The type VI secretion system in *Pectobacterium* spp: Role in virulence and regulation by Zur.

By

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DECLARATION

I, Alessandro Rino Gricia, 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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To see behind walls, to *draw* closer.

To find each other and *feel*.

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^{O}C:	degrees Celsius
µl:	microliter
µg:	microgram
Δt6:	(deleted *TssC*) mutated strain
Δzur:	(deleted *zur*) mutated strain
Gent:	gentamycin
Gent^{R}:	gentamycin resistance gene
Kan	kanamycin
Kan^{R}:	kanamycin resistance gene
Amp	ampicillin
Amp^{R}:	ampicillin resistance gene
bp:	base pairs
Δt6-p*t6*:	*TssC* complemented strain
Δzur-p*zur*:	*zur* complemented strain
cfu:	colony forming units
CV:	crystal violet
EPS:	extracellular polysaccharides
h:	hours
min:	minutes
LB:	Luria-Bertani broth
mM:	milimolar
OD:	optical density
Pcb 1692:	*Pectobacterium carotovorum* subsp. *brasiliense* 1692

PCWDE:	plant cell wall degrading enzymes
PNAG:	poly-N-acetyl-glucosamine
rpm:	revolutions per minute
spp.,:	species
SRE:	soft rot enterobacteriacea
subsp.:	subspecies
T6SS:	type VI secretion system
v/v:	volume to volume
w/v:	weight to volume

SUMMARY

Pectobacterium spp. together with *Dickeya* spp. belong to a group of soft rot disease causing pathogens known as the soft rot *Enterobacteriaceae* (SRE). *Pectobacterium carotovorum* subsp. *brasiliense* is an aggressive pathogen of potato within South Africa causing aerial stem rot, tuber soft rot and blackleg both in the field and during post-harvest storage. In this study, emphasis has been placed on characterization of the type VI secretion system as a virulence determinant for anti-bacterial competition in *Pcb* 1692, since this system has been linked to effector proteins capable of secretion affecting prokaryotes and in some cases eukaryotes.

Chapter 1, provides a review of literature on the role of the type VI secretion system within prokaryotes, including the T6SS's impact on virulence, disease progression and overall host fitness. The review discusses the T6SS as a virulence determinant through interacting with the host as well as neighbouring competing bacteria. The regulation thereof is also discussed. The zinc uptake regulator (Zur) is also described and its influence on the disease development and zinc uptake discussed.

In Chapter 2, 3 & 4, the role of the type VI secretion system as a virulence determinant in *Pcb* 1692 was investigated. Utilizing a previously generated mutant of the *TssC* gene (encoding ClpV, a core component of the type VI secretion system) this study generated data which indicated that the *Pcb1692Δt6* mutant strain's competitive fitness was hindered, losing the ability to outcompete neighbouring bacteria in potato tubers. Similarly, a reduction in virulence *in planta* as well as the inability to produce standard levels of EPS within the T6SS mutant was observed. These observations suggest that the type VI secretion system plays a key function in competitive fitness *in planta*, ensuring that nutrients available are utilized by *Pcb* 1692 as opposed to other competing endophytes resulting in efficient colonization of the host.

Similarly, the role of the zinc uptake transcriptional regulator (*zur*) as a regulator of the type VI secretion system as well as a virulence determinant was investigated. After iron, zinc is the most important transition metal, acquired through the ZnuABC zinc uptake system which is negatively regulated by the Zur protein. Zur has too been described as a multifunctional regulatory protein reported to regulate virulence determinants in various pathogens with a functional *zur* gene essential for virulence. This study identified a homolog of *zur* within *Pcb* 1692 and proceeded to disrupt the gene encoding for *zur*. Through bioinformatics procedures the binding site of Zur was shown to be located within the VipA promoter, responsible for transcribing the core components of the type VI secretion system. Data indicated that the *Pcb1692Δzur* mutant strain, where the zinc uptake regulator was non-functional, was deficient in production of a number of virulence determinants, namely the production of EPS, biofilm formation and oxidative stress protection resulting in a reduction of virulence *in planta*. In contrast to this, the competitive fitness of the mutant strain *in planta* was intensified due to the absence of the regulator Zur, resulting in the increased expression of the type VI secretion system. These observations suggest that through unknown mechanism/s, Zur directly or indirectly regulates virulence determinants and is required by *Pcb* 1692 for host colonization and disease progression.

CHAPTER ONE

Review of Literature

1.1 Introduction

Within a polymicrobial niche, bacteria compete in a continuous war between different array of bacteria for space and nutrient resources (Hibbing *et al.*, 2010). Bacteria's potential to persist and bourgeon in an environment depends on their ability to interact with the various components of that ecological niche (Gallique *et al.*, 2017). The mediation of these interactions with commensal and pathogenic bacteria within the surrounding environment are conducted by various mechanisms including quorum sensing, secretion systems which translocate proteins into the extracellular environment (Christie & Vogel, 2000). Secreted proteins perform key roles in the interactions of the bacterial pathogen with competing bacteria, host organisms as well as the abiotic environment (Joshi *et al.*, 2017). Within Gram-negative bacteria, such as SRE, six major secretion systems (referred to as Types I-VI) have been described to date (Desvaux *et al.*, 2009). SRE are able to use the anti-bacterial activity through the secretion of bacteriolytic effector proteins through the Type VI Secretion System (T6SS) in a single, cell contact dependent step (Kapitein & Mogk, 2013). The T6SS is an important virulence factor utilized to target host cells and, perhaps more commonly, an efficient mechanism to target and compete against opposing bacteria in polymicrobial environments (Silverman et al., 2012). This specialized protein transporter mediates toxic effector proteins, ranging from single-domain proteins to multi-domain proteins capable of affecting different components of the target cell, from a Gram-negative bacterial donor cell to prokaryotic or eukaryotic recipient cells (Miyata *et al.*, 2013). In *V. cholera* and *P. aeruginose* effector proteins are delivered directly into eukaryotic cells, resulting in the formation of actin protrusions and actin cross-linking most commonly providing protection against macrophages and predatory amoeba (Joshi *et al.*, 2017). More recent, studies have focussed their attention on mediation of inter and intra-bacterial interactions, through such competitive killing, whereby the pathogen in question is able to manipulate the polymicrobial environment to its benefit, therefore increasing its

capacity to survive and proliferate (Murdoch *et al.*, 2011). As a result, the T6SS mediates the killing of commensal and pathogenic bacteria to assimilate nutrients for the fittest pathogenic bacterial species, enabling the invasive colonization of the host (Russel et al., 2013).

To colonize and cause disease in plants, *Pectobacterium* species must utilize a variety of strategies and virulence determinants (Davidsson *et al.*, 2013). As a result of the unfavourable conditions bacterial pathogens need to proliferate in, virulence factors such as protein secretion systems, swimming motility, biofilm formation and plant cell wall-degrading enzymes (PCWDEs) are employed to evade, colonize, kill competing bacteria, acquire nutrients and successfully cause infection (Tanui *et al.*, 2017). The end game of these processes results in the ecological fitness enabling the bacterial pathogen to assimilate released nutrient elements from the cell cytoplasm such as zinc and iron released into the extracellular space during cell degradation (Dow *et al.*, 2003). The ability of *Pcb* to inhibit the growth of SRE as well as other related bacteria through anti-bacterial strategies improves *Pcb's* chance to thrive in a free-for-all situation (Tanui *et al.*, 2017). Extracellular polysaccharidesis an important virulence factor in numerous bacteria, including phytopathogens (Quiñones *et al.*, 2005, Marcotte *et al.*, 2007). Duarte and colleagues showed that *Pcb* 1692 secretes large amounts of EPS which occludes xylem vessels, resulting in stem rot and wilting of potato plants (Duarte *et al.*, 2004). Furthermore, extracellular polysaccharides constitute a major component to the formation of biofilms which aid in protection from plant antimicrobial agents, UV light as well as the efficient assimilation of nutrients. The formation of biofilms is too an important virulence determinant in many plant and animal pathogens (Rahme *et al.*, 2000, Prithiviraj *et al.*, 2005).

Transition metals such as iron and zinc acts as an essential trace elements needed by living organisms and functions as a structural or catalytic cofactor for a large number of enzymes and

proteins (Fones & Preston, 2013). However, if the intracellular zinc concentration exceeds a critical level it becomes lethal therefore bacteria must tightly regulate its uptake (Patzer & Hantke, 1998). The ZnuABC and PitA as well as the ZntA are important zinc uptake and export systems regulated by zinc-sensing metalloregulatory proteins which combine with cognate metals as co-repressors (Huang *et al.*, 2008). One such co-repressor protein is the zinc-uptake regulatory protein (*Zur*), which negatively regulates the ZnuABC zinc uptake system in a zinc-dependent manner (Huang *et al.*, 2009). Functional characterization of Zur proteins have been described in a number of bacteria, such as *Bacillus subtilis*, *Escherichia coli* and *Xanthomonas* spp. (Patzer & Hantke, 1998, Gaballa *et al.*, 2002, Tang *et al.*, 2005).

In this study, we aimed to functionally characterize the T6SS's role in virulence as well as its effect on *Pcb* 1692 virulence determinants, including EPS production and biofilm formation as well as the T6SS's role in anti-bacterial activity resulting in *Pcb* 1692's augmented virulence as opposed to certain other SRE. Furthermore, we aimed to establish the role of *Pcb* 1692 Zur in regulating the T6SS in *Pcb* 1692. The questions addressed in this study are: Will *TssC* gene disruption affect *Pcb* 1692's virulence *in planta* (in potato tubers)? If so, which virulence determinants are affected by the mutation of *TssC*? Does the T6SS mediate the killing of competing SRE? Is there a regulatory action imposed by Zur on the T6SS in *Pcb* 1692? We speculate that the *Pcb* 1692 strain defective for the T6SS will exhibit a reduction in its ability to macerate potato tuber tissue. Additionally, as per the literature, it is likely that the T6SS plays a role in anti-bacterial activity within *Pcb* 1692 and subsequent deactivation of the T6SS will result in the inability to outcompete neighbouring SRE. Moreover, we speculate that a *zur* deficient strain will affect the expression of the T6SS resulting in altered levels of anti-bacterial activity due to a regulatory affect it imposes.

Due to the recent emergence of more virulent *Pectobacterium carotovorum* subsp. *brasiliense*, genes involved in conferring this amplified pathogenicity as well as the drivers of disease

development remain a mystery. Therefore, through investigating the role of the type VI secretion system in competitive fitness as well as regulation of the system through the zinc uptake regulator (Zur) in *Pectobacterium carotovorum* subsp. *brasiliense*, our results has added new knowledge on the importance of the T6SS towards *Pcb* 1692 invasion, colonization and disease development. Together, this will broaden our knowledge on this economically important plant pathogen, by targeting genes such as *zur* in future control strategies.

1.1.1 Soft-rot *Enterobacteriaceae*

Potato blackleg is a seed-borne disease caused by two major genera of Soft Rot Enterobacteriaceae (SRE), namely *Pectobacterium* spp., and *Dickeya* spp., (Charkowski *et al.,* 2012*)*. The SRE are Gram-negative, non-spore forming, rod-shaped facultative anaerobes harbouring peritrichous flagella which was initially described in 1917 as *Erwinia* spp., (Gardan *et al.*, 2003, Czajkowski *et al.*, 2011). After decades of extensive phylogenetic work, SRE previously incorrectly classified, were placed into the new genera of *Pectobacterium* and *Dickeya* on the basis of 16S rDNA sequences and host ranges (Hauben *et al.*, 1998). These two genera consist of broad host-range pathogens responsible for the wilting and rotting symptoms associated with blackleg disease of plants, causing significant economic damage worldwide due to tissue maceration (Ma *et al.*, 2007). Characteristics such as blackleg symptoms are due to pathogens ability to synthesize and secrete active protopectinases as well as numerous plant cell-was degrading enzymes (PCWDEs) capable of macerating plant tissue within under 48 hours (Charkowski, 2015).

1.1.2 SRE classification

Pectobacterium spp., and *Dickeya* spp., were previously classified under a single genus along with all other plant pathogenic Enterobacteriaceae, namely *Erwinia* (Gardan *et al.*, 2003). Since

reclassification of several subspecies, there are five distinct species within the *Pectobacterium* genus derived from the *Erwinia carotovora* subspecies, namely *Pectobacterium atrosepticum* (*Pa*), *P. carotovorum* (*Pcc*), *Pectobacterium. betavasculorum, Pectobacterium. wasabiae* and *Pectobacterium. carotovora* subsp. *brasiliensis* (*Pcb*). Due to the thorough characterization of *P. atrosepticum*, where disease processes and pathogenicity determinants were identified and annotated, *Pa* was considered as a model organism for the identification of key genes involved in the progression and development of disease in closely related SRE pathogens (Bell *et al.*, 2004). As a result, genomes of a number of other *Pectobacterium* spp., have been sequenced, annotated and made available on NCBI, including the Brazilian *Pcb* 1692 strain (Glasner *et al.*, 2008).

1.1.3 *Pectobacterium* spp., virulence mechanism and disease symptoms

Pectobacterium spp., are widespread across the globe and are found in many agricultural regions infecting host plants within at least 50% of angiosperm families, the potato crop being the most affected. However, through phylogenetic analysis of all reported hosts of the SRE, a degree of host specificity was detected (Czajkowski *et al.*, 2011). *Pectobacterium* was observed to be prominent pathogen in *Brassica* crops however there has been no reports of other SRE such as *Dickeya* spp. on these crops, with the opposite being true for grain crops (Ma *et al.*, 2007). Blackleg disease caused by various *Pectobacterium* spp., can affect potatoes at three different stages within their agricultural cycle: at planting, resulting in the decay of seed pieces, during the growth season, resulting in the death of tubers and stems and in storage, resulting in the rotting of large volumes of potatoes (Charkowski et al., 2014). *Pectobacterium* spp. pathogens are often described as brute force, due to their ability to produce significant amounts of plant cell wall degrading enzymes (PCWDE) which result in the maceration and degradation of the plant cell wall and middle lamella in the leaves, stems, roots and tubers (Charkowski, 2015).

1.2 Bacterial type VI secretion system

1.2.1 Type VI secretion system as a virulence determinant

In various environmental niches, the secretion of proteins such as toxins and extracellular polysaccharides by bacterial pathogens can confer selective advantages resulting in survival within a host, progression of disease and proliferation (Tseng *et al.*, 2009). The survival advantages are conferred by the numerous virulence and colonization factors which pathogenic bacteria synthesize and secrete in the form of proteins (Alfano & Collmer, 2004). To date, bacteria have been found to use a number of diverse mechanisms to efficiently transport proteins into the extracellular compartment. The secreted proteins perform key roles in the interactions of the bacterial pathogen with competing bacteria, host organisms as well as the abiotic environment (Tseng *et al.*, 2009). Within Gram-negative bacteria, six major secretion systems (referred to as types I-VI) have been described based on the characteristics of their substrates, molecular mechanisms of protein transport and their conserved structural components (Costa *et al.*, 2015). One such system, namely the type VI secretion system (T6SS), is a specialized contact-dependent transporter of proteins mediated from a Gram-negative bacterial donor cell to a prokaryotic or eukaryotic recipient cell, having a role in both host interactions as well as pathogenesis. (MacIntyre *et al.*, 2010, Konovalova & Søgaard-Andersen, 2011). Effector proteins are translocated into target cells through phage-related, contractile nanomachinery composed of several sub-complexes (Cianfanelli *et al.*, 2016). Type VI secretion system gene clusters have been reportedly identified in the genomes of approximately 100 bacterial species (Zoued *et al.*, 2014). Of these bacteria, a large proportion harbour numerous T6SS and can be an important virulence factor utilized to target host cells or, perhaps more commonly, an efficient mechanism to target and compete against opposing bacteria in polymicrobial environments (Boyer *et al.*, 2009, Zoued *et al.*, 2014).

1.2.1.1 T6SS role in virulence

The T6SS was initially implicated in pathogenicity, contributing to virulence in several pathogenic bacteria such as *Vibrio cholera* and *Pseudomonas aeruginosa* where effector proteins, specifically VgrG and Hcp, are delivered directly into host cells, termed anti-eukaryotic (Durand *et al.*, 2012). Phenotypic consequences of prokaryotic bacteria-to-eukaryotic host effector protein transfer include adaptation to deoxycholic acid, the formation of actin protrusions, actin cross-linking and rounding as seen in the J774.1 murine macrophages, and killing of the *Dictyostelium* amoeba (Pukatzki *et al.*, 2006, Pukatzki *et al.*, 2007).

In order to assess the significance of the *hcp* gene in the progression of *in vivo* infection, infected control mice as well as mice immunized with rHcp with a sub-lethal dose of wild-type *Aeromonas hydrophila*. The authors reported that post inoculation, the sera of the infected control mice developed specific antibodies against Hcp whereas sera acquired prior inoculation was void of Hcp specific antibodies, inferring that during *in vivo* infection, Hcp was produced. It was observed that all of the immunized mice survived in contrast to the control mice who died within 48 hours. Authors too concluded that the *vasH* and *vasK* genes were required for the translocation and section of Hcp (Suarez *et al.*, 2008). VAS too displays homology to the *icmF* gene of *Legionella pneumophila*, which too is a core component of the T6SS. Authors reported that when the wild-type V52 *V. cholerea* was mixed with the *Dictyostelium* amoebae, a 250-fold reduction in the recovery of viable amoebae was observed. In contrast, when *Dictyostelium* amoebae was mixed with the *V. cholerae vasK* mutant, the mutant *V. cholerae* strain was attenuated in virulence and was efficiently used by *Dictyostelium* as a bacterial substrate (Pukatzki *et al*., 2006).

In *Pectobacterium atrosepticum*, Mattinen *et al.*, (2007) analysed secreted proteins with proteomics from culture supernatant of bacterial cells grown in minimal media that was supplemented with plant host extracts. Through quantitative real-time PCR, four homologous *hcp* genes found to be secreted by the T6SS were observed to be up-regulated when in the presence of plant extracts and were therefore considered to be putative virulence factors. Subsequently, authors generated a *Δhcp1* mutant strain and its ability to macerate potato tubers compared to the *P. atrosepticum* wild-type was assessed. The *Δhcp1* mutant strains tuber tissue and stem tissue maceration ability was observed to be reduced compared to that of the wild-type. Instead of mutating all four hcp genes observed to be up-regulated in the presence of plant extracts, a strain overexpressing Hcp1 was generated, resulting in double the amount of rot produced in tubers compared to that of the wild-type, providing evidence to support the notion that Hcp1 secreted by the T6SS is involved in the virulence *P. atrosepticum* (Mattinen *et al.*, 2007). Similarly, the T6SS was observed to be utilized for pathogenesis in *Pantoea ananatis* (Shyntum *et al.*, 2015). Authors generated a *ΔT6* mutant strain through inactivating the T6SS-1 gene cluster encoding for 13 core genes of the T6SS machinery and conducted pathogenicity assays on susceptible onion plants. The *ΔT6* mutant strain was unable to induce disease symptoms on onion leaves compared to that of the *P. ananatis* wild-type. Pukatzki *et al.*, (2006) identified a set of genes, which they named VAS (virulence-associated secretion) encoding for an exclusive protein secretion system which they concluded was the type VI secretion system due to the highly conserved nature of the gene cluster.

In *Agrobacterium tumefaciens*, Hcp was too observed to be secreted by the T6SS and subsequent deletion of either the entire operon encoding for the T6SS or the *icmF* gene (encoding for a core component of the T6SS) resulted in attenuated virulence in potato tuber disks. Authors observed a reduction of 80% in the ability of the *ΔT6SS* mutant strain to produce tumours in potato tubers (Wu *et al.*, 2008). Similarly, in pear flowers, Tian *et al.*, (2017)

observed an attenuation in virulence in an *Erwinia amylovora* strain with mutated T6SS gene clusters (T6SS-1 and T6SS-3). Authors generated 33 mutant strains each independently mutated for a component of the *E. amylovora* T6SS-1, T6SS-2 and T6SS-3. Of the 33 mutant strains, 19 were observed to be attenuated in their ability to produce necrotic lesions on immature pear fruit in comparison to the *E. amylovora* wild-type strain (Tian *et al.*, 2017).

1.2.1.2 Competitive activity of the T6SS

In order to persist within an ecological niche, where bacteria are involved in a perpetual war against numerous competitors, bacterial pathogens are required to possess weapons of destruction capable of inhibiting the proliferation of competing bacterial cells (Chassaing & Cascales, 2018). Through the utilization of protein secretion systems, bacteria can both interact and manipulate the abiotic environment, non-living chemical or physical parts of the environment that affect living organisms, through producing biofilm which directly aid in the efficient assimilation of nutrients as well as eukaryotic and prokaryotic cells (Chen *et al.*, 2015). Amongst the mechanisms utilized in aiding the struggle against neighbouring bacteria, the T6SSs relative ability to target effector proteins into competing bacterial cells within a eukaryotic host or within the eukaryotic host itself, greatly increases the pathogenic potential and competitive fitness of the bacterial pathogen in question (Jiang *et al.*, 2014).

P. aeruginosa, V. cholerae and *Burkholderia thailandensis* all encode specialized T6SS gene clusters vital for anti-bacterial competition (MacIntyre *et al.*, 2010, Schwarz *et al.*, 2010, Jiang *et al.*, 2014). *V. cholera* has been a model organism for studies conducted on the T6SS due to its employment in interactions within both eukaryotic and prokaryotic competitors (MacIntyre *et al.*, 2010). Within *V. cholera*, during its host and aquatic life cycles, the T6SS not only promotes its survival but also affects its evolution through facilitating horizontal gene transfer. Pukatzki *et al*., (2006) first observed the ability of *V. cholerae* to mediate protection from

macrophage and amoebae predation through two effector proteins secreted by the T6SS, namely VgrG-1 and VasX. The T6SS structural component, VgrG-1, harbouring an actin crosslinking C-terminal domain prevents predation through causing cytotoxic actin-crosslinking, compromise the integrity of the eukaryotic actin cytoskeleton resulting in cell rounding and ultimately death, within the amoeba predator *Dictyostelium discoideum* as well as J774 macrophages (Pukatzki *et al.*, 2006, MacIntyre *et al.*, 2010). Interestingly, VgrG-1 has been observed to attribute to the efficient colonization of the intestine of mice and rabbit causing diarrheal and inflammation symptoms.

The presence of the peptidoglycan-degrading effector protein, VgrG-3, too mediated the efficient colonization of rabbit and mouse intestinal tract (Joshi *et al.*, 2017). On the other hand, the cargo effector protein VasX, has demonstrated the ability to disrupt the cell membrane of both *Escherichia coli* as well as *D. discoideum*. However the VgrG-3 effector protein was observed to be anti-bacterial as opposed to anti-eukaryotic, eliminating the commensal host microbiota within the preferred niche of *V. cholera*, the lumen of the small intestine, promoting intestinal colonization and disease progression (Zheng *et al.*, 2011). This phenomenon of survival advantage has too been reported within *P. aeruginosa* and has been linked to the T6SS.

Within the hemolysin co-regulated secretion island I of the three T6SS clusters within *P. aeruginosa*, three effector proteins were identified namely, Tse1-3 (Hood et al. 2010). Of these effector proteins, all are anti-bacterial contributing to the competitive fitness of the pathogen with Tse1 and Tse3 able to degrade the peptidoglycan of neighbouring bacteria and Tse2 targeting the cytoplasm of competing bacteria. Intriguingly, *P. aeruginsa*'s T6SS-dependent anti-bacterial activity is elicited by the T6SS of other predatory bacteria. In 2013, Basler *et al*., reported that *P. aeruginosa* was unable to kill T6SS mutated strains of *Acinetobacter baylyi* or *V. cholerae*. However killing of *A. baylyi* and *V. cholerae* expressing a functional T6SS was

observed by *P. aeruginosa*, ensuring an effective counterattack on heterologous species expressing an active T6SS within the same ecological niche.

Although most reports have focused on the ability of the T6SS to eliminate competing bacteria, the T6SS has also been shown to contribute in response to stress, horizontal gene transfer and self-recognition (Weber *et al.*, 2009, Borgeaud *et al.*, 2015). Among the nine identified Gram-negative Proteobacteria, *P. aeruginosa* was the only genera encoding three different T6SS gene clusters (Chen *et al.*, 2015). In 2012, Sana *et al.*, reported a decreased survival of *Caenorhabditis elegans* when infected with wild-type *P. aeruginosa* (PAO1) compared to when infected with the clpV2 mutant strain (clpV2 encoded by the H2-T6SS gene cluster), inferring that an actively expressed H2-T6SS contributes to the virulence of *P. aruginosa* (Sana *et al.*, 2012). Similarly, infection of *C. elegans* with PAO1 ΔclpV3 mutant (clpV3 encoded by the H3-T6SS gene cluster) resulted in a delayed death compared to the wild-type. Therefore implying that an active H3-T6SS is too required for virulence in *P. aeruginosa* (Sana *et al.*, 2013). The T6SS of *Pantoea ananatis* was too observed to contribute to the killing of Gram negative competitors *in vitro*. Wild-type *P. ananatis* and a strain of *P. ananatis* mutated for the T6SS-1 (*ΔT6*) was co-inoculated independently with 30 Gram negative competitors. The *ΔT6* mutant was observed to be attenuated in its ability to kill competitors such as *E. coli* DH5α, *Salmonella enterica* and *Pantoea stewartii* compared to the wild-type strain (Shyntum *et al.*, 2015).

1.2.1.3 T6SS's effect on other virulence determinants

As previously stated, bacterial pathogens rely on a number of processes for the progression of disease within the host plant. Important virulence determinants including the release of toxins, interactions with the host cell, antibacterial activity and the production of biofilms and EPS are necessary for disease development (Piqué *et al.*, 2015). The release of toxins targeting either

the eukaryotic host or the prokaryotic competitor both require a dedicated protein secretion system, however many reports have linked the production and formation of biofilms as well as EPS with the secretion systems responsible for toxin-antitoxin transfer (type VI secretion system) (Southey-Pillig *et al.*, 2005).

The production of biofilms is a pathogenic mechanism that hinders the eradication of organisms due to the increased availability of nutrients as well as biofilm resistance against antimicrobial agents compared to their planktonic counterparts (Nesper *et al.*, 2001, Costerton *et al.*, 2005). Fux *et al.*, (2005) reported that approximately over half of bacterial infections are elicited by surface-associated microbial communities, otherwise known as biofilms and act as reservoirs of infection, protecting the bacterial inhabitants against harsh environmental conditions (Fazli *et al.*, 2014). Communities of bacteria embedded within an extracellular polymeric matrix produced by the bacteria represents switching from a planktonic state of existence to a sessile one, greatly increasing bacterial proliferation through creating complex bacterial communities.

Enteroaggregative *Escherichia coli* (EAEC) harbour a functional Sci-1 T6SS consisting of 21 genes, of which 13 are shared among all EAEC. A mutant strain defective for *sciZ*, one of the 8 genes not conserved, was generated and observed as unable to produce Hcp as well as produce a functional biofilm compared to the wild-type (Aschtgen *et al.*, 2010). Similarly, Tian *et al.*, (2015) identified a T6SS core gene cluster consisting of 17 genes within *Acidovorax citrulli*. The authors generated 17 T6SS mutants, each independently mutating one of the 17 core genes of the T6SS core cluster. Of the 17 mutants, 4 were attenuated for the production of mature biofilms with the wild-type strain producing significantly more biofilm under the same conditions. As previously mentioned, *Caenorhabditis elegans* infected with wild-type *P. aeruginosa* (PAO1) resulted in a significant decreased survival rate when compared to *Caenorhabditis elegans* infected with the clpV2 mutant strain (clpV2 encoded by the H2-T6SS gene cluster). Therefore an actively expressed H2-T6SS contributes to the virulence of *P.*

aruginosa. Furthermore, through mass spectrometry and two-dimensional gel electrophoresis the biofilm maturation stage of *P. aeruginosa* PAO1 was too analysed, confirming that Hcp forms part of the biofilm formation within *P. aeruginosa* (Southey-Pillig *et al.*, 2005) (Sana *et al.*, 2012). Moreover, the expression of Hcp1 in biofilm cells was observed to be higher than that of cells in planktonic state. The deletion of Hcp1 resulted in attenuation of biofilm-specific antibiotic resistance inferring a role of H1-T6SS in biofilm-specific antibiotic resistance (Southey-Pillig *et al.*, 2005).

Vibrio parahaemolyticus isolates display the ability to alternate between opaque and translucent colony morphology through OpaR, a quorum sensing transcriptional regulator. Such differences in morphology arise from the up-regulated production of extracellular polysaccharide in opaque colonies (Güvener & McCarter, 2003). Enos-Berlage et al., (2004) generated a *V. parahaemolyticus Δhcp* mutant strain inactivating the T6SS secreted haemolysin co-regulated protein. In both the opaque and translucent *Δhcp* mutant colonies, biofilm production was reduced in comparison to the wild-type with only single-cell attachment in translucent colonies and attenuation of biofilm formation in later stages of development in opaque colonies (Enos-Berlage *et al.*, 2005).

In *Burkholderia cenocepacia*, the atsR gene encoding for a sensor kinase-response regulator was mutated. Authors reported a T6SS dependent over-expression of Hcp resulting in a type VI hyperactive mutant strain exhibiting a higher resistance to *Dictyostelium* predation compared to the wild-type strain (Aubert *et al.*, 2008). Authors too observed an attenuation in adhesion as well as formation of biofilms however did not conclusively substantiate whether the attenuation in biofilm production was as a result of the T6SS. Similarly, the T6SS in *Pseudomonas. fluorescens* MFE01 too plays a role in biofilm formation. *P. fluorescens* strain MFE01 displays anti-bacterial activity towards competing bacteria such as rhizobacteria through the expression of *hcp2* however is non-virulent towards eukaryotic cells. Gallique *et*

al., (2017) generated a MFE01 mutant strain through mutating the *tssC* gene, a core component of the T6SS. Authors observed that when the MFE01 wild-type strain was co-inoculated with *P. fluorescens* MFP05 on a glass surface, MFP05 was unable to produce a biofilm. However when MFP05 was co-inoculated with *MFE01ΔtssC* mutant strain, normal biofilm biovolume was observed suggesting that the decrease in MFP05 biofilm is attributed to the T6SS as well as raising the possibility that anti-bacterial activity could be deployed under biofilm conditions. Authors too observed that the *MFE01ΔtssC* mutant strain's ability to form biofilm in flow cell chambers was attenuated compared to that of the MFE01 wild-type. Furthermore, through confocal microscopy, the level of biofilm maturation was too investigated. Authors observed that the *MFE01ΔtssC* mutant strain exhibited a low level of maturation in comparison to the MFE01 wild-type.

In avian pathogenic Escherichia coli (APEC) as well as *V. cholerae*, components of the T6SS such as Hcp, ClpV and the intracellular multiplication factor protein (IcmF) have been observed to be involved in the adherence to epithelial cells (Das *et al.*, 2002). De Pace *et al.*, (2011) generated an APEC SEPT362 mutant strain, *ΔicmF*, and observed that the T6SS component IcmF was both required for the adherence to HeLa cells as well as for the production of biofilms. Authors observed that the *ΔicmF* mutant strain produced 80% less biofilm on a polystyrene surface and was unable to produce biofilm on a glass surface in comparison to the wild-type strain, indicating that the *icmF* gene stimulates biofilm formation (Das *et al.*, 2002). Similarly, in *Acidovorax citrulli*, Tian *et al.*, (2015) identified a cluster composed of 17 genes encoding core T6SS proteins and generated 17 mutant strains by independently deleting the 17 core gene components of the T6SS. Authors observed that the *ΔimpL, ΔvasD, ΔimpK* and *ΔimpF* mutant strains produced significantly less biofilm than the *A. citrulli* wild-type strain (Tian *et al.*, 2015). Notably, VasD, a sciN-like protein, was required for biofilm formation. In enteroaggregative *E. coli*, SciN has been reported as an outer membrane lipoprotein which is

exposed to the periplasmic space. Lipoproteins are important components in almost all secretion systems and are involved in the assembly of secretion systems. Furthermore, SciN has also been implicated in the production of biofilm in Enteroaggregative *E. coli* (Aschtgen *et al.*, 2008).

The production of EPS has been reported to contribute to virulence of bacterial pathogens, protection from environmental stresses as a consequence of its highly hydrated and anionic consistency as well as bypassing plant defence systems and nutrient loss (Flemming *et al.*, 2007). *Erwinia amylovora*, a bacterial pathogen causing fire blight, harbours three T6SS gene clusters each contributing to the virulence and anti-bacterial fitness of the pathogen (Tian *et al.*, 2017). Tian *et al.*, (2017) generated 33 mutants each mutated for an individual core gene of the T6SS-1, T6SS-2 and T6SS-3. Of the 19 mutants displaying attenuated virulence, reduction in necrotic lesions, all were attenuated in the production of EPS. *E. amylovora* produces two varieties of EPS; amylovoran, which is considered as a pathogenicity factor and levan, a virulence factor (Vrancken *et al.*, 2013). Of the 19 mutants attenuated for virulence, 9 were deficient in the production of amylovoran, 3 were deficient in the production of levan and 7 were deficient in the production of both (Tian *et al.*, 2017). Although the T6SS has been previously implicated in virulence, prior to the above mentioned study, the connection between EPS and the T6SS has not been reported (Tian *et al.*, 2017).

Despite conclusions in numerous reports that the inactivation of the T6SS results in the attenuation of biofilm production and formation, the link connecting the two virulence determinants is never further explored. Leading to the question, is the formation of biofilms dependent on a functionally active T6SS or does the T6SS directly or indirectly regulate the production of biofilms? Enos-Berlage *et al.*, (2005) reported a novel function for the *Vibrio parahaemolyticus* VP1393 gene encoding a secreted protein highly similar to that of *V. cholerae* Hcp. Authors observed that VP1393 contributed to a more robust biofilm architecture

as well as three-dimensional expansion with the associated VP1393 mutant strain attenuated for the formation of biofilms. This report linked the production of biofilms to the T6SS dependent secretion of Hcp. Similarly, Sauer *et al.,* (2002) reported that within *P. aeruginosa*, during the late stages of biofilm formation and maturation, Hcp was observed to be secreted at an elevated rate resulting in an increased level of Hcp.

1.2.2 Type VI secretion system machinery

Reports have reached consensus that the T6SS is composed of thirteen highly conserved proteins as well as a complement of variable accessory, regulatory and secreted elements and proteins (Basler, 2015). Two-hybrid and co-immunoprecipitation interaction experiments and subcellular localization experiments performed on the thirteen core proteins revealed that the T6SS system consists of two main subassemblies: a membrane-associated assembly and an assembly which bears resemblance to that of a bacteriophage (Zoued *et al.,* 2013). The various components work together allowing for the translocation of effector proteins across the envelope of the bacterial donor cell and through the outer cell membrane of the recipient cell (Cianfanelli *et al.*, 2016).

The 13 conserved core genes, essential for encoding proteins that form the structure of the secretion system, are encoded within large, variable gene clusters (Shalom *et al.*, 2007, Leiman *et al.*, 2009, Shneider *et al.*, 2013). It is a common occurrence for additional copies of core components, particularly *hcp* and *vgrG,* to be found in orphan T6SS clusters outside of the main T6SS gene cluster. These orphan gene clusters are often found in close proximity to genes encoding putative effector proteins (De Maayer *et al.*, 2011). Both within and outside the main T6SS core gene cluster, antibacterial effectors are always encoded neighbouring to cognate immunity proteins. These immunity proteins function to protect the bacterial secreting cell

from its own toxins as well as the toxins secreted by neighbouring sibling cells (Russell *et al.*, 2014).

Majority of studies have focused on describing the various elements composed of the bacteriophage-like subassembly which is composed of five proteins, namely TssB, TssC, Hcp, ClpV and VgrG. Three of these proteins (TssC, Hcp and VgrG) have structural homology to that of the phage infection machinery (Kapitein & Mogk, 2013). The similarities between the T6SS and the bacteriophage tail structure has provided significant advancements in further understanding the mechanisms of the T6SS (Brunet *et al.*, 2014).

Through studying the effector protein delivery process to that of the bacteriophage infection process, a number of breakthroughs have been made. TssB and TssC form a bacteriophage contractile sheath which can be observed in a contracted or extended states *in vivo* by transmission electron microscopy (Lossi *et al.*, 2013, Bernal *et al.*, 2018). The energy required to penetrate a recipient cell is provided from the contraction of the TssB-TssC sheath. The recycling of the contractile sheath to its extended state is proposed to be performed by the ClpV AAA^+ ATPase (Bönemann *et al.*, 2009, Kube *et al.*, 2014). Contraction of the TssB-TssC sheathe exposes a Clp-binding motif which is recognized by ClpV, resulting in the recycling of the contracted sheathe to the extended state. TssE encodes for the production of the baseplate assembly protein. Encoding for proteins homologous to the bacteriophage tail tube and tail spike is the Hcp and VgrG proteins respectively (Lin *et al.*, 2013). Hexameric rings of haemolysin coregulated proteins (Hcp) form the intracellular tube with external and internal diameters of 8.5 and 4.0 ηm respectively, which mediates the movement of effector proteins through it (Leiman *et al.*, 2009). A trimer of valine-glycine repeat protein G (VgrG) is positioned at the distal end of the Hcp tube which mediates the puncturing of the target cell via a C-terminal β-helical spike.

Brunet *et al.,* (2014) observed that the Hcp and VgrG structural components were to exported proteins of the T6SS, Hcp in particular was observed to be a marker for T6S as it is reported to be secreted by the T6S apparatus in an abundant manner. The newly described proline-alanine-alanine-arginine repeat-containing protein (PAAR) caps the intracellular tube, interacting with VgrG in order to form a sharp tip on the spike. TssB/TssC heterodimers forms a contractile sheath which surrounds the Hcp complex existing in either a relaxed or contracted state upon the detection of an extracellular signal. These core components assemble to form a trans-envelope machinery whose function is to directly inject effector proteins secreted into the bacterial cytoplasm of the donor bacterial cell into a target cell (Pukatzki *et al.,* 2007).

Williams *et al.,* (1996) first detected the secretion of haemolysin coregulated protein (Hcp) and observed the absence of a signal sequence suggesting Hcp's involvement as a novel mechanism of protein secretion. This hypothesis was further cemented through a study conducted by Zheng & Leung (2007) demonstrating how Hcp secretion is dependent on other T6SS genes as well as how 13 of the 16 evp genes in *Edwardsiella tarda* require the Hcp-like EvpC for export. These finding led to the speculation that proteins are delivered to the extracellular space through an assembly of Hcp rings forming a channel. This was strengthened through the observation that Hcp hexamers stack in a head-to-tail orientation as well as the self-assembly of Hcp rings in vitro to form nanotubes (Brunet *et al.,* 2014). The nanotube structure of Hcp is too highly similar to that of phage λ and exhibits sequence similarity to the T4 gene product 19 (which forms the phage tail tube) as well as near identical internal and external diameters to that of the T4 gene product 19. The combination of the above mentioned facts strongly suggest the existence of an evolutionary relationship between the T6SS and phage tails (Mougous *et al.,* 2006).

1.2.3 Type VI secretion system secreted toxins

Through the transport of proteins with niche altering properties such as effector proteins, pathogens are able to interact with their biotic and abiotic surroundings allowing for disease progression and proliferation (Tseng *et al.*, 2009). T6SS$^+$ bacteria produce and secrete a range of effector proteins capable of targeting a number structural components of prokaryotic as well as eukaryotic organisms, namely membrane lipids, nucleic acids and peptidoglycan of competing bacteria (Pukatzki *et al.*, 2007, Heckel *et al.*, 2014). Effector proteins secreted by the T6SS are vastly diverse, ranging from single-domain proteins to multi-domain proteins which fall under four main families of effector proteins based on the domains present, namely: peptidoglycan glycoside hydrolase family, peptidoglycan amidase family, lipase/phospholipase family and the DNase/RNase nuclease family (Pukatzki *et al.*, 2009). In spite of this diversity, the common co-occurrence of effectors and cognate immunity proteins has become commonplace, underscoring the capacity of the T6SS to mediate co-ordinated antibacterial attacks (Benz *et al.*, 2012).

1.2.3.1 Bacteria cell wall-targeting T6SS effector proteins

The bacterial cell wall consists of a major component observed to be a preferential target for T6SS effector proteins, peptidoglycan (Russell *et al.*, 2011). Cell wall-targeting effector proteins targeting the peptidoglycan of bacterial cell walls are arguably the most thoroughly characterized effectors and are thought to be a principal component of the T6SS arsenal (Jiang *et al.*, 2014).

Peptidoglycan is composed of a glycan backbone comprised of alternating β1,4-linked *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) subunits (Meroueh *et al.*, 2006). Type VI secreted effectors belonging to the peptidoglycan glycoside hydrolase and peptidoglycan amidase families (Tge and Tae families respectively) are capable of digesting large marcromolecular structures such as peptidoglycan through targeting the peptide and

glycan moieties of the macromolecule (Flaugnatti *et al.*, 2016). Few effector proteins which have shown to supplement bactericidal activity through the T6SS have been biochemically characterized.

Brooks *et al.,* (2013) observed that T6SS structural proteins such as VgrG can to facilitate the translocation of specific effectors as well as containing C-terminal effector domains capable of degrading peptidoglycan and nucleic acids, as seen in *V. cholera* VgrG3. *P. aeruginosa* and *Serratia marcescens* secretes Tse1 and Ssp1 or Ssp2 from the Tae family respectively. Tse1 is an amidase capable of cleaving the γ-D-glutamyl-L-meso-diaminopimelic acid amide bond of peptidoglycan crosslinks (Benz *et al.,* 2012). Characterized bactericidal effectors from the Tge family includes Tge2 and Tge3 from *P. protegens* and Tse3 muramidase from *P. aeruginosa.* Tse3 is a muramidase capable of cleaving the γ-D-glutamyl-meso-2,6-diaminopimelic acid bond of peptidoglycan (Lu *et al.*, 2014).

1.2.3.2 Bacteria cell membrane-targeting T6SS effector proteins

Due to the conserved nature and essential function of the cell membrane, effectors secreted by the T6SS target this structure and are characterized as belonging to the lipase/phospholipase family (Tle). These effector proteins function through hydrolysing the lipid component of the membrane, more specifically, the phosphodiester bonds (Jiang *et al.*, 2014). Five Tle families have been characterized with familes, Tle1-4 utilizing a nucleophilic serine (GxSxG motif) and Tle5 utilizing dual catalytic histadine residues (HxKxxxxD motif) (Russell *et al.*, 2013). The Tle effector proteins belonging to all five families are hypothesized to degrade the cell membrane from the periplasm, similar to that of cell wall-degrading effector proteins. Such a hypothesis was made due to the observation that Tle anti-toxin proteins inactivate their cognate toxin proteins directly and were detected to localize to the periplasmic space (Russell *et al.*, 2014).

Due to the similarity between Prokaryotes and Eukaryotes in terms of their cell membranes (differing only in relative concentrations of their constituents), the mediation of interbacterial as well as Eukaryotic interactions by Tle effector proteins is a possibility (Russell *et al.*, 2013). Additional studies exploring this concept is required however eukaryotic infection models involving *V. cholerae tle2 and P. aeruginosa tle5* are being studied.

1.2.3.3 Nucleic acid-targeting T6SS effector proteins

Poole *et al.*, (2011) observed that within the C-terminal region of recombination hot spot (Rhs) elements, domains associated with endonucleases are present. Koskiniemi *et al.*, (2013) recently characterized the bactericidal activity of two effector proteins namely, RhsA and RhsB from *Dickeya dadantii*. The authors observed the presence of C-terminal Ns_2 and HNH endonuclease domains within RhsA and RhsB respectively resulting in the degradation of cellular DNA, conferring an intraspecies advantage.

1.2.4 T6SS effector protein delivery

The important question of how the T6SS recognises effector proteins to be secreted has yet to be answered. However recent reports have described how T6SS effector proteins are recruited and delivered by the T6SS. Classification of effector proteins are according to two broad mechanisms of effector translocation by the T6SS machinery: either through noncovalent interactions with a core component (cargo effectors) or fused to structural components (specialized effectors) (Durand *et al.*, 2014, Whitney *et al.*, 2014). Components of the expelled Hcp-VgrG-PAAR structure are associated with effector proteins in both mechanisms of effector translocation, implying that multiple effectors are delivered into a target cell simultaneously (Shneider *et al.*, 2013).

Specialized effectors are observed to harbour effector domains which are covalently fused to a core protein component of the extracellular puncturing assembly and are typically represented

as evolved VgrG proteins (Durand *et al.*, 2012). Exemplified through the observations of Ma et al., (2009) who observed evolved VgrG proteins within *V. cholerae,* (specifically VgrG-1) which was the first example of the fusion between a core component and an effector domain. VgrG-1 harbours a 446 amino acid extension at the C-terminus providing the ability to induce cell rounding through cross-linking actin (Durand *et al.*, 2012, Dong *et al.*, 2013). Two examples include a peptidoglycan hydrolase domain of VgrG-3 (anti-bacterial) and an actin cross-linking domain of VgrG-1 (anti-eukaryotic). The specific position of the fusion guarantees the exposure of the extra domain upon target membrane puncturing. Recently, type VI secreted effector proteins harbouring effector domains fused to PAAR domains have been described (Hachani *et al.*, 2014). This alternate translocation strategy of fusing effector domains to that of PAAR domains is particularly common within recombination hot spot (Rhs) proteins (Koskiniemi *et al.*, 2013). Rhs proteins associated with the T6SS are polymorphic toxins composed of N-terminal domain containing a PAAR motif, a central Rhs repeat domain and a C-terminal variable toxin domain (Hachani *et al.*, 2014).

In contrast to specialized effectors, cargo effector proteins are not observed to be linked with other T6SS genes and effector protein domains are not fused to any component of the secretion machinery (Russell *et al.*, 2013). Yet, reports have observed that genes encoding effectors are located adjacent or in close proximity to *hcp, vgrG* or *paar* genes, implying that the secretion of said effectors is associated with that of the neighbouring core component (De Maayer *et al.*, 2011). The first report of this phenomenon was observed within *E. tarda*, where the effector protein EvpP and Hcp was secreted by the T6SS simultaneously (Zheng & Leung, 2007). Yet, the interaction between Tse2 and Hcp1 within *P. aeruginosa* has been extensively studied. Authors observed Hcp1 plays a chaperone-like role, stabilizing effectors encoded by the H1-T6SS gene cluster, through localizing effectors to the internal surface of Hcp1 (Silverman *et al.*, 2013). The VgrG-1, harbouring an actin cross-linking domain, of *V. cholerae* has too been

observed to associate with effector proteins, specifically the phospholipase effector protein TseL.

1.3.1 Type VI secretion system regulation

Given the impressive array of Gram-negative pathogens harbouring T6SS gene clusters that infect humans, animals, plants and bacteria, it is not surprising that the T6SS gene expression is governed by a number regulatory mechanisms (Chen *et al.*, 2015). Common regulatory mechanisms include changes in environmental cue such as pH and temperature as well as iron, zinc, phosphate and magnesium concentration, quorum sensing (QS) and two-component regulatory systems. Majority of *V. cholerae* harbour the full complement of T6SS genes, however between strains, T6SS gene regulation differs with some strains restricting gene expression and others constitutively expressing the T6SS genes (Zheng *et al.*, 2010).

Quorum sensing has been reported to regulate various virulence determinants such as the T6SS within *V. cholera* and *P. aeruginosa* (Lesic *et al.*, 2009). So too is the expression of Hcp, where up-regulation occurs at high-cell density, deletion of the AI synthase genes (LuxS) results in the attenuation of Hcp production (Ishikawa *et al.*, 2009). Within *V. alginolyticus*, the expression of Hcp1 is up-regulated in the exponential phase at a low-cell density and mutation of LuxO (hapR repressor) decreased Hcp transcription. In contrast to *V. alginolyticus, V. cholerae* Hcp is up-regulated at a high-cell density (Miyata *et al.*, 2013). Within *V. parahaemolyticus*, the central output regulator (OpaR) represses transcription of Hcp1 indirectly, regulating two independent T6SS clusters with one active at a low-cell density and the other active at a high-cell density. Interestingly, OpaR too regulates cyclic-di-GMP, which has been reported to up-regulate T6SS gene expression in *P. aeruginosa* (Gode-Potratz & McCarter, 2011). Furthermore, LasR a homoserine lactone transcription factor, controls the expression of three T6SS clusters (HSI-I, HSI-II and HSI-III) in *P. aeruginosa* at a

transcriptional level. Suppressing gene expression of HSI-I whereas positively regulating HSI-II and HSI-III gene expression (Chen *et al.,* 2015).

Ishikawa *et al.,* (2012) reported that the T6SS of *V. cholerae* is important for interbacterial competition within the species native environment. Majority of its life cycle is spent in stagnant water or within the ocean, experiencing constant environmental condition fluxes. When grown in conditions with high concentrations of salt, certain *V. cholerae* strains up-regulate and secrete Hcp facilitating the killing of *E. coli*, which was observed to be enhanced in conditions of high salt concentration. In contrast, under standard conditions in the absence of salt, Hcp was observed to be produced however was not secreted (Ishikawa *et al.,* 2012).

Another transcriptional regulator positively regulating the T6SS in *P. aeruginosa* and *V. cholerae* is RpoN, an alternate σ^{54} factor (Pukatzki *et al.*, 2006, Chen *et al.*, 2015). RpoN as well as one of its activators VasH, which acts as a bacterial-enhancer-binding protein, have been reported as vital for *V. cholerae* virulence and killing of *E. coli* and *D. discoideum*. T6SS σ^{54} -dependent promoters have been described upstream T6SS operons in *P. aeruginosa, V. cholerae* as well as *Aeromonas hydrophila*, suggesting that bacterial-enhancer-binding protein mediated regulation is common in T6SS gene clusters. Similarly, *P. syringae* and *P. aeruginosa* HSI-II and HSI-III gene clusters harbour σ^{54} –binding sites, implying T6SS-mediated virulence in *Pseudomonas* is regulated in a σ^{54} –dependent manner (Bernard *et al.,* 2011).

1.3.2 Zinc uptake transcriptional regulator (Zur), a potential type VI secretion system regulator

1.3.2.1 Zinc uptake transcriptional regulator protein

During evolution microbial settlers have acquired a number of adaptations used to exploit nutritive sources through invading different ecological niches within the ecosystem of a host

(Shea & Chesson, 2002). Fungi, protozoa and intracellular bacteria for example search for host-cell compartments, gaining access to essential nutrients, which signifies a key model for their survival and evolution (Moulder, 1985, Ammendola *et al.*, 2007). In response, countermeasures are developed by the host in order to restrict the availability of nutrients to the pathogen while keeping them accessible to the host for its own vital functions. Therefore the capability of bacterial pathogens to adapt and colonize specific environments is measured by their ability to acquire sufficient amounts of specific nutrients that are crucial for their growth and so an imbalanced equilibrium between the nutritional needs of the host as well as the pathogen is established (Fatima & Senthil-Kumar, 2015).

Understanding how bacterial pathogens react to nutrient limitation within an infected host, where essential elements are not freely available, is of particular relevance for the health and improvement of the host plant (Schaible & Kaufmann, 2005). Iron acts as an important catalyst in a number of fundamentally important biological redox reactions, thus for bacterial survival, a sufficient supply of this transition metal is required (Andrews *et al.*, 2003). However the availability of iron within eukaryotes is strictly controlled by metal-binding proteins such as ferritin and lactoferrin as well as bound to organic acids and amino acids such as malate, citrate and nicotianamine (Skaar, 2010). Although the competition for iron between host and pathogen is considered to be one of the most important determinants for pathogen invasive success and disease progression, other transition metals such as zinc are too required for survival (Payne, 1993). Zinc's importance is highlighted by the fact that zinc binding proteins constitute approximately 10% of the human proteome with bacteria predicted to incorporate zinc into 6% of all proteins. Zinc too partakes in vital structural and/or catalytic roles in all six classes of enzymes as well as in replication and transcription factors, gene expression, cofactor of virulence factors, glycolysis, intracellular redox buffering of the cell, pH regulation and

biosynthesis of extracellular peptidoglycan and amino acids (Outten & O'halloran, 2001, Andreini *et al.*, 2006, Ammendola *et al.*, 2007)

The zinc uptake transcriptional regulator (Zur), is capable of sensing subfemtomolar concentrations of cytosolic Zn(II), implying that starvation of cellular Zn(II) occurs at exceptionally low Zn(II) concentrations. Outten and O'Halloran (2001), observed that the minimal cellular Zn(II) concentration required for growth within *E. coli* was 0.2 mM which is approximately 2000 times the concentration of Zn(II) found in LB medium. In the presence of Zn^{2+} sufficiency, Zur is capable of binding to a near perfect palindrome located in the intergenic region between the *znuA* and *znuBC* operons. This association results in the repression of expression of both transcriptional units.

Like other members of the Fur family of metalloregulatory proteins, *B. subtilis*, Zur is a dimer in solution and for protein folding and dimerization, requires a structural Zn^{2+}. In the case of Zn^{2+} sufficiency, Zur performs the role of an active repressor through the additional binding of Zn^{2+} to a regulatory site within each monomer, resulting in a fully metallated protein designated $Zur_2:Zn_4$ (Gaballa & Helmann, 1998, Ma *et al.*, 2011). The expression of *zur* is relatively uniform across a range of growth conditions, with its activity primarily, if not exclusively, regulated by the reversibly binding of Zn^{2+} to this regulatory binding site. In the transition from Zn^{2+} sufficiency to deficiency, Zur too transitions from a fully metallated protein ($Zur_2:Zn_4$) to a partially metallated dimer ($Zur_2:Zn_3$) and finally to the form with Zn^{2+} bound only at the structural sites designated the inactive resting form ($Zur_2:Zn_2$). The $Zur_2:Zn_3$ intermediate is 20 fold less efficient at associating with DNA in comparison to the fully metallated ($Zur_2:Zn_4$) protein (Outten *et al.*, 2001, Ma *et al.*, 2011).

1.3.2.2 Zinc uptake transcriptional regulator as a virulence determinant

The zinc uptake regulatory protein is known to regulate approximately seven operons which encode proteins facilitating adaption to Zn(II) limitation (Tang *et al.*, 2005). One of which, the ZnuABC zinc uptake system. Within many bacterial species, expression of the ZnuABC high-affinity zinc-uptake system is regulated by a Fur-family repressor known as Zur, sensing intracellular concentrations of zinc. Regulation occurs at the transcriptional level where metal-sensing metalloregulatory proteins, once combined with cognate metals as co-repressors, repress transcription through binding DNA (Huang *et al.*, 2009). The depletion of the specific co-repressor metal results in the induction of zinc acquisition genes whereas the presence of such metals induces metal efflux/sequestration genes as well as act as inducers for repressor proteins (Yang *et al.*, 2007).

Maintenance of zinc ion homeostasis not only prevents cellular toxicity, it too plays a role in virulence models. In *Haemophilus ducrei* and *Pasteurealla multocida*, *znuA* and *znuC* mutants were observed to be less virulent in comparison to their respective parent strains (Garrido *et al.*, 2003). Interestingly, in *P. multocida*, despite harbouring sequences that predict with high probability a ZnuABC zinc-uptake system, only one *fur*-like with no *zur*-like genes were detected. Due to the specialized nature of the mucosal parasite *P. multocida*, where the availability of zinc and iron is growth limiting, the regulation of multiple uptake systems by a single repressor could be beneficial (Garrido *et al.*, 2003).

Evidence from previous reports, particularly in *Xanthomonas* spp., have indicated that apart from regulating the ZnuABC zinc uptake system, Zur is a multifunctional regulatory protein capable of directly and indirectly regulating the expression of various virulence determinants. In *X. oryzae* pv. *oryzae*, by disrupting the *zur* gene, multiple virulence determinants of *X. oryzae* pv. *oryzae* were affected. Authors reported a significant decrease in the ability of the mutant strain to cause lesions within tobacco leaves compared to that of the wild-type strain (Yang *et al.*, 2007). Furthermore, the absence of a functional Zur protein resulted in an

attenuated production of EPS as well as a 500-fold reduction in cell number, post treatment with H_2O_2, when compared to the wild-type. Zur was too implicated in eliciting a hypersensitive response as well as regulating the *hrp* operon (*hrpA* to *hrpF*), which encodes for the type III secretion system responsible for secreting plant cell wall degrading enzymes.

Through an indirect mechanism, Zur regulates HrpX, a regulator which controls the expression of the *hrp* operon through interaction with the plant-inducible promoter (PIP) box (Huang *et al.,* 2009). A recent study conducted within Chinese radish reported that the presence of a functional *zur* gene was required for full virulence in *Xanthomonas campestris* pv. *campestris* (Huang *et al.,* 2008). Not only was the *zur* mutant strain attenuated for virulence, it was too deficient in the production of EPS. Interestingly, the authors reported Zur did not regulate the expression of the *hrp* genes within *X. campestris* pv. *campestris* suggesting that the Zur regulatory functions of virulence and pathogenicity within different strains could have evolved genetic diversity (Tang *et al.,* 2005, Huang *et al.,* 2008). Moreover, the attenuation in virulence observed in the *zur* mutant strain was not a result of zinc toxicity. The authors noted that the mutant strain was able to grow in zinc supplemented growth media with concentrations of zinc similar to that of the intercellular fluid extract found within host leaves. The observed reduction of growth and attenuation of virulence observed in the *zur* mutant strain was speculated to be the result of altered expression of virulence genes regulated by Zur.

Within *B. subtilis,* Zur too regulates the expression of the *sbo* operon, responsible for the production of subtilosin as well as the expression of *rocE* and *rocD*, which are genes involved in amino acid transport (Gaballa *et al.,* 2002, Yang *et al.,* 2007). The virulence-related flagellar gene fliZ is too regulated by Zur within *S. typhimurium*. In addition to genes involved in the uptake of Zn(II), a homolog of *zur*, np20, is too related to the virulence of the animal pathogen *P. aeruginosa* (Harvie *et al.,* 2005) (Ma *et al.,* 2011).

1.3.2.3 Zinc uptake transcriptional regulator's effect on the T6SS

The pathogenesis of bacterial pathogens is co-ordinated and controlled through the action of numerous virulence determinants such as the formation of biofilm, secreted toxins, adhesion and exopolysaccharide production. Therefore, such virulence determinants are required to be regulated in an efficient manner in order to optimize bacterial pathogenesis (Bernard *et al.*, 2010). Importantly, environmental cues too result in the up-regulation and down-regulation of the T6SS, with reports showing that when grow in nutrient limiting conditions (minimal media), the T6SS-2 gene cluster of *Burkholderia pseudomallei* was transcribed optimally. In contrast, when *B. pseudomallei* was grown in nutrient rich media, the T6SS-2 was expressed poorly (Burtnick *et al.*, 2011). In *B. thailandensis*, Si *et al.*, (2017) reported that 60 μM of exogenously Zn^{2+} resulted in the repression of the T6SS-2 gene cluster. It was found that the T6SS-2 promoter region contained a predicted Zur box, a regulatory sequence usually upstream genes responsible for Zn^{2+} acquisition. In the presence of excess zinc (zinc sufficiency), the Zn^{2+} binding zinc uptake transcriptional regulatory protein (Zur), represses transcription of T6SS-2 therefore hindering expression. However, as the intracellular concentration of Zn^{2+} decreases, Zn^{2+} no longer acts as a co-repressor with Zur and therefore does not repress the TSS-2 gene cluster (DeShazer, 2019). Si *et al*., (2017) reported that in *B. thailandesis*, the Zur box overlaps the T6SS-2 -10 promoter region and therefore Zur represses the expression of the T6SS-2 in conditions of excess nutrients seemingly through preventing the recognition of the -10 promoter element from RNA polymerase σ^{70} (Si *et al.*, 2017).

2.4 Justification of study

The aim of this study is to investigate the affect the type VI secretion system has on virulence in *Pcb* 1692 disease progression as well as the regulation of the secretion system through a mechanism not yet described. TssC, is a core component of the type VI secretion system, a reliable candidate for studying the involvement of the type VI secretion system in *Pcb* 1692 disease processes. Zur, is a zinc uptake transcriptional regulator previously observed to regulate virulence in phytopathogens as well as regulate the type VI secretion system and therefore is a reliable candidate for studying the involvement of the type VI secretion system in *Pcb* 1692 disease processes.

The *zur* and *tssC* mutant strains will be compared to the *Pcb* 1692 wild-type strain in order to answer the following research questions:

1. Does the type VI secretion system influence *Pcb* 1692 virulence determinants in the progression of disease?
2. Is type VI secretion system involved in the killing a neighbouring competing bacteria?
3. Does Zur influence *Pcb* 1692 virulence determinants in the progression of disease?
4. Does the Zur protein regulate the type VI secretion system, influencing its ability to kill competing bacteria?

The ability of *Pcb* 1692 to kill neighbouring competing bacteria is likely to contribute to the heightened virulence observed within this pathogen. Since the type VI secretion system has been reported to mediate anti-bacterial activity in a number of other pathogens as well as the detection of a putative Zur binding site within the VipA operon encoding the core components of the type VI secretion system, the hypotheses are as follows:

1. A type VI secretion system defective *Pcb* 1692 strain will affect the virulence of the pathogen, resulting in an attenuated ability to cause disease.
2. Similarly, a Zur defective *Pcb* 1692 strain will too affect the virulence of the pathogen, resulting in an attenuated ability to cause disease.
3. A Zur defective *Pcb* 1692 strain will result in the de-regulation of the type VI secretion system, leading to an up-regulated anti-bacterial activity.

Currently, the type VI secretion system has not been linked to anti-bacterial activity within the SRE. In addition, the role Zur plays on disease processes has not been studied. Therefore, this study will further our understanding on the amplified degree of virulence observed within *Pcb* 1692, an important topic requiring further elucidation.

CHAPTER TWO

Investigating the role of the type VI secretion system in *Pectobacterium* disease processes and its regulation by Zur.

Materials & Methods

2. Materials and Methods

2.1 Strains and growth conditions

The bacterial strains and plasmids utilized in this study are listed in Table 2.1. Bacterial strains were grown in Luria-Bertani (LB) broth or on nutrient agar at 37^{o}C. Where necessary, growth media was supplemented with either 50 µg/mL Kanamycin (Sigma-Aldrich), 100 µg/mL Ampicillin (Sigma-Aldrich) or 15 µg/mL Gentamycin (Sigma-Aldrich). All experimental assays conducted in this study were performed in technical triplicates, with three biological replicates. The *Pcb* 1692 wild-type, *Pcb*1692*Δt6, Pcb1692Δzur, Pcb*1692*Δt6*-p*t6* and *Pcb1692Δzur*-p*zur* strains were tested in these assays (unless stated otherwise). Growth media was supplemented with 50 µg/mL kanamycin in the assays where the mutant strain was used. For assays conducted with the complement strain, growth media was supplemented with 100 µg/mL ampicillin. For co-inoculation assays conducted with the targeted competing bacterial strains (*D. dadantii*, *D. chrysanthemi*, *Pcb* G4P5), growth media was supplemented with 15 µg/mL gentamycin.

Table 2.1 Bacterial strains and plasmids utilized in this study

BACTERIAL STRAINS	MODIFICATION	DESCRIPTION	SOURCE
***Pcb* 1692**	Wild-type	Initially isolated from potato in Brazil, sequenced strain	Lab collection
Pcb1692Δt6	*TssC* gene disrupted	*Pcb1692Δt6,* Kan^R	(Shyntum *et al*., 2018)
***Pcb1692Δt6*-pt6**	*TssC* complemented	*Pcb1692Δt6* expressing *TssC* ORF from the Trc99A plasmid, Amp^R	(Shyntum *et al*., 2018)
Pcb1692Δzur	*zur* gene disrupted	*Pcb1692Δzur,* Kan^R	This study
***Pcb1692Δzur*-pzur**	*zur* complemented	*Pcb1692Δzur* expressing *zur* ORF from the Trc99A plasmid, Amp^R	This study
Dickeya chrysanthemi	Harbouring pMP7605 conferring gentamycin resistance	*D. chrysanthemi*, $Gent^R$	This study
Dickeya dadantii	Harbouring pMP7605 conferring gentamycin resistance	*D. dadantii*, $Gent^R$	This study
Pectobacterium carotovorum subsp. carotovorum	Harbouring pMP7605 conferring gentamycin resistance	*Pcc*, $Gent^R$	This study
Pectobacterium atrosepticum	Harbouring pTRC99a conferring ampicillin resistance	*Pa*, Amp^R	This study
Pectobacterium carotovorum subsp. Brasiliense 1692 G4P5	Harbouring pMP7605 conferring gentamycin resistance	*PcbG5P4*, $Gent^R$	This study
PLASMIDS			
pTrc99A-Rat	Rat FABPI removed	Bacterial expression vector harbouring inducible lacI promoter; Ampicillin resistant.	(Velkov *et al*., 2005)
pTrc99A-*t6*		Bacterial expression vector containing the *Tssh* gene insert, Amp^R	This study
pTrc99A-*zur*		Bacterial expression vector containing the *zur* gene insert, Amp^R	
pKD4		37°C growth temperature; R6K promoter; carries FRT-kan-FRT cassette insert; Kanamycin and Ampicillin resistant.	
pKD20		Temperature sensitive replication ori (R101); native terminator (repA101ts); encodes lambda Red genes (*exo*, *bet*, *gam*); arabinose-inducible promoter (P_{araB}); encodes araC for P_{araB} promoter repression; Ampicillin resistant.	

2.2 *In vitro* growth assays

The *in vitro* growth assays for the wild-type, mutants and complement strains (*Pcb* 1692, *Pcb*1692*Δt6, Pcb1692Δzur*, *Pcb*1692*Δt6*-p*t6* and *Pcb1692Δzur*-p*zur*) were assessed through culturing each bacterial strain in LB broth. The cultures were incubated with aeration at 37^{o}C for 16 h at 120 rpm. The optical density of the overnight cultures was measured at 600 ηm (OD_{600}) and adjusted to an OD_{600} of 0.1 and resuspended in 1X phosphate buffered saline (PBS). A total of 1 mL was inoculated into 200 mL of LB broth and grown at 37^{o}C with aeration at 120 rpm. The OD_{600} was recorded at hourly intervals for a total of 16 h with the Multiscan GO spectrophotometer (Thermo-Scientific). The assay was performed in three technical repeats and three biological repeats.

2.3 Virulence assays

Potato tubers (cv. Mondial, a susceptible cultivar) were surface sterilized utilizing 10% sodium hypochlorite (bleach) for 15 minutes, washed three times with double distilled water then aerated for 30 minutes. Surface-sterilized potato tubers (cv. Mondial) were punctured with a sterile pipette tip to a depth of approximately 1cm. 10 µl of *Pcb* 1692, *Pcb*1692*Δt6, Pcb*1692*Δzur, Pcb*1692*Δzur*-p*zur* and *Pcb*1692*Δt6*-p*t6* standardized to an OD_{600} of 1 were independently inoculated into the wounded tubers. A negative control was included consisting of sterile 10 mM $MgSO_4$ buffer inoculated into the wounded tubers. The infected tubers were placed in moist containers and incubated for 24 h, 48 h and 72 h at 25^{o}C. At each post-inoculation time point the extent of the tuber maceration was quantified through scooping and weighing the macerated tuber tissue of each strain. The experiment was performed three independent times each consisting of three technical repeats.

2.4 Extracellular polysaccharide (EPS) production determination

The production of EPS was measured as previously described by (Tang *et al.*, 1991) with few modifications. The bacterial cultures (*Pcb* 1692, *Pcb*1692*Δt6*, *Pcb*1692*Δzur, Pcb*1692*Δt6*-p*t6* and *Pcb*1692*Δzur*-p*zur* were inoculated into 100 mL of LB broth and grown with aeration for 72 h at 37^{o}C and 120 rpm until an OD_{600} of 2.5 is reached. The bacterial cultures were transferred to 50 mL Greiner tubes followed by centrifugation at 10 000 rpm for 10 minutes. EPS was collected through precipitation of the supernatant using 96% ethanol followed by drying for 3 h at 37^{o}C and weighing. The assay was performed in triplicates.

2.5 Biofilm formation assay

Biofilm formation assay was performed as previously described by Tanui *et al.*, (2017) with minor alterations. The *Pcb* 1692, *Pcb*1692*Δt6, Pcb*1692*Δzur, Pcb*1692*Δt6*-p*t6* and *Pcb*1692*Δzur-zur* strains were grown in LB broth for 16 h followed by measuring and adjusting the OD_{600} to 0.4 and resuspended in 1X PBS. A total of 25 µL of the three bacterial cultures were inoculated into 40 mL of LB broth and incubated with aeration at 37^{o}C for 48 h at 80 rpm. The LB broth was discarded and the biofilm formation was visualized by washing the 50 mL Erlenmeyer flask three times with double distilled water followed by staining with 0.1% crystal violet (Sigma-Aldrich) for 30 minutes at room temperature. Biofilm formation was too quantitatively assayed through measuring the OD_{370} of the stained suspensions in 96-well plates utilizing the spectrophotometer.

2.6 *In planta* competition assay

In planta competition assays were performed as previously described (Marquez-Villavicencio *et al.*, 2011) with minor modifications. Briefly, potato tubers (cv. Mondial, a susceptible cultivar) were surface sterilized using 10% sodium hypochlorite (bleach) for 15 minutes, washed three times with double distilled water then aerated for 30 minutes followed by piercing the surface of the tuber to a depth of 1 cm with a sterile pipette tip. Targeted bacteria (*D.*

chrysanthemi, *D. dadantii*, and *Pcb* G4P5*)* were transformed with plasmid pMP7605 conferring gentamycin resistance (Lagendijk *et al.*, 2010), as previously described. *Pcb* 1692 wild-type, *Pcb*1692*Δt6* mutant, *Pcb*1692*Δzur* mutant, *Pcb*1692*Δt6-pt6* complement and *Pcb*1692*Δzur-pzur* complement strains as well as targeted bacterial strains were inoculated into LB broth supplemented with appropriate antibiotics and incubated for 16 h at their respective optimal temperatures (Table 2.1). The overnight cultures OD_{600} were recorded and adjusted to an OD_{600} of 1, resuspended in 1X PBS, mixed in a 1:1 ratio and inoculated into surface sterilized potatoes. Inoculated tubers were placed in moist plastic containers and incubated for 72 h at 25^{o}C. In order to standardize, 0.2 g of macerated tuber tissue was scooped out and the CFU/g of surviving competing bacteria (*D. dadantii*, *D. chrysanthemi* and *Pcb* G4P5) determined by serial dilutions on LB supplemented with appropriate antibiotics. The assays was performed three independent times each consisting of three technical repeats.

2.7 *In vitro* competition assay

Bacterial strains were grown on LB agar supplemented with appropriate antibiotics for 16 h followed by adjusting the OD_{600} to 0.2 and resuspending the culture in 1X PBS. Competitor strains were mixed at a ratio of 1 : 1 with the *Pcb* 1692 wild-type, *Pcb*1692*Δt6* mutant and *Pcb*1692*Δt6*-p*t6* strains independently to a final volume of 1 mL and inoculated on LB agar for 76 h at 37^{o}C in the absence of antibiotic selection. Serial dilutions were performed on LB agar plates supplemented with gentamycin at 0 h and 24 h.

2.8 Generation of a *Pectobacterium carotovorum* subsp. *brasiliense zur* mutant strain

One-step inactivation strategy previously described by (Datsenko & Wanner, 2000) involving a lambda recombineering system was used to generate the *Pcb1692Δzur* mutant strain (Fig S2.2). Primers were designed per *Pcb* 1692 sequence utilizing the CLC sequence design

software (Table 2.4). Firstly, utilizing the zur_F & zur_R; zur_F_2 & zur_R_2 and kan_F & kan_R primer pairs, two PCR amplicons (560 bp and 860 bp) flanking the *zur* gene as well as the kanamycin resistance gene (derived from the pKD4 plasmid) were generated through the polymerase chain reaction. Each 20 µl amplification reaction mixture was set up using 2X Phusion Flash PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions, using 0.5 µM of each respective primer and 150 ng template DNA. The thermal cycling conditions for the PCR reactions utilizing the T100 Thermal Cylcer (Bio-Rad) was programmed for 1 cycle of 95 ^{o}C for 10 seconds followed by 34 cycles of 95 ^{o}C for 5 seconds, 61 ^{o}C for 15 seconds and 72 ^{o}C for 45 seconds. A final extension step was carried out for 1 cycle of 72 ^{o}C for 60 seconds. The three PCR generated amplicons were gel extracted using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific) as per the manufacturer's instructions. Using the zur_F & zur_R_2 primer pair, kanamycin insertion was achieved through an overlap extension PCR technique by producing a gene mutagenesis cassette consisting of previously generated PCR amplicons of the upstream and downstream regions flanking the *Pcb* 1692 *zur* gene fused to the pKD4 kanamycin resistance gene. The overlap extension PCR step was step up using 2X Phusion Flash PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions, using 0.5 µM of each respective primer and 100 ng template DNA. The thermal cycling conditions for the PCR reactions utilizing the T100 Thermal Cylcer (Bio-Rad) was programmed for 1 cycle of 95 ^{o}C for 10 seconds followed by 34 cycles of 95 ^{o}C for 5 seconds, 61 ^{o}C for 15 seconds and 72 ^{o}C for 45 seconds. A final extension step was carried out for 1 cycle of 72 ^{o}C for 60 seconds. The mutagenesis cassette was then electroporated into a strain of electrocompetent *Pcb* 1692 harbouring the curable pKD20 plasmid. A volume of 2 µl of *Pcb* 1692 harbouring the pKD20 plasmid was inoculated into 100 µl of LB broth and incubated at 31 ^{o}C until a OD_{600} of 0.4 is reached. A volume of 10 µl of L-arabinose was added to the bacterial cells followed by incubation at 31 ^{o}C for 30 minutes. The

bacterial culture was centrifuged a total of five times, discarding the supernatant after each centrifuge step, at 6000 rpm for 8 minutes at 4 ^{o}C, resuspending the bacterial cells in 40 ml H_2O, 20 ml H_2O, 10 ml 10% glycerol, 4 ml 10% glycerol and 1 ml 10% glycerol respectively after each centrifuge step. Subsequently, 200 ng of the mutagenesis cassette was added to 1 µl of electrocompentent bacterial cells and were then electroporated using the MicroPulser Electropoator (Bio-Rad) followed by incubation for 16 hours at 24 ^{o}C. Transformants were selected for on 50 µg/ml kanamycin supplemented nutrient agar. Kanamycin insertion were confirmed by PCR utilizing internal kanamycin primers and flanking primers (Ztest_F and Km1; Ztest_R and Km2) (Table 2.4 and Fig S3.2). This confirmed the integration of kanamycin as well as the site of insertion for the mutant.

2.9 Complementation of mutant strain

The 764 bp DNA fragment containing the *zur* gene as well as its putative promoter region was amplified from the total genomic DNA of *Pcb* 1692 using 2X Phusion Flash PCR Master Mix (Thermo Fisher Scientific) and primer set zcomp_F and zcomp_R (Table 2.4). The thermal cycling conditions for the PCR reactions were programed as follows as per table 2.2 utilizing the T100 Thermal Cylcer (Bio-Rad). Using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, South Africa) the *zur* amplicon was excised from the agarose gel and purified according to the manufacturer instructions. Prior to sequencing, the amplicons were independently cloned into the pTRC99A expression vector followed by electroporation into the Pcb1692Δ*zur* mutant strain. Using agar plates supplemented with 100 µg/ml ampicillin, transformants (*Pcb1692*Δ*zur*-p*zur*) were selected.

Table 2.2. PRIMER SETS USED WITHIN THIS STUDY.

Primer	Sequence (5'-3')	Length (bp)	Source
Mutagenesis			
zur_F	CTCCACGCAGCCAAACGTCAG	21	This study
zur_R	CAGCTCCAGCCTACACAATCGTATCACCCTGACGCATTG	39	This study
kan_F	CAATGCGTCAGGGTGATACGATTGTGTAGGCTGGAGCTG	39	This study
kan_R	CACGGGTAACAAAGGTGATGTCCATATGAATATCCTCCTTAGTTCCTATTC	51	This study
zur_F_2	GAATAGGAACTAAGGAGGATATTCATATGGACATCACCTTTGTTACCCGTG	51	This study
zur_R_2	CGTAACGGTCAGATTGTCG	19	This study
zcomp_F	CGATCTGGTCGTGTAGCGCTTC	22	This study
zcomp_R	GAAACACTGGGAAGAGCACCAC	22	This study
zurT_F	GATCACACGAAGAGCAGC	18	This study
zurT_R2	CTTTGCCGATATCTTCATTCC	21	This study
Km_1	TCGTCCTGCAGTTCATTCAGG	21	(Shyntum *et al.*, 2018)
Km_2	CGCTATCAGGACATAGCGTTGG	21	(Shyntum *et al.*, 2018)
qRT-PCR			
ffhF	TGGCAAGCCAATTAAATTCC	20	(Tanui *et al*., 2017)
ffhR	TCCAGGAAGTCGGTCAAATC	20	(Tanui *et al*., 2017)
tsscF	TTCAGCACGCTGGCTTCCTG	20	(Shyntum *et al.*, 2018)
tsscR	TGAACTTCTCTAGGCATTCTGC	20	(Shyntum *et al.*, 2018)

2.10 Metal ion sensitivity test

In order to determine the effect of transition metals on the mutant strain, the *Pcb* 1692, *Pcb1692Δzur* and *Pcb1692Δzur*-p*zur* strains were grown in LB broth supplemented with appropriate antibiotics for 16 h followed by measuring and adjusting the OD_{600} to 1.0. The cultures were used to inoculate M9 minimal media 0.6% Na_2HPO_4 (w/v), 0.3% KH_2PO_4 (w/v), 2% NaCl (w/v), 0.1% NH_4Cl (w/v), 1mM $CaCl_2$, 0.2% $MgSO_4$ (w/v), supplemented with increasing concentrations of $ZnSO_4$ at a ratio of 1:100 followed by incubation for 24 h with aeration at 28^oC with agitation of 120 rpm. Cell density was measured and absorbance at OD_{600} was determined using the Multiscan GO spectrophotometer (Thermo Fisher Scientific).

2.11 qRT-PCR assay

The *Pcb*1692*Δzur* mutant and *Pcb* 1692 wild-type strains were independently inoculated in potato tubers for 72 h followed by scooping the macerated tissue and suspending in RNA stabilization buffer (Qiagen, Hilden, Germany). Using RNeasy mini kit (Qiagen, Hilden, Germany) the total RNA was extracted following the manufacturer's instructions. Genomic DNA was removed using DNaseI (Qiagen, Hilden, Germany) from the total RNA samples. NanoDrop1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to check the concentration of the total RNA extracted. First-strand cDNA was synthesized from 1 µg of the total RNA samples using the Superscript VI First-Strand Synthesis system (Invitrogen).The cDNA was used in real-time PCR reactions using the Quantstudio 12 flex thermocycler (Applied Biosystems). The list of primers used in qRT-PCR is provided in Table 2.2. The tsscF and tsscR as well as the ffhF and ffhR primer pairs used to target the *tssC* and *ffh* genes for the qRT-PCR included: *tssC* (encoding for the contractile sheath, a core component of the type VI secretion system) and the *ffh* gene, which was used as an internal normalization gene or the housekeeping gene (Table 2.4). The relative fold differences of gene expression was calculated according to the $2^{\Delta\Delta CT}$ method using the *ffh* gene as a reference (Takle *et al.*, 2007)

2.12 Gene neighbourhood screening

In order to determine the level of conservation in the genomic context surrounding *zur*, 100 SRE genomes were analysed by HMMR3 through inspecting the gene neighbourhood by integrating genomic annotation and co-ordinates with functional domain predictions.

2.13 Promoter binding site prediction

In order to determine the binding site of the transcriptional regulator *zur*, the nucleotide sequence of the promoter region upstream the Tss type VI secretion system core gene cluster (VipA operon) was subjected to MEME Suite analysis utilizing FIMO (Find Individual Motif

Occurrences). The software ran under the default parameters using the 'Combined Prokaryotes' database, which includes three external databases namely, CollecTF (Bacterial TF motifs), Prokaryotes (Prodoric Release 8.9), Prokaryotes (RegTransBase v4) and Combined Prokaryotes (Timothy *et al.*, 2009).

2.14 Statistical analysis

In this study, experiments were performed in triplicate, three independent times. Where applicable, a one-way Analysis of Variance (ANOVA) was performed to determine statistical significance with a *p*-value less than 0.05 ($p<0.05$) considered to be statistically different.

CHAPTER THREE

Investigating the role of the type VI secretion system in *Pectobacterium* disease processes and its regulation by Zur.

Results & Discussion

3. Results

3.1 Contribution of the *Pcb* 1692 T6SS to virulence *in planta*

The *Pcb*1692*Δt6* mutant, previously generated by (Shyntum *et al.*, 2018), was utilized within this study. The relative growth rates of the *Pcb* 1692 wild type, *Pcb*1692*Δt6* mutant and *Pcb*1692*Δt6*p-*t6* complement strains were assessed in order to determine whether disrupting the *tssC* gene, a core component of the T6SS, had an effect on the mutant strain's ability to grow resulting in a significant difference in growth rate. However, there was no significant difference in growth rate between the mutant strain and its respective complement strain. Therefore any phenotype observed in the subsequent assays was a result of the disruption of the gene of interest (*tssC*) as opposed to differing growth rates (Fig 3.1).

In order to determine whether the T6SS in *Pcb* 1692 plays a role in *Pcb* 1692 virulence in potato tubers, it was noted that the wild type *Pcb* 1692 strain and *Pcb*1692*Δt6*-p*t6* complement strain produced similar amounts of macerated tuber tissue at each respective time point. The *Pcb*1692*Δt6* was significantly attenuated as less macerated tuber tissue was recovered at each time point, particularly at the 72h time interval (Fig 3.2). Tubers mock-inoculated with the negative control (10mM $MgSO_4$) exhibited no tissue maceration. These results suggest that the T6SS in *Pcb* 1692 contributes to the virulence of the pathogen during *in planta* infection. However it is unlikely that the T6SS is directly involved in the maceration of potato tuber tissue, rather the T6SS contributes to virulence indirectly.

3.2 Extracellular polysaccharide production by *Pcb*1692*Δt6* mutant strain was attenuated

The results indicated that production of EPS by *Pcb*1692*Δt6* mutant strain was significantly attenuated (0.11 g/100mL) compared to *Pcb* 1692 wild-type strain and *Pcb*1692*Δt6*-p*t6*

complement strain which produced 0.66 g/100mL and 0.57 g/mL of EPS respectively (Fig 3.3). The results suggest that *Pcb*1692 type VI secretion system plays a regulatory role in the production of extracellular polysaccharides in *Pcb* 1692. Extracellular polysaccharides is an important virulence factor in numerous bacteria, including phytopathogens (Quiñones *et al.*, 2005, Marcotte *et al.*, 2007). *Pcb* 1692 secretes large amounts of EPS which occludes xylem vessels, resulting in stem rot and wilting of potato plants. Furthermore, extracellular polysaccharides constitute a major component to the formation of biofilms which aid in protection from plant antimicrobial agents, UV light as well as the efficient assimilation of nutrients (Tanui *et al.*, 2017).

3.3 Biofilm formation by the *Pcb*1692*Δt6* strain was significantly attenuated

The formation of biofilms is an important virulence determinant in many plant and animal pathogens (Rahme *et al.*, 2000, Prithiviraj *et al.*, 2005). In Enteroaggregative *Escherichia coli*, *A. citrulli*, *P. aeruginosa* and *V. parahaemolyticus*, reports have shown that through inactivation of the T6SS resulting in no expression and secretion of Hcp, biofilm formation is attenuated as opposed to the relative wild-type strains (Enos-Berlage *et al.*, 2005, Southey-Pillig *et al.*, 2005, Aschtgen *et al.*, 2010, Tian *et al.*, 2015). Therefore, the hypothesis was made that the *Pcb* 1692 type VI secretion system could contribute to biofilm formation during potato tuber colonization thus exacerbating development of disease. Therefore, we determined the influence of type VI secretion system in the formation of biofilms by *Pcb* 1692 using crystal violet (CV) staining technique. Results indicated that both *Pcb* 1692 wild-type and *Pcb*1692*Δt6*-p*t6* complement strains formed biofilm as seen on the surface of the conical flask (Fig 3.4**A**). The *Pcb*1692*Δt6* mutant strain was unable to produce a biofilm under the same conditions (Fig 3.4**A**). A quantitative biofilm assay was performed to further support the above

mentioned observation using the spectrophotometer. The absorption mean value of the biofilm formed by the *Pcb*1692*Δt6* mutant strain was significantly reduced compared to that of the *Pcb* 1692 wild-type and *Pcb*1692*Δt6*-p*t6* complemented strains (Fig 3.4**B**).

3.4 Contribution of the T6SS to anti-bacterial competition

3.4.1 The *Pcb* 1692 T6SS has no influence on competitive fitness in nutrient rich environments

To determine whether the T6SS of *Pcb* 1692 mediates killing of neighbouring competing bacteria within a nutrient rich environment, *in vitro*, standardized cultures of *Pcb* 1692, *Pcb*1692*Δt6* and *Pcb*1692*Δt6*-p*t6* strains were independently co-inoculated with three competitor strains (*D. dadantii*, *D. chrysanthemi* and *Pcb* G4P5) on LB agar and enumeration of CFU/mL of targeted competitor bacteria showed that there was no reduction in the CFU/mL of the two competing bacteria at 72 hpi when co-inoculated on nutrient rich LB agar with either *Pcb* 1692 wild-type, *Pcb*1692*Δt6* or *Pcb*1692*Δt6*-p*t6*. We can hypothesize that in the presence of excess nutrients, despite contact between *Pcb* 1692 and competing neighbouring bacteria, the T6SS is not deployed for competitive fitness.

3.4.2 The *Pcb* 1692 T6SS plays a role in inter-bacterial competition *in planta*

Given that the T6SS of *Pcb* 1692 did not play a role in bacterial *in vitro* competition, but 13 of the core genes in the tss cluster were shown to be up-regulated in potato tubers within the first 24 hours post inoculation, contributing to virulence *in planta* (Bellieny-Rabelo et al., 2019). Therefore, it was reasoned that the T6SS in *Pcb* 1692 might play a role in competition *in planta*. To determine the contribution of the *Pcb* 1692 T6SS in bacterial competition, *in planta*, standardized cultures of *Pcb* 1692, *Pcb*1692*Δt6* and *Pcb*1692*Δt6*-p*t6* were independently co-

inoculated with competitor strains (*D. chrysanthemi*, *D. dadantii* and *Pcb* G4P5*)* into surface sterilized potato tubers, and CFU/mL of competitor bacteria enumerated three days post-inoculation. Results showed no reduction in the CFU/mL of *Pcb* G4P5 when co-inoculated in potato tubers with either *Pcb* 1692 wild-type, *Pcb*1692*Δt6* and *Pcb*1692*Δt6*-p*t6* suggesting that the effectors secreted by the T6SS of *Pcb* 1692 cannot inhibit the growth of this strain (Fig 3.5 & 3.6). In contrast, while a three-fold reduction in the CFU/mL was observed when *D. chrysanthemi and D. dadantii* were co-inoculated with the *Pcb* 1692 wild-type and the *Pcb*1692*Δt6*-p*t6* complement, this inhibition was completely lost when competitor strains were co-inoculated with the *Pcb*1692*Δt6* mutant strain (Fig 3.5 & 3.6). In *P. ananatis,* the T6SS was also observed to contribute to anti-bacterial activity with a strain of *P. ananatis* mutated for the T6SS-1 (*ΔT6*) attenuated in its ability to kill competitors such as *E. coli* DH5α, *Salmonella enterica* and *Pantoea stewartii* compared to the wild-type strain in a co-inoculation study consisting of 30 Gram-negative competitors (Shyntum *et al.*, 2015). These results more definitively demonstrate the involvement of the *Pcb* 1692 T6SS in inter-bacterial competition *in planta*.

3.5 Bioinformatics analysis

In previous studies, *zur* homologs have been characterized in several plant and human pathogens, which include *Xanthomonas* spp., *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes* (Patzer & Hantke, 1998, Dalet *et al.*, 1999, Campoy *et al.*, 2002, Yang *et al.*, 2007, Huang *et al.*, 2008). The putative binding site of the transcriptional regulator *zur*, was found to be situated in *Pcb* 1692 within the Tss promoter region, VipA operon encoding for the core components of the T6SS, and is supported with a q-value and p-value of 0.0176 and 9.07E-06 respectively as well as a score of 8.85714 indicating statistical significance. The annotated *zur* gene from *E. coli* K-12 was acquired from Swis-PROT (POAC51) and used as a query to identify a potential *zur*

homolog in *Pcb* 1692. A protein BLAST with organism filtering to *Pcb* 1692 was performed which matched to the annotated PCBA-RS11860 [100% coverage; 98% identity], which was then verified by the presence of a 510 bp annotated transcriptional regulator named *zur* in *Pcb* 1692 found within the NCBI REFSEQ database with an identical locus tag. Furthermore, utilizing the BLASTn tools available on PATRIC and NCBI database as well as the OrthoMCL program (in order to determine conservation of the *zur* homologues between species) and data downloaded from the NCBI Refseq database, the complete genome sequence of *Pectobacterium carotovorum* subsp. *brasiliense* strain *Pcb* 1692 was analysed. A gene encoding a protein of 169 amino acid residues which shares a high amino acid similarity with the Zur proteins of 100 SRE strains most notably *D. dadantii* (DDA3937_RS02765), *P. carotovorum* (PC1_RS02560), *P. carotovorum* (PCC21_RS02680) and *P. atrosepticum SCRI1043* (ECA_RS03150) (Table S1) was identified. The *zur* gene product from the *Pectobacterium carotovorum* subsp. *brasiliense* strain 1692 (locus tag PCBA_RS11860) and the upstream gene PCBA_RS11865, encoding a hypothetical protein possibly unrelated to *zur*, were orientated in the same direction separated by 144 bp between the stop codon of PCBA_RS11865 and the start codon of PCBA_RS11860 (Fig 3.8**A**). The 144 bp intergenic space was hypothesised to contain the native promoter for PCBA_RS11860.

3.6 Generation of the *Pcb1692Δzur* mutant

This prompted us to functionally characterise Zur's role in the regulation of the T6SS of *Pcb* 1692. Using one step inactivation method, the *zur* gene was successfully disrupted (Fig S3.1). The mutant generated was confirmed using selected primers to ascertain the insertion of the kanamycin resistance marker by a series of PCR reactions (Fig S3.2). The zurT_F primer annealed 841 bp upstream of the *zur* mutation insertion site and the Km1 annealed internally to the kanamycin resistance gene, amplifying a 1,5 kb fragment (Fig S3.2), verifying the insertion of the kanamycin resistance gene. The *Pcb1692Δzur* mutant was complemented

through expressing the *zur* gene *in trans* using the pTrc99A expression vector. Mutant and complemented strains growth analysis indicated that the insertion of the kanamycin resistance gene as well as the plasmid insertion, did not negatively influence the growth of the respective strains. The *Pcb1692Δzur*-p*zur* complement strain was constructed by cloning a 718 bp fragment from 138 bp upstream of the start codon of PCBA_RS11860 (including the 144 bp intergenic region mentioned above) to 70 bp downstream of the stop codon of PCBA_RS11860 in the promoterless expression vector pTrc99A. Granted PCBA_RS11860 may be the last ORF of a polycistronic operon, *Pcb1692Δzur*-p*zur* was functional within the phenotypic assays performed, indicating that the 718 bp fragment contains a functional promoter.

3.7 Testing of *Pcb1692Δzur* mutant strain phenotypes

3.7.1 *In vitro* growth curve

The relative growth rates of the *Pcb* 1692 wild type, *Pcb*1692*Δzur* mutant and *Pcb*1692*Δzur*p-*zur* complement strains was assessed in order to determine whether disrupting the *zur* gene had an effect on the mutant strains ability to grow resulting in a significant difference in growth rate. However there was no significant difference in growth rate between the mutant strain and its respective complement strain. Therefore any phenotype observed in the upcoming assays is a result of the disruption of the gene of interest (*zur*) as opposed to differing growth rates (Fig 3.9).

3.7.2 Biofilm formation by the *Pcb*1692*Δzur* strain was significantly attenuated

The influences of *Pcb* 1692 Zur in the formation of biofilm was determined by using crystal violet (CV) staining technique. Results indicated that both *Pcb* 1692 wild-type and *Pcb*1692*Δzur*-p*zur* complement strains formed biofilm as seen on the surface of the conical

flask (Fig 3.12**A**). The *Pcb*1692*Δzur* mutant strain was unable to produce a biofilm under the same conditions (Fig 3.12**A**). A quantitative biofilm assay was performed to further support the above mentioned observation using the spectrophotometer. The absorption mean value of the biofilm formed by the *Pcb*1692*Δzur* mutant strain was significantly reduced compared to that of the *Pcb* 1692 wild-type and *Pcb*1692*Δzur*-p*zur* complemented strains (Fig 3.12**B**).

3.7.3 Virulence assay

In order to determine whether Zur in *Pcb* 1692 plays a role in *Pcb* 1692 virulence in potato tubers, standerdized cultures of *Pcb* 1692 wild-type, *Pcb*1692*Δzur* mutant and *Pcb*1692*Δzur*-p*zur* complement strains were independently inoculated into potato tubers. It was noted that the wild type *Pcb* 1692 strain and *Pcb*1692*Δzur*-p*zur* complement strain produced similar amounts of macerated tuber tissue at each respective time point. Whereas the *Pcb*1692*Δzur* was significantly attenuated as less macerated tuber tissue was recovered at each time point, particularly at the 72h time interval (Fig 3.11). Tubers mock-inoculated with the negative control (10mM $MgSO_4$) exhibited no tissue maceration.

3.7.4 Zur's impact on maintaining zinc homeostasis in *Pcb* 1692

Due to the paradoxical biological effect of varying concentrations zinc has on bacterial cells, the intracellular Zn(II) level must be precisely regulated (Huang *et al.*, 2008, Gilston *et al.*, 2014). Previously, studies have shown that X. *campestris* pv. *campestris*, employs zinc uptake regulator Zur, to repress the transcription of Zn(II) uptake systems in order to maintain zinc homeostasis. In order to determine whether *Pcb* 1692 Zur plays a role in maintaining Zn(II) homeostasis in *Pcb* 1692, *zur* gene was disrupted and characterized. M9 minimal media was supplemented with increasing concentrations of Zn(II) prior to being inoculated with standardized cultures of *Pcb* 1692*, Pcb1692Δzur* and *Pcb1692Δzur*-p*zur*. Our results indicated that the *Pcb1692Δzur* mutant maintained a similar growth rate to that of the *Pcb* 1692 wild-

type and *Pcb*1692*Δzur*-p*zur* complement until a Zn(II) concentration threshold of 1.25 mM was reached, at which point the *zur* mutant was unable to grow as convincingly as the wild-type strain (Fig 3.10).

3.8 *Zur*'s involvement in regulating the type VI secretion system in *Pcb* 1692

3.8.1 Gene expression

Studies have demonstrated a functional *zur* gene is required for the full development of disease in both *X. campestris* pv. *campestris* as well as *X. oryzae* pv. *oryzae* since *zur* has been linked to the regulation of genes associated with virulence (Yang *et al.*, 2007, Huang *et al.*, 2009). Through the use of bioinformatic tools such as OrthoMCL, MEME Suite (Bailey *et al.*, 2009) BLASTn tools available on PATRIC and NCBI database as well as data downloaded from the NCBI Refseq database, we observed that of the 100 SRE strains tested for the presence of the *zur* gene, all 100 harboured a conserved *zur* domain as well as being constrained to harbouring the presence of only one *zur* ortholog (Table S1). The putative binding site of the transcriptional regulator *zur*, was also found to be situated within the Tss promoter region of the type VI secretion system core cluster (Fig 3.7; Table S2). These results were supported by a q-value and p-value of 0.0176 and 9.07E-06 respectively as well as a score of 8.85714. Therefore due to the Zur protein's ability to negatively regulate other transcriptional processes as well as its ability to bind in the promoter region of the T6SS core cluster, we aimed to determine whether the Zur protein is involved in imposing a regulatory effect on the type VI secretion system, directly affecting the anti-bacterial activity of *Pcb 1692.* Therefore we investigated the role of Zur in *Pcb* 1692 in relation to regulation of a core gene in the type VI secretion system, t*ssC* in order to determine whether the absence of *zur* gene directly influences the expression of the

T6SS resulting in an altered competitive fitness, *in planta*, as opposed to influencing a mechanism unrelated to the T6SS.

From the qPCR results, it was found that the relative expression of the *TssC* gene was up-regulated eleven-fold in the *Pcb1692Δzur* mutant strain (Fig 3.13), indicating that *zur* gene negatively regulates the T6SS. Loss of Zur function results in over expression of the T6SS, therefore contributing to the increased competitive fitness observed *in planta* (Fig 3.15). These results suggest that expression of the type VI secretion system core gene cluster is under regulation of Zur, furthermore clarifying as to why the *Pcb1692Δzur* mutant strain was more efficient at outcompeting *D. dadantii* in the co-inoculation competition studies.

3.8.2 *In planta* competition

Standardized cultures of *Pcb 1692*, *Pcb1692Δzur* and *Pcb1692Δzur*-p*zur* were independently co-inoculated with *D. dadantii* within surface sterilized potato tubers and CFU/mL of the targeted competitor bacteria (*D. dadantii*) enumerated three days post inoculation. Interestingly, while the results show a one-fold reduction in the CFU/mL of the *D. dadantii* competitor when co-inoculated with the *Pcb1692Δzur* mutant strain, this increased inhibition was lost when the *D. dadantii* competitor was co-inoculated with the *Pcb 1692* wild-type strain and *Pcb1692Δzur*-p*zur* complement strain (Fig 3.15). Suggesting that the loss of function of Zur within the *Pcb1692Δzur* mutant strain amplified the competitive fitness of the strain through either increased production and secretion of effectors secreted by T6SS, resulting in a toxic accumulation of effectors or through a mechanism unrelated. From these results we can suggest that Zur acts as a repressor of the T6SS altering *Pcb 1692*'s ability to eliminate competing bacteria, possibly through regulation of the type VI secretion system.

3.9 Discussion

Type VI secretion systems have been described in a number of Gram-negative bacteria and have been shown to act against both prokaryotes and eukaryotes (Boyer *et al.*, 2009, Basler, 2015). Within bacterial pathogens, the T6SS was first implicated in virulence through secreting effector proteins directly into eukaryotic cells (Ho *et al.*, 2014). However the T6SS's reputation as a competitive aggressor has stolen the attention of many studies, focussing mainly on the T6SS's ability to outcompete neighbouring bacteria through its anti-bacterial activity (Ma *et al.*, 2014, Shyntum *et al.*, 2015). Here we show that the type VI secretion system present in *Pcb* 1692, contributes to virulence and is vital for interbacterial competition during *in planta* infection.

In the current study, the *Pcb*1692*Δt6,* T6SS deficient mutant strain was functionally characterized and the T6SS's role in disease progression, competitive fitness and virulence during host-pathogen interactions was assessed. Furthermore, the *Pcb*1692*Δzur* zinc uptake regulator deficient mutant strain was characterized to assess whether the T6SS is under regulation of the zinc uptake regulator (Zur). Our results demonstrated that the *Pcb1692Δt6* mutant strain was attenuated in EPS production, biofilm formation and virulence in potato tubers. Similarly, *Pcb*1692*Δt6* mutant strain was inefficient in anti-bacterial competition during *in planta* infection indicating that T6SS regulate some virulence factors directly or indirectly. This was in agreement with studies conducted by Shyntum *et al.*, (2015) where authors showed that mutation of T6SS in *Pontea ananatis* resulted in reduced virulence in onions (Shyntum *et al.*, 2015). Furthermore, our findings showed that the *Pcb* 1692 T6SS might be under the regulon of Zur, since the *Pcb1692Δzur* mutant strain demonstrated enhanced capabilities to outcompete targeted bacteria *in planta*. This result was further supported through the observation that the *TssC* gene was up-regulated eleven-fold in the *zur* mutant strain. Inferring

that in the absence of Zur, the T6SS is under less stringent regulatory action and is over-expressed resulting in the enhanced effective killing of neighbouring competitors.

The type VI secretion system has been a point of focus for study in recent years, however no emphasis has been directed to determining whether there is a direct link between the type VI secretion system and anti-bacterial activity in *Pectobacterium* species (Kamber *et al.*, 2017).

Kamber *et al.*, (2017) observed attenuation in virulence in *Erwinia amylovora* strain due to mutation of two T6SS gene clusters (T6SS-1 and T6SS-3). They showed that at 12 days post inoculation (DPI) inoculated pear flowers exhibited a significant attenuation in produced necrotic lesions of approximately 20% for the T6SS-1 and T6SS-3 double mutant compared to the wild-type strain. The T6SS-1 and T6SS-3 single mutants exhibited an attenuation in produced necrotic lesions of approximately 5% compared to the wild-type strain (Kamber *et al.*, 2017). Similarly, Sana *et al.*, (2012) reported that the H2-T6SS and H3-T6SS T6SS gene clusters of *P. aeruginosa* contributes to the virulence of the PAO1 wild-type strain, which was significantly more effective at killing *Caenorhabditis elegans* than that of the clpV2 and clpV3 mutants (H2-T6SS and H3-T6SS mutant strains). Interestingly, in this study, the *Pcb1692Δt6* mutant strain with a non-functional T6SS, was attenuated in its ability to macerate potato tuber tissue. Therefore, we hypothesize that the observed reduction in virulence of the *Pcb1692Δt6* strain, during *in planta* infection, was a result of the altered expression of virulence determinants, such as genes involved in the production of biofilms and extracellular polysaccharides that might be regulated or influenced by the T6SS.

According to studies by Tian and colleagues, they observed that *Erwinia amylovora* was able to produce two types of extracellular polysaccharide, amylovoran and levan. They reported that of the 19 strains of *E. amylovora* independently mutated for individual core genes of the T6SS, the authors reported 7 mutants with decreased amylovoran and levan production, 9 mutants

with decreased amylovoran and 3 mutants with decreased levan with all mutants attenuated in virulence (Tian *et al.*, 2017). The production of EPS has been reported to contribute to virulence of bacterial pathogens, protection from environmental stresses as a consequence of its highly hydrated and anionic consistency as well as bypassing plant defence systems and nutrient loss (Flemming *et al.*, 2007). Similarly, the *Pcb1692Δt6* mutant strain was also deficient in the production of extracellular polysaccharides. The subsequent decrease in the *Pcb1692Δt6* mutant strains ability to produce EPS could contribute to the attenuation in virulence observed within the mutant strain.

Biofilm formation is a survival strategy employed by many pathogens to prevent its eradication by antimicrobial agents (Nesper *et al.*, 2001, Costerton *et al.*, 2005). In this study, the *Pcb1692Δt6* mutant strain was affected in the production and formation of a functional biofilm compared *Pcb* 1692 wild-type strain. Southey-Pillig *et al.*, (2005) reported that through mass spectrometry and two-dimensional gel electrophoresis, the expression of Hcp was determined to be critical for the production of mature biofilms in *P. aeruginosa*. As expected, when the *Hcp1* gene of *P. aeruginosa* was mutated, the formation of biofilms was attenuated and biofilm specific antibiotic resistance was abolished, inferring that H1-T6SS is closely linked to biofilm production (Chen *et al.*, 2015).

Due to the dynamic, polymicrobial nature of the environmental niches bacteria inhabit, competition for space and nutrients is vital for invasion, adaptation, proliferation and successful colonization of its host (Hibbing *et al.*, 2010). As a result the T6SS has been implicated to play a role in bacterial competition killing any neighbouring bacteria.

The legume cowpea has been shown to be exclusively nodulated by the wild-type strain of *P. phymatum*, even when exposed to various β-rhizobia, inferring that *P. phymatum* employs a mechanism allowing it to outcompete other β-rhizobia and produce root nodules (Ahmad &

Morgan, 1994, Lardi *et al.*, 2017). De Campos *et al.*, (2017) concluded that the T6SS was found to be one of the factors responsible for the success of *P. phymatum* in cowpea as the authors observed that when the *P. phymatum* wild-type was co-inoculated with competing β-rhizobia within cowpea, 100% of nodules formed were occupied by *P. phymatum*. However, when the T6SS mutated strain was co-inoculated, a number of the root nodules formed were observed to be occupied by the competing β-rhizobia (de Campos *et al.*, 2017). *E. amylovora* too relies on the activity of the T6SS to outcompete competing bacteria. Tian *et al.*, (2017) examined the abilities of 33 T6SS *Erwinia amylovora* mutants in competitively killing *Escherichia coli DH5α in vitro*. Compared to the co-inoculation with *Erwinia amylovora* wild-type where survival of the competing *E. coli* was low, when co-inoculated with all 33 mutants, survival of the *E. coli* was significantly increased. The authors came to the conclusion that not only is the T6SS functional within *E. amylovora*, it too plays a role within bacterial competition (Tian *et al.*, 2017). The phenotype of the T6SS mutant within *Serratia marcescens* was similarly assessed (Murdoch *et al.*, 2011). The authors found that the inactivation of the T6SS did not have any evident influence on the motility, biofilm formation or anti-eukaryotic activity within *S. marcescens*. However the T6SS was observed to play a major role in anti-bacterial activity as the survival of competing *E. coli* was increased 10^4 fold when co-inoculated with the T6SS mutant strain compared to that of the co-inoculation with the *S. marcescens* wild-type and *E. coli*, which yielded significantly less viable *E. coli* cells. In the co-inoculation assays performed in this study where three SRE (*D. chrysanthemi*, *D. dadantii* and *Pcb*G4P5*)* were co-inoculated with the *Pcb* 1692, *Pcb*1692*Δt6* and *Pcb*1692*Δt6*-p*t6* strains, the antagonistic effect of competition the *Pcb* wild-type, mutant and complement strains exerted on the targeted competitors was quantified through enumeration of bacteria through CFU/mL. Our results showed that there was a significant reduction in survival of *D. chrysanthemi* and *D. dadantii* when co-inoculated with the *Pcb* wild-type and complement strains, however this competitive

fitness was lost when the *Pcb*1692*Δt6* was co-inoculated with the respective competitors. Interestingly, there was no evidence of competition between *Pcb* 1692 and *Pcb*G4P5, suggesting that the effectors secreted by the T6SS of *Pcb* 1692 cannot inhibit the growth of this strain. The absence of a functional type VI secretion system resulted in an increased survivability of the *D. chrysanthemi* and *D. dadantii* competitors when co-inoculated with the *Pcb*1692*Δt6* mutant strain, indicating a loss of competitive fitness within the mutant strain.

Cues such as pH, osmolarity, plant extracts, temperature and divalent metals such as iron are required by the T6SS of some bacteria in order to be induced (Miyata *et al.*, 2013). As mentioned before, when inoculated in potato tubers, the T6SS of *Pcb* 1692 was up-regulated (Bellieny-Rabelo *et al.*, 2019). Similarly to what was observed in the studies conducted with the type VI mutant strains of *P. Phymatum*, *E. amylovora* and *S. marcescens*, the type VI secretion system mutant in this study was too deficient in its ability to outcompete neighbouring competitor cells (Murdoch *et al.*, 2011, de Campos *et al.*, 2017, Kamber *et al.*, 2017). The *Pcb* 1692 wild-type was able to outcompete two of the three competing strains *in planta,* drastically decreasing their CFU as opposed to the *Pcb*1692*Δt6* mutant which was not able to outcompete any of the three competitors. Therefore, we can infer that within potato tubers the T6SS of *Pcb* 1692 is recruited to hinder the growth of various enophytes.

The regulation of zinc uptake has a key role in cellular function, understanding the manner in which bacterial pathogens regulate cellular processes in response to certain environmental queues is of the utmost relevance to plant health (Ammendola *et al.*, 2007). In *E. coli*, the *zur* protein negatively regulates the expression of the high-affinity ZnuABC zinc uptake system (Patzer & Hantke, 1998). Genes with clear similarity to ZnuABC were not detected in *Pectobacterium carotovorum* subsp. *brasiliense,* suggesting that the ZnuABC genes may be highly divergent. However, based on the *Pcb1692Δzur* mutant strain's inability to grow at

higher concentrations of zinc compared to the wild-type strain, it is reasonable to assume that isofunctional genes of ZnuABC exist in *Pectobacterium carotovorum* subsp. *brasiliense.*

Sequence analysis of the ORF, PCBA_RS11860 of *Pectobacterium carotovorum* subsp. *brasiliense* using BLASTn tools as well as the OrthoMCL program, indicated that the protein product is a possible homolog of the *zur* gene. A mutation of this *zur* gene through a method previously described by Datsenko *et al.,* (2000) resulted in an increased sensitivity to zinc, with the mutant strain unable to proliferate in growth medium with increased concentrations of zinc. These findings established a role for PCBA_RS11860 in zinc homeostasis and strongly support the hypothesis that PCBA_RS11860 encodes a *zur* protein.

Due to the finding that the putative binding site of the transcriptional regulator *zur*, was situated within the Tss promoter region of the type VI secretion system core cluster as well as the Zur protein's ability to negatively regulate other transcriptional processes, we aimed to identify a function for the *Pcb1692* Zur protein in anti-bacterial activity through regulating the type VI secretion system. In the co-inoculation assays performed, we observed that the *Pcb1692Δzur* mutant strain elicited a one-fold decrease in the CFU/mL of the competitor strain, *D. dadantii,* three days post inoculation when compared to the co-incubation of *D. dadantii* with the *Pcb1692* wild-type (Fig. 2.7). Intriguingly, absence of the Zur protein in the *Pcb1692Δzur* strain resulted in an increased level of anti-bacterial activity. We can hypothesize that Zur binds to the promoter region of the Tss core gene cluster (VipA operon), negatively regulating the type VI secretion system. Therefore, absence of Zur in *Pcb1692Δzur* mutant strain resulted in no such negative regulation of the T6SS, hence an amplified degree of anti-bacterial killing was observed compared to the *Pcb* 1692 wild-type strain.

In order to further support the hypothesis that Zur negatively regulates the type VI secretion system, the expression of the *TssC* gene, a T6SS core gene within the VipA operon, was

determined using real-time qPCR. We observed that the *TssC* gene was significantly up-regulated (evelen-fold up-regulation) in the *Pcb1692Δzur m*utant strain compared to the *Pcb*1692 wild-type strain, suggesting that in the absence of the transcriptional regulator Zur, genes within the VipA operon are significantly up-regulated. This up-regulation of genes involved in the type VI secretion system support the co-inoculation assay where the *Pcb1692Δzur* mutant was more capable of outcompeting the *D. dadantii* competitor.

CHAPTER FOUR

Investigating the role of the type VI secretion system in *Pectobacterium* disease processes and its regulation by Zur.

Concluding Remarks & Future Work

4.1 Conclusions and perspectives

The type VI secretion system is a specialized contact-dependent transporter of proteins mediated from a Gram-negative bacterial donor cell to a prokaryotic or eukaryotic recipient cell, having a role in both host interactions as well as pathogenesis.

The research presented in this dissertation suggests that the T6SS is an important virulence determinant in *Pcb* 1692, highlighting the importance of further investigation of the T6SS in the progression of disease in phytopathogens. Although, the exact role that the T6SS plays in SRE is largely unknown, the fact that this protein secretion system is upregulated when *Pcb* 1692 is inoculated in potato tubers is an indication that the pathogen recruits it for the progression of disease. In this study, the T6SS mutant strain was attenuated in its ability to macerate potato tuber tissue as well as being attenuated in the production of biofilm and EPS in comparison to the *Pcb* 1692 wild-type strain. The T6SS mutant strain was too attenuated in its ability to kill competing bacteria in comparison to the *Pcb* 1692 wild-type. Furthermore, a gene encoding the zinc uptake regulator (Zur) was also disrupted. The Zur mutant strain was unable to grow in M9 minimal media supplemented with increased levels of Zn(II) in coparison to the *Pcb* 1692 wild-type and was attenuated in its ability to macerate potato tuber tissue. The Zur mutant was too attenuated in its ability to produce biofilm, EPS as well as being observed to more effectively kill competing neighbouring bacteria due to a core gene of the T6SS machinery (*tssC*) up-regulated in the mutant strain in comparison to the *Pcb* 1692 wild-type strain. This study provides evidence to the importance of the T6SS in the development of disease in *Pcb* 1692 in the host plant and helped to elucidate the role T6SS plays in bacterial fitness and virulence.

In recent years the type VI secretion system has been a point of focus due to its growing reputation as a bacterial killing machine. The T6SS has been proven to facilitate the progression

of disease as well as aid in host colonization through influencing virulence determinants, manipulation of the host itself and competing microbes within the abiotic environment. In this study, the T6SS of *Pcb* 1692 was shown to contribute to disease processes such as the production of EPS, biofilm formation and killing of competing neighbouring bacteria. In the absence of a functional T6SS, *Pcb* 1692 loses the ability to produce EPS, an important virulence determinant. Similarly, the formation of biofilms as well as the ability to kill neighbouring bacteria *in planta* was attenuated in the T6SS deficient strain. Finally, although anti-bacterial competition was not affected *in vitro*, the observed reduction in targeted bacteria *in planta* may be the key to constructing more effective management strategies for the reduction and/or prevention of environmental spread in potato fields and post-harvest. In this study, the *Pcb* 1692 *zur* gene was too mutated in order to determine whether it imposed a regulatory effect on the *Pcb* 1692 T6SS. As is seen in the literature, we observed the *Pcb* 1692 Zur to regulate the expression of genes involved in zinc acquisition and storage systems. Such regulation of zinc uptake systems could be crucial for the longevity as well as virulence of *Pcb* 1692 in potato tubers, given that zinc is limiting in this environment in the early stages of development with *Pcb* 1692 having to compete with other bacteria for the limited zinc. Interestingly, we reported a novel mechanism of regulation exerted on the T6SS of *Pcb* 1692. We observed that Zur exerts a negative regulatory effect over the T6SS, overexpressing the core genes of the VipA operon in the absence of Zur.

Combining the findings of this research it can be concluded that the T6SS of *Pcb* 1692 is indeed recruited by this pathogen in the progression of disease development and could be a contributing factor to *Pcb* 1692 heightened degree of virulence.

In future studies, the link between the T6SS and virulence determinants such as biofilm production and EPS production can be further explored in ordered to determine the reason as to why in the absence of a functional T6SS the production of biofilm and EPS is attenuated.

The reason as to why the T6SS was up-regulated within potato tubers can also be explored, giving further insight into what triggers the pathogen to overexpress a mechanism which aids in the progression of disease development. As Zur has previously been reported to be a multifunctional regulator of many virulence determinants, a more extensive analysis of which virulence determinants Zur regulates in *Pcb* 1692 could be undertaken. Since Zur's putative binding site is within the promoter region of VipA and in the absence of a functional Zur a core gene of the T6SS, *tssC*, was up-regulated, further studies could assess whether all of the core genes within the VipA operon are up-regulated in the absence of Zur. Additionally, the relative fold change can be assessed at different time points post inoculation in order to assess at which time point post inoculation the T6SS would be most expressed.

Finally, targeting a secretion system with notable influence over numerous virulence determinants, such as the T6SS, may be the key to implement more effective management strategies. Reducing the spread of *Pcb* 1692 within the environment as well as post-harvest.

4.5 References

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Chapter three - Figures

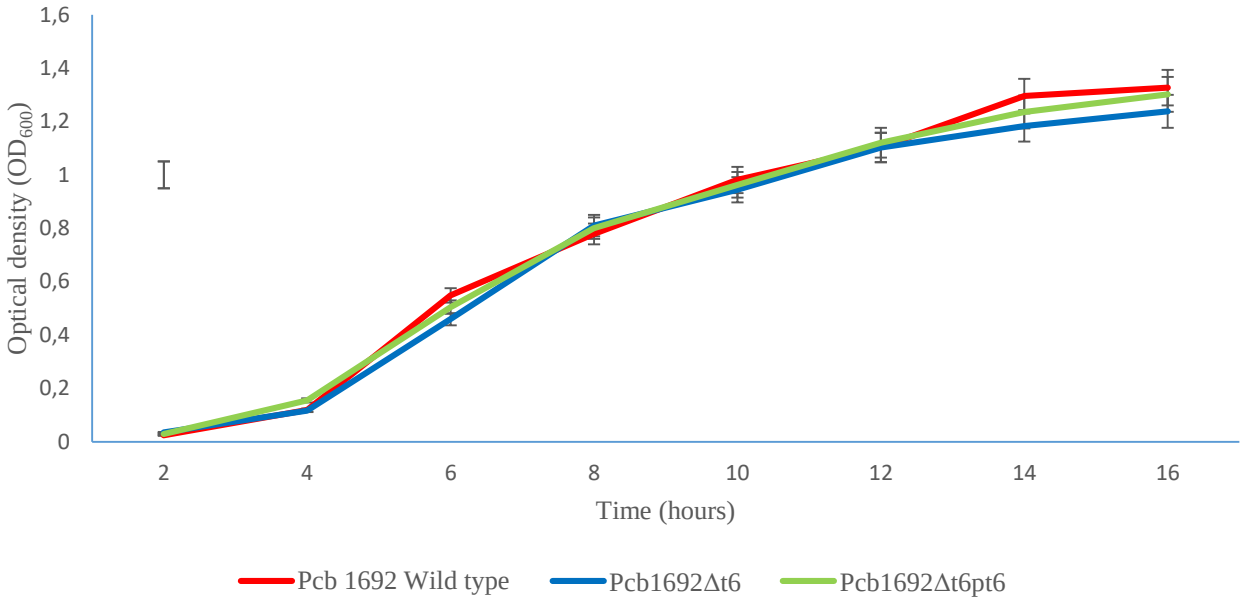


Fig 3.1. *In vitro* growth curve analysis of the wild-type *Pcb* 1692, *Pcb1692Δt6, Pcb1692Δt6*p-*t6* strains. All strains tested were grown for 16 h and measured at OD_{600} at one-hour intervals. Statistical analysis indicates no significant difference for growth between tested strains (*p*-value < 0.05).

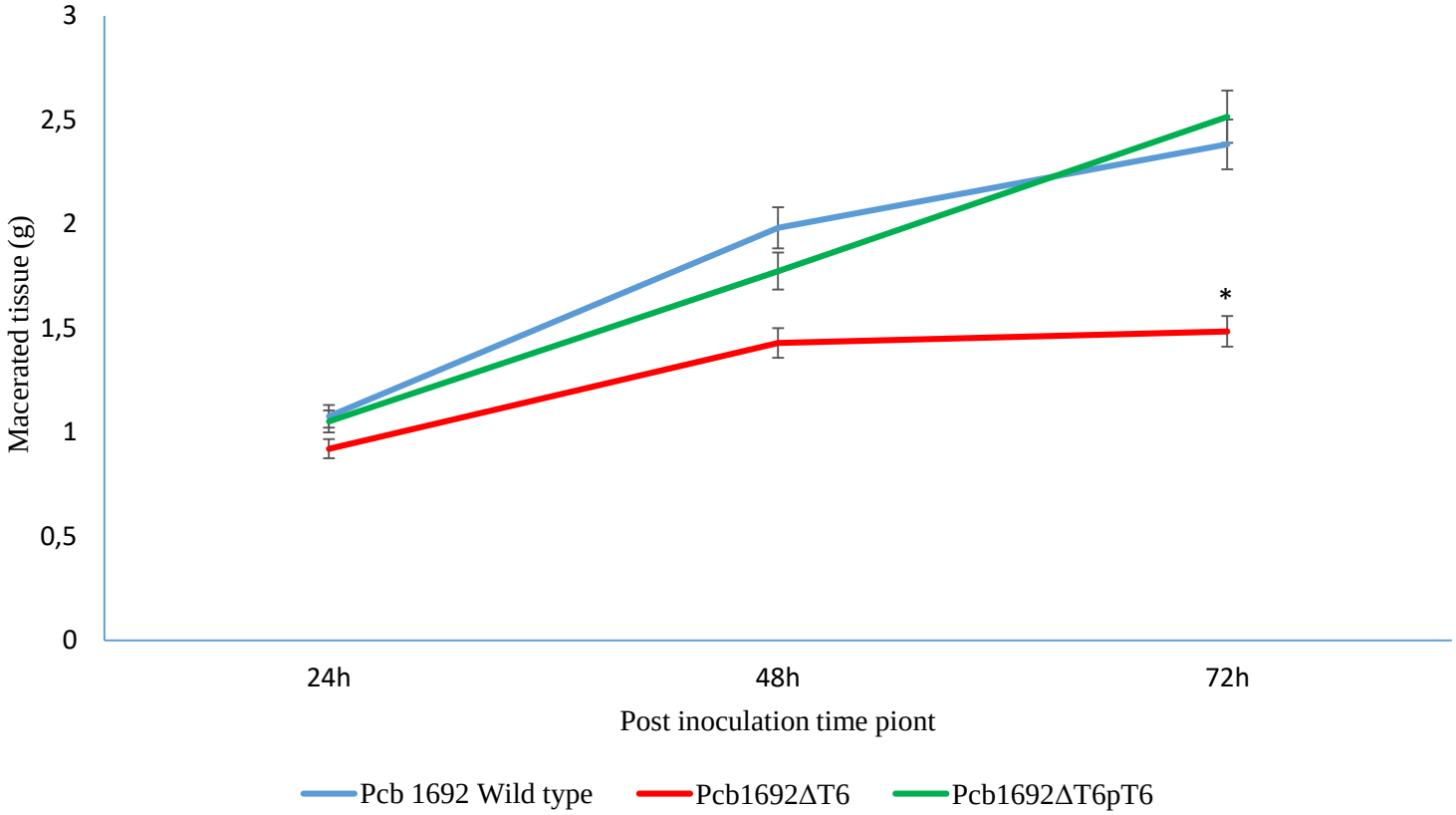


Fig 3.2. Effect of T6SS in *Pcb* 1692 virulence on potato tubers. Susceptible potato tubers (cv. Mondial) were inoculated with standardized cultures of *Pcb* 1692, *Pcb1692Δt6* and *Pcb1692Δt6*-p*t6*. $MgSO_4$ was used as a negative control. Macerated tissue was scooped and weighed at 24 hpi, 48 hpi and 72 hpi (*p*-value < 0.05).

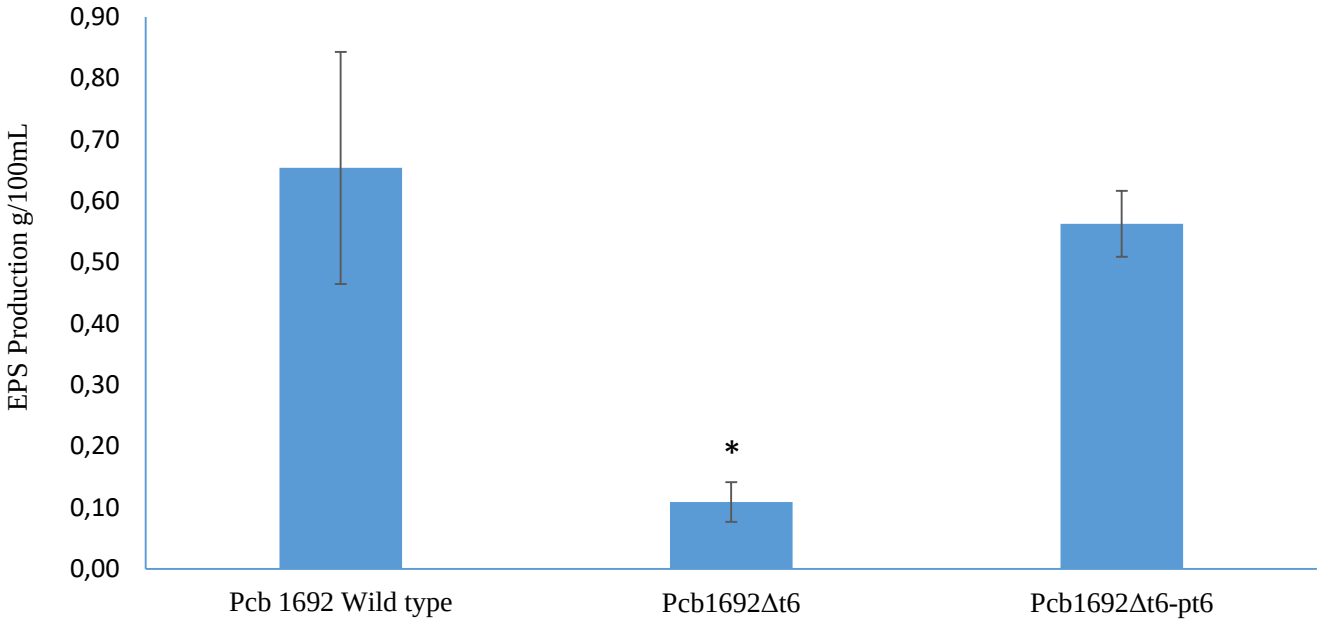


Fig 3.3. Comparison of EPS production by *Pcb1692Δt6 and Pcb* 1692 wild type strains. The EPS production in the *Pcb1692Δt6* mutant strain was significantly reduced compared to the *Pcb* 1692 wild type and *Pcb1692Δt6*-p*t6* strains (*p*-value < 0.05).

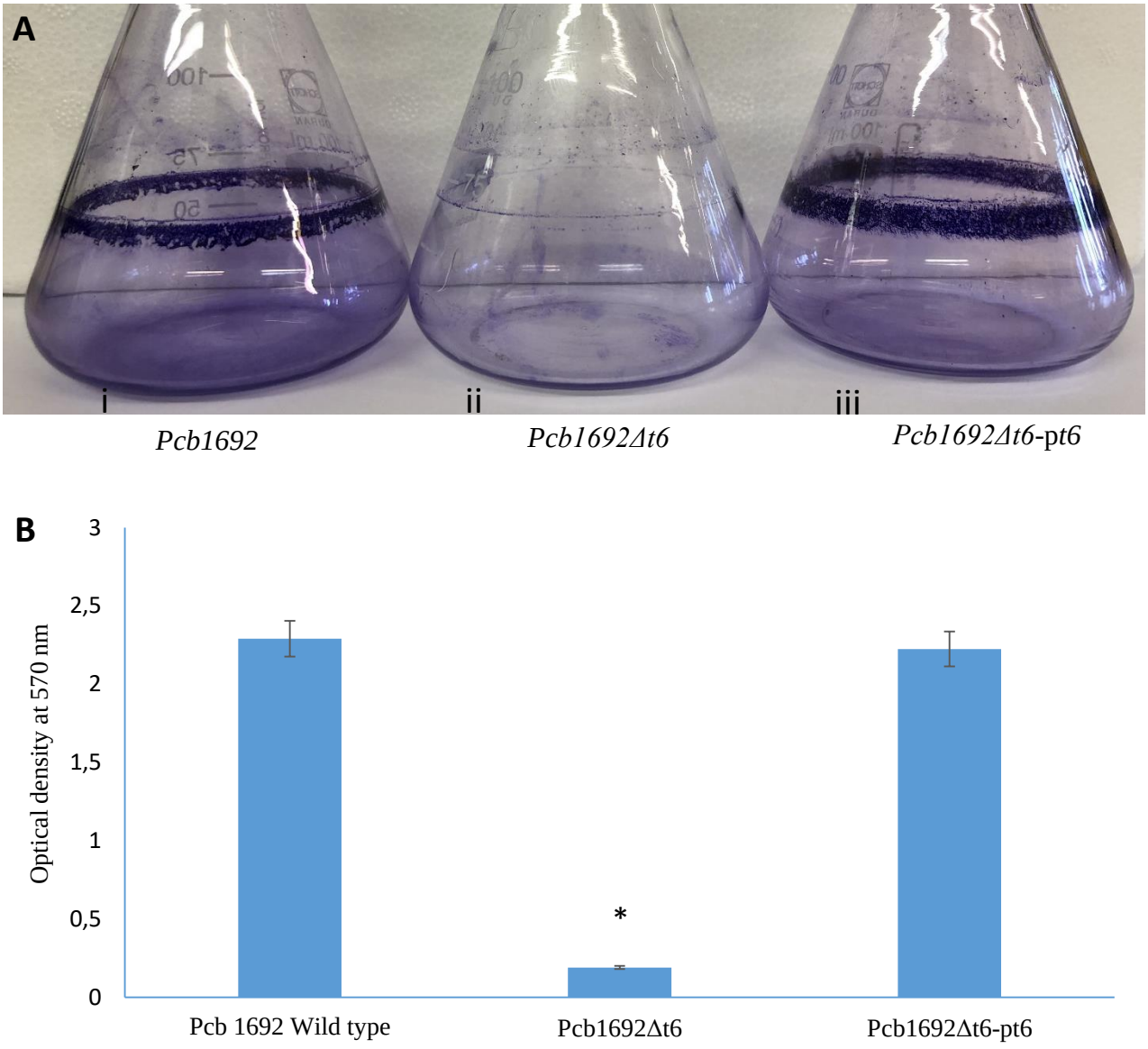


Fig 3.4. The effect of the type VI secretion system on biofilm formation within *Pcb* 1692. **A.** Qualitative formation of biofilm within conical flasks prior to staining with 0.1% crystal violet (Sigma-Aldrich). Flasks i, ii and iii represents the *Pcb* 1692 wild-type, *Pcb1692Δt6* mutant strain and *Pcb1692Δt6*-p*t6* strain respectively. **B.** Quantitative analysis of biofilm formation by the *Pcb* 1692 wild-type, *Pcb1692Δt6* mutant strain and *Pcb1692Δt6*-p*t6* complement strain. Data represents the averaged mean values the optical density at OD_{570} from three independent experiments (p-value < 0.05).

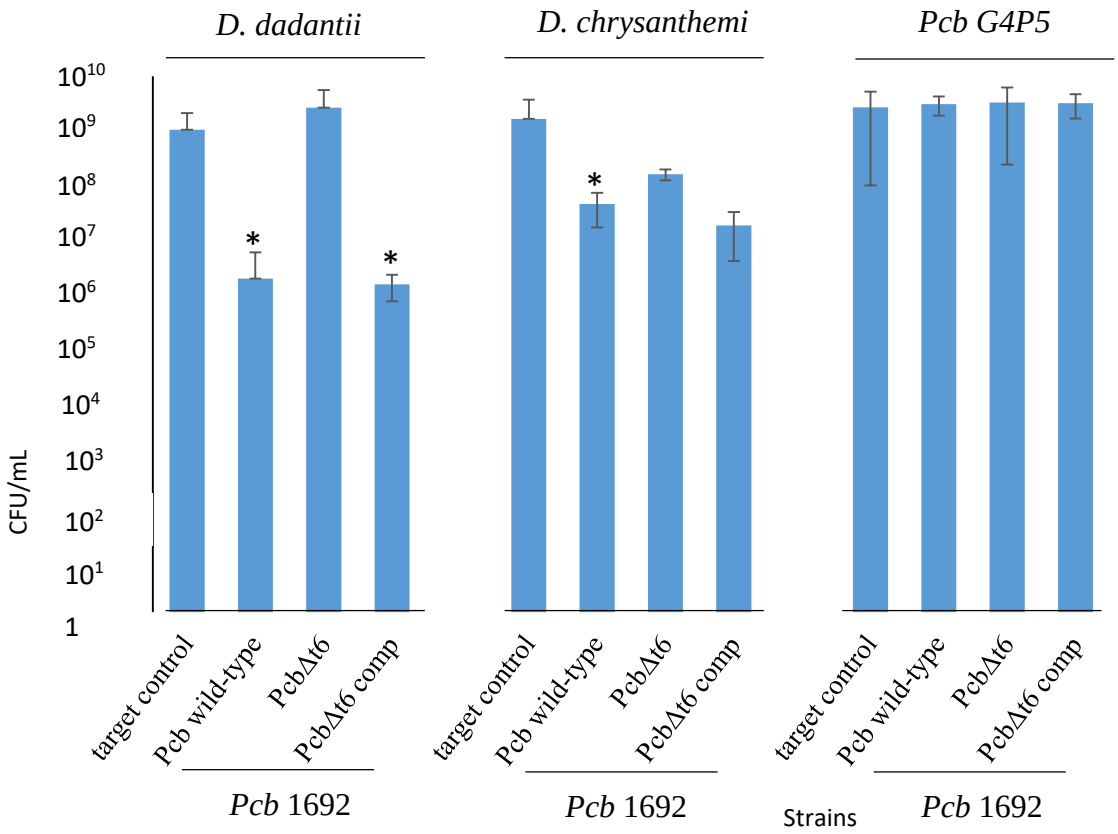


Fig 3.5. Effect of T6SS in *Pcb* 1692 anti-bacterial competition *in planta*. Susceptible potato tubers (cv. Mondial) were inoculated with standardized cultures of *D.dad*, *Pcb*G4P5, *D.chr*, *Pcb* 1692, *Pcb1692Δt6* and *Pcb1692Δt6pT6* as well as co-inoculated with independent cultures of targeted bacteria and *Pcb* 1692, *Pcb1692Δt6* and *Pcb1692Δt6pT6.* $MgSO_4$ was used as a negative control. Macerated tissue was scooped and weighed at 72 hpi and targeted bacteria enumerated through serial dilutions (p-value < 0.05).

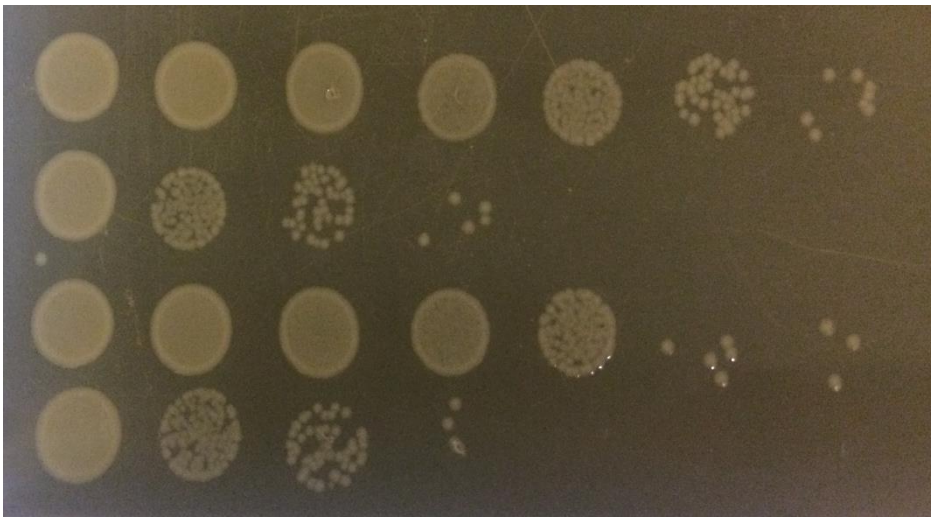


Fig 3.6. Effect of T6SS in *Pcb* 1692 anti-bacterial competition *in planta*. Susceptible potato tubers (cv. Mondial) were inoculated with separate as well as mixed cultures of *D.dad*, *Pcb* 1692, *Pcb1692Δt6* and *Pcb1692Δt6*-p*t6.* $MgSO_4$ was used as a negative control. Macerated tissue was scooped at 72 hpi and subjected to serial dilutions followed by plating on gentamycin supplemented agar, as previously described, selecting for *D. dadantii*.

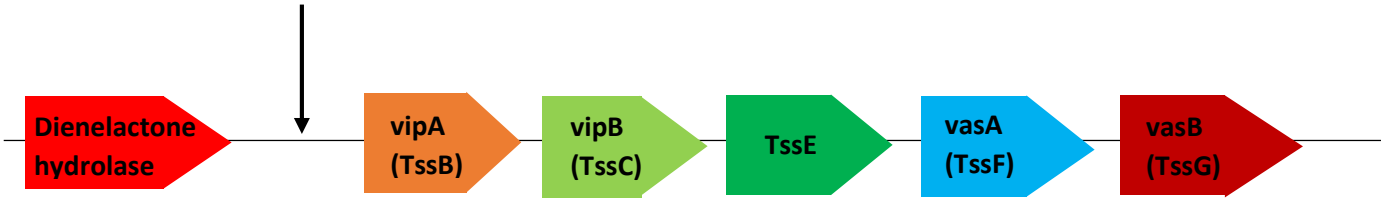


Fig 3.7. Diagrammatic depiction of the binding site of *zur* in the VipA operon (encoding the core components of the T6SS) in *Pcb* 1692. The arrow indicates the binding site of Zur and is supported with a *p*-value of 0.00000907. The intergenic region between dienelactone hydrolase and vipA is 935 bp, the Zur binding site is situated 629 – 647 bp upstream of vipA (encoding for TssB).

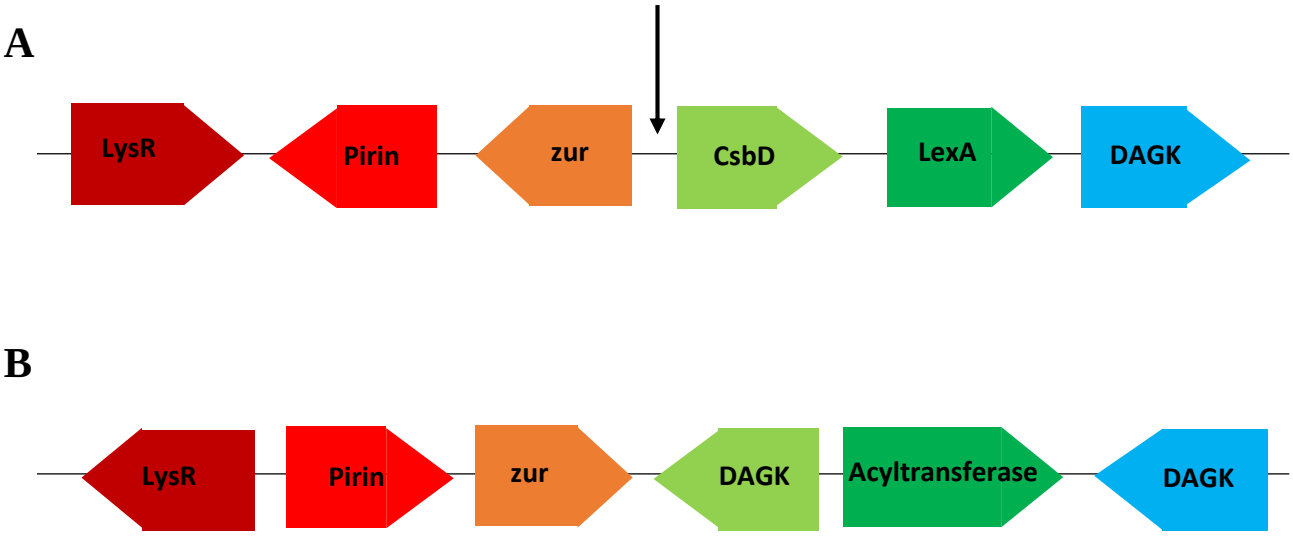


Fig 3.8. A. Diagrammatic depiction of *Pectobacterium* spp. *zur* gene and neighbouring gene region. The arrow indicates the 144 bp intergenic space between the *zur* gene and upstream gene containing *zur*'s native promoter identified on NCBI genome browser as (TGTCATATCACCCTGACGCATTGAGGAACAAGACGGATATCTTACCATGACTGAATAAAA). **B. Diagrammatic depiction of *Dickeya* spp. *zur* gene and neighbouring gene region.**

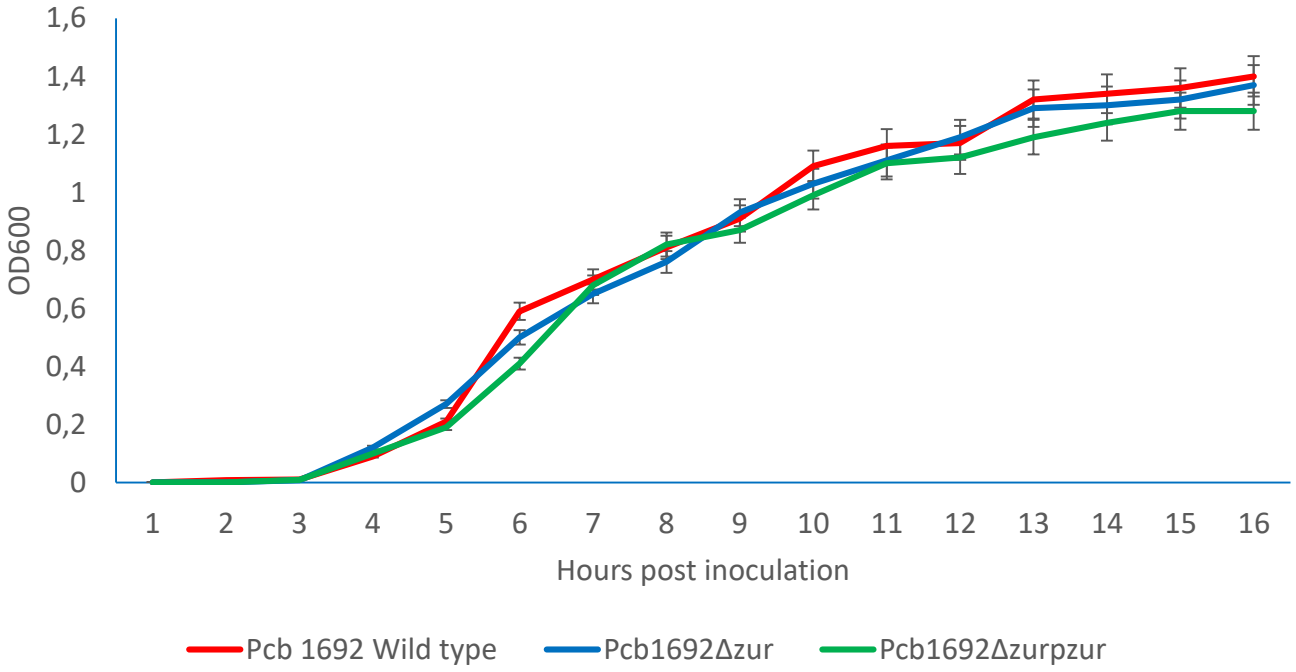


Fig 3.9. *In vitro* growth curve analysis of the wild-type *Pcb* 1692, *Pcb1692Δzur*, *Pcb1692Δzur*p-*zur* strains. All strains tested were grown for 16 h and measured at OD_{600} at one-hour intervals. Statistical analysis indicates no significant difference for growth between tested strains (*p*-value < 0.05).

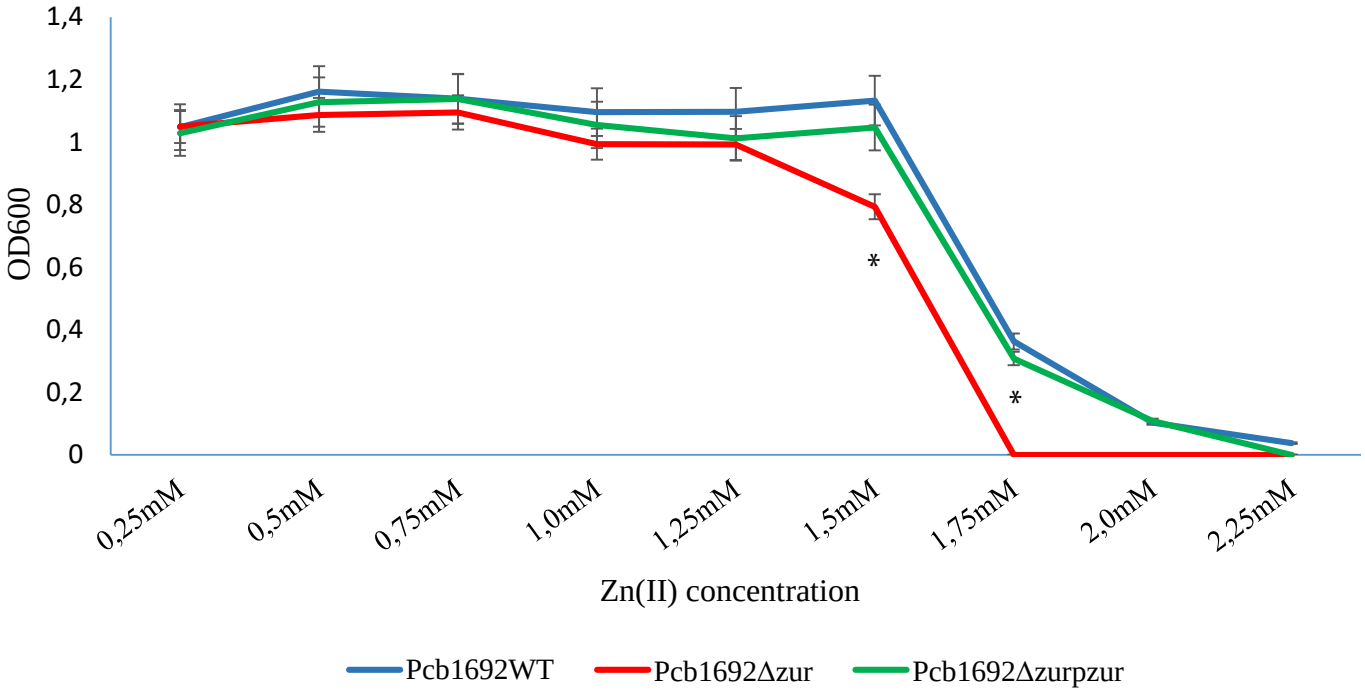


Fig 3.10. Increasing concentrations of transition metal Zn(II) affect mutant viability. M9 minimal media supplemented independently with increasing concentrations of Zn(II) (II) was inoculated with standardised cultures of *Pcb* 1692, *Pcb1692Δzur and Pcb1692Δzur*-p*zur*. The optical density (OD_{600}) was measured 24 hpi (*p*-value < 0.05).

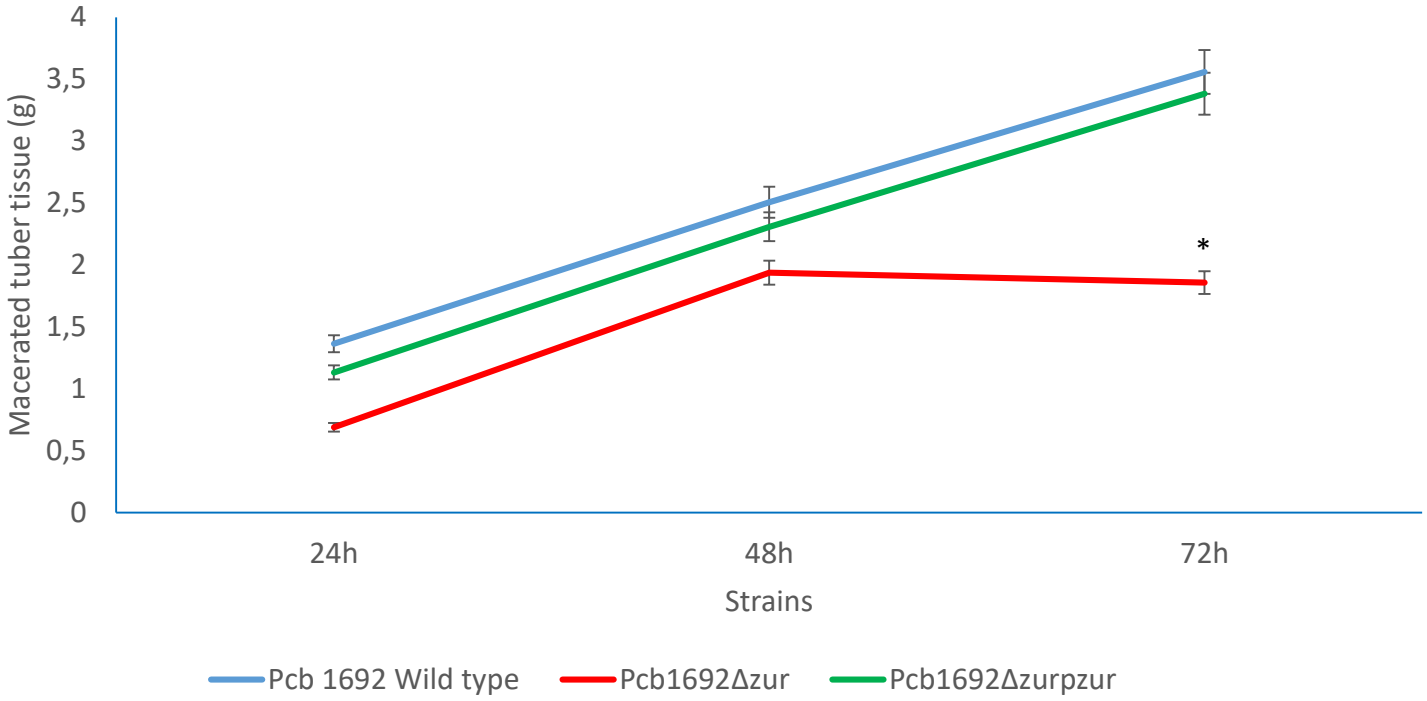


Fig 3.11. Effect of Zur in *Pcb* 1692 virulence on potato tubers. Susceptible potato tubers (cv. Mondial) were inoculated with standardized cultures of *Pcb* 1692, *Pcb1692Δzur* and *Pcb1692Δzur*-pzur. $MgSo_4$ was used as a negative control. Macerated tissue was scooped and weighed at 24 hpi, 48 hpi and 72 hpi (p-value < 0.05).

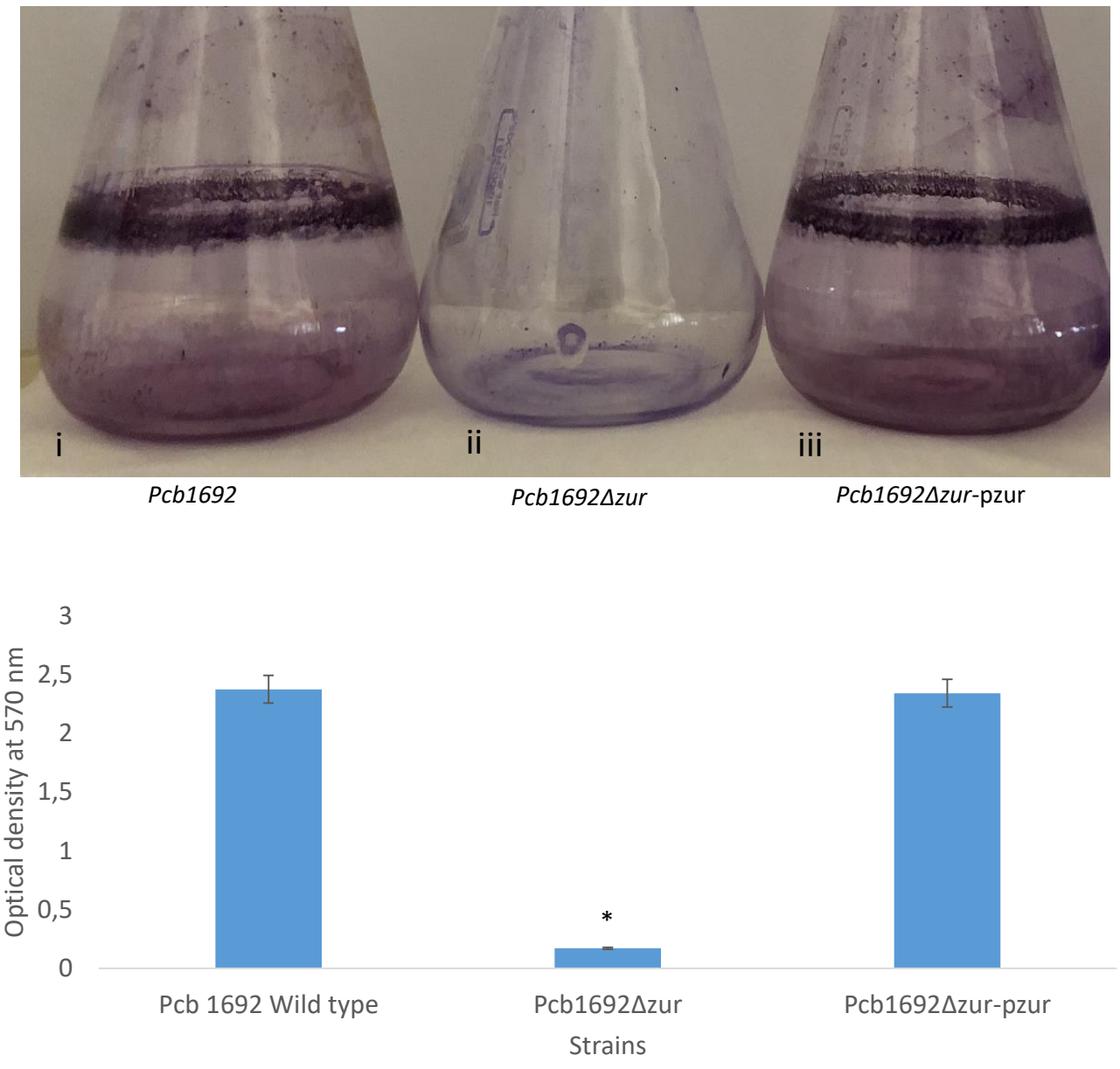


Fig 3.12. The effect of Zur on biofilm formation within Pcb1692 A. Qualitative formation of biofilm within conical flasks prior crystal violet staining. Flasks i, ii and iii represents the Pcb1692 wild-type, *Pcb1692Δzur* mutant strain and *Pcb1692Δzur*-p*zur* complement strain respectively. **B.** Quantitative analysis of biofilm formation by the *Pcb1692* wild-type, *Pcb1692Δzur* mutant strain and *Pcb1692Δzur*-p*zur* complement strain. Data represents averaged mean values of optical density at OD_{570} from three independent experiments (p-value < 0.05).

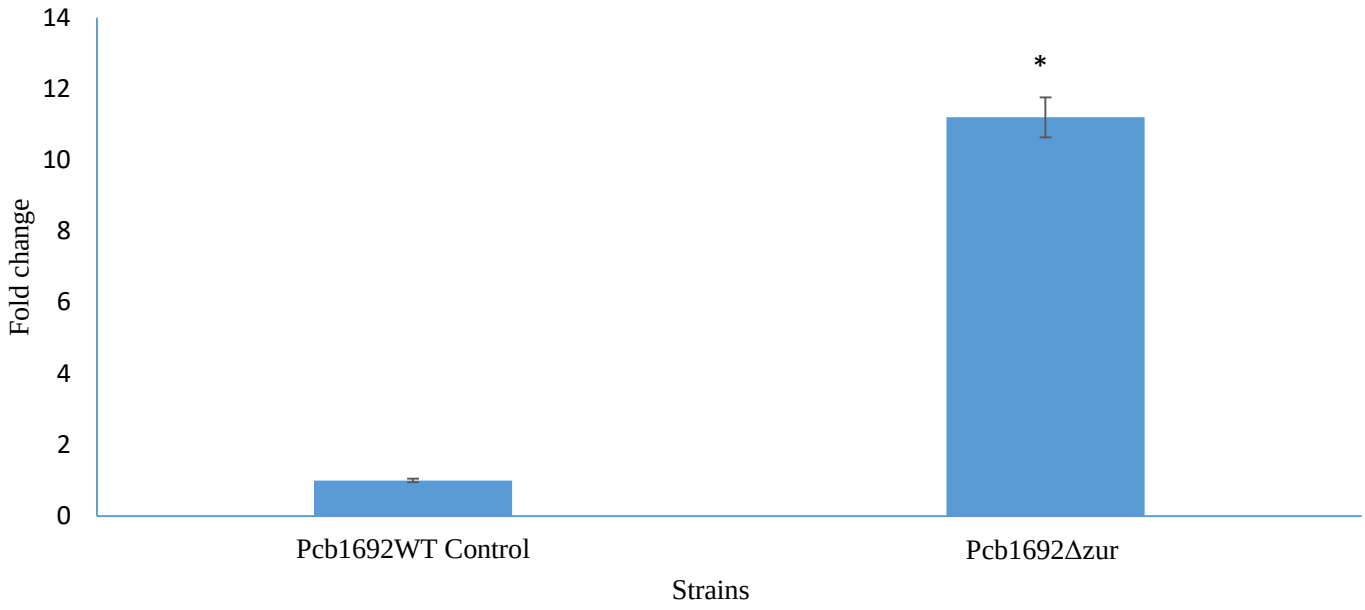


Fig 3.13. Differential expression of a type VI secretion system core gene, *TssC*. The expression of the *TssC* gene in the *Pcb1692Δzur* mutant strain was analysed under different conditions relative to the *Pcb* 1692 wild-type control. The *Ffh* gene was used as a housekeeping gene to normalize gene expression .

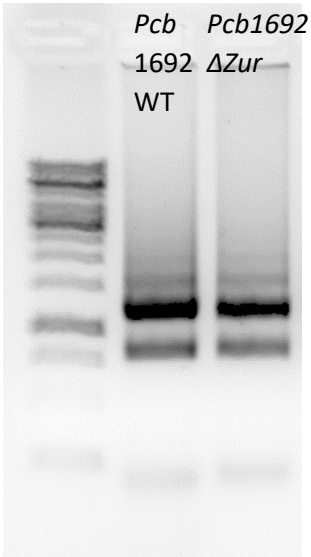
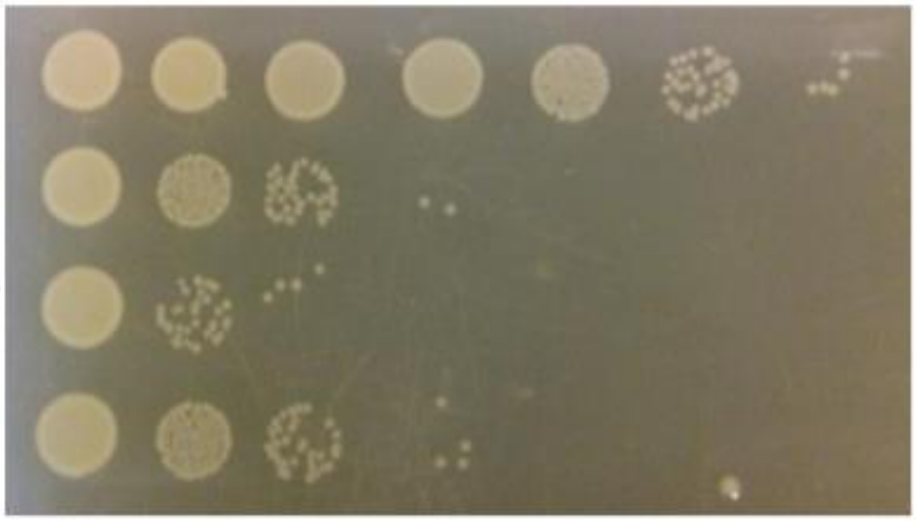


Fig 3.14. Gel electrophoresis image of extracted RNA. Extracted RNA used for the differential expression assy.



D.dad + H_2O

D.dad + *Pcb* 1692

D.dad + *Pcb*1692*Δzur*

D.dad + *Pcb*1692*Δzur*-p*zur*

Fig 3.15. Zur's contribution to anti-bacterial activity within *Pcb* 1692. Susceptible potato tubers (cv. Mondial) were inoculated with separate as well as mixed cultures of *D. dad, Pcb* 1692, *Pcb1692Δzur* and *Pcb1692Δzur*-p*zur*. $MgSO_4$ was used as a negative control. Macerated tissue was scooped and weighed at 72 hpi and subjected to serial dilutions followed by plating on gentamycin supplemented agar, as previously described, selecting for *D. dadantii*.

Chapter three – Supporting data

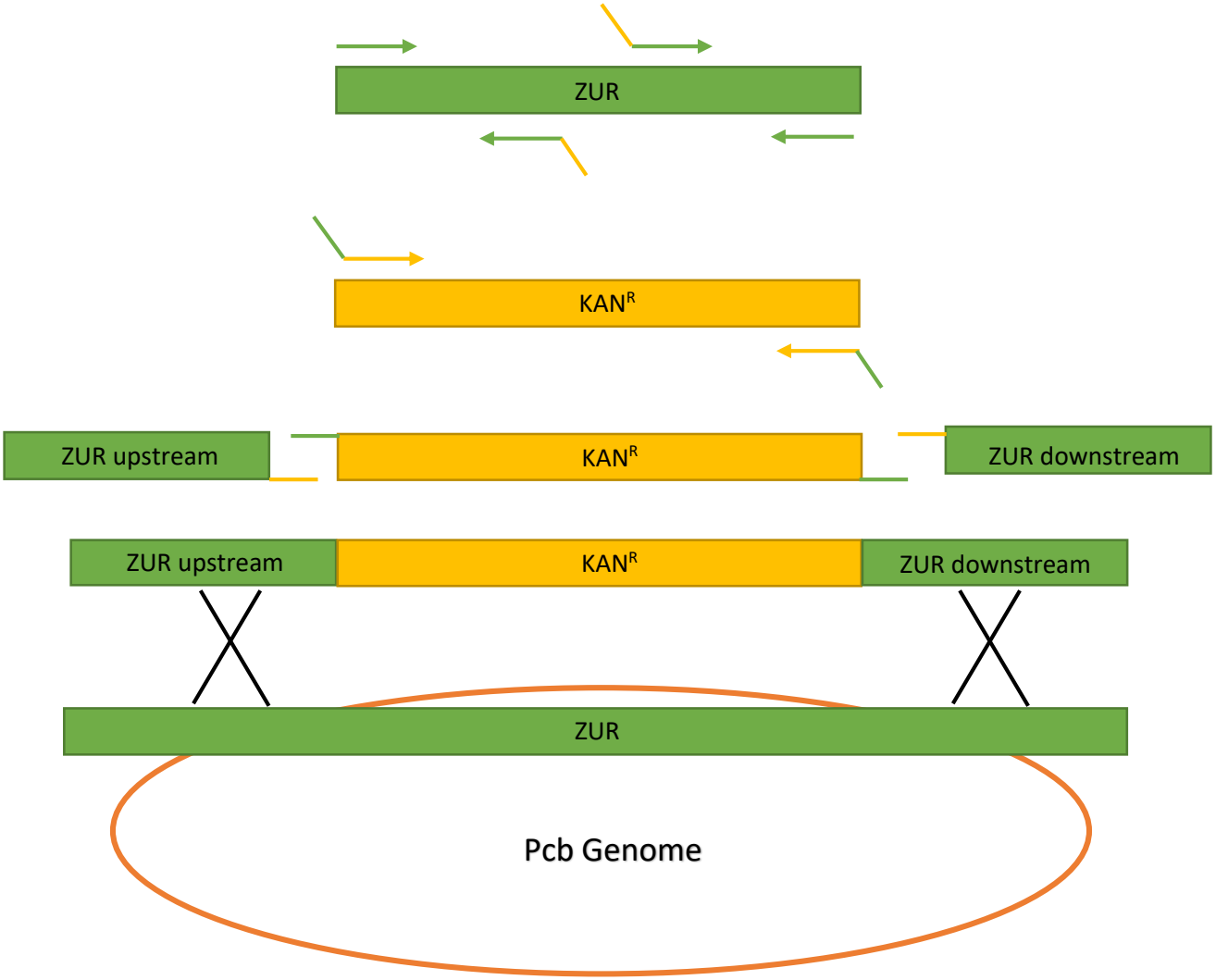
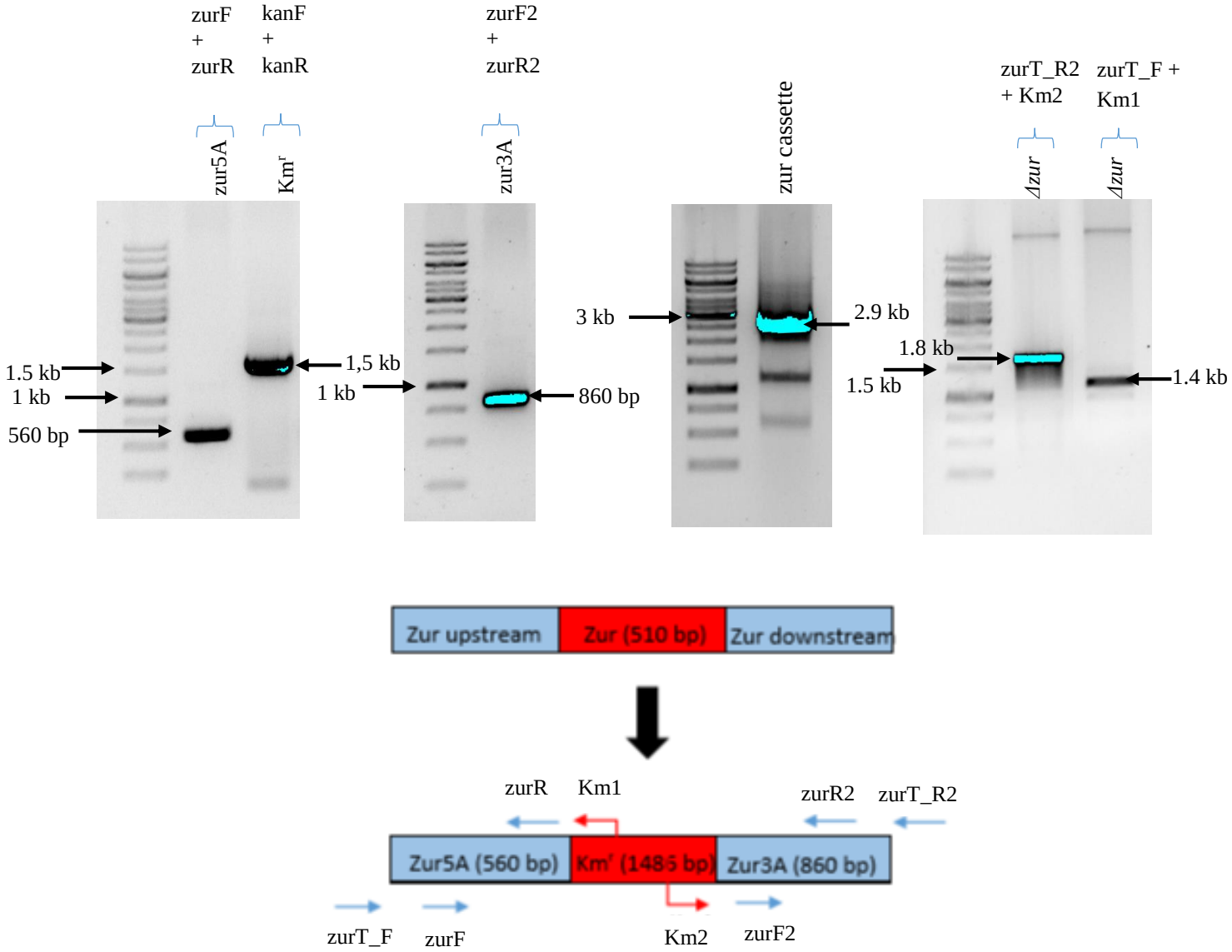


Fig S3.1. Mutation cassette construction and gene insertion technique. The *zur* gene was amplified utilizing two primer sets (one forward and reverse primer was designed to include overhangs for kanamycin). The kanamycin resistance gene was amplified utilizing one primer set designed to carry overhangs for the matching *zur* gene fragments. A master forward and reverse primer set was utilized to fuse the three fragments. The mutation cassette was electroporated into a *Pcb* 1692 strain harbouring the pKD20 plasmid.



Expected size: zurT_R2 + Km2 = 1793 bp

Expected size: zurT_F + Km1 = 1439 bp

Expected size: zur mutation cassette = 2926 bp

Fig S3.2. PCR verification of the *Pcb1692Δzur* mutation of ***Pcb1692.*** The zur gene was deleted and replaced by the kanamycin resistance gene (red rectangle) using a technique involving the Lambda Red-mediated homologous recombination. The Km1 and Km2 primers are internal kanamycin primers, while the gene-specific primers are zurF and zurR as indicated in the figure.

Supplementary data

Table S1. GENE NEIGHBOURHOOD SCREENING

upstream_2	upstream_1	target	downstream_1	downstream_2	Organism
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium atrosepticum;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium atrosepticum;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium atrosepticum;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. actinidiae;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	CsbD(-)	LexA_DNA_bind+Peptidase_S24(-)	*Pectobacterium carotovorum subsp. odoriferum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium atrosepticum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium atrosepticum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium atrosepticum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium atrosepticum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium atrosepticum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium betavasculorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. actinidiae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. actinidiae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*

HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. brasiliense;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum subsp. odoriferum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	CsbD(+)	LexA_DNA_bind+Peptidase_S24(+)	*Pectobacterium polaris;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya chrysanthemi;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya chrysanthemi;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya chrysanthemi;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya chrysanthemi;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya chrysanthemi;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya chrysanthemi;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya solani;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya solani;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya solani;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya solani;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya zeae;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Dickeya zeae;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Pectobacterium carotovorum;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	LexA_DNA_bind+Peptidase_S24(-)	DAGK_prokar(-)	*Pectobacterium parmentieri;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya dadantii subsp. dieffenbachiae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya dadantii;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya dadantii;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya dadantii;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya dianthicola;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya dianthicola;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya dianthicola;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya dianthicola;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya dianthicola;*

HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya solani;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya solani;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya solani;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya solani;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya solani;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya solani;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya solani;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya solani;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya solani;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya zeae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya zeae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya zeae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya zeae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya zeae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya zeae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya zeae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Dickeya zeae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Pectobacterium parmentieri;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Pectobacterium parmentieri;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Pectobacterium wasabiae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Pectobacterium wasabiae;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	DAGK_prokar(+)	*Pectobacterium wasabiae;*
HTH_1+LysR_substrate(-)	Pirin(+)	FUR(+)	No data	No data	*Dickeya dadantii;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	CsbD(+)	*Pectobacterium carotovorum subsp. carotovorum;*
HTH_1+LysR_substrate(+)	Pirin(-)	FUR(-)	LexA_DNA_bind+Peptidase_S24(+)	CsbD(+)	*Pectobacterium carotovorum subsp. carotovorum;*

Table S2. ZUR BINDING SITE ANALYSIS

Sequence name	gi|198441471|ref|NZ_ABVX01000021.1|:9287-10299
Start	629
Stop	647
Strand	-
Score	8,85714
p-value	9,07E-06
q-value	0,0176
Matched sequence	AAAACCTAATAATTTTAAT

Table S3. PAIRED COMPARISON SIGNIFICANT *p*-VALUE (TURKEY TEST)

***in planta* Competition**	*p*-value
Dickeya dadantii	
*Pcb*1692WT+Ddad against *Pcb1692Δt6*+Ddad	0.0041246
*Pcb*1692WT+Ddad against *Pcb1692Δt6p-t6*+Ddad	0.9999997
Pcb1692Δt6p-t6+Ddad against *Pcb1692Δt6*+Ddad	0.0058775
Dickeya chrysanthemi	
*Pcb*1692WT+Dchr against *Pcb1692Δt6*+Dchr	0.0601002
*Pcb*1692WT+Dchr against *Pcb1692Δt6p-t6*+Dchr	0.9439141
Pcb1692Δt6p-t6+Dchr against *Pcb1692Δt6*+Dchr	0.0409596