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Identification of antibacterial type 6 secretion system effectors in *Pectobacterium carotovorum* subsp. *brasiliense* 1692

By

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Declaration

I, Nikki Miguel, declare that this thesis/dissertation, which I hereby submit for the degree Magister of Scientiae (Microbiology) 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.

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Date:

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Thesis outputs

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Abstract

Pectobacterium carotovorum subsp. *brasiliense* 1692 is an economically important pathogen as it causes soft rot and blackleg of numerous agricultural crops, especially potato, in South Africa and in other parts of the world. In a multicellular environment, microorganisms interact with each other and protein secretion systems mediate many of these interactions. Survival often requires competitive interactions against closely and distantly related species. Although initially identified as an antieukaryotic virulence factor, the T6SS has mainly been implicated in interbacterial competition. Studies have shown that the T6SS of *Pectobacterium carotovorum* ssp. *brasiliense* 1692 (*Pcb1692*) is significantly upregulated upon *in planta* infection; however, the precise role during infection remains unknown. In this study, the role of the T6SS in *Pcb1692* was investigated. *In planta* potato leaf competition assays indicate that no observable competition was mediated by the T6SS in *Pcb1692*. As the majority of T6SSs are involved in bacterial competition, a bioinformatics approach was used to identify whether the lack of T6SS-mediated competition was due to an absence of antibacterial effectors within the genome. Upon discovery of a number of putative antibacterial effectors, these were cloned and ectopically expressed in *E. coli*. Results indicate that individual expression of effectors, except the AHH nuclease, does not contribute towards bacterial growth inhibition. Altogether, this study has provided insight into the antibacterial role of the T6SS of *Pcb1692*, and results indicate that although the T6SS is significantly upregulated during infection, bacterial competition is not its foremost role within potato leaves.

List of abbreviations

°C	degrees Celsius
Amp	ampicillin
bp	base pairs
CDI	contact-dependent inhibition
CFU	colony forming units
Cv.	cultivar
Dc	*Dickeya chrysanthemi*
Dd	*Dickeya dadantii*
ddH_2O	double distilled water
DUF	domain of unknown function
eDNA	extracellular DNA
g	grams
gDNA	genomic DNA
hr	hour
HSI	Hcp-secretion island
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan	kanamycin
kb	kilo base pairs
kV	kilovolts
LB	Luria-Bertani broth
MCS	multiple cloning site
μg	micrograms
min	minutes
ml	millilitres
μl	microlitres

mM	millimolar
μM	micromolar
ms	milliseconds
ng	nanograms
nm	nanometres
OD	optical density
ORF	open reading frame
Pa	*Pectobacterium atrosepticum*
Pcb	*Pectobacterium carotovorum* ssp. *brasiliense*
Pcc	*Pectobacterium carotovorum* ssp. *carotovorum*
PCWDE	plant cell wall degrading enzymes
PLA	phospholipase A
PLD	phospholipase D
Pw	*Pectobacterium wasabiae*
rpm	revolutions per minute
s	seconds
T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T3SS	Type 3 secretion system
T4SS	Type 4 secretion system
T5SS	Type 5 secretion system
T6SS	Type 6 secretion system
Tet	tetracycline
T_m	melting temperature

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Figure 2.1 Effect of variable attacker-to-target ratios in competition assays *in planta*

Two different attacker-to-target ratios were used (1:1 and 10:1) to determine whether there is a significant difference in the outcome of competition between two strains when variable attacker concentrations were used. Results indicate that there is no significant difference in target cell survival when a 1:1 ratio was used compared to 10:1 ratio, indicating that for short-duration assays, the attacker concentration does not significantly influence competition results for the strains used in this study. Statistical analyses were performed using one-way ANOVA with $\alpha = 0.05$. Statistical analysis was deemed significant if the p-value was smaller than 0.05 ($p < 0.05$) and insignificant when the p-value was larger than 0.05 ($p > 0.05$). Tukey's HSD post-hoc test was used to determine which comparisons were significant.

Competition of *Pcb1692* with:

A – *Pectobacterium atrosepticum* (*Pa*)

B – *Dickeya chrysanthemi* (*Dc*)

C – *Dickeya dadantii* (*Dd*)

D – *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*)

Figure 2.2 *In planta* interbacterial competition

Various SREs were competed against either *Pcb1692* or *Pcb*_ΔT6SS by co-infiltration into susceptible potato leaves (cv. Mondial). Both the target and the attacker strains were enumerated by serial dilutions. The first three bars (solid colour) correspond to the controls, and the remaining bars (striped) to co-inoculations. The colour of the bar indicates which strain from the co-infection was selected for. The survival for both wild type and mutant strains of *Pcb* remain relatively constant in all cases. However, target SRE titres do not differ significantly compared to each other in competition with either *Pcb1692* or *Pcb*_ΔT6SS, indicating that the T6SS does not contribute towards bacterial growth inhibition. Significant differences were indicated with different alphabet letters above each bar. Bars with the same letter do not differ significantly ($p > 0.05$), while bars with different letters differ significantly from each other ($p < 0.05$).

Competition of *Pcb1692* and *Pcb*_ΔT6SS with:

A – *Pectobacterium atrosepticum* (*Pa*)

B – *Dickeya chrysanthemi* (*Dc*)

C – *Dickeya dadantii* (*Dd*)

D – *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*)

Figure 2.3 Genomic organisation of T6SS components and associated genes in *Pcb1692*

Genes associated with the T6SS were dispersed throughout the genome in five distinct loci. **A** - The core genes (Tss core) encoding structural subunits are encoded in the same genomic locus. Directly adjacent to the core cluster lies the first Hcp-secretion island (HSI-1) encoding a Hcp-VgrG pair and a putative effector-immunity pair downstream thereof. **B** – HSI-2 encodes an Hcp-VgrG pair, followed by two putative effectors and a PAAR protein. **C** – HSI-3 lacks a *vgrG* gene, and the genes downstream are uncharacterised and have no known role with T6SSs. **D** – HSI-4 encodes two putative effector-immunity pairs and one gene with an unknown role. **E** – HSI-5 only encodes one *hcp* gene and two hypothetical genes downstream

Figure 2.4 Protein sequence alignment

Alignment of *P. atrosepticum* phospholipase, ECA3426, with the alpha/beta hydrolase AED_0003357 from *Pcb1692*. Sequences share 90% similarity, both conserving the GXSXG lipase motif (blocked in red).

Figure 2.5 Plasmid restriction maps

A – Plasmid map indicating unique restriction cut sites expected to be present on pTrc99A. The multiple cloning site (MCS) is demarcated from NcoI to HindIII. The MCS is preceded by a *trc* promoter. Plasmid pTrc99A is characterised by a ColE1 ori, ampicillin resistance gene. **B** – Plasmid map of pCH450 indicating the unique restriction cut sites. The multiple cloning site is demarcated from EcoRI to PstI. The MCS is preceded by an arabinose-inducible promoter. Plasmid pCH450 is tetracycline resistant and has a p15A ori, which is compatible for maintenance with colE1-ori plasmids.

Figure 2.6 Confirmation of restriction enzyme map in MCS for pTrc99A

First lane in all gels is a 1 kb GeneRuler ladder (Thermo Scientific). Restriction digests of pTrc99A were performed as follows: **A** – (1) NcoI did not linearise, as all three plasmid conformations are still present; (2) SacI and (3) SmaI did not linearise; (4) PstI and (5) HindIII linearised pTrc99A completely. **B** – (1) SalI, (2) XbaI and (3) SalI-XbaI double digest linearised plasmid DNA; (4) BamHI, (5) KpnI and (6) BamHI-KpnI double digest did not linearise plasmid DNA. (7) undigested control. Linearisation of plasmid DNA indicates that cut sites are present in the plasmid.

Figure 2.7 Schematic depicting plasmid modification strategy from pTrc99A to pTrc100

A – Hypothetical gene H5907 (*PCBA_RS05790*; arbitrarily chosen) was PCR-amplified from *Pcb1692* genomic DNA. The forward primer introduced three restriction sites at the 5' end – XbaI, KpnI, and BamHI. BamHI and SalI were incorporated at the 3' end of the gene by the reverse primer. Both the PCR product and pTrc99A were digested with XbaI and SalI and ligated using these compatible ends. **B** – Ligation of the PCR product to linearised pTrc99A yielded plasmid pTrc::H5907 with an expanded MCS as well as the hypothetical gene H5907 within the MCS. **C** – The hypothetical gene needed to be removed from the plasmid to make the plasmid usable for downstream cloning of selected effector and immunity genes. Digesting pTrc::H5907 with BamHI removed the gene and produced a linear plasmid with two BamHI ends, which were self-ligated to yield a circular plasmid, pTrc100, that included functional KpnI and BamHI cut sites. The MCS of pTrc100 possesses XbaI, KpnI, BamHI, and SalI cut sites downstream of the *trc* promoter (red triangle).

Figure 2.8 Construction of plasmid pTrc100 from pTrc99A

First lane in all gels is a GeneRuler 1 kb ladder (Thermo Scientific). **A** – PCR of gene H5907, which was 440 bp. **B** – Restriction digests of pTrc::H5907. (1) undigested control. (2) SalI-XbaI double digest and (3) BamHI-KpnI double digest completely linearised the plasmid and produced a ~440 bp insert and 4,2 kb backbone. **C** – Restriction digests of pTrc100 with (1) SalI, (2) XbaI, (3) SalI-XbaI double digest, (4) BamHI, (5) KpnI and (6) BamHI-KpnI double digests linearised plasmid pTrc100; (7) undigested pTrc100 control.

Figure 2.9 Confirmation of restriction enzyme map for pCH450

First lane contains 1 kb DNA ladder (NEB). Each lane corresponds to a single enzyme digest within the MCS of pCH450. In all cases the enzymes digested the plasmid DNA to linear DNA, with the exception in lane 4, where some open circular plasmid remained in addition to the linear DNA below it. Restriction digest of pCH450 with (1) EcoRI, (2) KpnI, (3) NcoI, (4) NdeI, (5) PstI, (6) PvuI, (7) SacI, (8) SmaI, and (9) XhoI.

Figure 2.10 PCR amplification of effector and immunity genes from *Pcb1692* gDNA

First lane in all images is a 1 kb GeneRuler ladder (Thermo Scientific). **A** - PCR amplification of putative effector genes. Phos – 1,4kb; WHH – 990 bp; Rhs1 – 470 bp; AHH – 470 bp; and D123 – 800 bp. These genes were subsequently cloned into pCH450, with the exception of Phos that still required an N-terminal secretion signal. **B** - PCR amplification of putative immunity genes. Ank – 700 bp; SUKH – 540 bp; Rhs1i – 480 bp; AHHi – 570 bp. These genes were subsequently cloned into pTrc100.

Figure 2.11 Insertion of N-terminal signal sequence

First lane is a 1 kb GeneRuler ladder (Thermo Scientific). **A** – (1) pET-26b::Phos digested with NdeI and XhoI. Upper two bands correspond to the plasmid backbone. The 1,5 kb fragment at the bottom corresponds to Phos fused with the PelB signal sequence. (2) Plasmid pCH450 was digested with NdeI and XhoI to yield compatible ends for ligation. The resitriction digest yielded only linear plasmid DNA, at ~ 3,8 kb. **B** – Digestion of pCH450::PelB-Phos to confirm insertion of the fused fragment into pCH450. (1) Undigested plasmid control. (2) Restriction digest of pCH450::PelB-Phos with NcoI yielded three fragments. Successful insertion of the desired segment produced three fragments with the following sizes: 4 kb, 1,1 kb, and 81 bp. The 81 bp fragment is very faint.

Figure 2.12 Growth of *E. coli* DH5-alpha expressing effectors from pCH450

Effector expression was induced with 0.2% L-arabinose after 30 min of growth, or effector and immunity proteins from pCH450 and pTrc100, respectively.

A – Expression of nuclease RhsA from *Dickeya dadantii* 3937 in *E. coli* as positive control. Expression of the effector alone shows strong inhibition of growth after 120 min. Simultaneous expression of both the nuclease and its associated immunity factor (RhsA+I) restores normal growth

B – Expression of AHH nuclease decreases the growth rate of *E. coli*. Expression of both AHH and its immunity protein (AHH+i) negates the toxic effect of the effector alone; however, after ~220 min a plateau in growth is reached due to protein overexpression.

C – Expression of phospholipase (Phos), WHH nuclease (WHH), and D123 protein (D123) and Rhs effectors in *E. coli* shows growth comparable to that of the empty vector control (pCH450). Individual expression of these effectors does not contribute to bacterial growth inhibition under the conditions analysed.

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CHAPTER ONE

Literature Review:

Protein secretion systems and other competitive strategies employed by *Pectobacterium* species

Introduction

There are two key genera in the soft rot *Enterobacteriaceae*, namely *Pectobacterium* and *Dickeya* (Charkowski *et al.*, 2012). Formerly, these species were all classified as *Erwinia carotovora* and *Erwinia chrysanthemi*, respectively (Falkow et al., 2006). Based on 16S sequences, the genus designation of *Erwinia carotovora* and *Erwinia chrysanthemi* was changed to *Pectobacterium* on account of an average of only 95.6% similarity shared with true *Erwinia* spp. (Hauben *et al.*, 1998). In 2005, discrepancies in 16S sequences from the *P. chrysanthemi* complex indicated that eight sequences clustered in a distinct clade, and were subsequently reclassified under the novel genus *Dickeya*, as *Dickeya chrysanthemi* (Samson *et al.*, 2005). *D. chrysanthemi* could be further divided into six distinct species according to DNA-DNA hybridisation. In 2016, a comprehensive comparative genomic assessment of the order *Enterobacteriales* indicated that its sole family *Enterobacteriaeceae* was to be divided into seven families. Consequently, *Pectobacterium* and *Dickeya* genera have been moved to the novel family, *Pectobacteriaceae*, along with *Brenneria, Sodalis*, and *Lonsdalea* (Adeolu *et al.*, 2016). Recently, the taxonomy of *Pectobacterium carotovorum* was revised using whole-genome sequencing data (Zhang *et al.*, 2016). Currently, *Pectobacterium carotovorum* is divided into four subspecies: *actinidiae, odoriferum, carotovorum,* and *brasiliense*. Numerous results including biochemical characterisation tests, multilocus sequence alignments, *in silico* DNA-DNA hybridisation, average nucleotide identity, and 16S rRNA sequences all reinforce that *Pectobacterium carotovorum* subsp. *brasiliense*, along with the other three subspecies, should be elevated to species level (Zhang *et al.*, 2016).

Soft rot *Enterobacteriaceae* (SRE) are rod-shaped, Gram-negative, facultative anaerobes, which are characteristically pectolytic (Perombelon & Van Der Wolf, 2002, Czajkowski *et al.*, 2015). The SRE cause soft rot and blackleg resulting in severe losses of a wide variety of agricultural crops worldwide including potatoes, carrots, celery, cucumber, turnips and chicory (Lebecka, 2005). Losses may occur both in the field as well as postharvest in storage. The

cultivar, soil conditions, management practices, climate, and temperature have varying effects on the degree of loss (Guchi, 2015). Disease progression is difficult to predict, as disease initiation and severity depends on a number of characteristics including water availability, tuber maturity, and the particular strains involved (Van der Merwe *et al.*, 2009, Marquez-Villavicencio *et al.*, 2011).

Potato is the most important non-cereal agricultural crop in the world and *Pectobacterium* spp. cause an estimated loss of 24% annually (Ragunath *et al.*, 2012, Wu, 2016). A battery of factors facilitate SRE pathogenicity, including plant cell wall degrading enzymes (PCWDE), motility, siderophore production, protein secretion systems, and adhesion (Perombelon, 2002). Most members of this group are broad host-range, necrotrophic plant pathogens infecting crops such as potatoes, carrots, celery, cucumber, turnips, and chicory, with the exception of *P. atrosepticum*, which is limited to potatoes (Lebecka, 2005, Waleron *et al.*, 2014). SRE colonise vascular tissue and intercellular spaces and are the main causal agents of soft rot and blackleg. Soft rot symptoms are characteristic of the SRE group; however, some pathogens, including *P. atrosepticum*, *P. wasabiae*, *Pcb*, *Dickeya dianthicola*, and *D. solani*, additionally cause potato blackleg (De Boer & Rubio, 2004).

First characterised in 2004 (Duarte *et al.*, 2004), *Pectobacterium carotovorum* subsp. *brasiliense* 1692 (*Pcb*) has been identified as being more aggressive than *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Pectobacterium atrosepticum* (*Pa*) (Van der Merwe *et al.*, 2009, Panda *et al.*, 2012). Initially discovered in Brazil, *Pcb* has been isolated in several different countries worldwide, including South Africa, Zimbabwe, Poland, Switzerland, Peru, Korea, USA, and Canada (Duarte *et al.*, 2004, Van der Merwe *et al.*, 2009, De Boer *et al.*, 2012, Nabhan *et al.*, 2012, Ngadze *et al.*, 2012, Waleron *et al.*, 2015, Werra *et al.*, 2015). *Pcb* is associated with a number of hosts, including potato, paprika, nepenthes, chrysanthemum, and eggplant (Van der Merwe *et al.*, 2009, Choi & Kim, 2013, Lee *et al.*,

2013, Lee *et al.*, 2014). In South Africa, *Pcb* is the main causal agent of potato blackleg (De Boer *et al.*, 2012).

P. atrosepticum, P. carotovorum subsp*. carotovorum and P. carotovorum* subsp*. brasiliense* share approximately 80% of their genomes (Glasner *et al.*, 2008). Co-infection of the same field and same plant is common among these pathogens, especially when the climate is conducive for multiple species. The similarity in soft rot symptoms likely lies in the comparable set of PCWDEs these pathogens produce (pectate lyases, pectin lyases, polygalacturonases, cellulases, and rhamnogalacturonases), with *Pcb* and *Pcc* genomes encoding more PCWDEs than *Pa* (Glasner *et al.*, 2008). Soft rot is not limited to the SREs, as soft rot clostridia often co-infect with SREs.

The success of a microbe in a given niche depends on a number of factors, including environmental aspects, its intrinsic characteristics, as well as the presence or absence of other microbial species within the community. Cells are required to interact with both the environment and cohabiting species. Such interaction is in part mediated through distinct protein secretion pathways, by pathogens and mutualists alike (Green & Mecsas, 2016). Phytopathogens make use of protein secretion systems to secrete various virulence factors. *Pectobacterium carotovorum* subsp. *brasiliense* possesses type 1-6 secretion systems, contributing towards virulence and other phenotypes (Nykyri, 2013).

Bacterial Protein Secretion Systems

Protein secretion systems can be divided into two groups, namely one-step and two-step secretion systems, depending on how they are transported across the cell wall. In Gram-negative bacteria, which possess two cell membranes, proteins can be secreted out of the cell in one of two ways. The first is via the Sec- and Tat pathways, which first transport proteins into the periplasm (Green & Mecsas, 2016). These proteins are further transported into the extracellular milieu via the type 2 or type 5 secretion system. Secretion out of the cell via the

type 2 or type 5 secretion system is termed two-step secretion due to the intermittent process. Alternatively, proteins may be transported in a Sec- or Tat-independent mechanism, utilising a one-step secretion system that translocates proteins from the cytoplasm directly into the extracellular space, or directly into a target cell. One-step secretion systems include the type 1-, 3-, 4-, and 6 secretion systems (Green & Mecsas, 2016).

The type 1 secretion system (T1SS) consists of three proteins; an ATP-binding cassette (ABC) transporter, an outer membrane factor (OMF), and a membrane fusion protein (MFP), which joins the ABC transporter to the OMF via a periplasmic channel (Tseng *et al.*, 2009, Green & Mecsas, 2016). A C-terminal signal sequence is common to all T1SS substrates (Lenders *et al.*, 2015). Substrates include lipases, proteases, toxins, and surface layer proteins. Soft rot *Enterobacteriaceae* encode multiple T1SSs (Charkowski *et al.*, 2012). In the SRE, the T1SS is used to transport metalloproteases and adhesins (Charkowski *et al.*, 2012). Multi-repeat adhesins facilitate binding of the pathogen to the host plant (Pérez-Mendoza *et al.*, 2011). Metalloproteases impede the manufacture of the plant cell wall or degrade enzymes secreted by the pathogen (Panda, 2014). Disruption in the regulation of the T1SS resulted in reduced virulence and root attachment in *Pectobacterium atrosepticum* (Pérez-Mendoza *et al.*, 2011). Recently, it has also been suggested that a T1SS is involved in the secretion of the antimicrobial carotovoricin from *Pcb* 1692 (Bellieny-Rabelo *et al.*, 2019).

The type 2 secretion system (T2SS) is only present in Gram-negative bacteria, as it is located in the outer membrane (Green & Mecsas, 2016). Therefore, the T2SS relies on the Sec- or Tat pathways to deliver proteins into the periplasm, where they are folded prior to export across the outer membrane. Due to its dependence on Sec/Tat pathways, T2SS substrates possess a cleavable N-terminal signal peptide. The T2SS translocon consists of at least 12 proteins, which form three distinct assemblies – the pseudopilus, inner membrane platform, and outer membrane secretin (McLaughlin *et al.*, 2012). The T2SS mainly secretes enzymes

that facilitate interaction with the environment (Tseng *et al.*, 2009, Green & Mecsas, 2016). Characteristic of *Pectobacterium* spp. is the secretion of its key virulence factor, plant cell wall degrading enzymes (Perombelon & Van Der Wolf, 2002). The T2SS, present in all pectobacteria, is dedicated to the delivery of these enzymes (Nykyri, 2013). Whereas the T1SS augments virulence in *Pectobacterium* spp., the T2SS system is a necessity for pathogenesis.

The type 3 secretion system (T3SS) is assembled from nine core proteins to form the injectisome, which spans both membranes (Green & Mecsas, 2016). Proteins may be secreted out of the cell, or directly into eukaryotic host cells (Tseng *et al.*, 2009). Proteins secreted by this system are called effectors, as they manipulate host functions. The SRE deliver relatively few T3 substrates, with only one known effector to date, DspA/E (Charkowski *et al.*, 2012). Substrates are targeted to the T3SS by an N-terminal, non-cleavable signal sequence, and many are directed to the translocon by chaperones (Green & Mecsas, 2016). The T3SS regulates the hypersensitive response, in which plants respond to effectors by initiating localised cell death, resulting in necrotic lesions (Johansson *et al.*, 2015). Thus, pathogens deficient in the T3SS do not elicit a hypersensitive response (Kim *et al.*, 2009, Tseng *et al.*, 2009). The T3SS in Gram-negative bacteria delivers effectors into plant cells to supress plant defence mechanisms (Diallo *et al.*, 2012). Although the T3SS is required for virulence in some pathogens, such as *Pseudomonas syringae* and *Ralstonia solanacearum*, in *Pectobacterium* spp. it is inessential, while facilitating leaf colonisation (Kim *et al.*, 2009, Morello & Collmer, 2009, Nykyri, 2013).

The type 4 secretion system (T4SS) is a conduit for DNA, as well as proteins, into target bacterial and eukaryotic cells (Davidsson *et al.*, 2013). Various T4SSs have different gene complements. *P. atrosepticum* possess a VirB-T4SS, similar to that in *Agrobacterium tumefaciens* (Bell *et al.*, 2004). This particular system encodes 12 genes that form a translocon

spanning from the inner membrane through the periplasm into the outer membrane (Green & Mecsas, 2016). Not much is known about the substrate transported by the T4SS in *Pectobacterium* spp., but attenuation of this system in *P. atrosepticum* diminishes virulence, which suggests that effectors may be transferred to the host (Bell *et al.*, 2004, Kraepiel & Barny, 2016).

The type 5 secretion system is the second Sec/Tat-dependent system. Of the known secretion systems, the T5SS is structurally the simplest, comprised of one or two proteins (Gerlach & Hensel, 2007). Autotransporter secretion relies on the presence of all the necessary domains within a single protein (Filloux & Sagfors, 2015). Proteins have an N-terminal domain that allows for Sec-dependent secretion into the periplasm. The C-terminus possesses a β-barrel domain forming a pore in the outer membrane. Once secreted to the surface of the cell, proteolysis is directed by an N-terminal protease domain. Two-partner secretion requires an outer membrane protein that delivers discrete substrates out of the cell. The T5SS does not noticeably contribute towards virulence in *Pectobacterium* spp. (Nykyri, 2013).

The Type 6 Secretion System

Numerous studies have alluded to the existence of the single-step type 6 secretion system (T6SS) many years before it was officially classified (Bingle *et al.*, 2008). The T6SS is a needle-like structure reminiscent of the phage tail that shoots out from the cell into an adjacent cell to translocate effector proteins into the penetrated cell. It was only in 2006 that Pukatzki and colleagues proved that this cluster of genes, present in many bacterial species, encoded for the T6SS in *Vibrio cholerae* (Pukatzki *et al.*, 2006). Bioinformatic analyses have shown that the T6SS is widely distributed, present in approximately a quarter of Gram-negative bacteria (Ho, 2014). Its ability to target both prokaryotic and eukaryotic cells allows for T6SS-bearers to eradicate competitors from their environmental niche as well as to promote virulence. Often the contribution of the T6SS towards pathogenesis is supplemental and not

a major virulence determinant (Bernard *et al.*, 2010). Bacteria may encode multiple type 6 clusters and in phytobacterial genomes, only 7% of strains contain more than two T6SS clusters

(Bernal *et al.*, 2018). The particular function of a T6SS depends on the species, however, the T6SS has been seen to play a role in biofilm formation, host colonisation, virulence, adhesion, and metal-ion acquisition (Bernard *et al.*, 2010). Gram-positive bacteria are not targeted by the T6SS due to the thickness of their cell wall (Hachani *et al.*, 2013, Joshi *et al.*, 2017).

Many T6SS effectors have been identified thus far, able to target eukaryotes, bacteria, or both (Pukatzki *et al.*, 2007, Russell *et al.*, 2011, Jiang *et al.*, 2016). Hood *et al.* (2010) first identified the ability of the T6SS to target bacterial cells. Since then it has been determined that the foremost target of the T6SS is bacterial cells (Schwarz *et al.*, 2010a, Russell, 2014, Russell *et al.*, 2014a, Alcoforado Diniz & Coulthurst, 2015, Robb *et al.*, 2016). In some cases, a single system may target both eukaryotic and bacterial cells, as is the case for *Vibrio cholerae* (Russell, 2014, Filloux & Sagfors, 2015). *V. cholerae* effector VasX targets bacterial membrane lipids as well as membranes within the eukaryote *Dictyostelium discoideum* (Miyata *et al.*, 2011). In other cases, distinct T6SS can differentially target bacterial or eukaryotic cells, as in *Pseudomonas aeruginosa* which encodes three distinct T6SSs: H1-T6SS, H2-T6SS, and H3-T6SS (Hachani *et al.*, 2011). Although effector Tse2 secreted by H1-T6SS displays toxicity towards both eukaryotic and bacterial cells, H1-T6SS exclusively targets bacterial cells *in situ* (Russell *et al.*, 2011).

Burkholderia pseudomallei, B. mallei, and *B. thailandensis* exemplify the use of multiple T6SS for disparate purposes, with some strains possessing up to six distinct systems (T6SS–1-6) (Schwarz *et al.*, 2010b). T6SS–1 has been studied considerably, but much information is lacking for the other five T6SSs. Compared to animal-pathogenic *Burkholderia* spp., environmental *Burkholderia* spp., have fewer T6SSs and none possess the T6SS-5 cluster,

which is involved in eukaryotic host cell manipulation by inducing cell membrane fusion (Schwarz *et al.*, 2010b, Angus *et al.*, 2014, Schwarz *et al.*, 2014, Bernal *et al.*, 2018). In *B. thailandensis*, T6SS-1 is involved in interbacterial competition in biofilms as well as to constrain quorum sensing cheaters within the community (Angus *et al.*, 2014, Majerczyk *et al.*, 2016). The T6SS-4 is involved in manganese uptake during oxidative stress by secreting a Mn^{2+}-binding effector extracellularly, in addition to its antibacterial role (Si *et al.*, 2017). In *B. pseudomallei*, T6SS–1-6 are expressed at different growth phases and regulated by different systems (Miyata *et al.*, 2013). T6SS–2 and T6SS–6 are negatively and positively regulated by the transcriptional regulator TctR, respectively (Losada *et al.*, 2018). T6SS–2 is expressed in nutrient-limiting conditions and by subinhibitory concentrations of many classes of antibiotics. The T6SS–5 is essential for virulence in *B. thailandensis* and all other T6SSs are dispensable, whereas the T6SS–1 is the only necessary T6SS for virulence in *B. pseudomallei* (Schwarz *et al.*, 2010b, Fisher *et al.*, 2012). This highlights that T6SSs have specific roles, and their requirement is determined by the ecological niche of the species.

There have been attempts to assign functions to a T6SS based on its phylogenetic grouping (Schwarz *et al.*, 2010b, Barret *et al.*, 2011). Some clades, however, comprise T6SS clusters that are either antibacterial or target the eukaryotic host, or single systems targeting both (Bernal et al., 2018). While phylogenetic analysis does not seem to indicate pathogenic or benign associations or whether a cluster is antibacterial or antieukaryotic, the phylogenetic data does suggest that there is a propensity for particular clusters to be horizontally acquired, and that the genomic arrangement of type 6 components remains similar for T6SSs belonging to the same cluster (Bernal *et al.*, 2018).

Type 6 secretion systems belong to one of four phylogenetic groups: $T6SS^{i}$, $T6SS^{ii}$, $T6SS^{iii}$, and $T6SS^{iv}$. Generally speaking, mention of the T6SS refers to the phylogenetic group $T6SS^{i}$, found in Proteobacteria. This T6SS is the hallmark of species such as *P. aeruginosa, V.*

cholerae, E. coli, Aeromonas hydrophila, and *Burholderia* spp. (Mougous *et al.*, 2006, Pukatzki *et al.*, 2006, Aschtgen *et al.*, 2008, Schwarz *et al.*, 2010b); however, atypical T6SSs have also been identified. *Francisella tularensis* encodes a T6SS that shares little sequence similarity to the canonical T6SS^{i} and has been designated T6SS^{ii} (Clemens *et al.*, 2018). T6SS^{ii} lacks the ClpV ATPase component that is essential for systems belonging to group T6SS^{i} (Bernal *et al.*, 2018). *Bacteroides fragilis* and *Flavobacterium johnsoniae* possess a T6SS that has been classified into a third distinct group, T6SS^{iii} (Russell *et al.*, 2014b). A fourth group, T6SS^{iv}, was recently identified in *Amoebophilus asiaticus* (Böck *et al.*, 2017). T6SS^{iii} and T6SS^{iv} systems lack homologues for core membrane components TssJLM, and T6SS^{iv} additionally lacks the ClpV ATPase. Phytopathogens only encode T6SS^{i} and will be the only T6SS discussed from here onward (Bernal *et al.*, 2018).

Structure of the T6SS

The T6SS bears a striking resemblance to that of the contractile phage tail system, albeit inversely directed. The T6SS is made up thirteen core structural proteins (TssA-M), of which many have corresponding homologues in the phage system. These proteins collectively extend from the cytoplasm and span the inner membrane, periplasm, and outer membrane. The structure can be divided into three subassemblies: (1) the shaft consisting of the Hcp tube and sheath, (2) the cytoplasmic baseplate, and (3) the membrane complex (Figure 1.1).

The shaft is composed of multiple Hcp (TssD) subunits, which form hexameric rings that are stacked to form a hollow tube (Durand *et al.*, 2014). The length of the shaft is limited by the availability of Hcp (Vettiger & Basler, 2016). The tube is tipped at the distal end by a VgrG trimer. A conical protein with a Pro-Ala-Ala-Arg (PAAR) motif is situated at the tip of the VgrG trimer and serves to sharpen the tip, facilitating penetration of target cells (Shneider *et al.*, 2013). Recently, studies have indicated that at least one PAAR protein is essential for T6SS activity (Cianfanelli *et al.*, 2016); consequently, the authors have proposed that the PAAR

protein be designated the 14th component of the T6SS. It is common for T6SS-bearing bacteria to possess multiple Hcp- and VgrG-encoding genes, which may be distributed across the genome (Coulthurst, 2013). TssB/C form the tubular sheath that surrounds the Hcp tube, which may span the entire diameter of the cell (Ho *et al.*, 2014). Secretion systems I-VI hydrolyse ATP to drive protein transfer (Filloux & Sagfors, 2015). However, the T6SS does not directly employ ATP for substrate loading and energising secretion. During the polymerisation of the sheath, the uncontracted state of TssB/C inherently possesses the required potential energy to propel the shaft upon cell contact (Ge *et al.*, 2015). Once discharged, the AAA+ ATPase ClpV only recognises sheath proteins that are in the contracted state (specifically, a motif only exposed in contracted TssB proteins) and proceeds to depolymerise the sheath for re-use.

The membrane complex acts as a point of attachment of the shaft to the cell, as well as a conduit for the tube to pass through (Alteri & Mobley, 2016, Zoued *et al.*, 2016). The first protein to be engaged for T6SS assembly is TssJ, which is localised to the outer membrane and is shortly followed by the attachment of inner membrane proteins TssM and TssL (Durand *et al.*, 2015). TssM bridges the interaction between TssL and TssJ. In many species, the T6SS is anchored to the cell wall through peptidoglycan-binding domains contained in some TssL proteins, TagL, or another T6SS-associated protein with a peptidoglycan-binding domain (Aschtgen *et al.*, 2010). In some species such as *V. cholerae* and *P. atrosepticum*, however, no equivalent protein has been identified. Once the membrane complex has been established, the baseplate is recruited.

Since tube and sheath polymerisation in phage requires a baseplate, a corresponding assembly in the T6SS was searched for. Brunet *et al.* (2015) identified TssE, TssF, TssG, TssK, and VgrG as the T6SS equivalent baseplate components. TssA is recruited to the cytoplasmic end of the membrane complex where it facilitates recruitment of the baseplate

(Zoued *et al.*, 2016). All components of the baseplate are located within the cytoplasm (Zoued *et al.*, 2014). To ensure cohesion of the subassemblies, TssK joins the shaft to the membrane complex, by interacting with TssL, Hcp and TssC (Zoued *et al.*, 2013). Once the baseplate has been assembled, TssA interacts with Hcp and TssC to assemble the shaft (Zoued *et al.*, 2016). Pushed along by the sheath, TssA continually remains at the apex of the shaft to incorporate the next set of proteins.

A number of T6SS components share similarity with phage contractile tail components. VgrG resembles the gp5-gp27 needle of bacteriophage T4, which forms part of the hub of the baseplate (Leiman *et al.*, 2009, Brunet *et al.*, 2015). TssE is homologous to phage gp25 which complexes with the gp5-gp27 hub (Brunet *et al.*, 2015). Hcp resembles the tail tube protein gpV of bacteriophage λ and gp19 of phage T4 (Pell *et al.*, 2009, Brunet *et al.*, 2015). The sheath proteins TssB/C are structurally and functionally similar to phage T4 sheath proteins (Brunet *et al.*, 2015). It is due to these similarities with phage that the assembly and activity of the T6SS is paralleled with its respective counterparts in the bacteriophage. Proteins from the membrane complex share no similarity to phage components; rather, TssM and TssL are resemble two membrane components of the T4SS, IcmF and IcmH/DotU, respectively (Thomas *et al.*, 2010, Brunet *et al.*, 2015). Due to this high similarity to the phage tail, the mechanism of action of the T6SS has been derived from the action of the tail complex in phages.

The membrane complex assembles randomly within the cytoplasmic membrane and each complex can serve as a point at which a complete T6SS could assemble within a few seconds (Brunet *et al.*, 2015, Durand *et al.*, 2015). Membrane complexes often occur in excess to the number of sheaths present, indicating that the sheath is polymerised on preformed membrane complexes (Durand *et al.*, 2015). Additionally, membrane complexes can be recycled for numerous tube polymerisation events. The number of T6SS structures per cell is limited to the

availability of tip complexes and the length of the sheath is dependent on the availability of Hcp (Ho *et al.*, 2014, Vettiger & Basler, 2016). The efficiency of a firing event is dependent on the relative position of the target cell to the T6SS apparatus and the regulation of the system (Ho *et al.*, 2014).

Regulation of the T6SS

Given the taxonomic diversity of bacteria employing their T6SS against an assorted range of targets including plant, humans, and animals, a number of regulatory mechanisms are involved in maintaining T6SS activity. Contact-dependent inhibition is observed once cells are grown on solid media, indicating that T6SS activity is enabled upon attachment and cells become sessile (Bernard *et al.*, 2010). The T6SS can exhibit either erratic or safeguarded firing (Ho *et al.*, 2014). In some species, such as *Vibrio* and *Acinetobacter*, the T6SS is highly active and aggressive, although less effective by firing unselectively (Basler *et al.*, 2013, Bernal *et al.*, 2017). In contrast, the *P. aeruginosa* T6SS, which is limited to counterattack, is largely inactive and launches more specifically. Tight regulation, especially during pathogen-host interactions is crucial to allow for pathogen survival and pathogenesis, as untimely expression of virulence factors could initiate a defensive host response in advance (Filloux *et al.*, 2008).

Stimuli activating the T6SS may arise from host factors or external conditions, such as nutrient limitation (Alcoforado Diniz *et al.*, 2015, Navarro-Garcia *et al.*, 2016). The T6SS is regulated by various stimuli in different species. In *P. aeruginosa*, T6SS activity is upregulated by perturbations in the outer membrane (Basler *et al.*, 2013). In *A. tumefaciens,* it is activated by acidic conditions (Ma *et al.*, 2014), in *P. atrosepticum* it is induced by plant extracts (Mattinen *et al.*, 2008). Most *V. cholerae* strains carry a complete T6SS cluster; however, regulation between strains can differ markedly. The T6SS in non-pandemic *Vibrio cholerae* is

constitutive; however, the system is more tightly regulated in the pandemic strains O1 and O139 (Shao & Bassler, 2014).

In *V. cholerae*, expression of the T6SS has been observed *in vivo* (Miyata *et al.*, 2013). The transcription of the majority of genes, such as housekeeping genes, is regulated by sigma-70 (σ^{70}). Alternative sigma factors regulate the expression of a subset of genes in response to specific stimuli. Sigma-54 (RpoN or σ^{54}) regulates genes that are involved in biofilm formation, nitrogen acquisition, and motility (Hao & Shearwin, 2017). Transcription factor σ^{54} together with the obligatory enhancer binding protein, VasH, are also necessary for expression of the T6SS in both pandemic and endemic *V. cholerae* strains (Pukatzki *et al.*, 2006, MacIntyre *et al.*, 2010, Kitaoka *et al.*, 2011). Flagellar regulatory sigma factor (FliA), σ^{28}, represses T6SS expression (Syed *et al.*, 2009). Quorum sensing is also involved in T6SS regulation through LuxO and HapR (Ishikawa *et al.*, 2009). Not only does quorum sensing regulate the degree of expression, but a *luxO*-deletion mutant induces expression of the T6SS during an earlier growth phase.

T6SS regulation has been extensively studied in *P. aeruginosa*. The regulatory cascade controlling the expression of the T6SS is quite complex, regulating the expression of three distinct systems, H1-T6SS, H2-T6SS, and H3-T6SS. Regulation is further complicated by the fact that these systems have different roles: H1-T6SS is involved in bacterial killing, H2-T6SS targets eukaryotic cells, and H3-T6SS exhibits antieukaryotic and -prokaryotic activity (Hood *et al.*, 2010, Sana *et al.*, 2012, Jiang *et al.*, 2014). H1-T6SS is activated by quorum sensing via LasR when cell titres are low (Silverman *et al.*, 2012). *In vitro* expression of H1-T6SS is induced in a Δ*retS* background, indicating that RetS negatively regulates expression of H1-T6SS (Basler & Mekalanos, 2012). Many two-component systems have been implicated in T6SS regulation (Filloux *et al.*, 2008). RetS is a hybrid sensory kinase that contains both a sensor and receiver domain (Laskowski & Kazmierczak, 2006). To date, the environmental

signal triggering RetS is still unknown (Francis *et al.*, 2018). RetS is involved in the expression of many phenotypes, especially virulence, and together with other sensory kinases, these phenotypes are regulated in response to a variety of environmental signals. Two-component system regulators GacS/GacA are negatively and positively regulated by RetS and LadS, respectively (Brencic *et al.*, 2009). This two-component system regulates only two small RNAs, RsmY and RsmZ, however the regulatory effects within the cell are far-reaching. RsmY and RsmZ modulate levels of the small RNA RsmA by directly biding to it; RsmA in turn regulates at least 500 genes including those of the H1-T6SS, which it represses at the post-transcriptional level (Records & Gross, 2010). RsmA regulates numerous phenotypes, including motility, metabolism, biofilm formation, secretion systems, and exopolymeric substance production.

Regulations at the post-translational level are mediated by disruptions in the cellular membrane, which activate H1-T6SS assembly. A signal cascade is initiated via the membrane-embedded protein complex TagQRST (Basler *et al.*, 2013). TagT transfers the signal upon membrane disruption through the complex to TagR, which in turn stimulates autophosphorylation of the kinase PpkA (Hsu *et al.*, 2009, Gallique *et al.*, 2017). PpkA is necessary for phosphorylation of FHA1 that in turn promotes H1-T6SS assembly (Hood *et al.*, 2010). PpkA phosphorylation is counteracted by phosphatase PppA. Loss of activity of PpkA completely abolishes T6SS activity, whereas inactivation of PppA results in a hyperactive T6SS (Basler *et al.*, 2013). The accessory protein FHA is absent from more than 50% of species possessing a T6SS (Ho *et al.*, 2014).

Regulation of H2-T6SS and H3-T6SS is much less studied than that of H1-T6SS. Transcriptional factor, MvaT, represses the expression of both H2-T6SS and H3-T6SS (Bernard *et al.*, 2010). In *P. aeruginosa*, σ^{54} represses H2-T6SS and one of the two H3-T6SS clusters (the right H3-T6SS operon) whereas the left H3-T6SS operon is activated by σ^{54}

(Sana *et al.*, 2013). Both H2-T6SS and H3-T6SS are positively regulated by quorum sensing via LasR and MvfR (Chen *et al.*, 2015). At stationary phase, H2-T6SS is significantly upregulated and mediates internalisation into epithelial cells (Sana *et al.*, 2012). H2-T6SS is further induced by iron-limiting conditions (Sana *et al.*, 2012).

Regulation has been less studied in *Pectobacterium* species. In *P. atrosepticum*, the T6SS is positively controlled by σ^{54}, unlike the H1-T6SS of *P. aeruginosa* (Bernard *et al.*, 2011). The T6SS of *P. atrosepticum* is upregulated by potato extracts *in vitro* (Mattinen *et al.*, 2008), whereas in *Pcb1692* potato extracts had no impact on T6SS competition (Shyntum *et al.*, 2018). The T6SS in *P. atrosepticum* is also positively regulated by quorum sensing via the AHL-synthesising protein, ExpI (Liu *et al.*, 2008). ExpI also regulates the expression of type 6 effectors in this species.

Important to note is that the T6SS is often implicated in a number of roles apart from cell killing. Global regulators are often implicated in the regulation of the T6SS; thus, other phenotypes are often associated with T6SS activity. For example, biofilm formation and T6SS activity are both regulated by networks such as the Gac/Rsm signal transduction pathway (Hood *et al.*, 2010, Valentini & Filloux, 2016). The Gac/Rsm pathway is further regulated by SuhB and the global regulator c-di-GMP (Valentini & Filloux, 2016, Li *et al.*, 2017). Myriads of cellular processes are regulated by c-di-GMP including motility, toxin production, antibiotic production and resistance, chemotaxis, biofilm formation, and other virulence phenotypes, in response to various environmental stimuli, which differ between species (Römling *et al.*, 2013). This hierarchical regulation indicates that numerous cellular processes can contribute towards a single phenotype, and that one cue can induce multiple phenotypes. Regulation of the T6SS is exceptionally diverse among bacteria. To highlight this, the Gac/Rsm regulatory pathway directly regulates the H1- and H3-T6SS of *P. aeruginosa*, but not the H2-T6SS (Bernard *et al.*, 2010). Thus, it is clear to see how the T6SS and other virulence factors can contribute

towards the same phenotypes, such as biofilm formation or virulence, when they are both regulated by the same pathways.

Type 6 effectors

Type 6 effectors target both eukaryotic and prokaryotic cells. Eukaryotic-targeting effectors are generally divided into two main groups: (1) mimics of host cellular proteins, and (2) proteins that covalently modify host cellular proteins (Toft & Andersson, 2010). Effectors have a range of cellular targets and manipulate host functions in several ways to impair defence response, such as mimicking or interacting with host transcription factors to interfere with gene expression or disrupting host cell structure by disturbing the cytoskeleton (Deslandes & Rivas, 2012). Effectors that target prokaryotes are often referred to as toxins, as they cause cell death by destroying conserved cell components such as the cell wall, cell membrane, or nucleic acids (Alcoforado Diniz *et al.*, 2015). All type 6-secreted proteins are referred to as effectors, whether they are eukaryotic- or prokaryotic-targeting. It is expected that a strain's effector repertoire is representative of its host and ecological niche (Dou & Zhou, 2012, Koebnik & Lindeberg, 2012, Puhar & Sansonetti, 2014).

All the characterised protein secretion systems deliver effectors out of the cell, albeit the requirements for secretion and targets differ (Benz & Meinhart, 2014). In Gram-negative bacteria, the T1- and T2SS secrete effectors into the surrounding medium, whereas the T3-, T4-, T5-, and T6SSs have the capacity to deliver effectors directly into a target cell (Zhang *et al.*, 2012). Furthermore, only the T5SS and T6SS are limited to mediating bacterial competition. The function of the T3-, T5-, and T6SSs is limited primarily to the secretion of toxins (Zhang *et al.*, 2012).

Type 6 effector transport

To reach the intended destination, secreted proteins need cross the cell wall through the appropriate channel. Signal peptides assist by directing proteins intended for secretion

towards their cognate secretion system, albeit, no dedicated signal sequence has been identified for type 6 effectors (Liang *et al.*, 2015). However, specificity is required to target dedicated type 6 effectors through the T6SS complex (Silverman *et al.*, 2013). Consequently, there are two classes of type 6 effectors: specialised- and cargo effectors. Specialised effectors, such as the structural Hcp, VgrG, and PAAR proteins, possess additional covalently-linked C-terminal toxin domains. For example, the C-terminal ends of VgrG1 and VgrG3 from *V. cholerae* possess an actin cross-linking domain and muramidase domain, respectively; VgrG1 of *A. hydrophila* contains a C-terminal vegetative insecticidal protein (VIP2) domain (Pukatzki *et al.*, 2007, Dong *et al.*, 2013, Durand *et al.*, 2014). Thus, specialised effectors have a dual function: as part of the structural complex and as an effector protein.

Cargo effectors on the other hand associate with VgrG, Hcp and PAAR proteins noncovalently to mediate their export (Alcoforado Diniz & Coulthurst, 2015, Alcoforado Diniz *et al.*, 2015). VgrG proteins are essential for effector delivery, as deletion of all VgrGs renders a cell incapable of T6SS-dependent killing (Hachani *et al.*, 2014). Thus, instead of type 6 effectors possessing distinct signal sequences they depend on interactions with structural components for their delivery. Although not associated with all type 6 effectors, the MIX motif has been identified at the N-terminus of some effectors (Salomon *et al.*, 2014). The MIX motif is found in both antibacterial and antieukaryotic effectors (Ray *et al.*, 2017). In some instances, chaperone proteins, such as DUF4123 and DUF1795, interact with the effector and Hcp/VgrG/PAAR as a bridge to mediate secretion (Lien & Lai, 2017).

Effector genes are often found neighbouring genes that encode the structural substrates Hcp, VgrG, PAAR, and chaperone proteins (Bernal *et al.*, 2017). The genomic association of effectors with these structural genes often determines their secretion specificity. Effectors downstream of VgrG are often more efficiently transported by that VgrG or exclusively by it (Silverman *et al.*, 2013, Cianfanelli *et al.*, 2016). Genes associated with the delivery of

effectors are often used to identify effector genes within the genome (Alcoforado Diniz & Coulthurst, 2015, Liang *et al.*, 2015, Whitney *et al.*, 2015). Effector genes are often dispersed throughout the genome in a locus distinct from the core cluster that encodes the T6SS apparatus; thus, using structural genes as genetic markers facilitates effector identification, especially as T6SS effectors have no dedicated marker sequence (Barret *et al.*, 2011, Hachani *et al.*, 2014, Ma *et al.*, 2018).

Effector-immunity protein interactions

Unlike antieukaryotic effectors, antibacterial effectors (often named Tse's) require a cognate immunity protein to protect producers from self-intoxication (Figure 1.2) (Hood *et al.*, 2010). Bioinformatic analyses indicate that immunity genes are mostly located directly downstream of their cognate effector. Exceptions occur, as in the case of *P. aeruginosa* where the immunity gene for effector Tse1 occurs ahead of the effector gene (Russell *et al.*, 2011). Bacteria may also encode additional immunity genes without a respective toxin, providing immunity to the host from competing species that produce the cognate effector (English *et al.*, 2012, Russell *et al.*, 2012). Immunity proteins (often named Tsi's) inhibit the activity of effectors by directly binding to their active site as mono- or multimers (Benz *et al.*, 2013, Chen *et al.*, 2015). Tsi2 from *P. aeruginosa* forms a dimer, which occludes the active site of the cytoplasmic effector Tse2 (Robb *et al.*, 2016). Although effectors may have the same antibacterial activity, immunity proteins may differ significantly (Tang, 2018).

Effector-immunity cross-reactivity has also been observed, although occurs rarely. Effectors that belong to the same family have similar structures, thus respective immunity proteins may be able to bind to effectors from other species belonging to the same family (Zhang *et al.*, 2013). These immunity proteins are limited to binding effectors from the same family only; however, immunity proteins are not able to engage in protein interactions with all effectors from the same family. The degree of effector inactivation is not as complete when non-cognate

immunity proteins bind. For example, although Tsi1 from *P. aeruginosa* and Tsi1 from *Burkholderia phytofirmans* are both able to bind to Tse1 from either species, neutralisation is not complete when the immunity protein is not bound to its cognate effector (Russell *et al.*, 2012). Although cross-reactivity has been observed, immunity proteins generally bind very specifically to neutralise their cognate effector.

Recently, it has been shown that shaft components transferred into sister cells can be reused for assembly of a functional T6SS structure in the recipient cells, indicating that the T6SS likely penetrates into the cytosol, as well as the periplasm (Vettiger & Basler, 2016). Due to the length of the sheath, which can span the diameter of the cell, calculations show that it could extend a substantial distance into a closely situated target cell, and that the firing event would provide the force required for penetration (Ho *et al.*, 2014). In addition to VgrG and Hcp proteins being transported across cells, Vettiger & Basler (2016) observed that effectors are also transported into and released by recipient cells. This is understandable, as effectors associate directly with these components for translocation.

Antibacterial effectors

Initially, four families of antibacterial effectors were identified (Alteri & Mobley, 2016). The peptidoglycan glycoside hydrolase (*tge* genes) and peptidoglycan amidase (*tae/ssp* genes) families both target peptidoglycan and act in the periplasm (Alcoforado Diniz *et al.*, 2015). The phospholipase family (*tle*) targets cell membranes, and the nuclease family (*tde*) targets DNA and RNA. Depending on their function, effectors are localised to different compartments within the target cell (Russell *et al.*, 2011, Deslandes & Rivas, 2012). By delivering an array of effectors into the target, they may act cumulatively towards cell death.

3.3.3.1. Peptidoglycan-targeting effectors

The bacterial cell wall is composed of glycan and peptide moieties (Vollmer *et al.*, 2008). The glycan component consists of repeating units of the N-acetylglucosamine (NAG) and

N-acetylmuramic acid (NAM) disaccharides. Tetrapeptides bound to NAM link adjacent chains to form a multi-layered structure. Peptidoglycan amidases (Tae effectors) cleave the peptide moiety and peptidoglycan glycoside hydrolases (Tge effectors) cleave within the glycan component (Russell, 2014). Peptidoglycan glycoside hydrolases include muramidases and glucosaminidases. Peptidoglycan amidases have been grouped into four families according to their cleavage specificity (Benz *et al.*, 2013). Tae1 cleaves the bond between D-glutamine and L-meso-diaminopimelic acid on the donor peptide in Gam-negative bacteria, whereas Tae4 cleaves the same bond, albeit on the acceptor peptide. Tae2 and Tae3 both cleave the bond between L-meso-diaminopimelic acid and D-alanine.

The first antibacterial effectors identified were from *P. aeruginosa* and both (Tse1 and Tse3) target peptidoglycan (Russell *et al.*, 2011). Tse1 has amidase activity and Tse3 has glycoside hydrolase activity. *Serratia marcescens* encodes two distinct amidase effectors, Ssp1 and Ssp2 (Alcoforado Diniz & Coulthurst, 2015). VgrG3 from *V. cholerae* is a muramidase, however, it groups distinctly from other Tge effectors (Dong *et al.*, 2013). All peptidoglycan-targeting effectors are active within the periplasm; cytosolic expression of these effectors in ectopic hosts does not cause growth inhibition.

3.3.3.2. Phospholipases

Phospholipases are widely distributed among microorganisms, plants, and animals (Richmond & Smith, 2011). They have diverse substrates and have different targets sites within their substrate (Wilton & Waite, 2002). In phospholipids, the glycerol molecule is attached to a fatty acid chain at the *sn*-1 and *sn*-2 carbons; the *sn*-3 carbon molecule is attached to a phosphate and a head group molecule. As each fatty acid chain may vary as well as the head group, much diversity exists within phospholipids. Phospholipases are categorised into four major groups depending on bond cleavage within the phospholipid:

phospholipase A (PLA), phospholipase B (PLB), phospholipase C (PLC), and phospholipase D (PLD).

The PLA group is divided into two subgroups: PLA1 and PLA2. Both phospholipases cleave the ester bonds that attach the fatty acids to glycerol; however, PLA1 cleaves the *sn*-1 ester linkage, whereas PLA2 cleaves the *sn*-2 ester linkage (El Alaoui *et al.*, 2016). Cleavage of phospholipids with PLA yields free fatty acids and lysophospholipids. PLB phospholipases cleave the phospholipid at both the *sn*-1 and *sn*-2 ester bonds (Richmond & Smith, 2011). PLC and PLD cleave on either side of the phosphate moiety within the phospholipid (Wilton & Waite, 2002). PLC cleaves at the *sn*-3 ester bond, whereas PLD cleavage removes the head group moiety from the phosphate moiety.

A review of literature indicates that type 6 phospholipases belong to either the PLA or PLD family. Five groups of type 6 phospholipase effectors (Tle1-5) have been identified, which are grouped according to sequence and phylogeny (Russell *et al.*, 2013). The enzymatic characterisation for Tle1, Tle2, and Tle5 have been experimentally determined and respectively have PLA2, PLA1, and PLD enzymatic activity (Hu *et al.*, 2014), whereas activities for Tle3 and Tle4 have not yet been experimentally identified (Russell *et al.*, 2014a, Flaugnatti *et al.*, 2016). Tle1-4 all have a GXSXG catalytic lipase motif, whereas Tle5 (phospholipase D) is characterised by a HXKXXXXD motif (Russell *et al.*, 2013). The diversity in phospholipid cleavage by different phospholipases is quickly illustrated by Tle1 from entero-aggregative *E. coli* (EAEC), which has PLA1 activity and shows specificity for phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine, but not phosphatidylglycerol (Flaugnatti *et al.*, 2016), whereas PldA from *P. aeruginosa* cleaves phosphatidylethanolamine (Sana *et al.*, 2016). No type 6 phospholipases have been characterised in SREs yet.

3.3.3.3. Nucleases

Nucleases are phosphodiesterases that cleave the phosphodiester bond in nucleic acids and can be grouped into two main categories: DNases that cleave deoxyribonucleic acid and RNases that cleave ribonucleic acid (Mishra, 1995). Both types of nucleases have been identified in type 6 effectors. The nuclease toxin domain is not unique to the T6SS bacterial targeting system and is also found among bacteriocins and the contact-dependent inhibition (CDI) system (Alcoforado Diniz *et al.*, 2015).

Type 6 nucleases are broadly distributed. A well-cited example are two nucleases identified in *Dickeya dadantii*: RhsA and RhsB, which effectively degrade genomic DNA (Koskiniemi *et al.*, 2013). In *A. tumefaciens*, two nucleases, Atu4350 and Atu3640, with a toxin_43 domain are secreted by the T6SS (Ma *et al.*, 2014). These nucleases rapidly digest DNA in the presence of Mg^{2+} and *in planta*, these toxins are required for leaf colonisation. *Proteus mirabilis* secretes a type 6-dependent HNH nuclease that contributes towards swarming motility by killing non-kin bacterial populations (Alteri *et al.*, 2013). Similar to *P. mirabilis*, a type 6 dependent nuclease secreted by *Myxococcus xanthus* contributes towards non-kin selection (Gong *et al.*, 2018). *M. xanthus* T6SS mutants are unable to discriminate between non-kin cells and subsequently merge with non-kin colonies. Another example of a type 6 nuclease is Tse7, which belongs to the HNH nuclease superfamily and is secreted by H1-T6SS in *P. aeruginosa* (Pissaridou *et al.*, 2018). Rhs2 from *Serratia marcescens* is another HNH endonuclease (Alcoforado Diniz & Coulthurst, 2015). Rhs2 is particularly involved in intraspecies killing – killing strains of S. marcescens that lack the immunity protein against this nuclease. Literature indicates that the HNH endonuclease domain is commonly found among type 6 nuclease toxins. Although the majority of type 6 nucleases are DNases, one effector represents the second category of nuleases: Tse2 from *P. aeruginosa* is an example of a ribonuclease (Li *et al.*, 2012).

Antieukaryotic effectors

Thus far, host targeting via T6SS effectors has been observed against fungi, protists and animals, however, no plant-targeting effector has been identified yet (Bernal *et al.*, 2018, Trunk *et al.*, 2018). Eukaryotic-targeting effectors target various cellular structures and have a diversity of implications. Antieukaryotic effectors affect adhesion, host internalisation, phagocytosis proficiency, membrane integrity, cytoskeletal rearrangements, survival and replication, competitive fitness, cytotoxicity, immune system responses, and virulence against host (Hachani *et al.*, 2016).

In *Vibrio cholerae*, VgrG1 and VgrG2 have actin-crosslinking activity in *Dictyostelium* (Sheahan *et al.*, 2004, Pukatzki *et al.*, 2006). In *P. aeruginosa*, VgrG2b is secreted by H2-T6SS and is required for host cell invasion (Sana *et al.*, 2016). TplE is also secreted by H2-T6SS and is a phospholipase, which interacts with the endoplasmic reticulum (ER) of host cells (Jiang *et al.*, 2016). Interaction of TplE with the ER leads to the disruption thereof which triggers cytotoxicity. A phospholipase D effector secreted by H3-T6SS of *P. aeruginosa*, PldB, facilitates internalisation of the pathogen into human epithelial cells via the phosphatidylinositol 3-kinase/Akt pathway (Jiang *et al.*, 2014). *Burkholderia cenocepacia* protects itself from host defences by interrupting the recruitment of NADPH oxidase components that are involved in oxidative burst in the macrophage (Rosales-Reyes *et al.*, 2012). Additionally, Hcp secreted by *B. cenocepacia* induces cytoskeletal rearrangement in macrophages (Aubert *et al.*, 2008). Hcp from *Aeromonas hydrophila* causes an increase in caspase 3 levels leading to apoptosis of host cells (Suarez *et al.*, 2008). EvpP from *Edwardsiella tarda* is required for pathogen internalisation in the fish host and for survival (Chen *et al.*, 2017). Other species that have eukaryotic-targeting T6SSs include, *Campylobacter jejuni*, *E. coli, Salmonella enterica*, and *Ralstonia solanacearum* (Hachani *et al.*, 2016).

Other effectors

Some effectors show both antibacterial and antieukaryotic activity. PldB from *P. aeruginosa* not only mediates internalisation into host epithelial cells, but hydrolyses the phosphodiester bond in bacterial cell membranes (Jiang *et al.*, 2014). TplE from *P. aeruginosa* also functions as a PLA1 phospholipase to target bacterial cell membranes (Jiang *et al.*, 2016). Phospholipases are good candidates for tans-kingdom effectors as both kingdoms possess phospholipid membranes within the cell.

Recent studies have indicated that T6SSs are not solely involved in the transport of toxic proteins. *Proteus mirabilis* uses the T6SS for self/non-self recognition in swarming motility by secreting self-identity protein D (IdsD) into sister cells (Saak & Gibbs, 2016). IdsD is neutralised by cognate immunity protein, IdsE. Transfer of IdsD to non-sister cells via the T6SS results in a negative interaction within the recipient (which lacks IdsE) causing unrelated bacteria to form boundaries between each other. Wang *et al.* (2015) have shown that T6SS-4 of *Yersinia pseudotuberculosis* transports a zinc-binding effector into the extracellular milieu to overcome oxidative stress (Wang *et al.*, 2015). Additional instances of metal ion uptake by the T6SS have been identified in *Burkholderia thailandensis* by TseM (Mn^{2+} uptake) and by TseF for iron uptake *P. aeruginosa* (Lin *et al.*, 2017, Si *et al.*, 2017).

Alternative Bacterial Killing Systems

The T6SS allows a bacterial cell to directly penetrate a target cell and to deliver toxins within this cell (Alteri & Mobley, 2016). There are, however, a number of different bacterial killing systems that can be employed to kill other bacteria. Bacteria can either kill rival cells by contacting them directly, or indirectly by secreting substances that eventually come into contact with the competitor and subsequently result in growth inhibition. The contact-dependent inhibition (CDI) system was the first system implicated in contact-dependent bacterial killing (Aoki *et al.*, 2011). Although the T6SS is also a contact-dependent killing system, the “CDI system” usually refers to the CdiA/CdiB toxin system that directly kills target

bacteria (Ruhe *et al.*, 2013). Contrary to the T6SS and CDI system, secreted antimicrobial compounds such as antibiotics, bacteriocins and antimicrobial peptides, kill bacteria indirectly by diffusing within the extracellular milieu (Sharp *et al.*, 2017). When bacteria interface with these antimicrobials, growth inhibition ensues via several mechanisms, including membrane pore formation, inhibition of cell wall synthesis and inhibition of protein synthesis (Snyder *et al.*, 2013). *Pectobacterium* spp. make use of both contact-dependent and -independent killing systems to free their niche of competitors.

Carbapenem

Carbapenem is an antibiotic produced by a number of bacteria including *Pectobacterium* spp. (Marquez-Villavicencio *et al.*, 2011). Carbapenem is a β-lactam antibiotic that targets the cell wall (Papp-Wallace *et al.*, 2011). Carbapenems have a broad spectrum of activity and are active against both Gram-positive and Gram-negative bacteria. Carbapenem synthesis is directed by a number of genes: *carRABCDEFGH* (McGowan *et al.*, 2005). CarR is a LuxR homologue which acts together with CarI to positively regulate carbapenem synthesis. The biosynthetic pathway is encoded by *carABCDE*. As carbapenem is antibacterial, the producing cell protects itself from self-intoxication through the products of *carFG*. The function of CarH is unknown.

Production of carbapenem in *Pectobacterium* is regulated by quorum sensing molecules N-(3-oxohexanoyl)-L-homoserine (OHHL) and synthesis occurs concomitantly with that of plant cell wall degrading enzymes (Põllumaa *et al.*, 2013). During stationary phase, the levels of OHHL are reduced and carbapenem synthesis is arrested (Durrant, 2016). Temperature and nutrient composition also affect carbapenem production. Carbapenem synthesis is also dependent on the presence of oxygen and is therefore not likely to be produced within plant tissues where oxygen levels are low (Shyntum *et al.*, 2018). Given the broad spectrum of activity, carbapenem production in *Pectobacterium* spp. is expected to target mainly epiphytes (Marquez-Villavicencio *et al.*, 2011).

Bacteriocins

Bacteriocins are antimicrobial peptides ubiquitously produced among bacteria and are generally narrow-spectrum antimicrobials (Riley & Gordon, 1999). They are produced under stress conditions such as nutrient limitation, high cell densities, and cell damage. Antibiotics and bacteriocins are often produced in response to competition-related stresses (Cornforth & Foster, 2013). The mechanism of activity for bacteriocins includes pore formation in membranes, inhibition of enzymes, inhibition of peptidoglycan synthesis, and nuclease activity (Rebuffat, 2016).

Several bacteriocins are produced by *Pectobacterium* spp. such as carotovoricin, phenazine, carocins and pyocin-like bacteriocins (Marquez-Villavicencio *et al.*, 2011). Carotovoricin is a phage tail-like bacteriocin encoded by a lysis cassette as well as a cluster of genes that make up the structural protein (Roh *et al.*, 2010). Expression of carotovoricin is induced by DNA damage, and is also regulated by temperature, with optimal production at 23°C (Nguyen *et al.*, 2002). *Pectobacterium* strains can produce several types of carotovoricin with different strain specificities (Nguyen *et al.*, 2001). In cells that are susceptible, carotovoricin induces a phospholipase A, which is normally situated in the cell membrane, and causes degradation of the cell membrane leading to cell death (Itoh *et al.*, 1981).

Colicins are narrow-spectrum bacteriocins that are produced by *E. coli* and targeted against closely related strains (Cascales *et al.*, 2007). They are modular, with an N-terminal secretion signal, a receptor-binding domain at the centre, and a C-terminal toxin domain. Colicins cause cell death through various mechanisms, which are determined by the C-terminal toxin domain. The toxin domains may cause pore formation, degrade nucleic acid, inhibit cell wall synthesis or hydrolyse existing cell walls in susceptible species (Braun & Patzer, 2013). Colicin-like bacteriocins are found in many other bacteria. Carocin D produced by *Pectobacterium carotovorum* ssp. *carotovorum* is a colicin-like bacteriocin with DNase activity (Roh *et al.*,

2010). Carocin D is a narrow-spectrum antimicrobial used for competition against closely related *Pectobacterium* strains. Carocin S2 shares considerable similarity to carocin D and colicin D from *E. coli* and possesses ribonuclease activity (Chan *et al.*, 2011).

Like colicins, pyocins are bacteriocins produced by *Pseudomonas aeruginosa* for intraspecies competition (Dingemans *et al.*, 2016). Pyocins have the same modular design as colicins: a secretion signal, a receptor-binding domain, and a C-terminal toxin domain. S-type pyocins have DNase, tRNAse or pore-forming activity. Carocin S1 is homologous to pyocin S3 and -AP41; and similarly possesses nuclease activity (Chuang *et al.*, 2007).

Bacteriocins specifically produced by *Pcb* include pectocin M1 and pectocin M2, which are similar to colicin M, which inhibits cell wall synthesis (Grinter *et al.*, 2012). Under iron-limiting conditions pectocin M1 and -M2 display enhanced antibacterial activity. Both pectocin M1 and pectocin M2 inhibit peptidoglycan synthesis by degrading the lipid II precursor (Grinter *et al.*, 2013). *Pcb1692* also produces carotovoricin and an S-type pyocin with tRNase activity (Shyntum *et al.*, 2018).

Other contact-dependent inhibition systems

The archetypal CDI system is comprised of three proteins: CdiA, CdiB, and CdiI. CdiA contains a C-terminal toxin domain, and is transported by the T5SS (Benz & Meinhart, 2014). Unlike T6SS effectors, antibiotics and bacteriocins, CDI toxins are not secreted from the cell into the surrounding space, but protrude from the cell surface (Ruhe *et al.*, 2013). CdiB is located in the outer membrane and forms a β-barrel through which CdiA can pass. Once CdiA has passed through the membrane, it is assembled on the cell surface into an extended filament. Cells are protected from toxicity by neutralising CdiA with the immunity protein, CdiI, which is likely located in the inner membrane (Ruhe *et al.*, 2013).

BamA, which is located in the outer membrane, is a receptor for the CDI system and is present in all Gram-negative bacteria (Ruhe *et al.*, 2013). CdiA from one cell recognises BamA on the target cell and binds to it. Once bound, the C-terminal toxin domain is cleaved and transported into the target cell where the toxin domain can exert its inhibitive function (Hayes *et al.*, 2014). All CDI toxins discovered so far have either nuclease or pore-forming activity. Few CDI toxins have been identified in SREs. In *Pectobacterium* spp. the T5SS is associated with delivery of CDI toxins (Nykyri, 2013). CdiA from *D. dadantii* 3937 has potent DNase activity (Webb *et al.*, 2013).

Rhs proteins function in a contact-dependent manner and many have C-termini with striking similarity to CDI C-termini (Hayes *et al.*, 2014). Likewise, Rhs proteins also have polymorphic C-terminal toxin domains (Poole *et al.*, 2011). Like CDI systems, Rhs toxins are also accompanied by immunity proteins. Rhs proteins are characterised by YD-repeats and the core regions are highly repetitive, which makes them amenable to recombination (Hill *et al.*, 1994). Downstream orphan toxin genes/domains can thus be incorporated with Rhs elements forming functional Rhs toxins (Koskiniemi *et al.*, 2014). The precise function of the Rhs domain still remains unknown (Bernal *et al.*, 2017).

Numerous type 6 effectors are Rhs proteins (Koskiniemi *et al.*, 2013, Alcoforado Diniz & Coulthurst, 2015). The EagR chaperone proteins (DUF1795) are often encoded upstream of Rhs effectors and mediate their interaction with VgrG for export (Alcoforado Diniz *et al.*, 2015). *Dickeya dadantii* exports three type 6 Rhs proteins: RhsA, RhsB, and RhsC (Koskiniemi *et al.*, 2013). Both RhsA and RhsB are endonucleases that degrade genomic and plasmid DNA, with a NS_2 family nuclease domain and HNH endonuclease domain, respectively. The toxin domain of RhsC is unidentified although it effectively inhibits the growth of *E. coli*. The metallopeptidase-4 domain in Rhs-CT1 from *E. coli* disrupts cellular integrity by targeting either the cell wall or cell membrane, although the precise mechanism is not known (Ma *et al.*,

2017). Type 6 effectors with Rhs domains have diverse C-termini and contribute towards bacterial killing in many ways.

The aim of this study was to determine the role of the T6SS of *Pcb1692* in interbacterial competition *in planta* in potato leaves as well as to identify T6SS effectors encoded in the genome of *Pcb1692*. The objectives of the study were to determine whether various attacker-target ratios have an effect on bacterial competition *in planta* and to determine how the T6SS contributes towards bacterial competition against various target species within potato leaves. The next set of objectives was to determine whether *Pcb1692* encodes T6SS effectors and to establish whether any of the identified effectors had antibacterial activity.

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CHAPTER TWO

Investigating the role of the type 6 secretion system in *in planta* competition and identification of type 6 effectors in *Pectobacterium carotovorum* subsp. *brasiliense* 1692

1. Introduction

Survival is arduous. Bacteria must compete with rival species, overwhelm the host to mount an infection, scavenge for limiting nutrients, and replicate to come out on top. Protein secretion systems play an important role in maintaining these interactions (Green & Mecsas, 2016). The type 6 secretion system (T6SS) is a transmembrane, one-step protein secretion system composed of thirteen core structural components, TssA-M, and functions as an inverted phage tail complex to inject effectors into adjacent target bacterial or eukaryotic cells (Coulthurst, 2013, Basler, 2015). The T6SS functions in a contact-dependent manner and is implicated in a number of roles, including meditating interbacterial competition, eukaryotic host interactions, virulence, biofilms, as well as the acquisition of metal ions from the extracellular milieu (Pukatzki *et al.*, 2006, Bernard *et al.*, 2010, Si *et al.*, 2017).

A quarter of Gram-negative species encode type 6 secretion systems, of which a third have multiple clusters in their genomes. A small number of phytobacteria possess more than two clusters (Coulthurst, 2013, Bernal *et al.*, 2018). A single system may be dedicated to targeting both eukaryotic and prokaryotic cells or either of the two (Filloux & Sagfors, 2015). Some species possessing multiple systems dedicate a system towards a particular role, as seen in *Burkholderia thailandensis*, which possesses five distinct T6SSs (Schwarz *et al.*, 2010b). Its T6SS-1 is involved in biofilms, the T6SS-4 is involved in manganese uptake during oxidative stress, and T6SS-5 contributes towards virulence in the eukaryotic host (Schwarz *et al.*, 2010b, Si *et al.*, 2017). Phylogenetics has been used to determine (1) the role of a T6SS, (2) whether it targets either eukaryotes or prokaryotes, and (3) the niche or host with which the bacterium is associated (Bernal *et al.*, 2018). Such endeavours, however, have proven to be erroneous, as a single system may have different targets, and bacteria in the same niche may have systems from different phylogenetic clades.

Genome arrangement, accessory genes, and regulation of T6SS components may differ, but essentially, all type 6 secretion systems have the same components and function similarly (Bernal *et al.*, 2018). Thus, insights into system specialisation can be gleaned from the effector

repertoire delivered, rather than phylogenetic placement of a T6SS. The majority of T6SSs are implicated in interbacterial competition (Russell, 2014, Robb *et al.*, 2016), and as such, much research has gone into the identification of antibacterial effectors. Antibacterial T6SS effectors target conserved structures within the cell, thus, effector-producing cells must encode a cognate immunity protein for protection from self-intoxication (Yang *et al.*, 2018). Thus far, six distinct types of antibacterial effectors have been classified: peptidoglycan hydrolases (English *et al.*, 2012, Benz *et al.*, 2013); amidases (Russell *et al.*, 2011); phospholipases (Russell *et al.*, 2013, Jiang *et al.*, 2014); nucleases (Koskiniemi *et al.*, 2013, Ma *et al.*, 2014); pore-forming effectors (Miyata *et al.*, 2011); and $NAD(P)^+$ glycohydrolases (Whitney *et al.*, 2015, Tang *et al.*, 2018).

Type 6 effectors lack a common secretion signal or motif, which challenges effector identification (Liang *et al.*, 2015). Experimental identification of effectors includes secretome analysis, screening for immunity proteins and computational identification (Russell *et al.*, 2012, Fritsch *et al.*, 2013). Identification is facilitated by searching for genes that consistently neighbour effectors, including DUF4123 and DUF1795/EagR (Alcoforado Diniz & Coulthurst, 2015, Liang *et al.*, 2015, Whitney *et al.*, 2015). Salomon *et al.* (2014) have identified a conserved N-terminal MIX motif in some effectors; however, this motif is not characteristic of all type 6 effectors. Although a number of host-targeting type 6 effectors have been discovered, to date no plant-targeting effectors have been identified (Bernal *et al.*, 2018). Bioinformatic searches using effector architecture can be used to find similar effectors in a large number of genomes (Ma *et al.*, 2017, Ma *et al.*, 2017).

Previous studies indicate that the T6SS of *Pcb1692* is significantly upregulated upon *in planta* infection (Bellieny-Rabelo *et al.*, 2019). It is not known whether this system is involved in eukaryotic or prokaryotic targeting, or whether it contributes towards virulence or another role. Given that the T6SS is mainly associated with antibacterial activity, this study aims to assess the interbacterial competitive capacity of the T6SS of *Pcb1692*. To this end, *in planta* competition assays determined whether the T6SS contributes towards bacterial targeting

within the plant. Thereafter, type 6-dependent effectors were identified bioinformatically and five were selected as putative effectors. Furthermore, to characterise the antibacterial capacity of selected effectors *in vitro*, plasmid pTrc99A was modified for cloning and then antibacterial activity of the selected effectors was assessed *in vitro* using ectopic expression assays in *Escherichia coli*.

2. Materials and Methods

2.1 Maintenance of cultures

All strains were grown at 37°C, unless otherwise stated. *Pectobacterium atrosepticum* was grown at 28°C. Antibiotics (Sigma-Aldrich) were supplemented where necessary at the following concentrations, unless otherwise stated: ampicillin, 100 μg/ml; kanamycin 50 μg/ml; and tetracycline, 10 μg/ml. Cultures were grown in LB broth [4% (w/v) Tryptone, 4% (w/v) yeast extract, 2% (w/v) NaCl]. Strains carrying pCH450 with effector inserts were maintained with the addition of 0.1% (w/v) glucose to liquid LB medium and 2% (w/v) glucose to LB agar plates. Expression of plasmids was induced with 1 mM IPTG and 0.2% (w/v) L-arabinose, for pTrc100 and pCH450, respectively. Strain stocks were maintained at -70 °C in 25% (v/v) glycerol. Refer to Table 1 for a list of strains and plasmids used in this study. The strains selected for competition assays in this study was based on literature specifying that soft rot species can co-inoculate the same plant; soft rot species available from the university culture collection were used.

2.2 *In planta* interbacterial competition

To determine whether the T6SS plays a role in *in planta* infection, the efficiency of killing was compared between *Pcb* wild type and *Pcb*_ΔT6SS strain against various target species. As the T6SS is upregulated *in planta* in potato tuber infections (Bellieny-Rabelo *et al.*, 2019), it became necessary to determine whether conditions in the host plant are necessary for T6SS activity in *Pcb1692*. Shyntum *et al.* (2018) determined the role of *Pcb1692* T6SS in bacterial competition in potato tubers, whereas this study assessed the effect of the *in planta* environment of potato leaves on T6SS-dependent bacterial competition in *Pcb1692*. Six- week old potato leaves (cv. Mondial) were infiltrated with bacterial cultures. *Pcb* wild type and *Pcb*_ΔT6SS were transformed with empty pET26 to confer kanamycin resistance. *Dickeya dadantii* 3937, *Dickeya chrysanthemi*, *Pectobacterium carotovorum* subsp. *carotovorum*, and *Pectobacterium atrosepticum* were transformed with empty pTrc100 to confer ampicillin resistance. Overnight cultures were adjusted to an OD_{600} of 1.0 and *Pcb* wild type was mixed

with each strain, respectively, in a 1:1 ratio and 10:1 ratio. The same was done for *Pcb*_ΔT6SS. As potato leaves are very difficult to infiltrate, a precise volume could not be administered into the leaf. Therefore, the entire leaf was pricked several times and infiltrated using a needleless syringe. Leaves were picked one day after infection, ground, and resuspended in 1 ml of 10 mM $MgSO_4$. Serial dilutions were performed to calculate the CFU/ml. These values were standardised to CFU/g tissue. Infections were performed in triplicate. Statistical analyses were performed using one-way ANOVA with $\alpha = 0.05$. Statistical analysis was significant if the p-value was smaller than 0.05 ($p < 0.05$) and insignificant when the p-value was larger than 0.05 ($p > 0.05$). Tukey's HSD post-hoc test was used to determine which comparisons were significant.

Nomenclature used for competition assays is as follows: assays are named according to the two competing strains used. The strain selected for on selective media is named first followed by the co-inoculated strain in parentheses. For example, *Pa* (WT) indicates that *Pectobacterium atrosepticum* was competed with *Pcb1692* wild type and that the survival of *Pectobacterium atrosepticum* was determined in this case. The converse, WT (*Pa*), refers to the same assay, however, in this case the survival of *Pcb1692* was determined by plating on different selective media. The inclusion of "10" indicates that a 10:1 *Pcb1692*-to-target ratio was used for that assay [E.g. *Pa* 10 (WT)]. Similarly, *Pcb1692*_ΔT6SS (abbreviated T6) competed against *Pa*, was denoted either as *Pa* (T6) or *Pa* 10 (T6) (1:1 ratio and 10:1 ratio, respectively).

2.3 Effector identification

Contextual analysis of type 6 secretion system gene neighbourhoods was performed using PATRIC and the ASAP databases to locate T6SS genes and associated effectors in *Pcb1692* (https://www.patricbrc.org/ and https://asap.genetics.wisc.edu/asap/home.php). Using the known sequence of a VgrG gene from *Dickeya* dadantii 3937 (YP_003883690.1), BLASTp was used to search the genome of *Pcb1692* for similar homologues. Using the identified VgrG genes in *Pcb*, the neighbourhoods were analysed for type-6 associated genes. Protein

sequences of the identified genes were submitted to the InterPro Scan database to functionally classify genes using conserved domains and motifs present in the genes. Genes that were located downstream of Hcp and VgrG genes were considered potential type 6 effectors.

2.4 DNA extraction and purification

Genomic DNA (gDNA) was extracted from overnight cultures using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, Massachusetts, USA). Cells were harvested by centrifugation of 1 ml of overnight culture for 10 min at 5000 rpm. The pellet was resuspended in 180 μl digestion solution, 20 μl Proteinase K and incubated at 56°C for 30 min. A volume of 20 μl of RNase A was added to the solution and incubated at room temperature for 10 min. After the addition of 200 μl of lysis solution the mixture was thoroughly vortexed followed by the addition of 400 μl of 50% (v/v) ethanol. The mixture was transferred to purification column and centrifuged at 6000 rpm for 1 min. The column was washed with 500 μl wash buffer I and centrifuged at 8000 rpm for 1 min, followed by the addition of 500 μl wash buffer II and centrifugation at maximum speed for 3 minutes. DNA was eluted by the addition of 200 μl of ddH_2O, incubation at room temperature for 2 min and centrifuged at 8000 rpm for 1 min. Purified genomic DNA was stored at -20°.

Plasmid DNA was extracted from overnight cultures using GeneJET Plasmid Miniprep Kit (Thermo Scientific). Cells were harvested by centrifuging 5 – 10 ml overnight cultures at 8000 rpm. All subsequent centrifugation steps were performed at 14500 rpm. Cells were resuspended in 250 μl resuspension solution followed by the addition of 250 μl lysis solution. A volume of 350 μl of neutralisation solution was added. If the resulting precipitate was very viscous (which would yield very low DNA concentrations) an additional 100 μl of lysis solution was added followed by 140 μl of neutralisation solution. The resulting solution was centrifuged for 5 min. The supernatant was transferred to a GeneJET spin column and centrifuged for 1 min. The column was washed with 800 μl wash solution and centrifuged for 1 min. Plasmid DNA was eluted in 40 μl of ddH_2O and stored at -20°C. For large scale plasmid purification,

cells were harvested from 30 – 40 ml of overnight culture as above. All steps remained the same, except that 2 ml of lysis buffer and 2.8 ml neutralization buffer were used.

DNA in agarose gel was excised from the gel and purified using GeneJET Gel Extraction Kit (Thermo Scientific), according to manufacturer instructions. All centrifugation steps were performed at maximum speed. Gel slices were melted at 60°C in 300 μl of binding buffer and the melted solution was transferred to a purification column and centrifuged for 1 min. The column was washed with 800 μl wash buffer and the DNA was eluted in 30 μl ddH_2O and stored at -20°C.

2.5 Preparation of electrocompetent cells

Overnight culture of *E. coli* DH5-alpha was added to 100 ml LB broth to a final OD_{600} = 0.01 without antibiotics and grown at 37°C to an OD_{600} between 0.4 to 0.6, measured with a Multiskan Go (Thermo Scientific™) spectrophotometer. The culture was transferred to two 50 ml Greiner tubes and centrifuged at 6000 rpm for 6 min at 4°C. The pellet was washed twice with 40 ml and 20 ml ice-cold ddH_2O, respectively. The pellets were pooled and subsequently washed with 8 ml of 10% (v/v) glycerol. The pellet was resuspended in 1.5 ml 10% (v/v) glycerol, and aliquots of 100 μl were frozen in liquid nitrogen and stored at -70°C. Plasmids were transformed into electrocompetent cells. Electroporation was performed using a MicroPulser electroporator (Bio-Rad) at 1.8 kV and 4.5 ms. Cells were recovered in LB broth for 1 hr then plated onto LB agar plates supplemented with the appropriate antibiotics and incubated overnight.

2.6 Preparation of chemically competent cells

Chemically competent cells were prepared using the calcium chloride method. Overnight culture (of all soft rot species) was added to 100 ml LB broth to a final OD_{600} = 0.01 without antibiotics and grown to an OD_{600} between 0.4 to 0.6. Cells were pelleted by centrifugation at 6000 rpm for 6 min at 4°C, and resuspended in half the volume of ice-cold, filter sterilised calcium chloride (50 mM). The suspension was centrifuged again at 6000 rpm for 6 min at 4°C. The pellet was resuspended in a mixture of 750 μl of calcium chloride (50 mM) and 750

μl of 50 % (v/v) glycerol. Cells were snap frozen in liquid nitrogen and stored at -70°C. To transform chemically competent cells, 100 μl aliquots were mixed with the DNA to be transformed, incubated on ice 30 min and subjected to heat shock at 42°C for 2 min. After heat shock, the cells were chilled on ice for 2 min followed by the addition of 600 μl of LB broth. Cells were recovered for 1 hr at 37°C with shaking and plated onto LB agar plates containing the appropriate antibiotics.

2.7 Restriction enzyme digestion

Thermo Scientific FastDigest enzymes were used for all restriction digests. For screening, approximately 500-1000 ng of plasmid DNA was used. For linearization of vectors and recovery of inserts for ligation reactions, approximately 3 μg of plasmid DNA was used. For screening reactions, plasmid DNA (~500 ng), 2 μl 10X FastDigest Buffer, 10 units of each appropriate enzyme, and nuclease-free water was added to a final volume of 20 μl. Restriction enzymes were heat inactivated at 80°C for 10 min. Reactions were electrophoresed on 1% (w/v) agarose gels and the appropriate band excised and purified. For preparation of ligation inserts, 3 μg plasmid DNA, 3 μl 10X FastDigest Buffer, 15 units of each appropriate enzyme, and nuclease-free water was added to a final volume of 30 μl. The reactions were heat inactivated at 80°C for 10 min and electrophoresed on 1% (w/v) agarose gels and the appropriate band excised and purified.

2.8 Polymerase chain reaction

2.8.1 Primer design

Primers were designed using CLC Main Workbench version 6.1 and genome sequences were obtained from the PATRIC database (https://www.patricbrc.org/) as well as the ASAP database (https://asap.genetics.wisc.edu/asap/home.php). Restriction cut sites were incorporated into the 5' end of the gene sequences to facilitate cloning into a suitable plasmid. Refer to Table 2 for the list of primers.

2.8.2 PCR amplification and colony PCR

All PCRs were carried out with Phusion High-Fidelity PCR Master Mix (Thermo Scientific). Each reaction was set up with 2X master mix, 0.5 μM each of the forward and reverse primers, ~37 ng template DNA, and ddH_2O to the final volume. The PCR was performed in a T100 Bio-Rad thermal cycler for 35 cycles, with the initial denaturation at 98°C for 30 s, denaturation at 98°C for 10 s, annealing at T_m + 3°C of the lower T_m primer for primers >20 nucleotides and at T_m of the lower T_m primer for primers ≤20 nucleotides for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. Samples were electrophoresed in 1% (w/v) agarose gel in 1X TAE buffer (40 mM Tris, 20 mM NaOAc; 1 mM EDTA; pH 8.5) for 45 min at 90 V and subsequently excised and purified. Colony PCR was performed as above, but the PCR was downscaled to a final volume of 10 μl as follows: 5 μl of 2X master mix, 10 μM each of the forward and reverse primers, 1 μl culture, and 3.4 μl ddH_2O. Cycling conditions and electrophoresis were performed as above, except that the initial denaturation was increased from 30 s to 3 min.

2.9 Nucleotide sequencing

To confirm the integrity of the DNA constructs, the nucleotide sequences of the construct was determined using the ABI-PRISM BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. Each reaction contained 100 ng of purified DNA per kb of plasmid to be sequenced, 3.2 pmol of sequencing primer per reaction, 1 μl of BigDye Ready Reaction mix, 1 μl of 5x BigDye Sequencing buffer, and ddH_2O to a final volume of 10 μl. The cycle sequencing reactions were performed in a T100 thermal cycler. Following initial denaturation at 94°C for 1 min, the reaction mixtures were subjected to 25 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 15 s, and extension at 60°C for 4 min. The products were mixed with 60 μl of 100% (v/v) ethanol, 11 μl ddH_2O and 2 μl of NaOAc (pH 4.3, 3 M), and incubated at -80°C for 15 min. Following centrifugation at 14 500 rpm for 30 min, the supernatant was discarded, and the pellets rinsed

twice with ice-cold 70% (v/v) ethanol. The samples were dried for 2 min at 60°C and submitted to the University of Pretoria's Sequencing Facility for sequencing.

2.10 Ligation reactions

Ligation was performed using T4 DNA ligase (Thermo Scientific). Blunt-end cloning was performed by adding 50 ng/μl linear vector DNA, insert DNA at a 5:1 molar ratio to the vector, 5 units of T4 DNA ligase, 1 μl PEG 4000 solution, and nuclease-free water to a final volume of 20 μl. The reaction mixture was incubated at 22°C for 1 hr and heat inactivated at 75°C for 5 min. Sticky-end ligations were performed as above, except insert DNA was added at a 3:1 insert-to-vector molar ratio, and PEG 4000 was omitted. Ligation reactions were transformed into chemically competent cells PCR products were first sub-cloned into the positive selection pJET 1.2/Blunt cloning vector (Thermo Scientific), using blunt end ligation as above and transformed into chemically competent *E. coli* DH5-alpha.

2.11 Construction of pTrc100

The expression vector pTrc99A was obtained from the FABI culture collection repository for the cloning of effector and immunity genes. Towards this end, it was transformed into *E. coli* DH5-alpha, and extracted using the alkaline lysis plasmid extraction method. To verify that the multiple cloning site (MCS) was still the same as the published plasmid map, a series of restriction digests with NcoI, EcoRI, SacI, SmaI, SalI, XbaI, KnpI and BamHI were performed. Once the remaining restriction sites were confirmed, the plasmid was modified to facilitate cloning into this plasmid by reintroducing KnpI and BamHI cut sites into the plasmid by their incorporation into the 5' region of the PCR primers (H5907_F/R) of an arbitrarily chosen gene, *AED-0003810*. PCR amplification of *AED-0003810* incorporated XbaI, KpnI, and BamHI upstream of the ORF and BamHI and SalI downstream of the ORF. The PCR product was subcloned into pJET1.2/blunt and excised with XbaI/SalI restriction digest. Plasmid Trc99A was digested with XbaI/SalI and ligated with the excised PCR product. The gene was removed using BamHI restriction digest and the linear plasmid purified from the gel and religated. In this manner BamHI and KnpI were reintroduced into the MCS. The resulting

plasmid is referred to as pTrc100 as its MCS differs slightly from parental pTrc99A, but the rest of the plasmid remains unchanged. This plasmid was used for the ligation of immunity genes.

2.12 Construction of pCH450 derivates and pTrc100 derivatives

Plasmids pCH450 and pCH450::RhsA$^{(D.\ dadantii)}$ were generously provided by Prof. C Hayes, University of California, Santa Barbara. The plasmids were transformed into *E. coli* DH5-alpha. Plasmid pCH450 was extracted for further cloning experiments. The putative effector genes identified were PCR amplified, purified, and subcloned into pJET 1.2/Blunt cloning vector and excised for ligation into arabinose-inducible expression plasmid pCH450 cut with suitable enzymes to allow for ligation. The resulting ligation reactions were transformed into *E. coli* DH5-alpha, colonies were selected, and screened with plasmid extractions and restriction digests. To assess the activity of cognate immunity genes against selected effectors, cognate immunity genes were PCR amplified as above and ligated into pTrc100 then transformed into *E. coli* DH5-alpha already carrying the respective pCH450::effector plasmid.

The phospholipase (Phos) is expected to be active only within the periplasm, thus, cytoplasmic expression will not cause any inhibition of bacterial growth when expressed in *E. coli*. To overcome this, Phos needed to be fused to a secretion signal. To achieve this, Phos was excised from pJET 1.2/Blunt with SacI and SalI restriction enzymes. The resulting gene fragment was then ligated into expression vector pET-26b+ which contains the PelB secretion signal prior to the MCS. Insertion of Phos into the MCS places the PelB secretion signal in front of this gene. The ORF of Phos together with the N-terminal secretion signal was then excised from pET-26b+ using NdeI and XhoI liberating a 1.5 kb fragment. This fragment was then ligated into the MCS of pCH450 that was cut with NdeI and XhoI to produce compatible ends for ligation. The resulting plasmid was transformed into *E. coli* DH5-alpha cells. To confirm the insertion of the PelB-signal sequence-flanked gene, the resulting plasmid

pCH50::Phos was extracted from the transformed cells and digested with NcoI, which cuts within the phospholipase gene, within pCH450 and also 5' of the PelB sequence.

2.13 Ectopic expression of effectors

Oxygen-limited overnight cultures were prepared by incubating test tubes filled to the brim with LB broth inoculated with *E. coli* pCH450::effector and supplemented with 10 µg/ml tetracycline and 0.1% (w/v) glucose to repress expression from the arabinose-inducible promoter. Cultures were washed once in 10 mM $MgSO_4$, adjusted to an OD_{600} of 0.05 in fresh LB broth supplemented with 5 µg/ml tetracycline, 50 µg/ml ampicillin, and 1 mM IPTG, as required. After 30 minutes, expression of the effector in pCH450 was induced with 0.2% (w/v) L-arabinose. For dual plasmid expression, effector expression from pCH450 was induced as above and expression of immunity genes from pTrc100 (overnight cultures grown as above) was induced with 1 mM of IPTG immediately. Optical density (600 nm) was measured hourly.

3. Results

3.1 *In planta* interbacterial competition

Currently, no data exist for the contribution of the T6SS in *Pcb1692* for *in planta* interbacterial competition. There is no set standard in the literature for the attacker-to-target ratio used for competition assays; these ratios typically vary from 1:1 to 10:1. Therefore, it was first necessary to establish whether different ratios significantly affect competition between two strains. For this study, a 1:1 and 10:1 attacker-to-target ratio was assessed. In all cases, the controls (*Pcb1692, Pcb1692*_ΔT6SS, *Pa*, *Dc*, *Dd*, and *Pcc*) which were infiltrated into the leaves individually, grew well, with cell titres well above 10^7 CFU/g tissue (Figure 2.1). Co-infiltration of target strains with *Pcb* at a 1:1 ratio was expected to reduce the cell titres of target strains due to competition. Indeed, this was observed in all cases (Figure 2.1 A-D). The reduction in target survival was significant compared to the respective control in all cases, except when *Pa* was co-inoculated with *Pcb1692* (Figure 2.1 A). Next, competing bacterial strains at 10:1 attacker-to-target ratios was expected to significantly reduce target survival compared to the 1:1 assays. Interestingly, although survival in the 10:1 assays was lower than the 1:1 assays [with the exception of *Dickeya dadantii* (Figure 2.1C)], the difference in survival of target species was not statistically significant in all instances at *p* value of 0.05 (Figure 2.1 A-D). In other words, the attacker concentration does not significantly play a role in competition assays *in planta* in one day post infection trials for the strains used in this study.

As the attacker-to-target ratio did not significantly influence the outcome of competition, a 1:1 attacker-to-target ratio was subsequently used to determine whether the T6SS of *Pcb1692* contributes towards *in planta* interbacterial competition. To this end, six-week-old susceptible potato leaves (cv. Mondial) were used for competition assays. In these bacterial competition assays, various SRE species were co-inoculated with either wild type *Pcb1692* or *Pcb1692*_ΔT6SS. As both the attacker (*Pcb1692* or *Pcb1692*_ΔT6SS) and target species (*Pa, Pcc, Dd, Dc*) have competitive abilities, either of the two strains could be out-competed during these assays, thus, cell survival of both the target and attacker strains was enumerated

after one day post infection to assess the degree to which each strain was affected during co-inoculations.

Attacker strains [*Pcb1692* (WT) or *Pcb1692*_ΔT6SS (T6)] and target strains (*Pa, Pcc, Dd, Dc*) were infiltrated into the leaves individually as negative controls. One day post infection, both attacker and target strains were enumerated to determine the extent of competition against each other (Figure 2.2). Cell titres of all the controls were expected, and subsequently observed, to be higher than co-inoculations, as no competitors were present to impede their growth. When inoculated individually (controls) or co-inoculated, *Pcb1692* (*Pcb* WT) and *Pcb1692*_ΔT6SS (T6) grew well with cell titres that exceeded 10^8 CFU/g tissue (Figure 2.2 A–D). However, co-inoculation of various SRE target species with either *Pcb1692* or *Pcb1692*_ΔT6SS showed a marked decrease in all target species titres compared to their respective controls. Selection for *Pcb1692* or *Pcb1692*_ΔT6SS attacker strains from co-inoculations shows that their survival does not differ significantly to their respective controls, indicating that the attacker species are outcompeting the target species in all instances, and that the target species do not significantly affect attacker titres. The “attacker” and “target” nomenclature are arbitrary, as either species can potentially compete with each other to a certain degree; however, for this study *Pcb1692* or *Pcb1692*_ΔT6SS were designated as the attacker species and *Pa, Pcc, Dd, Dc* as the target species. As the outcome of competition is unknown (i.e. it is possible that one of the target strains could outcompete one of the attacker strains), cell titres for both the attacker and target strains from co-inoculation assays were determined. Thus, as the attacker strain titres remained constant in both the controls and co-inoculations, the reduction in cell titre of target species is due to competitive interactions imposed by *Pcb1692* (wild type and mutant) during co-inoculations.

As the efficiency of a killing system is assayed, titres of target strains are expected to be higher when competed with the *Pcb1692*_ΔT6SS (as a killing system has been disabled) than when competed with wild type *Pcb1692*. Assays with *P. atrosepticum* show that competition against

Pcb1692 reduced *Pa* titres, however, not significantly compared to the *Pa* control; whereas competition against *Pcb1692*_ΔT6SS significantly reduced *Pa* titres (Figure 2.2 A). The opposite was expected, however, to determine whether this difference is significant in terms of T6SS-mediated competition, *Pa* titres from co-inoculations with *Pcb1692* or *Pcb1692*_ΔT6SS were compared to each other. Results indicate that there is no significant difference in *Pa* survival when competed with either of the *Pcb1692* strains. Thus, the T6SS does not contribute significantly towards bacterial growth inhibition.

Competition of *Dickeya chrysanthemi* against both strains of *Pcb1692* saw a significant decrease in target titres compared to the control (Figure 2.2 B); however, the difference in survival of *Dc* from co-inoculations with *Pcb1692* or *Pcb1692*_ΔT6SS was not significant, indicating that the T6SS does not contribute significantly towards bacterial growth inhibition. The same results were obtained for competition of *Dickeya dadantii* against *Pcb1692* and *Pcb1692*_ΔT6SS (Figure 2.2 C). Assays with *P. carotovorum* show that competition against *Pcb1692* wild type and mutant reduced *Pcc* titres; however, the reduction was only significant when *Pcc* was competed with *Pcb1692* wild type (Figure 2.2 D). There was no significant difference in survival when *Pcc* was competed with *Pcb1692* or *Pcb1692*_ΔT6SS, once again indicating that the T6SS does not significantly contribute towards bacterial growth inhibition.

Statistical analyses consistently indicate that there is no significant difference between competition of a target species with *Pcb1692* compared to *Pcb1692*_ΔT6SS. In other words, the reduction in titres observed from co-inoculations is not due to the T6SS. As both wild type and mutant *Pcb1692* titres remain relatively consistent throughout the co-inoculation experiments while target titres decrease, the results seem to indicate that *Pcb1692* may be interacting competitively with these target species, albeit using competitive strategies other than the T6SS in potato leaves.

Previous studies show that the T6SS is up-regulated *in planta* (Bellieny-Rabelo *et al.*, 2019), however, results from the current study indicate that bacterial killing is not the main role of the

T6SS for *Pcb1692* in plant leaves. The *Pcb1692* genome encodes several other antimicrobial compounds such as carbapenem, pyocin, and T5SS, which were upregulated during host infection in potato tubers (Shyntum *et al.*, 2018, Bellieny-Rabelo *et al.*, 2019). Since no role was determined for the T6SS effectors thus far, it was hypothesised that these other antimicrobial systems might be masking the effect of T6SS effectors. Thus, a different strategy that required expression of individual effector in *Escherichia coli* was undertaken. To achieve this, an *in silico* approach was adopted to determine the effector repertoire encoded by *Pcb1692*, and subsequently to determine whether any of the effectors are antibacterial effectors.

3.2 Identification of Hcp-secretion islands and T6SS effectors

To identify T6SS-dependent effectors within *Pcb1692*, an *in silico* bioinformatic approach was used. Genome architecture provides insight into the function and association of neighbouring genes: Genes adjacent to each other are often involved in the same process or combine with each other to form functional units (Huynen *et al.*, 2000). Genomic context and established protein-protein interactions were used to identify putative T6SS-effectors in *Pcb1692*. The genome was searched for both haemolysin-coregulated protein (Hcp) and valine-glycine repeat protein (VgrG) genes. *Pcb* encodes five Hcp genes (*AED_0004534; -4521; -6274; -4513; -3112*) and three VgrG genes (*AED_0002727; -3069; -3812*) (Figure 2.3). Using Hcp-VgrG gene islands as markers, downstream genes were evaluated for putative T6SS-dependent effectors and associated immunity genes.

The first region containing Hcp-VgrG genes [Hcp-secretion island (HSI-1)] is adjacent to the T6SS core cluster (Figure 2.3 A). The first gene downstream of VgrG (*AED_0003357*) contains an N-terminal PAAR domain, which marks it as a potential T6SS-dependent effector. Protein sequence analysis revealed an alpha-beta hydrolase fold in addition to the PAAR domain. Similarly, *Pectobacterium atrosepticum* strain SCRI1043 T6SS phospholipase, *ECA3426* (http://202.120.12.133/**SecReT6**/component.php?pid=50122350), possesses an N-terminal PAAR domain and alpha/beta hydrolase fold. Alignment of AED_0003357 and ECA3426

protein sequences show 90% similarity, with both proteins conserving the GXSXG motif, which is characteristic of lipases (Figure 2.4). In both *Pcb* and *P. atrosepticum*, the downstream gene is predicted to be the immunity genes (*AED_0003357* & *ECA3425*, respectively), both containing an ankyrin domain with four repeats.

The other two Hcp-secretion islands with an Hcp-VgrG pair occupy distinct localities in the genome separate from the core cluster. In HSI-2 (Figure 2.3 B), the first gene downstream of the Hcp-VgrG pair (*AED_0003811*) bears a DUF4123 domain that is associated with type 6 effector chaperones, further indicating that the genes in this region are associated with the T6SS. The next gene annotation is a hypothetical gene (*AED_0003810*), with no recognisable motifs or domains. This is followed by a putative effector-immunity pair; the effector (*AED_0003809*) contains an HNH/Endonuclease VII fold with a WHH nuclease motif and the downstream immunity gene (*AED_0003808*) belongs to the SUKH superfamily with a Smi1/Knr4 domain. The last putative effector in this cluster belongs to the cell division cycle protein 123 family (D123 domain) and is succeeded by a PAAR gene; thus, no immunity gene is associated with the D123 gene.

HSI-3 consists of seven genes; however, this island lacks a VgrG gene (Figure 2.3 C). All genes within this locus have no known function associated with the T6SS. Moreover, they do not resemble effector-immunity pairs, and are therefore most likely not antibacterial effectors. Likewise, HSI-5 lacks a VgrG gene, and Hcp is followed by two very small hypothetical genes with no known function (Figure 2.3 E).

HSI-4 encodes seven genes in this locus (Figure 2.3 D). The first gene downstream of VgrG (*AED_0003068*) contains a DcrB domain and is followed by two putative effector-immunity pairs. The first effector (*AED_0006270*) contains a PAAR domain, YD-repeats and a C-terminal toxin domain clearly defined by the PxxxxDPxGL motif present in Rhs genes. As with all Rhs genes, the downstream gene is the associated immunity gene (*AED_0004232*), however, no specific domains were detected in this gene. The next effector (*AED_0004231*)

CHAPTER TWO

Investigating the role of the type 6 secretion system in *in planta* competition and identification of type 6 effectors in *Pectobacterium carotovorum* subsp. *brasiliense* 1692

1. Introduction

Survival is arduous. Bacteria must compete with rival species, overwhelm the host to mount an infection, scavenge for limiting nutrients, and replicate to come out on top. Protein secretion systems play an important role in maintaining these interactions (Green & Mecsas, 2016). The type 6 secretion system (T6SS) is a transmembrane, one-step protein secretion system composed of thirteen core structural components, TssA-M, and functions as an inverted phage tail complex to inject effectors into adjacent target bacterial or eukaryotic cells (Coulthurst, 2013, Basler, 2015). The T6SS functions in a contact-dependent manner and is implicated in a number of roles, including meditating interbacterial competition, eukaryotic host interactions, virulence, biofilms, as well as the acquisition of metal ions from the extracellular milieu (Pukatzki *et al.*, 2006, Bernard *et al.*, 2010, Si *et al.*, 2017).

A quarter of Gram-negative species encode type 6 secretion systems, of which a third have multiple clusters in their genomes. A small number of phytobacteria possess more than two clusters (Coulthurst, 2013, Bernal *et al.*, 2018). A single system may be dedicated to targeting both eukaryotic and prokaryotic cells or either of the two (Filloux & Sagfors, 2015). Some species possessing multiple systems dedicate a system towards a particular role, as seen in *Burkholderia thailandensis*, which possesses five distinct T6SSs (Schwarz *et al.*, 2010b). Its T6SS-1 is involved in biofilms, the T6SS-4 is involved in manganese uptake during oxidative stress, and T6SS-5 contributes towards virulence in the eukaryotic host (Schwarz *et al.*, 2010b, Si *et al.*, 2017). Phylogenetics has been used to determine (1) the role of a T6SS, (2) whether it targets either eukaryotes or prokaryotes, and (3) the niche or host with which the bacterium is associated (Bernal *et al.*, 2018). Such endeavours, however, have proven to be erroneous, as a single system may have different targets, and bacteria in the same niche may have systems from different phylogenetic clades.

Genome arrangement, accessory genes, and regulation of T6SS components may differ, but essentially, all type 6 secretion systems have the same components and function similarly (Bernal *et al.*, 2018). Thus, insights into system specialisation can be gleaned from the effector

repertoire delivered, rather than phylogenetic placement of a T6SS. The majority of T6SSs are implicated in interbacterial competition (Russell, 2014, Robb *et al.*, 2016), and as such, much research has gone into the identification of antibacterial effectors. Antibacterial T6SS effectors target conserved structures within the cell, thus, effector-producing cells must encode a cognate immunity protein for protection from self-intoxication (Yang *et al.*, 2018). Thus far, six distinct types of antibacterial effectors have been classified: peptidoglycan hydrolases (English *et al.*, 2012, Benz *et al.*, 2013); amidases (Russell *et al.*, 2011); phospholipases (Russell *et al.*, 2013, Jiang *et al.*, 2014); nucleases (Koskiniemi *et al.*, 2013, Ma *et al.*, 2014); pore-forming effectors (Miyata *et al.*, 2011); and $NAD(P)^+$ glycohydrolases (Whitney *et al.*, 2015, Tang *et al.*, 2018).

Type 6 effectors lack a common secretion signal or motif, which challenges effector identification (Liang *et al.*, 2015). Experimental identification of effectors includes secretome analysis, screening for immunity proteins and computational identification (Russell *et al.*, 2012, Fritsch *et al.*, 2013). Identification is facilitated by searching for genes that consistently neighbour effectors, including DUF4123 and DUF1795/EagR (Alcoforado Diniz & Coulthurst, 2015, Liang *et al.*, 2015, Whitney *et al.*, 2015). Salomon *et al.* (2014) have identified a conserved N-terminal MIX motif in some effectors; however, this motif is not characteristic of all type 6 effectors. Although a number of host-targeting type 6 effectors have been discovered, to date no plant-targeting effectors have been identified (Bernal *et al.*, 2018). Bioinformatic searches using effector architecture can be used to find similar effectors in a large number of genomes (Ma *et al.*, 2017, Ma *et al.*, 2017).

Previous studies indicate that the T6SS of *Pcb1692* is significantly upregulated upon *in planta* infection (Bellieny-Rabelo *et al.*, 2019). It is not known whether this system is involved in eukaryotic or prokaryotic targeting, or whether it contributes towards virulence or another role. Given that the T6SS is mainly associated with antibacterial activity, this study aims to assess the interbacterial competitive capacity of the T6SS of *Pcb1692*. To this end, *in planta* competition assays determined whether the T6SS contributes towards bacterial targeting

within the plant. Thereafter, type 6-dependent effectors were identified bioinformatically and five were selected as putative effectors. Furthermore, to characterise the antibacterial capacity of selected effectors *in vitro*, plasmid pTrc99A was modified for cloning and then antibacterial activity of the selected effectors was assessed *in vitro* using ectopic expression assays in *Escherichia coli*.

2. Materials and Methods

2.1 Maintenance of cultures

All strains were grown at 37°C, unless otherwise stated. *Pectobacterium atrosepticum* was grown at 28°C. Antibiotics (Sigma-Aldrich) were supplemented where necessary at the following concentrations, unless otherwise stated: ampicillin, 100 μg/ml; kanamycin 50 μg/ml; and tetracycline, 10 μg/ml. Cultures were grown in LB broth [4% (w/v) Tryptone, 4% (w/v) yeast extract, 2% (w/v) NaCl]. Strains carrying pCH450 with effector inserts were maintained with the addition of 0.1% (w/v) glucose to liquid LB medium and 2% (w/v) glucose to LB agar plates. Expression of plasmids was induced with 1 mM IPTG and 0.2% (w/v) L-arabinose, for pTrc100 and pCH450, respectively. Strain stocks were maintained at -70 °C in 25% (v/v) glycerol. Refer to Table 1 for a list of strains and plasmids used in this study. The strains selected for competition assays in this study was based on literature specifying that soft rot species can co-inoculate the same plant; soft rot species available from the university culture collection were used.

2.2 *In planta* interbacterial competition

To determine whether the T6SS plays a role in *in planta* infection, the efficiency of killing was compared between *Pcb* wild type and *Pcb*_ΔT6SS strain against various target species. As the T6SS is upregulated *in planta* in potato tuber infections (Bellieny-Rabelo *et al.*, 2019), it became necessary to determine whether conditions in the host plant are necessary for T6SS activity in *Pcb1692*. Shyntum *et al.* (2018) determined the role of *Pcb1692* T6SS in bacterial competition in potato tubers, whereas this study assessed the effect of the *in planta* environment of potato leaves on T6SS-dependent bacterial competition in *Pcb1692*. Six- week old potato leaves (cv. Mondial) were infiltrated with bacterial cultures. *Pcb* wild type and *Pcb*_ΔT6SS were transformed with empty pET26 to confer kanamycin resistance. *Dickeya dadantii* 3937, *Dickeya chrysanthemi*, *Pectobacterium carotovorum* subsp. *carotovorum*, and *Pectobacterium atrosepticum* were transformed with empty pTrc100 to confer ampicillin resistance. Overnight cultures were adjusted to an OD_{600} of 1.0 and *Pcb* wild type was mixed

with each strain, respectively, in a 1:1 ratio and 10:1 ratio. The same was done for *Pcb*_ΔT6SS. As potato leaves are very difficult to infiltrate, a precise volume could not be administered into the leaf. Therefore, the entire leaf was pricked several times and infiltrated using a needleless syringe. Leaves were picked one day after infection, ground, and resuspended in 1 ml of 10 mM $MgSO_4$. Serial dilutions were performed to calculate the CFU/ml. These values were standardised to CFU/g tissue. Infections were performed in triplicate. Statistical analyses were performed using one-way ANOVA with $\alpha = 0.05$. Statistical analysis was significant if the p-value was smaller than 0.05 ($p < 0.05$) and insignificant when the p-value was larger than 0.05 ($p > 0.05$). Tukey's HSD post-hoc test was used to determine which comparisons were significant.

Nomenclature used for competition assays is as follows: assays are named according to the two competing strains used. The strain selected for on selective media is named first followed by the co-inoculated strain in parentheses. For example, *Pa* (WT) indicates that *Pectobacterium atrosepticum* was competed with *Pcb1692* wild type and that the survival of *Pectobacterium atrosepticum* was determined in this case. The converse, WT (*Pa*), refers to the same assay, however, in this case the survival of *Pcb1692* was determined by plating on different selective media. The inclusion of "10" indicates that a 10:1 *Pcb1692*-to-target ratio was used for that assay [E.g. *Pa* 10 (WT)]. Similarly, *Pcb1692*_ΔT6SS (abbreviated T6) competed against *Pa*, was denoted either as *Pa* (T6) or *Pa* 10 (T6) (1:1 ratio and 10:1 ratio, respectively).

2.3 Effector identification

Contextual analysis of type 6 secretion system gene neighbourhoods was performed using PATRIC and the ASAP databases to locate T6SS genes and associated effectors in *Pcb1692* (https://www.patricbrc.org/ and https://asap.genetics.wisc.edu/asap/home.php). Using the known sequence of a VgrG gene from *Dickeya* dadantii 3937 (YP_003883690.1), BLASTp was used to search the genome of *Pcb1692* for similar homologues. Using the identified VgrG genes in *Pcb*, the neighbourhoods were analysed for type-6 associated genes. Protein

sequences of the identified genes were submitted to the InterPro Scan database to functionally classify genes using conserved domains and motifs present in the genes. Genes that were located downstream of Hcp and VgrG genes were considered potential type 6 effectors.

2.4 DNA extraction and purification

Genomic DNA (gDNA) was extracted from overnight cultures using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, Massachusetts, USA). Cells were harvested by centrifugation of 1 ml of overnight culture for 10 min at 5000 rpm. The pellet was resuspended in 180 μl digestion solution, 20 μl Proteinase K and incubated at 56°C for 30 min. A volume of 20 μl of RNase A was added to the solution and incubated at room temperature for 10 min. After the addition of 200 μl of lysis solution the mixture was thoroughly vortexed followed by the addition of 400 μl of 50% (v/v) ethanol. The mixture was transferred to purification column and centrifuged at 6000 rpm for 1 min. The column was washed with 500 μl wash buffer I and centrifuged at 8000 rpm for 1 min, followed by the addition of 500 μl wash buffer II and centrifugation at maximum speed for 3 minutes. DNA was eluted by the addition of 200 μl of ddH_2O, incubation at room temperature for 2 min and centrifuged at 8000 rpm for 1 min. Purified genomic DNA was stored at -20°.

Plasmid DNA was extracted from overnight cultures using GeneJET Plasmid Miniprep Kit (Thermo Scientific). Cells were harvested by centrifuging 5 – 10 ml overnight cultures at 8000 rpm. All subsequent centrifugation steps were performed at 14500 rpm. Cells were resuspended in 250 μl resuspension solution followed by the addition of 250 μl lysis solution. A volume of 350 μl of neutralisation solution was added. If the resulting precipitate was very viscous (which would yield very low DNA concentrations) an additional 100 μl of lysis solution was added followed by 140 μl of neutralisation solution. The resulting solution was centrifuged for 5 min. The supernatant was transferred to a GeneJET spin column and centrifuged for 1 min. The column was washed with 800 μl wash solution and centrifuged for 1 min. Plasmid DNA was eluted in 40 μl of ddH_2O and stored at -20°C. For large scale plasmid purification,

cells were harvested from 30 – 40 ml of overnight culture as above. All steps remained the same, except that 2 ml of lysis buffer and 2.8 ml neutralization buffer were used.

DNA in agarose gel was excised from the gel and purified using GeneJET Gel Extraction Kit (Thermo Scientific), according to manufacturer instructions. All centrifugation steps were performed at maximum speed. Gel slices were melted at 60°C in 300 μl of binding buffer and the melted solution was transferred to a purification column and centrifuged for 1 min. The column was washed with 800 μl wash buffer and the DNA was eluted in 30 μl ddH_2O and stored at -20°C.

2.5 Preparation of electrocompetent cells

Overnight culture of *E. coli* DH5-alpha was added to 100 ml LB broth to a final OD_{600} = 0.01 without antibiotics and grown at 37°C to an OD_{600} between 0.4 to 0.6, measured with a Multiskan Go (Thermo Scientific™) spectrophotometer. The culture was transferred to two 50 ml Greiner tubes and centrifuged at 6000 rpm for 6 min at 4°C. The pellet was washed twice with 40 ml and 20 ml ice-cold ddH_2O, respectively. The pellets were pooled and subsequently washed with 8 ml of 10% (v/v) glycerol. The pellet was resuspended in 1.5 ml 10% (v/v) glycerol, and aliquots of 100 μl were frozen in liquid nitrogen and stored at -70°C. Plasmids were transformed into electrocompetent cells. Electroporation was performed using a MicroPulser electroporator (Bio-Rad) at 1.8 kV and 4.5 ms. Cells were recovered in LB broth for 1 hr then plated onto LB agar plates supplemented with the appropriate antibiotics and incubated overnight.

2.6 Preparation of chemically competent cells

Chemically competent cells were prepared using the calcium chloride method. Overnight culture (of all soft rot species) was added to 100 ml LB broth to a final OD_{600} = 0.01 without antibiotics and grown to an OD_{600} between 0.4 to 0.6. Cells were pelleted by centrifugation at 6000 rpm for 6 min at 4°C, and resuspended in half the volume of ice-cold, filter sterilised calcium chloride (50 mM). The suspension was centrifuged again at 6000 rpm for 6 min at 4°C. The pellet was resuspended in a mixture of 750 μl of calcium chloride (50 mM) and 750

μl of 50 % (v/v) glycerol. Cells were snap frozen in liquid nitrogen and stored at -70°C. To transform chemically competent cells, 100 μl aliquots were mixed with the DNA to be transformed, incubated on ice 30 min and subjected to heat shock at 42°C for 2 min. After heat shock, the cells were chilled on ice for 2 min followed by the addition of 600 μl of LB broth. Cells were recovered for 1 hr at 37°C with shaking and plated onto LB agar plates containing the appropriate antibiotics.

2.7 Restriction enzyme digestion

Thermo Scientific FastDigest enzymes were used for all restriction digests. For screening, approximately 500-1000 ng of plasmid DNA was used. For linearization of vectors and recovery of inserts for ligation reactions, approximately 3 μg of plasmid DNA was used. For screening reactions, plasmid DNA (~500 ng), 2 μl 10X FastDigest Buffer, 10 units of each appropriate enzyme, and nuclease-free water was added to a final volume of 20 μl. Restriction enzymes were heat inactivated at 80°C for 10 min. Reactions were electrophoresed on 1% (w/v) agarose gels and the appropriate band excised and purified. For preparation of ligation inserts, 3 μg plasmid DNA, 3 μl 10X FastDigest Buffer, 15 units of each appropriate enzyme, and nuclease-free water was added to a final volume of 30 μl. The reactions were heat inactivated at 80°C for 10 min and electrophoresed on 1% (w/v) agarose gels and the appropriate band excised and purified.

2.8 Polymerase chain reaction

2.8.1 Primer design

Primers were designed using CLC Main Workbench version 6.1 and genome sequences were obtained from the PATRIC database (https://www.patricbrc.org/) as well as the ASAP database (https://asap.genetics.wisc.edu/asap/home.php). Restriction cut sites were incorporated into the 5' end of the gene sequences to facilitate cloning into a suitable plasmid. Refer to Table 2 for the list of primers.

2.8.2 PCR amplification and colony PCR

All PCRs were carried out with Phusion High-Fidelity PCR Master Mix (Thermo Scientific). Each reaction was set up with 2X master mix, 0.5 μM each of the forward and reverse primers, ~37 ng template DNA, and ddH_2O to the final volume. The PCR was performed in a T100 Bio-Rad thermal cycler for 35 cycles, with the initial denaturation at 98°C for 30 s, denaturation at 98°C for 10 s, annealing at T_m + 3°C of the lower T_m primer for primers >20 nucleotides and at T_m of the lower T_m primer for primers ≤20 nucleotides for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. Samples were electrophoresed in 1% (w/v) agarose gel in 1X TAE buffer (40 mM Tris, 20 mM NaOAc; 1 mM EDTA; pH 8.5) for 45 min at 90 V and subsequently excised and purified. Colony PCR was performed as above, but the PCR was downscaled to a final volume of 10 μl as follows: 5 μl of 2X master mix, 10 μM each of the forward and reverse primers, 1 μl culture, and 3.4 μl ddH_2O. Cycling conditions and electrophoresis were performed as above, except that the initial denaturation was increased from 30 s to 3 min.

2.9 Nucleotide sequencing

To confirm the integrity of the DNA constructs, the nucleotide sequences of the construct was determined using the ABI-PRISM BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. Each reaction contained 100 ng of purified DNA per kb of plasmid to be sequenced, 3.2 pmol of sequencing primer per reaction, 1 μl of BigDye Ready Reaction mix, 1 μl of 5x BigDye Sequencing buffer, and ddH_2O to a final volume of 10 μl. The cycle sequencing reactions were performed in a T100 thermal cycler. Following initial denaturation at 94°C for 1 min, the reaction mixtures were subjected to 25 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 15 s, and extension at 60°C for 4 min. The products were mixed with 60 μl of 100% (v/v) ethanol, 11 μl ddH_2O and 2 μl of NaOAc (pH 4.3, 3 M), and incubated at -80°C for 15 min. Following centrifugation at 14 500 rpm for 30 min, the supernatant was discarded, and the pellets rinsed

twice with ice-cold 70% (v/v) ethanol. The samples were dried for 2 min at 60°C and submitted to the University of Pretoria's Sequencing Facility for sequencing.

2.10 Ligation reactions

Ligation was performed using T4 DNA ligase (Thermo Scientific). Blunt-end cloning was performed by adding 50 ng/μl linear vector DNA, insert DNA at a 5:1 molar ratio to the vector, 5 units of T4 DNA ligase, 1 μl PEG 4000 solution, and nuclease-free water to a final volume of 20 μl. The reaction mixture was incubated at 22°C for 1 hr and heat inactivated at 75°C for 5 min. Sticky-end ligations were performed as above, except insert DNA was added at a 3:1 insert-to-vector molar ratio, and PEG 4000 was omitted. Ligation reactions were transformed into chemically competent cells PCR products were first sub-cloned into the positive selection pJET 1.2/Blunt cloning vector (Thermo Scientific), using blunt end ligation as above and transformed into chemically competent *E. coli* DH5-alpha.

2.11 Construction of pTrc100

The expression vector pTrc99A was obtained from the FABI culture collection repository for the cloning of effector and immunity genes. Towards this end, it was transformed into *E. coli* DH5-alpha, and extracted using the alkaline lysis plasmid extraction method. To verify that the multiple cloning site (MCS) was still the same as the published plasmid map, a series of restriction digests with NcoI, EcoRI, SacI, SmaI, SalI, XbaI, KnpI and BamHI were performed. Once the remaining restriction sites were confirmed, the plasmid was modified to facilitate cloning into this plasmid by reintroducing KnpI and BamHI cut sites into the plasmid by their incorporation into the 5' region of the PCR primers (H5907_F/R) of an arbitrarily chosen gene, *AED-0003810*. PCR amplification of *AED-0003810* incorporated XbaI, KpnI, and BamHI upstream of the ORF and BamHI and SalI downstream of the ORF. The PCR product was subcloned into pJET1.2/blunt and excised with XbaI/SalI restriction digest. Plasmid Trc99A was digested with XbaI/SalI and ligated with the excised PCR product. The gene was removed using BamHI restriction digest and the linear plasmid purified from the gel and religated. In this manner BamHI and KnpI were reintroduced into the MCS. The resulting

plasmid is referred to as pTrc100 as its MCS differs slightly from parental pTrc99A, but the rest of the plasmid remains unchanged. This plasmid was used for the ligation of immunity genes.

2.12 Construction of pCH450 derivates and pTrc100 derivatives

Plasmids pCH450 and pCH450::RhsA$^{(D.\ dadantii)}$ were generously provided by Prof. C Hayes, University of California, Santa Barbara. The plasmids were transformed into *E. coli* DH5-alpha. Plasmid pCH450 was extracted for further cloning experiments. The putative effector genes identified were PCR amplified, purified, and subcloned into pJET 1.2/Blunt cloning vector and excised for ligation into arabinose-inducible expression plasmid pCH450 cut with suitable enzymes to allow for ligation. The resulting ligation reactions were transformed into *E. coli* DH5-alpha, colonies were selected, and screened with plasmid extractions and restriction digests. To assess the activity of cognate immunity genes against selected effectors, cognate immunity genes were PCR amplified as above and ligated into pTrc100 then transformed into *E. coli* DH5-alpha already carrying the respective pCH450::effector plasmid.

The phospholipase (Phos) is expected to be active only within the periplasm, thus, cytoplasmic expression will not cause any inhibition of bacterial growth when expressed in *E. coli*. To overcome this, Phos needed to be fused to a secretion signal. To achieve this, Phos was excised from pJET 1.2/Blunt with SacI and SalI restriction enzymes. The resulting gene fragment was then ligated into expression vector pET-26b+ which contains the PelB secretion signal prior to the MCS. Insertion of Phos into the MCS places the PelB secretion signal in front of this gene. The ORF of Phos together with the N-terminal secretion signal was then excised from pET-26b+ using NdeI and XhoI liberating a 1.5 kb fragment. This fragment was then ligated into the MCS of pCH450 that was cut with NdeI and XhoI to produce compatible ends for ligation. The resulting plasmid was transformed into *E. coli* DH5-alpha cells. To confirm the insertion of the PelB-signal sequence-flanked gene, the resulting plasmid

pCH50::Phos was extracted from the transformed cells and digested with NcoI, which cuts within the phospholipase gene, within pCH450 and also 5' of the PelB sequence.

2.13 Ectopic expression of effectors

Oxygen-limited overnight cultures were prepared by incubating test tubes filled to the brim with LB broth inoculated with *E. coli* pCH450::effector and supplemented with 10 µg/ml tetracycline and 0.1% (w/v) glucose to repress expression from the arabinose-inducible promoter. Cultures were washed once in 10 mM $MgSO_4$, adjusted to an OD_{600} of 0.05 in fresh LB broth supplemented with 5 µg/ml tetracycline, 50 µg/ml ampicillin, and 1 mM IPTG, as required. After 30 minutes, expression of the effector in pCH450 was induced with 0.2% (w/v) L-arabinose. For dual plasmid expression, effector expression from pCH450 was induced as above and expression of immunity genes from pTrc100 (overnight cultures grown as above) was induced with 1 mM of IPTG immediately. Optical density (600 nm) was measured hourly.

3. Results

3.1 *In planta* interbacterial competition

Currently, no data exist for the contribution of the T6SS in *Pcb1692* for *in planta* interbacterial competition. There is no set standard in the literature for the attacker-to-target ratio used for competition assays; these ratios typically vary from 1:1 to 10:1. Therefore, it was first necessary to establish whether different ratios significantly affect competition between two strains. For this study, a 1:1 and 10:1 attacker-to-target ratio was assessed. In all cases, the controls (*Pcb1692, Pcb1692*_ΔT6SS, *Pa*, *Dc*, *Dd*, and *Pcc*) which were infiltrated into the leaves individually, grew well, with cell titres well above 10^7 CFU/g tissue (Figure 2.1). Co-infiltration of target strains with *Pcb* at a 1:1 ratio was expected to reduce the cell titres of target strains due to competition. Indeed, this was observed in all cases (Figure 2.1 A-D). The reduction in target survival was significant compared to the respective control in all cases, except when *Pa* was co-inoculated with *Pcb1692* (Figure 2.1 A). Next, competing bacterial strains at 10:1 attacker-to-target ratios was expected to significantly reduce target survival compared to the 1:1 assays. Interestingly, although survival in the 10:1 assays was lower than the 1:1 assays [with the exception of *Dickeya dadantii* (Figure 2.1C)], the difference in survival of target species was not statistically significant in all instances at *p* value of 0.05 (Figure 2.1 A-D). In other words, the attacker concentration does not significantly play a role in competition assays *in planta* in one day post infection trials for the strains used in this study.

As the attacker-to-target ratio did not significantly influence the outcome of competition, a 1:1 attacker-to-target ratio was subsequently used to determine whether the T6SS of *Pcb1692* contributes towards *in planta* interbacterial competition. To this end, six-week-old susceptible potato leaves (cv. Mondial) were used for competition assays. In these bacterial competition assays, various SRE species were co-inoculated with either wild type *Pcb1692* or *Pcb1692*_ΔT6SS. As both the attacker (*Pcb1692* or *Pcb1692*_ΔT6SS) and target species (*Pa, Pcc, Dd, Dc*) have competitive abilities, either of the two strains could be out-competed during these assays, thus, cell survival of both the target and attacker strains was enumerated

after one day post infection to assess the degree to which each strain was affected during co-inoculations.

Attacker strains [*Pcb1692* (WT) or *Pcb1692*_ΔT6SS (T6)] and target strains (*Pa, Pcc, Dd, Dc*) were infiltrated into the leaves individually as negative controls. One day post infection, both attacker and target strains were enumerated to determine the extent of competition against each other (Figure 2.2). Cell titres of all the controls were expected, and subsequently observed, to be higher than co-inoculations, as no competitors were present to impede their growth. When inoculated individually (controls) or co-inoculated, *Pcb1692* (*Pcb* WT) and *Pcb1692*_ΔT6SS (T6) grew well with cell titres that exceeded 10^8 CFU/g tissue (Figure 2.2 A–D). However, co-inoculation of various SRE target species with either *Pcb1692* or *Pcb1692*_ΔT6SS showed a marked decrease in all target species titres compared to their respective controls. Selection for *Pcb1692* or *Pcb1692*_ΔT6SS attacker strains from co-inoculations shows that their survival does not differ significantly to their respective controls, indicating that the attacker species are outcompeting the target species in all instances, and that the target species do not significantly affect attacker titres. The “attacker” and “target” nomenclature are arbitrary, as either species can potentially compete with each other to a certain degree; however, for this study *Pcb1692* or *Pcb1692*_ΔT6SS were designated as the attacker species and *Pa, Pcc, Dd, Dc* as the target species. As the outcome of competition is unknown (i.e. it is possible that one of the target strains could outcompete one of the attacker strains), cell titres for both the attacker and target strains from co-inoculation assays were determined. Thus, as the attacker strain titres remained constant in both the controls and co-inoculations, the reduction in cell titre of target species is due to competitive interactions imposed by *Pcb1692* (wild type and mutant) during co-inoculations.

As the efficiency of a killing system is assayed, titres of target strains are expected to be higher when competed with the *Pcb1692*_ΔT6SS (as a killing system has been disabled) than when competed with wild type *Pcb1692*. Assays with *P. atrosepticum* show that competition against

Pcb1692 reduced *Pa* titres, however, not significantly compared to the *Pa* control; whereas competition against *Pcb1692*_ΔT6SS significantly reduced *Pa* titres (Figure 2.2 A). The opposite was expected, however, to determine whether this difference is significant in terms of T6SS-mediated competition, *Pa* titres from co-inoculations with *Pcb1692* or *Pcb1692*_ΔT6SS were compared to each other. Results indicate that there is no significant difference in *Pa* survival when competed with either of the *Pcb1692* strains. Thus, the T6SS does not contribute significantly towards bacterial growth inhibition.

Competition of *Dickeya chrysanthemi* against both strains of *Pcb1692* saw a significant decrease in target titres compared to the control (Figure 2.2 B); however, the difference in survival of *Dc* from co-inoculations with *Pcb1692* or *Pcb1692*_ΔT6SS was not significant, indicating that the T6SS does not contribute significantly towards bacterial growth inhibition. The same results were obtained for competition of *Dickeya dadantii* against *Pcb1692* and *Pcb1692*_ΔT6SS (Figure 2.2 C). Assays with *P. carotovorum* show that competition against *Pcb1692* wild type and mutant reduced *Pcc* titres; however, the reduction was only significant when *Pcc* was competed with *Pcb1692* wild type (Figure 2.2 D). There was no significant difference in survival when *Pcc* was competed with *Pcb1692* or *Pcb1692*_ΔT6SS, once again indicating that the T6SS does not significantly contribute towards bacterial growth inhibition.

Statistical analyses consistently indicate that there is no significant difference between competition of a target species with *Pcb1692* compared to *Pcb1692*_ΔT6SS. In other words, the reduction in titres observed from co-inoculations is not due to the T6SS. As both wild type and mutant *Pcb1692* titres remain relatively consistent throughout the co-inoculation experiments while target titres decrease, the results seem to indicate that *Pcb1692* may be interacting competitively with these target species, albeit using competitive strategies other than the T6SS in potato leaves.

Previous studies show that the T6SS is up-regulated *in planta* (Bellieny-Rabelo *et al.*, 2019), however, results from the current study indicate that bacterial killing is not the main role of the

T6SS for *Pcb1692* in plant leaves. The *Pcb1692* genome encodes several other antimicrobial compounds such as carbapenem, pyocin, and T5SS, which were upregulated during host infection in potato tubers (Shyntum *et al.*, 2018, Bellieny-Rabelo *et al.*, 2019). Since no role was determined for the T6SS effectors thus far, it was hypothesised that these other antimicrobial systems might be masking the effect of T6SS effectors. Thus, a different strategy that required expression of individual effector in *Escherichia coli* was undertaken. To achieve this, an *in silico* approach was adopted to determine the effector repertoire encoded by *Pcb1692*, and subsequently to determine whether any of the effectors are antibacterial effectors.

3.2 Identification of Hcp-secretion islands and T6SS effectors

To identify T6SS-dependent effectors within *Pcb1692*, an *in silico* bioinformatic approach was used. Genome architecture provides insight into the function and association of neighbouring genes: Genes adjacent to each other are often involved in the same process or combine with each other to form functional units (Huynen *et al.*, 2000). Genomic context and established protein-protein interactions were used to identify putative T6SS-effectors in *Pcb1692*. The genome was searched for both haemolysin-coregulated protein (Hcp) and valine-glycine repeat protein (VgrG) genes. *Pcb* encodes five Hcp genes (*AED_0004534; -4521; -6274; -4513; -3112*) and three VgrG genes (*AED_0002727; -3069; -3812*) (Figure 2.3). Using Hcp-VgrG gene islands as markers, downstream genes were evaluated for putative T6SS-dependent effectors and associated immunity genes.

The first region containing Hcp-VgrG genes [Hcp-secretion island (HSI-1)] is adjacent to the T6SS core cluster (Figure 2.3 A). The first gene downstream of VgrG (*AED_0003357*) contains an N-terminal PAAR domain, which marks it as a potential T6SS-dependent effector. Protein sequence analysis revealed an alpha-beta hydrolase fold in addition to the PAAR domain. Similarly, *Pectobacterium atrosepticum* strain SCRI1043 T6SS phospholipase, *ECA3426* (http://202.120.12.133/**SecReT6**/component.php?pid=50122350), possesses an N-terminal PAAR domain and alpha/beta hydrolase fold. Alignment of AED_0003357 and ECA3426

protein sequences show 90% similarity, with both proteins conserving the GXSXG motif, which is characteristic of lipases (Figure 2.4). In both *Pcb* and *P. atrosepticum*, the downstream gene is predicted to be the immunity genes (*AED_0003357* & *ECA3425*, respectively), both containing an ankyrin domain with four repeats.

The other two Hcp-secretion islands with an Hcp-VgrG pair occupy distinct localities in the genome separate from the core cluster. In HSI-2 (Figure 2.3 B), the first gene downstream of the Hcp-VgrG pair (*AED_0003811*) bears a DUF4123 domain that is associated with type 6 effector chaperones, further indicating that the genes in this region are associated with the T6SS. The next gene annotation is a hypothetical gene (*AED_0003810*), with no recognisable motifs or domains. This is followed by a putative effector-immunity pair; the effector (*AED_0003809*) contains an HNH/Endonuclease VII fold with a WHH nuclease motif and the downstream immunity gene (*AED_0003808*) belongs to the SUKH superfamily with a Smi1/Knr4 domain. The last putative effector in this cluster belongs to the cell division cycle protein 123 family (D123 domain) and is succeeded by a PAAR gene; thus, no immunity gene is associated with the D123 gene.

HSI-3 consists of seven genes; however, this island lacks a VgrG gene (Figure 2.3 C). All genes within this locus have no known function associated with the T6SS. Moreover, they do not resemble effector-immunity pairs, and are therefore most likely not antibacterial effectors. Likewise, HSI-5 lacks a VgrG gene, and Hcp is followed by two very small hypothetical genes with no known function (Figure 2.3 E).

HSI-4 encodes seven genes in this locus (Figure 2.3 D). The first gene downstream of VgrG (*AED_0003068*) contains a DcrB domain and is followed by two putative effector-immunity pairs. The first effector (*AED_0006270*) contains a PAAR domain, YD-repeats and a C-terminal toxin domain clearly defined by the PxxxxDPxGL motif present in Rhs genes. As with all Rhs genes, the downstream gene is the associated immunity gene (*AED_0004232*), however, no specific domains were detected in this gene. The next effector (*AED_0004231*)

encodes an AHH nuclease motif which belongs to the HNH/Endonuclease VII fold like the WHH-nuclease in HSI-2. This gene is also followed by a hypothetical gene without any distinguishable domains (*AED_0004230*). Due to the antibacterial nature of nucleases, the hypothetical gene is regarded as the cognate immunity gene for the AHH nuclease.

In total, five putative effector genes were identified within *Pcb*, of which four have the typical arrangement of antibacterial effectors. To determine whether the selected genes encode proteins with antibacterial activity, they need to be cloned into an expression vector and expressed ectopically in *E. coli*, which is inherently susceptible as it does not encode the cognate immunity genes. Ectopic expression of each effector will therefore likely kill *E. coli* cells if the effector has functional antibacterial activity. Co-expression of the effector protein with a cognate immunity protein will result in survival of *E. coli* cells confirming the effector/immunity pair as functional.

3.3 Plasmid modification

For ectopic expression of effector proteins, a dual plasmid system was required in which the effector and immunity genes are co-expressed from two distinct expression vectors. Effector genes were expressed from arabinose-inducible plasmid pCH450, whereas immunity genes were expressed from IPTG-inducible plasmid pTrc99A. This allows for the expression of the effector and immunity genes to be induced separately. In order to clone immunity genes into pTrc99A, a suitable MCS was required.

The restriction enzyme map of pTrc99A was confirmed using enzymes that cut within the MCS (Figure 2.5 A). Restriction cut sites that were still intact were expected to linearize the plasmid generating a single 4.2 kb band. The following enzymes were used: NcoI, SacI, KpnI, SmaI, PstI, HindIII, BamHI, SalI, and XbaI. In Figure 2.6 A, the first lane shows that digestion with NcoI did not yield linearised plasmid. Rather, the three bands present represent the three plasmid conformations obtained after plasmid extraction, namely, open circular plasmid DNA at the top, linear DNA at the centre, and a high concentration of supercoiled plasmid DNA at the bottom. The same was observed for single digests with SacI and SmaI, in lanes 2 and 3

(Figure 2.6 A), respectively. This indicates that NcoI, SacI, and SmaI restriction sites are no longer present in the MCS. Single digests with PstI and HindIII in lanes 4 and 5, respectively, yielded linearised plasmid approximately 4.2 kb in size, as expected, indicating that these cut sites are still present. In Figure 2.6 B, lanes 1 and 2 contain single digests with SalI and XbaI, respectively. The lower band corresponds to digested linear DNA approximately 4.2 kb in size; some undigested plasmid DNA remains above the linear DNA at the 4.2 kb mark. A double digest with SalI and XbaI in lane 3 generated a single band of linear DNA approximately 4.2 kb in size. Lanes 1 – 3 indicate that both SalI and XbaI cut sites are functional. The smaller fragment liberated from the MCS (between cut sites) by the digest is of such a small size (6 bp) that it migrates off the gel. In contrast, both KpnI and BamHI failed to cut the plasmid in both single and double digests (Figure 2.6 B; lanes 4-6, respectively), which indicates that these cut sites are no longer present. Lane 7 contains undigested plasmid DNA as a control; the upper band corresponds to undigested open circular DNA and the lower band corresponds to undigested supercoiled DNA. This series of restriction digests indicates that the MCS of pTrc99A only has SalI, XbaI, PstI, and HindIII restriction sites remaining.

The remaining cut sites in pTrc99A were deemed insufficient to allow for the cloning of genes identified in section 3.2, as some of the selected effector and immunity genes are cut internally by these enzymes. Internal cut sites in these genes will prevent ligation of the entire open reading frame into the plasmid. Thus, to facilitate cloning, additional restriction sites needed to be introduced into the MCS of pTrc99A. While designing primers for selected effector and immunity genes, a hypothetical gene located in HSI-2 (*PCBA_RS05790*) was arbitrarily selected as the vehicle to re-introduce KpnI and BamHI restriction sites into the MCS as none of the selected effector and immunity genes can be cut internally by these enzymes. This plasmid modification was achieved in a three-step process depicted in Figure 2.7. By introducing the required cut sites into the 5’ and 3’ regions of the PCR-amplified gene (Figure 2.7 A), the gene can be inserted into pTrc99A and later removed leaving an intact MCS for subsequent cloning strategies.

The first step involved incorporating the necessary cut sites into the forward and reverse primer sequences (Figure 2.7 A). The forward PCR primer was generated with three restriction sites 5' to the gene-specific sequence in the following order: XbaI, KpnI, BamHI; and the reverse PCR primer was generated with SalI and BamHI restriction sites 5' to the gene-specific sequence. PCR amplification of the gene from *Pcb1692* gDNA generated an approximate 440 bp fragment (Figure 2.8 A). This fragment was purified and subcloned into the pJET 1.2/Blunt cloning vector and transformed into *E. coli* DH5-alpha. This was used as the screening step to select a clone in which the sequence was maintained during PCR. A suitable clone was grown overnight and a plasmid extraction was performed. Restriction digestion efficiency was improved by inserting the PCR amplicon into the pJET subcloning vector first, instead of digesting the PCR product directly. The insert, which was flanked by the necessary restriction sites, was liberated from the pJET vector by restriction digest with both XbaI and SalI, which are located upstream and downstream of the of the gene, respectively. Next, to produce compatible ends for ligation, pTrc99A was also digested with SalI and XbaI (Figure 2.7 A), and treated with alkaline phosphatase to prevent spurious re-ligation of the vector. Ligation of the gene and the linearised vector generated the recombinant plasmid pTrc::H5907 (Figure 2.7 B). To confirm the insertion and maintenance of the sequence of the introduced cut sites, pTrc::H5907 was digested with SalI-XbaI double digest and KpnI-BamHI double digest, (Figure 2.8 B, lanes 2 and 3, respectively). Lane 1 (Figure 2.8 B) corresponds to the undigested control plasmid, pTrc::H5907. Generation of linear vector at 4.2 kb and a second fragment at 440 bp in lanes 2 and 3 confirms the insertion of gene H5907. Additionally, this indicates that this plasmid contains intact SalI, XbaI, KpnI, and BamHI restriction sites.

Plasmid pTrc::H5907, however, was not yet suitable for subsequent cloning experiments, as it still carried the gene within the MCS. Thus, it was necessary to remove this gene to rescue the multiple cloning site. This gene was removed by treating the plasmid with a BamHI restriction digest, which cuts at both ends of the gene, thereby releasing the gene from the plasmid backbone and generating compatible ends within the remainder of the plasmid. After

pTrc::H5907 was digested with BamHI, the linear backbone was purified and self-ligated. The resulting plasmid (Figure 2.7 C), pTrc100, was extracted and a series of restriction digests were performed to confirm the restriction sites that were present. The following cut sites are expected in the following order downstream of the *trc* promoter: XbaI, KpnI, BamHI, SalI, PstI, and HindIII (Figure 2.7 C). In Figure 2.8 C, lanes 1-6 contain single digests of pTrc100 with the aforementioned enzymes, respectively, and lane 7 contains the undigested plasmid control. All digests generated a single ~4.2 kb DNA fragment corresponding to the expected plasmid size. Thus, KpnI and BamHI were successfully reintroduced into the MCS generating a plasmid with a slightly modified MCS from plasmid pTrc99A. The modified plasmid, renamed pTrc100 was used in subsequent cloning steps.

The MCS of plasmid pCH450 was similarly confirmed. The plasmid was digested with EcoRI, KpnI, NcoI, NdeI PstI, PvuI, SacI, SmaI, and XhoI (Figure 2.9, lanes 1-9, respectively). Digestion with all nine enzymes yielded linearised DNA (migrating to ~ 3.8 kb). Some undigested plasmid DNA remains when cut with NdeI (lane 4) and is visualised high above the linear band. KpnI cuts twice within the MCS; however, the second piece of DNA liberated from the digest is 36 bp in size and migrates off the gel. These restriction digests indicate that all the cut sites are maintained on this plasmid.

Once the MCS of pCH450 was confirmed and pTrc100 was sufficiently modified, the bioinformatically identified type 6 effectors and their respective immunity proteins were PCR amplified from *Pcb1692* (Figure 2.10). In all cases, gene amplicfication was very specific, with only one band obtained. As expected, the phospholipase (Phos) amplicon migrated to approximately 1.4 kb, the WHH nuclease to 990 bp, both the Rhs1 and AHH nuclease effectors to 470 bp, and the D123 to 800 bp (Figure 2.10 A).

The immunity proteins were similarly amplified. The expected PCR fragments were obtained for the ankyrin (Ank) immunity protein for the phospholipase, which migrated to approximately

700 bp, the SUKH immunity protein for the WHH nuclease (540 bp), the AHH immunity protein (AHHi; 570 bp), and the Rhs1 immunity protein (Rhs1i; 480 bp) (Figure 2.10 B).

The effector and immunity gene amplicons were purified from the gels and used in subsequent ligation reactions with either pCH450 or pTrc100, with the exception of the Phos amplicon, which still needed to be fused to a suitable secretion signal.

3.4 Fusion of PelB signal sequence to phospholipase gene

To ensure periplasmic expression of the phospholipase gene during ectopic expression studies, a secretion signal had to be fused ahead of the phospholipase ORF. A suitable signal sequence is included in the expression plasmid pET-26b+. Phos was ligated into the MCS of pET-26b+, which placed it downstream of the signal sequence. The pET plasmid system could not be used for expression of effector genes as they are IPTG inducible; thus, the signal-fused gene had to be transferred to the arabinose-inducible plasmid pCH450. Restriction sites that flank both the signal sequence and the Phos ORF were selected: NdeI and XhoI. Digestion of pET-26b+::Phos liberated a 1.5 kb fragment (Figure 2.11 A) that contained the fused DNA fragment (lower band). This fragment was then ligated into pCH450 that was digested with the same enzymes to create compatible ends.

After transformation into *E. coli*, plasmid was extracted from these cells to confirm the insert. NcoI was chosen for this confirmation step as wild type pCH450 only contains one such site; whereas if the desired DNA fragment was successfully inserted, three NcoI cut sites would be present in the plasmid. The NcoI sites are situated in the MCS of pCH450, 5' to the PelB signal sequence, and within the phospholipase gene. Thus, three fragments resulting from a digest with NcoI confirm the insertion of the desired DNA fragment. Unsuccessful insertion would yield one fragment of ~3.8 kb in size; whereas digestion of recombinant plasmid results in three fragments 4 kb, 1.1 kb and 81 bp in length. The expected fragments were obtained upon digestion of recombinant plasmid (Figure 2.11 B; lane 2). Lane 1 in Figure 2.11 B contains undigested pCH450::PelB-Phos as a control. This confirms that the expression vector pCH450

contains the correct gene insert, and is ready to be used in ectopic expression assays in *E. coli*.

3.5 Evaluation of the antibacterial activity of T6SS-associated effectors

To investigate the potential of putative antibacterial toxin-encoding genes, these genes were cloned into expression vector pCH450 and ectopically expressed in *E. coli* DH5-alpha, as described by Koskiniemi *et al.* (2013). Ectopic expression of effectors allows the activity of these genes to be assayed without interference from inherent immunity factors produced by the host. Therefore, the selected effector genes, *AED_0003357* [Phospholipase (Phos)], *AED_0006270* (Rhs1), *AED_0003810* (WHH nuclease), *AED_0004231* (AHH nuclease), and *AED_0003807* (D123 protein), were cloned into pCH450 and expressed individually in *E. coli* DH5-alpha. To overcome the effect of any toxicity produced by effectors, immunity genes were expressed from pTrc100 concurrently with effector expression.

The ectopic expression system was first tested using the RhsA nuclease from *Dickeya dadantii* as a positive control transformed into *E. coli* DH5-alpha (obtained from C. Hayes, University of California, Santa Barbara (Koskiniemi *et al.*, 2013)). In Figure 2.12 A, ectopic expression of effector and immunity genes (RhsA and RhsA+I) were compared to *E. coli* with empty vector pCH450 or pCh450+pTrc100 negative controls (red and yellow lines, respectively). Effector expression was induced with 0.2% L-arabinose after 30 min growth (indicated by the red arrow). Expression of RhsA alone showed a distinct growth inhibition due to effector toxicity after 120 min growth (blue line). To counteract the activity of the effector, pTrc99A::RhsAi was introduced into the pCH450::RhsA background (generating the RhsA+I strain). Simultaneous expression of both the effector and its cognate immunity protein (green line) prevented growth inhibition. At 120 min growth, the optical density of the effector-inhibited strain (RhsA+I) considerably exceeded the effector-only (RhsA) strain. This confirms that introduction of heterologousT6SS effector genes into *E. coli* DH5-alpha can successfully inhibit the growth in the absence of cognate immunity genes. As this system has been established, the putative effectors identified in *Pcb1692* in this study were expressed accordingly.

No growth inhibition due to effector expression was observed for the selected genes, except for the AHH nuclease (Figure 2.12 B and Figure 2.12 C). As for the positive control experiment with RhsA, ectopic expression of *Pcb1692* effectors was compared to empty vector controls pCH450 and pTrc100+pCH450. Individual expression of the phospholipase, WHH nuclease, D123 protein, and Rhs1 showed growth comparable to the empty vector control (Figure 2.12 B), even after prolonged periods of expression. This is very distinct to the inhibition observed by RhsA expression in Figure 2.12 A. Individual expression of the AHH effector (Figure 2.12 C), however, resulted in slight growth inhibition, indicating that it has some toxic effect on susceptible cells.

Compared to RhsA in Figure 2.12 A, inhibition by AHH is not as pronounced. Whereas RhsA causes the optical density to decrease continually, the optical density continued to increase with the expression of AHH, however, distinctly slower than the controls. To confirm that this reduced growth rate was not due to random selection of a slow-growing colony, *E. coli* bearing both the pCH450::AHH effector and pTrc100::Ai immunity plasmids was grown using the same induction conditions, albeit immunity expression was induced immediately. Growth was initiated in the presence of IPTG to induce production of the immunity protein and thereby negate the result of effector production. For the first three hours of growth, the curve (AHH+i) displayed growth comparable to the controls (pCH450 and pTrc+pCH450), but thereafter plateaued at OD_{600} = ~0.47 for the remaining time points. To determine whether this plateau was caused due to excessive heterologous protein production, the effector-immunity strain was grown in uninduced conditions [AHH+I (U)]. This time, bacterial growth was able to surpass the plateau, indicating that protein overexpression was indeed causing the inhibition of cell growth even though the toxicity of the effector was negated by the immunity protein. In conclusion, no growth inhibition due to effector expression was observed for the selected genes (Figure 2.12 B), except for the AHH nuclease (Figure 2.12 C).

4. Discussion

Pectobacterium carotovorum subsp. *brasiliense* is found in most potato growing regions in South Africa; 77% of soft rot species isolated from diseased material in South Africa were identified as *Pcb* (Van der Merwe *et al.*, 2009). *Pcb* is the most aggressive SRE species on potato and given its broad distribution, it is a pressing concern for the potato industry, not only in South Africa, but worldwide (Charkowski, 2018). In recent years, soft rot induced by SRE has been recognised as a disease complex comprised of co-infecting SREs, but not limited to, as Gram-positive species from genera such as *Clostridium* and *Bacillus* also cause soft rot (Charkowski, 2018). *Pcb* has been isolated from infected tissue together with *Pcc, Pw*, and *Dickeya* spp. (Kim *et al.*, 2009, Van der Merwe *et al.*, 2009, De Boer *et al.*, 2012).

Contrary to *in vitro* conditions, the T6SS of *Pcb1692* is upregulated *in planta* (Bellieny-Rabelo *et al.*, 2019). The T6SS is a major contributor towards virulence in some species such as *P. aeruginosa*; it interacts with host cells as well as effectively outcompetes other bacteria (Hood *et al.*, 2010, Basler *et al.*, 2013, Jiang *et al.*, 2016). The role of the T6SS in *Pcb1692* was unknown, therefore, this study sought to determine whether the T6SS of *Pcb1692* contributes towards bacterial competition *in planta* in potato leaves. Previously, it has been noted that T6SSs can be ineffective *in vitro* and proficient *in planta* (Ma *et al.*, 2014). It was hypothesised that the same was true for the T6SS of *Pcb1692*. Contrary to expectation, the T6SS did not show significant contribution towards bacterial killing *in planta* in potato leaves. Due to the lack of antibacterial activity observed *in planta*, an absence of antibacterial effectors was expected within the genome of *Pcb1692*. Interestingly, a small number of putative antibacterial effectors (which follow the effector-immunity gene configuration typical of antibacterial effectors) were identified in the *Pcb1692* genome. Expected to kill *E. coli* in ectopic expression assays, all but one of these putative effectors displayed antibacterial activity. Altogether, this study indicates that the T6SS in *Pcb1692* does not play as large a role in bacterial targeting as expected, even though antibacterial effectors are encoded within the genome.

In a recent study, synergism between soft rot species has been demonstrated by co-infection of *P. wasabiae* with other *Pectobacterium* spp. *in planta* in potato tubers, which led to increased tissue maceration and lesion size compared to infection with *P. wasabiae* alone (Valente *et al.*, 2017). Although maceration is increased, co-infecting species sharing the same niche will compete with each other for nutrients liberated during tissue maceration. Competition can be mediated by numerous strategies including antibiotic production, motility, signal interference, reduced expression of metabolically taxing genes, disruption of competitor adhesion, and direct cell targeting strategies such as the T6SS (Hibbing *et al.*, 2010, Ghoul & Mitri, 2016).

No contribution of the T6SS of *Pcb1692* towards bacterial growth inhibition has been observed *in vitro* (Shyntum *et al.*, 2018); however, Bellieny-Rabelo *et al.* (2019) have shown that the T6SS in *Pcb1692* is up-regulated *in planta* in potato tubers. Similarly, *in vitro*, *Agrobacterium tumefaciens* was unable to outcompete *Pseudomonas aeruginosa* in a T6SS-dependent manner, rather *P. aeruginosa* outcompeted *A. tumefaciens* (Ma *et al.*, 2014). Interestingly, the converse was observed in an *in planta* setting when *A. tumefaciens* and *P. aeruginosa* were infiltrated into *Nicotiana benthamiana* leaves. This serves to demonstrate that competition results obtained *in vitro* are not always comparable with *in vivo* conditions. Given the lack of *Pcb1692* T6SS activity in bacterial targeting *in vitro* and that the T6SS is upregulated *in planta* in potato tuber infections (Bellieny-Rabelo *et al.*, 2019), it became necessary to determine whether conditions in the host plant are necessary for T6SS activity in *Pcb1692*. Shyntum *et al.* (2018) determined the role of *Pcb1692* T6SS in bacterial competition in potato tubers, whereas this study assessed the effect of the *in planta* environment of potato leaves on T6SS-dependent bacterial competition in *Pcb1692*. Although soft rot in potatoes is generally seed- or soil-borne, aerial rot may ensue when SRE enter through wounds in the stems and leaves. Thus, tissues other than tubers were also assessed; specifically, potato leaves were assessed in this study.

In this study, *in planta* infections (potato leaves) showed that both *Pcb1692* wild type and *Pcb1692*_ΔT6SS titres remained relatively constant throughout this experiment, indicating that these strains effectively compete against the target species, and in turn, are not readily harmed by the targeted species. Likewise, when *Pcb* ICMP19477 was co-inoculated with *P. atrosepticum* SCRI1043, growth of *Pcb* ICMP19477 was not affected by the co-inoculation (Durrant, 2016). As target species' titres were reduced in both *Pcb1692* and *Pcb1692*_ΔT6SS co-inoculations compared with the target-alone control infection, it is clear to see that *Pcb1692* employs some competitive strategies against the co-inoculated target species. Two aspects of *in planta* infection were assessed in this study, (1) the role of attacker-to-target ratio on the competitive outcome and (2) the role of the T6SS on bacterial competition in potato leaves.

Pathogen levels are often low in seed tissue, soil, and water, and pathogen distribution within the infected plant is commonly uneven (Fletcher *et al.*, 2006, Martinelli *et al.*, 2015). Titres are lower when plants are not in the vegetative growth phase and when plants are asymptomatically or latently infected (López *et al.*, 2009). In literature, competition assays are not conducted with standardised attacker-to-target ratios. These ratios typically range from 1:1 to 10:1 in favour of the attacker (Hood *et al.*, 2010, Brunet *et al.*, 2013, Ma *et al.*, 2014, Alcoforado Diniz & Coulthurst, 2015). As the T6SS is a contact-dependent cell-targeting system, an increase in attacker titre increases the likelihood of target cell penetration, and a reduced viability of target species is expected in these instances. Thus, two competitive ratios were selected in this study to determine whether attacker concentrations (*Pcb1692* wild type only) significantly affect competition against different SRE target species. To compare with literature, a maximum of 10:1 and a minimum of 1:1 attacker-to-target ratio was selected to determine whether such high attacker ratios would introduce bias into the experiment. Even though a decrease in target titre was observed in the 10:1 assays compared to 1:1 assays, it was not as extensive as expected, and statistical analyses indicate that there is no significant difference in target species titres between 1:1 and 10:1 competition assays. Thus, a 1:1 ratio was used for subsequent experiments. Next, the target species were co-inoculated with either

Pcb1692 or *Pcb1692*_ΔT6SS to assess the contribution of the T6SS in bacterial competition. Statistical analyses indicate that none of the target species titres in co-inoculations with either *Pcb1692* wild type or *Pcb1692*_ΔT6SS, compared to each other, were significant. In other words, the results indicate that the T6SS in *Pcb1692* does not contribute significantly towards competition *in planta* in potato leaves.

Although the T6SS does not contribute significantly to bacterial killing in potato leaves, target species' titres (except Pcc–T6 co-inoculation) drop significantly in competition with either strain of *Pcb1692* compared to their controls (Figure 2.2), indicating that *Pcb* interacts with the target species competitively, albeit using another mechanism. This is confirmed by many observations that *Pcb* is an accomplished competitor in the polymicrobial community, able to outcompete *Pcc*, *Pa*, and *Dd* (Van der Merwe *et al.*, 2009, Durrant, 2016). Although *Pcb* has been isolated from diseased tissue together with *Pcc*, *Pa* is not isolated from tissues infected with *Pcb* (De Boer & Rubio, 2004, Van der Merwe *et al.*, 2009). In this study, *Pa* was isolated from *Pcb*-infected material, however, this may be due to the short duration of the assay.

Bacteria are rarely present as a single, isolated cell and the natural environment is predominated by multispecies communities. The general mode of interaction between species in such environments is competitive, with few cooperative relations (Stubbendieck & Straight, 2016). Competitive behaviour can be grouped into two broad categories: exploitation and interference competition. Exploitation competition is the rapid assimilation of limiting nutrients by one species to the detriment of others that also require the resource in an indirect manner (Cornforth & Foster, 2013). Exploitation competition is especially prevalent in cell dense environments. In interference competition, one species directly targets another in an effort to harm it (Cornforth & Foster, 2013). Strategies that result in this type of competition include the production of quorum quenching molecules, antimicrobial compounds, direct cell targeting with contact-dependent inhibition (CDI) systems and the T6SS, and extracellular vesicles (Stubbendieck & Straight, 2016, Bauer *et al.*, 2018). Bacteria that are capable of multiple

strategies may be more equipped at outcompeting rivals in multispecies communities (Ghoul & Mitri, 2016).

The precise mechanisms involved in competition employed by *Pcb1692* in this study were not explored, however, studies have shown that *Pcb* is able to employ a number of competitive strategies. Exploitation competition in *Pcb1692* is mediated by the production of siderophores, which enables the pathogen to sequester iron (Tanui *et al.*, 2017). Iron is necessary for cellular signalling, as a co-factor for enzymes, and for protection from plant-induced oxidative burst mediated by reactive oxygen species (Durrant, 2016). Another exploitative strategy utilised by *Pcb* to rapidly acquire nutrients is through the production of plant cell wall degrading enzymes (PCWDEs) (Perombelon, 2002, Ghoul & Mitri, 2016). Exploitative competition then leads to interference competition (Cornforth & Foster, 2013).

In *Pcb1692*, interference competition may be mediated by CDI, antibiotic production, secretion of bacteriocins, and the T6SS. CDI is mediated by the T5SS, which is present in *Pcc, Pcb, Pa, and Pw*; however, the role thereof has hitherto not been assessed (Nykyri, 2013). Another player in bacterial competition mediated by *Pectobacterium* spp. is the broad-spectrum β-lactam antibiotic carbapenem, which was shown to inhibit growth of *Dd* and *Pcc in vitro* (Põllumaa *et al.*, 2013, Durrant, 2016, Shyntum *et al.*, 2018). A number of *Pectobacterium* spp., including *Pcb1692*, encode the carbapenem gene cluster; however, this cluster and its associated immunity gene are absent from *P. atrosepticum* SCRI1043 and *D. dadantii* 3937 (used in this study) as well as some other SRE (Shyntum *et al.*, 2018). When cells have reached a threshold density of ~10^7cells/g tissue, carbapenem together with PCWDEs are produced (Perombelon, 2002, Glasner *et al.*, 2008). Although carbapenem is effective against *Pa* and *Dd*, Shyntum *et al.* (2018) observed that carbapenem is only produced in *Pcb1692* in aerobic environments. Oxygen levels within plant tissues can be very low and insufficient for carbapenem production (Zabalza *et al.*, 2009); thus, carbapenem is not a likely contributor towards the competition observed in this study. Furthermore, the nature of carbapenem as a broad-spectrum antibiotic active under aerobic conditions, supports its role as a competitive

strategy against endophytes and opportunistic secondary infecting competitors (Glasner *et al.*, 2008, Shyntum *et al.*, 2018).

Pectobacterium spp. produce various bacteriocins that could contribute towards competition, such as carotovoricin, phenazine, carocin S1 and S2, and others (Durrant, 2016). *Pcb1692* also produces pectocin M1 and pectocin M2 which contribute towards competition against *P*cc and *P. atrosepticum* (Grinter *et al.*, 2012). Shyntum *et al.* (2018) observed that once induced with mitomycin C, carotovoricin and pyocin-S are produced by *Pcb1692 in vitro* (other bacteriocins were not assessed) and contribute towards bacterial killing of *Pcc* and *Dd.* Furthermore, these bacteriocins were also produced in anaerobic conditions when carbapenem is no longer produced (Shyntum *et al.*, 2018). Other stress conditions were not tested in that study. Bacteriocins are often produced under stress conditions including oxygen limitation, high temperatures, and competitive conditions (Durrant, 2016). Given the conditions of competition, it is likely that bacteriocins may contribute towards the observed competition in this study, especially as bacteriocins mostly target closely related species (Holtsmark *et al.*, 2008).

Interestingly, *in vitro,* Shyntum *et al.* (2018) observed killing of *Pa, Pcc, Dd*, and other species by *Pcb1692*, however, saw no competitive reduction of *D. chrysanthemi*. Data from this study shows that *in planta* (in leaves) wild type *Pcb1692* significantly contributes towards bacterial killing of *Dc* (Figure 2.2 B). This signifies that the host environment is an important factor contributing towards bacterial competition. Importantly, data from this study as well as from Shyntum *et al.* (2018) indicate that tissue type plays an important role in determining the contributing competitive mechanisms – in this study the T6SS does not contribute significantly towards bacterial competition in potato leaves, whereas *Pcb1692* significantly outcompeted *D. dadantii*, *D. chrysanthemi*, and *Pcc* in potato tubers (Shyntum *et al.*, 2018). Shyntum *et al.* (2018) noted that in potato tubers iron levels are a major contributor towards the competitive ability of *Pcb1692*. Nutrient content can differ markedly between different tissue types, which

may account for the variability in competition observed (Walworth & Muniz, 1993). Durrant (2016) noted that variable nutrient levels affect antibiotic production.

A number of the abovementioned competitive strategies may be responsible for the observed decrease in target species' titres. Although the T6SS proves to be very effective in many other species, this is not the case *Pcb1692* in potato leaves. For species such as *P. aeruginosa*, the T6SS assumes potent antibacterial capabilities in many different environments, whereas in *Erwinia amylovora*, the T6SS takes on a secondary role and marginally contributes towards virulence (Kamber *et al.*, 2017). Also, instead of reducing competitor viability directly, the T6SS of *E. amylovora* contributes to competition by affecting exopolymeric substance production. It is clear to see that the T6SS contributes towards bacterial competition variably, depending on the species.

Given the lack of antibacterial activity in leaves imparted by the T6SS in this study, it was of interest to determine the T6SS effector repertoire in *Pcb1692*, and to determine whether the T6SS-mediated competition observed by Shyntum *et al.* (2018) could be due to the presence of bacteria-targeting effectors in the genome. A bioinformatic approach was used to identify these T6SS effectors. The set of bacterial-targeting effectors provides insight into the effect of the T6SS in bacterial competition observed in these *in planta* competition assays.

Bioinformatic analyses indicated that only one type 6 secretion system cluster with all thirteen core genes is present in *Pcb1692*, suggesting that all five putative effectors identified are dependent on this particular T6SS system (Figure 2.3). In literature, a number of methods are employed to identify effector genes; however, a key difficulty in identification is that T6SS effectors have no characteristic motifs or secretion signal (Liang *et al.*, 2015). Thus, the approach used to identify effectors in this study made use of first locating genes that are consistently associated with type 6-dependent effectors (Alteri & Mobley, 2016). Hcp and VgrG genes are often found dispersed throughout the genome and used as indicators to locate T6SS effectors (Barret *et al.*, 2011, Hachani *et al.*, 2014, Ma *et al.*, 2018). It has been well

established that effectors downstream of Hcp and VgrG genes are T6SS effectors (Ma *et al.*, 2017). Hcp, VgrG, PAAR, and chaperone proteins are mainly used in this type of approach.

PAAR-bearing proteins make use of the PAAR domain to interact with VgrG to form a sharp tip, and a vast number of PAAR proteins contain C-terminal toxin domains (Shneider *et al.*, 2013, Unterweger *et al.*, 2017). A variety of effector toxin domains are associated with N-terminal PAAR domains including nucleases, phospholipases, and Rhs toxins (Sana *et al.*, 2016). *Pcb* encodes three PAAR genes each located in a different genomic region. The first is small and lacks a C-terminal toxin domain, and the other two are specialised effectors. The PAAR-encoding gene, *AED_0006105* (renamed PAAR2), has no additional domains and is located in HSI-2. This protein most likely serves in a structural capacity to complete the T6SS apparatus. Given its association with HSI-2 (which is distantly located from the core genes) and using the cognate VgrG in HSI-2, PAAR2 may bridge the connection of effectors in HSI-2 with those from other loci.

The first specialised PAAR-containing effector in *Pcb1692* (*AED_0003357*; Phos) is located in HSI-1 (Figure 2.3 A) and encodes a C-terminal alpha/beta hydrolase domain with a GXSXG catalytic motif. Enzymes of the alpha/beta hydrolase family have a G-X-nucleophile-X-G catalytic motif and are active on diverse substrates (Holmquist, 2000). In lipases, the nucleophile is represented by a catalytic serine residue. Given the high similarity of this gene to *P. atrosepticum* phospholipase (ECA3426) (90% similarity; Figure 2.4) and the presence of the PAAR domain, this gene was presumed to encode a type 6 effector, and specifically a phospholipase effector, as type 6-dependent lipase-targeting effectors have been shown to be phospholipases (Russell *et al.*, 2013)

Some phospholipases are only active against prokaryotes, such as Tle2 (TseL) from *V. cholerae* V5. Tle2-deficient strains lose most of their ability to outcompete *E. coli* (Russell *et al.*, 2013). PldA (Tle5) from *P. aeruginosa* is secreted by the H2-T6SS and was shown to have antibacterial activity against *Ps. putida* (Russell *et al.*, 2013), as well as

interacts with its eukaryotic host through Akt binding, showcasing the ability of effectors to target both eukaryote and prokaryote (Sana *et al.*, 2015). In addition to degrading phospholipids, phospholipases may also be involved in signalling in the host (Jiang *et al.*, 2014). The role of Phos in *Pcb1692* is still unknown, and the Tle group designation has also not yet been identified. Given that Phos contains a GXSXG motif indicates that it does not belong to Tle5, which are always PLD enzymes characterised by two HxKxxxxD motifs (Russell *et al.*, 2013). Thus, Phos is expected to have either PLA1 or PLA2 activity. Whether Phos is also involved within the plant host is also unknown.

The gene following Phos in *Pcb1692* (*AED_00002726*; Ank) contains four ankyrin repeats. The ankyrin domain is the most common protein-protein interaction domain, and in bacterial contexts, it is often delivered by protein secretion systems into eukaryotic cells to interfere with host functions (Al-Khodor *et al.*, 2010). This gene is found downstream of the putative phospholipase in both *Pcb* and *P. atrosepticum* and also share considerable similarity (Figure 2.4). Zhang *et al.* (2012) characterised a large number of novel toxins and immunity domains in bacteria using comparative genomics. They found that the ankyrin domain was present in a wide range of proteins including restriction endonuclease-like toxins, papain-like peptidases, and most notably, also found in immunity proteins of toxin systems. Ankyrin-containing immunity proteins were especially associated with toxins carrying the AHH nuclease domain, and are widely distributed throughout firmicutes, γ-proteobacteria, and planctomycetes (Zhang *et al.*, 2012). Consistent with their findings, the genomic location of Ank relative to the putative phospholipase toxin gene, indicates that Ank is the dedicated immunity protein for Phos.

The second specialised PAAR effector in *Pcb1692* (AED_0006270; Rhs1) lies in HSI-4 and is annotated as an Rhs protein. Indeed, sequence analysis with InterPro indicates that this gene possesses the typical YD/Rhs repeats. Rhs proteins are usually very large, ranging from approximately 1500 – 2000 residues, with a polymorphic toxin domain in their C-terminus, and in *Enterobacteriaceae,* the toxin domain is delimited by the PXXXDPXGL motif (Hayes *et al.*, 2014). Like other type 6-associated Rhs effectors, Rhs1 in *Pcb1692* has an N-terminal PAAR

domain, it is characteristically large (1449 residues), and possesses a toxin domain in the C-terminus demarcated by the PXXXDPXGL motif (Jiang *et al.*, 2018). Rhs1, like Rhs effectors Tke2 and Tke4 from *Ps. putida* (Bernal *et al.*, 2017) and RhsP1 and RhsP2 from *P. aeruginosa*, possesses an RVXXXXXXXXG motif. To date, the aforementioned citations are the only instances where this motif is mentioned. No information exists about the significance of this motif, except that its presence was mentioned in RhsP1, RhsP2, Tke2, and Tke4, and it seems to be situated in the N-terminal domain. In Rhs1, this motif occurs twice and sequence analysis of *Dickeya dadantii* RhsA, RhsB, and RhsC indicated that this motif is also present in all three Rhs proteins and occurs twice in RhsA and RhsB and once in RhsC. Bearing in mind that C-termini of Rhs proteins are polymorphic, the sequence analysis of *Pcb1692* and *Dd* Rhs proteins indicates that this motif is not associated with a particular toxin domain.

The toxin domain of Rhs1 is demarcated by the PXXXDPXGL motif, however, no identifiable domain was found; thus, the biochemical activity of this Rhs protein remains unknown. The downstream gene, *AED-0004232*, contains no identifiable domains, however, an associated immunity gene always resides adjacent to Rhs toxins; thus, *AED-0004232* (renamed Rhs1i) encodes the cognate immunity protein for Rhs1 (Koskiniemi *et al.*, 2014). Thus, the genetic neighbourhood, as well as the N-terminal PAAR domain, indicates that Rhs1 (AED_0006270) is a type 6-associated effector with a downstream cognate immunity protein, Rhs1i.

The first gene downstream of VgrG in HSI-2 in *Pcb1692* (*AED_0003811*) is unannotated. Sequence analysis indicates that it contains a DUF4123 domain that is found in many species. Thus far, DUF4123, DUF1795 (renamed EagR), and DUF2169 have been implicated as chaperones for type 6 effectors (Russell, 2014, Unterweger *et al.*, 2017). Chaperone (also called accessory or adaptor) proteins are not essential for T6SS activity, however, they are crucial for the delivery of some type 6-dependent effectors by mediating their interaction with type 6 translocon (Alcoforado Diniz *et al.*, 2015). Unterweger *et al.* (2015) showed that T6SS adaptor protein 1 (Tap-1) like VasW, which contain a DUF4123 domain, was necessary

for the secretion of *V. cholerae* effector TseL. The DUF4123 domain is responsible for the translocation of diverse effectors, including hydrolases, nucleases, lipases, and colicins, by loading them onto the T6SS tip through specific interactions (Liang *et al.*, 2015). It is hypothesised that the DUF4123 domain in *Pcb1692* is responsible for the translocation of the WHH-nuclease effector and possibly the D123 protein (if it proves to be a type 6 effector). Furthermore, it is unknown whether this DUF4123 protein is limited to translocation of effectors within HSI-2 (Figure 2.3 B) or is able to promote translocation of effectors from other HSIs. As effectors often show secretion specificity for a VgrG upstream thereof, likewise, this DUF4123 protein may also be dedicated to secretion of HSI-2 effectors only. In many cases, studies have shown that the gene upstream of DUF4123 genes are either VgrG or PAAR genes and effectors are often located downstream thereof (Unterweger *et al.*, 2017). Consequently, the DUF4123 domain has also be used as a marker for identifying effectors downstream of it (Lien & Lai, 2017). Likewise, the chaperone protein DUF1795 is also commonly associated with the T6SS.

DUF1795, renamed EagR, is often associated with the delivery of Rhs effectors and PAAR-domain effectors (Koskiniemi *et al.*, 2013, Alcoforado Diniz & Coulthurst, 2015, Cianfanelli *et al.*, 2016, Bernal *et al.*, 2017, Ma *et al.*, 2017). No DUF1795 domain was identified in *Pcb1692* in this study. Interestingly however, the gene upstream of Rhs1 in HSI-4 (Figure 2.3 D), *AED_0003068*, is annotated DcrB and has no clear function with the T6SS. DcrB is found in many enterobacteria. Recently, the structure of DcrB in *Salmonella enterica* was solved, and it was found to have a DUF1795 domain (Rasmussen *et al.*, 2018). In *E. coli*, DcrB is situated in the periplasm and together with BtuB and DcrA mediate phage C1 adsorption and DNA injection by forming openings in the membranes (Samsonov *et al.*, 2002). No connection to the T6SS has been made, although it was noted that DcrB was upregulated by the two-component regulator, PhoP, in *E. coli*. The PhoP-PhoQ two-component system has been implicated in T6SS regulation in *Edwardsiella* sp. (Miyata *et al.*, 2013). In *Pcb1692*,

no genes equivalent to BtuB and DcrA were found in these HSIs and the function of DcrB in *Pcb1692* remains unknown.

Immunity genes are generally located directly downstream of the associated effector; however, this is not always the case. For example, the immunity gene Tsi1 in *P. aeruginosa* lies upstream of effector Tse1 (Hood *et al.*, 2010, Russell *et al.*, 2011). In *Pcb1692*, a hypothetical gene lies on either side of the WHH nuclease (Figure 2.3 B); thus, either could potentially function as the cognate immunity gene. Sequence analysis indicates that the gene upstream of the WHH nuclease is a hypothetical gene with no identifiable domains and no linkage to the WHH nuclease could be identified; whereas analysis of the downstream gene (*AED_0003808*; SUKH) indicates that this gene encodes a SUKH Knr4/Smi1-like domain. SUKH domains are found in both eukaryotic and bacterial species, however, in bacterial contexts, these domains are associated with immunity genes of contact-dependent toxins (Zhang *et al.*, 2011). Specifically, SUKH immunity genes protect from nucleases and nucleic acids deaminases. SUKH-containing immunity genes are often associated with CDI toxins, with HNH nuclease superfamily domain proteins, and also with YD repeat proteins. In all cases, the linkage of SUKH domains with WHH domains occurs in a non-CDI context. Comparative with Zhang *et al.* (2011) findings, the SUKH gene in *Pcb1692* is associated with a putative nuclease (WHH). Therefore, given the linkage of SUKH to a diversity of nucleases, especially to HNH-like domains (such as WHH and AHH), AED_0003808 (SUKH) is the most probable cognate immunity protein for the WHH nuclease.

Downstream of Rhs1/Rhs1i in HSI-4 lies another putative effector-immunity pair (*AED_0004231-AED_0004230*). Similar to the WHH nuclease, *AED_0004231* encodes an HNH/Endo VII nuclease fold domain defined by an AHH catalytic motif. This motif was newly identified together with the WHH, LHH, NucA, DH-NNK and GH-E motifs, which all belong to the HNH/Endo VII nuclease fold (Zhang *et al.*, 2011). AHH domains are widely represented among α,β,γ,δ, and ε-Proteobacteria, other bacterial phyla, eukaryotes, and viruses (Zhang *et al.*, 2012). Like LHH and WHH domains, the AHH domain is associated with contact-

dependent inhibition systems (Zhang *et al.*, 2011). T6SS-associated AHH domain-containing genes have been identified in *Vibrio parahaemolyticus* and *Vibrio antiquaries* (Salomon *et al.*, 2014, Jiang *et al.*, 2018). In both instances, the effectors displayed toxic effects in *E. coli* and toxicity could be ameliorated by expression of cognate immunity proteins. AHH toxins have also been identified in substrates of the T4SS of *Xanthomonas citri* (Souza *et al.*, 2015). Characterisation of AHH toxins associated with the T6SS in other species strengthens the findings that the AHH-containing gene in *Pcb1692* is indeed a type 6 effector. AHH nucleases of *V. parahaemolyticus* and *V. antiquaries* were inhibited by Imm11 and ImmAnk immunity proteins (Jiang *et al.*, 2018), respectively, indicating that various immunity proteins are able to neutralise AHH toxins. Examination of the gene downstream of the AHH nuclease (*AED_0004230*) in *Pcb1692* shows that it contains no identifiable domains. Since AHH-domain-containing proteins are toxic nucleases, the producer (*Pcb1692*) needs to be protected from self-intoxication. Thus, although the downstream gene has no domain that classifies it as an immunity gene, the inherent nature of the AHH nuclease upstream thereof requires that this gene encode a cognate immunity protein, and subsequently renamed AHHi.

The final non-structural T6SS-associated gene identified lies downstream of the SUKH immunity gene in HSI-2 (Figure 2.3 B). Sequence analysis of AED_0003807 (renamed D123) indicates that it belongs to the CDC (cell division cycle) 123 family. In *Saccharomyces cerevisiae*, CDC123 regulates the abundance of Chf proteins and thereby regulates the transition from G1 to G2 cell cycle phase (Bieganowski *et al.*, 2004). CDC123 is a highly conserved gene present in most eukaryotes, yet bacterial homologues were recently identified (Burroughs *et al.*, 2015). In this study, it was shown that CDC123 belongs to a novel superfamily of ATP-grasp enzymes, which could be further divided into three families (R2K.1, R2K.2, R2K.3), with most bacterial representatives clustering in the R2K.1 clade. It is speculated that proteins in this family catalyse peptide bonds. Many bacterial R2K.1 entities are linked to loci encoding polymorphic toxins, and specifically with those encoding SUKH immunity genes (Burroughs *et al.*, 2015); however, the significance hereof is unknown.

Interestingly, *Sagitella stellata* encodes the precise genomic arrangement of *Pcb1692* HSI-2. One suggestion is that these genes may act as a minor toxin or a regulator of the adjacent toxin, immunity protein or T6SS apparatus. This protein is not expected to be a bacterial toxin as it does not conform with effector-immunity gene arrangement and lacks a cognate immunity gene beside it. Thus, D123 may play a regulatory or chaperone-like role, as suggested, or it could act within the plant host as a eukaryotic-targeting effector.

Antibacterial effectors are always accompanied by an immunity protein to protect producing cells from self-intoxication (Yang *et al.*, 2018). As the four of the five identified effectors appear to be bacteria-targeting, based on effector-immunity gene configuration and toxin domain analysis, it was surmised that the T6SS of *Pcb* functions mainly in an antibacterial fashion. To further investigate the interbacterial role of the T6SS of *Pcb*, *AED_0003357* [Phospholipase (Phos)], *AED_0006270* (Rhs1), *AED_0003810* (WHH nuclease), *AED_0004231* (AHH nuclease), and *AED_0003807* (D123 protein) were ectopically expressed in *E. coli* as putative antibacterial effectors.

Many T6SS effectors have been identified that are able to target eukaryotes, bacteria, or both (Pukatzki *et al.*, 2007, Russell *et al.*, 2011, Jiang *et al.*, 2016). Antibacterial effectors have been generally divided into three main categories depending on the cellular target: peptidoglycan-, cell membrane-, and nucleic acid-targeting effectors. However, additional effector targets are gradually being discovered. In 2011 and 2014, respectively, colicin-like pore-forming effectors have been identified in *Vibrio cholerae* (VasX) and *P. aeruginosa* (PA14_69520) (Miyata *et al.*, 2011, Hachani *et al.*, 2014). In 2015, another class of antibacterial effector was discovered targeting vital dinucleotides $NAD(P)^+$ (Whitney *et al.*, 2015). Soon thereafter, the elusive function of Tse2 from *P. aeruginosa* was linked to its NAD dependence (Robb *et al.*, 2016); and recently Tne2 from *Ps. protegens* was characterised as a $NAD(P)^+$ glycohydrolase (Tang *et al.*, 2018).

In *Pcb1692*, two putative effectors are classified as nucleic acid-targeting, one is cell membrane-targeting, and the remaining two effectors are unknown. Of the five putative effectors identified, only the AHH nuclease displayed growth inhibition during *in vitro* ectopic expression assays (Figure 2.12). Although individual expression of the AHH nuclease inhibited the growth of *E. coli* (Figure 2.12 C), it was not as considerable as the inhibition mediated by RhsA from *D. dadantii* (Figure 2.12 A). This demonstrates that the degree of antibacterial activity differs between effectors. Nonetheless, the AHH nuclease effector clearly demonstrates antibacterial activity. Simultaneous expression of the effector and immunity gene (AHH+i) initially restored growth of *E. coli* (Figure 2.12 C), confirming that the observed reduction in growth of the effector-alone expression was indeed due to bacterial growth inhibition. However, upon prolonged simultaneous expression of these two genes (AHH+i), growth inhibition, characterised by a plateau in the growth curve, ensued. This sudden inhibition may be due to the deleterious effects of protein overproduction.

A number of factors may contribute towards such deleterious effects. Heterologous protein production introduces foreign proteins into the cell and may interfere with cell processes. The cost of heterologous protein production may cause a metabolic burden on the cell, which may manifest as a reduced growth rate (Sauer, 2001, Moriya, 2015). Thus, although the toxic effects of AHH expression are initially eliminated by conjunct AHHi expression, the excessive protein production over prolonged periods may hinder normal cellular processes, and thus, a plateau is observed. Moreover, the rapid overexpression of foreign proteins may lead to the formation of inclusion bodies, which is also promoted by growth at higher temperatures (Fakruddin *et al.*, 2013). Temperatures are often reduced to 15-25°C during protein expression for this reason. These expression assays were conducted at the optimal growth temperature for *E. coli*, at 37°C, thus inclusion body formation is probable.

An excessive amount of heterologous proteins in the cell may also contribute towards a reduction in growth. Such high levels in these assays may be attained with additional expression from pTrc99A, as an 82X fold increase in protein production occurs after induction

of pTrc99A (Warren *et al.*, 2000). Thus, to determine whether the plateau was caused due simultaneous induction and expression of proteins, L-arabinose and IPTG inducers were omitted, which then restored normal growth to the *E. coli* strain bearing both plasmids [AHH+I (U)] (Figure 2.12 C). Altogether, it can be concluded that excessive protein production is harmful for cellular growth of *E. coli* expressing both AHH and AHHi, and although the precise contributing factors towards growth inhibition with simultaneous effector and immunity expression were not established, omission of inducers indicate that cellular growth is returned to normal. Thus, co-expression of the immunity factor with the AHH effector indicates that AHH indeed has toxic effect on cell growth in the absence of the cognate immunity protein.

The results, both *in planta* competition and *in vitro* expression, indicate a lack of antibacterial activity for the T6SS of *Pcb1692* in potato leaves, but not in potato tubers. Although T6SS antibacterial effector have been identified, *in vitro* results indicate that they do not contribute towards bacterial growth inhibition when expressed singly, except for the AHH nuclease. In *V. parahaemolyticus*, deletion of piocinS/colicin DNase and AHH nuclease effectors did not impede interbacterial killing capacity (Salomon *et al.*, 2014). Similarly, in *Vibrio cholerae* VC1418 the killing ability of single-effector deletion mutants was comparable to wild-type cells, indicating that these effectors do not contribute significantly to bacterial killing on their own (Dong *et al.*, 2013). The state of activity of the T6SS also affects the effectivity of its secreted effectors. Converse results were obtained when the same effector was deleted in *V. cholerae* VC1418 (tightly regulated T6SS) and in *V. cholerae* V52 (Dong *et al.*, 2013, Russell *et al.*, 2013). Deletion of this gene in the constitutive strain V52 almost failed to kill the competitor (Russell *et al.*, 2013). This means that singular activity of effectors varies among species and may not contribute significantly towards bacterial killing and that the regulation of a specific T6SS also affects the outcome of specific effector secretion. In contrast to these *in planta* assays, bacterial killing via the T6SS by *Pcb1692* was observed *in planta* in potato tubers (Shyntum *et al.*, 2018). It may be that a concerted effort of the identified effectors has a synergistic effect on bacterial growth inhibition. Clearly, tissue type plays an important role in

determining the activity of the T6SS, as in tubers the T6SS significantly competes against a number of species, whereas none are significantly impacted in potato leaves. *In vitro* expression results suggest that *Pcb1692* bacterial targeting effectors do not contribute much towards bacterial growth inhibition. Comparative to the T6SS of *E. amylovora* (Kamber *et al.*, 2017), there may be a possibility that the bacterial inhibition observed *in planta* in potato tubers may be indirectly mediated.

The specificity of vehicle selection by effectors is important for their secretion; however, type 6 effectors have no secretion signal directing them towards the T6SS. Instead, type 6 effectors are covalently linked to Hcp/VgrG or interact with Hcp/VgrG either directly or through a chaperone protein to be secreted (Alcoforado Diniz *et al.*, 2015). Thus, type 6-dependent effectors make use of physical interactions with structural components of the translocon to mediate their secretion. When multiple Hcp or VgrG proteins are associated with a T6SS, effectors often show a preference for interaction with the Hcp/VgrG upstream of it (Silverman *et al.*, 2013, Cianfanelli *et al.*, 2016). *Pcb1692* encodes three VgrG proteins and five Hcp proteins. By forming a heteromeric VgrG tip, effectors from multiple HSIs can be transported in a single firing event using this VgrG specificity.

Interestingly, DNase effector ET1 of *E. coli* STEC004 not only requires a cognate Hcp, but a heteromeric Hcp tube composed of Hcp2A, Hcp2B, and Hcp/DUF796 for its delivery; deletion of any one of these Hcps abolishes ET1-dependent toxicity (Ma *et al.*, 2017). The association of effectors to their conveyance out of the cell is specifically mediated: examination of binding specificity of *P. aeruginosa* Tse2 ($Tse2^{PA}$) to Hcp1 indicated that $Tse2^{PA}$ interaction was limited to specific residues within the Hcp ring (Silverman *et al.*, 2013). Moreover, such interactions were not limited to this effector nor to this species: similar results were observed for $Tse1^{PA}$ and $Tse3^{PA}$, as well as for *Methylomonas menthanica* Tse2 homologue (Silverman *et al.*, 2013). In some cases, effectors do not have a strict dependence on a specific VgrG at all; for example, Ssp1 and Ssp4 from *Serratia marcescens* are delivered by either VgrG1 or VgrG2 (Cianfanelli *et al.*, 2016). Thus, effector specificity to cognate VgrG or Hcp proteins is not

limited to a few closely related species and is observed in many bacterial species. Consequently, it can be assumed that the effectors located downstream of a VgrG or Hcp gene in *Pcb1692* are dependent on translocation by the VgrG/Hcp upstream of it.

The specificity of Hcp/VgrG dependence has not been established for many effectors, nor for the effectors identified in this study; however, all effectors require that the T6SS is assembled with functional VgrG and Hcp. Thus, the homo- or heteromeric arrangement of VgrG and Hcp influences the payload of a single firing event, and essentially effectors that show no preference may be secreted at all times. It thus follows that if Hcp-secretion islands are differentially activated in response to various stimuli, the type 6 effector repertoire may differ depending on the environment. This is especially significant considering that the T6SS contributed towards bacterial growth inhibition in potato tubers, but not in potato leaves. It may be that induction conditions for the T6SS in *Pcb1692* differ in different tissues types.

Due to specific reliance on cognate Hcp or VgrG proteins for secretion, a heteromeric T6SS shaft allows for effectors from different HSIs to be secreted simultaneously. This is an important consideration, as these ectopic expression assays examined the antibacterial capacity of effectors expressed individually. Thus, given the lack of a killing phenotype for all but one effector, it may be necessary that these effectors act synergistically to effect an observable phenotype. Alternatively, the *in vitro* conditions used in this study may not be conducive for observable growth inhibition. In a recent study, LaCourse *et al.* (2018) have shown in *P. aeruginosa* that effectors can function synergistically as well as conditionally. Conditions that contribute to effector activity and efficacy include pH, temperature, salinity and O_2 availability. Variable conditions caused as much as a sevenfold increase in activity of one effector and decreased the activity of another by half. It was also observed that the synergistic activity of two effectors exceeded the sum of their individual activity. Another example of environmental influences on the performance of effectors can be seen from Tse1 and Tse3 from *P. aeruginosa* which target peptidoglycan. Their toxicity may be ameliorated by

increments in medium osmolarity (Russell *et al.*, 2011). Therefore, it is very likely that conditions in this study are not conducive for T6SS activity.

Furthermore, it has been recently demonstrated that some effectors may need to undergo post-translational modification to become enzymatically active. Ectopic expression of full-length RhsP with a WHH-nuclease domain has no toxic activity unless the inhibitory N-terminus is removed upon delivery through the T6SS apparatus by auto-proteolysis (Jiang *et al.*, 2018). It is important to note, that although the C-terminal toxin domain is inhibited by the N-terminus of RhsP, the C-terminal toxin domain is also inhibited by its cognate immunity protein encoded by RhsPi to protect sister cells from intoxication. No additional domains were detected in the effector genes identified from *Pcb1692*, therefore, post-translational modification such as the above example may not be probable, but modification cannot be excluded altogether.

Although a number of factors may contribute towards the lack of growth inhibition observed in both *in planta* and *in vitro* assays, assumption that the T6SS of *Pcb* is mainly antibacterial may need to be reviewed, even considering the fact that Shyntum *et al.* (2018) observed T6SS-mediated growth inhibition. Although the T6SS contributed towards bacterial killing, it may not be the only role or the major role of the T6SS in *Pcb1692*. In potato leaves, T6SS activity may play a supplementary role, if not absent. Bernal *et al.* (2018) have noted that thus far, no plant-targeting effectors have been identified. A key difficulty in identifying effectors is that T6SS effectors have no recognisable secretion signal or motifs (Liang *et al.*, 2015). Therefore, it may be possible that a number of effectors are still to be identified. As the D123 protein has no identifiable function or downstream immunity protein, this protein could be a candidate for a plant-targeted effector. Given that some effectors have shown the capacity for inter-kingdom activity, it may be necessary to re-evaluate whether the effectors we have identified as putative antibacterial effectors may have key functions within the eukaryotic context. Additionally, a recent report has described the first account of antifungal effectors delivered by the T6SS of *Serratia marcescens* (Trunk *et al.*, 2018).

Importantly, the T6SS has also been associated with activities that interact with the environment. When manganese levels are low, *Burkholderia thailandensis* secretes a manganese-chelating effector (TseM) through T6SS-4 to scavenge Mn^{2+} in the extracellular space (Si *et al.*, 2017). The T6SS has also been implicated in biofilm formation in a number of species including enteroaggregative *E. coli* (Aschtgen *et al.*, 2008), *Vibrio parahaemolyticus* (Enos-Berlage *et al.*, 2005), *Pseudomonas fluorescens* (Gallique *et al.*, 2017), and *Acinetobacter baumanii* (Kim *et al.*, 2017). *Pcb1692* colonises vascular tissue through biofilm formation. The precise composition of the *Pcb1692* biofilm is currently unknown. Biofilm matrices are composed of various substances including carbohydrates, flagella, adhesins, proteins, and extracellular DNA (Kostakioti *et al.*, 2013). Extracellular DNA (eDNA) is a structural component in many biofilms, which are important for the establishment of many pathogens (Kostakioti *et al.*, 2013, Kumar *et al.*, 2017). Extracellular nucleases are used to modulate the eDNA component of biofilms and can be used to restrain biofilm formation or promote biofilm detachment (Seper *et al.*, 2011, Beenken *et al.*, 2012). T6SS effectors can be found in the supernatant (Basler, 2015); thus, if the T6SS interacts with environmental elements, T6SS nucleases could have an effect on biofilms of competing bacteria.

Using *Pcb1692* effector-deletion mutants for *in planta* potato tuber competition assays conducted by Shyntum *et al.* (2018) would shed much light on the contribution of the identified type 6 effectors in bacterial competition and whether the T6SS is perhaps contributing towards bacterial growth inhibition via another mechanism. If indeed, the type 6 effectors identified in this study are functional, bacteria-targeting effectors, these preliminary findings suggest that the T6SS in *Pectobacterium carotovorum* subsp. *brasiliense* does not play as large a role in antibacterial competition as expected in potato leaves, rather it provides a supplemental function during plant infection. It must also be taken into consideration that this T6SS could have functions other than contact-dependent antibacterial competition.

When assessing the antibacterial capacity of one species against the other, it would be preferable to choose ratios that are representative of the natural occurrences of the species

used. Various soft rot species were used as it has often been observed that they are able to co-infect the same plant. Using too high an attacker-to-target ratio may bias results in favour of the attacker, especially when target species grow significantly slower than attacker species.

The lack of bacterial growth inhibition observed *in planta* in leaves suggests that either the T6SS is not active in this tissue type, or that the T6SS is involved in functions other than bacterial killing. The role of D123 as a bacterial-targeting effector is doubtful, and therefore, it may have another function.

5 Concluding remarks

Pectobacterium carotovorum subsp. *brasiliense* is an emerging pathogen, and combined with its broad host range and highly virulent nature it is a formidable pathogen in the agricultural sector. When any of our research on *Pcb1692* is presented, a recurring question is “how will your research solve this problem”. It is important to remember that before the problem can be dealt with, we need to establish a repository of information that can aid in the development of antagonistic strategies. Understanding what *Pcb* is capable of and what it does in response to various cues is essential for launching a counterattack. Therefore, this study has focused on the building of this repository, advancing the understanding of the role of the T6SS in *Pcb1692*.

This study was conducted as a prior study found that the T6SS of *Pcb1692* is highly upregulated *in planta* in potato tubers. In short, my study has shown that the T6SS of *Pcb1692* is not chiefly involved in bacterial competition *in planta* in potato leaves. Unlike many other studies where the T6SS vigorously outcompetes opposing species, this study has shown that the T6SS assumes a supplemental role in bacterial competition in potato leaves. In the scientific community there is so much focus on the T6SS as a potent antibacterial targeting device; this study drives home that the T6SS does not always take on such an elevated role in target eradiation. Furthermore, five putative T6SS effectors were bioinformatically identified in the genome of *Pcb1692*. These putative effectors were then assessed for antibacterial activity. Only one of the five effectors displayed antibacterial activity. Together with other studies, this study has shown that environmental conditions play an important role in the contribution of the T6SS in bacterial competition; that the T6SS can take on a supplemental role in bacterial killing; and that T6SS effectors differ in their efficacy.

When it comes to answering the question “how will your research solve the problem”, the first thing that needs to be said is that no breakthrough has been made to overcome the ravages of *Pcb1692*; however, valuable insight has been gained that can aid future endeavours of developing a control strategy. The two main contributing points identified by this study are that (1) the effectivity of the type 6 effectors secreted by *Pcb1692* are influenced by environmental

conditions and (2) that the potency of the T6SS differs between species. Considering the environmental influence on effector activity, an approach can be used to identify compounds or conditions which would subvert effector activity and render *Pcb1692* less competitive and less likely to cause disease. Considering T6SS potency, by identifying a bacterial species that is competitively superior to *Pcb1692* and also benign to the agricultural crop, this species may be developed as a suitable candidate for biocontrol.

There is much potential for future study of the T6SS of *Pcb1692*. Effector identification in this study was limited to genes downstream of VgrG/Hcp genes only. Additional effectors that are uninvolved in bacterial targeting may lie unidentified elsewhere in the genome. An ever-increasing amount of information indicates that the T6SS is implicated in a number of roles in addition to bacterial and eukaryotic targeting. It would be interesting to determine whether the T6SS of *Pcb1692* is involved in plant host manipulation. Currently, the identified antibacterial effectors do not display *in vitro* activity. Future studies could elucidate the conditions required for effector activity and also to determine whether these effectors function synergistically. If this is the case, potential therapeutics could target a specific effector instead of all effectors or the entire T6SS.

All in all, this study has shown that in leaves, the T6SS of *Pcb1692* is not a major inhibitor of bacterial competitors. There is no one-size-fits-all role for the T6SS, even in the same species; the environment is a major player in determining the efficacy, and activity of the T6SS. Given the limitation of effector identification, more effectors may be encoded in the genome of *Pcb1692*. Thus, although bacterial competition has taken on a supplemental role in leaf tissue, plant host-targeting effectors may still be active. This study has paved a firm foundation in the study of bacterial targeting by *Pcb1692*. Future endeavours in this field, especially in *Pcb*, include determining the full repertoire of type 6 effectors, determining whether the type 6 effectors function synergistically, the activity of the T6SS in different tissues, and importantly, exploring the possibility of alternative roles for the T6SS, such as plant host targeting or interaction with the environment.

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6. Figures

Figure 1.1

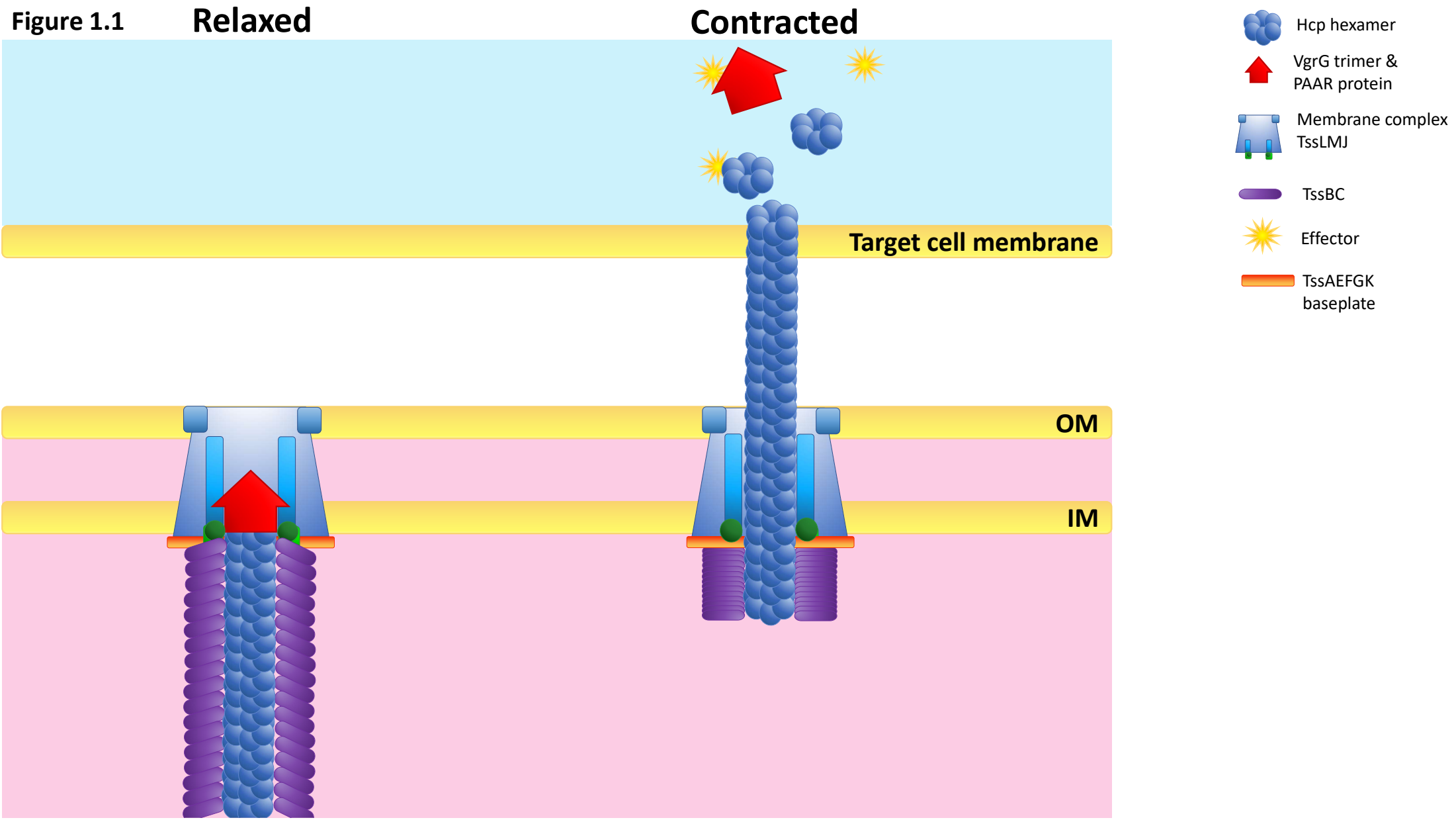

Relaxed
Contracted
Target cell membrane
OM
IM
Hcp hexamer
VgrG trimer &
PAAR protein
Membrane complex
TssLMJ
TssBC
Effector
TssAEFGK
baseplate

Figure 1.2

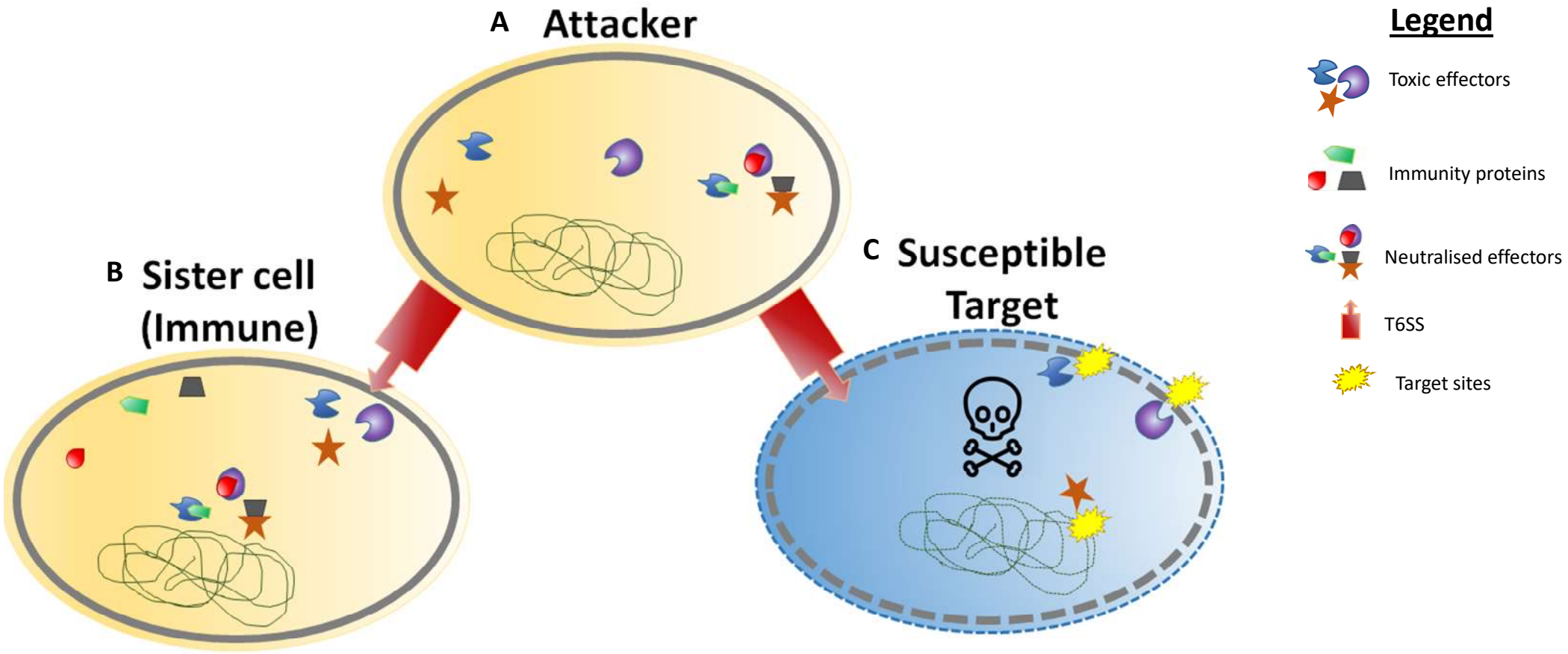

A Attacker
B Sister cell (Immune)
C Susceptible Target
Legend
Toxic effectors
Immunity proteins
Neutralised effectors
T6SS
Target sites

Figure 2.1

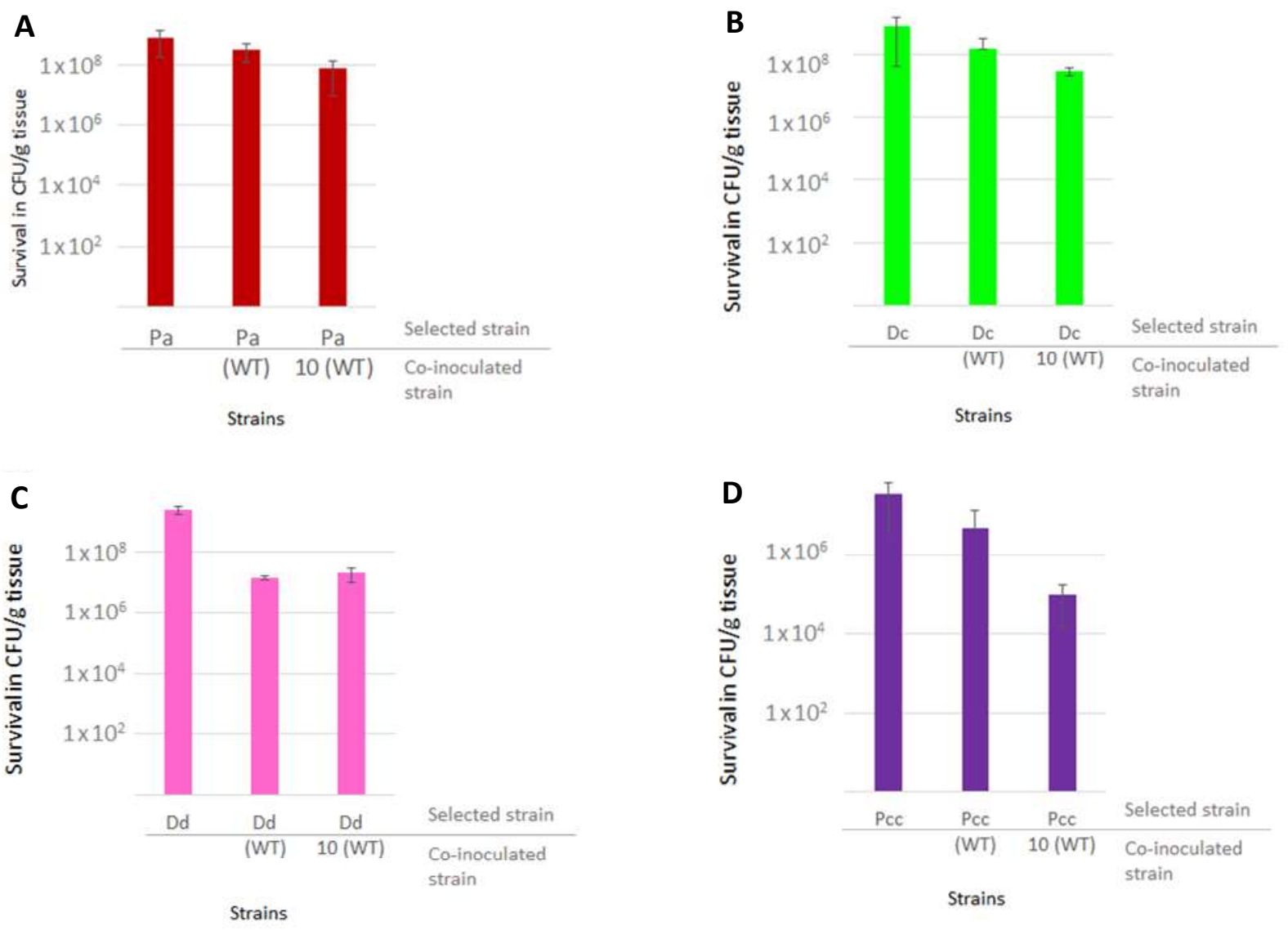
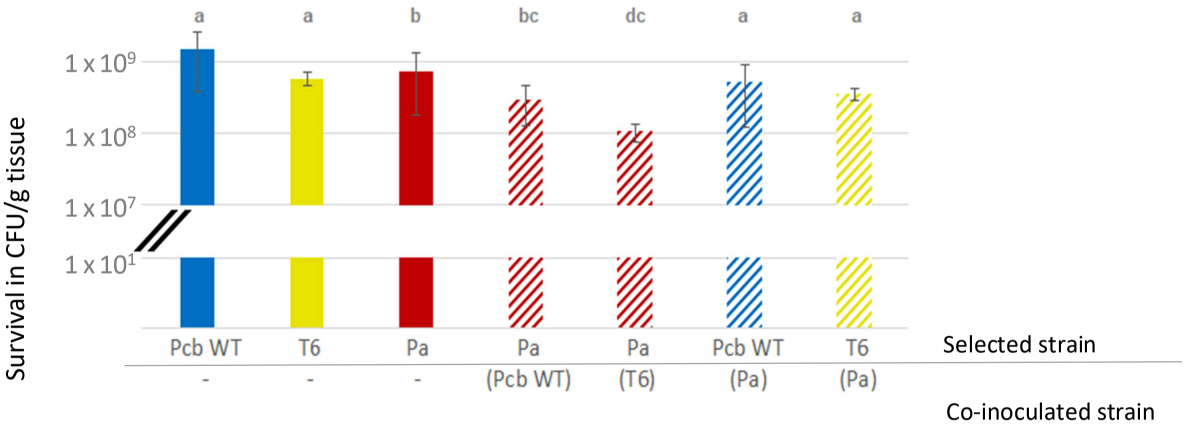

A
Survival in CFU/g tissue
1x10^8
1x10^6
1x10^4
1x10^2
Pa
Pa (WT)
Pa 10 (WT)
Selected strain
Co-inoculated strain
Strains
B
Survival in CFU/g tissue
1x10^8
1x10^6
1x10^4
1x10^2
Dc
Dc (WT)
Dc 10 (WT)
Selected strain
Co-inoculated strain
Strains
C
Survival in CFU/g tissue
1x10^8
1x10^6
1x10^4
1x10^2
Dd
Dd (WT)
Dd 10 (WT)
Selected strain
Co-inoculated strain
Strains
D
Survival in CFU/g tissue
1x10^6
1x10^4
1x10^2
Pcc
Pcc (WT)
Pcc 10 (WT)
Selected strain
Co-inoculated strain
Strains

Figure 2.2

A ***In planta*** **interbacterial competition of *Pectobacterium atrosepticum* against *Pcb1692* and *Pcb1692*_ΔT6SS**



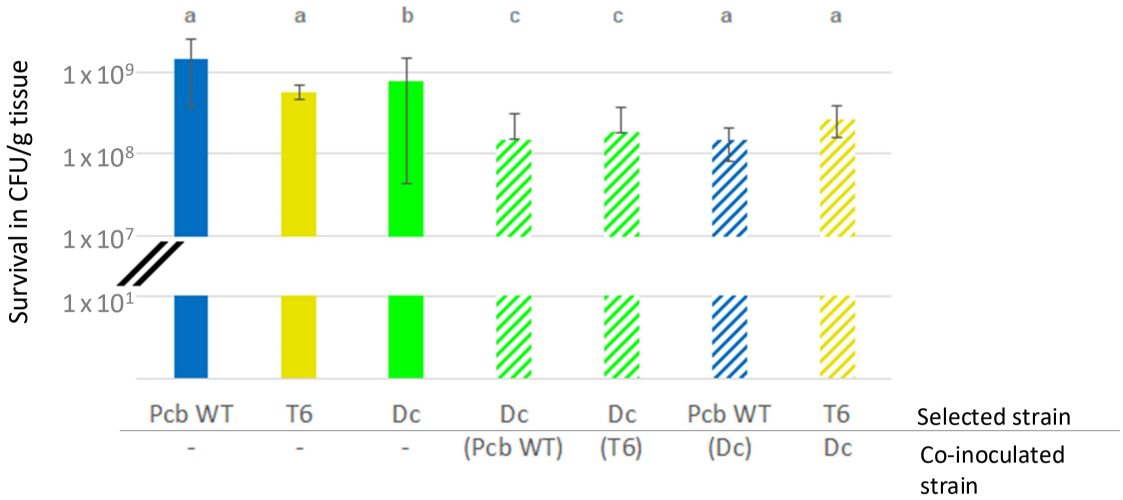
Key:

Pcb1692 (WT)

*Pcb1692*_ΔT6SS (T6)

P. atrosepticum (*Pa*)

B ***In planta*** **interbacterial competition of *Dickeya chrysanthemi* against *Pcb1692* and *Pcb1692*_ΔT6SS**



Key:

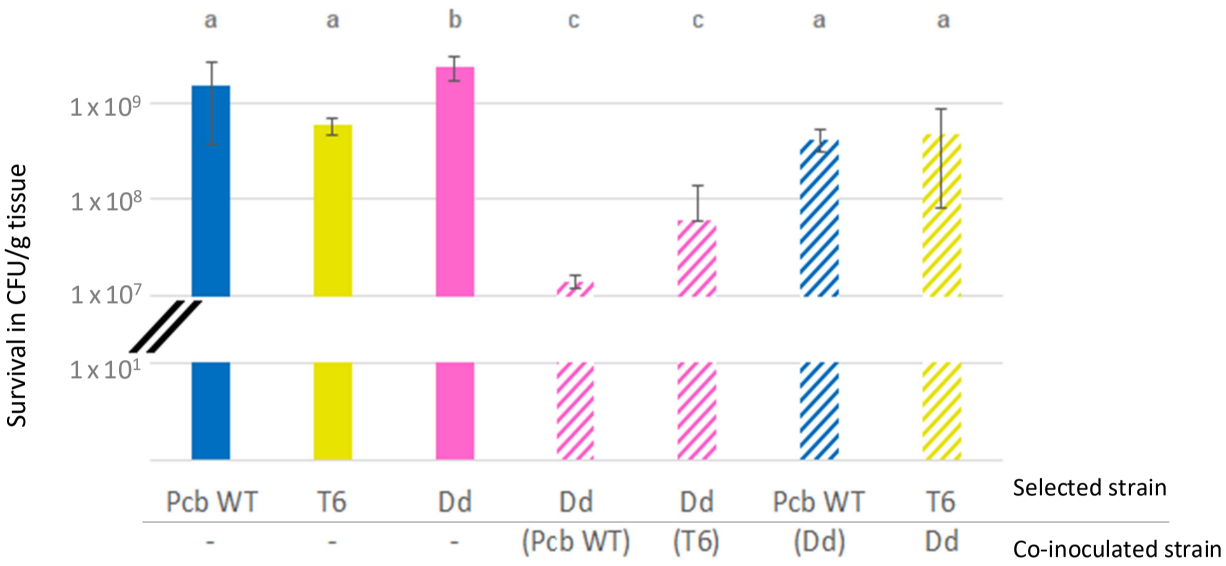
Pcb1692 (*Pcb* WT)

*Pcb1692*_ΔT6SS (T6)

D. chrysanthemi (*Dc*)

Figure 2.2

C ***In planta*** **interbacterial competition of *Dickeya dadantii* against *Pcb1692* and *Pcb1692*_ΔT6SS**



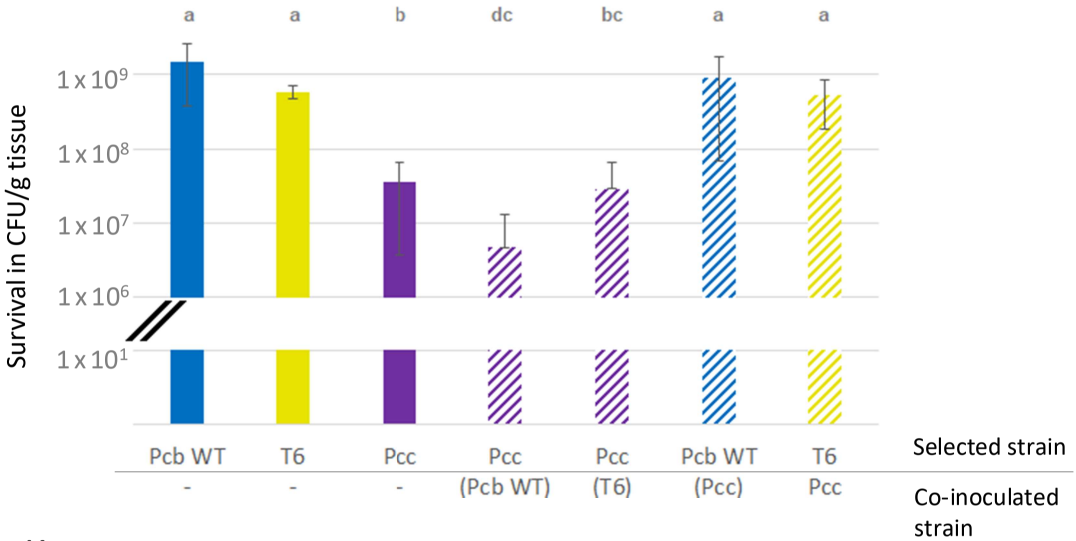
Key:

Pcb1692 (*Pcb* WT)

*Pcb1692*_ΔT6SS (T6)

D. dadantii (*Dd*)

D ***In planta*** **interbacterial competition of *Pectobacterium carotovorum* ssp. *carotovorum* against *Pcb1692* and *Pcb1692*_ΔT6SS**



Key:

Pcb1692 (WT)

*Pcb1692*_ΔT6SS (6)

P. carotovorum ssp. carotovorum (*Pcc*)

Figure 2.3

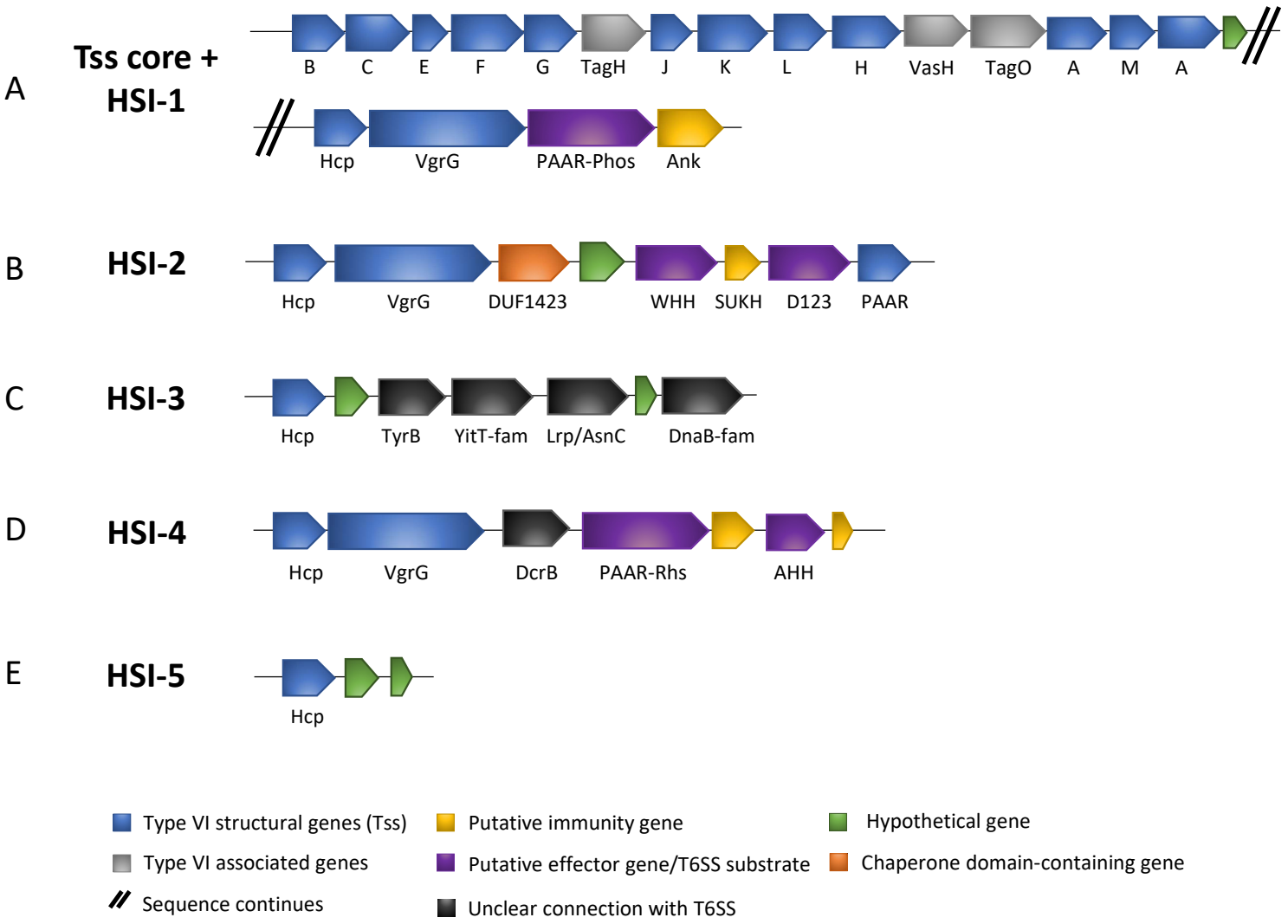
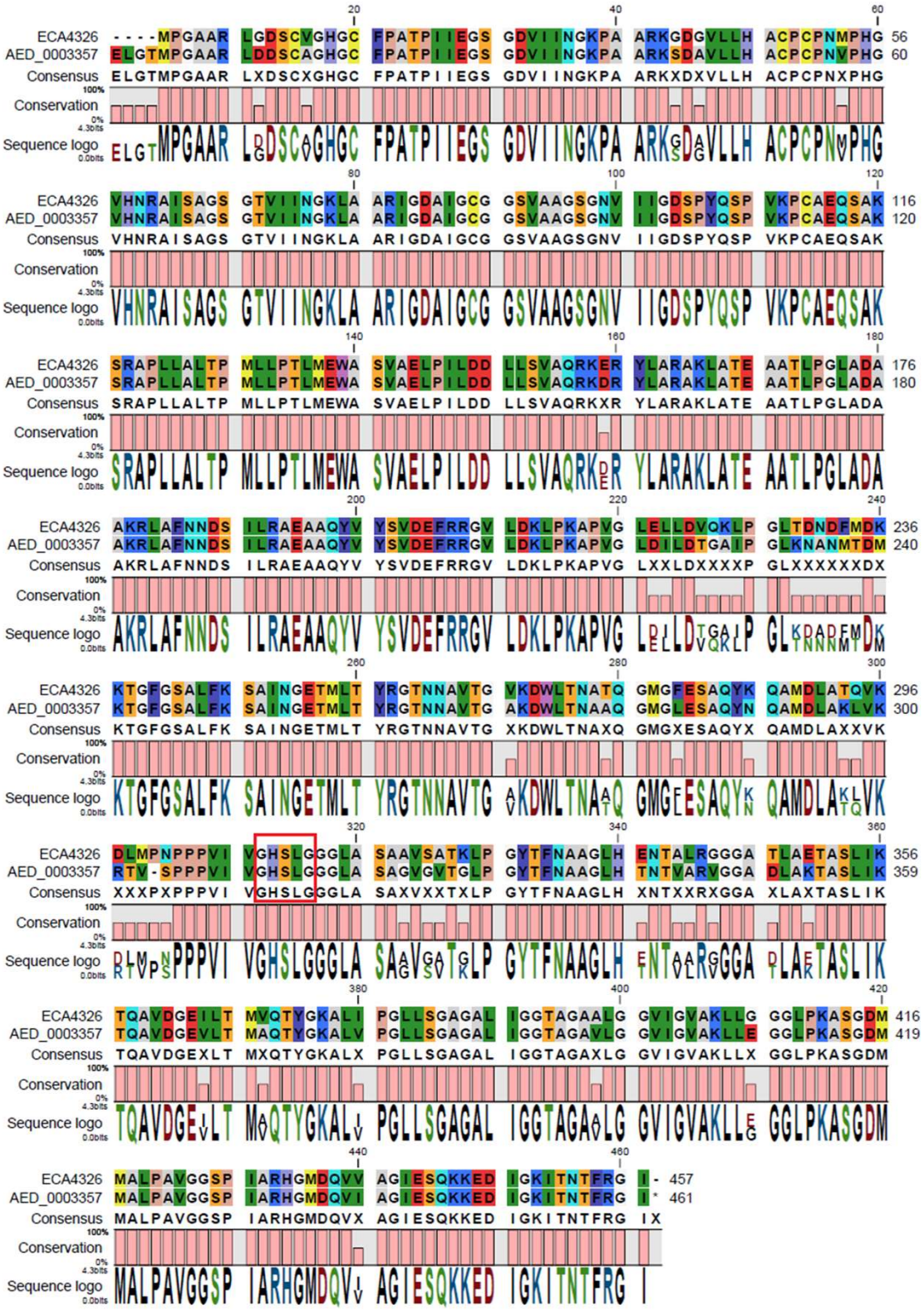

A
Tss core + HSI-1
B C E F G TagH J K L H VasH TagO A M A
Hcp VgrG PAAR-Phos Ank
B
HSI-2
Hcp VgrG DUF1423 WHH SUKH D123 PAAR
C
HSI-3
Hcp TyrB YitT-fam Lrp/AsnC DnaB-fam
D
HSI-4
Hcp VgrG DcrB PAAR-Rhs AHH
E
HSI-5
Hcp
Type VI structural genes (Tss)
Type VI associated genes
Sequence continues
Putative immunity gene
Putative effector gene/T6SS substrate
Unclear connection with T6SS
Hypothetical gene
Chaperone domain-containing gene

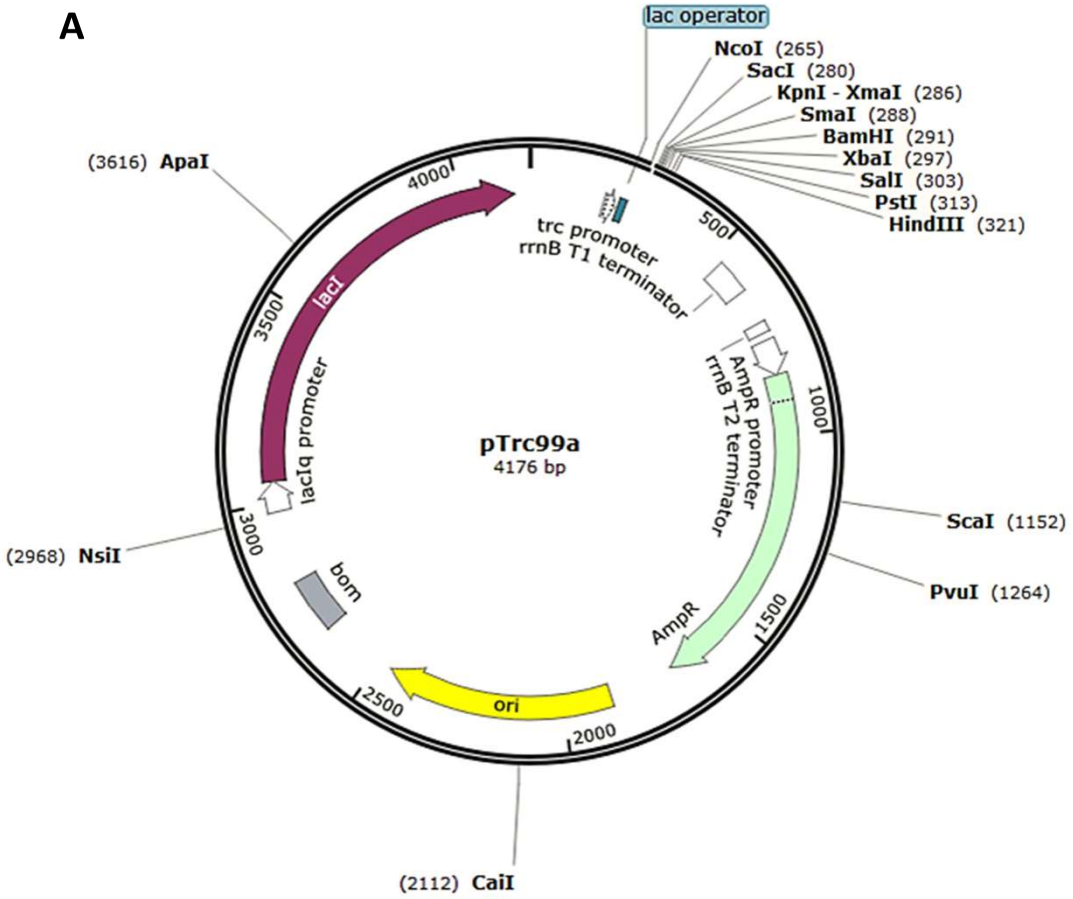
Figure 2.4

Protein sequence alignment of AED_0003357 and ECA4326



Score	Expect	Method	Identities	Gaps
800 bits(2067)	0.0	Compositional matrix adjust.	411/457(90%)	1/457(0%)

Figure 2.5


A
lac operator
NcoI (265)
SacI (280)
KpnI - XmaI (286)
SmaI (288)
BamHI (291)
XbaI (297)
SalI (303)
PstI (313)
HindIII (321)
(3616) ApaI
4000
500
3500
lacI
trc promoter
rrnB T1 terminator
lacIq promoter
AmpR promoter
rrnB T2 terminator
1000
pTrc99a
4176 bp
ScaI (1152)
(2968) NsiI
3000
bom
PvuI (1264)
AmpR
1500
2500
ori
2000
(2112) CaiI

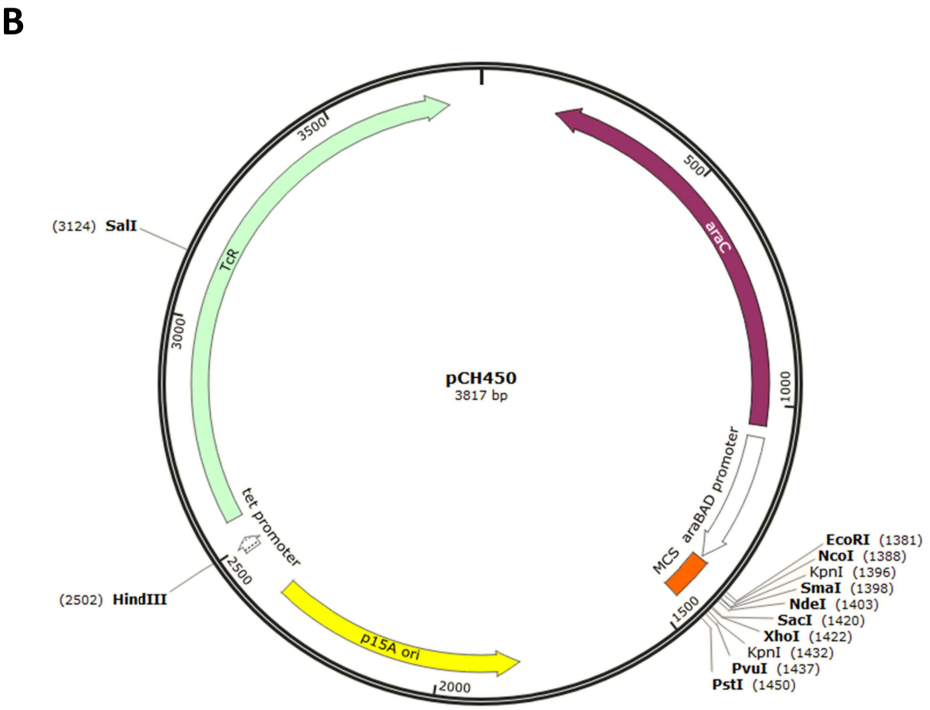
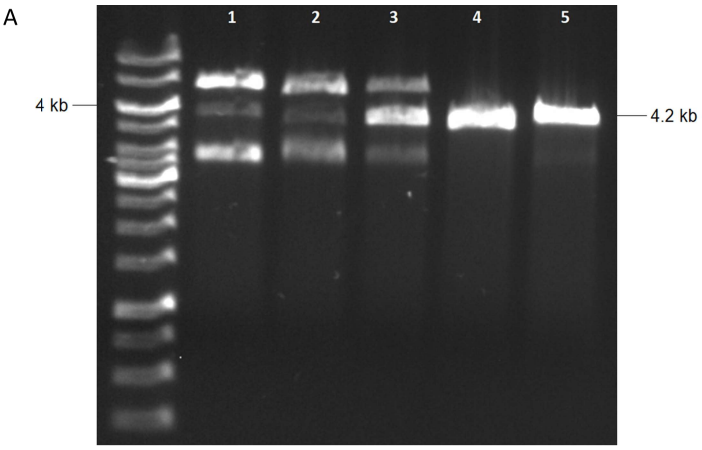

B
3500
500
(3124) SalI
araC
TcR
3000
pCH450
3817 bp
1000
araBAD promoter
MCS
EcoRI (1381)
NcoI (1388)
KpnI (1396)
SmaI (1398)
NdeI (1403)
SacI (1420)
XhoI (1422)
KpnI (1432)
PvuI (1437)
PstI (1450)
tet promoter
2500
(2502) HindIII
1500
p15A ori
2000

Figure 2.6



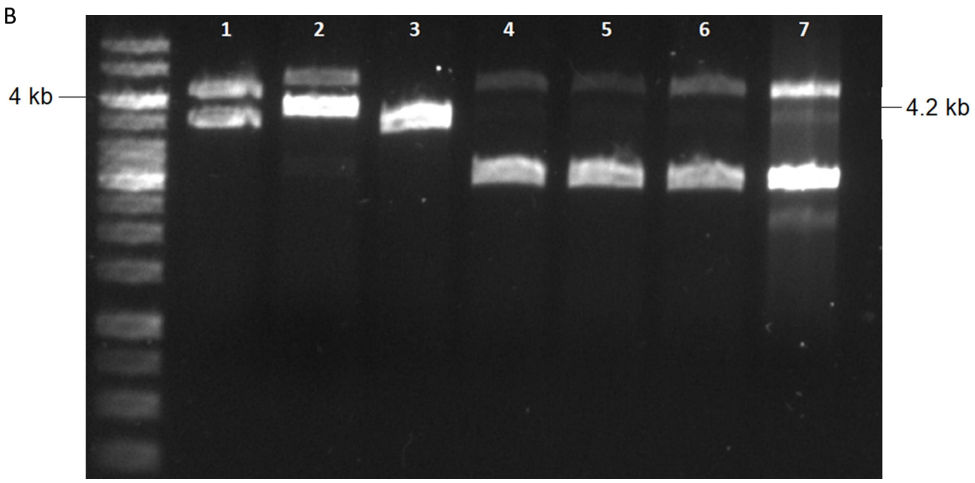


Figure 2.7

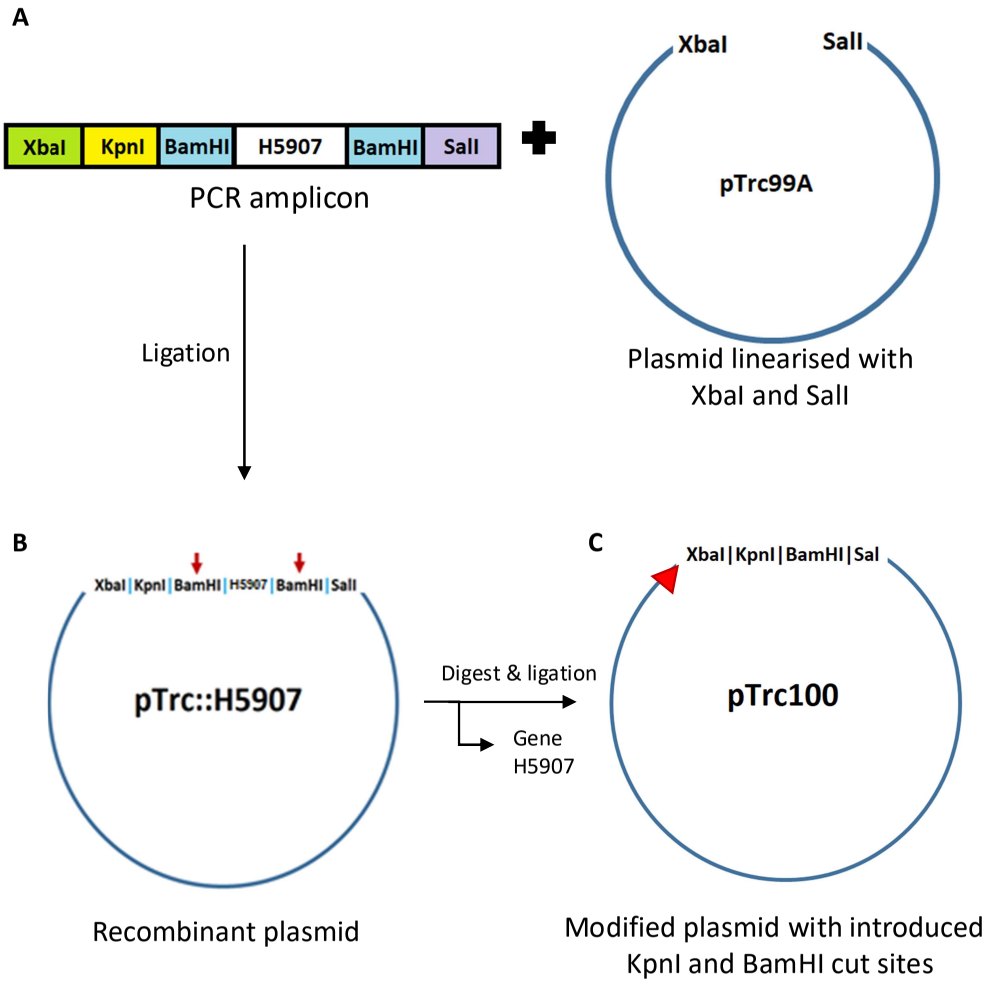

A
XbaI
KpnI
BamHI
H5907
BamHI
SalI
PCR amplicon
XbaI
SalI
pTrc99A
Plasmid linearised with
XbaI and SalI
Ligation
B
XbaI | KpnI | BamHI | H5907 | BamHI | SalI
pTrc::H5907
Recombinant plasmid
Digest & ligation
Gene
H5907
C
XbaI | KpnI | BamHI | SalI
pTrc100
Modified plasmid with introduced
KpnI and BamHI cut sites

Figure 2.8

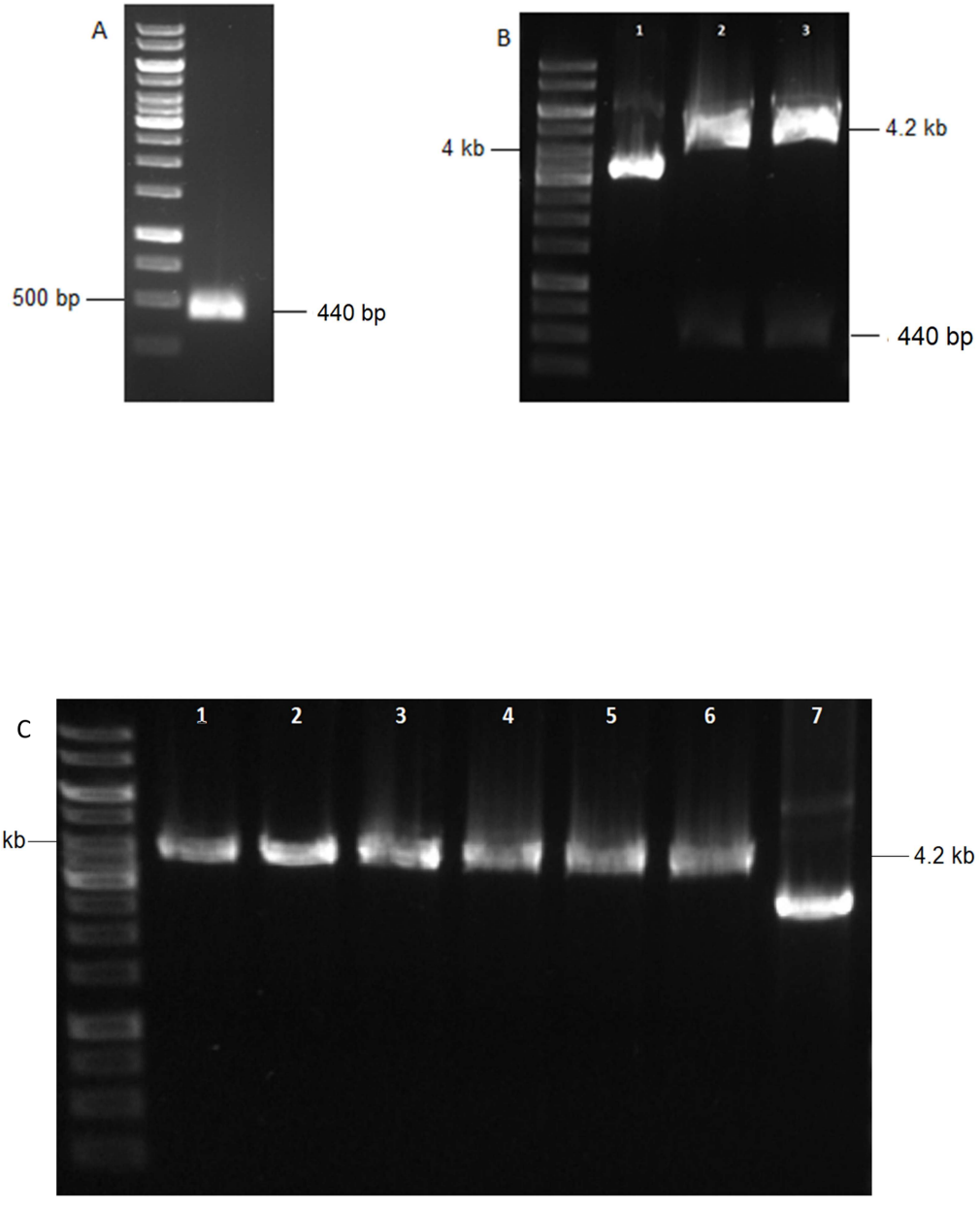


Figure 2.9

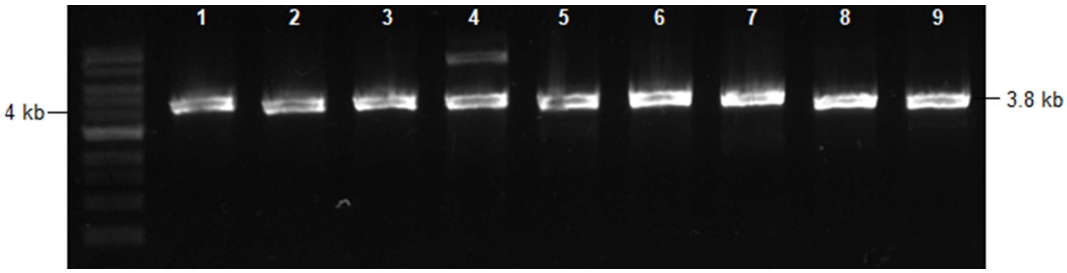


Figure 2.10

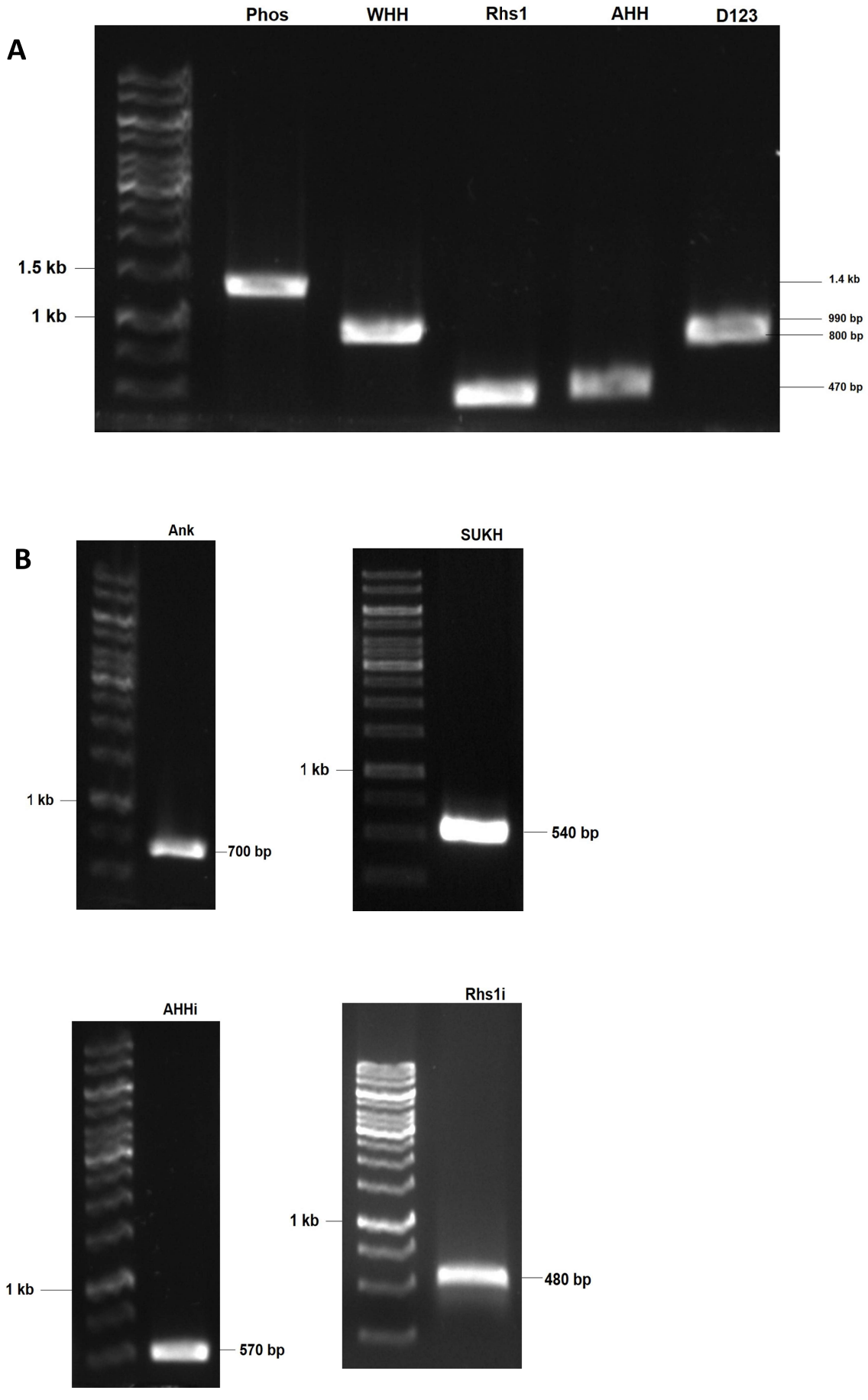
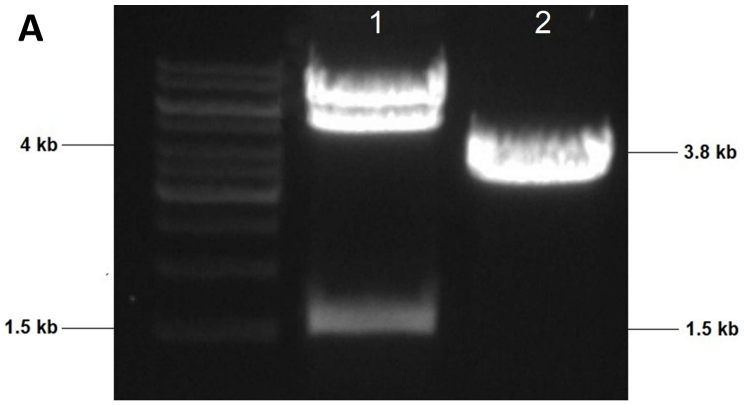

A
Phos
WHH
Rhs1
AHH
D123
1.5 kb
1 kb
1.4 kb
990 bp
800 bp
470 bp
B
Ank
1 kb
700 bp
SUKH
1 kb
540 bp
AHHi
1 kb
570 bp
Rhs1i
1 kb
480 bp

Figure 2.11



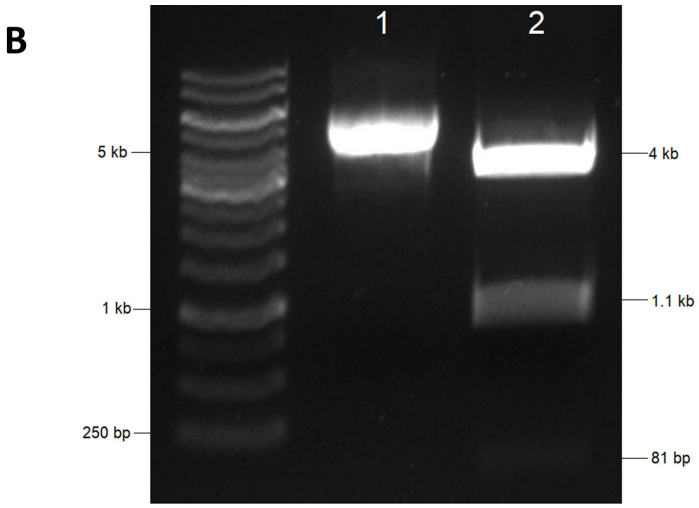
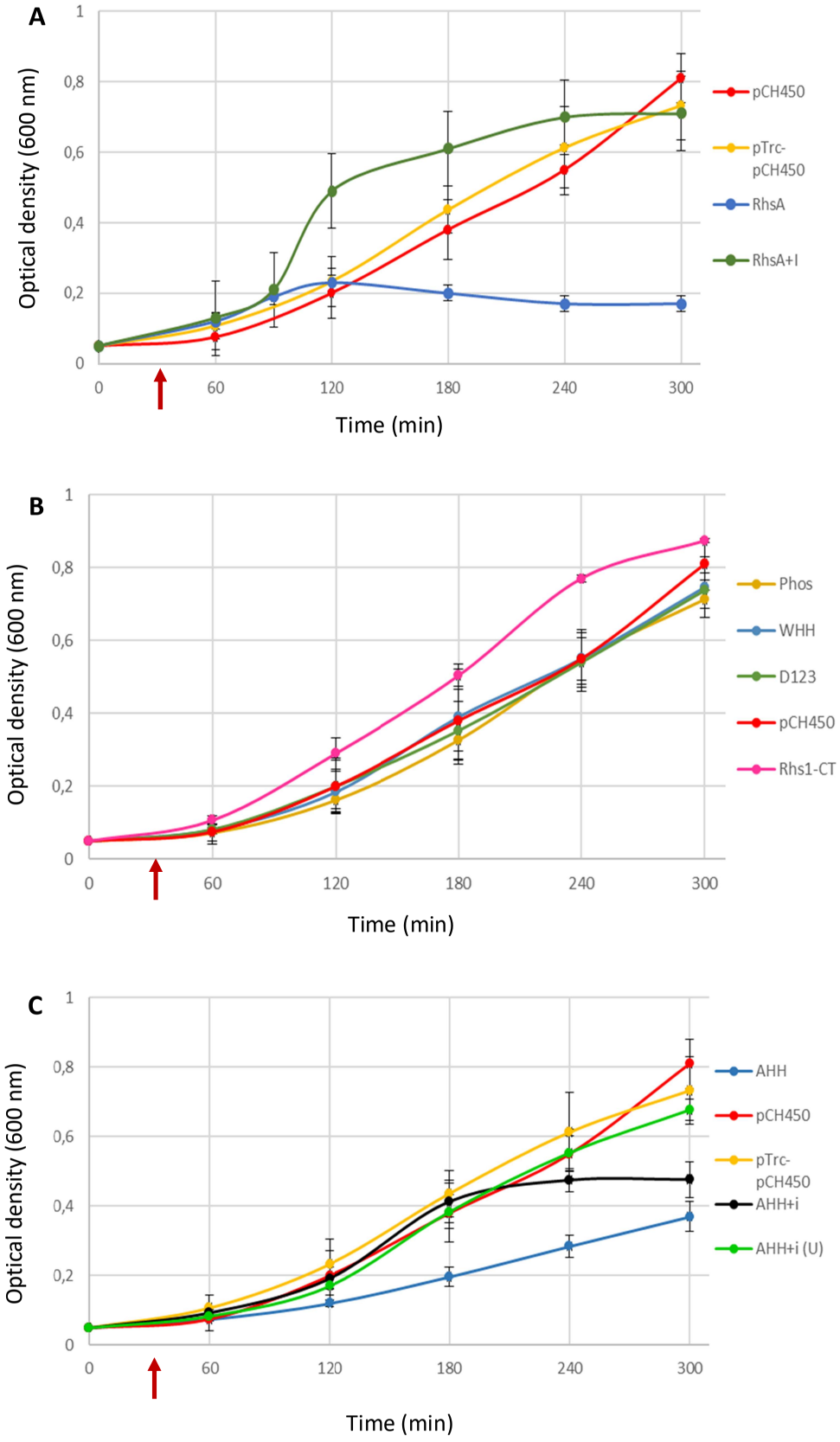


Figure 2.12


A
Optical density (600 nm)
1
0,8
0,6
0,4
0,2
0
0
60
120
180
240
300
Time (min)
pCH450
pTrc-pCH450
RhsA
RhsA+I
B
Optical density (600 nm)
Time (min)
Phos
WHH
D123
pCH450
Rhs1-CT
C
Optical density (600 nm)
Time (min)
AHH
pCH450
pTrc-pCH450
AHH+i
AHH+i (U)

7. Tables

Table 1: Strains and plasmids used in this study

Strain	Description	Obtained
Pcb1692	*Pectobacterium carotovorum* ssp*. brasiliense* strain 1692; wild type	FABI
*Pcb_*ΔT6SS	*tssC* deletion; T6SS inactivation	(Shyntum *et al.*, 2018)
E. coli DH5α	F⁻, *endA1*, *recA1*, *relA1*, *gyrA96*	Invitrogen
Pa	*Pectobacterium atrosepticum* SCRI1043	FABI
Pcc	*Pectobacterium carotovorum* ssp*. carotovorum*	FABI
Dc	*Dickeya chrysanthemi*	FABI
Dd	*Dickeya dadantii* 3937	FABI
Plasmid	**Description**	**Obtained**
pTrc99A	Amp^R; IPTG-inducible expression vector	FABI
pTRC99::H5907	Derivative of pTrc99 with hypothetical gene 5907 flanked by restriction sites to modify the plasmid to pTrc100	This study
pTrc100	Amp^R; IPTG-inducible expression vector; derived from pTrc99A with modified MCS	This study
pCH450	Tet^R; L-Arabinose-inducible (P_{BAD} promoter) expression vector	C.S Hayes (Koskiniemi *et al.*, 2013)
pET26b	Amp^R; IPTG-inducible; PelB signal sequence	Novagen
pET21a	Amp^R; IPTG-inducible	Novagen
pGEM-T Easy	Amp^R; pMB1 ori; f1 ori; lacZ'	Promega
pJET1.2/blunt	Amp^R, pMB1 ori,	Thermo Scientific
pTrc100_AHHi	IPTG-inducible expression of AHH immunity gene (AED-0004230)	This study
pTrc100_Ank	IPTG-inducible expression of phospholipase immunity gene (AED-0002728)	This study
pTrc100_SUKH	IPTG-inducible expression of WHH-nuclease immunity gene (AED-0003808)	This study
pTrc100_Rhs1i	IPTG-inducible expression of Rhs toxin immunity gene (AED-0004232)	This study
pCH450_$RhsA^{CT}$	L-Arabinose-inducible expression of $RhsA^{CT}$ from *Dickeya dadantii*	C.S Hayes (Koskiniemi *et al.*, 2013)
pCH450_Phos	L-Arabinose-inducible expression of type six phospholipase (AED-0003357)	This study
pCH450_WHH	L-Arabinose-inducible expression of type six WHH-nuclease (AED-0003809)	This study
pCH450_D123	L-Arabinose-inducible expression of D123 (AED-0003807)	This study
pCH450_AHH	L-Arabinose-inducible expression of type six AHH nuclease (AED-0004231)	This study
pCH450_AHH	L-Arabinose-inducible expression of type six Rhs toxin (AED-0006270)	This study

Table 2: List of primers used in this study

Primer name	Sequence (5' → 3')	Purpose
Trc_Test_F	TTCATTAATGCAGCTGGCACG	Sequencing; confirm inserts into pTrc100
Hcp2_F	GAGCTCGGTACCATGCCAACTCCATGCTATATCAGC	Open reading frame for expression
Hcp2_R	CTGCAGGTCGACTTACGCTTCGACCGGTGCA	
WHH_F	GAGCTCGGTACCATGGCGAATATCAGAAAGCAAAGCG	
WHH_R	CTGCAGGTCGACTTAATAATCCTCGCTACGCCACATCG	
Phos_F	GAGCTCGGTACCATGCCAGGTGCTGCACGTTTAG	
Phos_R	CTGCAGGTCGACTTATATTCCCCTGAATGTATTGG	
AHH_F	GAGCTCGGTACCATGGACTGGGTTGATCCGTTTGGGTTGGC	
AHH_R	CTGCAGGGTACCTCAATATTTAAACGTCCCTGAAACAAGTG	
Rhs1_F	GAGCTCGGTACCATGCCGCTGGATTGGGTCGATCCG	
Rhs1_R	CTGCAGGTCGACTCACCTTATTGGCGGATTGAAGAGGC	
D123_F	GTCGACGGTACCATGTATTCTGAACATAAGGCGAC	
D123_R	CTGCAGAAGCTTCTATAATAAATAGGGTCTTCTTTG	
H_5907_F	TCTAGAGGTACCGGATCCATGTGGCAGTTTAATGACCTGCA	Modification of pTrc99 to pTrc100
H_5907_R	GTCGACGGATCCTTAATCCAGACGGATAAATG	
ANK_F	GGTACCGAATTCATGCGGGCGAGTGAACTCTTTGAG	Immunity protein for cognate effectors
ANK_R	CTGCAGAAGCTTCTATACTGGCAGCCCTTTTTCCC	
SUKH_F2	TCTAGAATTACAACTCATCTTCCGATGTTGGC	
SUKH_R2	AAGCTTATGACTATTGAAAAATCTGATCTTGATAACTGG	
AHH-I_F	GAGCTCGGTACCATGAACATTTACACGCTAGAAGC	
AHH-I_R	CTGCAGGTCGACCTAATCTGATACTCCAACC	
Rhs1-I_F	GAGCTCGGTACCATGTCAAGTGTTTATTATATTGAACCCG	
Rhs1-I_R	CTGCAGGGTACCTTACACCTTTTCTATAATGGC	
pJET_156F	GGAGCAGGTTCCATTCATTG	Sequencing from pJET
pJET_156R	GAAAACCCACGCCACCTACA	

*Restriction cut sites underlined