any one the T6SS gene clusters found in the genome sequence of this strain. Bacterial growth competition assays demonstrated that the T6SS-1 of LMG 20103 and LMG 2665^{T} conferred a fitness advantage to when co-cultured with *Escherichia coli*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Salmonella* Typhimurium, *Pantoea stewartii* subsp. *indologens* and *P. ananatis* strains LMG 2664, LMG 2669 and LMG 5342. No phenotypes were observed following deletion of the T6SS-2 gene cluster of LMG 20103 and the T6SS-3 gene clusters of LMG 20103 and LMG 2665^{T}. This study presents the first experimental evidence demonstrating that T6SS-1 is a virulence determinant of *P. ananatis* strain LMG 2665^{T}. We also showed for the first time that the T6SS-1 of LMG 20103 and LMG 2665^{T} plays a role in bacterial competition.

3.2 INTRODUCTION

The genus *Pantoea* represents a wide collection of pathogenic and non-pathogenic isolates, including biocontrol agents and plant growth promoters (1, 2, 3, 4, 5, 6, 7). *Pantoea ananatis* is the most devastating species within this genus, causing diseases

on economically important plants such as rice, maize, onion, melon, *Eucalyptus* spp. cotton, Sudangrass, cantaloupe fruit, and pineapple (8). Diseases caused by *P. ananatis* decrease crop yield and lead to substantial economic loss (9, 10, 11). Therefore, considerable effort has been directed towards understanding the molecular basis of pathogenicity, disease epidemiology and ultimately, finding effective methods to limit the spread of these pathogens (13, 14, 15, 16). De Maayer *et al.* (2010) identified the type VI secretion system (T6SS) as a potential virulence determinant of *P. ananatis* strain LMG 20103, based on *in silico* analysis (17). This secretion system has been shown to be a virulence determinant of several Gram-negative bacteria (18, 19, 20, 21, 22, 23, 24, 25).

In Chapter 2, we identified three genetically distinct T6SS gene clusters which are differentially distributed amongst strains of *P. ananatis*. We also reported that the *rhsD* gene, located within the *vrgG* island of the *P. ananatis* T6SS-1 gene cluster, encodes a putative protein that shares structural homology to the insecticidal YenC2 toxin of *Yersinia entomophaga* (26). Furthermore, we identified genes located in the *hcp* island of the *P. ananatis* T6SS-1 cluster that encode proteins sharing sequence and structural homology to bacterial peptidoglycan-degrading enzymes. Finally, some of the gene products of the *P. ananatis* T6SS-2 gene cluster were found to share extensive structural homology to the insecticidal chitinase G toxin of *Streptomyces coelicolor* (17, 27). Together, these findings suggested that the gene products of *P. ananatis* T6SS-1 and T6SS-2 may encode bactericidal or insecticidal toxins, which may be secreted by the T6SS of these bacteria. In support of this hypothesis, the T6SSs of different bacteria have been associated with virulence, inter-bacterial competition, regulation of quorum sensing, stress responses, intracellular growth and colonization (28, 29, 30, 31, 32, 33, 34). The myriads of roles played by different bacterial T6SSs have been attributed various toxins and bacterial proteins, termed effectors that are delivered through the T6SS into eukaryotes, prokaryotes and plants. Some of these T6SS effectors include VgrG, Hcp, VasX, and a wide range of cell wall-degrading enzymes (amidases, lipases and peptidoglycan glycoside hydrolases) (18, 19, 35, 36, 37, 38, 39, 40, 41).

Several gene manipulation techniques have been used in gaining insight into the biological role of the T6SS, with the most widely used technique being mutagenesis (42). For example, Tn*5* transposon mutagenesis was used to identify T6SS mutants of *Rhizobium leguminosarum* with an expanded host range of nodulation to include clovers (43, 44, 45). This study demonstrated that the T6SS of *R. leguminosarum* plays a role in nodulation and symbiosis (44, 45). Similar, Dong *et al*. (2012) used a combination of saturation mutagenesis and proteomics to identify three T6SS effector-immunity pairs of *V. cholerae*, which played a role in inter-bacterial competition (37). Allele exchange mutagenesis has also been used for targeted deletion of T6SS genes using suicide vectors (46, 47). This technique was used to systematically delete all the genes located within the T6SS clusters of *Edwarsiella tarda*, *Vibrio cholerae* and *Agrobacterium tumefaciens* (20, 47, 48). The results of these studies consistently showed that 13 T6SS genes (*tssA* to *tssM*) were needed to assemble a functional T6SS and inactivation of any one these genes lead to a loss-of-function phenotype (20, 47, 48). A variation of allele exchange mutagenesis, called the lambda Red-mediated recombination technique, uses PCR-generated constructs to delete genes with the help of the lambda Red genes *gam*, *bet* and *exo* expressed from a plasmid (49). The lambda Red technique has been used for compound deletion of T6SS-2 (H2-T6SS) and T6SS-3 (H3-T6SS) gene clusters of *Pseudomonas aeruginosa*, and the T6SS gene cluster of *Salmonella enterica* serovar Gallinarum (50, 51). The lambda Red technique therefore, provides a fast and easy way to determine the biological role of individual T6SS genes and the function of bacterial T6SSs.

The aim of this study was to determine the biological role of the different T6SS gene clusters of *Pantoea ananatis*. This study was prompted by the finding that *P. ananatis* does not contain gene homologs of T2SS, T3SS and T4SS, which are well-known virulence determinants of several bacteria (52, 53, 54, 55, 56). Up to three T6SS gene clusters are differentially distributed in genome sequences of eight strains of *P. ananatis*, although none has been functionally characterized (14). Therefore, the aim of this study was to determine the contribution of the different T6SSs to the biology of *P. ananatis*. To achieve this objective we deleted the different T6SS gene clusters of *P. ananatis* strains LMG 20103 and LMG 2665^{T}, which are pathogenic to

Eucalyptus spp. and pineapple, respectively (57, 58). Herein, we present experimental evidence demonstrating that *P. ananatis* T6SS-2 is associated with virulence against prokaryotes and onion plants.

3.3 MATERIALS AND METHODS

3.3.1 Generation of *P. ananatis* T6SS mutants

3.3.1.1 Bacterial strains and plasmids

Two *P. ananatis* strains namely, LMG 20103 and LMG 2665^{T}, were selected to investigate the role that the T6SS plays in the biology of this pathogen. Both strains have a similar GC content (~ 53%), protein coding capacity (~ 4238) and harbour the LPP-1 megaplamid (280-352 kb) (59). The genome sequence of *P. ananatis* strain LMG 20103 contains three genetically distinct T6SS namely, T6SS-1, T6SS-2 and T6SS-3. The T6SS-1 and T6SS-3 gene clusters were found in the genome sequence of both strains of *P. ananatis*, while the T6SS-2 gene cluster is restricted to strain LMG 20103. Plasmid pRSFRedTER (Cm^{R}) expressing the lambda recombination genes *gam*, *bet* and *exo* under the control of the *lac* promoter was donated by Dr. Yoshihiko Hara (Ajinomoto Co., Inc. Japan), the helper plasmids pCP20 (for excision of the antibiotic marker gene from the genome) and pKD13 (which provides a kanamycin selection marker for gene disruption) were donated by Dr. Lucy Moleleki (University of Pretoria). All plasmids were propagated in *Escherichia coli* DH5α (Invitrogen) and stored in 15% (v/v) glycerol at -70^{o}C until required.

3.3.1.2 Culture conditions and reagents

Bacteria were grown at 30 - 37^{o}C on Luria-Bertani (LB) agar or in LB broth or minimum medium M9 salt supplemented with 1% glucose (60). When required, growth media were supplemented with antibiotics in the following concentrations: kanamycin (Km), 50 μg/ml, tetracycline (Tet), 15 μg/ml, chloramphenicol (Cm), 50 μg/ml and gentamycin (Gm), 20 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG), antibiotics and restriction enzymes (*Eco*RI and *Bam*HI) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Growth media and supplements were purchased from Merck Laboratory Supplies (Midrand, RSA). All reagents and chemicals were used according to the manufacturers' instructions.

3.3.1.3 DNA isolation and purification

A single bacterial colony was grown in LB broth at 32^{o}C with shaking (250 rpm). Overnight cultures were collected by centrifugation at 10 000 g (Centrifuge 5804R, Eppendorf) and the pellet was resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5). Chromosomal DNA and plasmids were extracted using the Quick-gDNA^{TM} MiniPrep (Zymo Research) and Zyppy^{TM} Plasmid Miniprep kits (Zymo Research), respectively. DNA was purified using the DNA Clean & Concentrator^{TM} (Zymo Research) and Zymoclean^{TM} Gel DNA Recovery kits (Zymo Research). All kits were used according to the manufacturer's instructions. Growth media were supplemented with appropriate antibiotics for plasmid extraction. The quality and quantity of DNA, PCR products and plasmids was determined using agarose gel electrophoresis and a NanoDrop$^{®}$ ND-1000 Spectrophotometer (NanoDrop Technologies).

3.3.1.4 DNA amplification and analysis

3.3.1.4.1 Primers

All primers used in this study are listed in Table 3.1. All primers were manually designed using the available draft genome sequence of *P. ananatis* LMG 2665^{T} and the fully assembled genome sequence of *P. ananatis* LMG 20103. Nucleotide sequences of strain LMG 20103 were downloaded from NCBI and aligned against the homologous sequence from strain LMG 2665^{T} using ClustalW in BioEdit (61). Primers were designed within conserved regions in both *P. ananatis* strains (where applicable). The quality of primers (GC content, hairpins, dimers and other secondary structures) were analyzed in OligoAnalyzer 3.1 (IDT Technologies). All primers were synthesized and purchased from Inqaba Biotech (RSA).

3.3.1.4.2 Polymerase chain reaction (PCR)

The PCR amplification mixture contained 30 – 45 ng genomic DNA, 1 × PCR buffer, 2.5 mM $MgCl_2$, 2.5 mM of each deoxynucleoside triphosphate (dNTP), 50 pmol of each primer, and 1 U of SuperTherm DNA polymerase (Southern Cross Biotechnology, RSA). PCR was performed using a Veriti^{R} Thermal Cycler (Applied Biosystems, USA) with the following amplification parameters: initial denaturation at 94^{o}C for 3 min, followed by 32 cycles of 30 s denaturation at 94^{o}C, 15 s annealing at

60 - 62^{o}C (depending on the T_m of the primers), and 1 - 4 min extension at 72^{o}C (depending on the predicted amplicon size) and a final extension at 72^{o}C for 7 min. Negative controls included all the PCR reagents without the DNA template. Aliquots of PCR products were electrophoresed on an 0.8% agarose gel to determine the fidelity of the PCR amplification. PCR products were purified as previously described, and the nucleotide sequence determined using the BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instruction. Sequencing was performed at the DNA Sequencing Facility of the University of Pretoria using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were compared to publicly available sequences at the National Center for Biotechnology Institute (NCBI) by BLASTN (62, 63).

3.3.1.5 Synthesis of gene disruption constructs

Gene disruption constructs were synthesized by fusion PCR, as described by Shevchuk *et al.* (2004) (44). These constructs were synthesized by PCR in two steps. In the first step, ~1000 nt corresponding to the 5' and 3' regions of the target locus were individually amplified from the genome using primers F1/R1 and F2/R2, respectively (Figure 3.1A). The kanamycin gene was amplified from plasmid pKD13 using primers KanF/KanR. The primer combinations R1/KanF and KanR/F2 were designed to be perfect reverse complements of each other. These primers provided complementary regions for fusion of the three constructs in a second round of PCR (Figure 3.1A). In this second round of PCR, 2 ng of each PCR product was added in a single PCR mixture and used as template DNA. PCR conditions were similar to the conditions used in the first round of PCR with the exception that only flanking primers F1 and R2 were included in the PCR reaction. The resulting PCR generated a 3.3 kb fused construct. This construct was purified from an agarose gel and re-amplified in four separate 50 µl PCR reactions to increase yield. Fused constructs were analyzed by gel electrophoresis and sequenced, as previously described. High Fidelity PCR Enzyme Mix (Fermentas, Life Sciences) with proofreading activity was used in all PCR reactions to minimize sequence changes usually associated with conventional Taq polymerases.

3.3.1.6 Preparation of electro-competent cells

Electroporation-competent cells were prepared as described by Sambrook *et al*. (1998) (65). In brief, a single bacteria colony was inoculated in 50 ml of LB broth and cultured overnight at 32^{o}C with shaking (250 rpm). Overnight cultures were diluted 1:100 in fresh LB broth (100 ml) and incubated with shaking (250 rpm) at 32^{o}C. At an OD_{600} of 0.6 (4 – 5 hrs) the cells were transferred to pre-chilled 50 ml centrifugation tubes and cooled on ice for an additional 5 min. Chilled cells were washed twice by repeated centrifugation and resuspended in 10% ice-cold glycerol. The centrifuge was maintained at 4^{o}C at all times and the cells kept on ice between washes. Following the final wash step, the cells were resuspended in 5 ml of ice-cold 10% glycerol. Aliquots of 60 µl were prepared in sterile 1.5 ml microcentrifuge tubes (Eppendorf), fast-frozen in liquid nitrogen and stored at -70^{o}C for future use.

3.3.1.7 Transformation of electro-competent cells

Prior to electroporation, electro-competent cells were thawed on ice. In a 1.5 ml microcentrifuge tube, 60 µl of electro-competent cells were mixed with 20 ng of plasmid or up to 500 ng of PCR product. The mixture was transferred to a 1 mm (gap size) pre-chilled electroporation cuvette (Bio-Rad Inc.), followed by electroporation using the Multiporator® device (Eppendorf). Electroporation conditions were set as follows: MODE = bacterial, E (electric field strength) = 2000V, τ (time constant) = 5 ms and resistance of 400 Ω. Electroporated cells were resuspended in 1 ml of LB broth and incubated at 32^{o}C for 1 h in a shaking incubator (250 rpm). After recovery of the cells, 100 µl of the cell suspension was spread onto LB agar containing appropriate antibiotics and incubated overnight at 32^{o}C. Transformants were verified by either plasmid extraction or PCR and analyzed on a 0.8% agarose gel.

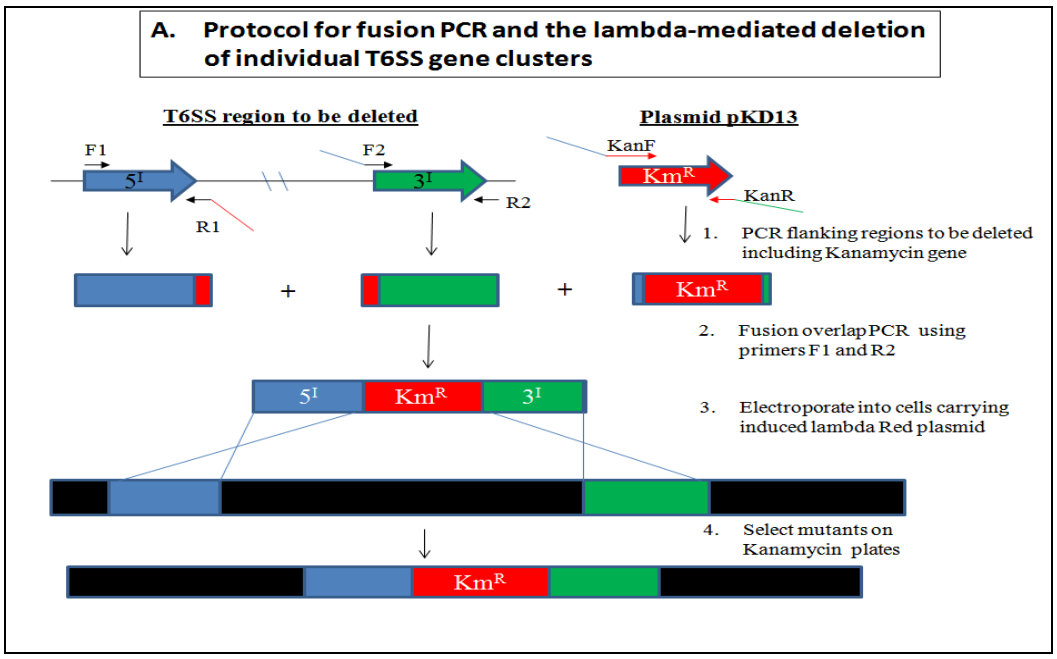
3.3.1.8 Generation of individual T6SS gene cluster deletion mutants

Pantoea ananatis strains LMG 20103 and LMG 2665^{T} were made electro-competent and transformed with the lambda plasmid pRSFRedTER (Cm^{R}) as previously described. Transformants were selected on LB agar plates supplemented with chloramphenicol (50 µg/ml). A single colony of the pRSFRedTER-transformed cells was grown overnight in 10 ml of LB broth supplemented with chloramphenicol (50 µg/ml). Overnight cultures were diluted 1:100 in fresh LB broth (100 ml)

supplemented with chloramphenicol (50 µg/ml) and 1.5 mM IPTG for induction of the λ genes. Growth was continued in a shaking incubator (250 rpm) at 32°C and the optical density monitored every hour. At an OD_{600} of 0.5, the induced cells were made electro-compentent and transformed with 300 – 500 ng of the PCR-generated gene disruption cassettes. Electroporated cells were resuspended in 1 ml of LB broth and incubated at 32°C for 1 h in a shaking incubator (250 rpm), followed by spread-plating onto LB agar supplemented with kanamycin (50 µg /ml).

3.3.1.9 Generation of double and triple T6SS gene cluster deletion mutants

Mutants generated from the first transformation event were Km^R and Cm^R due to the presence of the kanamycin cassette and plasmid pRSFRedTER, respectively (Figs 3.1A and 3.1B). In brief, pRSFRedTER was evicted by growth in 10% sucrose as previously described (66). The kanamycin gene was excised from the genome of the mutants by the FLP recombinase enzyme encoded by plasmid pCP20 (67, 68). The T6SS mutant strains of *P. ananatis* were made electro-competent and transformed with pRSFRedTER. Transformants were induced with IPTG and made electrocompetent, as previously described. Electro-competent mutant strains of *P. ananatis* were transformed with a second gene disruption construct and mutants selected on LB agar supplemented with kanamycin (50 µg /ml).



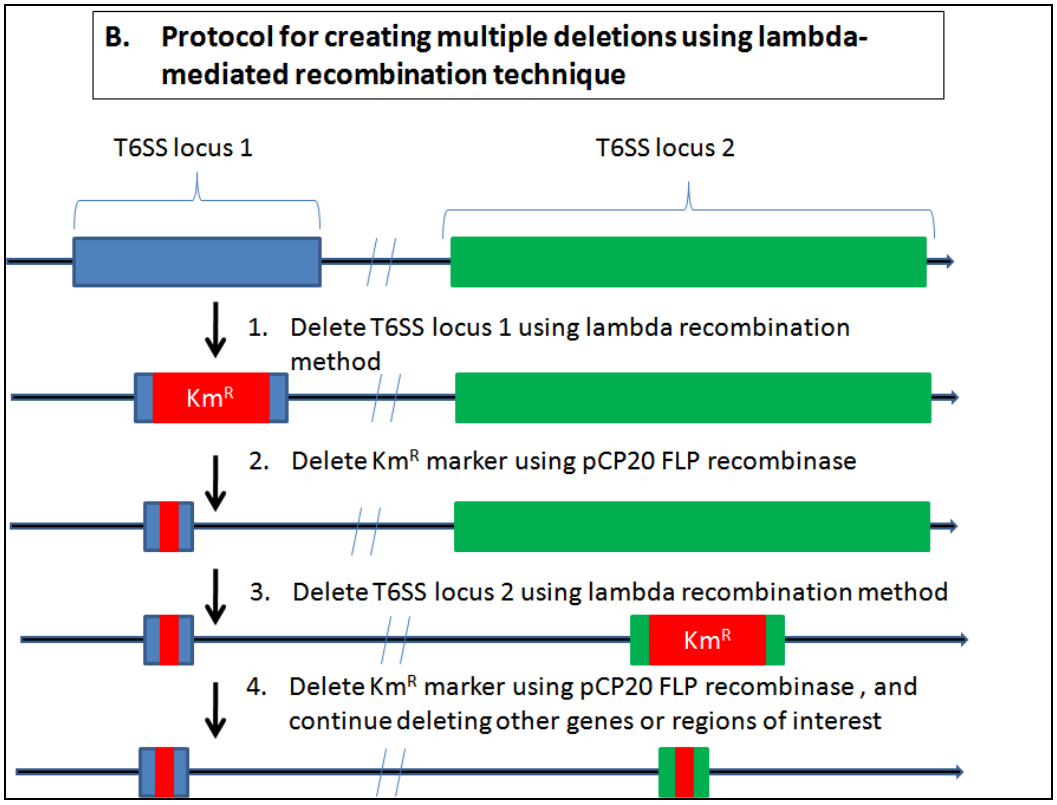


Fig 3.1: Protocol for lambda Red-mediated deletion of the different type VI secretion system (T6SS) gene clusters of *Pantoea ananatis*. Fig. 3.1A shows the overlap fusing PCR and the protocol used to delete individual T6SS gene clusters. Fig 3.1B outlines the procedure for deleting two or more T6SS gene clusters found in the genome sequence of *Pantoea ananatis*. // = figure was not drawn to scale.

3.3.2 Characterization of *Pantoea ananatis* T6SS mutants

3.3.2.1 PCR analysis

All mutants were verified by PCR using a combination of kanamycin internal primers, T6SS gene-specific primers and T6SS test primers (Table 3.1). Test primers (T6SS flanking primers) were designed to anneal at positions ~200 – 500 bp upstream and downstream of the T6SS gene cluster to be deleted. Importantly, the test primers did not bind to the fused PCR construct used for generating T6SS mutants. Test primers were also used in combination with the kanamycin internal primers Km1 (CAGTCATAGCCGAATAGCCT) and Km2 (CGGTGCCCTGAATGAACTGC) to verify the integration of PCR-generated disruption cassette at the expected T6SS locus (68). Gene-specific primers were designed to amplify selected genes within each T6SS gene cluster and the primers showed no cross-reaction with homologs from other T6SS gene clusters. All PCR products were analyzed by gel electrophoresis and the nucleotide sequences were determined by sequencing and BLASTN analysis, as previously described.

3.3.2.2 Southern blot analysis

Southern blot hybridization was performed according to the protocol outlined by Sambrook and Russell (2001) (60) using the DIG High Prime DNA labeling and detection Kit (Roche Biochemicals), according to the manufacturer's instructions. A 600 bp fragment of the kanamycin resistance gene was amplified by PCR using primers KanF and Km2, purified and used as positive control. Genomic DNA from wild-type strains of *P. ananatis* and kanamycin-resistant mutants was extracted using the Quick-gDNA^{TM} MiniPrep kit (Zymo Research). DNA (3 - 4 μg) was digested overnight with *Eco*RI and *Bam*HI restriction enzymes, and electrophoresed on a 0.8% agarose gel. DNA restriction fragments were transferred by upward capillary blotting onto a positively charged Hybond – Nylon membrane (Roche Biochemicals) and fixed to the membrane using UV light at 120 mJ. The fixed membrane was hybridized to a 600 bp fragment of the kanamycin gene labeled with alkali-labile DIG-11-dUTP (Roche Biochemicals) using the PCR DIG Probe Synthesis Kit (Roche Biochemicals). Hybridization and post-hybridization washes were performed under stringent conditions as recommended by the manufacturer. Restriction fragments that hybridized to the probe were detected calorimetrically with the NBT/BCIP colour

substrate (Roche Biochemicals). Colour development was allowed for 2 - 3 hrs and the membrane was scanned using a HP ScanJet 5590 (HP Solutions).

3.3.3 Phenotypic characterization of *Pantoea ananatis* T6SS mutants

3.3.3.1 *In vitro* growth assays

Pure cultures of wild-type and T6SS mutant strains of *P. ananatis* were grown overnight in 20 ml of LB broth in a shaking incubator (250 rpm) at 32^{o}C. Overnight cultures were diluted 1:100 in fresh LB broth (200 ml) and 200 ml of M9 minimum medium. All flasks were incubated with shaking (250 rpm) at 32^{o}C and growth was monitored by measuring the change in the optical density at 600 nm over 15 h. All assays were performed in duplicate and repeated three times. Data was exported to Excel, the average readings of all time points calculated and then used to draw a growth curve of OD_{600nm} vs Time (h).

3.3.3.2 *In planta* growth assays

Wild-type strains of *P. ananatis* and corresponding T6SS mutants were transformed with plasmid pMP7605 (Gm^{R}) as previously described. Pure cultures of transformed bacteria were grown overnight with shaking (250 rpm) at 32^{o}C in LB broth supplemented with gentamycin (15 μg/ml). Overnight cultures were diluted 1:100 into fresh LB broth and grown with shaking (250 rpm) to an OD_{600} of 0.4. At an OD_{600} of 0.4, bacteria were harvested by centrifugation and resuspended in double distilled auctoclaved water. Bacterial cultures were standardized to an OD_{600} of 0.1 (~6.2×10^{6} CFU/ml) and inoculated into the leaves of three-week-old onion seedlings (*Allium cepa* cv. Texas grando) using a sterile 1-ml hypodermal syringe. Inoculated leaves were cut were cut from onion seedlings 10 h, 24 h, 48 h and 72 h post-inoculation, using a sterile scalpel. Each leaf was macerated in 2 ml of TE buffer, using a mortar and pestle. Macerated plant tissue was serially diluted and plated onto LB agar supplemented with gentamycin (15 μg/ml). The assay was repeated three times.

3.3.3.3 Pathogenicity trials using onion seedlings

Pathogenicity trials were conducted on six week-old onion plants (cv. Texas grando) grown in the greenhouse at 25 - 28^{o} C with relative humidity of 35 to 40%. Cultures of wild-type and mutant strains of LMG 20103 and LMG 2665^{T} were grown

overnight in LB broth at 32^{o}C with shaking (250 rpm). Overnight cultures were standardized to an OD_{600} 0.1 (~ 6.2×10^{6} CFU/ml) in double distilled autoclaved water (ddH_2O) and inoculated into onion leaves using a hypodermal syringe (2 - 3 drops of inoculum). Two to six leaves/onion plant, including four biological replicates, were inoculated with each inoculum. Control plants were inoculated with double distilled autoclaved water. All assays were repeated under the same experimental conditions, as described above. Plants were inspected daily for a total of three days post-inoculation.

3.3.3.4 Bacterial growth competition assay

In vitro growth competition assays were conducted on solid LB agar to determine what role the different T6SS gene clusters of *P. ananatis* play in inter-bacterial interactions. The complete list of strains and plasmids used in this assay are provided in Table 3.2. To facilitate screening, we first conducted an *in vitro* competition assay between wild-type strains of *P. ananatis* and 34 different bacteria. Strains that were inhibited by the wild-type strains were then co-cultured with the different T6SS deletion mutants to determine which T6SS gene cluster was associated with the inhibition phenotype.

In summary, target bacterial strains were transformed with pMP7605 (Gm^{R}) as previously described. Pure cultures of transformed bacteria were grown overnight at 32^{o}C in LB broth containing appropriate antibiotics. Overnight cultures were washed twice by repeated centrifugation, resuspended in LB broth and the OD_{600} was adjusted to 0.1 with sterile LB broth. Inhibitor and target bacteria were mixed in a ratio of 1:1 and 20 µl of this mixture was spotted onto antibiotic-free LB agar and incubated overnight at 32^{o}C. Control experiments included target bacteria co-cultured with ddH_2O. Overnight cultures were harvested using a sterile loop and resuspended in 1 ml of LB broth. This was followed by serial dilutions in LB broth and spread plated onto LB agar supplemented with gentamycin (15 µg/ml) for bacterial enumeration. Competition assays were performed in duplicates and repeated three times.

3.3.3.4 Statistical analysis

An unpaired, two-tailed Student's *t*-test was used to compare the mean values of CFU/ml of recovered targeted bacteria following co-culture in LB broth (control), the different T6SS gene cluster mutants and wild-type strains of *Pantoea ananatis*. A *p*-value < 0.05 was considered to be statistically significant. Analyses were performed using JMP Statistical Discovery Software version 5.0.

Table 3.1 List of PCR primers used in this study

Primer names	Sequences 5' to 3'	Reference
PA T6SS-1 primers		
C1F1	CACAGCGCACCGCAATATCTG	This study
C1F2	AGCTCCAGCCTACACAATCGCAGATGGGCCATATTCAGCAGACGG	This study
C1KanF	CCGTCTGCTGAATATGGCCCATCTGCGATTGTGTAGGCTGGAGCT	This study
C1F2	GGTCCGACGGATCCCCGGAATTGCTCACCATTGTGTCATCAGTC	This study
C1KanR	GACTGATGACACAATGGTGAGCAATTCCGGGGATCCGTCGACC	This study
C1R2	ACTGTGCCATTCAACGCCTGAGTG	This study
C1TestF	TTGATCGCCATATCCGCCATCATC	This study
C1TestR	GCATGTTCCTCGACTGGACAGTG	This study
PA T6SS-2 primers		
C2FI	TCTTCAACAGCTCGTCATGAG	This study
C2R1	AGCTCCAGCCTACACAATCGCGCTGAAATAACACACCAGGGTTCAC	This study
C2KanF	GTGAACCCTGGTGTGTTATTTCAGCGCGATTGTGTAGGCTGGAGCT	This study
C2F2	GGTCGACGGATCCCCGGAATCGTGCCTTTGGCACTTCACTCTG	This study
C2KanR	CAGAGTGAAGTGCCAAAGGCACGATTCCGGGGATCCGTCGACC	This study
C2R2	GAATCTGGCCTGAGGTCGGACG	This study
C2testF	TACTGACGATTCTGACGGTACGTG	This study
C2testR	GCTGGCAGGCATTCTGTTCATC	This study
PA T6SS-3 primers		
C3FI	TGAATGTTGAACGTCACAGAGC	This study
C3R1	AGCTCCAGCTACACAATCGCCGTAACGACGAGCAGAATAACAGCG	This study
C3KanF	CGCTGTTATTCTGCTCGTCGTTACGGCGATTGTGTAGGCTGGAGCT	This study
C3F2	GGTCGACGGATCCCCGGAATTTGTCAGCAGGAATAATCACCTTGC	This study
C3KanR	GCAAGGTGATTATTCCTGCTGACAAATTCCGGGGATCCGTCGACC	This study
C3R2	ACACCCTGGTCACCAGTGATGTG	This study
C3testF	AGCGAAGCGATCTTCCGGAGC	This study
C3testR	AAGTCTTAGGTAGACTGAGCG	This study
Gene-specific primers		
tssAF (T6SS-1)	ATCGAGTTCGATAAGGCGCTG	This study
tssAR (T6SS-1)	CACGTTGCTGATACCGGTAAGC	This study
clpVF (T6SS-2)	CAGCTAAGGCTTAGCGACTGTG	This study
clpVR (T6SS-2)	CAGGAGCCACATACCGTATCG	This study
pknAF (T6SS-3)	CTAATTGGTGGGATTAATCAGC	This study
pknAR (T6SS-3)	TGGCAACCTGTCCGACGAAACC	This study
Kanamycin primers		
KanF	GCGATTGTGTAGGCTGGAGCT	This study
KanR	ATTCCGGGGATCCGTCGACC	This study
Km1	CAGTCATAGCCGAATAGCCT	Zhao (2009)
Km2	CGGTGCCCTGAATGAACTGC	Zhao (2009)

PA T6SS 1 to 3 = *Pantoea ananatis* type VI secretion system 1-3 gene clusters. All primers used were manually designed as described in the Materials and Methods section. KanF/KanR were used to amplify the kanamycin cassette from plasmid pKD13. Km1 and Km2 primers are internal kanamycin primers.

Table 3.2 List of bacterial strains and plasmids used in this study

Bacteria strain	Relevant characteristics or host of isolation	Collector (a) or Reference (b)
Inhibitor strains (Tc^R)		
Pantoea ananatis LMG 20103	Pathogen of *Eucalyptus* sp.,	Coutinho (1998)[b]
Pantoea ananatis LMG 2665^T	Pathogen of pineapple	Serrano (1928)[b]
LMG 2665^T ΔT6SS	ΔT6SS-1::Km^R, ΔT6SS-3::Km^R	This study
LMG20103 ΔT6SS	ΔT6SS-1::Km^R, ΔT6SS-2::Km^R, ΔT6SS-3::Km^R, ΔT6SS-(1&2)::Km^R, ΔT6SS-(1&3)::Km^R, ΔT6SS-(2&3)::Km^R, ΔT6SS-(1,2,3)::Km^R	This study
Targeted strains (Gm^R)		
Pantoea ananatis LMG 5342	Clinical isolate	De Baere (2004)[b]
Pantoea ananatis LMG 2669	Pathogen of pineapple	Spiegelber (1958)[b]
Pantoea ananatis LMG 2664	Pathogen of pineapple	Spiegelber (1958)[b]
Pantoea ananatis AJ13355	Isolated from soil in Japan	Hara (2012)[a]
Pantoea ananatis BD442	Pathogen of maize	Goszczynska (2004)[a]
Pantoea ananatis PA-4	Pathogen of onion	
Pantoea ananatis ICMP 10132	Pathogen of sugarcane	Almeida (1991)[a]
Pantoea ananatis ATCC 35400	Pathogen of honeydew melon	Wells (1981)[b]
Pantoea ananatis LMG 2678	Pathogen of wheat	Hayward (1961)[a]
Pantoea ananatis LMG 2101	Pathogen of rice	Fujii, Urano (2001)[a]
Pantoea ananatis LMG 20104	Pathogen of *Eucalyptus* sp.	Coutinho (1998)[b]
Pantoea ananatis Uruguay 40	Pathogen of *Eucalyptus* sp.	Wingfield (2004)[a]
Pantoea ananatis BD 301	Pathogen of onion	Goszczynska (2004)[b]
Pantoea ananatis BD 622	Pathogen of maize	Goszczynska (2004)[b]
Pantoea ananatis Mmir 9	Isolated from *Mirridiae* sp.	Roux (FABI)[a]
Pantoea vagans BCC006	Isolated from *Eucalyptus grandis*	Wingfield (FABI)[a]
Pantoea eucalypti LMG 24197^T	Isolated from *Eucalyptus grandis*	Wingfield (FABI)[a]
Pectobacterium atrosepticum LMG 6687	Pathogen of tomato	Samson (1985)[a]
Pectobacterium betavasculorum LMG 2398	Pathogen of potato	Lazar (1962)[a]
Pectobacterium carotovorum subsp. *carotovorum* LMG 2404^T	Pathogen of Irish potato	Hellmers (1952)[a]
Pectobacterium carotovorum subsp. *brasiliensis* 1692	Pathogen of potato	Van der Waals (FABI)[a]
Brenneria nigrifluens LMG 2696	Pathogen of walnut	Starr (1955)[a]
Brenneria quercina LMG 5952	Pathogen of oak	Hildebrand (1984)[a]
Salmonella Typhimurium	Clinical isolate	FABI
Pantoea stewartii subsp. indologens	Pathogen of maize	FABI
Klebsiella pneumonia TMA5	Isolated from *Eucalyptus* sp. in Thailand	Wingfield (FABI)[a]
Serratia marcescens LMG 2792^T	Isolated from pond water	FABI
Baccillus cereus Mn106-2a2c	Isolated from termite	Human (FABI)[a]
Bacillus subtilis A	Environmental isolate	Human (FABI)[a]
Bacillus subtilis B	Environmental isolate	Human (FABI)[a]
Burkholderia sp. P19	Isolated from palm tree	Wingfild (FABI)[a]
Pseudomonas putida WRB111	Isolated from *Eucalyptus* sp.	De Maayer (2005)[a]
Enterobacter sakazakii M658	Isolated from milkpowder in the UK	Forsythe (2008)[a]
Escherichia coli DH5α	F^- *recA*1 *endA*1 *hsdR*17 *deoR thi*-1 *supE*44 *gyrA*96 *rel*A1 Δ(*lacZYA argF*) U169 λ^-[Φ80*lacZ*Δ*M15*]	Invitrogen[b]
Plasmids		
pMP7604	pSV1 replicon, broad host range cloning vector, Tc^R	Lagendijk (2010)[b]
pMP7605	pBRR replicon, broad host range cloning vector, Gm^R	Lagendijk (2010)[b]

Tc^R = tetracycline resistance, Gm^R = gentamycin resistance, FABI = Forestry and Agricultural Biotechnology Institute. UK = United Kingdom

Table 3.3 List of *Pantoea ananatis* type VI secretion system deletion mutants (ΔT6SS) generated in this study

Bacteria strain	Relevant characteristics	Collector (a) or Reference (b)
Escherichia coli DH5α	F^- *recA*1 *endA*1 *hsdR*17 *deoR thi*-1 *supE*44 *gyrA*96 *relA*1 Δ(*lacZYA argF*) *U169* λ^- [Φ80*lacZ*Δ*M15*]	Invitrogen[a]
Pantoea ananatis LMG 20103	Pathogen of *Eucalyptus* sp., Ap^R, GeneBank CP001875.2. Genome sequence contains PA T6SS 1, 2 and 3.	De Maayer (2010)[b]
Pantoea ananatis LMG 2665^T	Pathogen of pineapple, Ap^R, partially assembled draft genome contains T6SS-1 and T6SS-3 GeneBank KF590027 and KF590026, respectively.	Serrano (1928)[b]
LMG 2665^T (0)	Derivative of LMG 2665^T expressing the lambda genes (*gam*, *bet* and *exo*) from plasmid pRSFRedTer (Cm^R)	This study
LMG 20103 (0)	Derivative of LMG 20103 expressing the lambda genes (*gam*, *bet* and *exo*) from plasmid pRSFRedTer (Cm^R)	This study
20103ΔT6SS-1	LMG 20103::Km^R (ΔT6SS-1) 30.8 kb deletion mutant	This study
20103 ΔT6SS-2	LMG 20103::Km^R (ΔT6SS-2) 30.7 kb deletion mutant	This study
20103 ΔT6SS-3	LMG 20103::Km^R (ΔT6SS-3) 9 kb deletion mutant	This study
20103 ΔT6SS-(1&2)	LMG 20103::Km^R Δ(T6SS-1::T6SS-2) 61.5 kb deletion mutant	This study
20103 ΔT6SS-(1&3)	LMG 20103::Km^R Δ(T6SS-1::T6SS-3) 39.9 kb deletion mutant	This study
20103 ΔT6SS-(2&3)	LMG 20103::Km^R Δ(T6SS-2::T6SS-3) 39.7 kb deletion mutant	This study
20103 ΔT6SS-(1,2&3)	LMG 20103::Km^R Δ(T6SS-1::T6SS-2::T6SS-3) 70.5 kb deletion mutant	This study
2665 ΔT6SS-1	LMG 2665^T::Km^R Δ(T6SS-1) 48 kb deletion mutant	This study
2665 ΔT6SS-3	LMG 2665^T::Km^R (ΔT6SS-2) 9 kb deletion mutant	This study
Plasmids		
pKD13	Broad host range plasmid containing the Km^R cassette with flanking FRT sequences	Datsenko (2000)[b]
pCP20	*FLP*$^+$, λCI857$^+$, λp_RRep^{ts}, Ap^R, Cm^R	Cherepanovad (1995)[b]
pRSFRedTer	Broad host range plasmid vector, expressing the lambda genes *gam*, *bet* and *exo* under the control of P-element P_{lacUV5}-lacI, levansucrase gene, Cm^R	Katashkina (2009)[b]

The table contains all T6SS deletion mutants of *P. ananatis* strains LMG 20103 and LMG 2665^T, including the plasmids (pKD13, pCP20 and pRSFRedTer) used to generate the mutants. Km^R, Cm^R and Ap^R correspond to resistance to kanamycin, choloramphenicol and ampicillin, respectively. Plasmids were maintained and propagated in *Escherichia coli* DH5α.

3.4 RESULTS

3.4.1 Generation of T6SS mutants in *P. ananatis*

The λ Red-mediated homologous recombination technique was successfully used to delete the different T6SSs encoded by two strains of *P. ananatis* (strain LMG 20103 and LMG 2665^{T}). All mutants generated in this study are listed in Table 3.3. No T6SS mutants could be obtained without prior transformation of wild-type strains LMG 20103 and LMG 26665^{T} with plasmid pRSFRedTer.

3.4.2 PCR verification of mutants

All mutants used in downstream applications were individually verified using locus-specific primers and internal kanamycin primers. Based on our previous *in silico* analysis, the T6SS-1 and T6SS-2 gene clusters are over 25 kb each, while the T6SS-3 gene cluster of *Pantoea ananatis* is ~9 kb. PCR primers flanking each T6SS gene cluster failed to produce an amplicon in wild-type strains but produced a 4 – 5 kb amplicon in the T6SS mutants (Fig 3.2 and results not shown). PCR primers targeting specific genes within each T6SS gene cluster produced amplicons of the predicted sizes in all *P. ananatis* wild-type strains but produced no amplicon in the corresponding mutant. PCR analysis of ΔT6SS-3 mutants from both strains of *P. ananatis* showed that 10/10 kanamycin-resistant colonies were true mutants. Sequencing of PCR products in the forward and reverse orientation confirmed replacement of the targeted T6SS gene cluster with the kanamycin gene.

3.4.3 Southern blot analysis of mutants

Southern blot analysis was used to determine the number of copies of the kanamycin cassette that inserted into the chromosome. Our results showed that the kanamycin probe did not hybridize to DNA restriction fragments of wild-type strains of *P. ananatis*. Importantly, the probe hybridized to the 600 bp kanamycin fragment which served as the positive control. The presence of a single hybridization signal in all mutants confirmed that a single copy of the PCR-generated kanamycin construct recombined in the genome (Fig. 3.3). This data together with our previous PCR verification results shows that the gene disruption cassettes transformed into wild-type strains of *P. ananatis* specifically recombined at the targeted T6SS gene cluster.

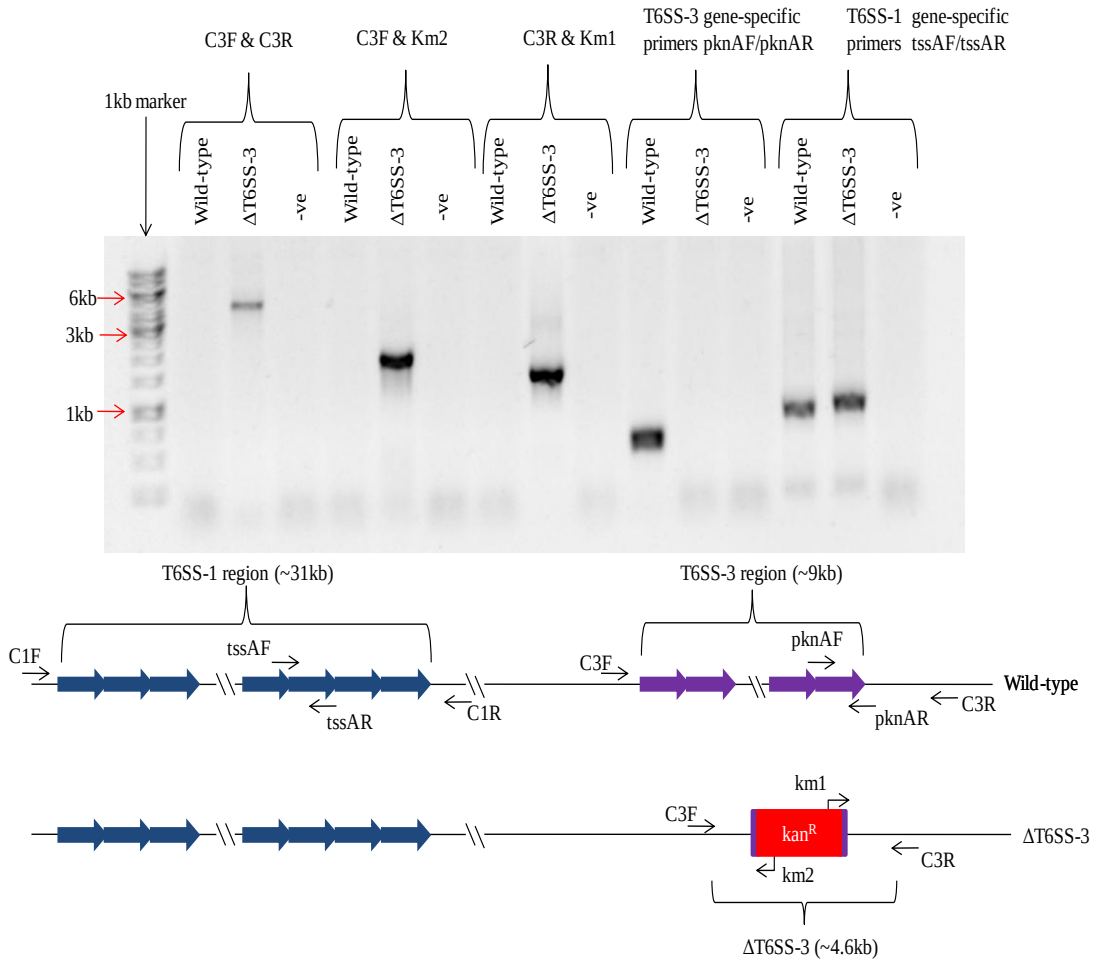


Figure 3.2: PCR verification of the type VI secretion system 3 (T6SS-3) mutant of *Pantoea ananatis*. PA = *Pantoea ananatis*, –ve = negative control. The combination of primers used for PCR verification are indicated as 1) Primers flanking T6SS-3 gene cluster (C3F & C3R); 2) Gene-specific primers pknAF & pknAR (T6SS-3), tssAF and tssAR (T6SS-1), and 3) A combination of flanking primers (C3F/C3R) and kanamycin primers (km1/km2).

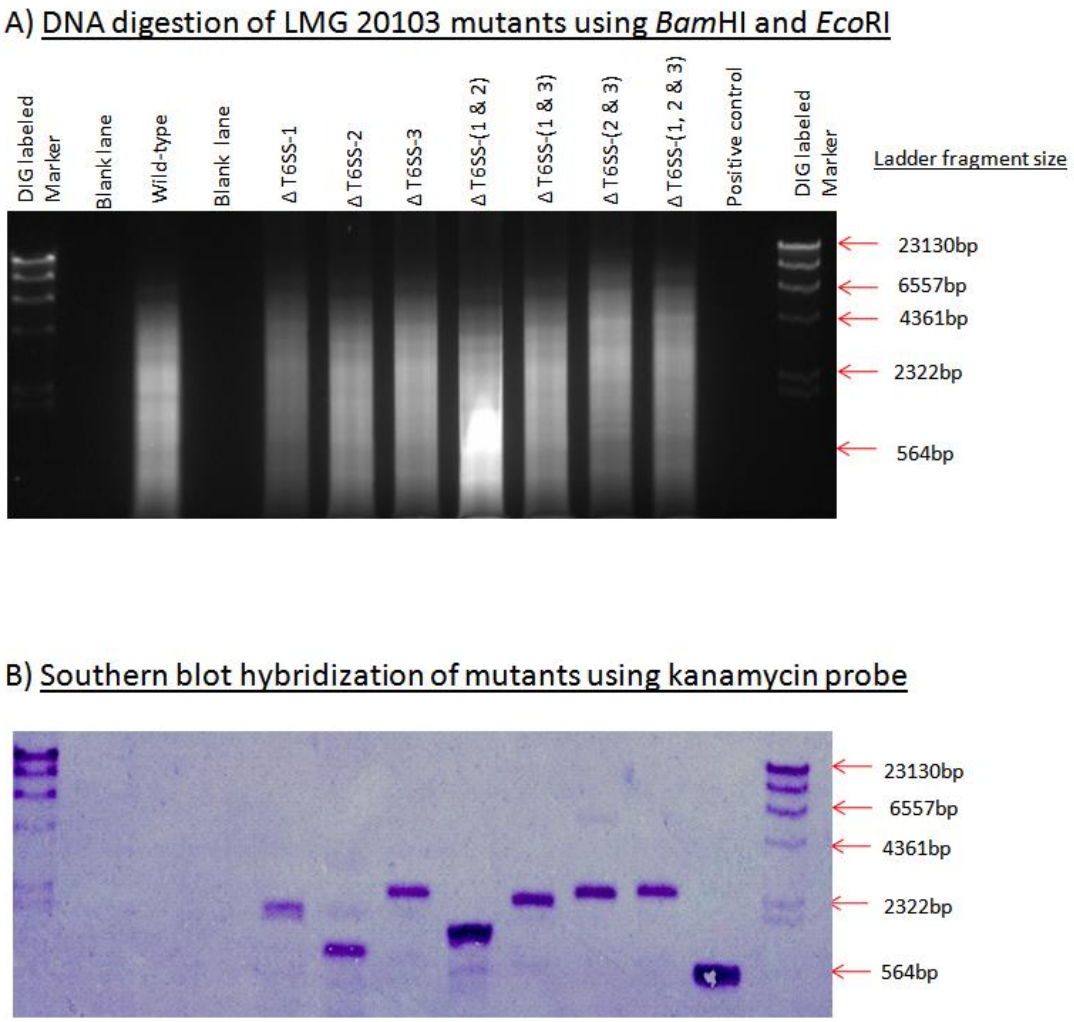


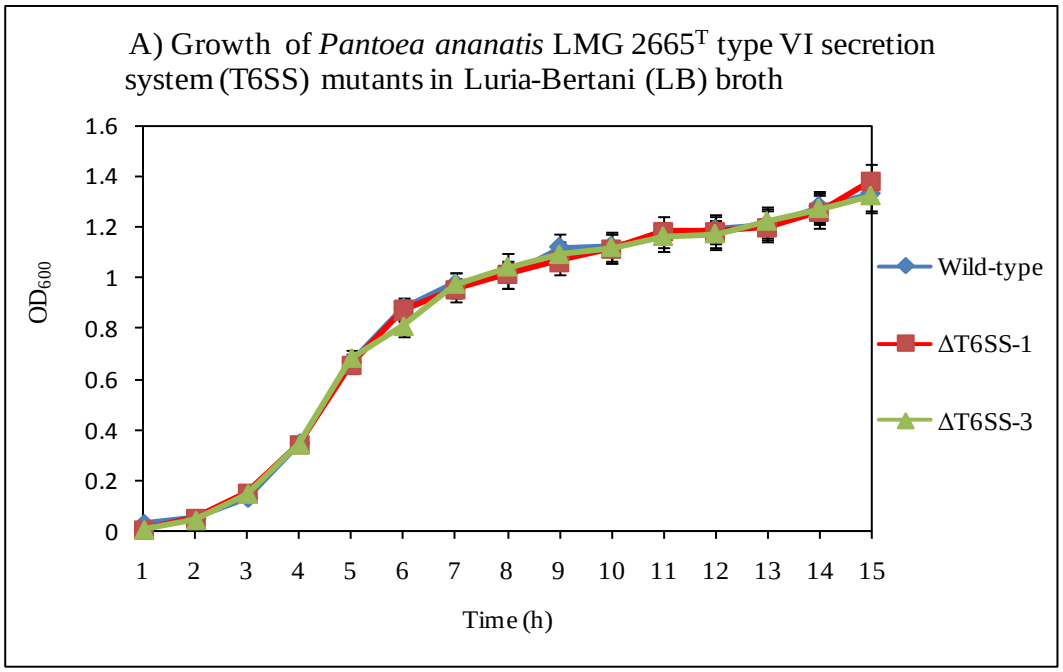
Figure 3.3 Southern blot hybridization of *Pantoea ananatis* strain LMG 20103 type VI secretion system (T6SS) mutants. Figure 3.5A shows complete digestion of the DNA of T6SS mutants and wild-type strains of *P. ananatis* using *Eco*RI and *Bam*HI restriction enzymes. Blank lanes represent wells in the agarose gel that were not loaded with restricted DNA samples. Positive control is a partial PCR sequence of the kanamycin gene. Fig 3.5B shows results from overnight hybridization with a DIG-dUTP labeled kanamycin probe.

3.4.4 Growth assay

Growth assays in rich and minimal media were used to determine the effect of the T6SS mutation on growth of *P. ananatis*. Our results demonstrated that wild-type strains of *P. ananatis* and their corresponding T6SS mutants had similar growth rates in both LB broth and M9 minimal medium (Figs. 3.4 and 3.5). Similarly, the T6SS mutants of strain LMG 20103 and LMG 2665^{T} grew at similar rates to the wild-type strains when inoculated into onion plants (Fig. 3.6).

3.4.5 Pathogenicity trials

To investigate the role of the different T6SSs in pathogenicity we inoculated the ΔT6SS mutants and wild-type strains of *P. ananatis* into onion seedlings (*Allium cepa* cv. Texas grando) and analyzed disease progression. Our results indicated that all ΔT6SS mutants of *P. ananatis* LMG 20103, when inoculated into onion plants, were as pathogenic as the wild-type strain (Fig. 3.7, and results no shown). These strains caused necrotic lesions which progressed and led to wilting of inoculated onion leaves 3 days post-inoculation. When comparing wild-type LMG 2665^{T} to its T6SS mutants, we observed that the ΔT6SS-3 mutant of LMG 2665^{T} was as pathogenic as the wild-type strain when inoculated into susceptible onion plants. Interestingly, the ΔT6SS-1 mutant of LMG 2665^{T} was non-pathogenic and produced no disease symptoms in onion seedlings.



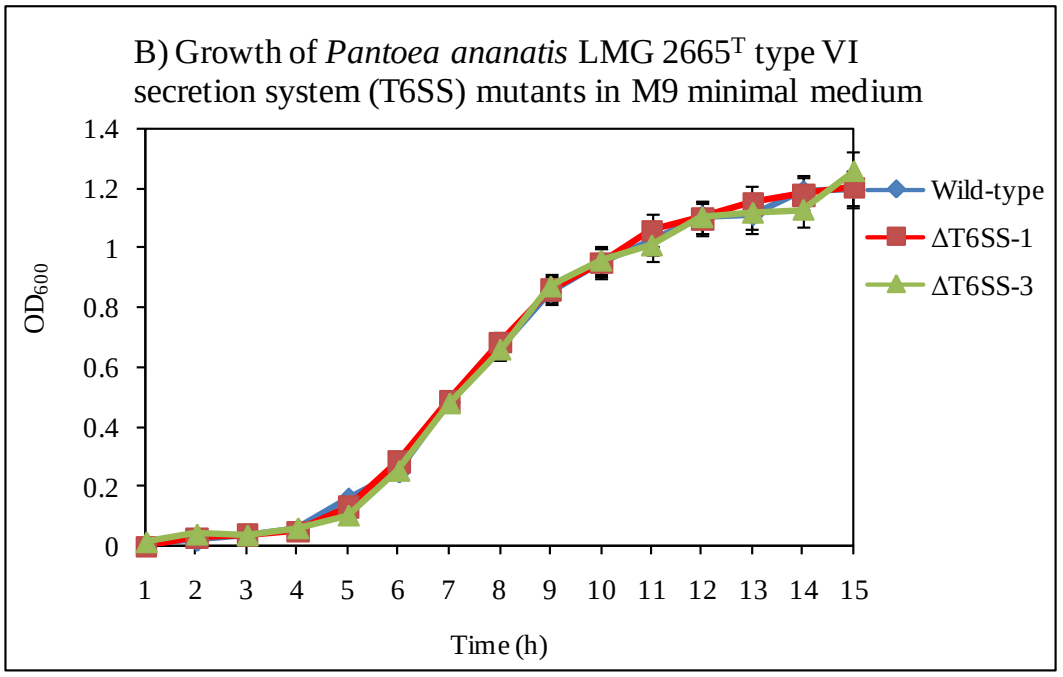
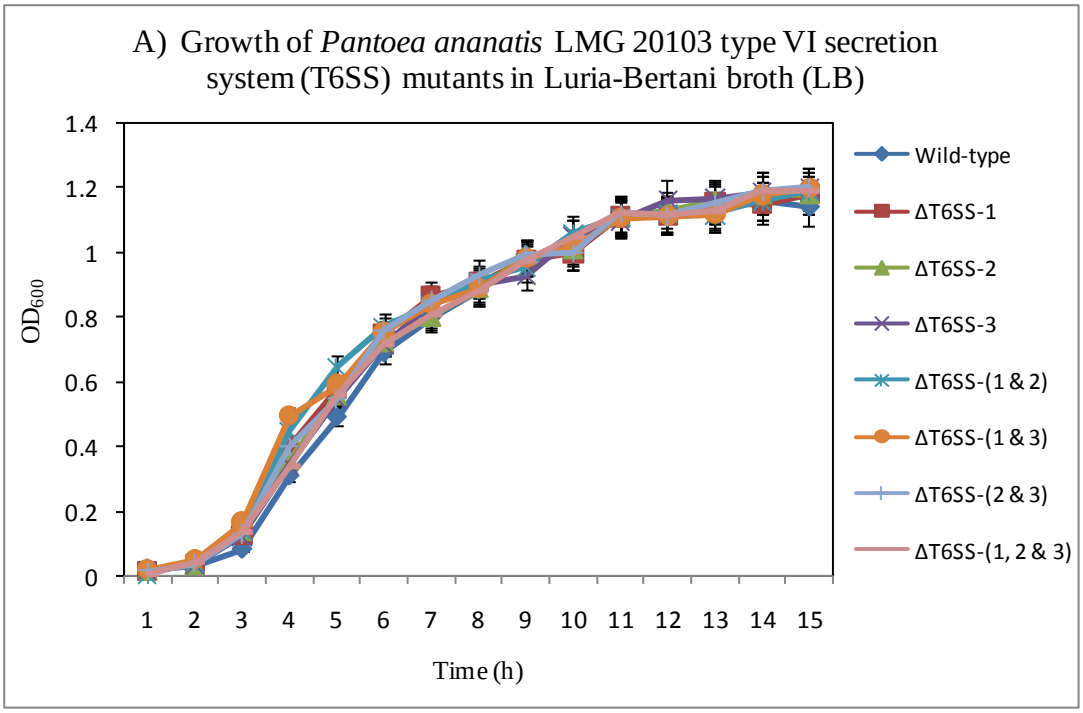


Figure 3.4 *In vitro* growth assay of *P. ananatis* LMG 2665^T type VI secretion system mutants. Figures A and B show growth of *P. ananatis* LMG 2665^T T6SS mutants in LB broth and minimum medium, respectively. All assays were performed in duplicates and repeated three times. Error bars represent ± SEM.



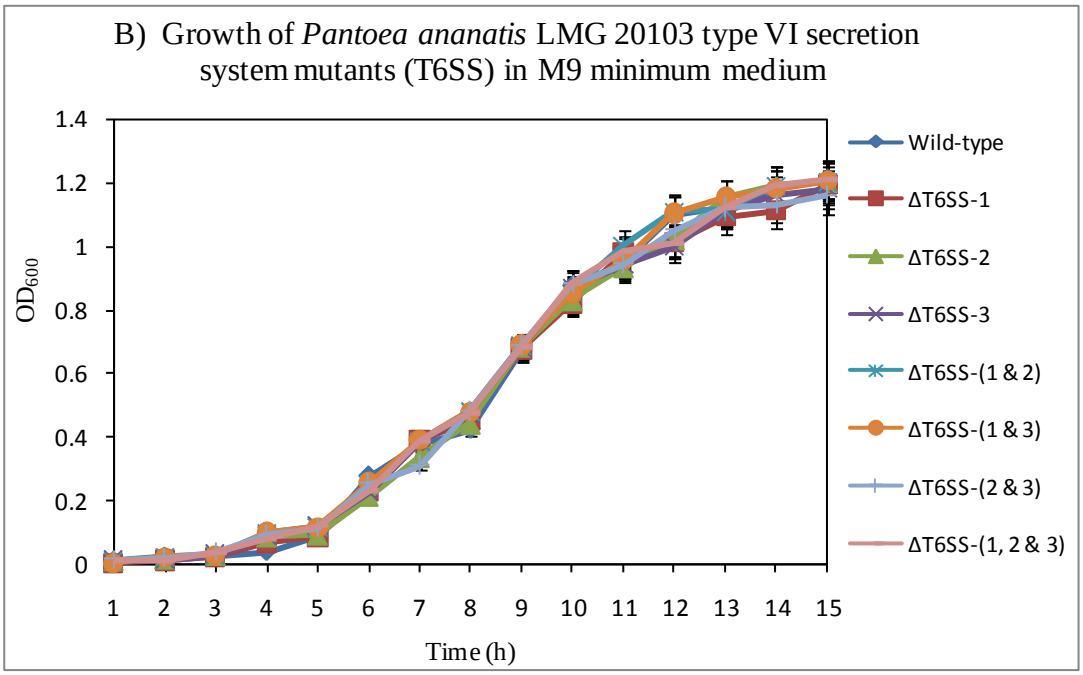
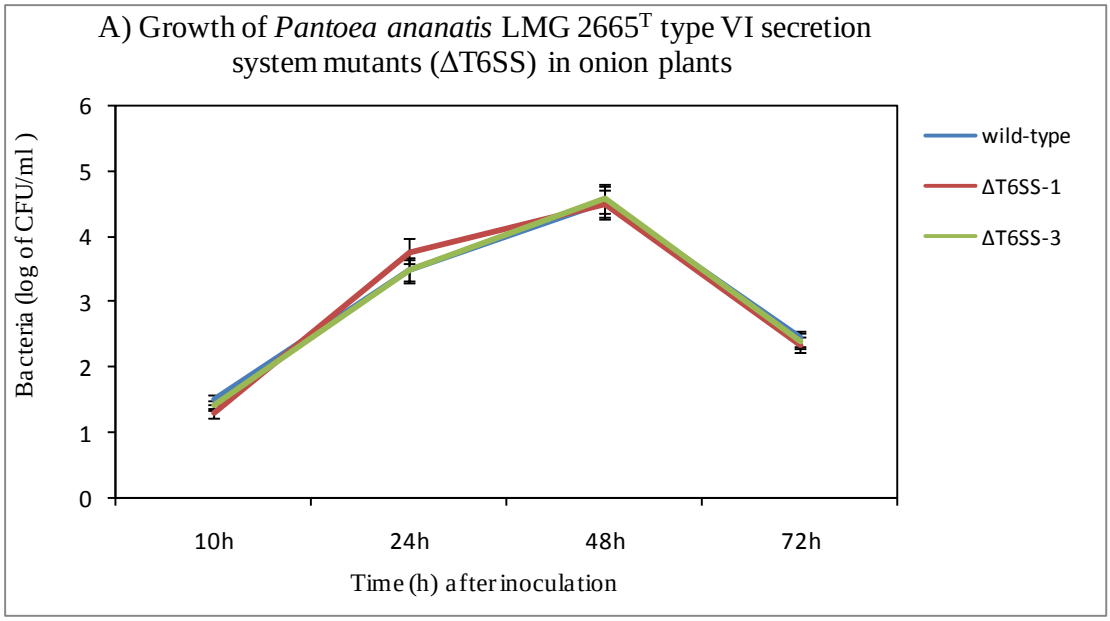


Figure 3.5 *In vitro* growth assay of *Pantoea ananatis* LMG 20103 type VI secretion system (T6SS) gene cluster mutants. Figures A and B show growth of the wild-type strain of *P. ananatis* LMG 20103 and its corresponding T6SS mutants in LB broth and minimum medium, respectively. All assays were performed in duplicate and repeated three times. Error bars represent the mean ± standard error.



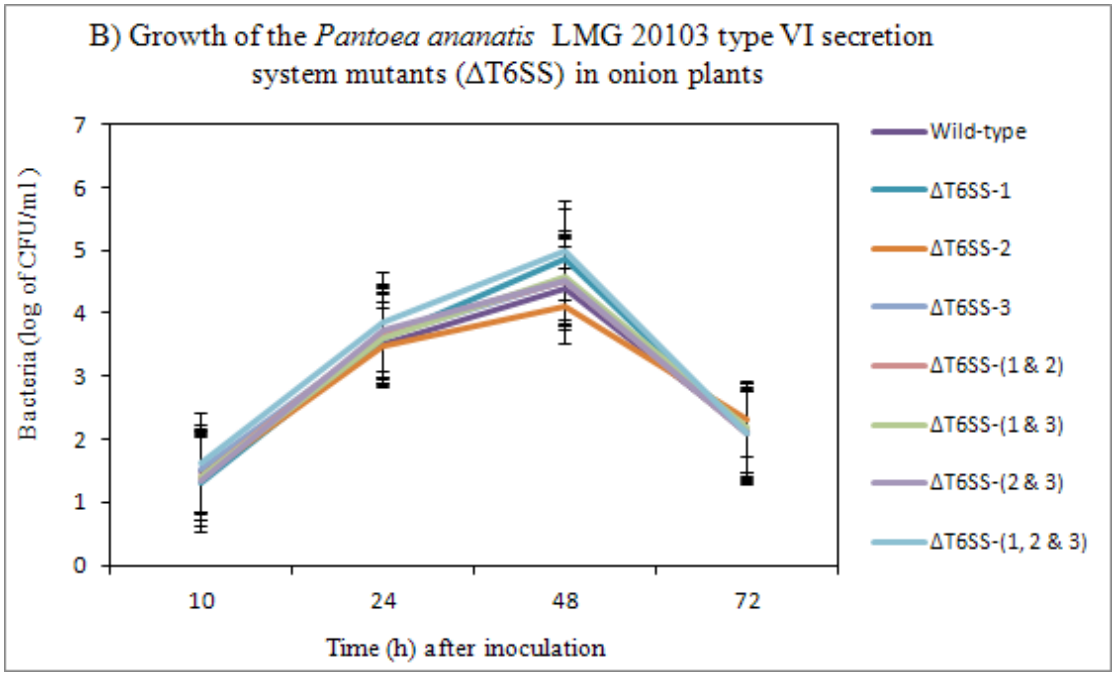
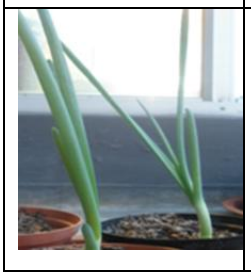


Figure 3.6 *In planta* growth assay of wild-type strains of *Pantoea ananatis* and their corresponding type VI secretion system mutants (ΔT6SS). Onion leaves were inoculated with 10^3 colony forming units (CFU/ml) of bacteria. Bacteria growth was determined by scoring the CFU/ml of bacteria recovered from onion leaf extracts. A shows the time course of growth of *P. ananatis* strain LMG 2665^T and the corresponding T6SS mutants, in onion plants. B shows the time course of growth of strain LMG 20103 and corresponding T6SS mutants, in onion plants. Experiments were repeated three times. Error bars represent the mean ± standard error.

ddH_2O	LMG 2665^T wild-type	2665^TΔT6SS-1	2665^TΔT6SS-3	LMG 20103 wild-type
				

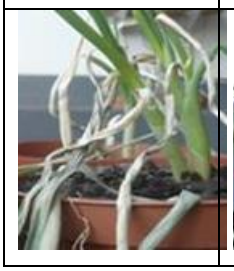
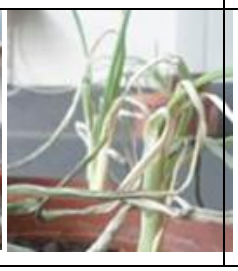
20103ΔT6SS-1	20103 ΔT6SS-2	20103 ΔT6SS-3	20103ΔT6SS-(1, 2 & 3)	ddH_2O
				

Figure 3.7 Results of virulence assay using *P. ananatis* strain LMG 2665^T and *Pantoea ananatis* strain LMG 20103 and type VI secretion system mutants. Onion plants (cv. Texas grando) were inoculated with 2 – 3 drops of bacterial suspension (OD_{600} of 0.1) using a sterile hypodermal syringe. Inoculated plants were maintained in the greenhouse for 3 – 4 days and disease severity recorded. Our results indicated that a) The ΔT6SS-1 deletion mutant of strain LMG 2665^T was non-pathogenic when inoculated into susceptible onion plants, b) The LMG 2665^T ΔT6SS-3 mutant was as pathogenic as the wild-type strain and 3) All T6SS deletion mutants of strain LMG 20103 were as pathogenic as the wild-type strain when inoculated into onion plants. In addition, the T6SS double mutants of LMG 20103 [ΔT6SS-(1 & 2), ΔT6SS-(1 & 3) and ΔT6SS-(2 & 3)] were as pathogenic as wild-type LMG 20103 (results not shown).

3.4.6 Bacterial growth competition assays

Recent studies have shown that some bacteria use the T6SS to engage in intrastrain and interspecies competition. This finding led us to hypothesize that the T6SS of *P. ananatis* could play a role in inter-bacterial competition. An *in vitro* growth competition assay was conducted on LB agar to determine whether or not the T6SS of *P. ananatis* has antibacterial properties. The results showed that 19.4% (7/36) of target bacteria were inhibited by wild-type LMG 20103 and LMG 2665^{T}. This inhibition represented a one log reduction ($p < 0.05$) in CFU/ml of the recovered target bacteria. Bacteria inhibited by these *P. ananatis* strains comprised of *Escherichia coli, Pectobacterium atrosepticum, Salmonellea enterica* serovar *Typhimurium* and three strains of *P. ananatis* (LMG 5342, LMG 2669 and LMG 2664). Target strains that were inhibited by wild-type strains of *P. ananatis* were also inhibited by the ΔT6SS-2 mutant strains of LMG 20103 and LMG 2665^{T}. Similarly, the ΔT6SS-3 and ΔT6SS-(2&3) mutants of LMG 20103 were as virulent as the wild-type. Susceptibility of target bacteria to growth inhibition by *P. ananatis* was completely lost following deletion of the T6SS-1 gene clusters of *P. ananatis* strains LMG 20103 and LMG 2665^{T} (Figs. 3.8 and 3.9). Furthermore, the double mutants, 20103ΔT6SS-(1&2), 20103ΔT6SS-(1&3) and the triple mutant 20103ΔT6SS-(1, 2, 3) all lost the ability to inhibit target bacteria. The results demonstrated that, growth inhibition exhibited by wild-type strains of *P. ananatis* was associated with T6SS-1. Results of the inter-bacterial competition assay are summarized in Table 3.4.

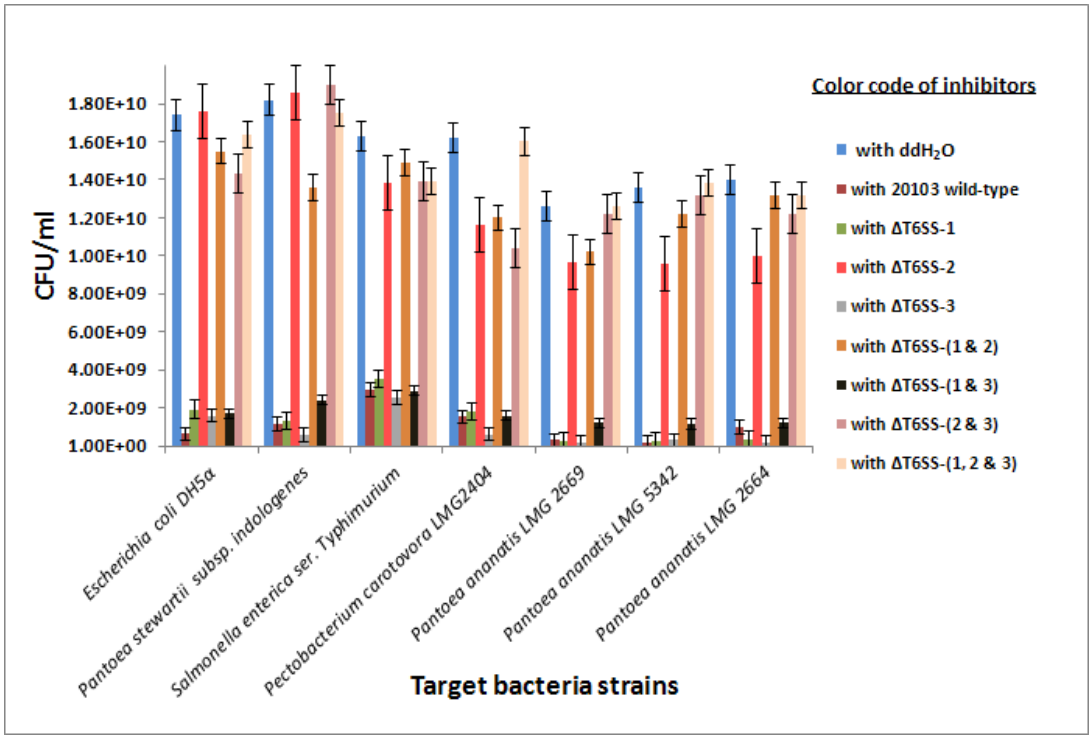


Figure 3.8 Bacterial competition assays between *Pantoea ananatis* LMG 20103 and indicated target bacteria. Graph shows the number of recovered competitor bacteria (CFU/ml) following 24 h co-cultured with wild-type and T6SS mutants of *P. ananatis* strain LMG 20103. Color of the bars represents the different bacteria targeted for inhibition by wild-type LMG 20103 and T6SS mutants. Blue bars represent controls, i.e., target bacteria co-cultured with ddH_2O (no LMG 20103 in the mixture). Height of the bars represents the CFU/ml of recovered target bacteria, error bars represent 1 standard deviation. All bars below 2.5E+09 CFU/ml are statistical significant reduction ($p < 0.05$) in CFU/ml of targeted bacteria as determined by an unpaired, two-tailed Student's *t*-test.

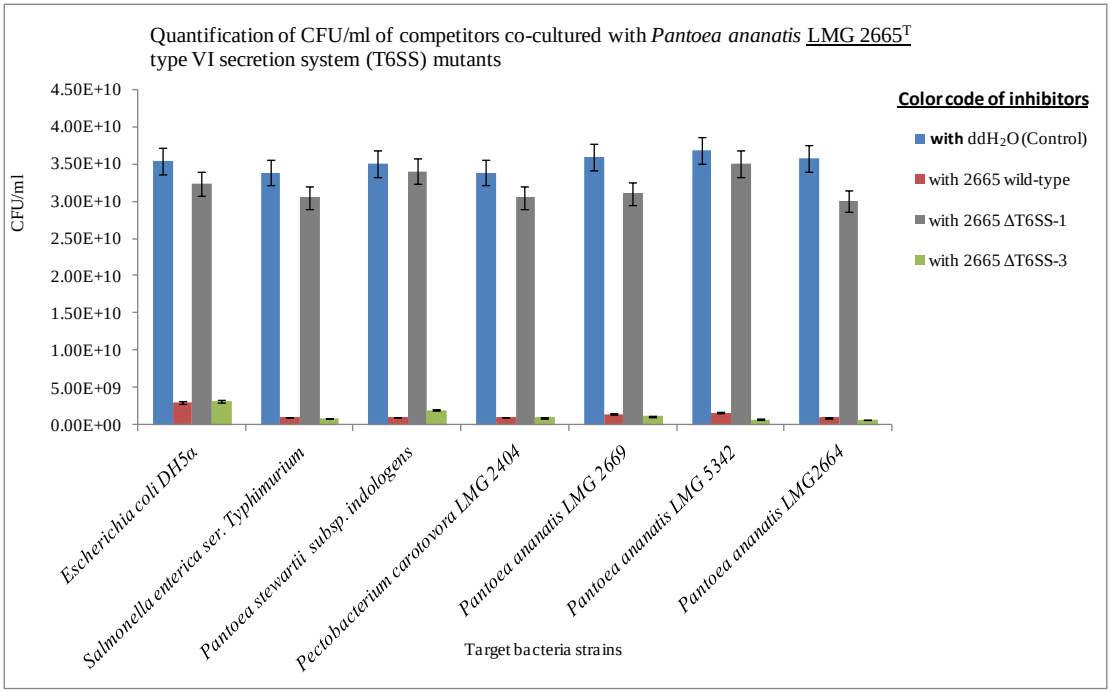


Figure 3.9 Bacterial competition assay between *P. ananatis* LMG 2665^T and the indicated target bacteria. Graph shows the number of recovered competitor bacteria (CFU/ml) following 24 h co-culture with wild-type and T6SS mutants of *P. ananatis* strain LMG 2665^T. Color of the bars represents the different bacterial species that were targeted for inhibition by *P. ananatis*. Height of the bars represents the CFU/ml of recovered target bacteria, error bars represent 1 standard deviation All bars below 5.00E+09 CFU/ml represent a statistical significant reduction ($p < 0.05$) in CFU/ml of targeted bacteria as determined by an unpaired, two-tailed Student's *t*-test.

Table 3.4 Summary of results from pathogenicity trials and inter-bacterial competition

***P. ananatis* strains**	**Pathogenicity trials**	**Competition assay**	**Significance of the results**
Wild-type strains (LMG 20103 & LMG 2665^T)	+	+	Suggest that *P. ananatis* strains LMG 20103 and LMG 2665^T are pathogenic to onion and inhibit growth of other bacterial species
20103 ΔT6SS-1	+	-	Suggests that T6SS-1 is not required for pathogenicity but plays a role in competition
20103 ΔT6SS-2	+	+	Suggests that T6SS-2 is not required for pathogenicity and competition
20103 ΔT6SS-3	+	+	Suggests that T6SS-3 is not required for pathogenicity and competition
20103 ΔT6SS-(1&2)	+	-	Confirms that T6SS-1 and T6SS-2 are not required for pathogenicity while T6SS-1 is required for competition
20103 ΔT6SS-(1&3)	+	-	Confirms that T6SS-1 and T6SS-3 are not required for pathogenicity and confirms that T6SS-1 is required for competition
20103 ΔT6SS (2&3)	+	+	Confirms that T6SS-2 and T6SS-3 are not required for pathogenicity and competition
20103 ΔT6SS (1, 2&3)	+	-	Confirms that T6SS-1 is required for competition and that none of the otherT6SS clusters of strain LMG 20103 are required for pathogenicity
2665^T ΔT6SS-1	-	-	Suggests that T6SS-1 is required for pathogenicity in onion plants and also plays a role in inter-bacterial competition
2665^T ΔT6SS-3	+	+	Suggests that T6SS-3 is not required for pathogenicity or inter-bacterial competition under our assay conditions

The results represent the average of three independent experiments done in the greenhouse under similar conditions. Positive pathogenicity reactions (+) indicates development of necrotic reactions at the point of inoculation, which later extended to the entire length of the onion leaf, leading to wilting. A positive inter-bacterial competition result (+) indicates the ability of the *P. ananatis* strain or mutant to inhibit growth of competing bacteria following 24 h co-culture on LB agar. Competitors used in inter-bacterial assays included *Escherichia coli* DH5α, *Salmonella enterica* serovar Typhimurium, *Pectobacterium carotovora* LMG 2404^T, *Pantoea ananatis* strains LMG 5342, LMG 2664 and LMG 2669.

3.5 DISCUSSION

The T6SS has been shown to be a virulence determinant of several different bacterial strains and species (69). More recently, the T6SS has been shown to target other bacteria, thus implicating the T6SS in inter-bacterial competition (30, 31, 32, 33, 34). Multiple T6SS gene clusters found in a given bacterial genome have been shown to play different roles (21, 29, 50). *Pantoea ananatis* is an important plant pathogen whose genome sequence contains up to three T6SS gene clusters (17). To date, it is unknown whether these gene clusters encode a functional T6SS and what such clusters play in the biology of *P. ananatis*. In this study, we systematically deleted the different T6SS gene clusters found in the genome sequence of *P. ananatis* strains LMG 20103 and LMG 2665^{T}.

Given that the T6SS of *Pectobacterium atrosepticum* contributes to virulence in potato plants, we decided to test if the T6SS of *P. ananatis* plays a role in onion pathogenesis (70). Our results showed that the LMG 2665^{T} T6SS-3 mutant was as pathogenic as the wild-type strain. However, the T6SS-1 mutant of LMG 2665^{T} was non-pathogenic and produced no disease symptoms, similar to the water-inoculated plants. Our results demonstrate for the first time that a T6SS (that of T6SS-1) is required for virulence of LMG 2665^{T} in onion plants. Interestingly, similar results were not obtained using *P. ananatis* strain LMG 20103. All individual T6SS mutants (ΔT6SS-1, ΔT6SS-2 and ΔT6SS-3) caused necrosis and wilting of onion plants similar to the wild-type strains. In *P. aeruginosa*, it was reported that H2-T6SS and H3-T6SS functionally compensated each other in virulence when either one was deleted (50). However, this was not the case in LMG 20103, as the double mutants ΔT6SS-(1&2), ΔT6SS-(1&3), ΔT6SS-(2&3) and the T6SS null mutant ΔT6SS-(1, 2&3) were as pathogenic wild-type LMG 20103. The reason for this discrepancy is unclear and it is currently unknown how this strain is capable of killing onion plants. The T6SS has not been associated with a virulence phenotype in several different bacteria. For example, no virulence defects were observed when the T6SS mutants of *V. anguillarum*, *Y. pestis*, Enteroaggregative *E. coli* and Uropathogenic *E. coli* were inoculated into different hosts (28, 71, 72, 73). Similarly, *P. syringae* pv. *syringae* T6SS mutants were able to colonize bean plants, multiply *in planta* and produce disease symptoms similar to the wild-type strain (74). In addition, the T6SS gene mutants of *P. atrosepticum*, when inoculated into potato tubers, were attenuated in

virulence, while deletion of the entire T6SS gene cluster of *A. tumefaciens* did not attenuate virulence of the T6SS mutant in potato plants, compared to the wild-type strain (70, 75). These findings suggest that while the T6SS is required for virulence of some bacterial species, it may not be in other bacteria.

We hypothesize that LMG 20103 and LMG 2665^T use different mechanisms to colonize and cause disease in onion plants. In support of this hypothesis, the enteric pathogens *S. enterica* serovars Enteritidis and Typhimurium cause systemic infections in mice using different mechanisms (76, 77). *S.* Typhimurium uses the T6SS encoded by *Salmonella* pathogenicity island 6 (SP-6) to colonize and cause disease in mice, however, this cluster is missing from the genome sequence of *S.* Enteritidis (25, 78). The genome sequence of *S.* Enteritidis contains a degenerate SP-19 T6SS gene cluster with an internal deletion of ~ 24 kb, suggesting that this serovar does not encode a functional T6SS (78). Despite the absence of the SP-6 T6SS and SP-19 T6SS gene clusters, *S.* Enterica can still colonize and infect mice using several serovar-specific genes (77). In addition, *S.* Typhimurium, and *S.* Enteritidis colonize poultry asymptomatically, while *S.* Gallinarum causes fowl typoid in in poultry (76). In order to colonize chicken organs, *S.* Typhimurium again uses the SP-6 T6SS (79), *S.* Gallinarum uses the SP-19 T6SS (51), while the mechanism of colonization of chicken by *S.* Enteritidis is largely unknown (51). In fact, *in trans* expression of the full-length SP-19 T6SS gene cluster from *S.* Gallinarum to *S.* Enteritidis resulted in defects in chicken colonization (51). It is, therefore, possible that the T6SS of LMG 20103 is not absolutely required for onion pathogenicity and was evolutionary conserved to play a general role in fitness in different environments or host plant. Alternatively, it is possible that our assay conditions were not sensitive enough to detect what role the T6SS of LMG 20103 plays in onion pathogenesis. Since LMG 20103 is a plant pathogen, the use of known alternative host plants, such as maize, could help unravel the biological role of the LMG 20103 T6SS in host-microbe interactions (12).

Recent studies have shown that some bacterial strains and species use their T6SS to inhibit growth of other bacteria (29, 30, 31, 32, 33, 34). We therefore conducted an *in vitro* growth competition assay to determine if the T6SS of *P. ananatis* plays a role in inter-bacterial interactions. Our results showed that LMG 20103 and LMG 2665^T

caused a 1 $\log_{10}$ reduction in CFU/ml of recovered *Pectobacterium carotovorum* subsp. *carotovorum*, *P. stewartii* subsp. *indologenes*, *E. coli*, *S.* Typhimurium, and three strains of *P. ananatis* (LMG 2669, LMG 2664 and LMG 5342). This inhibition was completely dependent on the T6SS-1 of *P. ananatis* and deletion of this gene cluster abrogated the growth defect of targeted bacteria. The magnitude of inhibition seen in this study is similar to that reported for *V. cholerae* strain A1552, which caused a 1.5 - 2 $\log_{10}$ reduction in the number of recovered target bacteria (80, 81). However, this degree of inhibition is vastly different from those reported for *S. marcescens* and *V. cholerae* strain V52 where up to a 4 $\log_{10}$ reduction in CFU/ml of recovered target bacteria was reported (30, 31). The main reason for this difference may be that the T6SS of *S. marcescens* and *V. cholerae* V52 are constitutively expressed and produce copious amounts of secreted Hcp, and therefore possibly similar amounts of antimicrobial effectors under laboratory conditions (18). On the other hand, the T6SS of *V. cholerae* A1552 is repressed under laboratory conditions and does not secrete high levels of Hcp into the culture supernatant under inducing conditions (340 mM NaCl and 37^{o}C) (80, 81). Similar findings have been reported for *P. aeruginosa* whose T6SS is repressed by the transcriptional regulator RetS and a post-transcriptional regulator PppA (19, 82, 83). Based on these studies, we speculate that our assay conditions may not have been optimal for induction of the T6SS of *P. ananatis*, however, this still needs to be experimentally verified. This notwithstanding, the magnitude of inter-bacterial inhibition seen in this study was statistically significant ($p < 0.05$) as determined by an unpaired, two-tailed Student's *t*-test, and thus indicates that the T6SS-1 of *P. ananatis* is active under our assay conditions (32^{o}C, LB agar).

Susceptibility to growth inhibition by the T6SS-1 of *P. ananatis* was not determined by the presence of a functional T6SS in target bacteria, since *E. coli* DH5α that lacks T6SS genes was inhibited alongside *S.* Typhimurium, which is known to encode a functional T6SS (51). We also noted that LMG 20103 and LMG 2665^{T} use T6SS-1 to inhibit growth of closely related strains *P. ananatis*. The biological significance of *P. ananatis* intraspecies competition is currently unknown however, similar findings have been reported for *S. marcescens*, *V. cholerae* and *V. parahaemolyticus* (31, 34, 84). Like *P. ananatis*, the T6SS of *S. marcescens* DB10 inhibits the growth of a closely related strain, *S. marcescens* ATCC 274, which was shown to encode an

active and functional T6SS (31). In that study, the authors proposed that the genome sequences of the two strains of *S. marcescens* could contain genes encoding different immunity factors, which confer resistance to a cognate T6SS effector (31). Using the same analogy, we speculate that *P. ananatis* strains that were inhibited by LMG 20103 and LMG 2665^T either 1) lacked the cognate immunity factor needed to neutralize the yet to be identified T6SS-1 effector, or 2) these *P. ananatis* strains encode a different effector/immunity system. In support of this hypothesis, 12 of the 15 strains of the *P. ananatis* (80%) analyzed in this study were not susceptible to growth inhibition by LMG 20103 and LMG 2665^T. In addition, while *P. ananatis* was able to inhibit growth of *P. carotovorum* subsp. *carotovorum*, it was unable to inhibit growth of *P. carotovorum* subsp. *brasiliensis*, *P. atrosepticum*, *Pantoea vagans*, *S. marcescens*, *Pseudomonas putida*, and *Bacillus subtillus* (results not shown). These findings demonstrate that the T6SS-1 of LMG 20103 and LMG 2665^T selectively targets some but not all bacterial strains and species. Similarly, the T6SS of *V. parahaemolyticus* inhibits growth of *E. coli* and *V. cholerae* but not *P. aeruginosa*, suggesting that bacterial resistance to T6SS-mediated cytotoxicity is widespread in different bacteria (34).

P. ananatis is ubiquitous in nature (8), suggesting that different strains will encounter and compete with other bacterial species for nutrients and niche. The test organisms used in our competition assay represent typical species that *P. ananatis* may encounter in these different environments. *S.* Typhimurium is a human pathogen (76), *P. carovotorum* is a pathogen of cucumber, onion, potato and cabbage (85), while *P. stewartii* subsp. *indologenes* has been isolated from symptomatic millets, pineapple, Sudangrass, Eucalyptus and onion (86, 87). With the exception of cabbage, *P. ananatis* has been isolated from all these plants (88, 89, 90, 91). In addition, the clinical isolate of *P. ananatis*, LMG 5342, was isolated from a bacteremic patient, suggesting that *P. ananatis* could encounter *S.* Typhimurium in humans (92). The ability to inhibit growth of these bacterial species could, therefore, provide *P. ananatis* a fitness advantage in terms of nutrient availability and niche colonization.

In conclusion, we have shown for the first time that the T6SS-1 of LMG 2665^T plays a role in the pathogenicity of *P. ananatis* in onion plants. We also showed that both LMG 20103 and LMG 2665^T use T6SS-1 to inhibit growth of several enteric pathogens including plant pathogens. While the roles of the *P. ananatis* T6SS-2 and

T6SS-3 gene clusters were not determined in this study, we have provided compelling evidence that LMG 20103 and LMG 2665^T do encode a functional T6SS. The results of our findings could in part explain why *P. ananatis* is a versatile and successful bacterial species. Some pathogenic strains could use the T6SS to cause disease symptoms in different susceptible host plants, while environmental strains could use the same gene cluster to compete with other bacterial species for nutrients and niche colonization. Future studies will be directed towards determining 1) the role of *P. ananatis* T6SS-2 in survival, proliferation and colonization of host plants, 2) optimal conditions for induction and expression of the T6SS of *P. ananatis*, 3) which T6SS effectors are associated with inter-bacterial competition, and 4) whether or not T6SS-2 and T6SS-3 gene clusters encode a functional T6SS and what role they play in the biology of *P. ananatis.* Addressing these issues will further our understanding of the role played by *P. ananatis* T6SS in virulence, fitness and inter-bacterial interactions.

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Chapter 4

TssD and TssA are components of the type VI secretion system-1 of *Pantoea ananatis* strain LMG 2665^{T} and are required for virulence and inter-bacterial competition

4.1 ABSTRACT

In Chapter 3, we showed that the type VI secretion system-1 (T6SS-1) of *Pantoea ananatis* strain LMG 2665^T was involved in virulence and inter-bacterial competition. Because the entire T6SS-1 gene cluster of LMG 2665^T was deleted in the above study, the contribution of individual genes found in the T6SS-1 gene cluster to the observed phenotypes could not be determined. Therefore, in this study, we generated isogenic mutants of LMG 2665^T T6SS-1 deficient in either *tssD* (encodes Hcp protein, an effector and structural protein) or *tssA* (encodes TssA, a conserved T6SS protein of unknown function). We functionally characterized these mutants with regards to pathogenicity in onion plants and their ability to inhibit growth of other Gram-negative bacteria. Our results show that Δ*tss*A and Δ*tssD* mutants were non-pathogenic on susceptible onion plants. Ectopic expression of the *tssA* and *tssD* genes in the corresponding T6SS mutants restored pathogenicity on onion to wild-type levels. In addition, *tssA* and *tssD* mutants lost the ability to inhibit growth of closely related bacterial species, while the complemented mutant strains were restored in their ability inhibit growth of *Escherichia coli*, *Pectobacterium carotovorum*, *Salmonella* Typhimurium and *P. ananatis* strains LMG 2664, LMG2669 and LMG 5342. Our results provide the first genetic evidence showing that the *tssA* and *tssD* genes located within the T6SS-1 gene cluster of LMG 2665^T are required for pathogenicity and inter-bacterial competition. We hypothesize that the *tssA* and *tssD* genes may encode conserved proteins which are required for secretion or biosynthesis of a functional T6SS-1 of LMG 2665^T.

4.2 INTRODUCTION

Pantoea ananatis is known to cause disease on several economically important crop plants (1). In the previous chapter, the role of the T6SS-1 of *P. ananatis* strain LMG 2665^T in the pathogenicity of onion and inter-bacterial competition was demonstrated. However, the homologous T6SS-1 of strain LMG 20103 was shown to play a role in inter-bacterial competition but was not required for onion pathogenesis. The dual role in pathogenicity and inter-bacterial competition demonstrated for the T6SS-1 of strain LMG 2665^T was particularly interesting and prompted further investigation. Unless otherwise stated, all references to the T6SS-1 gene cluster in this chapter will represent the T6SS-1 cluster of *P. ananatis* strain LMG 2665^T. The limitation of our

previous study was that, the T6SS-1 mutant was not complemented and the contribution of individual T6SS-1 genes to these phenotypes was not determined. Therefore, in this study, we focused on the *tssA* and *tssD* genes located within the T6SS-1 gene cluster of strain LMG 2665^{T}. The objective of this study was to determine whether *tssA* and *tssD* are required for virulence and inter-bacterial competition.

The T6SS gene clusters usually contain 15-30 different genes, of which 13 are conserved in different bacteria (2, 3, 4). These conserved T6SS genes have been designated *tssA* to *tssM* (type six secretion A-M) (5). Putative functions have been assigned to the protein products of *tssA* to *tssM* based on bioinformatics analysis, functional studies and protein-protein interactions (6, 7, 8, 9). One of the best studied proteins associated with the T6SS is Hcp (hemagglutin co-regulated protein), which is encoded by the *tssD* gene (10). Hcp forms hexameric rings that polymerize *in vitro* to form tube-like structures similar to the T4 bacteriophage tail tube (11, 12, 13, 14). This protein is believed to be both an effector and a structural component of the T6SS (10, 14). Functional studies showed that the *tssD* mutant of *Edwardsiella tarda* is attenuated in virulence against the blue gourami fish, while the *tssD* mutant of *Vibrio cholerae* was attenuated in virulence against amoeba and *E. coli* cells (15, 16). Similarly, the *tssD* mutant of *P. syringae* pv. *tomato* was unable to inhibit growth of other Gram-negative bacteria and the mutant also lost the ability to grow in mixed bacterial cultures (17). All *tssD* mutants studied to date are unable to secrete Hcp into the cell culture supernatant. These findings have led to the speculation that Hcp is an important constituent of the T6SS and inactivation of this protein or deletion of the *tssD* gene causes either defects in assembly or secretion of effectors, which ultimately compromised virulence or other phenotypes related to the T6SS (16). Remarkably, contradicting results have been associated with deletion of the *tssD* gene of some bacterial species. For example, the *tssD* mutant of *Pectobacterium atrosepticum* was not attenuated in virulence when inoculated into potato tubers, while the *tssC* and *tssK* mutants were attenuated in virulence (18, 19). In *Agrobacterium tumefaciens*, the *tssD* mutant was attenuated in virulence when inoculated into potato discs, while deletion of the entire T6SS gene cluster or the *tssM* gene did not produce similar results (20). Similarly, the *tssD* mutant of *V. cholerae* was avirulent in mice, while the *tssM* mutant lost the ability of produce diarrhea-like symptoms in mice compared to the

wild-type (21, 22). These findings suggest that while the *tssD* gene may be conserved in T6SS gene clusters found in bacterial genomes, the role of Hcp as an important structural component of the T6SS remains largely unknown.

TssA is predicted to be a cytoplasmic protein and contains an *impA*_like domain of unknown function (3, 9). It is speculated that TssA plays a regulatory role or is associated with proteins destined for secretion (3, 23). However, there is no experimental evidence showing a direct interaction between TssA and T6SS effectors such as Hcp and VgrG (24). Recent studies have shown that TssA interacts with TssK, which is predicted to be a cytoplasmic protein required for the formation of the TssB/TssC sheath complex (25). However, the biological significance of the TssA-TssK interaction is currently unknown. Functional studies have shown that the *tssA* mutant of *P. aeruginosa* was attenuated in virulence in a mouse model (26). Similarly, the *tssA* mutants of *Edwarsiella tarda* and *Vibrio cholerae* were attenuated in T6SS-related phenotypes, including Hcp secretion, virulence and inter-bacterial competition (15, 16). In addition, the *tssA* mutant of *Agrobacterium tumefaciens* was unable to secrete Hcp into the cell culture supernatant (24). These findings strongly suggest that the protein product of *tssA* could play an important role in either secretion or assembly of a functional T6SS. Unlike Hcp, no contradictory results have been reported for the TssA protein, which is encoded by the *tssA* gene found within the T6SS gene cluster of several bacterial species.

In this study, we investigated the role of the *tssA* and *tssD* genes located within the T6SS-1 gene cluster of *P. ananatis* strain LMG 2665^{T}. While deletion of the entire T6SS-1 gene cluster of strain LMG 2665^{T} resulted in loss of pathogenicity and attenuated virulence gainst other bacteria, these mutants were not complemented in that study. In addition, none of the 13 conserved genes (*tssA-M*) found in the T6SS-1 gene clusters of *P. ananatis* have been functionally characterized. Therefore, in this study, we constructed mutant and complemented mutant strains of two conserved T6SS genes, *tssA* and *tssD*. We functionally characterized the mutant strains based on pathogenicity and inter-bacterial competition to determine if they are absolutely required for these T6SS-related phenotypes. This study, therefore, presents the first genetic analysis of the *tssA* and *tssD* genes found within the T6SS-1 gene cluster of *Pantoea ananatis* strain LMG 2665^{T}.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial culture conditions

All bacterial strains and plasmids used in this study are presented in Table 4.1. Bacteria were routinely cultured at 32^{o}C in Luria-Bertani (LB) agar, LB broth or M9 minimal medium. When appropriate, antibiotics were supplemented into the growth medium in the following concentrations: kanamycin (50 µg/ml), tetracycline (15 µg/ml) and gentamycin (15 µg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) and restriction enzyme (*Eco*RI) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Growth media and supplements were purchased from Merck Laboratory supplies (Midrand, RSA). All reagents and chemicals were used according to the manufacturers' instructions.

4.3.2 Cloning of DNA fragments into plasmid vectors

4.3.2.1 Restriction endonuclease digestion

All reactions were performed in a microcentrifuge tube in a final reaction volume of 20 µl. Restriction digestion reactions included 2 µl of 10 x FastDigest buffer, 1 U of *Eco*RI restriction enzyme and 300 ng of plasmid pBRRMCS-5. The reaction was incubated for 1 hr at 37^{o}C and analyzed by agarose gel electrophoresis.

4.3.2.2 Klenow fill-in reaction

The Large Fragment of DNA Polymerase I (Klenow fragment) was used to polish the ends of DNA products. Klenow fill-in reactions included 200 – 500 ng of template (plasmid or PCR products), 2 µl of 10 x Klenow buffer, 0.5 mM dNTP mix (2 µg/ml stocks) and 0.5 U of Klenow enzyme in a final reaction volume of 20 µl. The reaction was incubated at room temperature for 15 min after which the DNA was purified using the DNA Clean and Concentrator^{TM} kit (Zymo Research), as directed by the manufacturer.

4.3.2.3 Ligation reaction

Ligation reactions contained 500 ng of PCR product, 100 ng of vector (pBRRMCS-5), 2 µl of 10 x ligation buffer and 1.5 U of T4 DNA ligase (Roche) in a final reaction volume of 20 µl. All ligation reactions were incubated overnight at 16^{o}C. Overnight

ligation reactions were transformated into electro-competent *E. coli* JM109. Transformants were selected on LB agar supplemented with gentamycin (15 µg/ml) following overnight incubation at 37^{o}C.

Table 4.1 List of bacterial strains and plasmids used in this study

Bacterial strains	Relevant characteristics	Source (a) or Reference (b)
Escherichia coli DH5α	F^{-} *recA*1 *endA*1 *hsdR*17 *deoR thi*-1 *supE*44 *gyrA*96 *relA*1 Δ(*lacZYA argF*) U169 λ^{-} [Φ80*lacZ*ΔM15]	Invitrogen[a]
Pantoea ananatis LMG 5342	Clinical isolate	De Baere *et al.* (2004)[b]
Pantoea ananatis LMG 2669	Pathogen of pineapple	Spiegelberg (1958)[a]
Pantoea ananatis LMG 2664	Pathogen of pineapple	Spiegelberg (1958)[a]
Pectobacterium carotovorum subsp. *carotovorum*	Pathogen of irish potato	Hellmers (1952)[a]
Salmonella enterica serovar Typhimurium	Clinical isolate	FABI[a]
Pantoea stewartii subsp. *indologenes*	Epiphyte	FABI[a]
Pantoea ananatis LMG 2665^{T}	Pathogen of pineapple	Serrano (1928)[b]
LMG 2665T (0)	Derivative of *P. ananatis* strain LMG 2665^{T} expressing the lambda recombination plasmid (pRSFRedTER), Cm^{R}	This study
Δ*tssA*	LMG 2665^{T}::Kan^{R} Δ*tssA*	This study
Δ*tssD*	LMG 2665^{T}::Kan^{R} Δ*tssD*	This study
Plasmids		
pRSFRedTER	Broad-host-range plasmid, expressing the λ *red*, *gam* and *bet* genes under the control of the P-element P_{lacUV5}-lacI, Cm^{R}, expressing the levansucrase gene	Katashkina *et al.* (2009)[b]
pKD13	Broad-host-range plasmid, containing the Km^{R} cassette with flanking FRT sequence	Datsenko and Wanner (2000)[b]
pBRRMCS-5	Broad-host-range promoterless cloning vector, multiclonal site, Gm^{R} for *in trans* expression of T6SS genes from endogenous promoters	Kovash *et al.* (1995)[b]
pBRRMCS-*tssA*	pBRRMCS-5 vector expressing the *tssA* gene	This study
pBRRMCS-*tssD*	pBRRMCS-5 vector expressing the *tssD* gene	This study

Ap^{R}, Cm^{R}, Km^{R}, Tc^{R} = resistance to ampicillin, chloramphenicol, kanamycin and tetracycline, respectively. All bacterial strains were transformed with plasmid pMP7604 and used for inter-bacterial competition. Unless otherwise stated, all isolates designated "a" under the column labelled "Source or Reference", were obtained from the Bacterial Culture Collection Facility located in the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

4.3.3 Generation of *tssA* and *tssD* mutants

All primers used in this study are provided in Table 4.2. Pure cultures of *P. ananatis* strain LMG 2665^{T} were made electro-competent and transformed with the lambda Red recombination plasmid (pRSFRedTER) as previously described (27, 28). Gene replacement cassettes were made by three-way fusion extension PCR, as described by Shevchuk *et al.* (2004), and used to replace the *tssA* and *tssD* genes of LMG 2665^{T} with the kanamycin resistance cassette (29). The *tssA* and *tssD* mutants of strain LMG 2665^{T} were selected on LB agar supplemented with kanamycin (50 μg/ml).

Table 4.2. List of PCR primers used in this study

Primers	Primer sequence 5' to 3'	References
***tssA* primers**		
TssARKan	AGCTCCAGCCTACACAATCGCGACTAAGCTAACAGTCAAGCCTTTACG	This study
TssAFKan	GGTCGACGGATCCCCGGAATCATCCGCTTCGCCTCAGGATTCAAACATCG	This study
KanAF	CGTAAAGGCTTGACTGTTAGCTTAGTCGCGATTGTGTAGGCTGGAGCT	This study
KanAR	CGATGTTTGAATCCTGAGGCGAAGCGGATGATTCCGGGGATCCGTCGACC	This study
TssAF1	AAGCAGGCTGTTTAGTGGATCG	This study
TssAR1	TGGTACCACCGATGTTGGGTAC	This study
TssAR2	TCCAGCAGGCGCAGTCGATTCG	This study
TssAF2	TGGTTGTTGAGAAGTCTGGTTC	This study
***tssD* primers**		
TssDFKan	GGTCGACGGATCCCCGGAATGCTATTGATATGTATTTGAAGGTAGAC	This study
TssDRKan	AGCTCCAGCCTACACAATCGCCTGGCTGGAATATCAAAGAGAACAAAGAAG	This study
KanDF	CTTCTTTGTTCTCTTTGATATTCCAGCCAGGCGATTGTGTAGGCTGGAGCT	This study
KanDR	GTCTACCTTCAAATACATATCAATAGCATTCCGGGGATCCGTCGACC	This study
TssDF1	ATTAAGTAGAACTTCTAATTCATTG	This study
TssDR1	TGCGAGCAACTGCACTAAAGCATG	This study
TssDF2	CTTATCCATGATTAAGTCTACAGC	This study
TssDR2	ACCTGCGGAGTACCACGATGCTGAC	This study
Kanamycin-specific primers		
KanF	GCGATTGTGTAGGCTGGAGCT	This study
KanR	ATTCCGGGGATCCGTCGACC	This study
Km1	CAGTCATAGCCGAATAGCCT	Zhao *et al.* (2009)
Km2	CGGTGCCCTGAATGAACTGC	Zhao *et al* .(2009)

The long primers are chimeric primers, that provide homologous regions for a three-way fusion PCR. KanF and KanR were use to amplify the kanamycin cassette from plasmid pKD13, while Km1 and Km2 are internal kanamycin primers.

4.3.4 Complementation of the *tssA* and *tssD* mutants

The promoterless, broad-host-range cloning vector pBRRMCS-5 was used for complementation of mutant strains (30). The full-length *tssA* and *tssD* genes, including 200 – 500 bp of the putative upstream promoter regions, were PCR amplified from the genome sequence of strain LMG 2665^{T}. Plasmid pBRRMCS-5 was digested with *Eco*RI and dephosphorylated with alkaline phosphatase, as previously described (31). PCR products and the linearized pBRRMCS-5 vector were polished with Klenow polymerase, ligated and transformed into electro-competent *E. coli* JM109. Cloned inserts were verified by PCR with primers flanking the cloning site of pBRRMCS-5. PCR amplicons were analyzed by agarose gel electrophoresis and sequencing. The plasmids containing the full-length genes were electroporated into the corresponding *tssA* and *tssD* mutants of *P. ananatis* LMG 2665^{T}.

4.3.5 Verification and phenotypic characterization of mutants

Characterization of the *tssA* and *tssD* mutant strains of LMG 2665^{T} were done as previously described in Chapter 3. In summary, these included 1) PCR verification of the *tssA* and *tssD* mutants, using gene-specific primers and the kanamycin internal primers, to ensure deletion of the targeted T6SS-1 gene, 2) Southern blot hybridizations to verify single integration of the antibiotic resistance marker into the chromosome, 3) *In vitro* growth assays in minimal and rich media to ensure that deletion of targeted genes did not affect the growth rate of the mutants, 4) *In planta* growth assays in onion plants to determine the effect of the mutations on growth, 5) Pathogenicity trials to verify the effect of the mutations on the ability of cause disease in susceptible onion plants, and 6) Bacterial competition assays to determine the effect of the individual mutations on T6SS-dependent killing of target bacterial species. Bacteria targeted for inhibition included *Escherichia coli* DH5α, *Pantoea stewartii* subsp. *indologenes*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Salmonella enterica* serovar Typhimurium, and three strains of *P. ananatis*, i.e. LMG 5342 (clinical isolate), LMG 2669 (pineapple isolate) and LMG 2664 (pineapple isolate). Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test and a *p*-value less than 0.05 were considered to be statistically significant. Analysis were performed using the JMP Statistical Discovery Software version 5.0

4.4 RESULTS

4.4.1 Verification of mutants

4.4.1.1 PCR verification

PCR amplification confirmed that the *tssA* and *tssD* genes were replaced with the kanamycin resistance cassette in the chromosome of *P. ananatis* strain LMG 2665^{T} (Figure 4.1). In addition, PCR using primers flanking the multi-clonal site of plasmid pBRRMCS-5 showed that only single copies of the *tssA* and *tssD* genes inserted into the vector (results not shown). Agarose gel electrophoresis and sequencing confirmed that the *tssA* and *tssD* genes were deleted from the genome sequence of strain LMG 2665^{T}. In addition, sequencing also confirmed that the *tssA* and *tssD* genes expressed from plasmid pBRRMCS-5 contained no stop/nonsense codons.

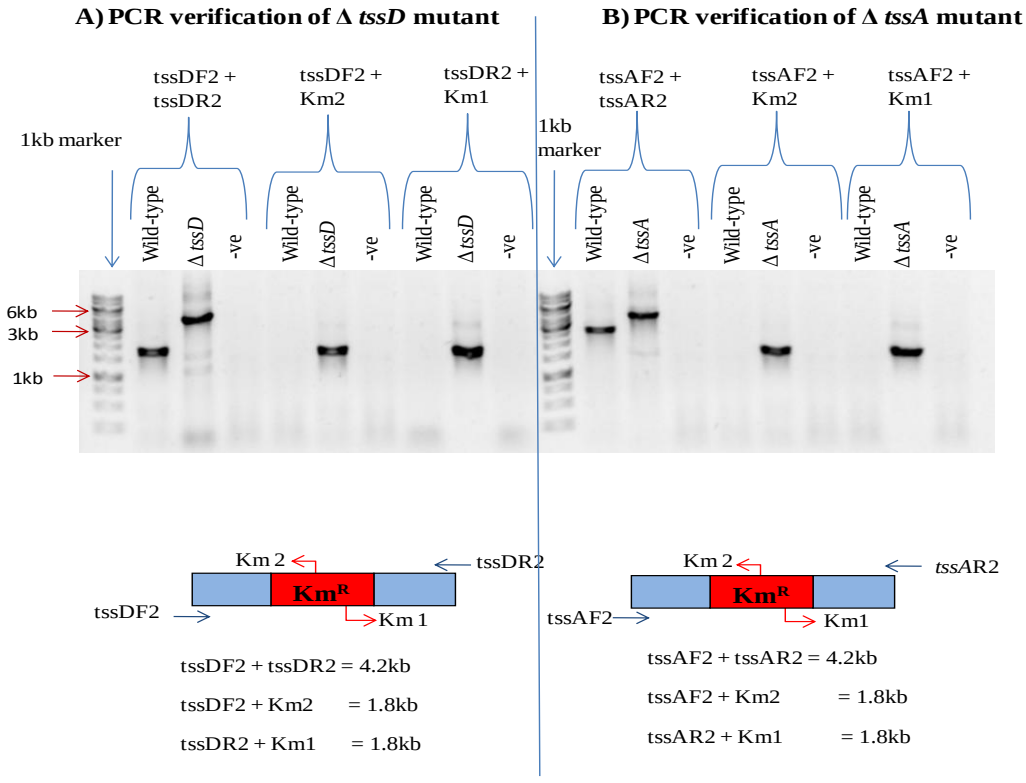

Figure 4.1 PCR verification of the Δ*tssA* and Δ*tssD* mutants of *Pantoea ananatis* strain LMG 2665^{T}. The *tssA* and *tssD* genes were deleted and replaced by the kanamycin resistance gene (red rectangle) using the Lambda Red-mediated homologous recombination technique. Km1 and Km2 primers are internal kanamycin primers, while gene-specific primers are indicated in A) as tssDF2/tssDR2 for the *tssD* gene, and in B) as tssAF2/tssAR2 for the *tssA* gene.

4.4.1.2 Southern blot analysis

The results indicated that the 200 bp kanamycin probe hybridized once to the DNA restriction fragments of the *P. ananatis* Δ*tssD* and Δ*tssA* mutants. The kanamycin probe also hybridized to the full-length PCR-generated kanamycin gene, which served as the positive control (results not shown). Importantly, the probe did not hybridize to the wild-type *P. ananatis* strain LMG 2665^T. These results showed that the kanamycin resistance cassettes were inserted into the chromosome of the Δ*tssD* and Δ*tssA* mutants of strain LMG 2665^T. These results, together with our PCR analysis, confirmed that only the targeted genes of *P. ananatis* strain LMG 2665^T were deleted.

4.4.2 Growth assays

All mutants grew at a similar rate to the wild-type *P. ananatis* LMG 2665^T in both LB broth and M9 minimal medium (Figure 4.2 and Figure 4.3). In addition, the mutants grew at a similar rate to the wild-type strain when inoculated into onion plants (Figure 4.4). These results therefore indicated that over the course of the 15 h, growth of the mutants was not affected, suggesting that there were no pleiotropic effects due to inactivation of the selected T6SS genes.

4.4.3 Pathogenicity trials

Onion plants inoculated with either the Δ*tssD* or Δ*tssA* mutant did not result in any disease symptoms, while the complemented mutants were restored in their ability to kill onion plants three days post-inoculation, similar to the wild-type strain (Figure 4.5). Importantly, control plants inoculated with water did not develop any disease symptoms.

4.4.4 Bacterial growth competition assay

Bacterial competition assays demonstrated that the wild-type *P. ananatis* strain LMG 2665^T caused a one log reduction ($p < 0.05$) in the number of recovered *Escherichia coli* DH5α, *Pantoea stewartii* subsp. *indologenes*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Salmonella enterica* serovar Typhimurium, and three strains of *P. ananatis*, which included LMG 5342 (clinical isolate), LMG 2669 (pineapple isolate) and LMG 2664 (pineapple isolate). The *tssA* and *tssD* mutants lost their ability to inhibit growth of targeted bacterial species compared to the wild-type.

Ectopic expression of the *tssA* and *tssD* genes from plasmid pBRRMCS-5 restored the ability of the complemented mutant strains to inhibit growth of the targeted bacterial species (Figure 4.6 and Figure 4.7). Bacteria co-cultured with double distilled autoclaved water (ddH_2O) served as controls and retained full viability during the entire course of the experiment.

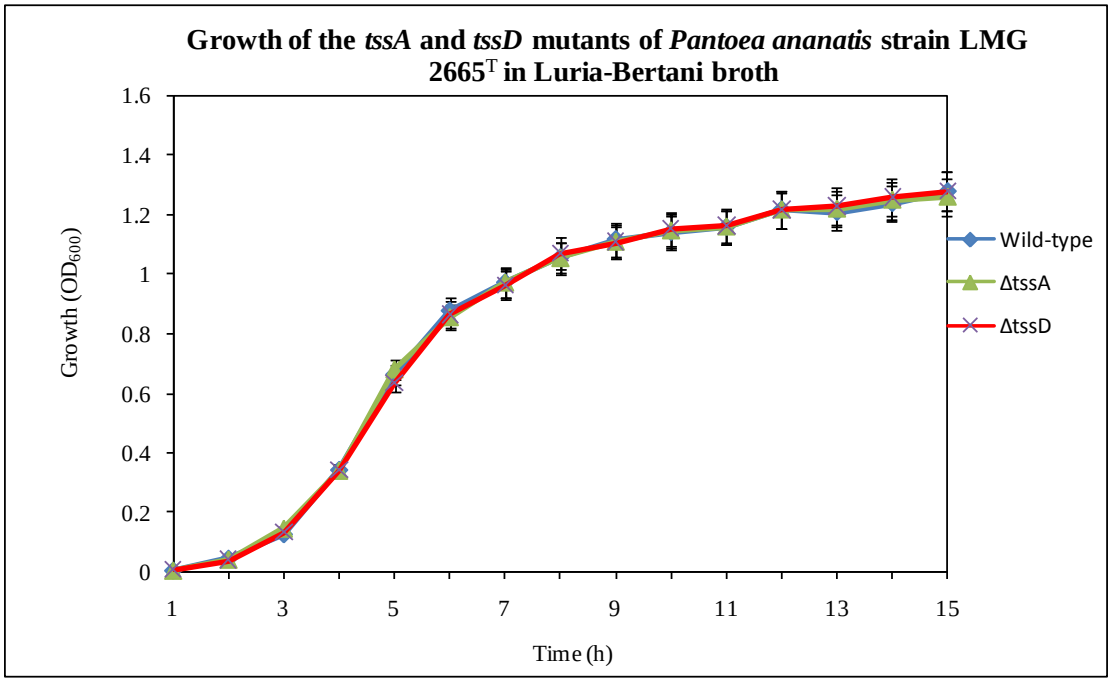

Figure 4.2 Growth of the *tssA* and *tssD* mutants of *Pantoea ananatis* in LB broth. Growth was monitored over 15 h and OD_{600} readings taken every hour. All assays were performed in duplicate and repeated three times. Error bars represent ±SEM.

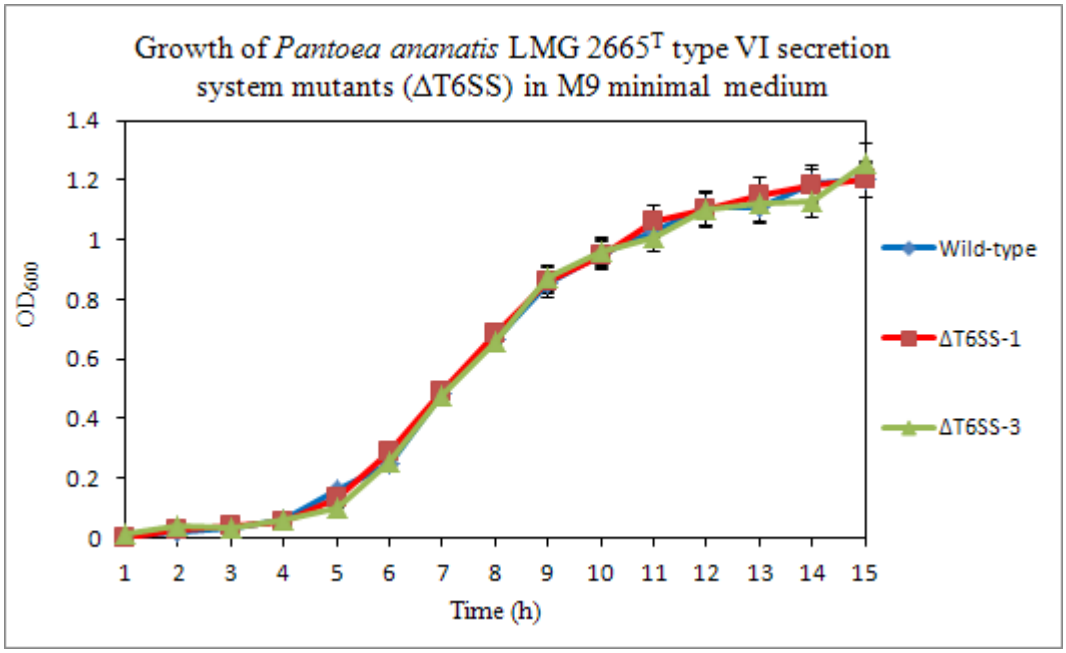

Figure 4.3 Growth of the *tssA* and *tssD* mutants of *Pantoea ananatis* in M9 minimum medium. Growth was monitored over 15 h and OD_{600} readings taken every hour. All assays were performed in duplicate and repeated three times. Error bars represent ±SEM.

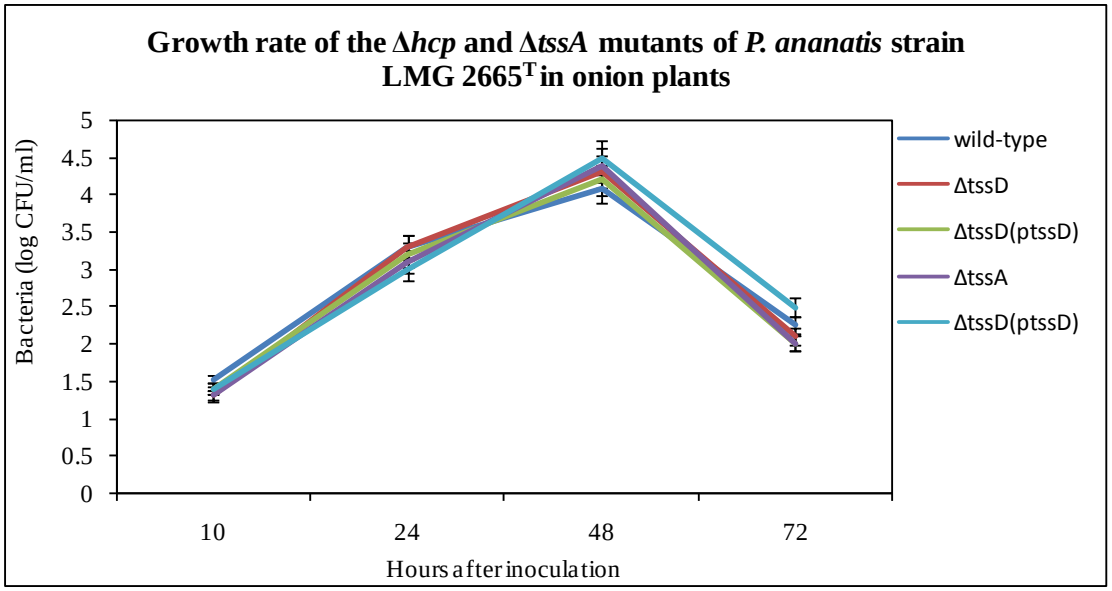

Figure 4.4 Growth of the *tssA* and *tssD* mutants of *Pantoea ananatis* in onion plants. Onion leaves were inoculated with 10^3 colony forming units (CFU/ml) of bacteria. Growth was monitored over 72 h and OD_{600} readings taken at the indicated time points in the figure. Experiments were repeated three timesE error bars represent the mean ± standard error.

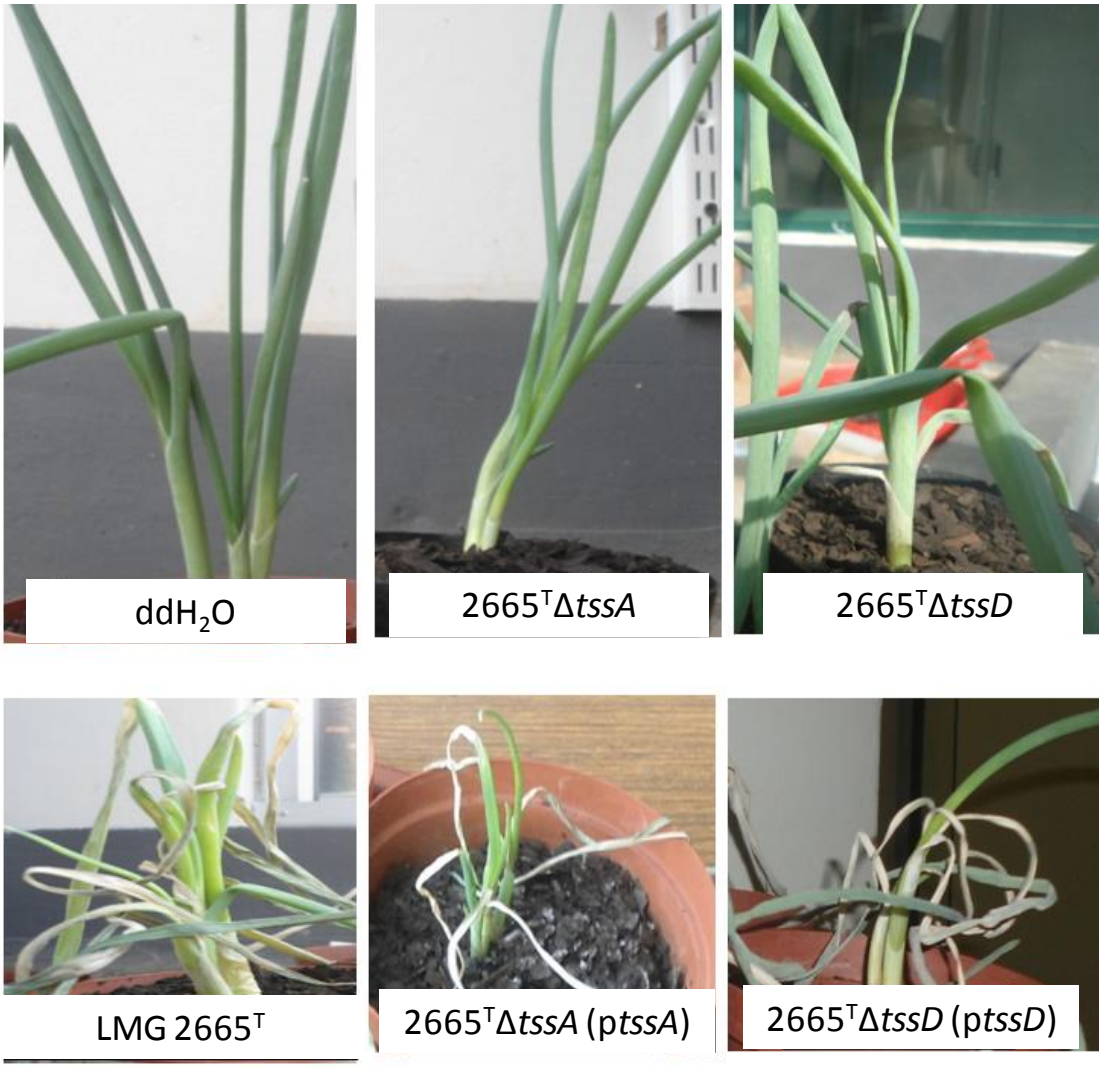

Figure 4.5 Pathogenicity trials using the Δ*tssA* and Δ*tssD* mutant of *Pantoea ananatis* strain LMG 2665^{T}. Onion plants (cv. Texas grando) were inoculated with 2 – 3 drops of the bacterial suspension (OD_{600} of 0.1) using a sterile hypodermal syringe. Inoculated plants were maintained in the greenhouse for 3 – 4 days and disease severity recorded. The *tssA* and *tssD* mutants represent isogenic mutants of the T6SS-1 gene cluster of *P. ananatis* strain LMG 2665^{T}. The 2665^{T}Δ*tssA*(p*tssA*) and 2665^{T}Δ*tssD*(p*tssD*) strains represent the complemented mutant strains. Pathogenicity trial were performed three times and produced the same results.

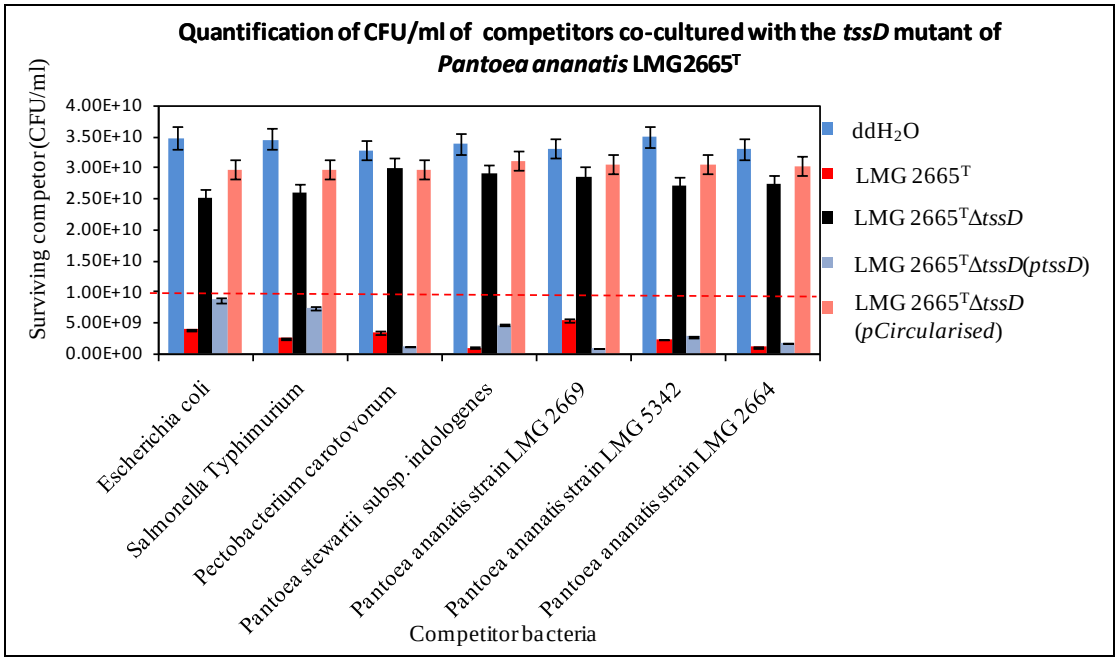

Figure 4.6 B**acterial competition assay using the *tssD* mutant of *Pantoea ananatis* strain LMG 2665^{T}**. Bacteria strains were mixed in a 1:1 ratio, spotted onto LB agar and grown for 24 h. Targeted bacteria were recovered and enumerated by spread plating. p(Circularised) corresponds to the LMG 2665^{T}Δ*tssD* mutant strain containing plasmid pBRRMCS-5 but not expressing the *tssD* gene. The bars in the figure represent the average number of recovered competitors, error bars represent the standard deviation. All values less than 1.00E +10 CFU/ml (indicated by the horizontal red line) represent a statistically significant ($p < 0.05$) inhibition of targeted strains by *P. ananatis*, as determined by an unpaired, two-tailed Student's *t*-test.

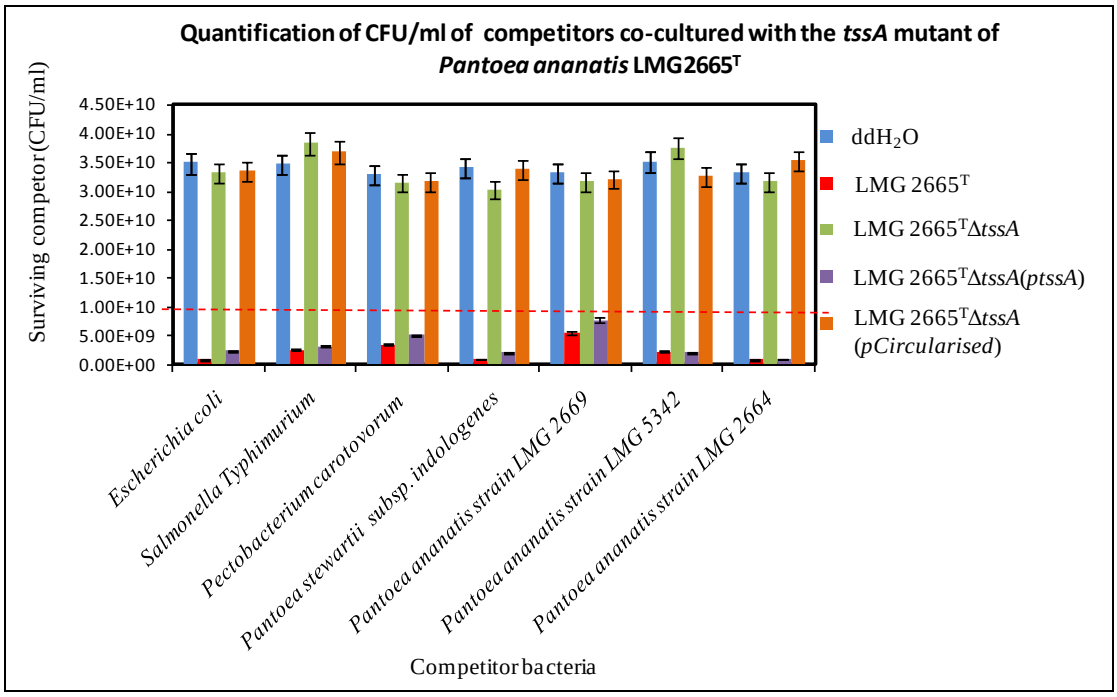

Figure 4.7 Bacterial competition assay using the *tssA* mutant of *Pantoea ananatis* strain LMG 2665^{T}. Different bacterial strains and species were co-cultured with either 1) the wild-type *P. ananatis* strain LMG 2665^{T}; 2) the Δ*tssA* mutant of LMG 2665^{T}; 3) the Δ*tssA* mutant expressing the *tssA* gene from a plasmid (complement); 4) the Δ*tssA* mutant carrying an empty vector (pCircularised) or 5) double distilled water (no *P. ananatis* in the mixture). Bacterial strains were mixed in a 1:1 ratio, spotted onto LB agar and grown for 24 h. Targeted bacteria were recovered and enumerated by spread plating. The bars in the figure represent the average number of recovered competitors, error bars represent the standard deviation. All values less than 1.00E +10 CFU/ml indicated by a dashed red line represent a statistically significant (p < 0.05) inhibition of targeted strains by *P. ananatis*, as determined by an unpaired, two-tailed Student's *t*-test.

4.5 DISCUSSION

Systematic deletion of all the genes found within the T6SS gene clusters of *E. tarda*, *V. cholerae* and *A. tumefaciens* showed that *tssA* - *tssM* are required for Hcp secretion, virulence and inter-bacterial competition (15, 16). This finding has led to the speculation that *tssA* - *tssM* encode proteins that are required for either secretion or biosynthesis of a functional T6SS (16, 32). Therefore, deleting any one of these genes could likely result in a non-functional T6SS. In a previous study, we showed that the T6SS-1 deletion mutant of strain LMG 2665^{T} was non-pathogenic in onion and also lost the ability to inhibit growth of several Gram-negative bacteria, compared to the wild-type strain. These findings demonstrated, for the first time, that the T6SS-1 gene cluster of strain LMG 2665^{T} encoded a functional T6SS. However, because of the size of the LMG 2665^{T} T6SS-1 gene cluster (~40 kb), the T6SS-1 mutant was not complemented. In addition, the contribution of conserved T6SS genes to the observed phenotypes was not determined in the above study. Therefore, in this study, we deleted the *tssA* and *tssD* genes found in the T6SS-1 gene cluster of strain LMG 2665^{T}. The isogenic mutants and their corresponding complemented mutant strains were characterized based on their ability to cause disease in onion plants and their ability to inhibit growth of other Gram-negative bacteria.

The *tssA* and *tssD* genes were specifically targeted because *tssA* homologs have not been extensively studied in bacteria known to encode a functional T6SS, while *tssD* has been functionally characterized in several bacterial species. The *tssA* gene encodes TssA, which is a cytoplasmic protein of unknown function, while *tssD* encodes Hcp, which is secreted by the T6SS of several bacteria and also forms a structure analogous to the phage tube, suggesting that Hcp is both a T6SS effector and structural protein (9, 10, 11). In addition, while both genes are required for Hcp secretion, only *tssA* has consistently been associated with T6SS-mediated virulence against eukaryotes and prokaryotes (18, 16, 21). In this study, we therefore considered *tssA* as a benchmark gene that is required for the synthesis of a functional T6SS. Our results demonstrated that the *tssA* gene of LMG 2665^{T} is required for pathogenicity in onion and inter-bacterial competition. We showed that the Δ*tssA* mutant of strain LMG 2665^{T} was non-pathogenic when inoculated into susceptible in onion plants. In addition, the Δ*tssA* mutant lost the ability to inhibit growth of *Escherichia coli* DH5α, *Pantoea stewartii* subsp. *indologenes*, *Pectobacterium carotovorum* subsp.

carotovorum, *Salmonella enterica* serovar Typhimurium, and *P. ananatis* strains LMG 5342 (clinical isolate), LMG 2669 (pineapple isolate) and LMG 2664 (pineapple isolate). Furthermore, the effect of the Δ*tssA* mutant on virulence and inter-bacterial competition could be genetically complemented by *in trans* expression of the *tssA* gene from plasmid pBRRMCS-5. These findings are in complete agreement with our previous study showing that the T6SS-1 of LMG 2665^T played a role in onion pathogenicity and inter-bacterial competition.

Having established that the *tssA* gene was required for T6SS-mediated phenotypes of strain LMG 2665^T, we then investigated the role of the *tssD* gene under similar assay conditions. Our results demonstrated that the *tssD* gene was required for virulence and inter-bacterial competition. This finding is in agreement with several studies that have shown that the *tssD* gene is required for T6SS-related virulence (10, 15, 16, 17). Based on these findings, we conclude that the *tssA* and *tssD* genes located within the T6SS-1 gene cluster of strain LMG 2665^T, encode proteins that are associated with a functional T6SS.

Hcp is believed to form a channel that allows passage of effectors in a single step from the cytoplasm to the exterior of the bacterial cells or directly into eukaryotic or neighbouring bacterial cells (9, 14). Functional studies have shown that the Hcp1 of *P. aeruginosa* and the Hcp (EvpP) of *E. tarda* form hexameric rings, which polymerize *in vitro* into nanotubes with an internal diameter of ~40 Å (11, 12). We showed that the Δ*tssD* mutant of strain LMG 2665^T was non-pathogenic when inoculated into onion plants and the mutant also lost the ability to inhibit growth of other Gram-negative bacteria. It is therefore possible that deletion of the *tssD* gene of strain LMG 2665^T disrupted formation of the Hcp channel, which is required for secretion of a yet to be identified T6SS-1 effector. This speculation is in agreement with studies showing that an intact *tssD* gene was required for secretion of T6SS effectors such as Tse 1-3, which are antimicrobial effectors of *Pseudomonas aeruginosa*, and VgrG1 and VgrG3 proteins of *V. cholerae*, which target eukaryotes and prokaryotes, respectively (10, 33, 34). In addition, the Hcp-1 protein of *P. aeruginosa* was recently shown to act as a receptor and a chaperone of Tse1-3 (35). These effectors bind inside of the Hcp-1 hexameric rings and deletion of Hcp dramatically reduced intracellular levels of Tse3 (35). It remains to be experimentally determined whether the *tssD* gene located within the T6SS-1 gene cluster of *P.*

ananatis strain LMG 2665^T encodes an Hcp homolog that plays a role in chaperoning proteins destined for secretion.

Our results also showed that the *tssA* gene of strain LMG 2665^T was required for virulence in onion and growth inhibition of other Gram-negative bacteria. Like *P. ananatis*, homologs of *tssA* found in most bacterial T6SS gene clusters do not contain predicted transmembrane domains or signal peptides, suggesting that *tssA* encodes a cytoplasmic protein (3, 9). TssA was recently shown to interact with TssK, a cytoplasmic protein that mediates formation of the TssB-TssC contractile sheath (25). However, the biological significance of the TssA-TssK interaction is currently unknown. While this study does not provide direct evidence for the role of the *tssA* gene of strain LMG 2665^T, we have provided experimental evidence demonstrating a direct association between *tssA* and the T6SS-1 phenotypes of *P. ananatis* strain LMG 2665^T. Therefore, consistent with a gene encoding a conserved T6SS protein, deletion of *tssA* resulted in loss of virulence phenotypes associated with the T6SS-1 of starin LMG 2665^T.

In conclusion, we have functionally characterized *tssA* and *tssD* mutants of strain LMG 2665^T and shown, for the first time, that *tssA* and *tssD* are required for pathogenicity in onion and inter-bacterial competition. Our results suggest that the *tssA* and *tssD* genes located within the T6SS-1 gene cluster of strain LMG 2665^T encode proteins that are required for a functional T6SS-1. Future perspectives will be to determine if the *tssD* gene found in the T6SS-1 gene cluster of strain LMG 2665^T encodes an Hcp homolog that can be secreted into the cell culture supernatant. In addition, we further need to functionally characterize all the genes found in the T6SS-1 gene cluster of LMG 2665^T to determine which of the *tss* genes are required for secretion or biosynthesis of a functional T6SS.

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CHAPTER 5

Summary

Pantoea ananatis is an important plant pathogen that causes disease symptoms in different plants worldwide. However, most of the virulence determinants of this pathogen have not been identified and functionally characterized. A previous study identified the type VI secretion system (T6SS) as a putative virulence determinant of *P. ananatis* strain LMG 20103, based on *in silico* analysis. This secretion system has been shown to play different roles in bacteria, including virulence, fitness and inter-bacterial competition. Therefore, the overall objective of this study was to determine the biological role (s) of the T6SS of *P. ananatis*.

The first chapter of this thesis is a review of the literature, dealing with the different secretion systems used by Gram-negative bacteria to secrete effectors (toxins and proteins) from the cytoplasm to the exterior of the cell. Six different secretion systems have been identified in Gram-negative bacteria, i.e. T1SS to T6SS. These secretion systems have been functionally characterized and shown to play different roles related to virulence, fitness and inter-bacterial interactions. The T6SS represents the most recently described secretion system found in Gram-negative bacteria. Gene clusters encoding the T6SS are widespread in several pathogenic and non-pathogenic bacteria, with up to six genetically distinct T6SS gene clusters found in some bacterial species. This secretion system has been associated with different processes such as virulence, fitness, biofilm formation, niche colonization, and inter-bacterial competition. The T6SS can target cytotoxic effectors into either eukaryotes, prokaryotes or both. Genes encoding bactericidal and bacteriostatic effectors have been identified in the T6SS gene clusters of different bacteria. However, only a few of these effectors such as Hcp, VgrG, VasX, Tse, Tae, Tge, Tle, Ssp and Rhs toxins have been functionally characterized.

In Chapter 2, a comparative analysis of the different T6SS gene clusters found in *P. ananatis* was undertaken. The T6SS-1 and T6SS-3 gene clusters of LMG 20103 were found to be conserved and syntenic in eight strains of *P. ananatis* of which genome sequences have been determined. Using PCR and probes, we also identified homologs of genes found in the T6SS-1 and T6SS-3 gene clusters in all 36 additional strains of *P. ananatis* of which the genome sequences have not been determined. The third cluster, T6SS-2 was found to be restricted to only three out of eight sequenced strains of *P. ananatis*, which included LMG 20103 (a pathogen of *Eucalyptus* spp.), PA-4 (a rice pathogen) and AJ13355 (a non-pathogenic strain isolated from the soil).

Furthermore, T6SS-3 gene homologs were also identified in 12 out of 36 (33%) environmental strains of *P. ananatis* analyzed in this study.

In Chapter 3, we functionally characterized the different T6SS gene clusters found in strains LMG 20103 and LMG 2665^{T}. Our results indicated that the T6SS-1 of LMG 2665^{T} plays a role in onion pathogenicity and growth inhibition of other bacteria. We also showed that the homologous T6SS-1 of strain LMG 20103 played a role in bacterial competition but was not required for pathogenicity in onion plants. Based on our assay conditions, no discernable phenotype was observed following deletion of the T6SS-2 and T6SS-3 gene clusters found in the genome sequences of either strains of *P. ananatis*.

In Chapter 4, we carried out a genetic analysis of the *tssA* and *tssD* genes found in the T6SS-1 of strain LMG 2665^{T}. This was done in part to validate results from Chapter 3, because the whole cluster deletion mutants were not complemented and secondly, to determine if these genes were required for T6S. Deletion of these genes abrogated pathogenicity in onion plants compared to the wild-type. In addition, the Δ*tssA* and Δ*tssD* mutants of strain LMG 2665^{T} were unable to inhibit growth of other Gram-negative bacteria following co-culture on LB agar. *In trans*-expression of the full-length *tssA* and *tssD* genes on a plasmid restored pathogenicity and inter-bacterial competition of the complemented T6SS mutants to near wild-type levels. These results, for the first time, demonstrated that the *tssA* and *tssD* genes of strain LMG 2665^{T} are required for pathogenicity and inter-bacterial competition. We hypothesize that these genes encode proteins that are essential for the biosynthesis of a functional T6SS.