Investigation of Pseudomonas aeruginosa Species-Specific Resistance to Type 6 Secretion System Attack

Investigation of *Pseudomonas aeruginosa* Species-Specific Resistance to Type 6 Secretion System Attack

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A thesis submitted to the University of London for the degree of Doctor of Philosophy

March 2025

COVID-19 impact statement

The work presented herein started in October 2020, amidst the COVID-19 pandemic. Due to this unforeseen event, I faced significant disruptions in my research project. These disruptions were reflected in prohibited or restricted access to laboratory facilities and limited access to transportation. Between December of 2020 and February of 2021, research students were not allowed access to the department, including laboratory facilities, offices and library. Following this period, I was granted limited access to laboratories on a shift-basis, allowing work for four hours per day, five days a week. During this time, I had to abide by social distancing rules and could not engage in any personal contact with other researchers, including senior postdoctoral researchers, lab managers and project supervisors. As a result, the work I was able to complete during this period was mostly done independently, and without direct support or guidance.

Normal access to the laboratories and resumption of social interactions, including direct supervision and group meetings, were reinstated in the Summer of 2021. This was approximately nine months after I had enrolled as a student, considerably shortening the period during which I was able to work in the laboratory during my 3.5 years studentship. The delays caused by the pandemic had a significant impact on the progress of my experimental work, the whole research project and my overall development as a research student. Furthermore, I was not granted any extensions to my studentship, including a thesis submission deadline. Despite these challenges, I remained committed to my project and resumed laboratory work as soon as it was feasible. However, due to the early disruptions, I had limited opportunities to analyse data or develop new hypotheses.

Abstract

The Type 6 Secretion System (T6SS) is a contact-dependent mechanism employed by many Gram-negative bacteria to deliver toxic proteins into the extracellular milieu or neighbouring cells. *Pseudomonas aeruginosa* is an opportunistic pathogen that encodes three T6SS apparatuses, one of which (H1-T6SS) specifically assembles and fires in response to exogenous T6SS activity. This response is lethal to competitors and provides *P. aeruginosa* a competitive advantage within microbial communities. Here, we demonstrated that inactivating H1-T6SS increases the susceptibility of *P. aeruginosa* to *Acinetobacter baylyi* T6SS attacks of than to those of *Vibrio cholerae*. This observation greatly motivated our exploration of species-specific behaviour mediated by the T6SS. Next, we investigated whether *P. aeruginosa* was selectively more resistant to *V. cholerae* T6SS due to effector toxicity by endogenously expressing *V. cholerae* effectors into *P. aeruginosa*. Unlike cytosolic expression, periplasmic expression of *V. cholerae* effectors results in significant toxicity, suggesting that proper cellular effector localisation is critical for toxicity and that *P. aeruginosa* is not inherently resistant to *V. cholerae* effectors. We then investigated whether *V. cholerae* T6SS could deliver effectors directly into the cytosol of *P. aeruginosa* by using fluorescence- and antibiotic-based reporters for cytosolic delivery. Despite our efforts, these reporter systems were ineffective in determining T6SS-mediated cytosolic delivery. We thus asked instead, whether *P. aeruginosa* cell wall could specifically prevent *V. cholerae* T6SS attacks. We revealed that *P. aeruginosa* mutants lacking one or more genes encoding for exopolysaccharides or lipid transport pathway proteins were not more susceptible to *V. cholerae* T6SS attacks. This suggests that *P. aeruginosa* cell wall might not play a role in species-specific resistance against *V. cholerae* T6SS. Lastly, we investigated the role of *P. aeruginosa* retaliatory H1-T6SS in a multispecies community consisting of T6SS-aggressor and T6SS-sensitive species. Our results revealed that *P. aeruginosa* is able to protect T6SS-sensitive *E. coli* from T6SS-aggressor *V. cholerae* in a H1-T6SS-dependent manner and by creating a physical barrier between aggressors and their victims. Collectively, our study underscores the pivotal role of *P. aeruginosa* retaliatory H1-T6SS in modulating interbacterial competition and shaping population dynamics by influencing species-specificity and the spatial organisation of microbial communities.

Acknowledgements

Completing this research project was by no means an individual accomplishment. No (wo)man is an island and I had the privilege of being supported by so many people to whom I would like to sincerely express my gratitude:

To Dr Brian Ho, for his scientific contributions and project guidance, and for consistently motivating me to improve my critical and communication skills.

To Professor Sanjib Bhakta, who has provided important advice for a prolific academic life, as well as enthusiastically recognising my achievements.

To Professor Katherine Thompson, for her unwavering encouragement during challenging moments and for pushing the boundaries of my critical thinking.

To Pilar, who has taught me so much in the lab but also outside of it. With whom I shared many of my concerns but also my victories. Throughout this journey, her patience and reassurance have been indispensable.

To Chris, who unexpectedly came into my life and quickly became an invaluable friend. Her wittiness, kindness and support have been a beacon of light. We are indeed a dynamic duo.

To my friends. In particular, to Francisco and Joana who were always curious, engaging and motivational about my project. Through the past 30 years, they have been unparalleled friends.

To my aunt, uncle and cousin, who have always been models for academic excellence and continually encourage my personal and professional development.

To my parents, for their unconditional love, for giving me more than I could have asked for and for always strongly encouraging my academic endeavours. They have stood by me still not fully grasping what I do as a scientist.

Last, but certainly not least, a heartfelt thanks to my husband Fábio. He has been my constant supporter and advocate, lifting me up when things are difficult and celebrating my successes as if they were his own. I am honoured and proud to have him as a best friend and a partner in life.

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List of abbreviations

ACD	Actin crosslinking domain
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair(s)
CDI	Contact-dependent growth inhibition
CFU	Colony forming units
CFU/ml	Colony forming units per millilitre
DNA	Deoxyribonucleic acid
EAEC	Entero-aggregative *E. coli*
eDNA	Extracellular DNA
EPS	Exopolysaccharides
ETEC	Enterotoxigenic *E. coli*
FLP	FLP or “flip” tyrosine recombinase
FRT	FLP recombinase recognition target
HA	Human influenza hemagglutinin
HGT	Horizontal-gene transfer
IM	Inner membrane
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth
Lox	Locus of crossover (x) in P1
LPS	Lipopolysaccharides
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
OM	Outer membrane
OMPs	Outer membrane proteins
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PTLBs	Phage tail-like bacteriocins
QS	Quorum sensing

RBP	Receptor-binding protein
RNA	Ribonucleic acid
Sec	General secretion pathway
SOC	Super optimal broth with catabolite repression
SSR	Site-specific recombination
Tat	Twin-arginine secretion pathway
TCS	Two-component system
TPP	Threonine phosphorylation pathway
TPS	Two-Partner Secretion
tRNA	Transfer RNA
T4P	Type IV pili
T4SS	Type 4 Secretion System
T6SS	Type 6 Secretion System
VAS	Virulence-associated secretion
WT	Wild-type

1 Introduction

1.1 Microbial communities

Bacteria living in our bodies play crucial roles in maintaining health and contributing to disease [1]. In healthy conditions, bacteria are in a symbiotic relationship with the host. However, disrupting this state of equilibrium can lead to the onset of disease. Pathogenic bacteria are responsible for a variety of infections that can be arduous to treat due to the surge in antimicrobial resistance [2]. In order to deal with infection it is critical to better understand how bacteria behave within their environment. Most bacteria associate in microbial communities, which can be encountered in many different bodily locations like the gut, lungs or skin [3,4]. The cooperative and competitive behaviours within these microbial communities shape their stability and function, and changes within these communities can imbalance bodily functions and lead to disease [1,5]. A growing body of evidence has shown that dysregulation of the human microbiota is associated with the development of respiratory, cardiovascular and metabolic diseases, among others [1]. Understanding community dynamics can help manage microbiomes to prevent or treat disease by modulating the microbiota.

Bacterial interactions can have a positive, negative or neutral impact for one or more species within a microbial community [6]. Interactions can be advantageous for the interacting microorganisms (mutualism) or have a negative impact on a species (competition). A body of evidence drawing from various experiments, converges on the conclusion that bacteria cells most often antagonise each other within a microbial community [7]. Insights into population dynamics can help clarify how microbial communities participate in disease and potentially help discover new antimicrobial targets. Investigating the mechanisms bacteria employ to attack one another (“bacterial weapons") or to defend themselves from aggressors (“bacterial armours”), can be used to our advantage in disease control and prevention.

For example, bacterial weapons may include extracellularly secreted antimicrobial molecules which could potentially be engineered to be delivered as prophylactic or therapeutic drugs. An example is nisin, an antimicrobial peptide that has been historically used as a food preservative and recently has shown to be able to modulate the porcine gut microbiome [8]. Phage-derived endolysins are another example of enzymes that can degrade the peptidoglycan and are currently being engineered to breach the bacterial outer

membrane in order to exert their antimicrobial effects [9]. Another potential strategy involves repurposing antimicrobial effectors secreted by bacterial secretion machineries as therapeutic or prophylactic drugs. If these antimicrobial effectors are harmless to host cells, they could be directly used to prevent or treat of infections caused by species sensitive to their effects.

Conversely, understanding how bacteria use their armours to protect themselves from external threats might uncover new ways of breaching these defences. New antimicrobials could be developed to overcome these barriers and exert a toxic effect on pathogens without damaging the host. For example, efflux pump inhibitors have been studied due to their potential as targets for battling antimicrobial resistance [10]. Efflux pumps are one of the most common mechanisms bacteria use to export antimicrobials, and thus can lead to antimicrobial resistance [10]. Hence, targeting these mechanisms presents a novel approach to improve the success of antimicrobial treatments. Additionally, given that bacterial secretion machineries participate in interbacterial competitions and niche occupation, they could also be suitable targets for antimicrobial drugs.

1.2 Bacterial interactions

1.2.1 Cooperative behaviour

It can be advantageous for bacterial cells to associate in a community rather than remain as individuals. Bacteria in communities can resist external threats, such as the effects of antimicrobials, enzyme degradation, erosion and desiccation [11]. Within a community, bacteria have better access to nutrients, either by confining such nutrients within a polymeric matrix or withdrawing them from cells that want to exploit them [12]. Furthermore, bacteria can also exchange metabolites that allow for metabolic pathways to be complemented by other members of the community [13].

Spatial structure can stabilise cooperation within these communities, by excluding freeloading “cheaters” or mutually sharing metabolites which are beneficial to species within the community [14]**.** Cooperation between bacteria typically occurs when a cooperator produces public goods that benefit both the producer and the receiver [13]. This cooperative behaviour can occur through the secretion of nutrient chelators, digestive enzymes, surface

adhesins, wetting agents, structural polymers and signalling molecules [15]. A recent example of cooperative behaviour that benefits the survival of a population was found in a thorough analysis of microbial communities [16]. The presence of auxotrophs within these microbial communities creates a metabolically rich environment which allows species to withstand drug treatment above their minimal inhibitory concentration.

In summary, bacterial communities can greatly benefit from cooperative behaviours, such as better access to nutrients or metabolites exchange, which enhance their metabolic capacity and the ability to withstand physical and chemical threats. This cooperation plays a crucial role in their stability and resilience to external stressors, including drug treatment.

1.2.2 Competitive behaviour

Despite their ability to exhibit cooperative behaviours, bacterial cells commonly compete with each other [17]. Bacterial antagonism is driven by the need to obtain spatial and nutrient dominance over a niche habitat [15]. Bacteria need to arm themselves to outcompete their neighbours, and thus interbacterial antagonism is believed to have shaped the evolution of bacterial physiology [7].

The race for resources and space between bacteria can present in a multitude of ways. Bacterial cells can release deleterious molecules either into their surrounding environment or directly into competitors, or they can expand to occupy specific niches. One of the prime examples of antagonistic bacterial interactions is antibacterial compounds produced by certain species of bacteria. *Streptomyces* species have long been a source of many antibiotics like streptomycin, chloramphenicol and tetracycline [18]. Lactic acid bacteria, like *Lactobacillus* spp. and *Lactococcus lactis*, can also produce harmful antimicrobial compounds, such as bacteriocins and organic acids [19]. Another way bacteria can achieve a competitive advantage is by releasing proteins into their surroundings or into target cells through secretion apparatuses [20].

Bacterial competition can take on different forms. For example, when a species is the initial coloniser of a niche, it can dominate a space and physically prevent competitors from taking over [21]. Similarly, one species can indirectly prevent another species from growing by dominating its competitors at the boundary between cells, appropriating space and restricting

the competitors' access to resources [21]. These forms of competition have been shown to impact the success of habitat colonisation [22]. It has been shown that the success of the competition when two distinct *Escherichia coli* cells compete for space can be analysed by measuring the area occupied by the prevailing strain. The competitive success can be influenced by cell density, cell lag time and the physical pressure of microcolonies at the boundary where bacterial cells meet [22]. Another mode of bacterial competition is whereby a group of species compete to re-occupy a newly released space by another species [21]. The competitive success is measured by the ability of a species to be the first to colonise and hold the new space by expanding and surviving. A computational model that analysed species distribution amongst microbiome samples showed that this form of competition can happen between different species of the gut microbiome [23].

1.2.3 Contact-independent

Interbacterial antagonism can occur through contact-independent mechanisms. One such way is the synthesis and secretion of bacteriocins, which are ribosomally-synthesised peptides with antimicrobial properties [24]. Bacteriocins have diverse mechanisms of action and high potency against several bacterial species. For example, the bacteriocin nisin targets peptidoglycan biosynthesis and exhibits antimicrobial activity against clinically relevant species like *Streptococcus pneumoniae* [24].

Another example is phage tail-like bacteriocins (PTLBs), which are large proteins structurally homologous to bacteriophage tails, and are widespread in Eubacteria [25]. PTLBs are highly potent, but their targets are precise, as they require the recognition of a receptor in the target cell for specificity [25,26]. PTLBs bind to the target cell through their tail fibres, which act as receptor-binding proteins (RBPs). Examples of PTLB receptors are the O-antigen and type IV pili in *Pseudomonas aeruginosa* [25].

Depending on their structure, PTLBs can be broadly divided into R-type (rigid-type) and F-type (flexible-type). R-type PTLBs are contractile particles composed of a tube terminating in an iron-loaded tailspike [26,27]. The tube is encased by a sheath and ends in a complex baseplate with tail fibres [26]. R-type PTLBs bind to the target cell's lipopolysaccharide (LPS) through tail fibres, and tube contraction drives the tailspike through the cell envelope.

A channel is formed, allowing ions to flow, which disrupt the membrane gradient and lead to cell death [25]. Conversely, F-type PTLBs are particles composed of a non-contractile tube and, due to their non-contractile form, cannot penetrate the cell envelope [25]. The mechanism of action of F-type PTLBs has not yet been fully characterised, but they are able to efficiently kill bacterial cells, possibly by forming a channel through the inner membrane and disrupting cell respiration [26,28]. In vitro, the production of PTLBs can be triggered by SOS response induction, like DNA damaging agents [25]. Even though PTLBs can contribute to a biological advantage, their action requires the sacrifice of the producing cell, *i.e.* the producing cell must die to release PTLBs for kin protection - a phenomenon that has been described as a form of altruism [25].

Colicins are another type of bacteriocins produced by *E. coli* that can kill closely related strains of *E. coli* [29]. Colicins are large proteins that exhibit a narrow target range of action by specifically binding to receptors of closely related strains. Colicins are expressed as a result of the activation of SOS response, and their release is followed by the synthesis of a colicin lysis protein [29,30]. A single colicin is sufficient to kill a prey bacterial cell, either through pore formation or enzymatic degradation [30].

Colicins have a similar modular structure: an N-terminal domain for transfer across the outer membrane (OM), a central receptor-binding domain and a C-terminal cytotoxic domain [30]. The C-terminal domain is the key part for the colicin toxicity and is either a pore-forming enzyme or an enzyme that cleaves peptidoglycan, RNA, tRNA or DNA [30]. Colicins can be classified into Group A, which are encoded by small plasmids and translocated through the Tol system, or Group B, which are encoded by large non--mobilisable plasmids and translocated through the TonB system [29]. To penetrate the outer membrane of a target cell, colicins first bind with high affinity to an outer membrane receptor through their receptor-binding domain [30]. They then assemble a translocon, which consists of the OM receptor and translocator proteins and at least one periplasmic or inner membrane translocator protein. A region of the colicin's N-terminal domain recruits the OM translocator protein to present signalling epitopes to the periplasm, whereupon proteins from the Ton or Tol system are captured.

In pore-forming colicins, the C-terminal domain is inserted into the inner membrane, creating a pore [29]. In contrast, the C-terminal domain of enzymatic colicins is translocated

through the inner membrane into the cytoplasm. In some cases, once an enzymatic colicin reaches the periplasm, it undergoes endoproteolytic cleavage of the C-terminal toxic domain in order to interact with inner membrane proteins and be translocated into the cytoplasm of the target cell [31,32]. Although it is thought that enzymatic colicins must undertake endoproteolytic processing prior to inner-membrane translocation, little is known about the proteolytic cleavage and C-terminal translocation through the inner membrane.

The action of bacteriocins depends on the recognition of a receptor on the target cell, which may create an opportunity for possible resistance mechanisms. Therefore, modifications on the target cell's surface or restricted receptor accessibility could increase bacteriocin resistance [24]. In microbial communities, bacteriocin-producing strains can gain dominance within a particular niche. Previously, a model has been proposed to describe bacteriocin dynamics within microbial communities [33]. Species either compete for spatial occupation of a site in a head-to-head competition, or a producing strain kills a sensitive strain by bacteriocin-induced lysis. When a sensitive strain is killed, resources are released to be taken up by the producing strain. However, bacteriocin immune strains can also benefit from freed-up resources. These strains can act as "cheaters" as they benefit from bacteriocins without contributing to their production [13].

Another way of interbacterial antagonism is achieved by the production of nonribosomal peptides and polyketides, which are two distinct families of natural products with many biological activities. Actinobacteria are known for producing a variety of secondary metabolites with antibacterial activity, like tetracyclines produced by *Streptomyces aureofaciens* [34,35]. Another example is colibactin, a hybrid polyketide-non-ribosomal peptide produced by *E. coli* and some Enterobacteriaceae [36]. The potential antimicrobial efficacy of colibactin has been attributed to the presence of cognate immunity genes not only in the producing species, but also in species commonly encountered in the human gut, suggesting that the immunity gene is being transferred to confer colibactin resistance [36,37]. In the context of microbial communities, the presence of colibactin immunity proteins could be ecologically advantageous since the producing and immune strain can gain dominance over commensal strains.

1.2.4 Contact-dependent

Bacteria can also antagonise each other through contact-dependent mechanisms by secreting proteins into their environment or neighbouring cells. Examples of secretion systems that play a role in bacterial competition and pathogenicity are contact-dependent growth inhibition (CDI), and Type 4 and Type 6 Secretion systems.

CDI is a two-partner secretion (TPS) system that is a subfamily of the Type 5 Secretion System (T5bSS) [38]. CDI was first discovered in *E. coli* as a mechanism whereby bacteria suppress the growth of other bacteria by direct cell-to-cell contact [39,40]. CDI is mediated by a pair of secretion proteins (CdiB/CdiA) and an outer membrane receptor (BamA, OmpC, OmpF or Tsx) [38]. CdiA is a large protein that extends outward from the surface of the inhibitor cell, and CdiB is an OM protein that contributes to the secretion and assembly of CdiA [41]. This system has also been described as "toxin on a stick" since CdiA acts as a "stick" delivering different toxins to a recognised receptor on the target cell membrane.

CdiA is an adhesin composed of three domains: a triple stranded beta-helix N-terminal, a receptor binding domain and a C-terminal effector domain. The N-terminal domain of CdiA has a Sec-secretion signal sequence and the protein is exported by OM protein CdiB. CdiA extends and binds to a specific receptor on the target cell, whereupon its C-terminal (CdiA-CT) domain is cleaved in order to be translocated into the target cell. The CdiA-CT is composed of two different domains with distinct function: a C-terminal domain with toxic activity and an N-terminal domain that is responsible for transport [42]. Therefore, it has been proposed that once the C-terminal toxic domain of CdiA-CT is in the periplasm, the N-terminal binds to inner membrane receptors for cytoplasmic translocation of the C-terminal domain [42,43]. Similar to colicin translocation, CdiA-CT requires periplasmic proteolytic processing prior to translocation into the cytoplasm of the target cell [43]. To protect from the effects of their own CDI system, bacterial cells encode a small immunity protein (CdiI) that specifically binds and inactivate its cognate CdiA-CT [41].

The C-terminal domain of CdiA can have different types of toxin modules that act in the target cell either by disrupting the cell membrane integrity or degrading nucleic acids [38]. Presumably, CDI systems are activated in densely populated environments, where close cell-to-cell contact is more likely to occur. [40]. CDI systems play a role in interbacterial competition

by inhibiting the growth of adjacent bacteria. However, the system's range of action varies with the expression of CdiA proteins. In *E. coli*, Class I CdiA proteins are species-specific, and inhibition is limited to same species population [38]. In contrast, Class II CdiA proteins have a wider range of action and can take part in interspecies competitions. In addition to *E. coli*, this system has been identified in other Gram-negative bacteria, like *P. aeruginosa* and *Acinetobacter* spp., as well as Gram-positive bacteria, like *Bacillus* and *Listeria* [38,41].

The contact-dependent and contact-independent mechanisms described above present effective ways of interbacterial antagonism. However, these mechanisms present some limitations compared to a bacterial machinery like the Type 6 Secretion System (T6SS). These systems have a narrow spectrum of activity by targeting species closely related to the ones which produce them. Additionally, they require a receptor in order to recognise and bind to the target cell, and their effects are usually triggered by SOS response mechanisms. On the other hand, the T6SS can target a multitude of different species, does not require a receptor on the target cell to exert its action and, in most cases, fires indiscriminately. Additionally, the T6SS delivers a repertoire of effector toxins with distinct activities, whilst other antagonistic mechanisms, like CDI, carry a single toxin domain.

Another mechanism by which bacteria antagonise each other in a contact-dependent manner and that does not require a receptor on the target cell surface is the type 4 Secretion System (T4SS). The T4SS is primarily recognised for its role in mediating Horizontal Gene Transfer (HGT) amongst bacterial cells [44]. HGT is the exchange of DNA between cells and has been associated with many bacterial evolution traits, such as antibiotic resistance and pathogenesis [45]. Through this mechanism, bacteria can share antibiotic-resistance genes with each other or secrete effector proteins and protein-DNA complexes that facilitate survival within the host. By increasing bacterial fitness and survival, the T4SS plays an important role in bacterial conjugation and evolution [46]. Recently, the T4SS has been associated with bacterial competition. Species from the *Xanthomonadaceae* family can use a subtype of T4SS to secrete toxic effectors into prey bacteria and kill them [47]. Moreover, the opportunistic pathogen *Stenotrophomonas maltophilia* is able to kill other Gram-negative species in a T4SS-dependent manner [48]. It has been shown that the periplasmic expression of a putative *S. maltophilia* T4SS effector greatly reduced the growth of *E. coli*. However, toxicity was reverted by the simultaneous expression of the cognate immunity protein. Delivering the

same *S. maltophilia* effector via the T4SS of another species, *Xanthomonas citri*, has also inhibited the growth of *E. coli*. Similar to CDI, T4SS-wielding bacterial cells produce specific immunity proteins that inactivate their cognate effectors, thereby protecting themselves against their own effectors and incoming effectors [46].

The systems described above can significantly impact microbial population dynamics (Figure 1.1). While CDI allows producing bacteria to inhibit the growth of neighbouring bacteria, the T4SS can secrete toxic effectors into target bacteria and kill them. However, CDI acts solely by inhibiting cell growth, requires the presence of a specific receptor on the surface of the target cell and targets closely related species. Bacterial antagonism through CDI is restrictive in terms of action and cell target, limiting the attacker cell's range of action. Furthermore, the T4SS antibacterial action has not been further explored and seems confined to only a few bacterial species, which restrains its action as an antibacterial weapon.

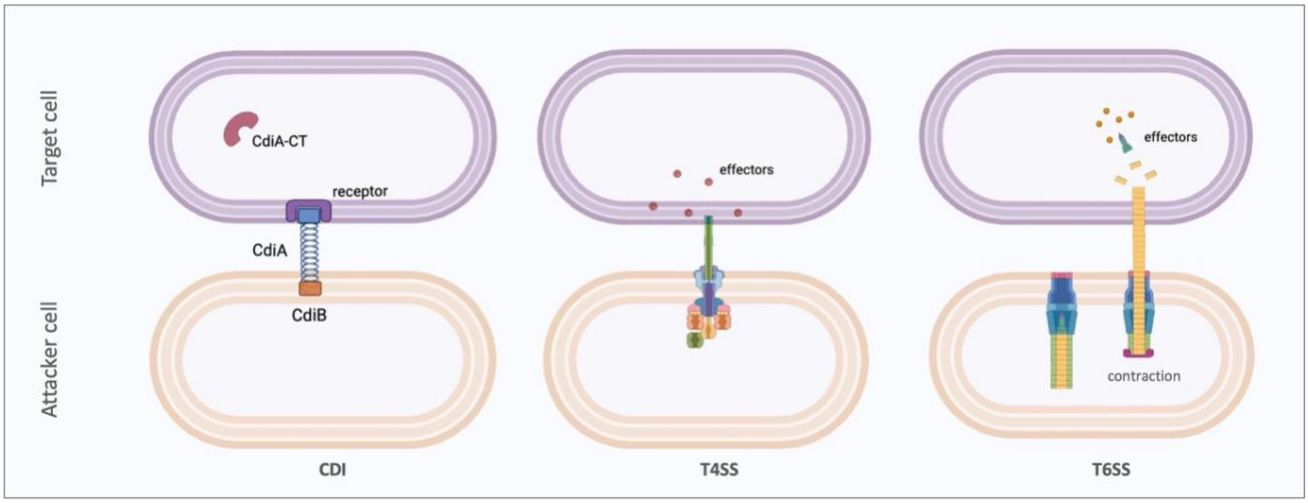

Figure 1.1. **Simplified depiction of Contact-dependent interbacterial antagonism.** Contact-dependent growth inhibition (CDI) acts like a "toxin on a stick", where toxins are delivered into target cells after being recognised by a specific receptor. The adhesin CdiA is exported by outer membrane protein CdiB through interaction of its N-terminal domain, which contains a Sec-secretion signal sequence. CdiA extends and binds to a specific receptor on the target cell, whereupon its C-terminal (CdiA-CT) domain is proteolytically cleaved so it can be translocated into the target cell. (image adapted from [49]). The T4SS, in addition to playing an important role in bacterial conjugation, can deliver toxic effectors into neighbouring bacterial cells. Many *Xanthomonas* species have a T4SS that can inject toxic effectors into competitive bacteria (image adapted from [46]). The T6SS is a cell envelope spanning nanomachine composed by a membrane complex, a baseplate and a tube/sheath spear topped up by a spike. Upon contraction, the T6SS punctures and delivers toxic effectors into target neighbouring bacteria (image adapted from [50]). In CDI-mediated competition, CdiA extends towards a receptor on the recipient cell to exert its killing effect, whereas, in T4SS- and T6SS-mediated competition, the systems deliver effectors into the recipient cell without requiring a receptor. For all three systems to effectively kill their competitors, target bacterial cells must lack immunity proteins for the cognate toxic effectors delivered.

1.3 Bacterial secretion systems in Gram-negatives

The world of bacterial secretion systems is incredibly vast. Up to date, 11 different types (T1SS-T11SS) have been identified [51]. Bacterial secretion systems have different structures, though they all share an important characteristic – the transport of proteins across one or more bacterial membranes. They can either localise to one of the cell membranes or stretch across the double membrane, transporting proteins directly from the cytosol, across the periplasm and into the extracellular environment. Due to double-membrane cell envelope, Gram-negative bacteria pose a challenge for substrate secretion compared to Gram-positive bacteria.

In Gram-negative bacteria, some secretion systems span both the inner and outer membranes, while others are aided by the general secretion (Sec) and twin-arginine (Tat) secretion pathways. These pathways first export molecules into the periplasm before facilitating their export into the extracellular space [51,52]. Whilst Sec transports unfolded proteins, Tat allows folded proteins to be translocated [20].

The current work will focus on the Type 6 Secretion System (T6SS), a complex nanomachine that plays a crucial role in both pathogenesis and bacterial competition. The T6SS can eliminate competitive bacteria and drive niche occupation by delivering toxic proteins into neighbouring cells. Unlike other contact-dependent mechanisms, such as CDI, the T6SS attacks are mostly indiscriminate, have a broad range of action and do not require recognition of a specific receptor on the target cell. These competitive characteristics have shown to be critical for shaping the structure and dynamics of microbial communities and will be discussed further.

1.4 The Type 6 Secretion System

1.4.1 Structure and Function

The T6SS was identified in a transposon screen that looked for virulence factors in *V. cholerae* strain V52 [53]. This screen identified a cluster of genes, termed virulence-associated secretion (VAS), as being responsible for *V. cholerae* virulence. The VAS gene cluster encoded a prototypic secretion system responsible for extracellular protein export. Because genes within the cluster lacked N-terminal signal sequences, this secretion system was proposed as the T6SS, with its primary function predicted to be the secretion of virulence factors into eukaryotic cells. It was later demonstrated that protein translocation into target cells required close cell-to-cell contact, demonstrating that the T6SS was also a contact-dependent system [54]. The T6SS has been found in more than 25% of genome-sequenced Gram-negative bacterial species [55], especially in Proteobacteria, such as *Vibrio* [53], *Pseudomonas* [56], *Acinetobacter* [57], *Yersinia* [55], *Campylobacter* [58]. A whole-genome study of 92 different bacteria identified 13 core T6SS genes which are highly conserved in both pathogenic and non-pathogenic bacteria [59]. However, the genes required for the T6SS assembly and function can differ between species and strains [60].

The T6SS can be further divided into different sub types according to phylogenetic analyses: T6SS^{i} is the canonical T6SS found in Proteobacteria, T6SS^{ii} is found in *Francisella* and T6SS^{iii} is found in Bacteroidetes [61,62]. Unlike the canonical T6SS^{i}, the assembly of T6SS^{ii} requires 17 core components [61], while the T6SS^{iii} requires 12 core components [63]. Another contractile apparatus similar to R-type pyocins has been identified in *Amoebophilus asiaticus* and named T6SS^{iv} [64]. Although structurally different from T6SS$^{i\text{-}iii}$, by lacking a membrane components and ClpV for example, T6SS^{iv} shares a similar evolutionary origin with the other T6SSs [64]. These distinctions between the different subtypes of T6SSs may be linked to the evolutionary adaptability of species and the diversity of species interactions.

Furthermore, the number of assembled T6SS apparatuses differs between species. For example, *Yersinia pestis* encodes a total of five T6SS clusters whereas *P. aeruginosa* encodes three [65]. Although there are many similarities between T6SSs of different species, each species adapts its attack strategy in response to environmental cues or interbacterial interactions [60].

Despite the genetic differences and number of assembled T6SS apparatuses among species, the T6SS structural features and mechanism of action are generally well-conserved [66]. The T6SS apparatus comprises three distinct sub-structures: membrane complex, cytoplasmic baseplate and sheath-tube complex [67] (Figure 1.2). The assembly of a functional structure begins with the addition of ten copies of each Type 6 subunit (Tss) that form the membrane complex: TssL, TssM and TssJ [68]. The inner membrane subunits TssL and TssM stretch across the inner membrane and periplasmic space and are anchored to the outer membrane by the N-terminal lipid moiety of TssJ. The base of the TssJLM membrane complex is then docked to the cytoplasmic baseplate, which is comprised of six subunits of TssE and TssG and twelve subunits of TssF [69]. The cytoplasmic baseplate forms around the VgrG-PAAR tip and connects to the membrane complex via TssK, which is followed by tube-sheath polymerisation.

The VgrG-PAAR tip is formed by a trimeric complex of valine-glycine repeat protein G (VgrG) and a conical extension formed by PAAR (proline, alanine, alanine, arginine) repeat superfamily [70,71]. The inner tube assembles to the base of the VgrG-PAAR tip and is formed by stacked hexameric rings of Hcp (hemolysin coregulated protein) enclosed by TssBC subunits (also known as VipA/VipB) [56,67]. The base of the membrane complex forms a hole of 15-20 Å diameter that is not sufficiently wide to take in the 110 Å wide Hcp tube and so it has been proposed that the membrane complex base changes conformation to allow loading of the inner tube [68].

Another component of the T6SS apparatus is TssA, which can play distinct roles in different bacterial species. In *P. aeruginosa*, TssA1 was found to interact with baseplate and tube-sheath components and thus proposed to be a component of the baseplate complex [72]. Whilst in *E. coli*, TssA2 was found to interact with tube-sheath components and to be required for Hcp and sheath formation [73]. TssA2 is initially recruited to the membrane complex, followed by the formation of the baseplate. Subsequently, tube and sheath polymerisation initiates, pushing TssA2 to the distal end of the sheath [73]. These two mentioned classes of TssA proteins are distinct in their structure, function and consequently on how they participate in the T6SS biogenesis. TssA1 is a structural component of the baseplate whilst TssA2 appears to coordinate tube-sheath assembly and its stability. These differences in function have been associated with the domain architecture of TssA proteins, and hence why they have been

classified into two different classes. All TssA proteins share a similar N-terminal domain (ImpA_N domain), but their C-terminal domain differs amongst classes [74]. The TssA1 C-terminal domain forms a dodecameric ring and has partial structural homology to the T4 phage baseplate component gp6 [72], whereas the TssA2 C-terminal domain (VasJ) forms two-stacked hexameric rings [73].

An additional class of TssA-like proteins with a different C-terminal domain but identical N-terminal domain has also been identified [75]. This class has been named TagA (Type 6 secretion accessory gene with ImpA domain) because proteins might act as accessory components of the T6SS. The C-terminal has a hydrophobic region that might work for membrane attachment and a region of unknown function (VasL). In *E. coli* and *V. cholerae*, TagA interacts with TssA2 by being recruited to the distal end of the sheath when the it is extended [76,77]. TagA stabilises the extended sheath and maintains its extended conformation. It has been observed that in a *V. cholerae* TssA mutant, TagA inhibits sheath assembly and TssM aids T6SS assembly [78]. This indicates that T6SS sheath initiation and termination is controlled by the interactions of TssA-TssM-TagA.

TssA proteins have been later categorised into two different forms instead of classes: a long form ($TssA_L$), found in *E. coli* and *V. cholerae*, and a short form ($TssA_S$), found in *P. aeruginosa* [79]. Although the function of $TssA_L$ has been well studied, it was unclear how $TssA_S$ interacted with the T6SS apparatus. It was then determined that $TssA_S$ interacts with accessory proteins TagB/TagJ which are recruited to the baseplate during sheath polymerisation [79]. Immediately after stabilising the polymerising sheath at the distal end, the sheath then contracts. In summary, TssA proteins are crucial in the early stages of the T6SS assembly, regulate sheath polymerisation and play a role in stabilising the sheath at the distal end of the apparatus.

Another accessory protein has also been shown to contribute to the T6SS biogenesis in entero-aggregative *E. coli* (EAEC) [80]. In EAEC, TssL lacks a peptidoglycan-binding domain, and thus requires an accessory anchoring protein, TagL. TagL is an inner membrane-anchored protein with an N-terminal peptidoglycan-binding domain that associates with the membrane complex and is dispensable for docking of the baseplate to the membrane complex.

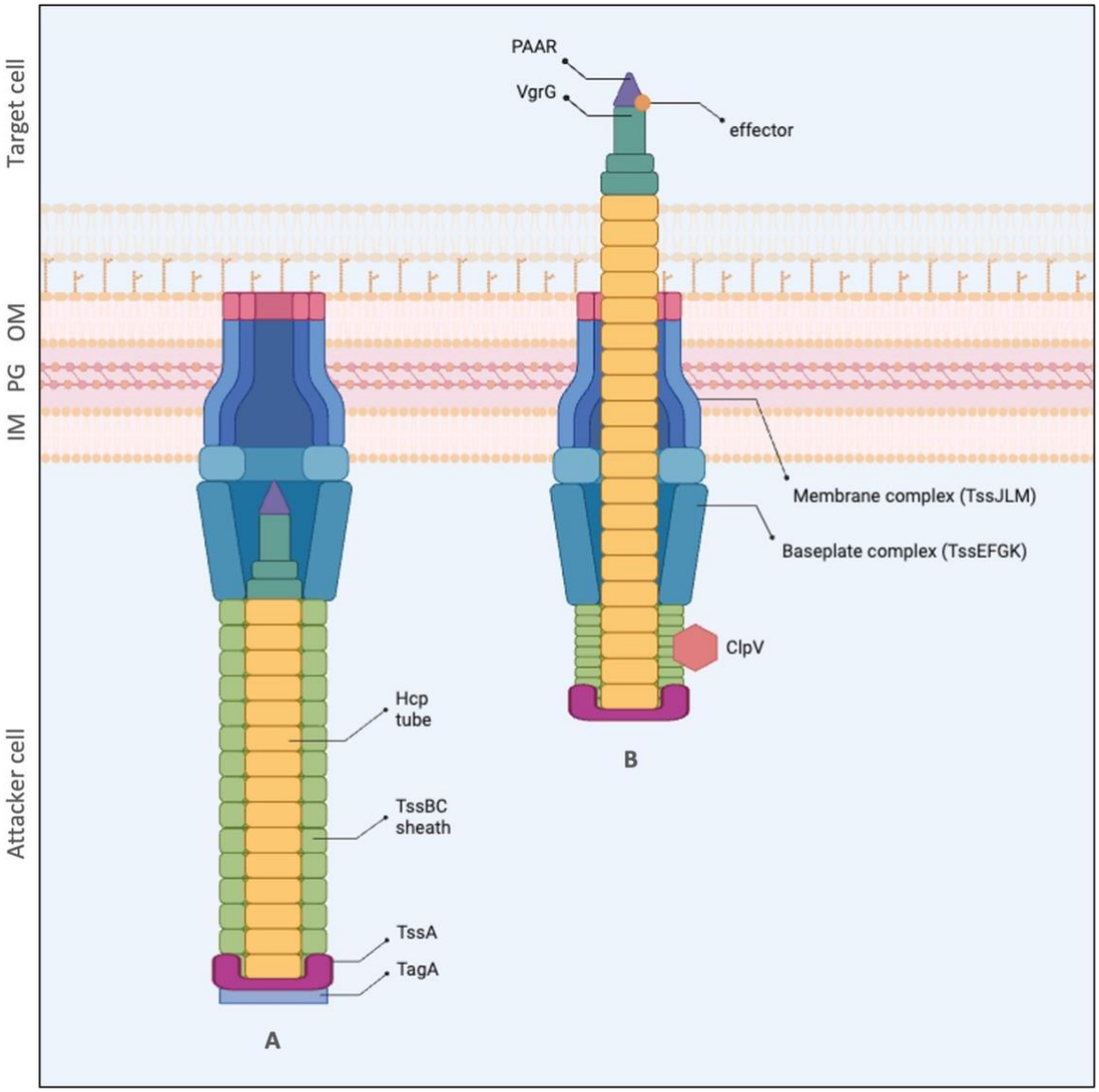

Figure 1.2. Schematic depiction of the Type 6 Secretion System (T6SS) in (A) extended or (B) contracted conformations. The T6SS is a multiprotein nanomachine that stretches through the cell inner membrane (IM), across the peptidoglycan-containing periplasm (PG) to the outer membrane (OM). The biogenesis of the T6SS starts with the assembly of the membrane-complex (dark blue) followed by formation of the baseplate (light blue). The inner Hcp tube (yellow) is topped up by a VgrG-PAAR spike (green-purple) and encased by TssBC subunits (light green). Upon contraction of the TssBC sheath, the Hcp tube is propelled outside of the attacker cell, and the VgrG-PAAR spike punctures and delivers effector proteins into a target cell. In *V. cholerae* TssA (purple) is recruited to the baseplate and pushed to the distal end of the tube-sheath upon polymerisation. TagA (lavender) is recruited to the distal end of the sheath where it associates with TssA to stabilise the sheath and maintain its extended conformation. After a contractile event, the sheath disassembles and the TssBC sheath can be remodelled and recycled by ClpV (pink) in the presence of ATP. Adapted from [50,66] using BioRender.

Due to their structural similarities to phage-tails, both R-type pyocins and the T6SS have been called “phage-tail like particles” [81]. The contractile mechanism of the T6SS is similar to the one observed in contractile bacteriophages, suggesting a common evolutionary origin. VgrG proteins were first described to have homology to the T4 spike complex (gp5 and gp27) and PAAR proteins to be structurally homologous to the gp5.4 spike tip protein [70,82,83]. Additionally, the Hcp tube was shown to be related to the bacteriophage tail tube protein gp19, the VipA/VipB sheath with the tail sheath protein gp18, and the cytoplasmic baseplate with gp25, gp6 and gp7 wedge units [82,84–86]. TssK, which docks the cytoplasmic baseplate to the membrane complex, is also thought to act similarly to gp10 in T4 phage by initiating wedge assembly [84]. The homology between the T6SS and contractile bacteriophages seems to be restricted to the contractile mechanism of the T6SS. While similarities exist between the tube-spike structure and the cytoplasmic baseplate component, no homology has been identified with the T6SS membrane complex. Despite the many structural and functional similarities between the T6SS and bacteriophages, the major difference lies in that the T6SS can assemble and reassemble many times by the same cell.

In *V. cholerae*, the assembly-contraction cycles of the T6SS can be visualised by fluorescently labelling VipA [87]. Fluorescence microscopy visualisation of the T6SS assembly in *V. cholerae* determined that the sheath assembles in 20-30 sec/μm and spans the entire width of the cell (0.75-1μm) [67]. The sheath quickly contracts to about half its length in less than 2 ms, then disassembles in the following 30-60 sec before reassembling. The cytoplasmic protein ClpV disassembles the contracted sheath and recycles VipA/VipB subunits to assemble a new sheath.

ClpV belongs to a family of AAA+ ATPases and is thought to be essential in T6SS function [88]. ClpV is not necessary for T6SS assembly but instead acts by remodelling VipA/VipB in the presence of ATP. This was first demonstrated in *P. aeruginosa*, when ClpV was only localised to the extended VipA sheath rather than in the contracted one [67]. After assembly, the VipA/VipB sheath rapidly contracts and propels the Hcp tube, VgrG-PAAR spike and associated effectors into the extracellular space or a neighbouring target cell [66].

1.4.2 Regulation of T6SS assembly and firing

The regulation of T6SS gene expression is species-specific, can be mediated by environmental signals, and can occur at the transcriptional, posttranscriptional and posttranslational levels [89]. In different strain of *V. cholerae*, the T6SS regulation can be mediated by abiotic factors, like temperature and osmolarity, and biotic factors, such as chitin and mucins [90–93]. At the transcriptional level, *V. cholerae* chitin recognition is also regulated by a two-component system (TCS) composed of a histidine kinase and a chitin-binding protein [89]. While in *P. aeruginosa*, the TCS GacS/GacA regulates gene expression in all three encoded T6SS clusters [94]. At the posttranscriptional level, the T6SS regulation in *P. aeruginosa* can also be controlled by the RetS-GacS-LadS system, which also regulates the expression of genes involved in biofilm formation [89].

The T6SS is regulated by a variety of mechanisms which reflect the distinct biological niches of T6SS-wielding bacteria. One important T6SS regulatory mechanism is the threonine phosphorylation pathway (TPP), which is controlled at the posttranslational level. This pathway has been first described in *P. aeruginosa*, though it is conserved in other species, like *Agrobacterium tumefaciens* and *Serratia marcescens* [95–98]. The TPP is mediated by the PpkA/PppA system, Fha (Forkhead-associated domain-containing) proteins and TagF. PpkA (Ser/Thr protein kinase) acts as a phosphorylation activator, whereas PppA (Ser/Thr protein phosphatase) and TagF act as repressors [66].

It has been previously shown that *P. aeruginosa* H1-T6SS is used as a defensive mechanism rather than an offensive one [99]. This duelling effect has been shown to be regulated by TagQRST-PpkA-Fha1-PppA cascade. In *P. aeruginosa*, the assembly of H1-T6SS requires Fha1 phosphorylation by PpkA, which can be antagonised by PppA [95]. A previous report has shown that whilst H1-T6SS activity is blocked by the inactivation of PpkA, it is markedly increased by the inactivation of PppA [99]. However, although inactivating PppA resulted in an increased H1-T6SS activity, cells had lost the ability to retaliate and kill *V. cholerae* [99]. Furthermore, the activation of PpkA and the phosphorylation of Fha1 have been shown to require a cell envelope regulatory system [100,101]. This system is composed of OM lipoprotein TagQ, and periplasmic and inner membrane proteins TagRST, which control the assembly and function of the H1-T6SS in response to environmental signals. The TagQRST system acts upstream of PpkA-Fha1-PppA and influences the assembly and function of the

H1-T6SS apparatus [100]. When TagT is inactivated, *P. aeruginosa* still assembles and fires H1-T6SS, but not in response to *V. cholerae* T6SS attacks, *i.e.* its duelling effect is lost [99]. Moreover, the loss of duelling ability in the TagT mutant was not limited to *V. cholerae* T6SS attacks and was also observed for *A. baylyi* T6SS attacks [99].

In *A. tumefaciens*, PpkA directly phosphorylates a component of the membrane complex (TssL) and this process is required for the secretion of Hcp [96]. The assembly of the membrane complex is initiated when PpkA phosphorylates TssL, which binds to Fha and ultimately activates the secretion of the T6SS apparatus. In *S. marcescens*, PpkA phosphorylates Fha, which is required for Hcp and effector secretion, whereas dephosphorylation is mediated by PppA, which promotes the rapid reassembly of the T6SS apparatus in a new location of the cell [97,98]. Moreover, TagF acts as a negative regulator by repressing the assembly of the T6SS membrane complex, although its effect can be overcome by PpkA [97].

1.4.3 Effector loading and mode of delivery

Effectors can be classified based on their interaction with the puncturing structure into cargo or specialised effectors (Figure 1.3). Cargo effectors bind non-covalently to one of the tube or spike components (Hcp, VgrG or PAAR), whereas specialised effectors are proteins where additional effector domains are covalently attached to the C-terminus of one of the tube-spike components [60].

Cargo effectors delivered through the Hcp bind to the interior of the tube and are stabilised within it. Examples of Hcp-dependent cargo effectors are Tse1-3 in *P. aeruginosa* [102]. The Hcp tube can only accommodate effector proteins smaller than its internal diameter thus Hcp-dependent effectors are usually small. However, the cavity formed by the cytoplasmic baseplate where the VgrG-PAAR complex docks into, is able to accommodate larger effector proteins [50,69]. Such is the case of effectors like *V. cholerae* VgrG1 and VgrG3 [103]. An example of a VgrG-dependent cargo effector is Tle1 in EAEC, which sits on the outside of VgrG and has phospholipase activity [104]. Interestingly, Tle1 is inactive when bound to VgrG, possibly as a mechanism to prevent deleterious effects to the attacking cell, suggesting that dissociation of VgrG-Tle1 is necessary for effector activity upon delivery [105]. An example of a PAAR-dependent

effector has been identified in *P. aeruginosa* [106]. TseT is delivered by *P. aeruginosa* H2-T6SS and directly interacts with PAAR and a chaperone (TecT) for delivery.

Furthermore, a class of polymorphic T6SS cargo effectors has been recently identified in *Vibrionaceae* [107]. These proteins contain an N-terminal domain named RIX (aRginine-rich type sIX) with C-terminal extensions with antibacterial, anti-eukaryotic or adaptor binding activities. Another class of polymorphic T6SS effectors has also been identified in Enterobacterales [108]. These effector have an N-terminal domain termed PIX (Pantoea type sIX) which is required for interaction with VgrG.

Specialised VgrG effectors with C-terminal toxin domains are also known as "evolved" VgrGs. One example is that of *V. cholerae* effector VgrG1, which has a C-terminal actin cross-linking domain and is essential for Hcp secretion and T6SS-mediated killing [70]. PAAR-specialised effectors are widespread and include a group of polymorphic proteins termed Rhs (rearrangement hot spot) [109]. Rhs proteins contain an N-terminal PAAR or VgrG domain, a conserved Rhs repeat containing domain and a C-terminal polymorphic effector domain [66]. Rhs proteins undergo autocleavage at the N-terminal and at the C-terminal to expose the effector domains required for interaction with VgrG. Structural analysis has shown that the conserved core domain forms a closed β-barrel spiral that enclose the C-terminal toxic domains in "cocoon" [110–112].

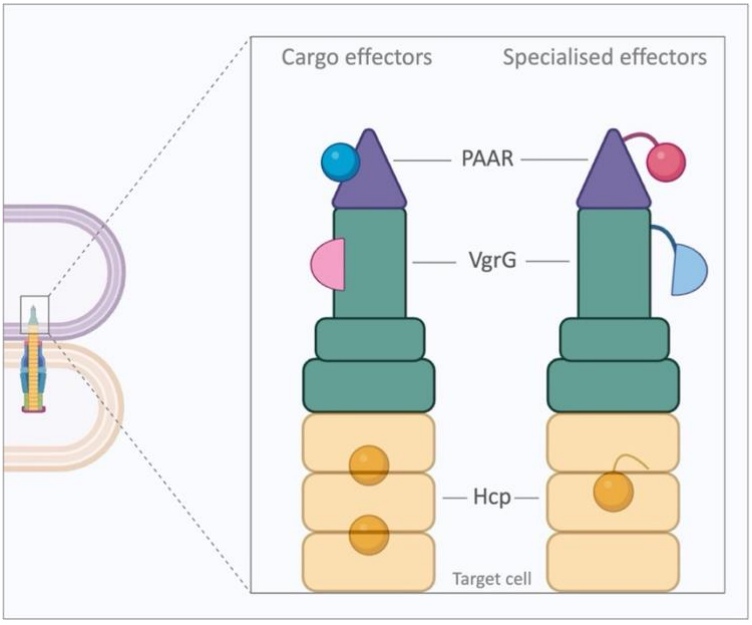

Figure 1.3 Simplified schematic depiction of T6SS effector loading. Effectors are classified into cargo and specialised effectors. Cargo effectors bind non-covalently to the inner tube of the Hcp or to the outside of VgrG or PAAR. Specialised effectors are proteins where additional effector domains covalently bind to the C-terminal of Hcp, VgrG or PAAR. Some effectors require chaperone or adaptor proteins for loading effectors into the T6SS apparatus, but those effectors are not currently depicted in this schematics.

Occasionally, chaperone or adaptor proteins are required to guide and stabilise effectors associated with structural components of the T6SS. For example, a family of chaperone proteins with conserved domain DUF4123 is required for effector delivery by binding the cognate VgrG proteins to an effector in *Aeromonas hydrophila* [113] and *V. cholerae* [114]. Recent work has demonstrated that rather than representing a single protein family, DUF4123 contains sub-families each of which have a unique C-terminal that recognises a unique effector family [115]. These findings suggest that DUF4123 allows the T6SS to recognise different effectors and diversify the repertoire of secreted effectors.

Another family of accessory proteins with conserved domain DUF1795 has been shown to bind to the N-terminal of PAAR-containing Rhs proteins in *S. marcescens* [109] and Tse6 effector in *P. aeruginosa* [116]. These accessory proteins are not required for effector delivery but instead necessary for effector loading onto VgrG. *S. marcescens* effector Rhs1 requires translocation factor EagR1, a chaperone with a DUF1795 domain, in order to exert its toxicity[117]. Furthermore, the C-terminal domain of VgrG has been shown to be the key determinant for the delivery of Tde effectors in *A. tumefaciens* [118]. The C-terminal is required for binding to a PAAR protein and a chaperone that directly interact with the toxic effector.

A detailed analysis of the distribution of effectors containing N-terminal transmembrane domains identified a conserved prePAAR motif in over 6000 putative T6SS effectors [119]. Effectors containing prePAAR motifs have been shown to require an Eag chaperone for T6SS export. Eag chaperones stabilise the transmembrane domains of prePAAR effectors in the cytoplasm prior to effector secretion. These putative effectors containing prePAAR motifs can be found in many Proteobacteria and have been divided into class I and II according to their transmembrane domains. Class I effectors have one transmembrane domain and a toxic C-terminal domain encased in a Rhs cage, whereas Class II effectors have two transmembrane domains, but they lack a Rhs cage [119]. A model has been proposed for the interaction between these effectors and their cognate chaperones: effectors might require the prePAAR motif for proper folding of the PAAR domain and effector loading onto the T6SS apparatus, whereas the transmembrane domain of the effectors possibly acts as a coating for the VgrG spike to penetrate the target cell membrane [119].

In Proteobacteria, various T6SS effectors have a conserved motif named MIX (marker for T6SS effectors) [120]. The MIX motif is located in the N-terminus of proteins that have a C-

terminal cytotoxic effector domain. It is suggested that MIX-containing proteins can target both eukaryotic and prokaryotic cells, like *V. cholerae* effector VasX. Because MIX is a conserved motif found near the T6SS gene cluster in many Proteobacteria, it has been proposed as a marker to identify new T6SS effectors. Moreover, a conserved motif was also found in a *V. parahaemolyticus* effector PoNe (polymorphic nuclease effector) [121]. PoNe has C-terminal DNase toxin domain and an N-terminal domain with a conserved motif named FIX (Found in Type six effector).

There is an ongoing debate about which cellular compartment the T6SS delivers its toxic effectors (Figure 1.4). A previous study identified a cryptic periplasmic localisation signal in *V. cholerae* VgrG3, which could explain effector translocation from the cytosol of the target cell into the periplasm [122]. This study suggests that *V. cholerae* T6SS might deliver effector VgrG3 directly into the cytosol of the target cell whereupon it is then translocated into the proper cellular compartment. Moreover, it has also been reported that *V. cholerae* T6SS tube-spike components and effector proteins can be exchanged amongst sister cells [123]. Sheath-deficient recipient cells can reuse incoming puncturing components from sister cells and assemble their own T6SS sheath. Because the T6SS apparatus assembles from the cytosol, this suggests that *V. cholerae* T6SS delivers the tube-spike and tip-associated effectors directly into the cytosol of sister cells.

On the other hand, various reports have shown that other species T6SSs might deliver effectors directly into the periplasm. It has been previously shown that *P. aeruginosa* and *A. baylyi* are able to deliver effectors into the periplasm of prey cells [124]. A prior study also showed that *A. tumefaciens* effector Tde1 is a DNase delivered into the periplasm of a recipient cell from where it is subsequently translocated into the cytosol [125]. The N-terminus of Tde1 binds to adaptor protein Tpa1 and this complex is delivered into the periplasm. There, Tpa1 permeabilises the cytoplasmic membrane so Tde1 can be translocated into the cytosol. This effector translocation through the inner membrane is attributed to a glycine zipper motif present in the N-terminus of Tde1 [125]. It has also been proposed that *P. aeruginosa* effector Tde6 is delivered into the target cell's periplasm, whereupon transmembrane segments of the effector create an inner membrane passage that allows cytoplasmic access [126]. Furthermore, a recently identified domain has been shown to be conserved amongst nuclease bacteriocins and T6SS effectors. Despite differences in sequencing, the inner membrane translocation

(IMT) domain showed a strong structural similarity to T6SS effectors [127]. Since the IMT domain is required for *P. aeruginosa* bacteriocin pyocin G to import its toxic nuclease domain into the cytoplasm, it was suggested that T6SS effectors with cytoplasmic activity share a similar mechanism of inner membrane translocation [127].

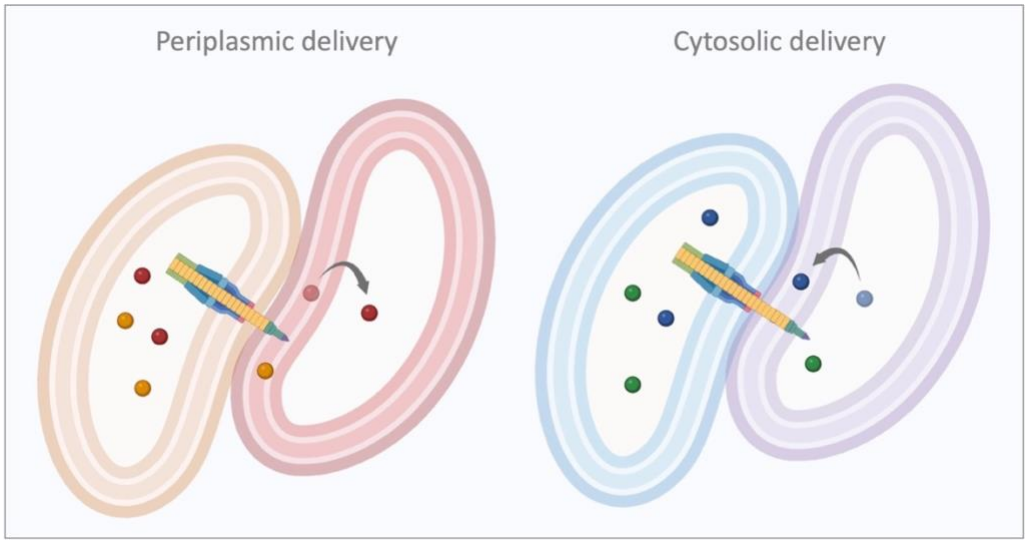

Figure 1.4 Schematic depiction of T6SS effectors modes of delivery. Effectors can be injected directly into the periplasm and exert their toxicity therein, or be posteriorly translocated into the cytosol. Conversely, effectors can be directly delivered into the cytosol, or subsequently translocated into the periplasm.

1.4.3.1 Antibacterial effectors

1.4.3.1.1 Effectors targeting the cell wall

The repertoire of T6SS-delivered effectors is vast and remains an active field of study. The T6SS can exert an antibacterial effect through the delivery of specific effector proteins that act on distinct parts of the target cell. Toxic effectors can act as phospholipases that cleave specific chains within membrane phospholipids. One example is Tle1 in both *Burkholderia thailandensis* and *P. aeruginosa* [128], Tle2 (TseL) in *V. cholerae* [129] and Tle1 in EAEC SC-1 [104]. Another example is *P. aeruginosa* effector Tle5 (PldA) which can destabilise, depolarise and bleb target cells [129]. Moreover, periplasmic expression of the C-terminal domain of *P. aeruginosa* effector VgrG2b (VgrG2b$_{c\text{-}ter}$) in *E. coli* results in a cellular morphological defect characterised by membrane blebbing [124]. Blebbing occurs in the middle of the cell, whereby the membrane cracks before the cell acquires a round shape. This effect is similar to that observed for beta-lactam antibiotics.

Effectors can also insert into the inner membrane of the target cell, by forming pores and thus disrupting membrane integrity. However, pore-forming effectors inhibit bacterial growth instead of lysing the target cell. Examples of pore-forming effectors are *Vibrio parahaemolyticus* Tme family [130], *P. aeruginosa* effector Tse4 [131] and *S. marcescens* effector Ssp6 [132].

In the periplasm, effectors can act on the peptidoglycan by hydrolysing the glycan backbone or the peptide chains. Glycoside hydrolases cleave the glycan backbone between the N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). Such is the case of Tge1 in *P. aeruginosa*, Tge2 in *Pseudomonas protegens* [133] and VT5 in enterotoxigenic *E. coli* (ETEC) [134]. Specifically, some of these effectors have lysozyme-like activity, like muramidases. Examples of these are effectors Tse3 in *P. aeruginosa* [135] and VgrG3 in *V. cholerae* [103].

On the other hand, effectors that hydrolyse peptide chains act as peptidases that cleave peptide stems, or as amidases that cleave peptidoglycan cross-links. Peptidases include *P. aeruginosa* effectors Tae1-4 [135,136], *Acinetobacter baylyi* effector TagX [137] and *Klebsiella pneumoniae* newly identified effector Tke [138]. Examples of effectors with amidase activity can be found in different species. Like effectors VT1 in ETEC [134], Tlde1 in *Salmonella enterica* [139], Tae1 in *A. baylyi* [137], TseH in *V. cholerae* [140] and Tse1 in *P. aeruginosa* [135]. Other effectors can

also act as zinc metallopeptidases, such as *A. baylyi* Tpe1 [137] and *P. aeruginosa* VgrG2b C-terminal domain [124].

Periplasm-targeting effectors have been shown to require disulphide bond formation in order to be active. Disulphide bond formation plays a crucial role in the stability and function of secreted proteins. In bacteria, disulphide bond formation is mediated in the periplasm by thiol disulphide oxidoreductase DsbA. In T6SS-mediated antibacterial competition, DsbA has been shown to have a dual role in the secreting cell and in the target recipient cell [141]. In the secreting cell, DsbA facilitates the assembly of a functional T6SS. This was demonstrated when a *S. marcescens dsbA1* and *dsbA2* mutant strain showed reduced secretion and killing effect against *E. coli*. Additionally, DsbA is essential for proper effector function in the recipient cell. The periplasmic toxicity of T6SS-delivered effectors Ssp2 and Ssp4 is only observed if DsbA is functional in the recipient cell [141]. Disulphide bond formation has also shown to be required for proper function of a *P. aeruginosa* effector in the periplasm of a recipient cell [124]. When *P. aeruginosa* periplasmic toxic effector VgrG2b$_{c\text{-}ter}$ is expressed in *E. coli*, cell viability is greatly reduced. In *E. coli* lacking DsbA, the periplasmic levels of VgrG2b$_{c\text{-}ter}$ cognate immunity protein decrease and bacterial growth is hindered by the periplasmic expression of VgrG2b$_{c\text{-}ter}$. Here, DsbA disulphide bond formation is required for immunity protection against the expression of a toxic effector.

1.4.3.1.2 Effectors targeting the cytosol

Effectors can target components of the cell cytosol, for example by acting as DNases or NADases. In *P. aeruginosa*, H1-T6SS is able to secrete both a DNase (Tse7) and a NADase (Tse6). Tse7 expression induces an SOS response, inhibits cell growth and results in complete DNA degradation [142]. While Tse6 consumes the target cell's $NADP^+$, causing growth inhibition without affecting cellular structural integrity [126]. Notably, Tse6 requires a housekeeping protein in order to exert its function [126]. Tse6 binds to translation elongation factor Tu (EF-Tu), forming a complex which is required for effector access to the cytosol of the recipient cell. Another effector with potent $NAD(P)^+$ hydrolase activity has also been found in *P. protegens* and classified as Tne2 (Type VI secretion NADase effector family 2) [143]. Along with this finding, effector Tse6 has later been classified as belonging to NADase family Tne1. A family of Tde DNases has also been found in *A. tumefaciens* and revealed a potent antibacterial effect in

interbacterial competitions [144]. Furthermore, a pyocin-like effector with DNase activity has been recently found to be secreted by *Yersinia pseudotuberculosis* T6SS-3 [145]. This effector (YPK_0952) has an N-terminal PAAR domain and a C-terminal S-type pyocin domain and can exert an antibacterial effect through contact-dependent and contact-independent mechanisms.

Other effectors with nuclease activity are encoded by Rhs genes, which usually encode an N-terminal PAAR or VgrG domain [66]. Examples are RhsAB in *Dickeya dadannii* [146], and Rhs2 in both *S. marcescens* [117] and *Acinetobacter baumannii* [147]. It has been recently shown that the C-terminal of *S. marcescens* effector Rhs1 is an $NAD(P)^+$ glycohydrolase which depletes essential cellular cofactors NAD^+ and $NADP^+$ in the recipient cell [148]. Interestingly, a Rhs hybrid effector has been identified in *Salmonella tennessee* as having a predicted C-terminus with DNase and RNase domains [149]. This hybrid DNase and RNase activity had been previously recognised in TseTBg, an endonuclease effector in *Burkholderia gladioli* [150].

A novel mechanism for antibacterial toxicity by a T6SS effector has also been identified in *P. aeruginosa* [151]. When effector *P. aeruginosa* effector Tsa1 is released into the target cell, it synthesises (p)ppApp, depletes ADP and ATP, which ultimately causes cell death. Tas1 has a (p)ppApp-synthetase domain at the C-terminus and a PAAR domain at the N-terminus, which is responsible for T6SS delivery. An hydrolase has been found to prevent the deleterious effects of Tas1 and thus might act as a defence mechanism for interbacterial competition [152].

In *Burkholderia cenocepacia*, a cytidine deaminase effector (DddA) has been found to preferentially target double stranded DNA in recipient cells [153]. However, DddA-mediated killing effect is dependent on recipient cell [154]. Whereas for example *A. baumannii* was resistant to T6SS-delivered DddA, *P. aeruginosa* was highly sensitive to its killing effect. Interestingly, species resistant to DddA killing acquired mutations, suggesting that this effector could drive species adaptation and have consequences in microbial population dynamics [154]. In *Salmonella* spp., nuclease effector TseV3 specifically recognises DNA, induces DNA double-strand breakage and activates SOS response when delivered into the target cell [155].

Furthermore, T6SS effectors can target protein synthesis and impair cell division. For example, *P. aeruginosa* H1-T6SS effector Tse8 targets a complex required for protein synthesis in bacteria that lack asparagine or glutamine tRNA synthases [156]. *P. aeruginosa* H2-T6SS delivers effector RhsP2, which resembles an ADP-ribosyltransferase and takes part in bacterial

antagonism by targeting the RNA of the recipient cell [157]. Another effector with ADP-ribosylating activity (Tre1) has been found in *Serratia proteamaculans* and it is capable of interrupting cell division when expressed in *E. coli* [158].

The early prevalence of identified periplasm-targeting effectors initially led to speculation that T6SS activity was limited to Gram-negative bacteria [60]. However, recent studies have challenged this belief after demonstrating that T6SS aggressors could also target Gram-positive species. The T6SS of *Pseudomonas chlororaphis*, which is homologous to H2-T6SS of *P. aeruginosa*, stimulates *Bacillus subtilis* sporulation when close cell-to-cell contact occurs [159]. Another report showed that the plant pathogen *Acidovorax citrulli* can deploy its T6SS to kill *B. subtilis* and *Mycobacterium smegmatis* via secretion of an Rhs-family nuclease [160]. Moreover, a strain of *A. baumannii* can kill *B. subtilis*, *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* in a T6SS-dependent manner [161]. *A. baumannii* can secrete D-lysine into the extracellular milieu, increasing the environmental pH. In turn, alkalinisation of the extracellular environment potentiates the peptidoglycan-degrading activity of *A. baumannii* effector Tse4.

1.4.3.1.3 Extracellular effectors

The T6SS is also able to secrete effectors in a contact-independent manner into the extracellular milieu. The T6SS of some species, like *B. thailandesis* and *Y. pseudotuberculosis*, can export metal scavenging proteins in response to oxidative stress [162–164]. The scavenged metals, such as copper, zinc and manganese, are imported back into the secreting cell conferring it a growth advantage.

Moreover, *P. aeruginosa* H3-T6SS secretes effector TseF, which is involved in iron acquisition in iron-deficient media [165]. TseF is incorporated in outer membrane vesicles where it interacts with a signalling compound to bring iron to receptors on the cell surface. The T6SS is a known contact-dependent mechanism for antibacterial antagonism, but these novel contact-independent mechanisms appear to also provide T6SS-wielding species with a fitness advantage and another way to overcome competitors.

Interestingly, *Y. pseudotuberculosis* T6SS-3 secretes a nuclease effector that can exert toxicity in a contact-dependent and contact-independent manner [166]. T6SS-3 can directly

deliver effector Tce1 into a neighbouring cell or into the extracellular milieu. When delivered extracellularly, Tce1 requires OM proteins OmpF and BtuB for entry in the target cell. Therein, Tce1 acts as a Ca^{2+} and Mg^{2+}-dependent endonuclease that cleaves DNA.

1.4.3.2 Anti-eukaryotic effectors

The repertoire of T6SS-delivered effectors is very vast and versatile, and T6SS-wielding species might have adapted their response according to their competitors. A T6SS firing event is able to deliver a payload of effectors into neighbouring cells that can impair their growth or directly kill them. Besides targeting competitive bacteria, the T6SS can also target eukaryotic cells. In *V. cholerae*, VgrG1 is required for toxicity against eukaryotic cells, such as *Dictyostelium discoideum* amoebae and J774 macrophages [53,70]. This cytotoxic effect was ascribed to the C-terminal actin-crosslinking domain (ACD) of VgrG1, which albeit being dispensable for secretion, it is required for anti-host toxicity [54]. In *Francisella*, T6SS-delivered effectors PdpC and PdpD are required for phagosomal escape [167], whereas effector OpiA acts in the *Francisella*-containing phagosome, leading to efficient escape of the bacteria into the cytoplasm [168].

The T6SS can simultaneously target prokaryotic and eukaryotic cells. For example, *V. cholerae* VasX is required to kill both amoeba and bacteria [169–171]. Similarly, T6SS-delivered effectors with both antibacterial and anti-eukaryotic activity have been identified in *Vibrio proteolyticus* [172]. Another example are phospholipases PldA and PldB in *P. aeruginosa* which target eukaryotes and prokaryotes and have therefore been described as “trans-kingdom” effectors [173,174]. Moreover, *P. aeruginosa* H2-T6SS effector TplE is a trans-kingdom effector that not only targets bacterial phospholipids but also induces stress response in the endoplasmic reticulum [175].

1.4.4 Exchange of genetic information

It has been previously shown that DNA can be transferred between cells following a T6SS attack. When *V. cholerae* T6SS is expressed on a chitinous surface, neighbouring cells die and release DNA, which can then be taken up by other cells [93,176]. Moreover, competent

V. cholerae can take up large genomic regions released from a cell that has been killed in a T6SS-dependent manner [177].

Similarly, the T6SS has been implicated in the acquisition of plasmids by predatorial *A. baylyi* when a prey releases DNA through cell lysis [178]. It has been confirmed that the efficiency of T6SS-dependent HGT is influenced by the activity of lytic effectors which cause prey release of DNA [137]. In *A. baylyi*, phospholipase effector Tle1 induces prey-cell lysis. The predator strain can take up the genetic material released by the prey at higher rates than if the prey had been subjected to the effect of a non-lytic effector (Tse1). Therefore, each predator-prey pair transfers DNA with a specific efficiency depending on how the predator kills its prey.

As mentioned previously, *V. cholerae* is also able to acquire secreted proteins from sister cells following a T6SS attack [123]. *V. cholerae* VgrG2/Hcp mutants are unable to assemble a T6SS sheath, secrete Hcp or kill *E. coli*. However, when sheath-deficient recipient cells are mixed with T6SS-wielding donor cells, recipients were able to restore their sheath. Furthermore, recipient cells lacking T6SS tip-associated effectors VgrG1/VgrG3/VasX were also able to restore sheath assembly after co-incubation with T6SS-wielding donors.

1.4.5 Defences against the T6SS

1.4.5.1 Immunity

Interbacterial competitions mediated by the T6SS are complex, as are the mechanisms by which bacteria employ their T6SSs for defence. Bacteria with an active T6SS encode cognate immunity proteins to protect them from the toxic effects of their own T6SS effectors. Naturally, immunity proteins are typically found in the target site of their cognate effectors [60]. Immunity proteins are commonly encoded alongside their corresponding effectors and prevent self- or kin-intoxication by directly binding to effectors and physically preventing their action. The first effector-immunity pair to be identified in *P. aeruginos*a was proposed to act through direct physical association [179]. Interestingly, an immunity protein in *S. proteamaculans* (Tri1) has been found to have a dual inhibitory function [158]. In addition to binding to the active site of its cognate effector Tre1, Tri1 removes ADP-ribose moieties from

Tre1. Tri1 exhibits ADP-ribosylhydrolase activity, which removes the moieties added by the ADP-ribosyltransferase effector Tre1.

Later on, many other effector-immunity pairs were also identified in other bacterial species, like *V. cholerae* [103,140,180], *E.coli* [104], *S. marcescens* [132] and *A. baylyi* [137], among others. The protective effect of immunity proteins is typically confirmed by deleting the immunity gene, which results in cell sensitivity to effector toxicity. This was first observed in *P. aeruginosa*, when cell growth was inhibited upon expression of effector Tse2 in mutants lacking the immunity protein Tsi2 [179]. Another example was seen in *V. cholerae*, when mutants lacking individual immunity genes became sensitive to T6SS-dependent killing by sister cells [103,181]. However, mutant survival increased when the immunity gene is expressed in trans.

Notably, *V. cholerae* effector TseH and its cognate immunity protein TsiH seem to be an exception [140]. As expected, TseH is highly toxic upon periplasmic expression in *E. coli* lacking its cognate immunity protein, although its toxicity is neutralised upon co-expression of the immunity protein. However, a *V. cholerae* TseH/TsiH mutant is less susceptible to T6SS killing by the wild-type strain [140]. Moreover, a *V. cholerae* mutant strain with all effectors inactivated except TseH is unable to kill *E. coli* as well as *V. cholerae* lacking TsiH [182]. *V. cholerae* TseH-mediated toxicity may be species-specific, with a preference for targeting susceptible species rather than *V. cholerae* sister cells. However, *Edwardsiella* and *Aeromonas* species, which can share colonising niches with *V. cholerae*, are highly susceptible to TseH [182]. Additionally, *E. coli* is resistant to T6SS-delivered TseH but susceptible to the periplasmic expression of TseH [182]. This phenomenon could be due to the ability of *E. coli* to respond to cell wall damage caused by the T6SS-delivered effector. Since *Aeromonas* is susceptible to TseH but *E. coli* has repair mechanisms that mitigate effector toxicity, it is possible that *V. cholerae* specifically employs TseH against species it may encounter or compete within the environment.

Certain Bacteroides species present in the human gut encode immunity genes in the absence of a cognate effector gene [183]. These orphan immunity genes are encoded in mobile elements that can be transferred to naïve species and provide protection against T6SS effectors. It has been hypothesised that orphan immunity genes could be a product of duplication or effector acquisition from neighbouring cells to protect against T6SS killing [184]. A large genomic and metagenomic study also revealed that gut Bacteroides can transfer mobile T6SS loci amongst their members [185].

1.4.5.2 Retaliation

Defence mechanisms against T6SS are not limited to the presence of immunity proteins in the target cell. Other potential adaptive protective pathways have been described, like the T6SS-dependent retaliatory behaviour observed in *P. aeruginosa*. Whereas the T6SS of *V. cholerae* and *A. baylyi* appears to assemble and fire randomly, *P. aeruginosa* H1-T6SS specifically assembles and fires when a threat is sensed [87,99]. *P. aeruginosa* propels its H1-T6SS and punctures an adjacent sister cell. In turn, the punctured cell responds by assembling its own T6SS at the exact point of attack and counterattacks [87]. This intra-species retaliation is not lethal, given that cells produce cognate immunity proteins to the effectors delivered by the others. *P. aeruginosa* increases its H1-T6SS activity only when it feels threatened rather than by its own volition. *P. aeruginosa* H1-T6SS retaliatory behaviour was also observed upon co-incubation with *V. cholerae* and *A. baylyi* [99]. *P. aeruginosa* H1-T6SS does not kill T6SS-deficient *V. cholerae* and *A. baylyi*. Instead, it counterattacks if these species express a functional T6SS. *P. aeruginosa* detects the point of attack and assembles a new T6SS that can fight back, emphasising that *P. aeruginosa* H1-T6SS is defensive instead of offensive. As described previously, this duelling ability observed in *P. aeruginosa* H1-T6SS is mediated by the TagQRST-PpkA-Fha1-PppA cascade.

Stress response genes have also been implicated in the protection against T6SS effectors [186]. *E. coli* displays resistance to *V. cholerae* T6SS-delivered effector TseL, but not to its periplasmic expression. A set of *E. coli* genes that help maintain membrane integrity during stress seem to be responsible for TseL-mediated response. The toxic effect of TseL towards *E. coli* could be sufficient if the effector is expressed in the cell periplasm, but insufficient if the effector is delivered in a T6SS-dependent manner. Similarly, *P. aeruginosa* can also use stress response mechanisms to defend against the effects of T6SS-delivered TseL [186].

1.4.5.3 Physical protection

The dynamics of T6SS within dense multicellular populations such as biofilms has also been explored. Biofilms are resilient polymicrobial communities that play an essential role in bacterial survival by creating a physical barrier to external factors [187]. The correlation between T6SS dynamics and biofilm formation was first demonstrated in EAEC [188]. Deletion of SciN

(TssJ), prevents Hcp secretion and biofilm formation. However, it remains unknown whether biofilm formation is dependent exclusively on SciN, the T6SS apparatus or secreted effectors. The T6SS has also been shown to confer a fitness advantage within multispecies biofilms [189]. In mixed species flow cell biofilm assays, wild-type *B. thailandensis* can overgrow *Pseudomonas putida*. However, *P. putida* readily displaces a T6SS-deficient *B. thailandensis*. Similarly, in a mixed biofilm of two *Pseudomonas fluorescens* strains, strain MFE01 strongly reduces the biovolume of strain MFP05 biofilm [190]. Though, if strain MFE01 lacks TssC1, strain MFP05 biofilm biovolume is unaffected.

The correlation between the T6SS regulation and biofilm formation has been studied in different *P. aeruginosa* strains. Hcp has shown to be implicated in biofilm formation in *P. aeruginosa* strain PAO1 [191] and to exhibit higher expression in *P. aeruginosa* strain PA14 biofilm cells compared to their planktonic counterparts [192]. Furthermore, the expression of Hcp1 is also higher in *P. aeruginosa* isolates that form biofilms compared to non-biofilm formation isolates [193].

The production of exopolysaccharide (EPS) has been shown to play a role in protecting against T6SS attacks [194]. In *V. cholerae*, EPS production does not prevent T6SS firing nor does it confer resistance to specific effectors. Instead, EPS acts as a physical barrier to exogenous T6SS attacks. *V. cholerae* prey lacking *vpsA*, a gene that encodes an essential component in EPS production, was more susceptible to T6SS-dependent killing by wild-type sister cells. However, *V. cholerae* predators lacking *vpsA* showed no impaired T6SS-dependent killing. A recent study has investigated the effect of capsule production on T6SS activity [195]. The capsule does not prevent *A. baumannii* from firing its own T6SS, albeit reducing its efficacy. However, deleting a gene essential for capsule production in *A. baumannii* increases its susceptibility to *Enterobacter cloacae* T6SS-mediated killing.

1.5 T6SS-wielding species

T6SS-wielding species are ubiquitous and play important roles in interbacterial competition and host colonisation [196–198]. Many T6SS-wielding species are important causative agents of disease, such as *P. aeruginosa* and *V. cholerae*, two of the species used in this work. However, many of these species have distinct niches and do not come across each other in the environment or in the human body. Nevertheless, they can be used as model organisms to study T6SS-mediated interbacterial interactions and to help study the complexity of microbial population dynamics. Herein, we briefly discuss the T6SS-wielding species used as model organisms for T6SS-mediated interactions in this work.

1.5.1 *Acinetobacter baylyi*

A. baylyi ADP1 is a Gram-negative non-motile bacillus commonly found in soil and water environments [199]. *A. baylyi* is a model organism commonly used in the laboratory for natural competence and genetic engineering in bacteria [200]. Although *A. baylyi* is a non-pathogenic species, it is closely related to *A. baumannii*, an opportunistic pathogen responsible for nosocomial infections [201].

A. baylyi encodes a single constitutively active T6SS apparatus which is able to kill a variety of other bacteria, such as *E. coli* [71,99,202]. A thorough genetic analysis of the T6SS cluster in *A. baylyi* revealed that it contains five effectors with distinct activities, most of which are necessary for antibacterial activity against *E. coli* [137]. While the identified effectors are not required for Hcp secretion [137], the three PAAR proteins within the T6SS cluster are necessary for Hcp secretion and for *E. coli* killing [71].

A. baylyi is a valuable model organism, not only due to its genetic ability but also for encoding a constitutively active T6SS, which is able to eliminate other bacteria. Bacterial interactions mediated by the T6SS of *A. baylyi* have been previously reported, and as such *A. baylyi* represents a robust laboratory model species for bacterial antagonism. Due to its genetic similarity with *A. baumannii* it can also serve as a proxy to study interbacterial interactions for clinically relevant species.

1.5.2 *Vibrio cholerae*

V. cholerae is a Gram-negative motile bacillus found in aquatic environments and the causative agent of severe diarrhoeal disease cholera [203]. *V. cholerae* has been responsible for causing many cholera pandemics, which have had devastating effects on public health. The first cholera pandemic dates from the 19th century, and since then, seven more pandemics have been reported [203]. There are up to 4 million cases and 143,00 deaths from cholera each year, and as of date, only three available oral vaccines [204]. Cholera is often difficult to differentiate from other diarrhoeal diseases and remains a cause for high morbidity and mortality in many developing countries [204].

V. cholerae spreads through the faecal-oral route and can be fatal if not rapidly treated [203]. Prevention and control can be achieved through improved surveillance, sanitation and hygiene, and access to preventative or treatment measures [204]. Symptoms usually appear within 12 hours to 5 days post-exposure and can be treated using rehydration solutions or antibiotics [203,205].

Once ingested through the consumption of contaminated water, *V. cholerae* cells make contact with the small intestine and express cholera toxin (CT) and toxin coregulated pili (TCP), which are responsible for the diarrhoeal disease and gut colonisation, respectively [206]. In order to survive and persist in the environment, *V. cholerae* has developed a variety of virulence and colonisation factors, like motility, quorum-sensing (QS), biofilm formation and antibacterial weapons like the T6SS [207]. Previous reports have shown that the T6SS can confer enhanced fitness to pandemic *V. cholerae* strains, suggesting its important role in cholera pathogenesis [208].

V. cholerae can efficiently deploy its T6SS to kill both eukaryotes and prokaryotes [53,209]. *V. cholerae* T6SS is encoded in the large cluster of the major chromosome, where the majority of the components are encoded, and three auxiliary clusters of the minor chromosome [210]. The extensive repertoire of *V. cholerae* T6SS-delivered effectors has been thoroughly studied and will be addressed in detail in Chapter 2.

1.5.3 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative bacillus found in a variety of environments as well as in healthy individuals [211]. However, it mostly manifests as an opportunistic pathogen and is commonly associated with multi-drug resistant (MDR) acute and chronic infections in immunocompromised patients [212]. Infections are predominantly hospital-associated and can affect the respiratory tract, like chronic obstructive pulmonary disease (COPD) or ventilator-associated pneumonia (VAP), the urinary tract, wounds, and soft tissue, among many others [212–216]. *P. aeruginosa* is responsible for 20% of hospital-associated infections in Europe and the USA, and is the most common cause of infection in patients with cystic fibrosis (CF), a genetic condition that can affect the lungs, deeming them highly susceptible to infection [211,217,218].

P. aeruginosa has a repertoire of mechanisms that contribute to its resistance and persistence in clinical settings, challenging the efficacy of antimicrobial treatments. *P. aeruginosa* fitness and recalcitrance can be attributed to a variety of functions: its ability to form biofilms, communicate through QS, produce exoproducts and secondary metabolites, up-regulate efflux pumps for antibiotic export, and use pili and flagella for motility and adherence [219]. Investigating ways to overcome these mechanisms will shed light on new antimicrobial targets, either by exploiting *P. aeruginosa* antimicrobial weapons or breaching its defence barriers.

P. aeruginosa is notoriously known to form biofilms, which are surface-adhering bacterial aggregates encased in a matrix of extracellular polymeric substances [220]. The biofilm matrix creates a physical barrier to environmental stresses and the host immune defences [221]. Therefore, biofilms are extremely difficult to eradicate and can be up to 1000 times more resistant to antibiotics than planktonic cells [222]. *P. aeruginosa* biofilms can act as diffusion barriers to the penetration of antibiotics, complicating the treatment of biofilm-associated chronic conditions [223,224]. Considering the protective barrier biofilms create around bacterial cells, it is reasonable to consider that *P. aeruginosa* biofilms could offer protection against exogenous T6SS attacks. If T6SS aggressors are unable to reach prey cells within the biofilm, toxic effector delivery will be prevented, and prey cells can survive. *P. aeruginosa* can also use its own T6SS to persevere within microbial populations, as the apparatus only assembles and fires when there is a threat of an exogenous T6SS attack [87,99].

P. aeruginosa encodes three distinct T6SS clusters, H1-T6SS, H2-T6SS and H3-T6SS [65]. An analysis of 122 *P. aeruginosa* genomes revealed that half of the genomes studied encoded more than one T6SS cluster [225]. The H1-T6SS is commonly associated with antibacterial activity since it secretes a multitude of antibacterial effectors and participates in interbacterial competition [99,126,135,179]. On the other hand, the H2- and H3-T6SSs have demonstrated both antibacterial and anti-eukaryotic activity [129,157,173,175,226]. Recently, the H3-T6SS has also been associated with virulence factors of *P. aeruginosa* PAO1 [227]. A *P. aeruginosa* H3-T6SS-deficient mutant (Δ*clpV3*) exhibited increased H1-T6SS activity. However, this mutant showed lower expression of H2-T6SS and H3-T6SS and other relevant virulence factors like biofilm formation, motility and T2SS and T3SS expression. More recently, a fourth T6SS apparatus has been reported in *P. aeruginosa* [228]. Upon analysing a large dataset of phylogenetically diverse *P. aeruginosa* strains, a gene cluster was found in a small subset of strains. This gene cluster was later named H4-T6SS to follow the previously adopted convention and has been shown to encode 12 out of the 13 genes required for the T6SS apparatus.

P. aeruginosa H1-T6SS delivers a variety of effectors with distinct antibacterial activities (Tse, Type 6 secretion exported). Effectors Tse1 and Tse3 are proposed to be directly delivered into the periplasm of the target cell, where they hydrolase the peptidoglycan [135]. On the other hand, Tse2 targets the cytoplasm where it is proposed to have ADP-ribosyltransferase activity [179,229]. Tse4 is a pore-forming effector that targets the periplasm, permeabilising the inner membrane by disrupting the proton motive force [131,230]. Effector Tse5 has also been named RhsP1 because it encodes a protein of the Rhs family that is associated with antibacterial activity through CDI [231]. As mentioned previously, Tse6 and Tse7 both target the cell cytosol acting as NADase and DNase, respectively [126,142]. In order to be delivered, H1-T6SS effectors interact with the T6SS machinery in different ways: Tse1-4 interact with the Hcp tube, whereas Tse5-7 interact with their cognate VgrG proteins, either directly or through a PAAR motif or adaptor proteins [116,126,142,230]. H2-T6SS and H3-T6SS are also associated with delivering antibacterial effectors that target the periplasm, specifically phospholipase D enzymes, PldA and PldB [129,173].

Transcription regulation of *P. aeruginosa* T6SS expression is mediated by QS regulator LasR, and posttranscriptional regulation is mediated by RNA-binding protein RsmA. LasR acts as a suppressor of H1-T6SS gene expression, but in H2-T6SS and H3-T6SS, it acts as a positive

regulator [232]. However, RsmA acts as a repressor and coordinator of all three *P. aeruginosa* T6SSs [94]. At the posttranslational level, *P. aeruginosa* H1-T6SS regulation is mediated by TPP, which has been previously described in this chapter and is a regulatory cascade that mediates *P. aeruginosa* retaliation.

1.5.4 Bacterial interactions through the T6SS

Multiple reports have shown that the T6SS can be used as an antimicrobial weapon in interbacterial competition between many different species [62,87,99,135,137,179,209]. There has been recent interest in understanding how T6SS interactions shape microbial community dynamics. Pathogens can employ their T6SS to take over a niche and eliminate commensal bacteria, and on the other hand, commensals can use their own T6SS to defend from pathogen predation.

To study the impact of T6SS on bacterial fitness a simulation model of interbacterial competition between different T6SS-wielding species has been recently developed [233]. This model has revealed that when *A. baylyi* T6SS firing increases, the amount of dead *E. coli* cells also increases at the barrier between competing cells ("corpse barrier effect"). However, the killing rate was dependent on the speed by which the victim cells lysed. There appears to be a killing effect saturation: if victim cell lysis is slow, T6SS aggressors encounter a barrier of dead cells and cannot exert their attacks in cells beyond that barrier; conversely, if victim cell lysis is rapid then space is freed for T6SS aggressors to find new victims. This effect was then confirmed by cell recovery assays when an engineered *A. baylyi* delivered single slow- or rapid-lysis toxins into *E. coli*. Thus, T6SS aggressors benefit from potent effector toxins that rapidly lyse their prey. The advantage of a T6SS aggressor carrying more than one potent toxin might be that a synergistic effect can be achieved with a cocktail of toxins, rather than just a single toxin. A similar "corpse barrier effect" was observed when two different T6SS aggressors were co-incubated [234]. T6SS-wielding *V. cholerae* and *A. hydrophila* formed segregated clusters with a death barrier at their interface. A barrier of dead cells and debris prevents aggressors from contacting one another. It is also possible that cells behind this protective barrier have the chance to replicate and make up for the damage incurred. Interestingly, when both species T6SSs are inactive they can coexist in a mixed culture, which suggests that T6SS strongly drives bacterial antagonism. Type IV pili (T4P) have also been associated with

interbacterial interactions mediated by the T6SS [235]. Naturally, aggregation leads to direct cell-to-cell contact and can potentially impact T6SS interactions. A previous study has shown that, under liquid conditions, *V. cholerae* T6SS predators can efficiently kill sister prey when T4P are active. The T4P is an appendage that mediates bacterial aggregation, enabling direct contact between cells which is crucial for T6SS-mediated antagonism.

The T6SS has been recognised as an offensive or defensive mechanism in interbacterial interactions. However, another model has shown that the T6SS can be used to pre-emptively deliver effectors in the context of community behaviour [236]. In *Proteus mirabilis*, T6SS-dependent killing has been linked with the Dienes line phenomenon [237]. The Dienes phenomenon is characterised by the ability of swarming strains to form a visible barrier between each other on a surface. Formation of this visible barrier happens between different *Proteus* strains and depends on the ability to recognise self from non-self [236]. When *P. mirabilis* swarming strains meet on a surface, their T6SSs fire and the dominant strain can penetrate the swarm barrier and carry on killing [236]. Social recognition associated with *P. mirabilis* swarming strains leads to constant T6SS firing, thus preventing cooperative behaviour.

There is growing evidence that bacteria have defence mechanisms against T6SS at the single-cell level, but further research is required on community protection against exogenous T6SS attacks [184,194]. In addition to protecting at the single-cell level against T6SS attacks, EPS-producers can offer community protection. Using an agent-based model for competition has shown that EPS can confer collective protection [238]. In a competition assay with *A. baylyi*, EPS-producing *E. coli* can survive at higher rates than its non EPS-producing counterpart. Protection was T6SS-dependent since no difference was observed in *E. coli* survival after competition with a T6SS-deficient *A. baylyi*. Furthermore, EPS-producing cells can not only protect themselves from T6SS attacks, but also shield surrounding non-producers in what is referred to as “flank protection”. These observations represent population dynamics in a small-scale and controlled laboratory environment. However, T6SS-wielding bacteria can encounter each other in environmental habitats, such as water or plants [239,240] or in body compartments, such as the gut [241]. To better understand natural processes, these models need to be scaled up to larger environments.

There has been a recent interest in investigating the role of the T6SS during colonisation and infection *in vivo*. For example, a murine model has been previously used to

study the T6SS effect in gut colonisation by different *Bacillus fragilis* strains [242]. T6SS-wielding non-toxigenic *B. fragilis* can exclude enterotoxigenic *B. fragilis*, preventing colonisation and protecting the host from intestinal inflammatory processes. An earlier investigation into the function of the T6SS in the mammalian gut contributed to delineating a model for *V. cholerae* gut colonisation [243]. This study revealed that in the middle and proximal small intestine *V. cholerae* cells are found in a planktonic state, where there is no close contact between cells. In this initial portion of the small intestine, single-state *V. cholerae* cells use their T6SS to compete with host microbiota. However, in the distal small intestine and cecum, *V. cholerae* cells aggregate and engage in close cell-to-cell contact and T6SS competition amongst *V. cholerae* population. Furthermore, in a model of infant mice pre-colonised with two commensal *E. coli* strains and infected with *V. cholerae*, the load of intestinal *E. coli* was lower in mice challenged with the wild-type compared to the T6SS mutant [198]. *V. cholerae* deploys its T6SS against gut commensal microbiota, increasing the expression of virulence factors and activating the host's immune response. *V. cholerae* might use its T6SS for interbacterial competition in the gut by clearing existing commensals, driving niche occupation, and thus enhancing pathogenicity.

The presence of commensal bacteria in the gut has been shown to influence how *V. cholerae* T6SS contributes to pathogenesis in a *Drosophila* model [197]. T6SS-wielding *V. cholerae* has a lethal effect against *Drosophila*. When T6SS-deficient *V. cholerae* colonises *Drosophila*, host colonisation decreases and survival increases. However, T6SS toxicity appears to be dependent on the presence of commensal bacteria. Removal of commensal species *Acetobacter pasteurianus* increases host survival, but its re-introduction restores T6SS-mediated host killing. On the other hand, removing all intestinal host bacteria did not further increase T6SS-mediated killing. Since T6SS-mediated killing is attenuated in the absence of commensal bacteria, this suggests that *V. cholerae* T6SS acts on commensals for pathogenesis. Furthermore, the T6SS has shown to be a potent weapon for interbacterial competition, used not only by predators that eliminate commensal species, but also by commensal to defend their niches. *Citrobacter rodentium*, a model for human pathogenic *E. coli*, competes for host gut colonisation with commensal *E. coli* Mt1B1 [244]. Both species encode two T6SS apparatuses and engage in T6SS-mediated competition to colonise the murine gut. Zebrafish have also been used as a model to study how *V. cholerae* T6SS can defeat commensal bacteria in the

gut [196]. Zebrafish pre-colonised with symbiotic *Aeromonas* species and subsequently infected with *V. cholerae* can excrete large aggregates of *Aeromonas* from the host gut. A functional T6SS in *V. cholerae* strongly stimulates the peristaltic movement of the zebrafish gut, which leads to the excretion of commensal bacteria.

1.5.5 Interbacterial competition with *P. aeruginosa*

It has been previously proposed that *P. aeruginosa* H1-T6SS retaliatory behaviour was triggered by mechanisms that disrupt the cell membrane [99]. One such mechanism is the T4SS. *E. coli* cells carrying the RP4 conjugative plasmid have demonstrated more susceptibility to being killed by *P. aeruginosa* T6SS than non-conjugative *E. coli* [245]. Moreover, exposing *P. aeruginosa* to polymyxin B, which is a membrane-disrupting antibiotic, also increases the cell's T6SS activity [245]. *P. aeruginosa* T6SS activity also readily increases in the presence of sputum isolated from CF patients. This has been attributed to the presence of eDNA (extracellular DNA) in CF sputum, which can disrupt the cell membrane by chelating membrane-bound cations [246]. Furthermore, if all *A. baylyi* T6SS effectors are removed, *P. aeruginosa* H1-T6SS retaliates as it would against the wild-type *A. baylyi* [137]. A T6SS deficient *A. baylyi* does not elicit a counterattack from *P. aeruginosa* H1-T6SS. However, when *A. baylyi* retains a functional T6SS but lacks effectors, *P. aeruginosa* H1-T6SS readily retaliates. This suggests that *P. aeruginosa* detects the T6SS physical firing rather than the delivery of toxic effectors.

However, a recent study has challenged the belief that *P. aeruginosa* H1-T6SS retaliation is triggered by membrane disruption mechanisms and instead is triggered by specific heterologous effectors. When all four *V. cholerae* T6SS effectors are inactivated, *V. cholerae* T6SS behaves like blank ammunition: it still fires, albeit not delivering toxic effectors [186]. When *P. aeruginosa* is preyed upon by a *V. cholerae* strain lacking the four effectors, its survival is comparable to that observed during an attack by a *V. cholerae* T6SS mutant. This observation indicates that *P. aeruginosa* did not sense an exogenous T6SS activity. Notably, it was shown that effector TseL was the key activator needed for *P. aeruginosa* H1-T6SS retaliation against *V. cholerae*. Here, it appears that *P. aeruginosa* specifically detects *V. cholerae* toxic effectors rather than the T6SS firing. *P. aeruginosa* H1-T6SS retaliates in response to *A. baylyi* effector-

less mutant but does not retaliate against a *V. cholerae* effector-less mutant. This suggests that *P. aeruginosa* adapts its H1-T6SS retaliatory behaviour depending on the species that it is attacked by.

To the contrary, a recent study has shown that *P. aeruginosa* H1-T6SS retaliation occurs irrespective of incoming effectors [247]. In this report, researchers have corroborated the earlier finding that *P. aeruginosa* H1-T6SS counterattacks against *A. baylyi* effector-deficient strain. However, unlike what was previously observed, *P. aeruginosa* H1-T6SS also retaliates against *V. cholerae* effector-deficient strain. The difference between the two studies could be attributed to the *V. cholerae* strain tested: whilst the first study used strain V52, the latter used strain 2740-80. These strains are similar, though it is unclear whether there are differences in the regulation of effector expression, or in the proportion and amount of effectors delivered into a target cell.

A process called PARA (*P. aeruginosa* response to antagonism) has been implicated as a mechanism for *P. aeruginosa* to survive bacterial antagonism and possibly thrive in multispecies communities [248]. Interestingly, this response mechanism appears to not require TPP. When all TPP components were deleted, *P. aeruginosa* H1-T6SS was activated in response to two other T6SS-wielding species. Moreover, *P. aeruginosa* cells that are lysed resulting from bacterial antagonism can activate PARA in the remainder *P. aeruginosa* cells [248]. Lysed kin cells act as a signal for the Gac/Rsm pathway, enhancing the expression of H1-T6SS genes. It is important to note that, whereas TPP is a posttranslational regulation mechanism essential for "tit-for-tat", PARA is mediated by the Gac/Rsm pathway, which regulates the expression of H1-T6SS genes. This suggests that *P. aeruginosa* has distinct and effective mechanisms to respond to bacterial antagonism.

1.6 Aims and Research questions

In this thesis we investigated the species-specific resistance of *P. aeruginosa* to exogenous T6SS attacks through the following aims and research questions:

Aim 1: *Vibrio cholerae* T6SS-effector toxicity towards *Pseudomonas aeruginosa*

Is *P. aeruginosa* selectively resistant to the *V. cholerae* effectors?

Aim 2: *Vibrio cholerae* T6SS-effector delivery into *Pseudomonas aeruginosa* cytosol

Does *V. cholerae* T6SS deliver effectors into *P. aeruginosa* cytosol?

Aim 3: *Pseudomonas aeruginosa* cell wall barriers as means to defend against exogenous T6SS attacks

Do individual *P. aeruginosa* cells have protective mechanisms against exogenous T6SS attacks?

Aim 4: *Pseudomonas aeruginosa* H1-T6SS shapes the population dynamics of a multispecies community

Can *P. aeruginosa* retaliatory H1-T6SS protect a T6SS-sensitive species from the attacks of T6SS-aggressor species?

2 *Vibrio cholerae* T6SS-effector toxicity towards *Pseudomonas aeruginosa*

2.1 Introduction

P. aeruginosa deploys its H1-T6SS apparatus in a specifically targeted retaliatory manner. This behaviour was first seen in T6SS-mediated interactions between homologous species [87]. Using time-lapse fluorescence microscopy it was possible to observe that *P. aeruginosa* increases H1-T6SS activity in response to increased T6SS activity in a sister cell. This T6SS-mediated bacterial interaction has been termed "T6SS duelling", since *P. aeruginosa* assembles and propels H1-T6SS at the exact position where it was attacked by a sister-cell. This intra-species retaliation is not fatal, given that one cell produces cognate immunity proteins to the effectors delivered by the other. Hence, *P. aeruginosa* increases H1-T6SS activity only when it is threatened rather than by its own volition. This behaviour was also observed when *P. aeruginosa* was mixed with two other Gram-negative species, *V. cholerae* and *A. baylyi* [99]. Wild-type *P. aeruginosa* does not kill T6SS-deficient strains of *V. cholerae* or *A. baylyi*. Instead, it counterattacks when these species express a functional T6SS in a phenomenon termed "tit-for-tat". *P. aeruginosa* detects the point of attack and assembles a new H1-T6SS apparatus that is able to fight back, suggesting that *P. aeruginosa* H1-T6SS is defensive instead of offensive.

It remains disputable whether *P. aeruginosa* "tit-for-tat" is a response to T6SS physical attacks or to specific T6SS-delivered effectors. A previous study investigated whether *P. aeruginosa* H1-T6SS retaliatory behaviour was due to *V. cholerae* T6SS-delivered effectors or to the physical puncture of the T6SS apparatus [186]. A *V. cholerae* mutant that had all effectors inactive but still assembled a functional T6SS did not evoke a counterattack from *P. aeruginosa* H1-T6SS. This suggests that *P. aeruginosa* recognises T6SS-delivered effectors rather than the T6SS stabbing. *P. aeruginosa* counterattacks were also attributed to a particular *V. cholerae* T6SS effector [186]. *P. aeruginosa* H1-T6SS only retaliated when *V. cholerae* effector TseL was delivered. In addition, *P. aeruginosa* viability decreased when TseL was expressed in the cell's periplasm. However, another report has shown that *P. aeruginosa* H1-T6SS retaliates regardless of incoming T6SS effectors [247]. Since each study used different

V. cholerae strains as the predator, it is unclear whether *P. aeruginosa* H1-T6SS retaliation is triggered by a specific strain or effector.

2.1.1 The Type 6 Secretion System as a weapon for bacterial competition

Competition is a natural occurrence within microbial communities, where bacteria race to obtain spatial and nutritional dominance over their neighbours [15]. In addition to other mechanisms discussed in Chapter 1, Gram-negative bacteria can use the T6SS as a means for environmental fitness and niche colonisation.

The T6SS was identified in *V. cholerae* as a mechanism for the extracellular secretion of proteins in the absence of an N-terminal secretion signal [53]. *D. discoideum* amoeba, a model eukaryotic cell, was readily killed when plated with *V. cholerae* serogroup O37 strain V52. To understand which *V. cholerae* V52 genes were responsible for virulence against *D. discoideum*, a library of *V. cholerae* colonies sensitive to amoeba predation was created and analysed. A thorough genetic analysis revealed that the VAS pathway was responsible for the secretion of proteins that lacked a secretion signal and that it also mediated amoeba and macrophage toxicity [53]. The VAS gene cluster was also found in many Gram-negative species, and the T6SS primary function was first attributed to the extracellular transport of virulence factors into eukaryotic cells.

The T6SS virulence against macrophages was ascribed to VgrG1, a T6SS protein which carries a C-terminal domain responsible for actin crosslinking in the host cell [70,171]. Two other VgrG proteins were identified (VgrG2 and VgrG3), and together with VgrG1 they form a trimeric complex analogous to the T4 phage tail spike, which can be used to puncture and export molecules into host cells [70,82].

The antimicrobial function of the T6SS was explored by employing *V. cholerae* to prey upon different Gram-negative and Gram-positive bacteria, as well as different yeast species [209]. This report showed that none of the tested Gram-positive bacteria or yeast were susceptible to T6SS-mediated killing. The lack of toxic effect against Gram-positives could be due to their membrane structure. Given that Gram-positive bacteria have a thick peptidoglycan layer, T6SS might not have been able to penetrate the peptidoglycan layer and deposit its toxic effectors inside the cell. Conversely, *V. cholerae* T6SS was highly toxic towards

other Gram-negative species, including *E. coli*, *Salmonella typhimurium* and *Citrobacter* [209]. However, recent reports have shown that certain species can also deploy their T6SSs against Gram-positive bacteria and Mycobacteria [159–161].

2.1.2 Antibacterial activity of T6SS-secreted effectors

The T6SS antibacterial activity has been largely attributed to the secretion of toxic effectors into target cells. T6SS-delivered antibacterial effectors can either kill or hinder the growth of competitors, so they are critical in interbacterial competition. The repertoire of T6SS antibacterial effectors is immense, and each effector can exert varying activities and target different parts of the recipient cell. T6SS effectors have been discussed in detail in Chapter 1.

2.1.3 *V. cholerae* antibacterial effectors

Antibacterial effectors secreted by *V. cholerae* T6SS have been widely studied for their activity and function. *V. cholerae* encodes three VgrG proteins (VgrG1-3) that have structural functions in the T6SS puncturing spike. A closer analysis of VgrG3 revealed a C-terminal peptidoglycan-binding domain, suggesting a potential antimicrobial function [70]. This potential antibacterial activity was investigated by co-culturing a *V. cholerae* VgrG3 mutant strain with *E. coli*. It has been shown that the VgrG3 mutant strain was unable to kill *E. coli* as effectively as wild-type *V. cholerae*. However, the complementary expression of VgrG3 in the mutant strain restored its ability to kill *E. coli* [180]. The VgrG3 mutant strain has also shown decreased levels of Hcp secretion (a hallmark of T6SS function), which further suggests that VgrG3 is not only essential for killing *E. coli* but also for T6SS functionality [180].

The antibacterial effect of VgrG3 is attributed to its C-terminus peptidoglycan binding domain, which is responsible for a lysozyme-like effect on target cells [103]. When VgrG3 is expressed in *E. coli*, cells acquire a spherical shape, suggesting that the cell wall has been impaired. Another study analysed the toxicity of VgrG3 towards *E. coli* by expressing the full-length protein, N-terminal core and C-terminal extension within the cell's cytosol [180]. VgrG3 was not toxic when expressed in the cytosol of *E. coli* despite reducing cell growth. However, expression of full-length VgrG3 or the C-terminal extension in the periplasm of *E.*

coli led to cell lysis. These disparate results could be because one of the studies employed optical density to assess effector toxicity, which does not evaluate cell viability or visualise effects at the cell level. The fact that VgrG3 is expressed in the cytosol of *E. coli* but still finds its way to the periplasm was attributed to a cryptic secretion signal found in a linker sequence between the protein core and its lysozyme domain [122]. This cryptic secretion pathway allows VgrG3 to be translocated from the cytosol to the periplasm, where it exerts its lytic activity.

VasX was identified as an antibacterial effector that interacts with membrane lipids [169]. VasX is regulated by VasH, it is not required for T6SS assembly, but it requires Hcp and VgrG2 to be secreted. Unlike Hcp, which localises to all cellular compartments, VasX can specifically be found in the cytoplasm and membrane fractions. VasX was described as having an N-terminal PH domain, a feature usually associated with signal transduction in eukaryotic cells, which effector might use to mimic host cell proteins and target host cell membranes. Bioinformatic analysis revealed that VasX has weak structural homology to colicins [171]. Colicins are proteins produced by specific strains of *E. coli* that are toxic to closely related strains of *E. coli* and can act in many deleterious ways towards the target cell, including DNA and RNA degradation and pore formation [29]. Therefore, it was suggested that VasX interacts with the target membrane lipids in a colicin-like manner. In order to be toxic, pore-forming colicins must be presented to the inner membrane from the periplasmic space to allow ion leakage and membrane disruption. The fact that VasX is only toxic in the periplasm of a producing cell lacking its cognate immunity protein could support this finding [170].

It has been previously shown that VasX can target *D. discoideum* amoeba and interact with membrane lipids [169]. A prior study has shown that VasX is insufficient for killing *E. coli*, perhaps due to a compensatory effect of the two other T6SS-secreted effectors (TseL and VgrG3). However, when *V. cholerae* lacked TseL and VgrG3, VasX was capable of killing *E. coli*, though not to the same extent as its wild-type counterpart [170]. It was initially suggested that VasX was loaded into the Hcp tube in order to be extracellularly secreted and deposited within the target cell [169]. However, a different mechanism for protein translocation via the T6SS has been proposed: effectors are not loaded into the Hcp tube, but instead bind to cognate VgrG orthologs or heterodimers of these proteins [70,103].

TseL is another *V. cholerae* T6SS-delivered effector, named after its lipase domain [103]. Inactivating a residue in the lipase domain of TseL prevented the effector from restoring the

antibacterial effect of a TseL mutant strain. Therefore, the lipase domain has been credited for the effector antibacterial ability. However, the TseL mutant strain was able to kill *E. coli* as efficiently as wild-type *V. cholerae*, suggesting that TseL is not a crucial effector for *V. cholerae* T6SS antibacterial toxicity [103,171].

It has been previously shown that *V. cholerae* single effector mutants are as toxic to *E. coli* as wild-type *V. cholerae* [103]. However, the simultaneous deletion of all three effectors decreases *V. cholerae* antibacterial activity against *E. coli*. These observations suggest that no single effector is responsible for *V. cholerae* T6SS-mediated antibacterial activity. Instead, a combination or "cocktail" of effectors is required for efficient antibacterial activity.

Previous studies have investigate how *P. aeruginosa* responds to *V. cholerae* T6SS attacks and its delivered effectors, with the caveat that *P. aeruginosa* with a functional H1-T6SS is expected to retaliate. We thus questioned whether *P. aeruginosa* with an inactive H1-T6SS is more sensitive to *V. cholerae* T6SS attacks. Here, we demonstrated that *V. cholerae* T6SS has a mild effect on the survival of H1-T6SS-deficient *P. aeruginosa*. To the contrary, H1-T6SS-deficient *P. aeruginosa* is significantly affected by *A. baylyi* T6SS, suggesting that *P. aeruginosa* is not inherently resistant to all exogenous T6SS attacks. Additionally, since each species secretes a unique set of effectors and the T6SS antibacterial activity has been largely attributed to effectors, we investigated whether *P. aeruginosa* is selectively resistant to *V. cholerae* effectors.

2.2 Results

2.2.1 *P. aeruginosa* is mildly affected by *V. cholerae* T6SS

V. cholerae strains encode accessory virulence factors that could be important for environmental fitness and disease transmission, such as HlyA hemolysin, HapA hemagglutinin/protease and RtxA toxin [54]. A T6SS active *V. cholerae* strain V52 lacking these three accessory toxins (*rhh* mutant) is highly virulent towards *Dictyostelium* amoeba and many Gram-negative species [53]. This indicates that *V. cholerae* toxicity towards eukaryotes and prokaryotes is T6SS-dependent [209]. However, *V. cholerae* V52 *rhh* is not toxic towards *P. aeruginosa*, which is primarily attributed to the ability of *P. aeruginosa* to counterattack by deploying H1-T6SS in a "tit-for-tat" manner [99]. To inactivate H1-T6SS in *P. aeruginosa* we deleted *tssB1* (PA0083), a homolog for *vipA* [99]. Since all assays in this chapter were conducted with H1-T6SS-deficient *P. aeruginosa* (*ΔtssB1*), it will henceforth be referred simply as *P. aeruginosa*.

The effects of the T6SS on *P. aeruginosa* were determined by assessing the survival of prey *P. aeruginosa* after a competition with predator *V. cholerae*. We observed that the survival of *P. aeruginosa* was only mildly affected by *V. cholerae* T6SS (Figure 2.1 A). We then questioned whether *P. aeruginosa* was resistant to all heterologous T6SS attacks. To test this we used *A. baylyi* as the predator strain in the competition assay. We observed that *A. baylyi* T6SS can significantly reduce the survival of *P. aeruginosa* by more than 100-fold (Figure 2.1 B). Whilst there is a statistically significant difference between *P. aeruginosa* recovery when preyed upon by wild-type (WT) compared to T6SS-deficient *V. cholerae*, the effect is minor especially when compared to *A. baylyi* T6SS killing effect. These observations suggest that *P. aeruginosa* is specifically more resistant to *V. cholerae* T6SS attacks.

2.2.2 *P. aeruginosa* resistance to T6SS attacks is independent of *V. cholerae* strain

Under laboratory conditions, *V. cholerae* serogroup O37 strain V52 has a constitutively active T6SS and exhibits toxicity in a T6SS-dependent manner [53,209]. To investigate whether *P. aeruginosa* resistance to T6SS attacks depended on the predator *V. cholerae* strain we have

employed environmental *V. cholerae* O1 El Tor strain 2740-80 in our competition assays against *P. aeruginosa*. Much like *V. cholerae* strain V52, the T6SS of strain 2740-80 has a mild effect on the survival of *P. aeruginosa* (Figure 2.1 C). This indicates that *P. aeruginosa* ability to resist *V. cholerae* T6SS attacks is independent of a specific strain.

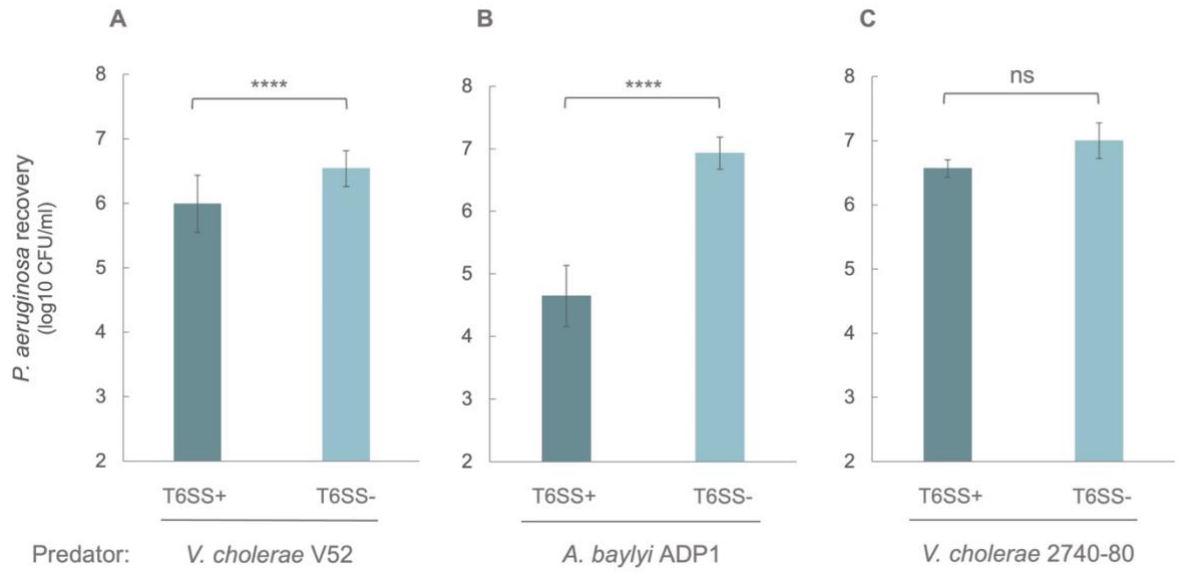

Figure 2.1 *P. aeruginosa* with an inactive H1-T6SS is more resistant to T6SS attacks from *V. cholerae* than from *A. baylyi*. Data represent the recovery of *P. aeruginosa ΔtssB1* following a pairwise competition assay (10:1 predator to prey ratio). Predators were either T6SS+ or T6SS- (A) *V. cholerae* V52, (B) *A. baylyi* ADP1, and (C) *V. cholerae* 2740-80. Data are presented as mean $\log_{10}$CFU/ml ± SD of at least three experiments with two technical replicates. Statistical significance was determined using a two-tail unpaired student's t-test to compare prey recovery (**** $p \leq 0.0001$).

2.2.3 *V. cholerae* effectors VgrG3, VasX and TseL are not toxic when expressed in the cytosol of *P. aeruginosa*

V. cholerae has a unique set of T6SS-delivered effectors that can be toxic when delivered into other Gram-negative species [103,171,180]. As such, we considered whether *P. aeruginosa* is selectively resistant to *V. cholerae* T6SS-delivered effectors. To test this we have cloned *V. cholerae* effector genes *vgrG3*, *vasX* and *tseL* into pPSV37 and induced effector expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG). Upon endogenous gene expression or repression, we observed that recovered *P. aeruginosa* colonies exhibited phenotypical differences amongst experimental replicates (colony size, colour and growth rates). Sanger sequencing analysis of individual colonies revealed genetic mutations within

the effector genes. These observations suggest that phenotypical inconsistencies could be due to leaky plasmid expression rather than effector toxicity. An insufficient gene repression could have led to expression of toxic effectors in the cytosol of *P. aeruginosa*, causing pressure for species adaptation.

To address this issue, we cloned each effector gene into a newly engineered arabinose-inducible vector, which we have named pPGA (after *Pseudomonas* Gentamicin Arabinose-inducible plasmid) (Figure S 2.1). This novel expression vector allows tightly controlled gene expression, unlike IPTG inducible vectors. To avoid the occurrence of resistance mutations against any of the toxic effectors, all experiments were performed in the presence of glucose to repress gene expression. Additionally, gene expression assays were performed immediately after obtaining transconjugant *P. aeruginosa* containing each effector to minimise passaging (Figure S 2.2 illustrates a detailed diagram of experimental procedures). Moreover, we cloned a non-toxic fluorescent protein (mCherry2) into pPGA to certify that the vector was non-toxic towards *P. aeruginosa* and that it was suitable for gene expression (Figure S 2.3A).

When each of the three effector genes was individually expressed in the cytosol of *P. aeruginosa*, cell recovery was similar to when gene expression was repressed (Figure 2.2). This suggests that *V. cholerae* effectors VgrG3, VasX and TseL are not toxic when expressed in the cytosol of *P. aeruginosa*.

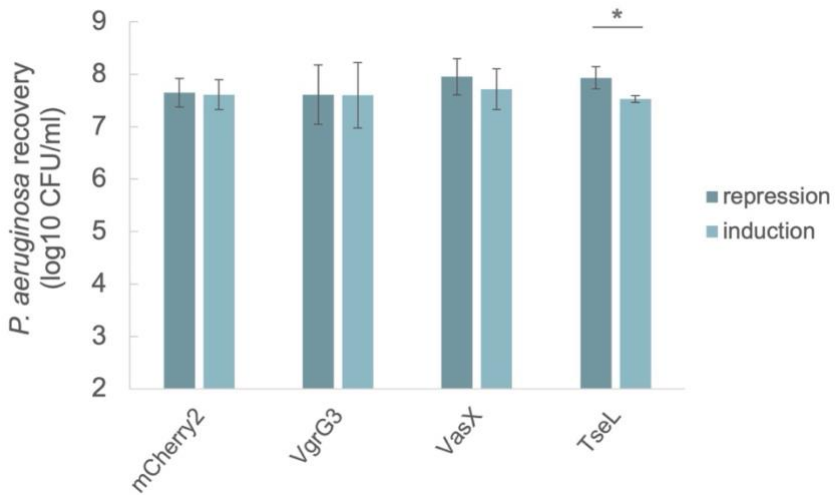

Figure 2.2 *V. cholerae* effectors are not toxic when expressed in the cytosol of *P. aeruginosa*. Data represent the recovery of *P. aeruginosa ΔtssB1* following the cytosolic expression of each *V. cholerae* T6SS-effectors (Vgrg3, VasX and TseL). Each effector was expressed under the newly engineered arabinose-inducible plasmid pPGA. Gene expression was induced with the addition of 0.2% arabinose or repressed with 0.2% glucose, after which colonies were recovered and enumerated. Data are presented as mean $\log_{10}$CFU/ml ± SD of at least three experiments with two technical replicates. Student's t test was performed to compare *P. aeruginosa ΔtssB1* recovery after induction or repression of each effector (* $p \leq 0.05$).

2.2.4 *V. cholerae* effectors VasX and TseL are toxic when expressed in the periplasm of *P. aeruginosa*

Each of the three *V. cholerae* effectors targets different components of prey bacterial cells. TseL and VasX disrupt membrane integrity whilst VgrG3 degrades peptidoglycan in the periplasm [103,122,169]. *V. cholerae* effectors might not be toxic *to P. aeruginosa* because they are not being localised to the cellular compartment where they would be enzymatically active. Therefore, we investigated whether *V. cholerae* effectors were toxic when expressed in the periplasm of *P. aeruginosa*. In order to localise *V. cholerae* effectors to the periplasm of *P. aeruginosa* we fused a Sec-secretion signal (MKKIWLALAGLVLAFSASA|AQYED) from *E. coli* DsbA to the N-terminus of each effector protein using a flexible linker (LEGPAG) (Figure S 2.4).

To test whether vector expression in the periplasm was toxic to *P. aeruginosa* we also expressed a non-toxic fluorescent protein (sfGFP) as a control. Unlike other fluorescent proteins, the super folding variant of GFP can be used for subcellular localisation studies. Previous reports have shown that sfGFP can be efficiently translocated into the periplasm and emit fluorescence predominantly through a cotranslational pathway [249,250]. Using fluorescence microscopy, we observed that *P. aeruginosa* expressing sec-sfGFP emitted bright green fluorescence around the rim of the cells, indicating periplasmic localisation of sfGFP (Figure S 2.3B). However, periplasmic expression of sfGFP led to a small reduction in cell viability, which could be due to the cost of over-expressing a protein (Figure 2.3).

Periplasmic expression of effectors TseL and VasX led to a reduction of more than 100-fold in *P. aeruginosa* cell viability (Figure 2.3). Notably, despite many attempts, we were unable to clone a sec-secretion signal to the N-terminus of VgrG3. Prior reports showed that VgrG3 is toxic when expressed in *E. coli* [103]. As such, a likely explanation is that the effector is extremely toxic, which could not be counteracted by gene repression throughout all cloning steps. Collectively, our results suggest that *V. cholerae* effectors are toxic when localised to the periplasm of *P. aeruginosa*. Therefore, *P. aeruginosa* resistance may be due to the inability of *V. cholerae* T6SS to properly deliver toxic effectors into the correct subcellular compartment of *P. aeruginosa*.

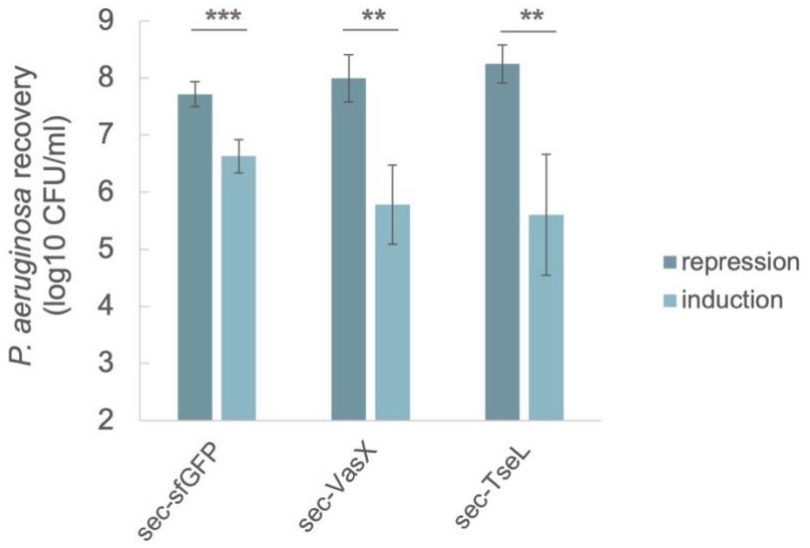

Figure 2.3 *V. cholerae* effectors are toxic when expressed into the periplasm of *P. aeruginosa*. Data represent the recovery of *P. aeruginosa ΔtssB1* following the periplasmic expression of each *V. cholerae* T6SS-effectors (VasX and TseL). Each effector was fused to the Sec-secretion signal (sec) of *E. coli* DsbA and expressed under the newly engineered arabinose-inducible plasmid pPGA. Gene expression was induced with the addition of 02.% arabinose or repressed with 0.2% glucose, after which colonies were recovered and enumerated. Data are presented as mean $\log_{10}$CFU/ml ± SD of at least three experiments with two technical replicates. Student's t test was performed to compare the recovery of *P. aeruginosa ΔtssB1* after induction or repression of each effector (** $p \leq 0.01$, *** $p \leq 0.001$).

2.2.5 Abrogating single *V. cholerae* effectors does not increase *P. aeruginosa* susceptibility to *V. cholerae* T6SS attacks

It has been previously reported that *V. cholerae* effector TseL was responsible for triggering *P. aeruginosa* H1-T6SS retaliation [186]. As such, we questioned whether a specific *V. cholerae* effector was responsible for *P. aeruginosa* resistance to *V. cholerae* T6SS attacks. To test this we determined the survival of *P. aeruginosa* after being preyed upon by either *V. cholerae* WT or single effector mutant strains. We observed that none of *V. cholerae* single effector mutant strains further reduced the survival of *P. aeruginosa* (Figure 2.4). This result suggests that *P. aeruginosa* resistance is independent of which effectors are being delivered by *V. cholerae* T6SS.

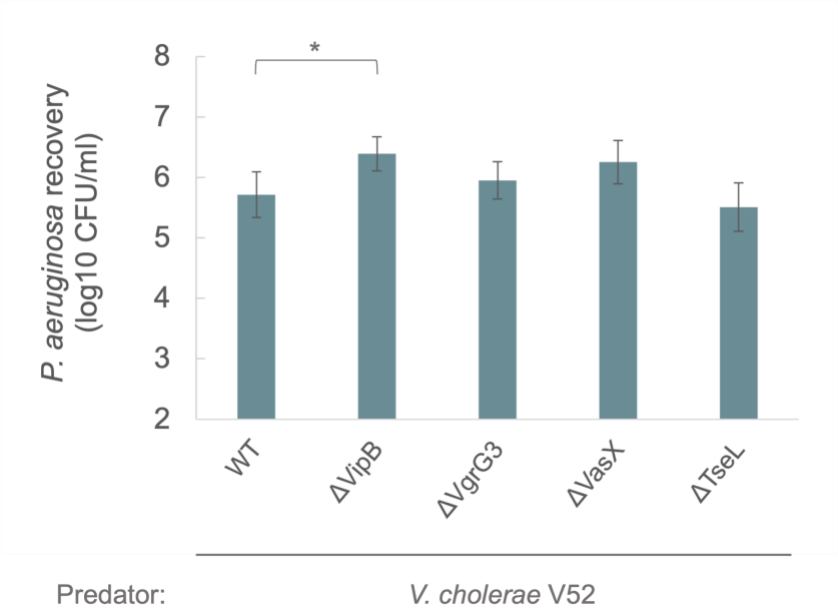

Figure 2.4. Abrogating single *V. cholerae* effectors does not increase *P. aeruginosa* susceptibility. Data represent the recovery of *P. aeruginosa ΔtssB1* following a competition assay with predator *V. cholerae* V52 (10:1 predator to prey ratio). Predators were *V. cholerae* WT, T6SS-deficient mutant (ΔVipB) and single effector mutant strains (ΔVgrG3, ΔVasX and ΔTseL). Data are presented as mean $\log_{10}$CFU/ml ± SD of at least three experiments with two technical replicates. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test to compare the recovery of *P. aeruginosa* after being preyed by *V. cholerae* mutant strains to the WT (* $p \leq 0.05$).

2.3 Discussion

Despite killing many Gram-negative species in a T6SS-dependent manner, *V. cholerae* is unable to effectively kill *P. aeruginosa* [209]. *P. aeruginosa* has an efficient retaliatory T6SS (H1-T6SS) that assembles and attacks when it detects T6SS activity in a neighbouring cell [87]. This suggests that a signal is being transferred between cells at the precise location where an aggressor's T6SS apparatus is being assembled. This retaliatory behaviour has been previously attributed to cell envelope alterations due to the physical puncturing of the T6SS. *P. aeruginosa* might detect exogenous T6SS components in its vicinity which may induce a protective response that results in *P. aeruginosa* assembling H1-T6SS to counterattack [87]. Whilst *V. cholerae* T6SS assembles and strikes indiscriminately [87], *P. aeruginosa* specifically directs its H1-T6SS attacks to prey cells with a functional T6SS [99]. *P. aeruginosa* entirely disregards neighbouring cells that are unable to assemble a T6SS apparatus and only attacks if it feels threatened. A previous study showed that neither *V. cholerae* nor *A. baylyi* T6SSs were able to kill a T6SS-deficient *P. aeruginosa* [99]. Therefore, we were motivated to further investigate the resistance of *P. aeruginosa* to exogenous T6SS attacks by *V. cholerae* and *A. baylyi*.

In previous studies, bacterial competition assays were performed at a low predator-to-prey ratio of 1:1 [99]. At this competitive ratio, prey cells might not be able to directly contact their aggressors sufficiently for a killing effect to be observed. Therefore, we increased predator cells by 10-fold, amplifying the amount of aggressors surrounding prey and increasing the probability of T6SS puncturing. Despite higher numbers of predator cells, we observed that *P. aeruginosa* was only mildly affected by *V. cholerae* T6SS (Figure 2.1 A), though highly susceptible to *A. baylyi* T6SS attacks (Figure 2.1 B). These results suggest that *P. aeruginosa* is not resistant to all heterologous T6SS attacks but rather specifically more resistant to *V. cholerae* T6SS attacks.

To investigate whether *P. aeruginosa* resistance to *V. cholerae* T6SS was specific to strain V52 serogroup O37, we further determined the survival of *P. aeruginosa* to environmental O1 El tor *V. cholerae* strain 2740-80. Both *V. cholerae* strains exerted comparable low toxicity against *P. aeruginosa* (Figure 2.1 A and C), indicating that *P. aeruginosa* resistance to *V. cholerae* T6SS is not strain-specific.

It has been previously shown that VgrG3 is highly toxic when expressed in the cytosol of *E. coli* [122]. The entirety of this mechanism is unknown, but a determinant VgrG3 protein sequence has been implicated in this cryptic secretion pathway. VgrG3, though not having a Sec or Tat secretion signal, can still be translocated from the cytosol of *E. coli* into its periplasm. This might explain why, despite many efforts, we were unable to clone VgrG3 with an N-terminal Sec-secretion signal into an *E. coli* cloning strain. The repression of VgrG3 expression in the cytosol *E. coli* was sufficient to prevent cell death and obtain a stable construct. However, VgrG3 is likely highly toxic if localised to the periplasm of *E. coli*, even when gene expression is tightly repressed.

P. aeruginosa survival to periplasmic expression of VasX and TseL has been previously tested by cloning a Tat secretion signal to the N-terminus of each effector. In that study, authors have reported that *P. aeruginosa* viability was affected by the periplasmic expression of TseL but not that of VasX [186]. Herein, we observed a significant reduction in *P. aeruginosa* viability when both TseL and Vasx were expressed in the periplasm (Figure 2.3). These disparate results might be attributed to differences between cloning vectors. Whilst effector genes were cloned in an IPTG-inducible vector previously, herein we cloned effectors into a new engineered arabinose-inducible vector with tight regulation. IPTG-inducible vectors can have leaky gene expression since they lack tight regulation. On the other hand, gene repression under arabinose-inducible vectors can be tightly controlled by the addition of D-glucose. Furthermore, whereas Kamal and colleagues have used the Tat secretion pathway to translocate effectors to the periplasm, we relied on the Sec secretion pathway. The Tat pathway requires proteins to be fully folded for translocation whereas the Sec pathway translocates unfolded proteins. VasX is considered to interact with the lipids of the target membrane in a colicin-like manner. Pore-forming colicins are usually unfolded in the periplasm and are presented to the inner membrane of the periplasmic space to exert their activity [29]. Given its homology to colicins, it is possible that VasX requires translocation in an unfolded state by the Sec pathway. Here, we showed that Sec-mediated expression of VasX was toxic to *P. aeruginosa*, though a similar observation has been reported previously in *V. cholerae*. The Sec-mediated periplasmic expression of VasX was toxic to the producing *V. cholerae* in the absence of cognate immunity protein, suggesting that VasX toxicity in the periplasm is dependent of Sec translocation [170].

Since *V. cholerae* effectors are not toxic when expressed in the cytosol of *P. aeruginosa* but highly toxic when localised to the periplasm, it is possible that *P. aeruginosa* has evolved resistance mechanisms that prevent effector toxicity in the cytosol or their translocation to the periplasm via an unknown mechanism. Confirming that the reduction of *P. aeruginosa* viability was due to the expression of *V. cholerae* effectors in the periplasm would require the co-expression of the effector along with the immunity protein. If toxicity of the effector was alleviated by the co-expression of the immunity protein compared to effector alone, this would suggest that *P. aeruginosa* viability was reduced due to the effector expression. Importantly, plasmid-based expression of effectors is dissimilar to direct T6SS-delivery of effectors. Plasmid expression allows a higher number of effector proteins within the target cell than the one achieved by a direct T6SS-mediated delivery. It is possible that *V. cholerae* T6SS is unable to deliver a sufficiently toxic load of effectors into *P. aeruginosa* in order for a sufficiently high killing effect to be observed.

V. cholerae effector TseL has previously shown to be a key player for triggering *P. aeruginosa* H1-T6SS retaliation [186]. In a competition assay between *P. aeruginosa* and multiple *V. cholerae* effector mutant strains, *P. aeruginosa* H1-T6SS readily retaliates and kills all *V. cholerae* mutants that specifically delivered TseL. Any *V. cholerae* mutants lacking TseL showed a comparable recovery to a T6SS-deficient *V. cholerae*. Given that a specific *V. cholerae* effector can trigger *P. aeruginosa* H1-T6SS counterattacks, a single effector may also elicit *P. aeruginosa* resistance to *V. cholerae* T6SS. Furthermore, *V. cholerae* T6SS effectors could compete for delivery into prey cells, hence inactivating a single effector could increase *V. cholerae* T6SS toxic effect. However, we observed that *P. aeruginosa* was not more susceptible after being preyed upon by single *V. cholerae* effector mutants compared to WT. These results suggest that specific *V. cholerae* effectors are not responsible for *P. aeruginosa* resistance to T6SS attacks, and that *P. aeruginosa* resistance is not due to effector competition for delivery.

In conclusion, our results show that a T6SS-deficient *P. aeruginosa* is more resistant to *V. cholerae* than to *A. baylyi* T6SS attacks. Additionally, *V. cholerae* T6SS effectors need to be localised to the proper cellular compartment in *P. aeruginosa* to exert antibacterial effect.

Table 2.1. Bacterial strains and plasmids used in this chapter.

Strain or plasmid	Relevant characteristics	Source
Vibrio cholerae		
V52	Parental strain, *rtxA hlyA hapA* (Δrhh), Str^R	[53]
V52 Δ*vipB*	In frame deletion of amino acid 12 to 486 of *vipB* (VCA0108)	[171]
V52 Δ*vgrG3*	In frame deletion of *vgrG3* (VCA0123)	[70]
V52 Δ*vasX*	In frame deletion of amino acid 3 to 1077 of *vasX* (VCA0020)	[171]
V52 Δ*tseL*	In frame deletion of amino acid 2 to 626 of *tseL* (VC1418)	[171]
2740-80	Parental strain, clpV::clpV-mCherry, $lacZ^-$, Str^R	[87]
2740-80 Δ*vipA*	In frame deletion of *vipA* (VCA0107)	[87]
***Acinetobacter baylyi* ADP1**		
WT	Wild type, Str^R (ATCC 33305)	[99]
T6SS-	Genes *aciad2688* to *aciad2694* replaced with Kan^R cassette	[99]
Pseudomonas aeruginosa		
PAO1	Wild type, Irg^R	Lab collection
PAO1 *ΔtssB1*	In frame deletion of amino acid 11 to 164 of *tssB1* (PA0083)	This study
Escherichia coli		
NEB® 10-beta	$DH10B^{TM}$ derivative, *recA1 relA1 endA1 rpsL* (Str^R)	New England Biolabs
SM10 λpir	*thi thr leu tonA lacy supE recA-RP4-2-Tc-Mu pir*	[251]
Plasmids		
pBAD33	Expression vector, p15A origin, araC, araBAD promoter, Cm^R	[252]
pPSV37	Expression vector, colE1 origin, PA origin, Gm^R	[253]
pPGA	Expression vector, PA origin, araC, araBAD promoter, Gm^R	This study
pEXG2	Allelic exchange vector, colE1 origin, oriT, *sacB,* Gm^R	[254]
pEXG2-tssB1	Suicide vector for *tssB1* deletion in *P. aeruginosa*	This study
pPGA-VgrG3	Expression vector with *vgrG3* (VCA0123)	This study
pPGA-VasX	Expression vector with *vasX* (VCA0020)	This study
pPGA-TseL	Expression vector with *tseL* (VC1418)	This study
pPGA-mCherry2	Expression vector with mCherry2	This study
pBAD33-sec-sfGFP	Expression vector with sec-secretion signal from *E. coli* DsbA and sfGFP	Lab collection
pPGA-sec-sfGFP	Expression vector with sec-secretion signal from *E. coli* DsbA and sfGFP	This study
pPGA-sec-TseL	Expression vector with sec-secretion signal fused to *V. cholerae* effector TseL	This study
pPGA-sec-VasX	Expression vector with sec-secretion signal fused to *V. cholerae* effector VasX	This study

Table 2.2. Oligonucleotides used in this chapter.

Amplicon	ID	Oligonucleotide sequence (5' to 3')
Primers to amplify *V. cholerae* effector genes		
vgrG3 (VCA0123)	oRR005_KpnI-VCA0123_F	ATAGGTACCAGGAGGAATTCACCATGGCAAGGTTACAGTTTCAATTAAAG
	oRR006_VCA0123-PstI_R	ATACTGCAGTTATTTTATATCAACCTCCAAACCGTCA
vasX (VC0020)	oRR001_KpnI-VCA0020_F	ATAGGTACCAGGAGGAATTCACCATGAGTAATCCCAATCAAGCTGC
	oRR002_VCA0020-HindIII_R	CGGAAGCTTAACCTTTTCCTACAACGAGATTTCT
tseL (VC1418)	oRR009_KpnI-VC1418_F	CAGGGTACCAGGAGGAATTCACCATGGATTCATTTAATTATTGCGTGCAG
	oRR010_VC1418-PstI_R	CATCTGCAGCTTATTTGCACCTTGATTTCATCTGGTA
Primers to amplify secretion signal from *E. coli* DsbA		
DsbA sec signal	oRR022_dsbA_sfGFP_F	AAAGGTACCAGGAGGAATTCACCATGAAAAAGATTTGGCTGGCG
	oRR023_dsbA_sfGFP_R	TTTCTGCAGAAGCTTATTTGTAGAGTTCATCCATGCCG
sec-vgrG3	oRR026_XhoI_VCA0123_F	ATACTCGAGGGTCCGGCTGGTCTGGCAAGGTTACAGTTTCAATTAAAGGTG
	oRR027_VCA0123_PstI_R	TTTCTGCAGTCATTTTATATCAACCTCCAAACCGTC
sec-vasX	oRR024_XhoI_VCA0020_F	ATACTCGAGGGTCCGGCTGGTCTGAGTAATCCCAATCAAGCTGC
	oRR025_VCA0020_HindIII_R	CGGAAGCTTAACCTTTTCCTACAACGAGA
sec-tseL	oRR028_XhoI_VC1418_F	ATACTCGAGGGTCCGGCTGGTCTGGATTCATTTAATTATTGCGTGCAGTGTAAC
	oRR029_VC1418_PstI_R	TTTCTGCAGTCATCTTATTTGCACCTTGATTTCATCT
Primers to generate *P. aeruginosa* PAO1 *tssB1* mutant (*ΔtssB1*)		
tssB1 deletion	oRR040_LF-TssB1_F_1	AAAGGTACCGTACTGGGACGGCGTCTATC
	oRR041_LF-TssB1_R_2	AGGCTCGTCACTGCTGGTAGTGCTTCCCAT
	oRR042_RF-TssB1_F_3	ACCAGCAGTGACGAGCCTCAGGCGTAA
	oRR043_RF-TssB1_R_4	CATAAGCTTGAAGGAGCGGTTGATGTTGA
Primers to create pPGA vector		
P. aeruginosa origin of replication + Gm^R	oRR074_NheI-PAori.Gm_F	CATGCTAGCACGCGTAATTCTCGAATTGACA
	oRR075_PAori.Gm-BspDI_R	CATATCGATGTGCGTTTTTTGCGTTTCCAC
araC	oRR076_BspDI-araC_F	CTTATCGATGCATAATGTGCCTGTC
	oRR077_araC-SpeI_R	CATACTAGTCAACAGATAAAACGAAAGGCCCA

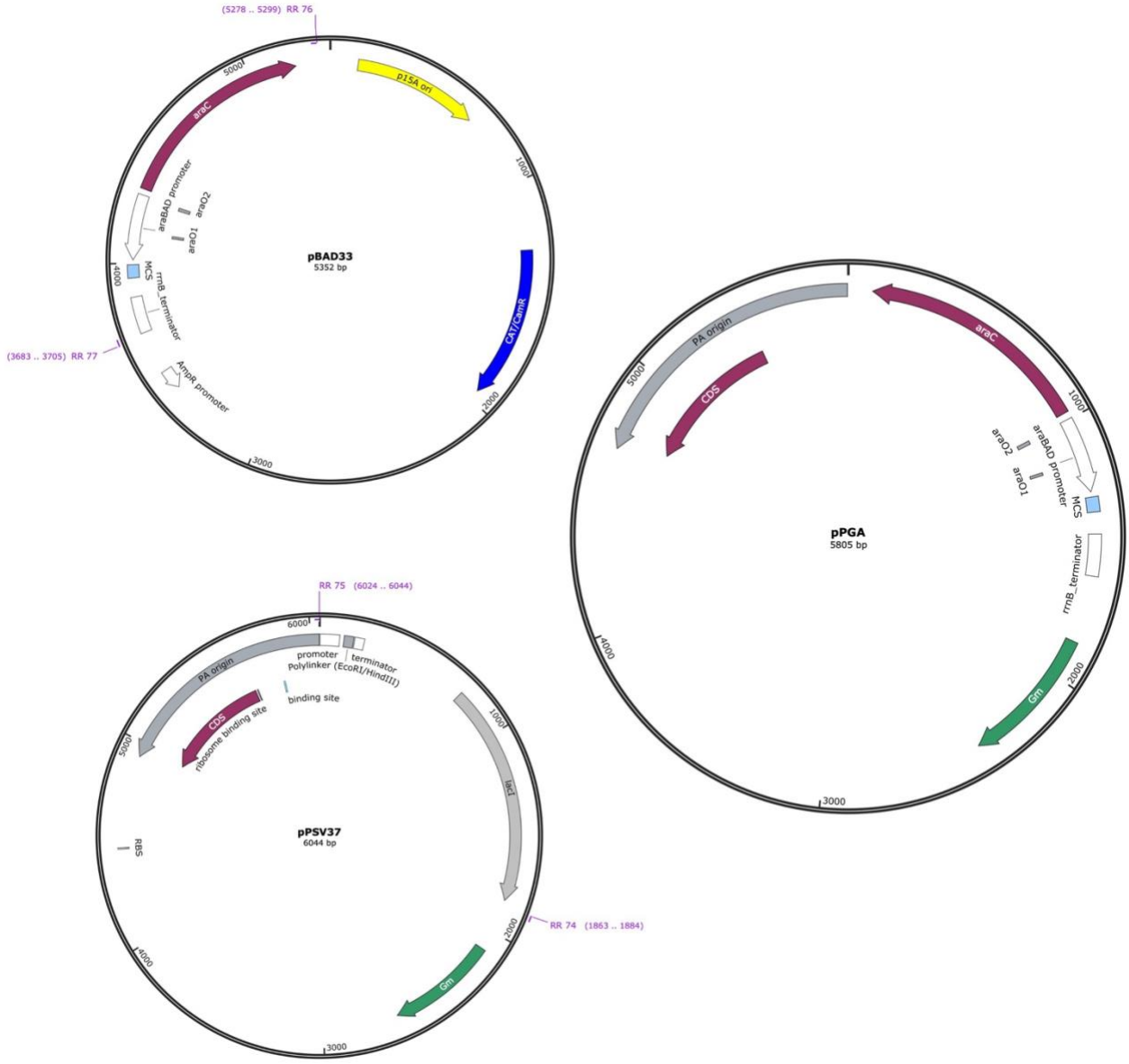

Figure S 2.1 Design and assembly of pPGA vector (*Pseudomonas* Gentamicin Arabinose-inducible plasmid). pPGA was constructed by cloning the L-arabinose regulatory gene *araC* of pBAD33 with the *P. aeruginosa* origin of replication and gentamicin resistance gene (Gm^R) of pPSV37.

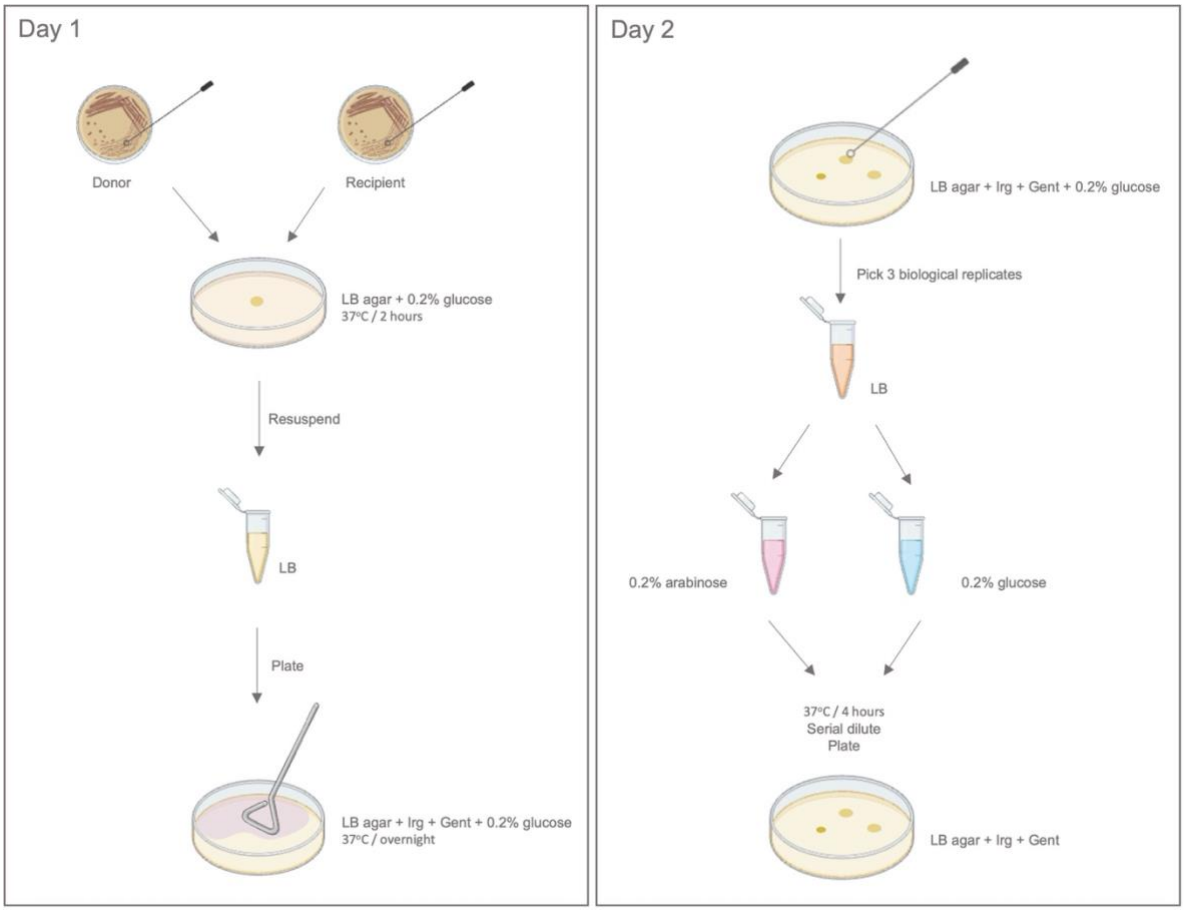

Figure S 2.2. Illustrated diagram of *V. cholerae* pPGA-based effector expression in *P. aeruginosa*. *E. coli* donors containing the conjugative plasmid pPGA with each *V. cholerae* effector are swirled together with recipient *P. aeruginosa ΔtssB1* onto pre-warmed LB agar + 0.2% glucose and incubated at 37°C for 2 hours. Following incubation, cells are scraped from the agar surface, resuspended in 1 ml LB and plated onto LB + 0.2% glucose and antibiotics to select for *P. aeruginosa* cells that have received pPGA. Plates are incubated at 37°C overnight, following which 3 colonies are individually picked into 1 ml of LB, resuspended by vortexing and the bacterial suspension is equally divided into two tubes. One of the tubes receives 0.2% glucose whilst the other receives 0.2% glucose. Both tubes are incubated at 37°C for 4 hours, after which suspensions are serially diluted and plated in LB agar with antibiotics to select for *P. aeruginosa* cells with pPGA. Details of this experimental procedure can be found in the Materials and Methods Chapter (Chapter 7).

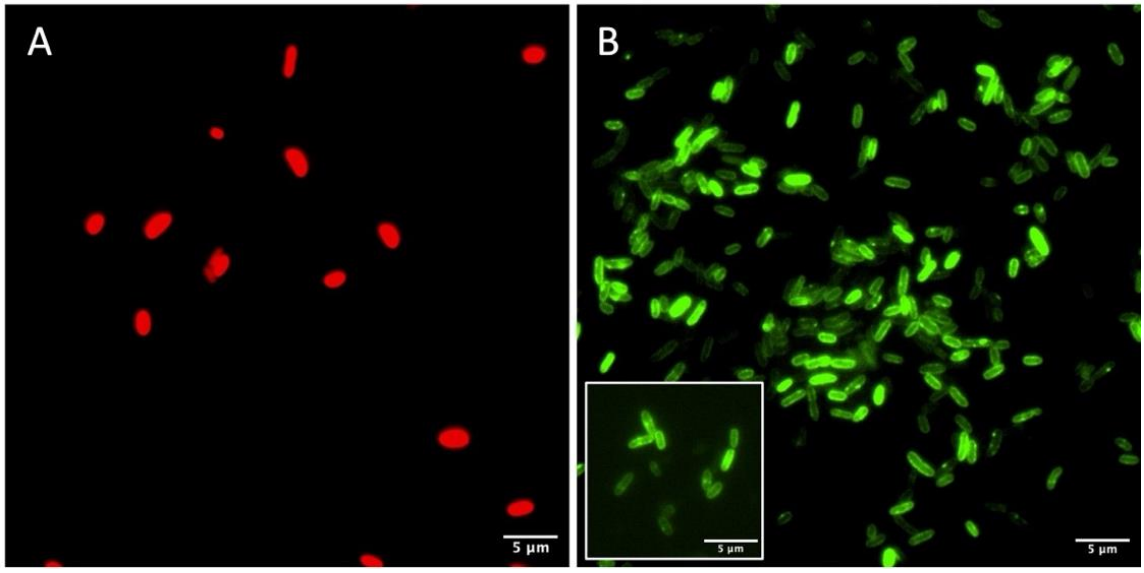

Figure S 2.3 Expression of (A) mCherry2 and (B) sfGFP fused to the sec-secretion signal of *E. coli* DsbA under the expression of newly engineered pPGA vector.

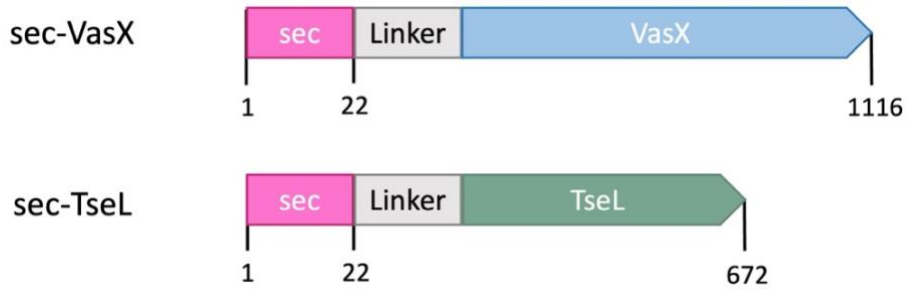

Figure S 2.4. Depiction of the fusion of the Sec-secretion signal of *E. coli* DsbA (MKKIWLALAGLVLAFSASA|AQYED) to *V. cholerae* effectors VasX (blue) and TseL (green) by a flexible linker (LEGPAG) (grey). Picture is not to scale.

3 *Vibrio cholerae* T6SS-effector delivery into *Pseudomonas aeruginosa* cytosol

3.1 Introduction

In Chapter 2 we investigated whether *P. aeruginosa* was specifically resistant to *V. cholerae* T6SS effectors. Our results indicate that *V. cholerae* effectors need to be properly localised to the periplasm of *P. aeruginosa* to exert toxicity. Given that *P. aeruginosa* is resistant to direct *V. cholerae* T6SS attacks but not to specific T6SS effectors, we hypothesised that either *V. cholerae* T6SS is not breaching *P. aeruginosa* outer membrane or *V. cholerae* effectors are delivered into the cytosol of *P. aeruginosa* but are unable to be translocated to the periplasm.

Previous work has shown that *P. aeruginosa* H1-T6SS is able to breach the outer membrane and deliver bacteriolytic effectors into the periplasm of sister cells [135]. In acompetition assay, *P. aeruginosa* lacking the Tse3 effector-immunity pair was susceptible to being killed by the wild-type strain. Considering that Tse3 immunity protein is found in the periplasm as an outer membrane lipoprotein, this observation suggests that *P. aeruginosa* H1-T6SS directly injects effector Tse3 into the periplasm of sister cells. Furthermore, when the outer membrane of *P. aeruginosa* was permeabilised, cells became more susceptible to the lytic effects of effector Tse1, suggesting that this effector targets the periplasm [135]. In *V. cholerae*, effectors and structural components can be shared and reused amongst sister cells, implying that both tube and spike complexes can directly reach the cytosol [123]. When recipient *V. cholerae* cells deficient in tube-spike assembly proteins (VgrG and Hcp) were mixed with donor sister cells with a functional T6SS, the recipients were able to restore tube-spike assembly. This effect depended on a functional T6SS delivery from donor cells. Moreover, recipient cells incubated with culture supernatant containing tube-spike components were unable to directly uptake them and reassemble their own T6SS [123]. This suggests that recipient cells reuse tube-spike components upon direct T6SS-delivery. However, the efficiency of this process depends on the amount of components delivered by donor cells and their ability to precisely aim the T6SS at recipient cells.

While these reports shed light on how bacteria deliver effectors into sister cells, it is still unclear where effectors are delivered into non-sister cells: whether they are directly delivered into the cytosol or instead into the periplasm. Previous studies have addressed this question by analysing in which cellular compartment toxic effectors are delivered. A previous report has shown that *V. cholerae* effector VgrG3 can be translocated from the cytosol of the target cell into its periplasm [103]. When *V. cholerae* effector VgrG3 was expressed in the cytosol of *E. coli*, cells rounded up and died. VgrG3 has a lysozyme domain that targets peptidoglycan, so it was proposed that VgrG3 could traffic from the cytosol of *E. coli* into its periplasm to exert toxicity. It was later confirmed that VgrG3 has a cryptic internal periplasmic signal that allows effector translocation into the periplasm [122]. After a series of VgrG3 truncation constructs were endogenously expressed in *E. coli*, a domain was identified as being critical for effector translocation and toxicity. Interestingly, another study has shown an opposing way of effector translocation in *P. aeruginosa* [116]. *P. aeruginosa* effector Tse6 acts in the recipient cell's cytosol and appears to be delivered first into the periplasm before being translocated across the inner membrane to then exert its toxicity in the cytosol. There is still ambiguity about which cellular compartment effectors are delivered in the recipient cell. However, this process does not appear be universal and instead depends on the organism.

3.1.1 T6SS-mediated heterologous protein delivery

The delivery of heterologous proteins into target cells through the T6SS has been accomplished before, in both *V. cholerae* and *P. aeruginosa*. In *V. cholerae*, a beta-lactamase enzyme (Bla) can be delivered in a T6SS-dependent manner by replacing the ACD of VgrG1 [54]. VgrG1-bla fusions can be effectively secreted and delivered into the cytosol of macrophage cells. Furthermore, *V. cholerae* effector VgrG3 can also be used as a scaffold for protein delivery [122]. Replacing the C-terminal lysozyme domain of VgrG3 with a nuclease domain from a heterologous species has been shown to efficiently target and kill prey bacteria.

A previous study showed that *P. aeruginosa* canonical VgrG proteins can secrete heterologous effector domains, effector proteins and T6SS-unrelated proteins in a T6SS-dependent manner [255]. However, a chimeric construct of a canonical VgrG and an effector protein (Tse2) could not be delivered into bacterial prey cells [255]. Although secretion assays

revealed that a chimeric construct of effector Tse2 fused to canonical VgrG1a was detected in the supernatant of T6SS-wielding cells, its absence in the supernatant of T6SS-deficient cells is indicative that secretion occurred in a T6SS-dependent manner. Nevertheless, the band corresponding to the secreted chimera in the supernatant was similar to that of the original canonical VgrG1a, suggesting that the chimera was perhaps susceptible to proteolytic cleavage. Furthermore, *P. aeruginosa* aggressors expressing the VgrG1a-Tse2 chimera were unable to kill sister prey cells susceptible to Tse2 toxicity [255]. These observations suggest that even though the VgrG1a-Tse2 chimera could be moderately extracellularly exported, it was unable to be directly delivered into target cells.

Another approach for potentially detecting the cytosolic delivery of heterologous proteins in recipient cells is by employing reporter systems. Herein, we designed a reporter system that is composed of a) T6SS+ donor cells that deliver a heterologous recombinase fused to a T6SS component, and b) recipient cells that contain a cytosolic reporter that undergoes a gene expression change mediated by the delivery of recombinase. We elected to deliver the recombinase through *V. cholerae* T6SS by fusing the full-length protein with PAAR protein and the core domain of effector VgrG3, by replacing its C-terminal lysozyme domain.

Recipient cells contain an excision construct with recombinase recognition sites that can switch between the expression of two genes upon recombinase expression. Recombinase mediates the switching of either a fluorescent signal or antibiotic resistance acquisition in the recipient cell. Since the reporter is located in the cytosol of recipient cells, DNA recombination will occur when the recombinase is specifically delivered into this compartment. A cytosolic reporter system will inform whether the T6SS of a heterologous species is able to puncture the recipient cell and deliver effectors into its cytosol.

Earlier studies revealed the potential of heterologous protein delivery using the T6SS, but at the expense of damaging the target cell [54,122,255]. Given that the delivered protein is toxic to the target cell, the detection of heterologous protein delivery was determined by quantifying prey cell survival. An advantage of our reporter system is that it presents a non-toxic approach to detect heterologous protein delivery. To detect heterologous cytosolic delivery we have engineered two similar reporter systems that rely on site-specific recombination (SSR), FLP-FRT and Cre-lox.

3.1.2 FLP-FRT reporter system

The FLP-FRT system is a member of the bidirectional recombinase superfamily, in which FLP is a tyrosine recombinase that is able to recombine (or "flip") specific DNA sequences [256,257]. FLP recombinase originates from the yeast 2 µ plasmid of *Saccharomyces cerevisiae* and recognises specific 34 base pair (bp) DNA sequences called FLP recombinase recognition targets (FRT) that flank the gene of interest [258].

The recombinase effect depends on the orientation and location of the FRT sites in the target DNA [259]. The DNA is excised when recognition target sites have the same orientation and are located in the same chromosome; is inverted when recognition target sites have opposite orientations and are located in the same chromosome; or is integrated when recognition target sites have similar orientation but are located in different chromosomes (Figure 3.1).

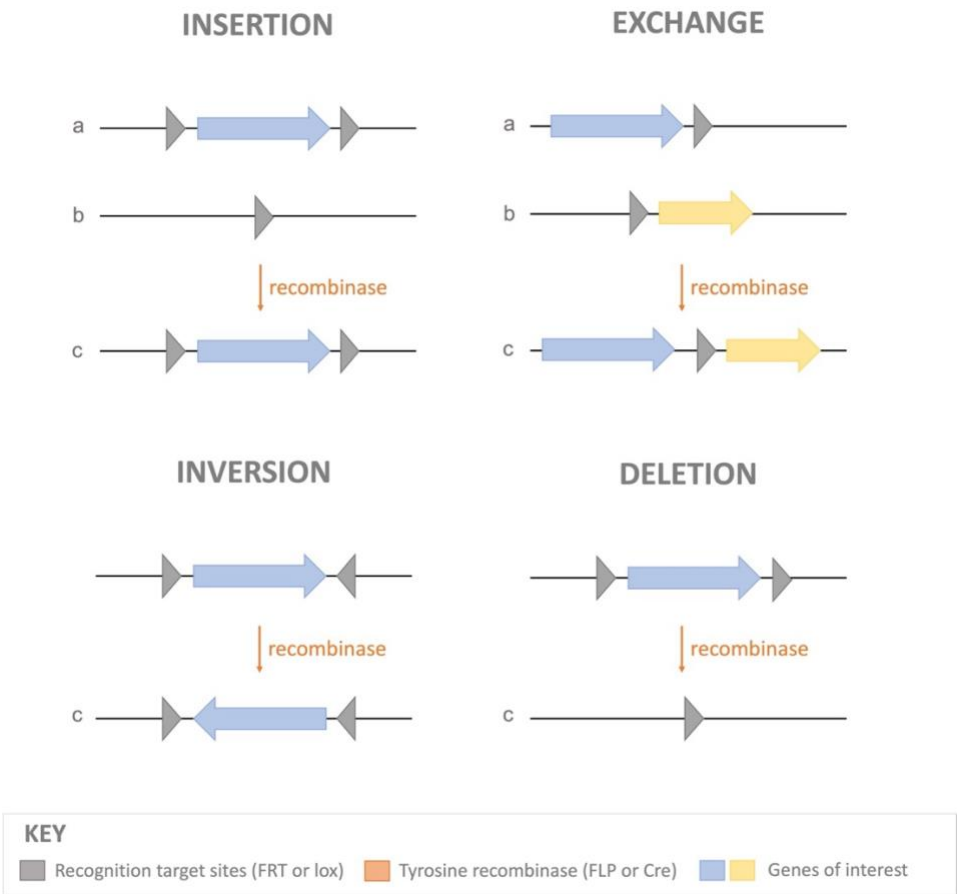

Figure 3.1 Site-specific recombination by Tyrosine recombinase. Exogenous DNA (a) can be introduced into chromosomal DNA (b) by site-specific tyrosine recombinases. The final product derived from DNA recombination (c) depends on the orientation and location of the recombinase recognition target sites.

The FLP-FRT recombination system has been widely used in eukaryotes, demonstrating high recombination efficiencies, and has also been applied in prokaryotes. FLP recombinase was used for gene editing to excise a chromosome-integrated resistance gene flanked by two FRT sites in *E. coli* [260] and cyanobacteria [261]. The FLP-FRT system has also been used for unmarked deletions of large DNA fragments in *H. pylori* [262] and to excise unwanted plasmid sequences after site-specific integration in *P. aeruginosa* chromosome [263]. To our knowledge, the FLP-FRT recombination system has not been used to detect cytosolic delivery using the T6SS or any other bacterial secretion system. As such, our reporter system presents a potential novel approach for detecting cytosolic T6SS delivery into unrelated target cells.

To understand whether *V. cholerae* T6SS can puncture and deliver effectors into the cytosol of *P.* aeruginosa, we constructed a fluorescent reporter system based on FLP-FRT site-specific recombination. The reporter consists of *mCherry2* flanked by two in tandem FRT sites, interrupting the translation of *mNeonGreen* (Figure 3.2). When FLP is expressed in the target cell, site-specific recombination occurs and the two FRT sites form a synaptic loop that excises *mCherry2*. This reporter allows the detection of FLP expression by signalling a recombination event through fluorescence emission on the target cell. This novel reporter system will be integrated into the chromosome of *P. aeruginosa* using two-step allelic exchange. This process consists of replacing a core component of *P. aeruginosa* T6SS (*tssB1*) with the FLP-FRT fluorescent reporter. To determine whether *V. cholerae* T6SS can deliver its effectors into *P. aeruginosa* cytosol we will fuse FLP to *V. cholerae* PAAR and the core domain of effector VgrG3.

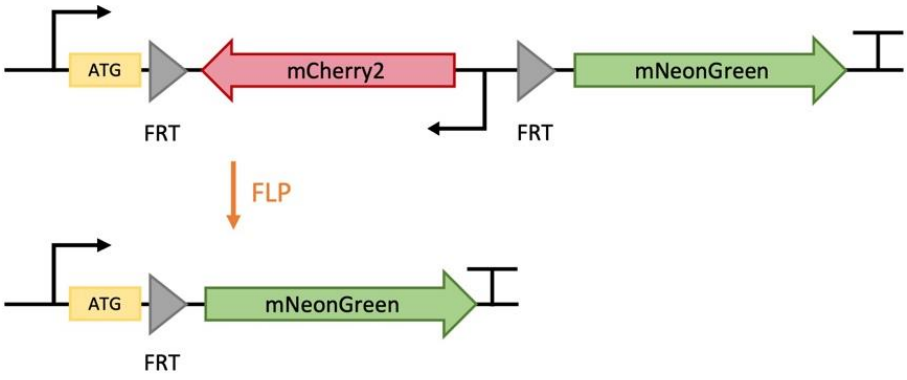

Figure 3.2. Depiction of the FLP-FRT fluorescent reporter construct (not to scale), before and after FLP-mediated recombination. The gene encoding for mCherry2 (red) is flanked by two FRT sites which interrupt the translation of mNeonGreen (green). Promoters are represented by arrows, FRT sites are represented in grey, and transcription terminator is represented by a T.

3.1.3 Cre-lox reporter system

The Cre-lox system is another member of the bidirectional recombinase superfamily, similar to the FLP-FRT system. In this system, Cre is a tyrosine recombinase derived from the P1 bacteriophage and lox is the recognition target sequence (locus of crossover (x) in P1) [257,258,264]. The Cre-lox recombination system has been previously used for gene replacement in *Lactobacillus plantarum* [265] and for cloning large DNA fragments in *Photorhabdus luminescens* and *A. tumefaciens* [266].

Previous studies have shown that Cre recombinase can be delivered via the T3SS [267] and the T6SS [268]. T6SS-mediated Cre delivery between *V. cholerae* sister cells can be detected by a cytosolic antibiotic construct with lox sites [268]. Recipient cells containing an antibiotic cassette construct flanked by lox sites were preyed on by donor cells with VgrG- and PAAR-Cre fusions. Though T6SS delivery of Cre was achieved with variable rates of efficiency, there were some limitations to this reporter system: VgrG-Cre constructs appeared to be toxic to the donor cell and elicit toxicity in recipient cells.

FLP and Cre are similar site-specific recombination technologies, and albeit having different nucleotide sequences, their recognition target sites (FRT and lox) share a similar general structure [258]. Both FRT and lox have 13 bp palindromic sequences that flank an 8 bp asymmetric core. There are a multitude of FRT and lox variants, which differ in the sequence of the asymmetric core. Nevertheless, recombination can still occur between variant recognition targets when the palindromic sequences are similar and only the asymmetric core, or spacer region, is modified [269,270].

To investigate whether *V. cholerae* T6SS is able to deliver effectors into *P. aeruginosa* cytosol we also constructed a reporter based on the Cre-lox recombination system. This reporter is conceptually similar to the FLP-FRT reporter system described previously. However, the lox reporter consists of an antibiotic-resistance gene flanked by two in tandem lox sites that interrupt the translation of a second antibiotic-resistance gene (Figure 3.3). Upon Cre expression in the recipient cell, the two lox sites are brought together, and the gene between them is excised. As previously, the detection of T6SS delivery occurs when recombination takes place in the recipient cell. The lox reporter will be integrated into the chromosome of *P. aeruginosa* to allow cytosolic detection of Cre delivery in a T6SS-dependent manner.

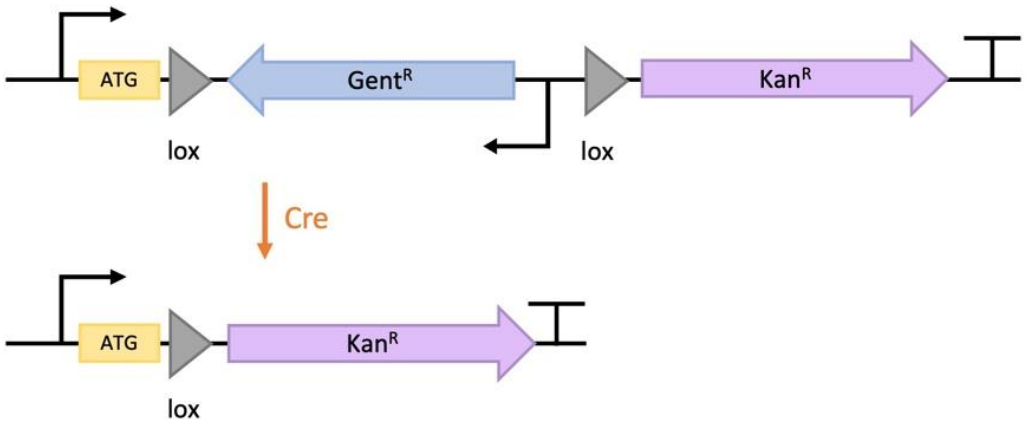

Figure 3.3. Depiction of the Cre-lox antibiotic reporter system (not to scale), before and after Cre-mediated recombination. The gentamicin resistance gene (blue) is flanked by two lox sites which interrupt the translation of the kanamycin resistance gene (purple). Promoters are represented by arrows, lox sites are represented in grey, and transcription terminator is represented by a T.

Unlike the FLP-FRT reporter, the Cre-lox reporter was engineered with antibiotic-resistance genes instead of fluorescent genes. The FLP-FRT reporter detects the emergence of cells that emit a different fluorescent signal after recombination. In contrast, the Cre-lox reporter specifically quantifies the number of cells that have undergone a recombination event, thereby acquiring a new antibiotic resistance. Therefore, the antibiotic-based Cre-lox reporter system might enable a more accurate and quantifiable detection of recombinase delivery.

3.2 Results

3.2.1 T6SS-FLP cytosolic delivery

To detect T6SS-mediated cytosolic delivery, we engineered a reporter system that emits a signal when FLP is specifically delivered into the cytosol of recipient cells. Recipient cells containing the FRT reporter initially emit red fluorescence. Following FLP delivery or endogenous expression, recipient cells should emit green fluorescence. Thus, a change in fluorescence indicates that recombination has occurred. This change in fluorescence is permanent and non-lethal to the recipient cell. When plated in LB agar, fluorescent colonies can be visualised and quantified using an imaging system set for the appropriate emission channels.

To confirm the functionality of the FLP-FRT reporter system, we endogenously expressed FLP in recipient cells that contained the FRT reporter. Endogenous expression was achieved by introducing FLP into a replicative plasmid which was then transformed and expressed in recipient cells. Endogenous expression of FLP in *E. coli*, *V. cholerae* and *P. aeruginosa* led to a permanent and detectable change in the fluorescence emitted by the cells (Figure S3.1). It is to be noted that none of the recipient cells emitted a different fluorescent signal without endogenous expression of FLP, suggesting that spontaneous recombination of the FRT reporter did not occur.

To deliver FLP in a T6SS-dependent manner we used a flexible linker (DGPAG) to fuse FLP to the core domain of effector VgrG3 (truncated at residue 648) and to spike component PAAR2 (Figure 3.4). A recombination assay was performed to detect T6SS cytosolic delivery: donor and recipient cells were first co-incubated in solid media to allow for T6SS activity, and then recovered on LB agar supplemented with the appropriate antibiotics to select for the growth of only recipient cells.

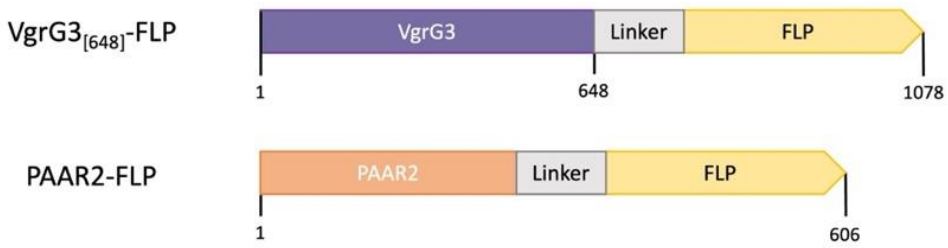

Figure 3.4. Depiction of FLP recombinase (yellow) fusions through a linker (grey) to: *V. cholerae* VgrG3 truncated at residue 648 (purple) and PAAR2 (orange). Picture not to scale.

V. cholerae T6SS can puncture and deliver effector proteins into sister cells without killing them due to the presence of cognate immunity proteins. As proof of principle that FLP can be delivered in a T6SS-dependent manner, *V. cholerae* T6SS was used to deliver FLP into *V. cholerae* sister cells containing the FRT reporter. *V. cholerae* recipient cells were recovered following co-incubation with *V. cholerae* FLP donors and plates were imaged on a ChemiDoc MP Imaging system using the Rhodamine (red) and Alexa 488 (green) channels. We observed that whilst the majority of colonies emitted red fluorescence, some colonies had lost their ability to emit fluorescence (Figure 3.5). The colonies that were no longer fluorescent were pale rather than bright fluorescent, which is likely a result of background fluorescence (controls can be seen in Figure S 3.1). Given that the plates with only *V. cholerae* recipient colonies (Figure 3.5 A) showed a mixture of red and pale green colonies, it is likely that the recipients have lost the plasmid containing the FRT reporter. To prevent plasmid loss in *V. cholerae* recipients, we integrated the FRT reporter into the chromosome of the cell. This was done by cloning the FRT reporter into a transposon plasmid (pBAMD1-6), which was randomly allowed to integrate into the chromosome of *V. cholerae*.

Additionally, to prevent re-secretion of the fusion proteins by the T6SS of recipients, we used T6SS-deficient strains as recipients. When we co-incubated *V. cholerae* donors containing the T6SS-FLP constructs with either *V. cholerae* or *P. aeruginosa* recipients containing a chromosomal FRT reporter, all recovered recipients emitted only red fluorescence. As such, these results suggest that if recombination occurred in recipient cells, it was below the detection limit.

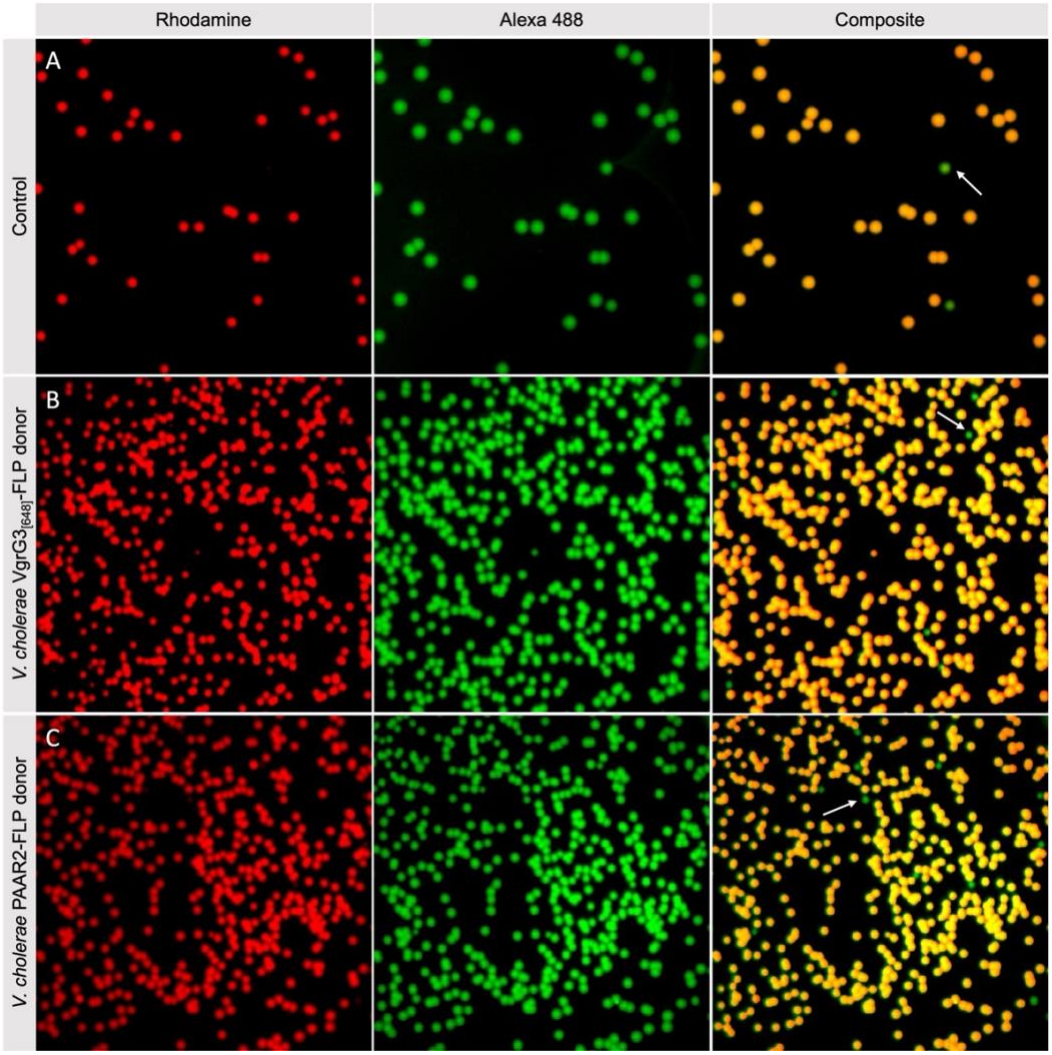

Figure 3.5. *V. cholerae* recipient cells with the FRT reporter after co-incubation with *V. cholerae* FLP donors. LB agar plates were imaged on ChemiDoc MP Imaging System (Biorad) using Rhodamine and Alexa 488 channels, and composite images were obtained by superimposing both channels. Images display colonies of (A) *V. cholerae* 2740-80 recipients expressing the FRT reporter (control) and *V. cholerae* 2740-80 recipients recovered after co-incubation with *V. cholerae* 2740-80 donors expressing (B) VgrG3$_{[648]}$-FLP and (C) PAAR2-FLP. In the composite images, pale green colonies represent cellular background of *V. cholerae* recipient cells that have lost the plasmid containing the FRT reporter.

3.2.2 *V. cholerae* VgrG3-FLP fusion constructs might hinder T6SS activity

Since recombination was not detected in recipients following incubation with *V. cholerae* T6SS-FLP donors, we hypothesised that T6SS-FLP constructs might hinder *V. cholerae* T6SS activity. *V. cholerae* T6SS is able to successfully kill *E. coli* but should spare sister cells due to the presence of immunity proteins. Therefore, we initially verified whether *V. cholerae* donors expressing the VgrG3$_{[648]}$-FLP construct could kill *E. coli* without self-damage. Our observations confirmed that *V. cholerae* donors expressing the VgrG3$_{[648]}$-FLP

construct were able to effectively kill *E. coli*, indicating that the construct does not compromise T6SS activity and toxicity. Additionally, the survival of *V. cholerae* sister cells remained unaffected when preyed upon by *V. cholerae* donors expressing the VgrG3$_{[648]}$-FLP construct.

However, it remained unclear whether the VgrG3$_{[648]}$-FLP construct hindered the T6SS functionality of *V. cholerae* donor cells. To investigate this we performed T6SS secretion assays. We started by detecting the presence of Hcp, a hallmark of a functional T6SS, and collected and prepared cultures of *V. cholerae* donors expressing VgrG3$_{[648]}$-FLP after incubation with different concentrations of arabinose. We observed that Hcp secretion was lowest when the VgrG3$_{[648]}$-FLP construct expression was highest (Figure 3.6A), which suggests that the VgrG3$_{[648]}$-FLP construct is hindering *V. cholerae* T6SS function.

To test whether the VgrG3$_{[648]}$-FLP construct was being secreted despite low levels of T6SS activity, we detected the presence of VgrG3$_{[648]}$-FLP in the culture pellet and supernatant. Our results show that the VgrG3$_{[648]}$-FLP construct was only detected in the cell culture supernatant induced with the lowest concentration of arabinose (Figure 3.6B). It would be expected to achieve higher expression levels with increased arabinose induction. As such, this suggests that the construct might be degraded or cleaved when strongly expressed. However, it should be noted that, despite many attempts, the detection of VgrG3$_{[648]}$-FLP construct could only be observed in one of the experimental replicates and this result was not reproducible. This observation suggests that *V. cholerae* T6SS is unable to properly secrete VgrG3$_{[648]}$-FLP construct.

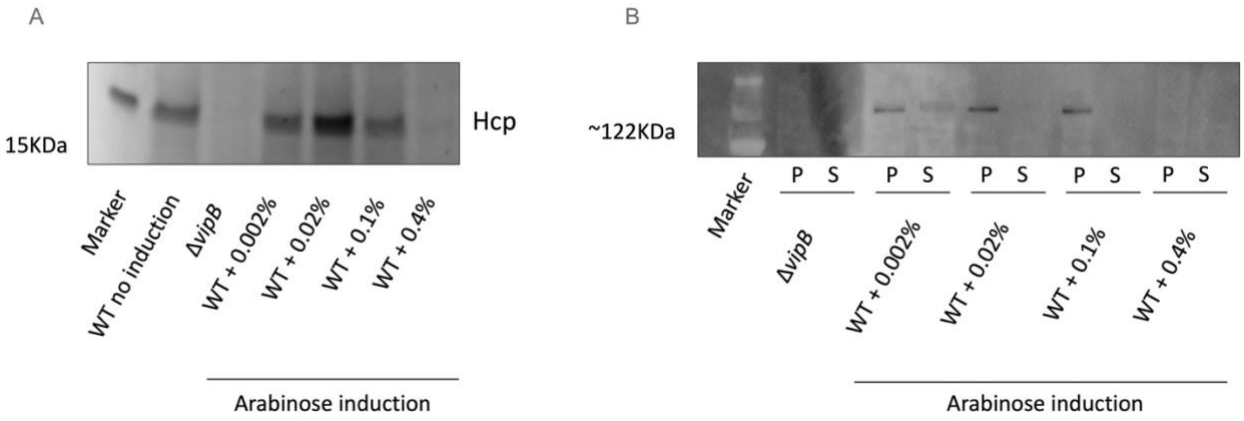

Figure 3.6. Protein secretion assays to assess T6SS functionality of *V. cholerae* expressing the VgrG3$_{[648]}$-FLP construct (*V. cholerae* V52 pBAD24oriT-VgrG3$_{[648]}$-FLP-HA). A Coomassie protein assay (A) showed that *V. cholerae* V52 expressing the VgrG3$_{[648]}$-FLP-HA construct is able to secrete Hcp, a hallmark of T6SS functionality. A Western-blot assay (B) revealed that, whilst the VgrG3$_{[648]}$-FLP-HA construct could be detected in the culture pellet (P) it was not detected in the supernatant (S). The blot was incubated with an anti-HA antibody to detect the presence of the VgrG3$_{[648]}$-FLP-HA construct. Cells were grown under increasing concentrations of arabinose to induce expression of the VgrG3$_{[648]}$-FLP-HA construct under a pBAD24oriT vector.

3.2.3 Cre-lox mediated T6SS cytosolic delivery

Given that VgrG3$_{[648]}$-FLP construct might hinder *V. cholerae* T6SS function and protein secretion, we engineered a Cre-lox reporter system to detect T6SS protein delivery into the cytosol of target cells. The Cre-lox reporter system works similarly to the FLP-FRT reporter system, whereby donor *V. cholerae* T6SS delivers Cre into the cytosol of a recipient cell containing a lox reporter. We engineered multiple antibiotic-based lox reporter constructs, but ultimately only two of them were stable and functional. We named one of these reporters gent-kan reporter because it confers recipients gentamicin resistance before Cre recombination, and kanamycin resistance following recombination. Another reporter was named kan-gent reporter and grants recipients kanamycin resistance before Cre recombination, and gentamicin resistance following recombination. For further details on the engineering of these reporter please refer to Tables 3.1 and 3.2.

3.2.4 The gent-kan reporter spontaneous recombination is minimal in *E. coli*

Recombinase mediated reporters could potentially spontaneously recombine in the absence of Cre. Therefore, we first determined the level of reporter spontaneous recombination in recipient cells by quantifying naïve recipients (cells that maintain the original antibiotic resistance) and recombined recipients (cells that lose the original antibiotic resistance and acquire new antibiotic resistance) in the absence of Cre recombinase. We observed that spontaneous recombination in the gent-kan reporter occurs in a small number of cells (counted on LB agar with kanamycin), though they are largely outweighed by the number of cells that remained naïve (counted on LB agar with gentamicin) (Figure 3.7). These results indicate that the gent-kan reporter is stable in *E. coli* and spontaneous recombination events are improbable.

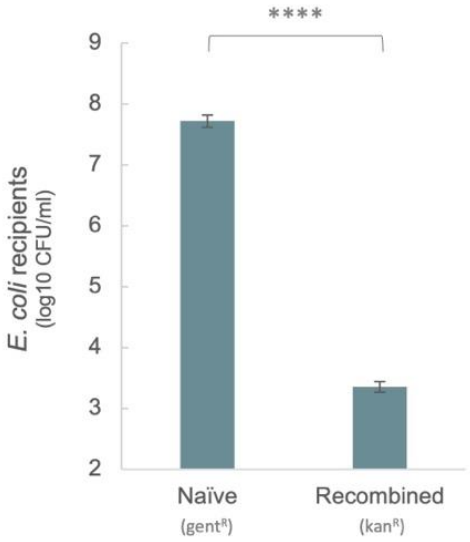

Figure 3.7. Spontaneous recombination of the gent-kan reporter in *E. coli*. Data represent the recovery of recipient *E. coli* cells containing the gent-kan reporter. Colonies were recovered and enumerated in LB agar with gentamicin (naïve colonies) and kanamycin (recombined colonies). Data are presented as mean $\log_{10}$CFU/ml ± SD of three experimental replicates. Statistical significance was determined using Student's t-test (**** $p \leq 0.0001$).

3.2.5 Gent-kan reporter recombines following Cre endogenous expression

To demonstrate the functionality of the gent-kan reporter we first determined whether recombination occurred in the presence of Cre or T6SS-Cre fusions in *E. coli*. Effector VgrG3 is composed of a core domain linked to a C-terminal lysozyme domain [122]. To address whether the length of the linker region was critical for T6SS-mediated secretion we performed a series of truncations within the effector linker, removing the C-terminal domain while preserving the conserved core domain. Each of these truncations were then fused to Cre using a double Serine linker (Figure 3.8).

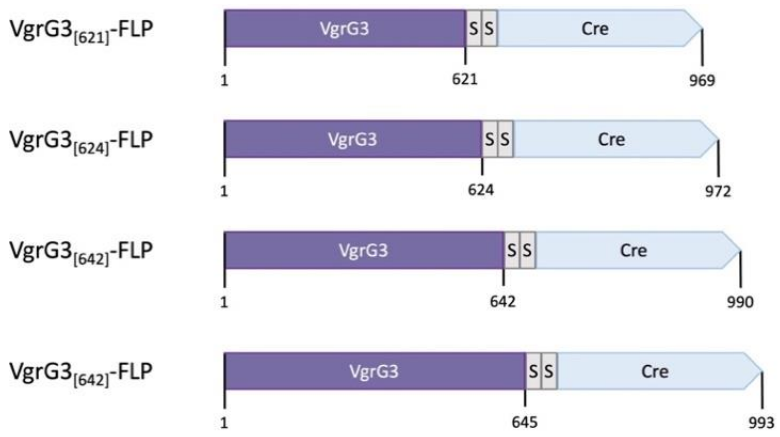

Figure 3.8. Depiction of Cre recombinase (blue) fusions through a double Serine linker (grey) to *V. cholerae* VgrG3 truncations (purple) at residues 621, 624, 642 and 645. Picture not to scale.

To account for spontaneous recombination we also endogenously expressed an empty plasmid in recipients containing the gent-kan reporter. The donors expressed Cre and VgrG3-Cre constructs under a Chloramphenicol-resistant plasmid (Cm^R), whereas recipients expressed the gent-kan reporter under an Ampicillin-resistant plasmid (Amp^R). This allowed donors and recipients to be differentially selected under when recovered and effectively quantified. To determine the recombination efficiency, the number of recombined recipient cells (LOG_{10} Amp^RKan^R) was divided over the total number of recipient cells (LOG_{10} Amp^RCm^R). After 3 hours of incubation with Cre and all VgrG3-Cre constructs, *E. coli* recipients containing the gent-kan reporter acquired kanamycin resistance, a result of Cre-mediated recombination

(Figure 3.9A and B). After 24 hours of incubation, the number of recombined *E. coli* cells was slightly higher than after 3 hours of incubation (Figure 3.9C and D). Since recombination efficiency is similar for both incubation periods, this is likely due to a greater number of cells resulting from extended growth during longer incubation periods.

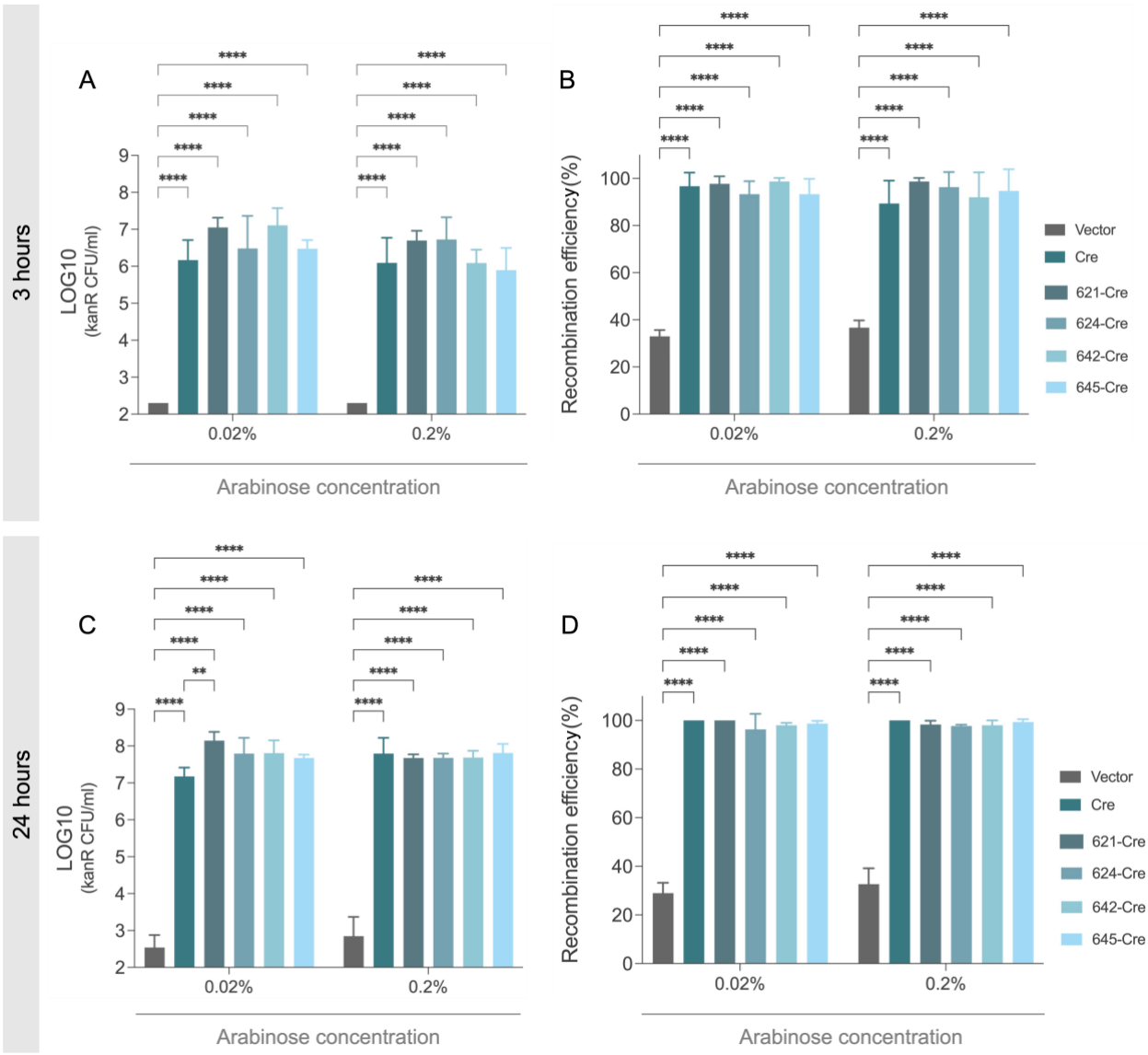

Figure 3.9. Endogenous expression of Cre and VgrG3-Cre constructs in *E. coli* containing the gent-kan reporter. Data represent recombination in log_{10}CFU/ml after endogenous expression of Cre and VgrG3-Cre constructs 0.02% under 0.2% arabinose induction after 3 hours (A and B) and 24 hours (C and D). Cre and VgrG3-Cre constructs were cloned in pBAD33oriT (Cm^R), whereas the gent-kan reporter was cloned in vector pBAD24oriT (Amp^R) to allow for selective enumeration of donors and recipients. Data are presented as mean log_{10}CFU/ml ± SD of three experimental replicates. Statistical analysis was performed using two-way ANOVA followed by Tukey's post-hoc test (** $p \leq 0.01$, **** $p \leq 0.0001$).

3.2.6 The gent-kan reporter is unstable in *V. cholerae*

Given that the gent-kan reporter was stable and could recombine in *E. coli*, we analysed its stability and function in *V. cholerae* donors. However, we observed that *V. cholerae* donors containing the gent-kan reporter acquired kanamycin resistance in the absence of Cre recombinase (Figure 3.10). We questioned whether this phenomenon was due to readthrough transcription, whereby *V. cholerae* cells can become resistant to both gentamicin and kanamycin, or instead due to spontaneous recombination of the reporter. To verify which of these scenarios occurred we picked a "recombined" colony from a kanamycin plate and performed a colony PCR to amplify the full length of the reporter. After running the amplified product in an agarose gel, we identified the presence of different sized products, which indicates amplification of the full-length reporter as well as the recombined reporter. This observation suggests that the gent-kan reporter spontaneously recombines in *V. cholerae*.

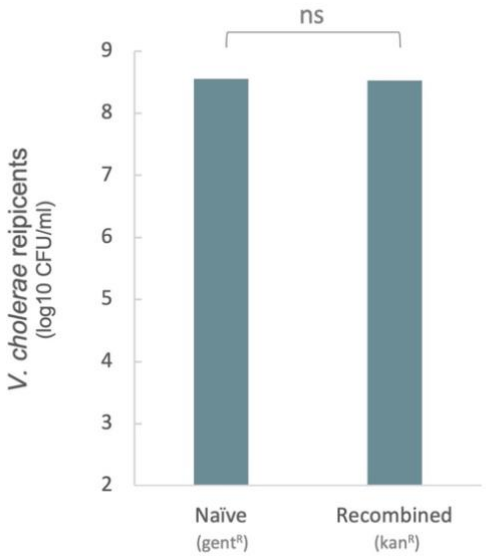

Figure 3.10. Spontaneous recombination of the gent-kan reporter in *V. cholerae*. Data represent transformed *V. cholerae* cells containing the gent-kan reporter. Colonies were recovered and enumerated on LB agar with gentamicin (naïve colonies) and kanamycin (recombined colonies). Data are presented as mean $\log_{10}$CFU/ml ± SD of three experimental replicates. Statistical significance was determined using Student's t-test (ns. non-significant, $p > 0.05$).

3.2.7 The kan-gent reporter is stable in *V. cholerae*

As with the previous reporter, we started by assessing the stability of the kan-gent reporter in *E. coli*. We observed that the kan-gent reporter had a high rate of spontaneous recombination in the absence of Cre recombinase (Figure 3.11A). Interestingly, the kan-gent reporter underwent minimal spontaneous recombination in *V. cholerae* (Figure 3.11B).

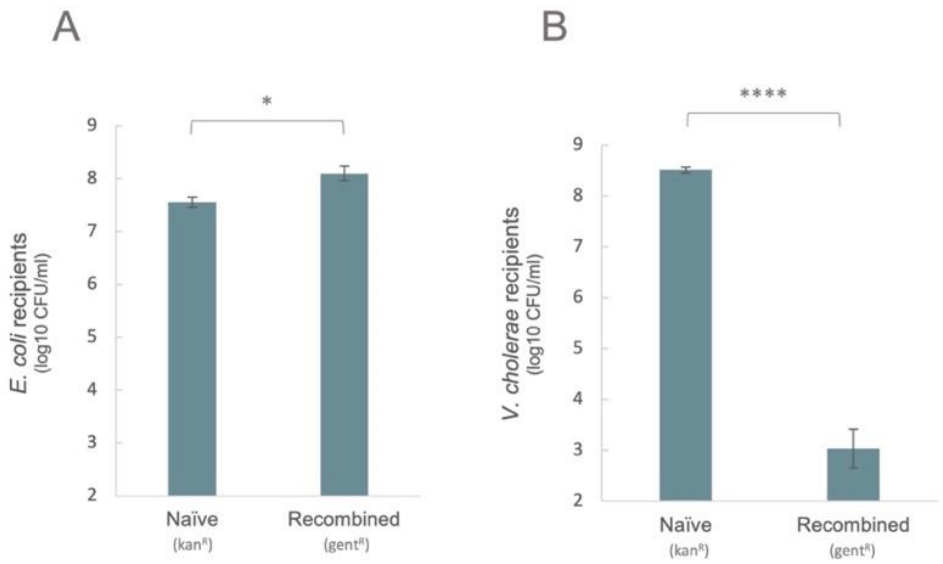

Figure 3.11. Spontaneous recombination of the kan-gent reporter in *E. coli* and *V. cholerae*. Data represent the recovery of recipient cells containing the kan-gent reporter in (A) *E. coli* and (B) *V. cholerae*. Colonies were recovered and enumerated on LB with kanamycin (naïve colonies) and gentamicin (recombined colonies). Data are presented as mean $\log_{10}$CFU/ml ± SD of three experimental replicates. Statistical significance was determined using Student's t-test (* $p \leq 0.05$; **** $p \leq 0.0001$).

3.2.8 Cre delivery through *V. cholerae* T6SS is undetectable

Since the kan-gent reporter was stable in *V. cholerae*, we performed a proof-of-concept recombination assay to detect T6SS-mediated cytosolic delivery of Cre recombinase between *V. cholerae* donors and recipients. In this assay, *V. cholerae* donor containing $VgrG3_{[645]}$-Cre construct were co-incubated with *V. cholerae* recipients containing the kan-gent reporter to allow for T6SS-mediated delivery of Cre. We have considered that spontaneous recombination of the kan-gent reporter might occur or that the $VgrG3_{[645]}$-Cre construct might be delivered by other systems. Therefore, to account for both of these occurrences during the T6SS-mediated Cre delivery recombination assay, we used WT

V. cholerae containing an empty vector as a control. Additionally, to account for effector competition for delivery we also used a *V. cholerae* VgrG3 mutant strain as donor for the VgrG3-Cre construct. The T6SS-mediated Cre delivery recombination assay showed that Cre-mediated recombination did not occur after incubation with any of *V. cholerae* donors containing the VgrG3-Cre construct (Figure 3.12). Collectively, these results suggest that either *V. cholerae* is unable to deliver Cre into sister cells in a T6SS-dependent manner or that the kan-gent reporter is unable to recombine after Cre T6SS delivery.

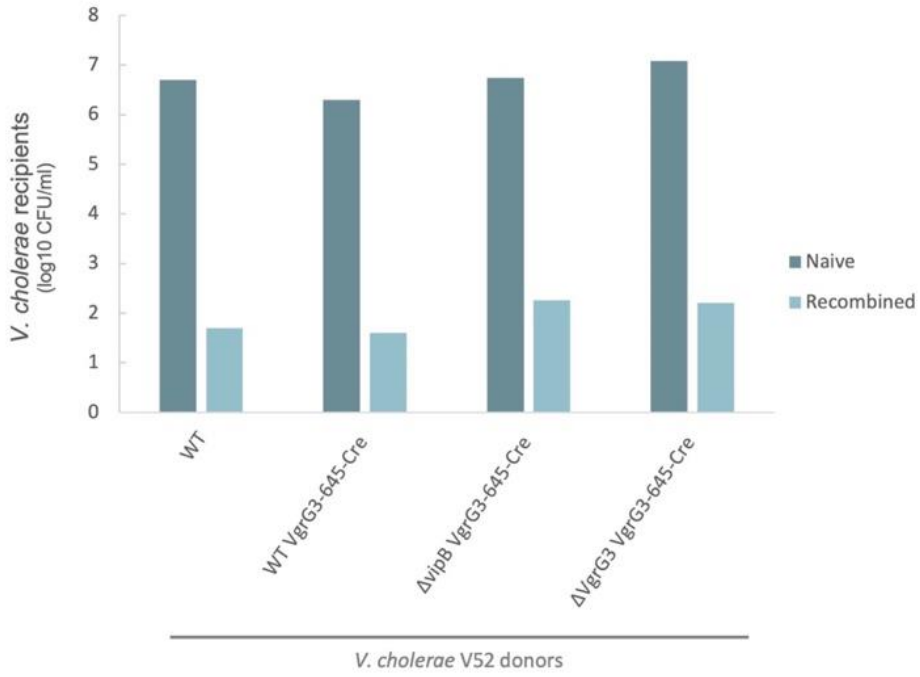

Figure 3.12. T6SS-mediated delivery of Cre between *V. cholerae* sister cells. Data represent the recovery of *V. cholerae* recipients containing the kan-gent reporter following co-incubation with *V. cholerae* donors expressing the $VgrG3_{[645]}$-Cre construct. Recipients were *V. cholerae* V52 *ΔvipB* expressing the kan-gent reporter and donors were either *V. cholerae* V52 WT, *vipB* or *vgrG3* mutants expressing the $VgrG3_{[645]}$-Cre construct. Data are presented as log_{10}CFU/ml of one single biological replicate.

3.3 Discussion

In this Chapter, we sought to understand whether *V. cholerae* T6SS can puncture and deliver toxic effectors into the cytosol of *P. aeruginosa*. To explore this, we engineered reporter systems to detect heterologous protein delivery into the cytosol using site-specific recombination. We first engineered a reporter system using FLP-FRT site-specific recombination, where FLP recombinase is delivered into a recipient cells by *V. cholerae* T6SS donor cells. The recipients contained a non-fluorescent reporter construct with FLP recognition target sites (FRT). We observed a detectable and permanent change in recipients upon FLP expression, indicating the functionality of the FRT fluorescent construct (Figure S 3.1). However, we did not endogenously express the VgrG3- or PAAR-FLP fusions in any recipients containing the FRT reporter. Hence, it is possible that neither of these T6SS-FLP constructs can be expressed, or that recombination cannot be detected when constructs are endogenously expressed in recipients.

When attempting to deliver FLP recombinase through *V. cholerae* T6SS into *V. cholerae* recipients, recombination could not be detected. We hypothesised that this might be due to *V. cholerae* inability to secrete functional T6SS-FLP constructs. Here, we demonstrated that the secretion of Hcp, a hallmark of a functional T6SS, decreases as the expression of VgrG3$_{[648]}$-FLP construct increases (Figure 3.6A). Additionally, we were unable to detect the secretion of VgrG3$_{[648]}$-FLP construct in *V. cholerae* culture supernatants (Figure 3.6B). Collectively, these results suggest that the VgrG3$_{[648]}$-FLP construct might hinder T6SS function in *V. cholerae* or that the construct fails to be properly folded for secretion through T6SS.

Heterologous protein delivery through the T6SS of *V. cholerae* has been achieved previously [122]. By replacing the lysozyme domain of VgrG3 with a *Salmonella* nuclease effector, *V. cholerae* was able to kill *V. cholerae* cells not expressing a nuclease cognate immunity protein. This demonstrates that *V. cholerae* T6SS could be used as a scaffold for heterologous protein delivery between the same species. However, our observations indicate that FLP recombinase could not be delivered through the T6SS of *V. cholerae* amongst sister cells. These discrepancies may stem from differences in size and structure between the *Salmonella* nuclease and FLP recombinase. It is possible that *V. cholerae* T6SS spike is unable to

accommodate the tetrameric structure of FLP recombinase, or that the VgrG3$_{[648]}$-FLP fusion fails to properly fold in order to be secreted.

Since *P. aeruginosa* is resistant to the majority of antibiotics available in our laboratory, we were motivated to engineer a reporter system that did not rely on acquired antibiotic resistance. However, the FLP-FRT reporter system has proven to be ineffective, as FLP recombinase cannot be delivered through *V. cholerae* T6SS. Moreover, the detection of recombination through this reporter system has shown to be time-consuming and resource-intensive. Hence why we were motivated to instead engineer an antibiotic reporter system to detect T6SS cytosolic delivery.

To demonstrate proof-of-concept before detecting T6SS-delivery into *P.* aeruginosa, we first assessed the stability and functionality of the lox reporters in *E. coli* and *V. cholerae*. Here, we demonstrated that spontaneous recombination of the gent-kan reporter is minimal in *E. coli* (Figure 3.7) and that recombination is highly efficient following endogenous expression of Cre recombinase (Figure 3.9). In contrast, the gent-kan reporter displayed a high spontaneous recombination rate in *V. cholerae* (Figure 3.10). The differences between reporter functionality between species could be attributed to the fact that the *E. coli* strain used is a *recA* mutant and thus the reporter is less likely to spontaneously recombine in this strain compared to *V. cholerae.*

Despite spontaneously recombining in *E. coli*, the kan-gent reporter is stable in *V. cholerae* (Figure 3.11). However, recombination was undetected when we attempted T6SS-mediated Cre delivery into *V. cholerae* recipients expressing the kan-gent reporter (Figure 3.12). We are conscious that this assay presents some limitations. One of them being that neither Cre nor the T6SS-Cre constructs were endogenously expressed in *V. cholerae* recipients expressing the kan-gent reporter to assess functionality. It is entirely possible that the kan-gent reporter is unable to recombine in *V. cholerae* recipients. Hence, our results could suggest that the kan-gent reporter is not functional, rather than that *V. cholerae* T6SS is unable to deliver Cre. Due to time constraints, the recombination assay for the kan-gent reporter was only performed once, which raises another limitation regarding experimental replicability. Importantly, an efficient fusion between a trimer (VgrG) and a tetramer (FLP or Cre recombinases) would be challenging given their distinct oligomerisation states.

However, T6SS-mediated Cre delivery has been accomplished before [268]. Therefore we considered that there could be sufficient structural flexibility for the fusion to be efficient. It is important to note that in the previous report of T6SS-mediated Cre delivery, recombination was achieved with low levels of efficiency [268]. Similar to the Cre-lox reporter system developed herein, an antibiotic reporter construct was engineered to detect cytosolic delivery of Cre. *V. cholerae* donors expressing VgrG3- and PAAR2-Cre fusions could be delivered into recipients, albeit with an efficiency of around 1%. Notably, T6SS-Cre fusions appeared to be toxic to *V. cholerae* donors, a challenge partially overcome by deleting all four *V. cholerae* toxic effectors. In our study, we have not accounted for this possibility and used WT *V. cholerae* as the donor in our experiments. As such, deleting all four toxic effectors might have improved T6SS-delivery and Cre-mediated recombination. However, we accounted for competition in effector delivery by using a *V. cholerae* VgrG3 mutant strain as a donor in our recombination assays (*V. cholerae* V52 Δ*vgrG3* pBAD33oriT-VgrG3$_{[645]}$-Cre).

Furthermore, T6SS-mediated heterologous protein delivery has also been attempted amongst different species. A chimeric construct of *V. cholerae* PAAR fused to a toxic effector from *Aeromonas dhakensis* was highly toxic when delivered into *V. cholerae* recipients [268]. In contrast, delivering this chimeric construct into *P. aeruginosa* recipients showed minor levels of toxicity. It could be argued that the toxicity observed from *V. cholerae* TseC delivery into *P. aeruginosa* is comparable to our previous data showing the recovery of *P. aeruginosa* after being preyed upon by WT *V. cholerae* (Chapter 2). It is to be noted that while we have shown that *P. aeruginosa* is more resistant to *V. cholerae* T6SS attacks*, P. aeruginosa* is not entirely unaffected.

Taken together, our data challenges the previous research on heterologous protein delivery through *V. cholerae* T6SS. Although it has been accomplished before, it is notable that protein size and structure are important for the effectiveness of T6SS delivery. Though our efforts to deliver recombinase proteins through *V. cholerae* T6SS were unsuccessful, both the FLP-FRT and Cre-lox reporter systems could be repurposed. We demonstrated that FLP- and Cre-mediated recombination can be achieved in *E. coli*. Therefore, our reporter systems could be used for assessing both conjugation efficiency and the stability and expression of chimeric recombinase fusions. For example, the FLP-FRT reporter system may be used to assess conjugation efficiency using real-time fluorescence

microscopy and the Cre-lox reporter system may be utilised to accurately determine the suitability of chimeric fusions upon endogenous expression with the lox reporter. These reporters are highly stable, non-toxic and efficiently report recombination, and therefore could be valuable tools in the laboratory.

Table 3.1. Bacterial strains and plasmids used in this Chapter

Strain or plasmid	Relevant characteristics	Source
***Vibrio cholerae* V52**		
WT	Parental strain, *rtxA hlyA hapA* (Δrhh), Str^R	[53]
ΔvipB	In frame deletion of amino acid 12 to 486 of *vipB* (VCA0108)	[171]
ΔvgrG3	In frame deletion of *vgrG3* (VCA0123)	[171]
***Vibrio cholerae* 2740-80**		
WT	Parental strain, clpV::clpV-mCherry, $lacZ^-$, Str^R	[87]
ΔvipA	In frame deletion of *vipA* (VCA0107)	[87]
***Pseudomonas aeruginosa* PAO1**		
WT	Wild type, Irg^R	Lab collection
ΔtssB1	In frame deletion of amino acid 11 to 164 of *tssB1* (PA0083)	Chapter 1
Escherichia coli		
NEB 10-beta	DH10B derivative, *recA1 relA1 endA1 rpsL* (Str^R)	New England Biolabs
One Shot PIR1	*endA recA pir* gene	ThermoFisher
SM10 λ*pir*	*thi thr leu tonA lacy supE recA-RP4-2-Tc-Mu pir*	[251]
MFD *pir*	Mu-free donor, SM10 λ*pir* and S17-1 λ*pir* derivative	[271]
Plasmids		
pBAD33	Expression vector, p15A origin, araC, araBAD promoter, Cm^R	[252]
pBAD24	Expression vector, pBR322 origin, araC, araBAD promoter, Amp^R	[252]
pBAD24oriT	pBAD24 with origin of Transfer (oriT)	Lab collection
pBAD33oriT	pBAD33 with origin of Transfer (oriT)	Lab collection
pPSV35	Expression vector, PA origin, $lacI^q$	[254]
pCOLADuet	Expression vector, colA replicon, kan^R	Lab collection
pPGA	Expression vector, PA origin, araC, araBAD promoter, Gm^R	Chapter 1
pEXG2	Allelic exchange vector with pBR origin, Gm^R, *sacB*	[254]
pBAMD1-6	Mini-Tn5 delivery plasmid, ori(R6K), Amp^R Gm^R	[272]
FLP-FRT Constructs		
pBAD24-mCherry2	*mCherry2* cloned into pBAD24	Lab collection
pBAD24-mNeongreen	*mNeonGreen* cloned into pBAD24	Lab collection
pBAD24-FRT reporter	Fluorescent FRT reporter (pRNA-FRT-[mCherry2]-pTac-mNeonGreen-rrnb)	This study
pPSV35-FRT reporter	Fluorescent FRT reporter (pRNA-FRT-[mCherry2]-pTac-mNeonGreen-rrnb)	This study
pEXG2-LF-FRT reporter-RF	Allelic exchange vector to replace *tssB1* with FRT reporter	This study
pBAMD1-6-FRT reporter	Fluorescent FRT reporter	This study
pBAD24-FLP	FLP recombinase cloned into pBAD24	Lab collection
pBAD24oriT-FLP	FLP recombinase cloned into pBAD24oriT	This study
pBAD33-FLP	FLP recombinase cloned into pBAD33	This study
pPGA-FLP	FLP recombinase cloned into pPGA	This study
pBAD24ori-sfGFP	sfGFP cloned into pBAD24	Lab collection
pBAD24ori-sfGFP-HA	HA tag cloned downstream of sfGP	This study
pBAD24oriT-VgrG3$_{[648]}$	First 648 residues of *V. cholerae* VgrG3 (VCA0123)	Lab collection
pBAD24oriT-VgrG3$_{[648]}$-FLP	First 648 residues of *V. cholerae* VgrG3 (VCA0123) fused to FLP by linker (DGPAG)	This study
pBAD24oriT-VgrG3$_{[648]}$-FLP-HA	pBAD24oriT-VgrG3$_{[648]}$-FLP fused with HA tag	This study
pBAD24oriT-PAAR2	PAAR2 cloned into pBAD24	Lab collection
pBAD24oriT-PAAR2-FLP	Full-length *V. cholerae* PAAR2 (VCA0284) fused to FLP by linker (DGPAG)	This study
Cre-lox Constructs		
pBAD24oriT-gent-kan	Gent-kan reporter (pJ23103-lox-[$gent^R$]-lox-kan^R	This study

pBAD24oriT-kan-gent	Kan-gent reporter (pBAD-lox-[kan^{R}]-lox-prrna-gent^{R})	This study
pBAD33oriT-Cre	Cre recombinase cloned into pBAD33oriT	This study
pBAD33oriT-VgrG3$_{[621]}$	First 621 residues of *V. cholerae* VgrG3 (VCA0123)	Lab collection
pBAD33oriT-VgrG3$_{[624]}$	First 624 residues of *V. cholerae* VgrG3 (VCA0123)	Lab collection
pBAD33oriT-VgrG3$_{[642]}$	First 642 residues of *V. cholerae* VgrG3 (VCA0123)	Lab collection
pBAD33oriT-VgrG3$_{[645]}$	First 645 residues of *V. cholerae* VgrG3 (VCA0123)	Lab collection
pBAD33oriT-VgrG3$_{[621]}$-Cre	VgrG3$_{[621]}$ fused to Cre by two Serines	This study
pBAD33oriT-VgrG3$_{[624]}$-Cre	VgrG3$_{[624]}$ fused to Cre by two Serines	This study
pBAD33oriT-VgrG3$_{[642]}$-Cre	VgrG3$_{[642]}$- fused to Cre by two Serines	This study
pBAD33oriT-VgrG3$_{[645]}$-Cre	VgrG3$_{[645]}$ fused to Cre by two Serines	This study

Table 3.2 Oligonucleotides used in this Chapter

Amplicon	ID	Oligonucleotide sequence (5' to 3')
Engineering the FLP fluorescent reporter		
mCherry2	1_pBAD_seq	AGTCCACATTGATTATTTGCACGG
	97_mCherry2_XbaI_HindIII_R	CCGAAGCTTATCTAGACTTGTACAGCTCGTCCATGC
	2_ pMMB_seq_R	TTCACTTCTGAGTTCGGCATGG
	109_Ptac_f	CGAGAGCTCTTGACAATTAATCATCGGCTCGTATAATGCATGCACGCGTTTTTTTGGGCTAGCAGGAG
mNeonGreen	oRR097_mNeonGreen_F	TCCTATTCTCTAGAAAGTATAGGAACTTCTGGTATCGAAGGGCGAAGAAG
	PMG 30	CCCAAGCTTATTATCTAGACTTGTACAACTCATCCATTC
	oRR096_Prrna_FRT_ultramer	CATGGATCCTTGCCAATATAACTAGGTTCTCTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAGGAATTCACCATGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTTAACTAGTATCAGAGCTCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTG
Out-of-frame fix	oRR115_SpeI_FlpFRT_fix_r	TTTACTAGTTCCTCCTTCGATACCAGAAGTTCCTATACT
	oRR116_SpeI_FlpFRT_fix_f	TTTACTAGTATGGTATCGAAGGGCGAAGA
Integrating FLP reporter into *P. aeruginosa* PAO1		
tssB1::FLP reporter	oRR040_LF-TssB1_F_1	AAAGGTACCGTACTGGGACGGCGTCTATC
	oRR043_RF-TssB1_R_4	CATAAGCTTGAAGGAGCGGTTGATGTTGA
	oRR117_BamHI-TssB1.LF_r	TACTTCGATTGCTATTGTACGGATCCACTGCTGGTAGTGCTTCCC
	oRR118_TssB1.RF-SalI_f	GTACAATAGCAATCGAAGTAGTCGACGACGAGCCTCAGGCGTAAG
Primers to amplify FLP recombinase		
FLP	oRR119_NheI-SalI-FlpE_f	GCTAGCAGGAGGATACTAGTATGGTCGACGGTCCGGCTGGTCTGAGCCAATTTGATATATTATGTAAAACAC
	oRR120_FlpE-HindIII_r	ATAAAGCTTATATGCGTCTATTTATGTAGGATGAAAG
Primers to introduce HA tag downstream of target gene		
sfGFP-HA	AF 4	AAAGAATTCAACATGTCTAAAGGTGAAGAACTGTTCA
	oRR134_sfGFP-XhoI-HA-HindIII_r	CATAAGCTTACGCGTAATCTGGCACATCGTACGGATACTCGAGTTTGTAGAGCTCATCCATGCC
Engineering Cre-lox reporters and Cre fusions		
Lox reporter	oRR220_loxP reporter_ult_f	GACGAATTCACCATGATAACTTCGTATAATGTATGCTATACGAAGTTATCTCGAGTAAGGTACCCATGTGATTTCTCCTGCTACCCCAAAAAAACGCGTGCATTCATTATAGAGAACCTAGTTATATTGGCAAACTAGTATAACTTCGTATAATGTATGCTATACGAAGTTATTCTAGACCCAAGCTTGTGATCAGGA
	oRR221_loxP reporter_r	TCCTGATCACAAGCTTGGGTCTAGA
kan^R	oRR214_KpnI-kanR_f	CTTGGTACCCTGAGCCATATTCAACGGGAA
	oRR215_kanR-SalI_r	CATGTCGACTTAGAAAAACTCATCGAGCATCAAAT
gent^R	oRR229_NheI-gentR_f	CATGCTAGCCTGTTACGCAGCAGCAACGATGTTACGC
	oRR230_gentR-HindIII_r	CCGAAGCTTAGGTGGCGGTACTTGGGTCG
pJ23103	DV_109	CGAGAGCTCTGATAGATATAACTAGGTTCTCGATTATGCATGCACGCGTTTTTTTGGGCTAGCAGGAG
kan^R promoter	oRR243_NheI-pmt kanR_f	CATGCTAGCATGCCTATTTGTTTATT
Cre	oRR207_NheI-Cre_f	CATGCTAGCCTGGTCCAAACTTCACTGCTC
	oRR208_Cre-XbaI-HindIII_r	CATAAGCTTATCTAGAGTCACCATCTTCTAAGAGGCG
	oRR209_Cre-HindIII_r	CATAAGCTTAGTCACCATCTTCTAAGAGGCG
	oRR239_EcoRI-Cre_f	AAAGAATTCACCATGGTCCAAACTTCACTGCTC

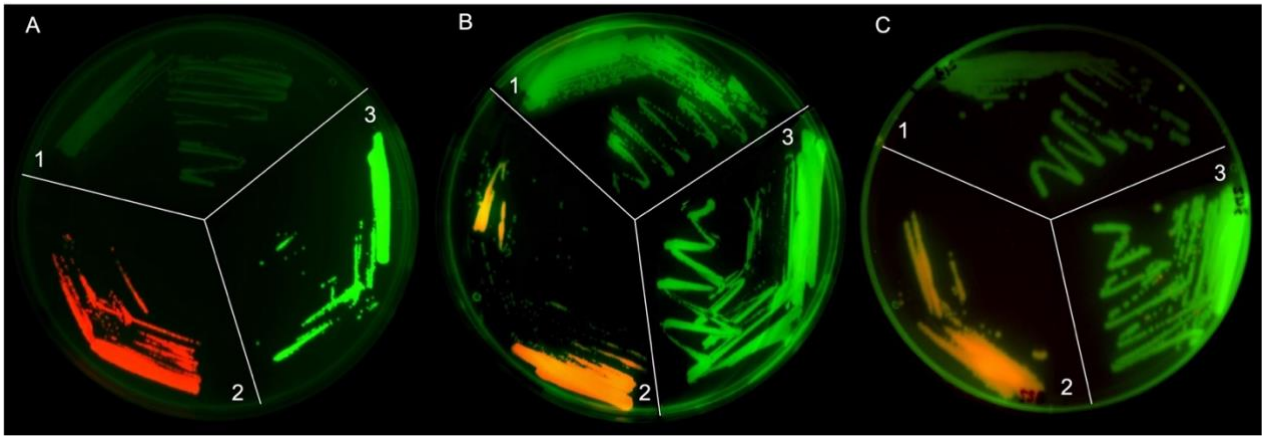

Figure S 3.1. LB plates streaked with (A) *E. coli*, (B) *P. aeruginosa* and (C) *V. cholerae* before and after FLP endogenous expression. Pictures represent *E. coli* NEB10b with empty vector pBAD24 (A1), pBAD24-FRT reporter (A2) and after endogenous recombination of pBAD33-FLP in the pBAD24-FRT reporter background (A3). *P. aeruginosa* PAO1 *tssB1* (B1), *P. aeruginosa* PAO1 *tssB1* chromosomally expressing the FRT reporter (B2) and after endogenous recombination of pPGA-FLP in the FRT reporter background (B3). *V. cholerae* V52 WT only (C1), V52 *vipB* chromosomally expressing the FRT reporter (C2) and after endogenous recombination of pBAD24oriT-FLP in the FRT background (C3). Plates were imaged with ChemiDoc MP Imaging System (Biorad) using Alexa488 channel (532/28) to detect green fluorescence and Rhodamine channel (602/50) to detect red fluorescence.

4 *Pseudomonas aeruginosa* cell wall barriers as means to defend against exogenous T6SS attacks

4.1 Introduction

In Chapter 3 we investigated whether *V. cholerae* T6SS could puncture the cell wall of *P. aeruginosa* and deliver effectors into the cytosol. However, it remains unclear whether *V. cholerae* T6SS can effectively deliver effectors into the cytosol of *P. aeruginosa* or if *P. aeruginosa* is more resistant to *V. cholerae* T6SS puncturing than to other species. Since the T6SS functions as a contact-dependent mechanism, prey cells may evade attacks by avoiding direct contact with the aggressors. This has been previously seen in planktonic cultures, where cells are suspended in a liquid, making direct contact less frequent and more challenging [179,209]. Since bacterial cells can create spatial distance from their aggressors, they might also be able to employ physical barriers to avoid T6SS attacks.

Numerous reports have demonstrated how T6SS effectors disturb the target cell [70,103,140,169,180], but less is known about the physical barrier each species provide to T6SS attacks. Target cells do not require T6SS-specific receptors to be recognised by aggressors, which allows T6SS great versatility. *P. aeruginosa* has a multitude of mechanisms that can confer protection to external pressures and may use these mechanisms to shield itself from exogenous T6SS attacks.

4.1.1 Exopolysaccharides

Bacteria can protect themselves from external factors by associating and forming biofilms. These aggregates comprise self-produced exopolysaccharides, proteins, lipids and eDNA [11]. This complex matrix forms a scaffold for surface attachment and provides a physical barrier to abiotic stresses, the host immune system and antimicrobials [273]. Interestingly, at the individual cell level, exopolysaccharides (EPS) have been shown to act as an armour that confers protection against exogenous T6SS attacks [194]. *V. cholerae vpsA* encodes an essential component in EPS production, and deleting this gene makes *V. cholerae* 10 times more vulnerable to *A. baylyi* and 100 times more vulnerable to *P. aeruginosa* T6SS. In *V. cholerae*,

EPS act similarly to “arrow slits in castle walls” – EPS shield *V. cholerae* from exogenous T6SS attacks without blocking its own T6SS from firing. Exopolysaccharides are key components of bacterial biofilms, but their shielding effect appears to be associated with individual cell protection rather than matrix protection provided by an established biofilm.

P. aeruginosa is able to secrete three major exopolysaccharides: alginate, Pel and Psl, each of which has a distinct chemical structure and role in biofilm formation [274]. Alginate is possibly the most well-studied exopolysaccharide in *P. aeruginosa* and plays a key role in biofilm stability and hydration. For example, in CF lungs there is an overproduction of alginate, which is responsible for creating large colonies and thick mucoid biofilms [275]. Alginate is encoded by an operon containing 14 structural genes, one of which is *alg44*. The product of *alg44* is a transmembrane protein important for alginate polymerisation, since the deletion of *alg44* prevents *P. aeruginosa* from producing alginate [276,277].

Psl is crucial for the initial attachment of planktonic cells to biotic and abiotic surfaces and for structural maintenance in the later stages of biofilm formation [275]. Besides its relevance as a matrix scaffold component, Psl acts as a signal molecule to control exopolysaccharide production and as a public good that benefits other cells within a biofilm [278,279]. Pel is important for bacterial aggregation that might be complementary to Psl. The *psl* locus contains 12 genes and the *pel* locus contains 7 genes, which respectively encode for the polysaccharides Psl and Pel synthesis and export [280]. Deleting *pslBCD* and *pelA* in *P. aeruginosa* leads to its inability to produce both Psl and Pel, thus impacting biofilm growth [281].

4.1.2 Lipid transport pathways

The cell envelope of Gram-negative bacteria is characterised by a double-membrane structure: an inner membrane (IM) that is in contact with the cell cytoplasm, and an outer membrane (OM) that is exposed to the extracellular milieu [282]. The double membrane is separated by the periplasmic space, which accounts for up to 20% of the whole cell volume and contains a thin layer of peptidoglycan. The OM is an asymmetric lipid bilayer that contains phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet [283]. Additionally, the OM has transmembrane proteins, often referred to as outer membrane proteins (OMPS), and lipoproteins that are anchored to the membrane through a lipid moiety.

Components of the OM are synthesised in the cytoplasm and must be transported through the IM and periplasm to reach the OM [284].

Lipoproteins are globular proteins with an N-terminal signal peptide and are synthesised in the cytoplasm [282]. Most lipoproteins cross the inner membrane in an unfolded state via the Sec pathway, but some cross it while folded via the Tat pathway. When lipoproteins reach the periplasm they undergo maturation steps and either remain in the IM or are transported to the OM by the Lol System [282]. The Lol system is composed of the ABC transporter LolCDE, which brings lipoproteins from the IM to chaperone protein LolA. LolA then facilitates the transport of lipoproteins across the periplasm to OM, where LolB mediated the insertion of lipoproteins in the OM.

Similarly, LPS are synthesised in the cytoplasm and transported to the OM across the periplasm by the Lpt system [285]. In *E. coli*, the Lpt system spans the whole cell envelope and is composed of seven essential proteins (LptABCDEFG). This trans-envelope protein complex is formed by two membrane assemblies in the IM and OM, which are interconnected by a periplasm protein (LptA). The LPS is formed by three distinct regions: the lipid A, the core oligosaccharide, and the O-antigen [286]. Interestingly, proper LPS formation in the aggressor is required to enable T6SS activity [287]. Environmental isolates of rough *V. cholerae* lacking O-antigen were unable to kill *E. coli* in a T6SS-dependent manner. Conversely, smooth *V. cholerae* isolates displayed a constitutively active T6SS that could kill *E. coli* and other environmental species.

Proteins of the MCE superfamily (originally named mammalian cell entry) are responsible for transporting hydrophobic molecules between the inner and outer membranes and are thus limited to double-membrane bacterial cells. In *E. coli*, the MCE superfamily has been associated with the import of molecules from the OM to the IM [288]. However, it has recently been suggested that this transport can also occur bidirectionally [289–291]. One of the most well-known protein complexes in the MCE superfamily is the Mla system (Maintenance of the OM Lipid Asymmetry). The Mla system is a tri-protein complex formed by a periplasmic protein (MlaC) that shuttles lipids between an IM complex (MlaFEDB) and an OM complex (MlaA-OmpF/C), preventing phospholipid accumulation in the OM [288]. YebT and PqiB are two other members of the MCE superfamily that have also been reported in *E. coli* [289]. These two large proteins form elongated tubes between the IM and OM and can transport lipids without

the aid of a periplasmic shuttle protein like MlaC [289]. YebT was later renamed as LetB (Lipophilic Envelope-spanning Tunnel B) and characterised as a trans-envelope conduit sufficiently long and wide for lipid transport between the IM and OM [291]. The OM plays a key role not only in the architecture of the cell, but also in its protection from external factors. Deleting genes in the Mla system has been shown to destabilise the OM, which further highlights the relevance of MCE proteins in the OM architecture [288,289,292].

Similar to what has been observed in *V. cholerae*, *P. aeruginosa* exopolysaccharides could also have a protective effect against exogenous T6SS attacks. Thus, manipulating *P. aeruginosa* exopolysaccharide expression could affect its resistance to exogenous T6SS attacks at the individual cell level. Here, we investigated *P. aeruginosa* resistance to exogenous T6SS attacks at the average cellular level rather than at population level. To test whether exopolysaccharides impact *P. aeruginosa* resistance to exogenous T6SS attacks we exposed *P. aeruginosa* mutant strains deficient in one or more genes encoding for exopolysaccharide production to *V. cholerae*. Additionally, we tested whether destabilising the OM of *P. aeruginosa* could sensitise cells to *V. cholerae* T6SS puncturing and effector delivery. Therefore, we also investigated whether the OM takes part in protecting *P. aeruginosa* against *V. cholerae* T6SS attacks. Similarly, we tested this by exposing *P. aeruginosa* mutant strains lacking a set of candidate genes that play a role in lipid transport to the OM to *V. cholerae*. In this Chapter, we used the previously generated H1-T6SS-deficient *P. aeruginosa* PAO1 (*ΔtssB1*) as parental strain. Henceforth, it will be referred simply as *P. aeruginosa*.

4.2 Results

4.2.1 *P. aeruginosa* EPS do not confer resistance to *V. cholerae* T6SS attacks

To investigate whether *P. aeruginosa* EPS is able to provide a physical barrier to exogenous T6SS attacks, we constructed in-frame deletions of specific genes in each of the loci encoding for the three essential exopolysaccharides in *P. aeruginosa*: alginate, Pel and Psl [220]. The alginate biosynthesis operon contains 14 structural genes encoding for alginate (Figure 4.1A). *P. aeruginosa* mutants resulting from the deletion of *alg44* have shown to be unable to produce alginate [276]. The *psl* locus contains 12 genes (Figure 4.1B) and the *pel* locus contains 7 genes (Figure 4.1C), which respectively encode for the exopolysaccharides Psl and Pel synthesis and export [280]. Deleting *pslBCD* and *pelA* in *P. aeruginosa* leads to its inability in producing both Psl and Pel, which impacts biofilm growth [281]. Therefore, we made single deletions of *alg44*, *pelA* and *pslBCD*, double deletions of a combination of these genes, and a triple deletion of these genes which we named EPS mutant.

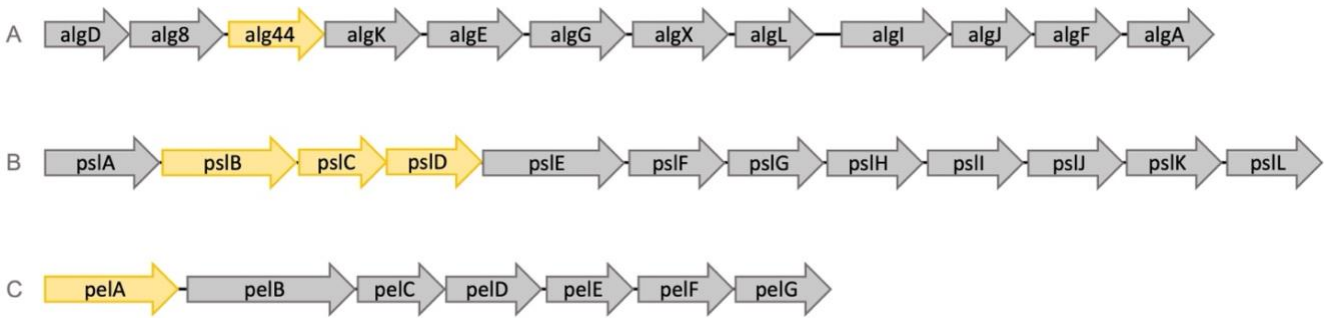

Figure 4.1 Genes responsible for encoding exopolysaccharides in *P. aeruginosa* PAO1: (A) Alginate operon, (B) Psl locus and (C) Pel locus. Genes deleted in this study are identified in yellow: *alg44* (PA3542), *pslB* (PA2232), *pslC* (PA2233), *pslD* (PA2234) and *pelA* (PA3064).

Since H1-T6SS-deficient *P. aeruginosa* was mildly susceptible to *V. cholerae* T6SS, we tested whether each of the *P. aeruginosa* exopolysaccharides mutants were increasingly affected by *V. cholerae* T6SS attacks. However, our observations indicated that none of *P. aeruginosa* mutants showed an increased sensitivity *to V. cholerae* T6SS-mediated killing (Figure 4.2).

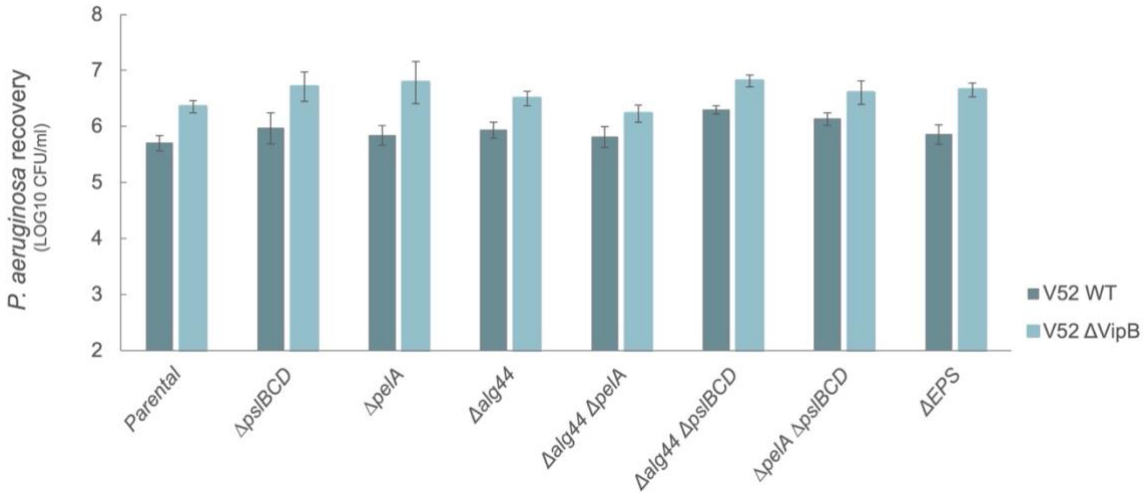

Figure 4.2. *P. aeruginosa* EPS do not confer resistance to *V. cholerae* T6SS attacks. Data represent the recovery of *P. aeruginosa ΔtssB1* (Parental) and EPS mutant strains following a competition assay with *V. cholerae* V52 WT or T6SS- (10:1 predator to prey ratio). Data are presented as mean $\log_{10}$CFU/ml ± SD of at least three experiments with two technical replicates. Statistical analysis using two-way ANOVA determined there was no significance between the recovery of *P. aeruginosa* mutants strains and the Parental strain ($p > 0.05$).

Considering that *P. aeruginosa* is capable of growing at a range of temperatures as high as 42°C, the temperature at which *P. aeruginosa* grows could differentially influence the production of exopolysaccharides. To investigate this, we grew overnight cultures of *P. aeruginosa* at different temperatures (30, 37 and 42°C), following which they were sub-cultured and grown to exponential phase at 37°C. We then performed our competition assays and assessed the recovery of *P. aeruginosa* after being preyed by either *V. cholerae* or *A. baylyi* T6SS. We observed that *P. aeruginosa* resistance to *V. cholerae* T6SS attacks remained unchanged regardless of the growth temperature at which *P. aeruginosa* grew overnight (Figure 4.3A). However, the susceptibility of *P. aeruginosa* to *A. baylyi* T6SS was significantly different depending on which temperature the cultures had grown overnight (Figure 4.3B).

In particular, we saw a significant reduction in the recovery of *P. aeruginosa* when the overnight culture had been grown a 42°C as opposed to 37°C. This temperature-dependent sensitivity to T6SS attacks could be associated with the fact that *P. aeruginosa* biofilm formation, including exopolysaccharide production, is most pronounced at lower temperatures [293].

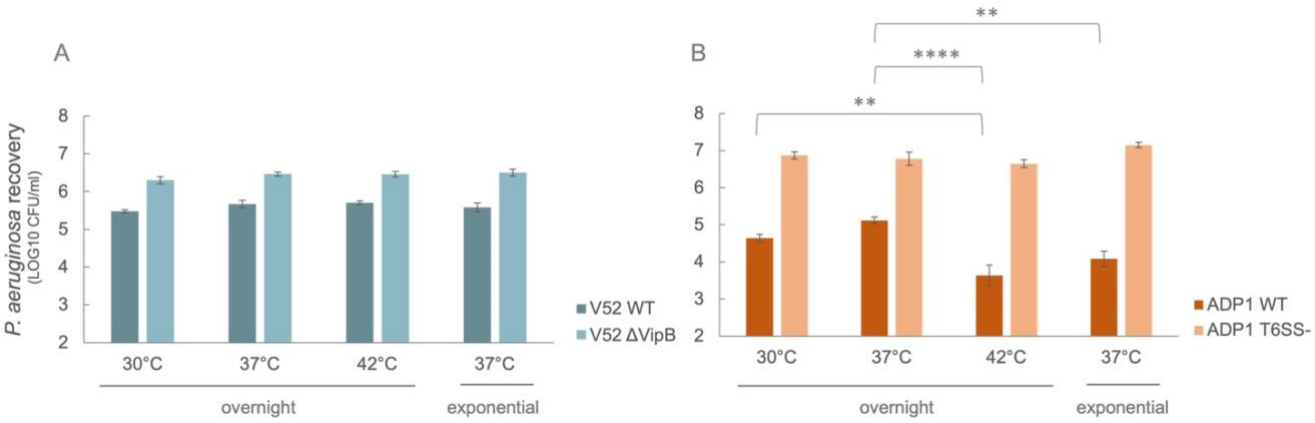

Figure 4.3. *P. aeruginosa* susceptibility to *A. baylyi* T6SS depends on the overnight growth temperature. Data represent the recovery of *P. aeruginosa ΔtssB1* following a competition assay (10:1 predator to prey ratio). Predators were (A) *V. cholerae* V52 WT or T6SS- (ΔVipB), and (B) *A. baylyi* ADP1 WT or T6SS-. Data are presented as mean $\log_{10}$CFU/ml ± SD of at least three experiments with two technical replicates. Statistical analysis was performed using two-way ANOVA followed by Tukey's post-hoc test (** $p \leq 0.01$, **** $p \leq 0.0001$).

4.2.2 Overnight growth temperature influences *P. aeruginosa* sensitivity to *A. baylyi* T6SS

Since *P. aeruginosa* resistance to *A. baylyi* T6SS was dependent on the overnight growth temperature of *P. aeruginosa* cultures, we investigated whether temperature differentially impacted each *P. aeruginosa* mutant to *A. baylyi* T6SS attacks. We have previously observed a significant difference between overnight cultures grown at 37 °C and 42°C. Therefore, we determined the survival of each *P. aeruginosa* mutant to *A. baylyi* T6SS following overnight growth at these temperatures (Figure 4.4). Our observations reveal that *P. aeruginosa* mutants were not significantly affected by *A. baylyi* T6SS when compared to the parental strain. This suggests that *P. aeruginosa* resistance to *A. baylyi* T6SS attacks depends on *P. aeruginosa* overnight growth temperature, but this is irrespective of the exopolysaccharide genes deleted.

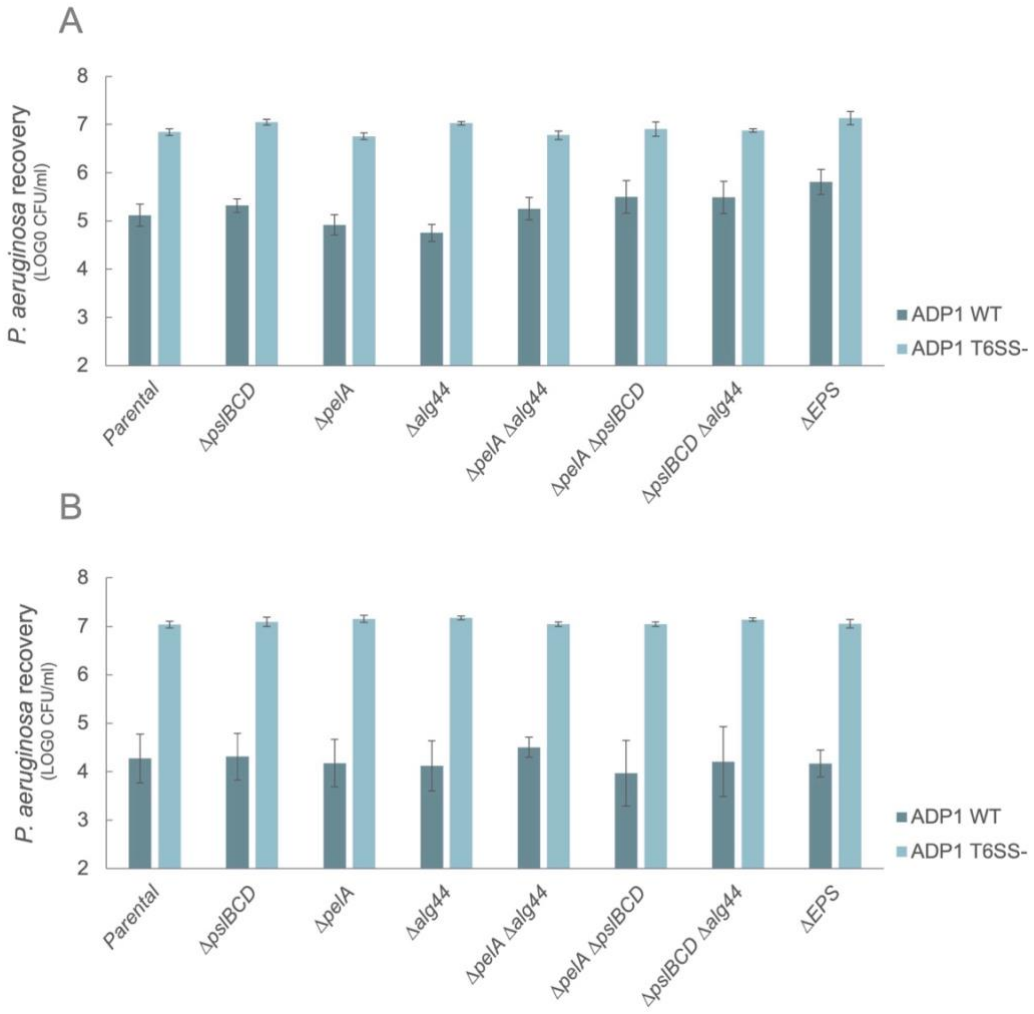

Figure 4.4. Overnight growth temperature influences *P. aeruginosa* susceptibility to *A. baylyi* T6SS. Data represent the recovery of *P. aeruginosa ΔtssB1* (Parental) and EPS mutant strains following a competition assay with *A. baylyi* ADP1 WT or T6SS- (10:1 predator to prey ratio). *P. aeruginosa* cultures before subculturing had grown overnight at (A) 37°C or (B) 42°C. Data are presented as mean $\log_{10}$CFU/ml ± SD of three experiments with two technical replicates. Statistical analysis using two-way ANOVA determined there was no statistically significant differences between the recovery of *P. aeruginosa* mutants strains and the Parental strain ($p > 0.05$).

4.2.3 Destabilising lipid transport systems does not increase *P. aeruginosa* susceptibility to *V. cholerae* T6SS attacks

In *P. aeruginosa*, the orthologous Mla system is encoded in the PA4452-4456 operon and MlaA (*vacJ*) is encoded by PA2800 (Figure 4.5) [294,295]. Because there is homology between the Mla operon and the toluene tolerance operon (*ttg2*), genes have been renamed as *ttg2A* (MlaF), *ttg2B* (MlaE), *ttg2C* (MlaD), *ttg2D* (MlaC) and *ttg2E* (MlaB) [295]. Deleting genes in the Mla operon has been shown to impact the cell membrane integrity. For example, a *ttg2D* mutant exhibits a weakened OM that deems the cell more susceptible to several membrane-disrupting agents [296]. Moreover, a *vacJ* mutant is more susceptible to cell wall stress and immune clearance [297].

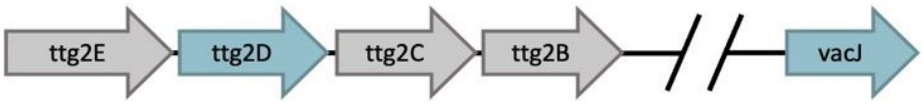

Figure 4.5. Mla operon in *P. aeruginosa* PAO1. Genes deleted in this study are identified in blue: *ttg2D* (PA4455) and *vacJ* (PA2800).

A BLAST (Basic local alignment search tool) search to find PqiB and LetB protein homology in *P. aeruginosa* has found a PqiB family protein orthologue encoded by gene PA4689. PqiB and LetB form trans-envelope conduits for lipid transport in *E. coli*. Therefore, we hypothesised that an orthologous protein in *P. aeruginosa* could function similarly. Here, we investigated whether destabilising lipid transport systems could sensitise *P. aeruginosa* to *V. cholerae* T6SS attacks. To test this we deleted three genes that play a role in the transport of molecules that make up the OM: *vacJ*, *pqiB* and *ttg2D*. Considering that *vacJ* and *ttg2D* belong to the Mla system we also tested whether deleting both genes could have a synergistic deleterious effect and further sensitise the OM to T6SS attacks. Our results showed that none of the individual mutants or the double mutants were more sensitive than the parental strain to *V. cholerae* T6SS killing (Figure 4.6).

It is possible that a specific *V. cholerae* effector is responsible for *P. aeruginosa* resistance to T6SS attacks. Alternatively, there might be competition for effector delivery, resulting in an insufficient effector payload to effectively reduce *P. aeruginosa* viability.

To test this, we analysed the survival of each *P. aeruginosa* mutant strain to *V. cholerae* T6SS attacks when aggressors delivered a pair-wise combination of toxic effectors. We observed a lethal effect comparable to that seen for *V. cholerae* WT, suggesting that destabilising lipid transport systems does not increase *P. aeruginosa* sensitivity to *V. cholerae* T6SS killing.

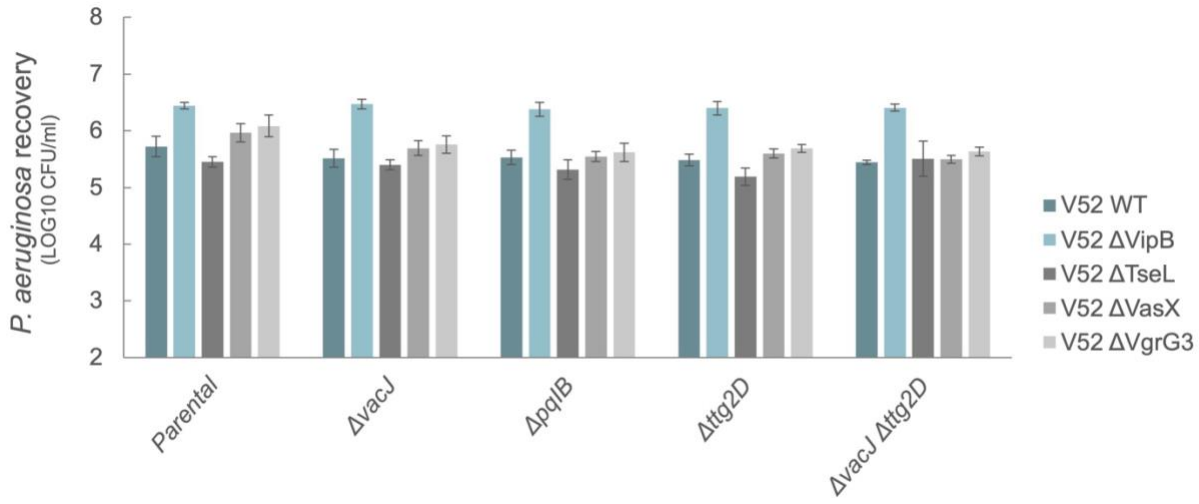

Figure 4.6. Destabilising lipid transport systems does not increase *P. aeruginosa* susceptibility to *V. cholerae* T6SS attacks. Data represent the recovery of *P. aeruginosa ΔtssB1* (Parental) and lipid transport mutant strains following a competition assay with *V. cholerae* V52 WT, T6SS- and single effector mutant strains (ΔTseL, ΔVasX and ΔVgrG3) (10:1 predator to prey ratio).Data are presented as mean $\log_{10}$CFU/ml ± SD of three experiments with two technical replicates. Statistical analysis using two-way ANOVA determined there was no significance between the recovery of *P. aeruginosa* mutant strains preyed by different *V. cholerae* strains ($p > 0.05$).

We also attempted to delete *surA*, a periplasmic chaperone that carries synthesised proteins from the IM to the OM; *lptH*, a functional homologue of *lptA*; and *lolA*, a chaperone protein in the Lol system. However, we were unable to successfully delete *surA*, *lptH* and *lolA* using a two-step allelic exchange approach, since all three genes are essential for the growth of *P. aeruginosa*. To potentially overcome our experimental setbacks, previously described methods could be used to construct conditional mutants for essential genes in *P. aeruginosa* [298,299].

4.3 Discussion

With a single contractile event, the T6SS can release toxic effectors that are deposited into neighbouring target cells. Exopolysaccharides produced by *P. aeruginosa* may create an obstruction to T6SS puncturing by other species. Here, we investigated whether specific putative genes encoding for EPS production in *P. aeruginosa* could confer protection against exogenous T6SS attacks by *V. cholerae* or *A. baylyi.* We hypothesised that by deleting a specific set of EPS genes in *P. aeruginosa*, the production of exopolysaccharides could be diminished. However, we demonstrated that by deleting genes encoding for EPS, *P. aeruginosa* was not significantly more susceptible to *V. cholerae* T6SS attacks (Figure 4.2).

P. aeruginosa undergoes temperature variations during its infection and transmission cycles, and is able to grow at temperatures as high as 42°C . These temperature variations have been shown to impact biofilm formation. The expression of *alg* and *pel* genes is increased at a lower temperature due to the increased levels of c-di-GMP, while the expression of *psl* genes increases with higher temperatures [293]. The growth temperature of *P. aeruginosa* might not have been ideal for EPS production, which could explain why a protective effect was not observed. Therefore, we tested whether *P. aeruginosa* growth temperature impacted resistance to *V. cholerae* and *A. baylyi* T6SS killing. We observed that there was no significant difference in the survival of *P. aeruginosa* to *V. cholerae* T6SS attacks, regardless of which temperature *P. aeruginosa* cultures had grown overnight (Figure 4.3A). Interestingly, we observed that *P. aeruginosa* was significantly more susceptible to *A. baylyi* T6SS attacks when the prey had grown overnight at 42°C rather than at 37°C (Figure 4.3B). This suggests that *P. aeruginosa* is differentially affected by *A. baylyi* T6SS attacks depending on its overnight growth temperature. A potential avenue to explore the effects of growth temperature in *P. aeruginosa* resistance to *A. baylyi* T6SS attacks could be through transcriptomic analysis of *P. aeruginosa*.

Because the survival of *P. aeruginosa* to *A. baylyi* T6SS was highest when prey had grown overnight at 37°C, but lowest when grown at 42°C, we hypothesised that *P. aeruginosa* resistance to *A. baylyi* T6SS could be because EPS confers higher protection at lower temperatures. Therefore, we tested whether *P. aeruginosa* mutants were less resistant to *A. baylyi* T6SS attacks when grown overnight at these two different temperatures (37 and

42°C). However, we observed that none of *P. aeruginosa* mutants were less resistant to *A. baylyi* T6SS at both of these temperatures (Figure 4.4). These results indicate that none of the EPS genes tested herein are involved in the protection of *P. aeruginosa* against *A. baylyi* T6SS attacks.

Nevertheless, other EPS genes may contribute to *P. aeruginosa* species-specific resistance to T6SS attacks. It has been previously shown that *alg44* encodes for an inner membrane co-polymerase that binds to c-di-GMP [276,277]. This co-polymerase is essential for alginate synthesis in *P. aeruginosa*, and its deletion abolishes alginate production. However, it has been shown that Alg44 might not be the only essential protein for alginate production and acts cooperatively with glycosyltransferase Alg8 for the polymerisation of alginate [300]. As such, under our test conditions, the single deletion of *alg44* might be insufficient to abolish alginate polymerisation.

Interestingly, a counter-cooperative effect has been suggested for Pel and Psl, which shows that Pel production is highest in the absence Psl [301]. The dependency of Pel and Psl in *P. aeruginosa* biofilm formation has been comprehensively studied. It has been suggested that in PAO1 strain, these two exopolysaccharides act in a complementary way. During biofilm formation, *psl* mutant strains adapt and acquire mutations to up-regulate Pel production in a compensatory way that drives biofilm survival [280]. However, this could not explain why *P. aeruginosa pslpel* mutants are still able to survive *V. cholerae* T6SS.

We have previously shown that *V. cholerae* effectors are toxic when expressed in the periplasm of *P. aeruginosa*. This suggests that *P. aeruginosa* is not inherently resistant to *V. cholerae* T6SS-effectors. However, *P. aeruginosa* is only mildly affected by *V. cholerae* direct T6SS attacks. It is possible that only a limited set of T6SS attacks can reach *P. aeruginosa* and/or that a limited amount of effector proteins can reach the periplasm of *P. aeruginosa* to exert a toxic effect. We have demonstrated that, unlike what was observed in *V. cholerae* [194], *P. aeruginosa* EPS is unable to confer protection against exogenous T6SS attacks. A possible explanation is that *V. cholerae* T6SS might be unable to puncture and deliver toxic effectors into *P. aeruginosa*. Alternatively, the deletion of these specific genes in *P. aeruginosa* might not have a considerable effect on EPS production and consequently may not compromise the cell's physical protection to exogenous T6SS attacks.

We then questioned whether disrupting *P. aeruginosa* lipid transport systems could sensitise the cells to exogenous T6SS attacks. Components of the bacterial OM are transported from the cytosol across the IM and periplasm by specialised systems. Disrupting these systems can destabilise the OM and make it more susceptible to disrupting agents and cell wall stresses [288,289,292]. It is possible that by impairing *P. aeruginosa* lipid transport systems, the cell is incapable of forming a proper OM and therefore becomes more susceptible to the puncturing and effector toxicity of *V. cholerae* T6SS. However, *P. aeruginosa* mutants lacking one or more genes important for molecule membrane translocation were not more susceptible to *V. cholerae* T6SS attacks (Figure 4.6).

A possible explanation is that the genes deleted may not affect the integrity of *P. aeruginosa* OM. These results raised the question as to whether deleting these specific genes involved in lipid transport effectively impairs the OM integrity. For example, we have reported for the first time a gene that encodes a PqiB family protein orthologue (PA4689) in *P. aeruginosa* PAO1. Future studies could inform on the role of this and other genes used in this Chapter in the membrane integrity of *P. aeruginosa*. This could be further explored with additional experimental work that assessed cell membrane integrity, like the 1-N-phenylnaphthylamine (NPN) assay [302]. Performing this assay could shed light on whether these genes are essential for *P. aeruginosa* OM integrity and further support our findings that disrupting the OM might not sensitise *P. aeruginosa* to exogenous T6SS attacks.

Table 4.1 Bacterial strains and plasmids used in this Chapter

Strain or plasmid	Relevant characteristics	Source
***Vibrio cholerae* V52**		
WT	Wild type, Str^R	[53]
ΔvipB	In frame deletion of amino acid 12 to 486 of *vipB* (VCA0108)	[171]
ΔvgrG3	In frame deletion of *vgrG3* (VCA0123)	[70]
ΔvasX	In frame deletion of amino acid 3 to 1077 of *vasX* (VCA0020)	[171]
ΔtseL	In frame deletion of amino acid 2 to 626 of *tseL* (VC1418)	[171]
***Acinetobacter baylyi* ADP1**		
WT	Wild type, Str^R (ATCC 33305)	[99]
T6SS-	Genes *aciad2688* to *aciad2694* replaced with Kan^R cassette	[99]
***Pseudomonas aeruginosa* PAO1**		
ΔtssB1	In frame deletion of amino acid 11 to 164 of *tssB1* (PA0083)	Chapter 2
Δalg44	*ΔtssB1*, in frame deletion of 8 to 375 of *alg44* (PA3542)	This study
ΔpelA	*ΔtssB1*, in frame deletion of amino acid 8 to 933 of *pelA* (PA3064)	This study
ΔpslBCD	*ΔtssB1*, in frame deletion of amino acid 5 of *pslB* to amino acid 253 of *pslD* (PA2232-PA2234)	This study
Δalg44 ΔpelA	*ΔtssB1, Δalg44, ΔpelA*	This study
Δalg44 ΔpslBCD	*ΔtssB1, Δalg44, ΔpslBCD*	This study
ΔpelA ΔpslBCD	*ΔtssB1, ΔpelA, ΔpslBCD*	This study
Δalg44 ΔpelA ΔpslBCD ("ΔEPS")	*ΔtssB1, Δalg44, ΔpelA, ΔpslBCD*	This study
ΔvacJ	*ΔtssB1*, in frame deletion of amino acid 11 to 229 of *vacJ* (PA2800)	This study
Δttg2D	*ΔtssB1*, in frame deletion of amino acid 7 to 259 of *ttg2D* (PA4455)	This study
ΔpqiB	*ΔtssB1*, in frame deletion of amino acid 8 to 754 of *pqiB* (PA4689)	This study
ΔvacJ Δttg2D	*ΔtssB1*, *ΔvacJ, Δttg2D*	This study
Escherichia coli		
NEB 10-beta	$DH10B^{TM}$ derivative, *recA1 relA1 endA1 rpsL* (Str^R)	New England Biolabs
SM10 λpir	*thi thr leu tonA lacy supE recA-RP4-2-Tc-Mu pir*	[251]
Plasmids		
pEXG2	Allelic exchange vector with pBR origin, Gm^R, *sacB*	[254]
pEXG2-alg44	Suicide vector for *alg44* deletion	This study
pEXG2-pelA	Suicide vector for *pelA* deletion	This study
pEXG2-pslBCD	Suicide vector for *pslBCD* deletion	This study
pEXG2-vacJ	Suicide vector for *vacJ* deletion	This study
pEXG2-pqiB	Suicide vector for *pqiB* deletion	This study
pEXG2-ttg2D	Suicide vector for *ttg2D* deletion	This study

Table 4.2. Oligonucleotides used in this Chapter

Amplicon	ID	Oligonucleotide sequence (5' to 3')
Primers to generate *P. aeruginosa* PAO1 exopolysaccharides mutants		
pslBCD	oRR155_KpnI-LF.pslBCD_f	ATAGGTACCCCGAATACCTGGTACGCAAC
	oRR156_LF.pslBCD_r	CGATCATTGGACGGCGTTCATCAGTAGAC
	oRR157_RF.pslBCD_f	AACGCCGTCCAATGATCGCTGAGGAGCGACA
	oRR158_RF.plsBCD-HindIII_r	CATAAGCTTGCGTCGTCGGTGTAGGTGCG
	oRR159_pslBCD_conf_f	TGGGCCTGTTTCCCTACCTC
	oRR160_pslBCD_conf_r	TTCGGCGAGCTGCTTTTCCT
pelA	oRR149_EcoRI-LF.pelA_f	ATAGAATTCTCCAGGCTGTGTTGGCGGT
	oRR150_LF.pelA_r	CGGCAACTCTCCTTTCTTGCTGAACCGCAT
	oRR151_RF.pelA_f	AAGAAAGGAGAGTTGCCGATGGAGCAGGT
	oRR152_RF.pelA-HindIII_r	CATAAGCTTGGCCATGTTCAGACGCAGGT
	oRR153_pelA_conf_f	ATTCCTGTGCTCTATCTGGC
	oRR154_pelA_conf_r	TTGCTTCCAAAAGCCCAGC
alg44	oRR161_EcoRI-LF.alg44_f	ATAGAATTCCGTGATGAGCGAGTGGCACA
	oRR162_LF-alg44_r	GCAGGGTGCTGACGTTGACGGCTGTATTCAT
	oRR163_RF.alg44_f	TCAACGTCAGCACCCTGCTGAACAAGGCC
	oRR164_RF.alg44-HindIII_r	CATAAGCTTATCTTTTCCAGGTCGCCCTG
	oRR165_alg44_conf_f	TATGGCTTCCGCGCGATTTC
	oRR166_alg44_conf_r	CGGTAGATCTGCCCCAGGTA
Primers to generate *P. aeruginosa* PAO1 lipid transport mutants		
vacJ	oRR169_KpnI-LF.vacJ_f	CATGGTACCGGATGGGGCTGGAGGACAG
	oRR170_LF.vacJ_r	CCTCCACCTGTTCGATCCAGTTCACGCCG
	oRR171_RF.vacJ_f	GGATCGAACAGGTGGAGGACGACTTCTAAGT
	oRR172_RF.vacJ-HindIII_r	CCGAAGCTTCCGAATGCTCGAGCACCG
	oRR173_vacJ_conf_f	GCAGCAATTGGAAACCGAAG
	oRR174_vacJ_conf_r	GAATGATCCGGTGGGAAAACT
pqiB	oRR181_XbaI-LF.pqiB_f	CGGTCTAGACGCCATCCTGGTATTCCTGT
	oRR182_LF.pqiB_r	CGCCCACTCCGGACTTGGCAGATCACTCA
	oRR183_RF.pqiB_f	CCAAGTCCGGAGTGGGCGCCGAAGATTC
	oRR184_RF.pqiB-HindIII_r	CATAAGCTTGTACTCCTGGCACTGTGCTT
	oRR185_pqiB_conf_f	CGAGAAAGCCAATTGTCCGC
	oRR186_pqiB_conf_r	CCAGGTGTTCCTGCGGGTAA
ttg2D	oRR175_KpnI-LF.ttg2D_f	CATGGTACCCTATAGTCCGCGCCCATTCG
	oRR176_LF.ttg2D_r	AAACATCAAGGGAGAGACTCTACGCATCAG
	oRR177_RF.ttg2D_f	GTCTCTCCCTTGATGTTTGGAGATTTCTGAATGC
	oRR178_RF.ttg2D-XbaI	CGGTCTAGACTGTCACGGATCTCCATGTT
	oRR179_ttg2D_conf_f	GGACTGCAATGCTCTGTTAC
	oRR180_ttg2D_conf_r	TCTTCCGCTTTCGCGGTT

5 *Pseudomonas aeruginosa* retaliatory H1-T6SS shapes the population dynamics of a multispecies community

Abstract

In microbial communities, bacterial antagonism can occur through the deployment of secretion systems that deliver toxic effectors into competitor cells. One such system is the Type 6 Secretion System (T6SS). *P. aeruginosa* encodes three T6SSs, one of which (H1-T6SS) specifically acts as a retaliatory weapon against exogenous T6SS activity of neighbouring bacteria. *P. aeruginosa* assembles and propels H1-T6SS at the exact point of an incoming T6SS attack, enabling it to efficiently eliminate those attackers without collateral damage. This phenomenon is called "tit-for-tat". Although previous studies have described the role of H1-T6SS in simple microbial communities, these studies have focused on pairwise combinations of organisms, ignoring the multispecies complexity of naturally occurring communities. Here, we investigate the role of "retaliator" *P. aeruginosa* H1-T6SS in shaping population dynamics of bacterial communities with multiple species, each with distinct behaviours. We examine population dynamics of communities containing *P. aeruginosa* along with a T6SS-attacker species or "aggressor" (*V. cholerae*) and a T6SS-sensitive species or "bystander" (*E. coli*). We observed that *P. aeruginosa* is able to protect bystanders from T6SS-mediated killing by the aggressors in a H1-T6SS-dependent manner. Interestingly, this protection was still observed even when *P. aeruginosa* was a minor part of the population, suggesting that H1-T6SS may be shaping the spatial organisation of the community whereby bystanders can avoid making contact with aggressors. Next, we used fluorescence microscopy to examine the spatial organisation of this multispecies community and observed that *P. aeruginosa* can also provide physical protection by shielding bystanders from incoming T6SS attacks. We further explored whether *P. aeruginosa* H1-T6SS-mediated protection requires recognition of specific effectors delivered by *V. cholerae* T6SS. Lastly, we investigated whether species-specificity influenced the T6SS-mediated protection of bystanders using *Acinetobacter baylyi* as a T6SS aggressor that is capable of efficiently eliminating H1-T6SS deficient *P. aeruginosa*. Our results showed that *P. aeruginosa* H1-T6SS can also protect bystanders from *A. baylyi* T6SS attacks. Our findings underscore the pivotal role of retaliatory T6SS behaviour in shaping population dynamics in multispecies communities that include *P. aeruginosa*.

5.1 Introduction

Microbial communities are extensively found in nature, and within them, cell-cell interactions play important roles in shaping the composition, productivity, and function of their population [6]. As discussed in Chapter 1, bacteria within these communities can exhibit cooperative and competitive behaviours. Competition can occur directly, whereby bacteria compete for niche colonisation and occupation by dominating the space, or indirectly by restricting the growth of competitors [21]. An example of bacterial competition within microbial communities is the deployment of secretion machineries that deliver toxic effectors into their competitors. One such machinery is the T6SS, which has been implicated in providing bacteria a competitive growth advantage within microbial communities. An example can be seen in microbial communities like biofilms, whereby T6SS-wielding species can overgrow and dominate other species. However, when these species T6SSs are inactivated, the competitive advantage is lost [189,303].

5.1.1 The T6SS shapes the spatial and structural organisation of microbial communities

Studying microbial communities is challenging due to the complexity of interactions that influence their structure and function [304–307]. In recent years, *in silico* methods, like agent-based models, have become valuable tools to investigate how T6SS-mediated microbial dynamics shape the spatial and structural organisation of microbial communities. Agent-based models, or individual-based models, are computational models that simulate the properties, activities and interactions between each individual agent (bacteria) with others and their environment [308]. For example, a model simulating a competition between T6SS-wielding and T6SS-sensitive species showed that killing occurs at the interface of both species, whereas growth occurs everywhere [309]. If a population of T6SS-sensitive cells is sufficiently large, it can continue to grow when being preyed by T6SS attackers and even outgrow their attackers. Another model showed that different *V. cholerae* T6SS-wielding strains undergo rapid phase separation between them, whereas T6SS-deficient strains remained well-mixed within the population [310]. This study later explored the effects of T6SS in public good cooperation. They showed that, by introducing a cooperator that secretes an exoproduct,

T6SS-mediated killing allows cooperators to resist invasion from non-producing "cheaters" due to their numerical advantage. However, without T6SS-mediated killing, cheaters outcompeted cooperators [310].

Another computational model was developed to simulate T6SS-mediated competitions between T6SS-wielding and T6SS-sensitive strains under increasing T6SS firing rates. This model revealed that the amount of dead cells increased with T6SS firing events which led to cell accumulation at the boundary between attackers and victims, in a phenomenon named the "corpse barrier effect" [233]. Similarly, another study described the effect of dead cells in a T6SS-mediated competitions within a microbial community. Using time-lapse microscopy they observed that a T6SS-proficient *V. cholerae* was able to kill its T6SS-sensitivecounterpart, but the accumulation of dead cell debris derived from the antagonistic attacks created a physical barrier to further contact killing. However, when these debris barriers were removed, the contact killing efficiently resumed [311].

Another study investigated the spatiotemporal organisation dynamics resulting from T6SS antagonism. Observations made using time-lapse microscopy showed that a mixed culture of T6SS-active *A. hydrophila* and *V. cholerae* formed segregated clusters [234]. Conversely, when the T6SS of the two species was inactive they co-existed in a well-mixed population. Moreover, when the clusters of both species came into direct contact, extensive cell death occurred at the interface between them, although cells remained protected from exogenous T6SS attacks inside each cluster [234]. This observation was also seen using an agent-based model and corroborates the previous findings on T6SS-mediated barrier killing effect [309].

This T6SS-mediated segregation effect was also observed in other species. Different T6SS-wielding *Vibrio fischeri* strains separated into microcolonies when co-incubated, but were well-mixed if their T6SSs were inactive [312]. A similar effect was also seen when *P. aeruginosa* was co-cultured with *S. maltophilia* [313]. *P. aeruginosa* formed large cell agglomerates which created a physical separation from the other species. However, when *P. aeruginosa* was co-cultured with a T6SS-deficient *S. maltophilia*, both species formed a well-mixed culture. Interestingly, despite analysing different species, these studies reached the same conclusion: the presence of an active T6SS drives a pattern of spatial organisation among microbial communities.

These studies underscore how T6SS-mediated killing can influence the spatial organisation of a microbial community. The deployment of T6SS shapes how cells arrange and compete within the community while T6SS-wielding species can also exhibit spatial behaviours that mitigate the effects of exogenous T6SS attacks. These observations suggest that the T6SS can have a dual role within a microbial community. In addition to driving cell overgrowth and spatial occupation, the T6SS also determines cell organisation within a community.

5.1.2 T6SS-specific behaviours within a microbial community

The dynamics of a microbial community is influenced not only by cell growth and spatial organisation but also by specific behaviours, including the secretion of proteins. For example, T6SS attackers that secrete fast-lysing effectors are more efficient in killing *E. coli*. This was observed after co-incubating T6SS-sensitive *E. coli* with *A. baylyi* mutants that secreted individual T6SS effectors [233]. An *A. baylyi* mutant carrying a fast-lysing effector is able to effectively clear *E. coli* whereas an *A. baylyi* mutant carrying a slow-lysing effector could not effectively clear *E. coli* despite its killing ability. Previously, researchers in this study had used a simulation model of T6SS competition that would remove dead cells that had been lysed. They proposed that the T6SS is an effective weapon by delivering lytic effectors that not only kill competitors but also cause their lytic disintegration [233].

As discussed in Chapter 1, bacterial cells can carry multiple weapons that allow for an effective competitive advantage. The range of such weapons dictates the outcome of bacterial interactions and how they influence a microbial community as a whole. Using an established agent-based model for bacterial competition it was predicted that the use of short- and long-range weapons might serve different purposes [314]. Short-range weapons, like T6SS and contact-dependent growth inhibition (CDI), provide a competitive advantage even if the producing cells are outnumbered. On the other hand, long-range weapons are effective when the producing cells are in abundance compared to their competitors. These predictions were later validated *in vitro* using *P. aeruginosa* as a model species for short- and long-range weapons. The efficacy of each weapon depends on the cell density it encounters: CDI acts efficiently across various cell densities, providing greatest advantage at initial low cell

densities, whilst R-type pyocins, as long-range weapons, are more effective at higher cell density [314].

Another example of specific behaviours that influence a microbial community is the interplay between T6SS and quorum sensing (QS). In a microbial community consisting of *V. cholerae* cells, both T6SS and QS have an impact on spatiotemporal cell death [315]. *V. cholerae* T6SS secretes lytic effectors that kill competing cells, thereby driving selective pressure for QS mutations. These mutations alter the expression of T6SS components or vibrio polysaccharide (Vps) production which allow cells to evade T6SS-mediated killing.

P. aeruginosa has distinct mechanisms to respond to bacterial antagonism within a microbial community. *P. aeruginosa* encodes three T6SSs (H1, H2 and H3), one of which (H1-T6SS) is specifically triggered by the presence of exogenous T6SS activity within a population. This specific response to antagonism was initially observed in a population of T6SS-active *P. aeruginosa* [87]. When attacked by a sister-cell, *P. aeruginosa* H1-T6SS assembles and propels at the exact point of attack and counterattacks. Similarly, this behaviour was observed when *P. aeruginosa* was also mixed with other T6SS-wielding species and has been referred to as "tit-for-tat" [99]. *P. aeruginosa* H1-T6SS can sense the activity of an exogenous T6SS, effectively counterattack and kill its competitors. Moreover, *P. aeruginosa* also employs an additional mechanism in response to bacterial antagonism. When *P. aeruginosa* cells die as a result of exogenous T6SS attacks, lysed cells release a signal that triggers the remaining surviving *P. aeruginosa* cells to activate the PARA system (*P. aeruginosa* response to antagonism) [248]. PARA acts as a signalling defence mechanism that increases the expression of H1-T6SS, allowing *P. aeruginosa* a competitive advantage over their neighbours.

The previous studies have focused on pairwise combinations of bacterial species, including those that investigated the role of the T6SS within a microbial community. However, it has been shown that pairwise combinations of species struggle to coexist, even though the same species thrive within a multispecies community [316]. Although *P. aeruginosa* is commonly associated with opportunistic infections, it lives ubiquitously in many environments and co-exists with other species in multispecies communities [317]. As such, models of pairwise combinations might be limited and inaccurate in providing a clear depiction of the population dynamics within complex multispecies communities.

Here, we explored the role of *P. aeruginosa* retaliatory H1-T6SS in the dynamics of a multispecies population. We considered whether *P. aeruginosa* H1-T6SS can effectively eliminate T6SS aggressors whilst sparing bystanders, thereby protecting bystanders from being eliminated. Furthermore, we examined how these T6SS-mediated species-specific interactions influenced the spatial organisation of multispecies communities. To test this we measured population dynamics of multispecies communities consisting of: a T6SS-attacker species or "aggressor", a T6SS-sensitive species or "bystander" and either a H1-T6SS-wielding *P. aeruginosa* ("retaliator") or its H1-T6SS-deficient counterpart. We have used *V. cholerae* and *E. coli* as representative species of aggressor and bystander behaviours, respectively. Additionally, we used fluorescence microscopy to get a more detailed picture of the mechanistic aspect of population dynamics and further investigated the role of *V. cholerae* T6SS effectors in the dynamics of these multispecies community. Lastly, we replaced *V. cholerae* with *A. baylyi* to investigate whether other T6SS attackers and thus species-specificity influence the dynamics of a multispecies population with *P. aeruginosa.* For the purposes of this chapter, when referring to *P. aeruginosa* T6SS we are specifically mentioning *P. aeruginosa* H1-T6SS.

5.2 Results

5.2.1 *P. aeruginosa* H1-T6SS protects *E. coli* from *V. cholerae* T6SS attacks

Bacteria regulate their T6SS assembly and firing in different ways. Whilst *V. cholerae* and *A. baylyi* deploy their T6SSs as aggressive weapons, *P. aeruginosa* employs its H1-T6SS in response to exogenous T6SS attacks as a retaliatory weapon [99]. These behaviours have been previously described at the level of pairwise competitions, but how they manifest in a multispecies community has not been explored. Here, we examined multispecies communities over time to determine the influence of a retaliatory T6SS in microbial community dynamics. Based on previous findings that *P. aeruginosa* T6SS specifically retaliates against exogenous T6SS attacks, we hypothesised that *P. aeruginosa* T6SS could protect T6SS-sensitive victims from T6SS-aggressors.

To test this, we selected *V. cholerae* 2740-80 as a T6SS-aggressor and *E. coli* MG1655 as a T6SS-sensitive species or bystander. As an initial test, we mixed equal proportions of aggressor and bystander species (1:1) and co-incubated this mixture with 25% of either T6SS+ or T6SS- *P. aeruginosa* PAO1 for 2 and 4 hours. Following incubation, we observed that *E. coli* survival significantly increased when *P. aeruginosa* T6SS+ was part of the initial population (Figure 5.1). This result suggests that *P. aeruginosa* H1-T6SS protects *E. coli* from *V. cholerae* T6SS attacks.

P. aeruginosa retaliatory H1-T6SS attacks are known to selectively antagonise T6SS aggressors without causing collateral damage [99]. Therefore, *P. aeruginosa* H1-T6SS could be directly antagonising *V. cholerae*, allowing *E. coli* to grow unimpeded. Additionally, *P. aeruginosa* might be creating a physical barrier between aggressor and bystander, preventing *V. cholerae* to reach and eliminate its victims.

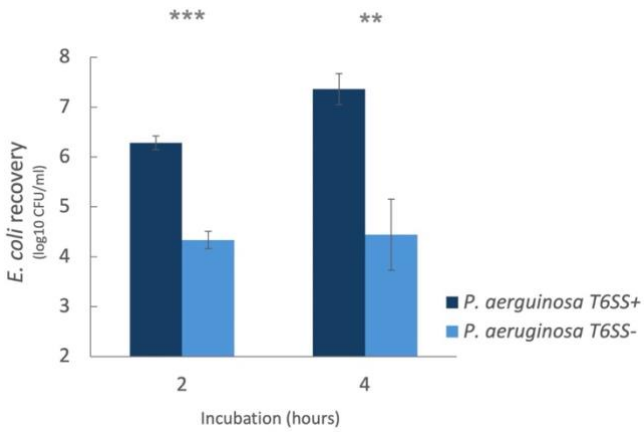

Figure 5.1 In a multispecies population, the survival of *E. coli* increases when *P. aeruginosa* T6SS+ is part of the population. Data represent the recovery of *E. coli* following a multispecies competition assay that involved combining a 1:1 ratio of a T6SS-aggressor (*V. cholerae* 2740-80) and a T6SS-sensitive species (*E. coli* MG1655), and 25% of either T6SS+ or T6SS- *P. aeruginosa* PAO1. Data is presented as mean $\log_{10}$CFU/ml ± SD of at least three experimental replicates with two technical replicates. Statistical significance was determined using a two-tail unpaired student's t-test (** $p \leq 0.01$, *** $p \leq 0.001$).

5.2.2 *E. coli* protection from *V. cholerae* T6SS attacks depends on the abundance of *P. aeruginosa* H1-T6SS in the initial population

Considering that *P. aeruginosa* H1-T6SS can protect *E. coli* from being eliminated, we examined how much *P. aeruginosa* was required to confer this protective effect. As such, we expanded our competition assay to include varying amounts of *P. aeruginosa* in the initial population and to take place during increasing incubation periods. Briefly, a defined 1:1 ratio of *V. cholerae* 2740-80 and *E. coli* MG1655 were mixed with different amounts of either H1-T6SS+ or T6SS- *P. aeruginosa* PAO1 (5, 10, 25, 35, 50 and 75%). Each multispecies population was plated onto LB agar and incubated for a defined time period (2, 4, 8 and 21 hours), after which cells were recovered and plated to isolate and enumerate each individual species in the final population (Figure 5.2).

5.2.2.1 Large amounts of *P. aeruginosa* H1-T6SS in the population

When *P. aeruginosa* T6SS+ constitutes a greater portion of the initial population (50–75%), it largely outnumbers both *V. cholerae* and *E. coli*. This suggests that large amounts of *P. aeruginosa* T6SS+ have the potential to greatly protect *E. coli* (bystander) by eliminating *V. cholerae* (aggressor). After determining the survival of each species in the multispecies population, we observed that *E. coli* survival was significantly higher when *P. aeruginosa* T6SS+ was a large part of the initial population. *V. cholerae* was consistently eliminated earlier in the incubation, which indicates that *E. coli* survived because *V. cholerae* was eliminated by *P. aeruginosa* in a H1-T6SS-dependent manner. However, at later timepoints (21h), *V. cholerae* was no longer eliminated by *P. aeruginosa* H1-T6SS, suggesting that an alternative protective mechanism occurred (Figure 5.2).

5.2.2.2 Moderate amounts of *P. aeruginosa* T6SS+ in the population

It would be expected that lowering the amount of *P. aeruginosa* T6SS+ in the initial population would also reduce *V. cholerae* elimination, and thus the survival of *E. coli*. Surprisingly, when *P. aeruginosa* T6SS+ represented 10-35% of the initial population, results showed a significant survival of *E. coli* earlier in the incubation. However, at these timepoints,

V. cholerae was not being eliminated by *P. aeruginosa* in a H1-T6SS-dependent manner, as no significant differences where observed in the recovery of *V. cholerae* when incubated with *P. aeruginosa* T6SS+ orT6SS-. Therefore, *P. aeruginosa* could be protecting *E. coli* by creating physical a barrier between *V. cholerae* and *E. coli* (Figure 5.2).

5.2.2.3 Minimal amounts of *P. aeruginosa* T6SS+ in the population

When *P. aeruginosa* T6SS+ is minimal within the initial population (5%) it is largely outnumbered by both *V. cholerae* and *E. coli*. This means that for every *P. aeruginosa* cell there are more than eight cells of each of the other species. Thus, it was unlikely that *P. aeruginosa* T6SS+ would be able to protect *E. coli* from *V. cholerae* T6SS attacks due to being greatly outnumbered. Notably, we observed that *E. coli* survival significantly increased in the presence of *P. aeruginosa* T6SS+ after longer incubation periods. *P. aeruginosa* was able to outcompete *V. cholerae* in a H1-T6SS-dependent manner and allow *E. coli* to survive. This suggests that *P. aeruginosa* T6SS+ must be allowed to grow in order to compete against *V. cholerae* (Figure 5.2).

In summary, *P. aeruginosa* T6SS+ significantly protects *E. coli* when it makes up a large part of a multispecies community with *V. cholerae* as a T6SS-aggressor. This occurs either because *P. aeruginosa* T6SS+ is initially a large constituent of the population (50-75%, 2-8h), or because cells had time to replicate and grow until they represented a large amount of the population (5%, 21h). In both instances, *P. aeruginosa* H1-T6SS protects *E. coli* through mass elimination of *V.* cholerae. On the other hand, when *P. aeruginosa* T6SS+ is present in moderate amounts within the multispecies community, the survival of *E. coli* is not due to *V. cholerae* mass elimination but rather by an unidentified mechanism.

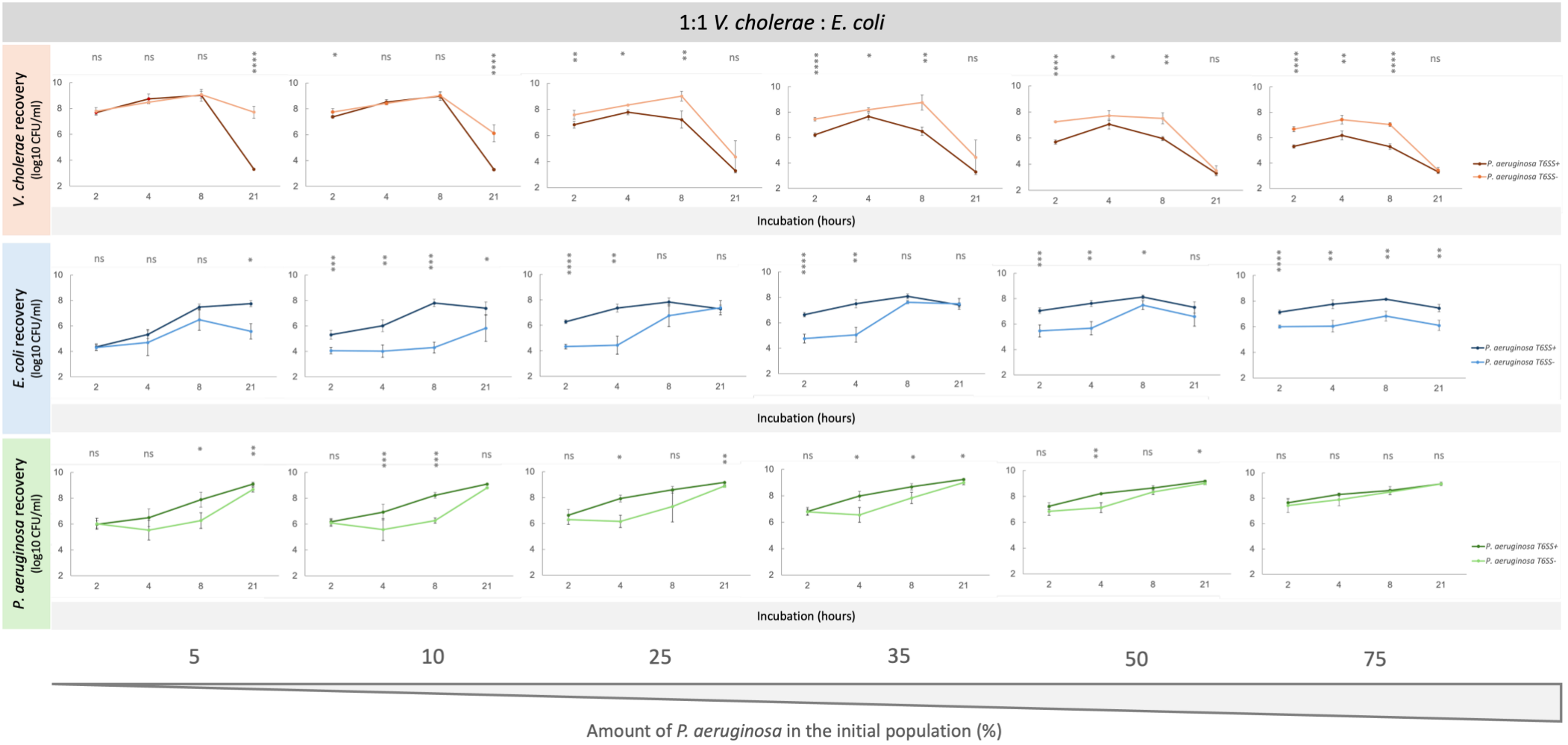

Figure 5.2 *E. coli* protection from *V. cholerae* T6SS attacks depends on the abundance of *P. aeruginosa* in the initial population. Data represent the recovery of each species following a multispecies competition assay where a 1:1 ratio of a T6SS-aggressor (*V. cholerae* 2740-80) and a T6SS-sensitive species (*E. coli* MG1655) were mixed with varying amounts of either T6SS+ or T6SS- *P. aeruginosa* PAO1. The multispecies population was incubated over increasing time periods, following which cells were recovered in the appropriate antibiotics to allow enumeration of each individual species. Data is presented as mean $\log_{10}$CFU/ml ± SD of at least three experimental replicates with two technical replicates. Statistical significance was determined using a two-tailed unpaired t-test to compare species recovery when either *P. aeruginosa* T6SS+ or T6SS- was part of the population at each timepoint (ns. non-significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

5.2.3 Large amounts of *V. cholerae* in the population

It is unclear whether *P. aeruginosa* H1-T6SS is able to eliminate almost all the viable *V. cholerae* cells within the population because both aggressor and bystander species are present in equal proportions. Therefore, we investigated whether *P. aeruginosa* H1-T6SS is able to eliminate *V. cholerae*, and thus protect *E. coli*, when aggressors outnumber bystanders by 100 times.

We considered that *P. aeruginosa* T6SS+ might not be able to compete against larger amounts of *V. cholerae*, which would result in a reduced protection of *E. coli*. To test this, we reproduced the previous experimental setup, but increased the amount of *V. cholerae* within the multispecies community. Herein, a defined 100:1 ratio of *V. cholerae* 2740-80 and *E. coli* MG1655 were mixed with different amounts of either H1-T6SS+ or T6SS- *P. aeruginosa* PAO1 (5, 10, 25, 35, 50 and 75%). Each multispecies population was plated onto LB agar and incubated for a defined time period (2, 4, 8 and 21 hours), after which cells were recovered and plated to isolate and enumerate each individual species in the final population (Figure 5.3).

The results of this multispecies competition showed the same previously observed pattern, whereby large amounts of *P. aeruginosa* T6SS+ in the population were able to eliminate almost all *V. cholerae*, thus allowing *E. coli* to survive. Interestingly, unlike what was observed previously, small amounts of *P. aeruginosa* T6SS+ in the initial population (5%) could significantly protect *E. coli* earlier during the incubation. However, as observed before, *P. aeruginosa* T6SS+ only eliminated almost all *V. cholerae* after over 8 hours of incubation. These observations indicate that the survival of *E. coli* is initially due to an unknown protective mechanism, but later on the incubation, it is due to mass elimination of *V. cholerae* by *P. aeruginosa* T6SS+. In summary, regardless of the amount of *V. cholerae* in the initial multispecies population, *P. aeruginosa* H1-T6SS is able to protect *E. coli* from being killed.

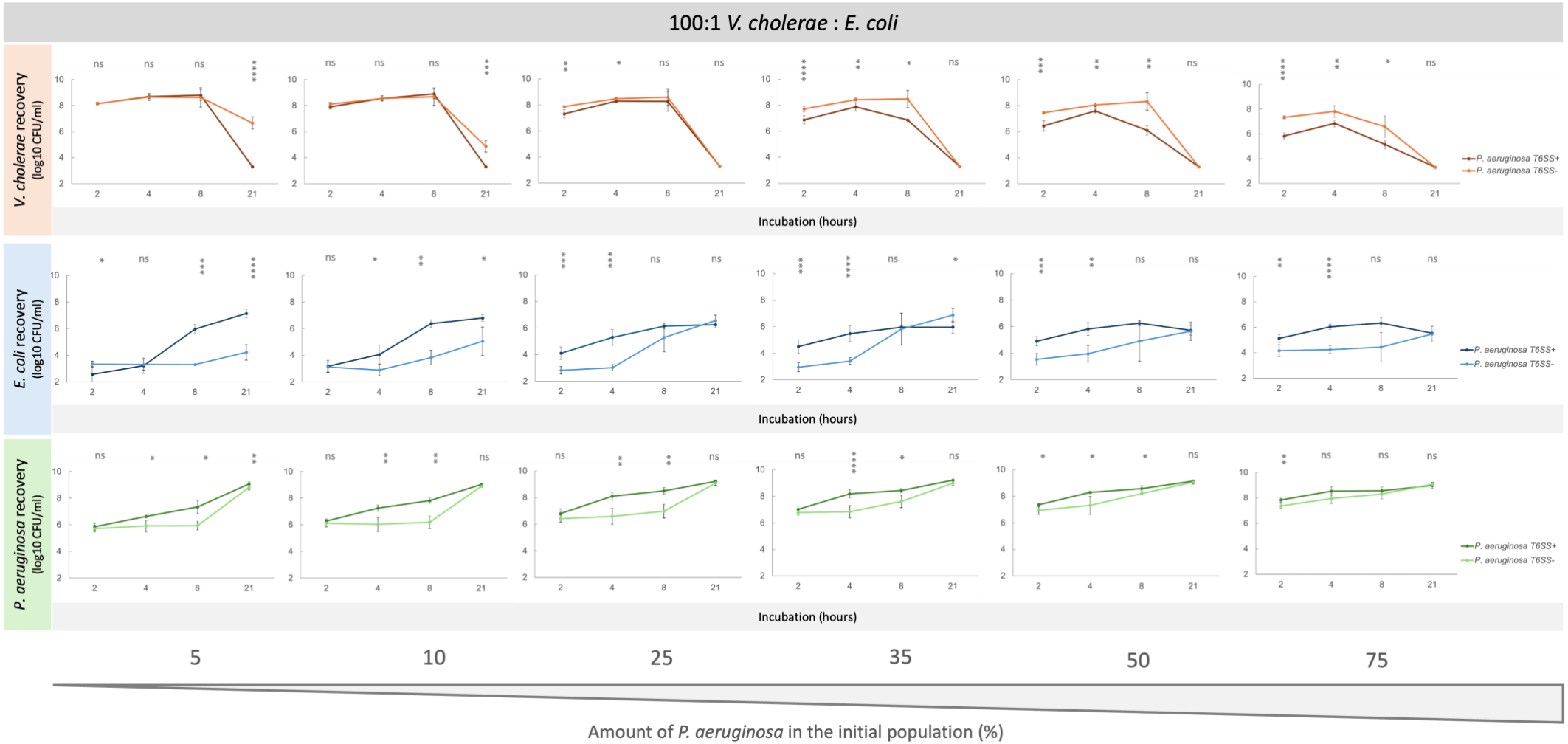

Figure 5.3 *P. aeruginosa* H1-T6SS is able to protect *E. coli* even when *V. cholerae* constitutes large amounts of the multispecies population. Data represent the recovery of each species following a multispecies competition assay where a 100:1 ratio of a T6SS-aggressor (*V. cholerae* 2740-80) and a T6SS-sensitive species (*E. coli* MG1655) were mixed with varying amounts of either T6SS+ or T6SS- *P. aeruginosa* PAO1. The multispecies population was incubated over increasing time periods, following which cells were recovered in the appropriate antibiotics to allow enumeration of each individual species. Data is presented as mean log_{10}CFU/ml ± SD of at least three experimental replicates with two technical replicates. Statistical significance was determined using a two-tailed unpaired t-test to compare species recovery when either *P. aeruginosa* T6SS+ or T6SS- was part of the population at each timepoint (ns. Not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

5.2.4 *P. aeruginosa* physically protects *E. coli* from *V. cholerae* T6SS attacks

The previous experimental data represents the bulk average of a multispecies population without specific details of the spatial organisation of the population. Therefore, it is challenging to determine the specific mechanism behind *E. coli* protection within the population. As previously observed, one way *P. aeruginosa* H1-T6SS can protect bystanders is by extensively eliminating aggressors. However, in certain instances, *P. aeruginosa* H1-T6SS is not protecting *E. coli* through mass elimination of *V. cholerae*, but rather by an unknown mechanism (Figure 5.2).

It is entirely possible that *P. aeruginosa* cells are providing physical protection, and thus aggressors are unable to reach bystanders in order to eliminate them. One such example is the "corpse barrier effect", whereby T6SS aggressors kill their victims and the resulting corpses accumulate at the boundary between aggressors and victims [233,311]. This barrier physically prevents the aggressors from reaching the remaining victims and continuing the killing. Another mechanism of physical protection can occur when T6SS-wielding species segregate into clusters, whereby aggressors kill each other only at the boundary were the clusters meet, thus sparing the cells within the cluster [234,309].

To understand whether physical protection is occurring and which mechanism is behind it, we used fluorescence microscopy to visualise the cellular organisation of the multispecies community. To do this, we expressed different fluorescent proteins in each species of the community: *V. cholerae* expressed sfCherry2 (red), *E. coli* expressed mTagBFP2 (blue) and *P. aeruginosa* expressed scGFP (green). We previously observed that when *P. aeruginosa* T6SS+ made up 25% of the initial population, the survival of *E. coli* significantly increased without mass elimination of *V. cholerae* (Figure 5.2). Therefore, we decided to further explore this scenario in the microscopy analysis. We mixed equal proportions of *V. cholerae* and *E. coli* (1:1) with 25% of *P. aeruginosa* T6SS+ or T6SS-. We then imaged the multispecies community immediately after mixing all three species, and 2 hours post incubation.

The multispecies suspension was initially seeded at a high density onto an agarose pad on a glass slide to allow visualisation of the population when cells were in direct physical contact. However, after 2-hours of incubation the population was overcrowded to allow

appropriate visualisation. Therefore, we seeded the multispecies suspension at a lower density to analyse the community dynamics of a population when cells are initially growing and expanding.

When the multispecies population was imaged immediately after mixing all three species, our observations revealed that *V. cholerae* (red) formed clusters of aggregated cells (Figures 5.4 A and B). This phenotype was expected, as *V. cholerae* cells naturally clump (indicated by arrows in the figure). On the contrary, both *E. coli* (blue) and *P. aeruginosa* (green) cells remained dispersed within the population. Following a 2-hour incubation period, we observed a consistent phenomenon of cell segregation within the population, whereby each species grew and formed a subpopulation of kin cells within the general population (Figures 5.4 C and D). These segregated clusters were more noticeable in crowded areas of the population. These observations were similar whether *P. aeruginosa* T6SS+ or T6SS- were part of the population. Considering that our aim was to investigate the role of H1-T6SS in population dynamics, we further focused our observations in populations containing *P. aeruginosa* T6SS+.

Furthermore, we also observed a phenomenon whereby a debris of dead cells accumulated at the barrier between cells (Figure 5.4 E). By creating a physical hindrance, this barrier of debris (pointed by triangles in the figure) can physically prevent aggressors from reaching and killing their victims. All the while, bacterial cells that are not in direct contact with aggressors can continue to grow unhindered.

A question arose when we compared these observations with our multispecies competition assay (Figure 5.2). How was *V. cholerae* not being eliminated? Reasonably, *P. aeruginosa* T6SS+ eliminates *V. cholerae* cells at the boundary where both species encounter. All the while, *V. cholerae* cells within the cluster will continue to replicate and grow. Therefore, it is possible that *V. cholerae* cells exist in a state of equilibrium, where its growth rate matches its death rate. Collectively, our results indicate that *P. aeruginosa* is able to physically protect *E. coli* from *V. cholerae* T6SS attacks, by creating a defence barrier between T6SS aggressors and bystanders.

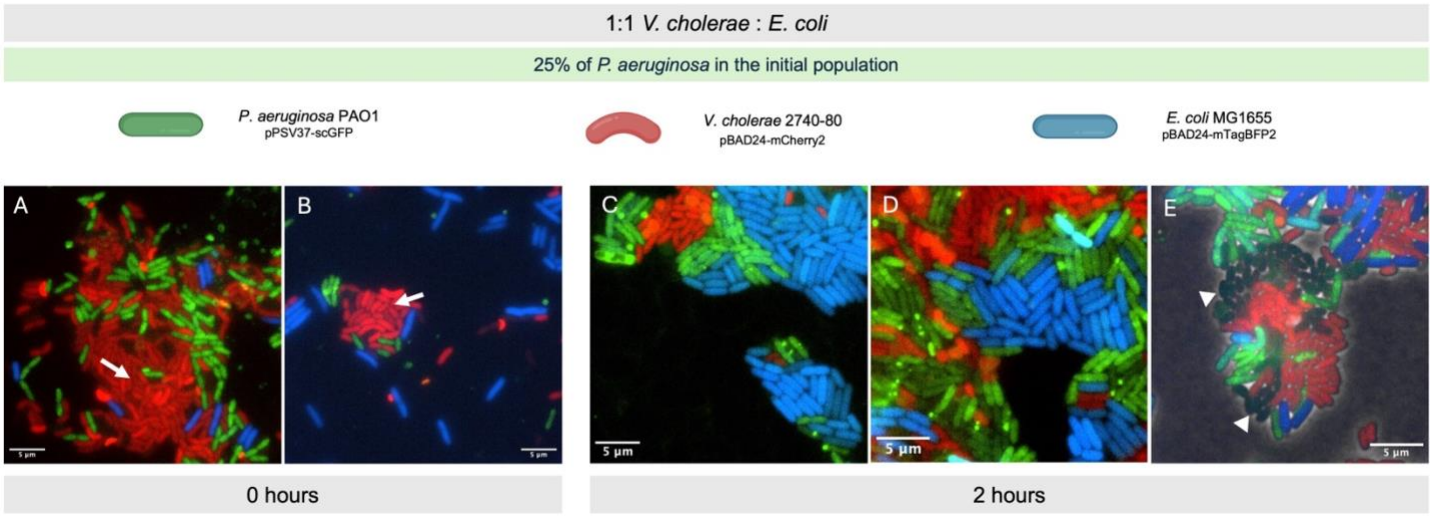

Figure 5.4 *P. aeruginosa* physically protects *E. coli* from *V. cholerae* T6SS attacks. Microscopy analysis of a multispecies population consisting of *P. aeruginosa* PAO1 expressing scGFP (green), *V. cholerae* 2740-80 expressing sfCherry2 (red) and *E. coli* MG1655 expressing mTagBFP2 (blue). Equal proportions of *V. cholerae* and *E. coli* (1:1) were mixed with 25% *P. aeruginosa* T6SS+ and the multispecies population was imaged immediately after species were mixed, and after 2h of incubation at 37ºC. A-B) Initially, *V. cholerae* aggregates into cells clusters (pointed out by arrows) whereas *P. aeruginosa* and *E. coli* cells remain dispersed in the population. C-D) Following incubation, *P. aeruginosa* protects *E. coli* from being killed by *V. cholerae* T6SS by creating a physical barrier between aggressors and victims. E) This killing effect results in a debris of dead cells (pointed out by triangles) that physically prevents *P. aeruginosa* T6SS+ to continue on killing. *V. cholerae* cells that are not directly in contact with *P. aeruginosa*, can continue to grow unimpeded. Images are representative of 4 independent replicates with at least 10 fields of view.

5.2.5 *P. aeruginosa* T6SS protects *E. coli* from *V. cholerae* T6SS attacks regardless of incoming effectors

Previous studies have reported that *V. cholerae* T6SS effectors contribute differentially to *P. aeruginosa* H1-T6SS retaliation and *E. coli* elimination [186,247]. If a single *V. cholerae* T6SS effector drives *P. aeruginosa* H1-T6SS retaliation, its absence would reduce *E. coli* protection. Similarly, if a single effector is responsible for killing *E. coli*, then its absence would prevent *E. coli* from being eliminated. As such, we examined whether a single *V. cholerae* T6SS effector could influence the dynamics of a multispecies community and impact the protection of *E. coli*.

To test this, *E. coli* was individually mixed with *V. cholerae* WT or mutant strains lacking a single T6SS-effector (VgrG3, VasX or TseL) and co-incubated with either 5 or 25% of *P. aeruginosa* T6SS+ or T6SS-. Since we previously observed a significant difference in *E. coli* protection from earlier during the incubation (Figure 5.2), we incubated these multispecies population for 2 hours (Figure 5.5). Our results showed that the absence of each individual *V. cholerae* T6SS effector did not decrease the survival of *E. coli* in the multispecies population

(Figure 5.5 C and D). This observation suggests that *P. aeruginosa* H1-T6SS is able to protect *E. coli* regardless of which *V. cholerae* T6SS effectors are being secreted.

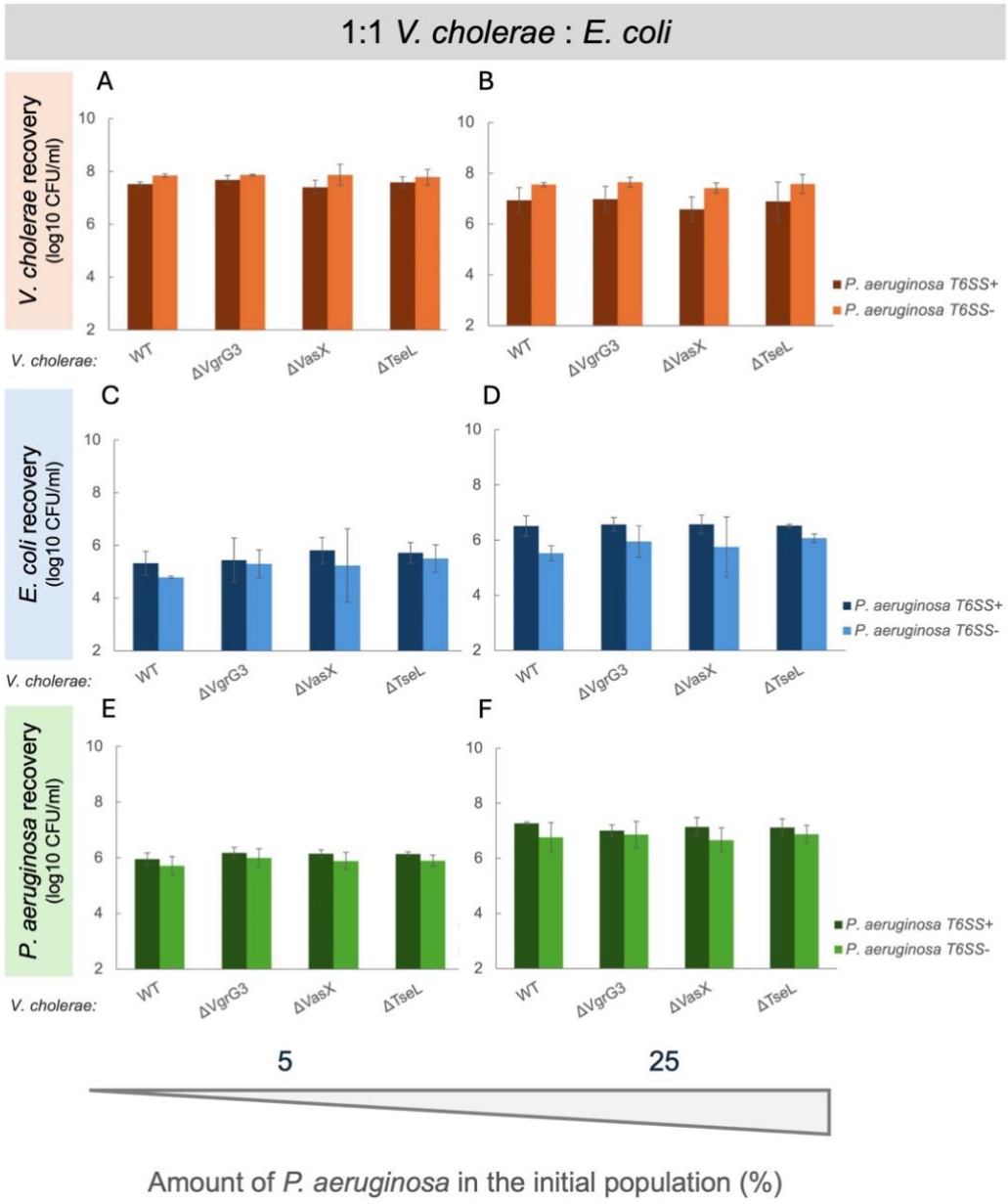

Figure 5.5 *P. aeruginosa* T6SS protects *E. coli* from *V. cholerae* T6SS attacks regardless of incoming effectors. Data represent the recovery of each species following a multispecies competition assay that involved combining a 1:1 ratio of a T6SS-aggressor (*V. cholerae* 2740-80) with a T6SS-sensitive species (*E. coli* MG1655) with 5 or 25% of *P. aeruginosa* PAO1 T6SS+ or T6SS-. T6SS-aggressors were either *V. cholerae* WT or single effector mutant strains (ΔVgrG3, ΔVasX and ΔTseL). The multispecies population was incubated at 37°C for 2h incubation, following which, cells were recovered in the appropriate antibiotics to allow enumeration of each individual species. Data represent the recovery of *V. cholerae* (A and B), *E. coli* (C and D) and *P. aeruginosa* (E and F) as mean $\log_{10}$CFU/ml ± SD of three experimental replicates with two technical replicates. Statistical analysis was performed using a two-way ANOVA to determine the effects of each strains' recovery when incubated with *P. aeruginosa* T6SS+ or T6SS-. No statistically significant differences were observed ($p > 0.05$).

However, it was unclear whether a single *V. cholerae* T6SS effector is responsible for eliciting retaliatory attacks by *P. aeruginosa* H1-T6SS or for eliminating *E. coli*. Previous studies suggest differential retaliatory and killing effect depending on the *V. cholerae* aggressor strain [186,247]. To help clarify whether *P. aeruginosa* H1-T6SS or *E. coli* respond differentially to *V. cholerae* effectors we performed 1:1 pairwise competitions. Briefly, competition assays were performed between *V. cholerae* 2740-80 WT or single effector mutant strains against either *E. coli* (Figure 5.6), to assess the elimination of a T6SS-sensitive species, or *P. aeruginosa*, to assess H1-T6SS-mediated retaliation (Figure 5.7).

Under our experimental conditions, we observed that *V. cholerae* single effector mutant strains were unable to kill *E. coli* as efficiently as wild-type *V. cholerae*, and that *E. coli* elimination was similar by each of the single effector mutants (Figure 5.6 A). Moreover, the effect observed was not caused by defects in growth or recovery of *V. cholerae* strains (Figure 5.6 B).

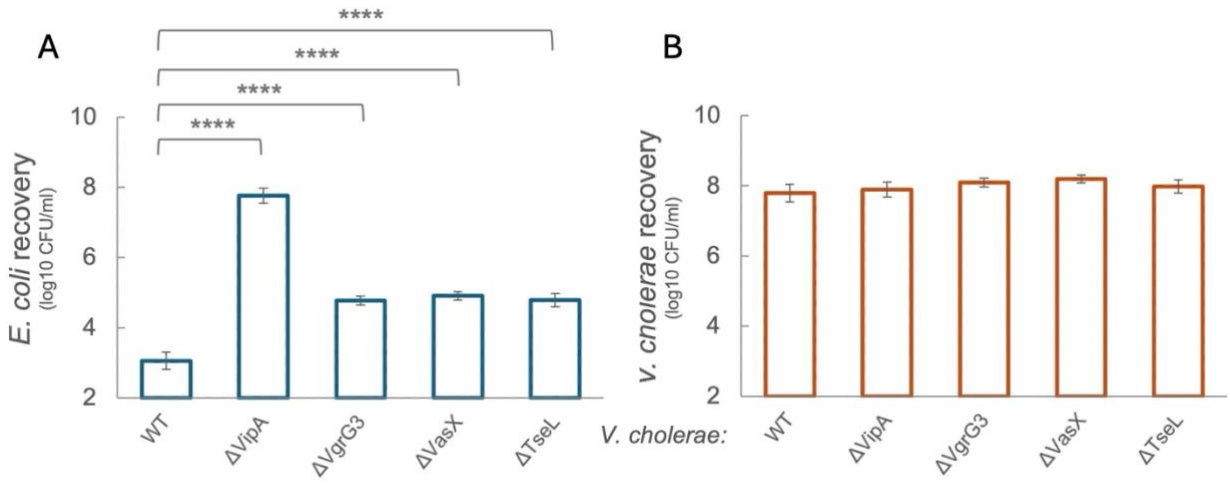

Figure 5.6 *V. cholerae* 2704-80 T6SS effectors are required for efficient killing of *E. coli*. Data represent the species recovery following a competition between *V. cholerae* and *E. coli* (1:1 predator to prey ratio). Predators were *V. cholerae* 2740-80 WT, T6SS-deficient mutant (ΔVipA) or single effector mutant strains (ΔVgrG3, ΔVasX and ΔTseL). Data represent the recovery of (A) *E. coli* and (B) *V. cholerae* as mean $\log_{10}$CFU/ml ± SD of three experimental replicates with two technical replicates. Statistical analysis was performed using one-way ANOVA. (A) One-way ANOVA determined there were statistically significant differences and post hoc comparisons were conducted using Tukey's test to compare each group means (**** $p \leq 0.0001$). (B) One-way ANOVA determined there was no statistically significant difference between group means ($p > 0.05$).

On the other hand, *P. aeruginosa* H1-T6SS was able to kill *V. cholerae* single effector mutants with similar efficacy (Figure 5.7 A) without incurring damage (Figure 5.7 B). This result suggests that *P. aeruginosa* H1-T6SS is triggered regardless of which *V. cholerae* T6SS effectors are being secreted through, aligning with what has been previously observed by George and colleagues [247]. Collectively, these results suggest that *V. cholerae* T6SS effectors are required for effectively eliminating *E. coli*, but dispensable for eliciting *P. aeruginosa* H1-T6SS retaliation.

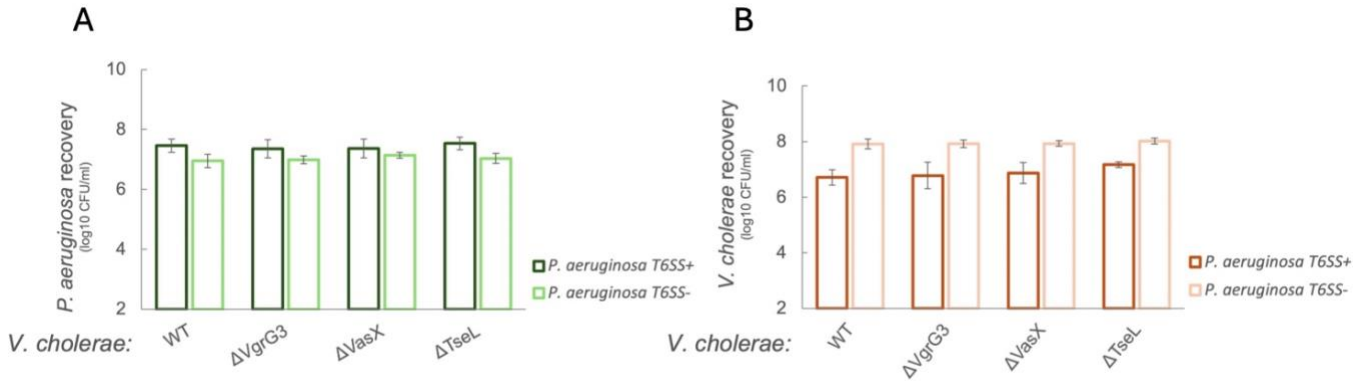

Figure 5.7 *V. cholerae* 2704-80 T6SS effectors do not differentially trigger *P. aeruginosa* H1-T6SS. Data represent species recovery following a competition assay between *V. cholerae* and *P. aeruginosa* PAO1 T6SS+ or T6SS. Predators were *V. cholerae* 2740-80 WT, T6SS-deficient mutant (ΔVipA) or single effector mutant strains (ΔVgrG3, ΔVasX and ΔTseL. Data represent the recovery of (A) *P. aeruginosa* and (B) *V. cholerae* as mean $\log_{10}$CFU/ml ± SD of three experimental replicates with two technical replicates. Statistical analysis was performed using a two-way ANOVA to determine the effects of each strains' recovery when incubated with *P. aeruginosa* T6SS+ or T6SS-. No statistically significant differences were observed ($p > 0.05$).

5.2.6 *P. aeruginosa* H1-T6SS protects *E. coli* when *A. baylyi* is the T6SS aggressor and constitutes a small part of the initial population

Next, we investigated whether *E. coli* protection could be influenced by T6SS species-specific behaviours by replacing *V. cholerae* 2740-80 with another T6SS aggressor in the multispecies competition assay. Unlike *V. cholerae* T6SS, *A. baylyi* T6SS can efficiently kill H1-T6SS-deficient *P. aeruginosa* (data presented in Chapter 2). Therefore, we employed *A. baylyi* as a T6SS aggressor in the multispecies competition assay by mixing defined ratios of *A. baylyi* and *E. coli* (1:1 or 100:1) with either 5 or 25% of *P. aeruginosa* PAO1 T6SS+ or T6SS- (Figure 5.8).

Our results showed that, when *A. baylyi* and *E. coli* constituted equal proportions (1:1) of the initial population and *P. aeruginosa* constituted 5% of the initial population, the survival of *E. coli* was independent of *P. aeruginosa* retaliatory H1-T6SS (Figure 5.8 C). Although *E. coli* was mildly protected from *A. baylyi* T6SS attacks after 4 hours of incubation (Figure 5.8 C), this protective effect was not due to total elimination of *A. baylyi* by *P. aeruginosa* T6SS+ (Figure 5.8 A), suggesting that physical protection could be occurring as observed in multispecies competitions where *V. cholerae* was the T6SS aggressor.

On the other hand, moderate amounts of *P. aeruginosa* T6SS+ in the initial population (25%) were able to significantly eliminate *A. baylyi*, which was reflected in an increased survival of *E. coli* (Figures 5.8 B and D). This suggests that in order for *P. aeruginosa* H1-T6SS to protect *E. coli* from *A. baylyi* T6SS attacks, it must be present in a similar proportion to the T6SS aggressor *A. baylyi*.

Moreover, we investigated whether *P. aeruginosa* H1-T6SS was able to protect *E. coli* against larger amounts of *A. baylyi*. To assess this, we increased *A. baylyi* by 100x in the initial multispecies population and observed that *P. aeruginosa* T6SS+ was unable to protect *E. coli* when it was greatly outnumbered by *A. baylyi* (Figures 5.8 I and K). These results suggest that *P. aeruginosa* H1-T6SS is able to offer protection only when both *E. coli* and *A. baylyi* constitute a similar amount of the multispecies population. Collectively, our observations reveal that *P. aeruginosa* H1-T6SS can protect *E. coli* from exogenous attacks by another T6SS aggressor. Notably, the efficiency of this protective effect is dependent on the aggressor and the its proportion within a multispecies community.

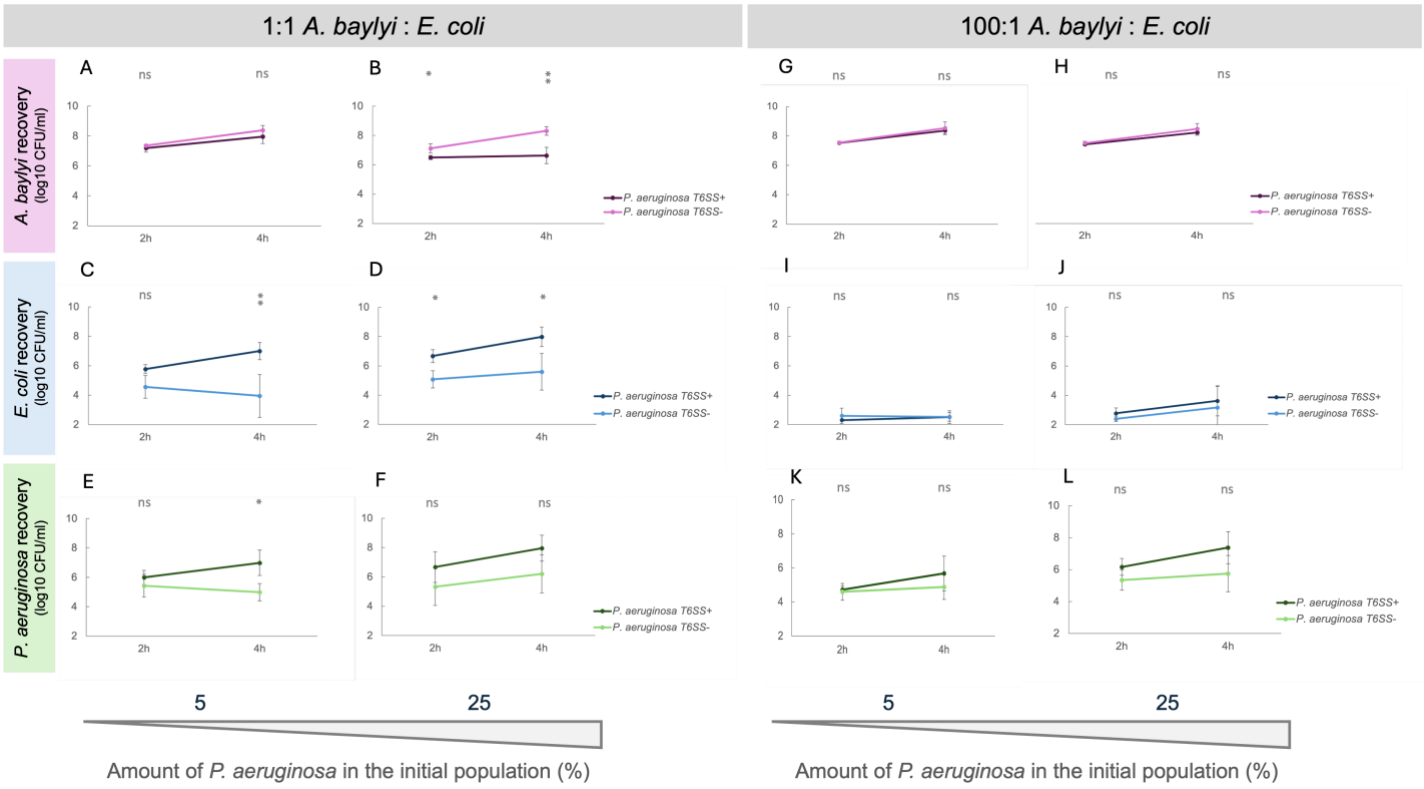

Figure 5.8 *P. aeruginosa* T6SS protects *E. coli* when *A. baylyi* constitutes a small part of the initial population. Data represent the recovery of each species following a multispecies competition assay that involved combining a 1:1 or 100:1 ratio of a T6SS-aggressor (*A. baylyi* ADP1) and a T6SS-sensitive species (*E. coli* MG1655) with 5 or 25% of *P. aeruginosa* PAO1 T6SS+ or T6SS-. The multispecies population was incubated at 37°C for 2h, following which, cells were recovered in the appropriate antibiotics to allow enumeration of each individual species. Data represent the recovery of *A. baylyi* (A, B, G and H), *E. coli* (C, D, I and J), and *P. aeruginosa* (E, F, K and L) as mean $\log_{10}$CFU/ml ± SD of three experimental replicates with two technical replicates. Statistical significance was determined using a two-tailed unpaired student's t-test to compare species recovery when either *P. aeruginosa* T6SS+ or T6SS- was part of the population at each timepoint (ns. non-significant, * $p \leq 0.05$, ** $p \leq 0.01$).

5.3 Discussion

Microbial community dynamics are influenced by numerous factors that directly or indirectly shape the spatial and structural organisation of the population. The T6SS has been implicated as a contributing element within these communities, either by providing T6SS-wielding bacteria a competitive advantage over other members of the population, or by driving organisational changes within the community [246,309,311,318]. *P. aeruginosa* is an opportunistic pathogen often found in multispecies communities that encodes three distinct T6SS clusters (H1, H2 and H3-T6SS) [65,317]. Unlike other T6SS-wielding species that deploy their T6SS aggressively, *P. aeruginosa* deploys H1-T6SS in response to exogenous T6SS attacks as a retaliatory weapon [99]. Here, we explored how this species-specific T6SS behaviour can influence population dynamics and shape multispecies microbial communities.

We leveraged *P. aeruginosa* to investigate how its retaliatory H1-T6SS influences the dynamics of a multispecies bacterial population. *P. aeruginosa* H1-T6SS attacks T6SS-aggressors without causing collateral damage [99]. Therefore, we asked whether *P. aeruginosa* H1-T6SS could protect a T6SS-sensitive species from the attacks of a T6SS-aggressor. To examine this, we performed a multispecies competition assay that involved a T6SS-retaliator (*P. aeruginosa*), a T6SS-aggressor (*V. cholerae*) and a T6SS-sensitive species (*E. coli*). We observed that when *P. aeruginosa* H1-T6SS is part of this tripartite community, *E. coli* was significantly protected from *V. cholerae* T6SS attacks (Figure 5.1). This suggests that *P. aeruginosa* retaliatory H1-T6SS antagonised and eliminated *V. cholerae* allowing *E. coli* survival.

We then determined how much *P. aeruginosa* T6SS+ would be required to eliminate *V. cholerae* and thus confer protection to *E. coli* (Figure 5.2). As expected, higher amounts of *P. aeruginosa* T6SS+ in the initial population (50-75%) offered more protection to *E. coli* due to elimination of almost all *V. cholerae* cells. As the amount of *P. aeruginosa* T6SS+ decreased in the initial population, we observed an interesting phenomenon. Rather than observing a reduction in *E. coli* protection, we saw that moderate amounts of *P. aeruginosa* T6SS+ (10-35%) could still significantly protect *E. coli*. Interestingly, this protective effect was not solely dependent on *P. aeruginosa* H1-T6SS. Albeit modestly, *P. aeruginosa* T6SS+ still protected *E. coli* at 8h of incubation by eliminating *V. cholerae*. However, at earlier timepoints *E. coli* was

significantly protected but not due to total elimination of *V. cholerae* by *P. aeruginosa* H1-T6SS. Moreover, as *P. aeruginosa* T6SS+ was reduced to minimal amounts in the population (5%), *E. coli* was only protected in a T6SS-dependent manner after longer incubation.

Notably, *E. coli* protection within this multispecies community appears to be time- and concentration-dependent. *P. aeruginosa* T6SS+ can protect *E. coli* from *V. cholerae* T6SS attacks when aggressors and bystanders each constitute equal parts of the population. Thus, we question whether *P. aeruginosa* T6SS+ could still confer protection to *E. coli* when the aggressor *V. cholerae* outnumbered bystanders. After increasing *V. cholerae* by 100x in the initial population, we observed that *P. aeruginosa* T6SS+ could still protect *E. coli* from being eliminated by *V. cholerae* T6SS (Figure 5.3). However, it was more evident that protection was H1-T6SS-dependent only when *P. aeruginosa* T6SS+ was a large part of the population. But as *P. aeruginosa* T6SS+ decreased in the population, *E. coli* was significantly protected even though *V. cholerae* was not being eliminated.

It is entirely possible that small amounts of *P. aeruginosa* T6SS+ in the initial population offer local physical protection to *E. coli*. This protection could be happening by the physical presence of *P. aeruginosa* at the boundary between aggressors and bystanders. Additionally, protection could be occurring due to a phenomenon called "corpse barrier effect", whereby dead cells accumulate at the boundary between competitor species and create a physical barrier to further killing [311]. The corpse barrier effect could explain why total elimination of *V. cholerae* by *P. aeruginosa* T6SS+ was not occurring in certain instances. *P. aeruginosa* retaliatory H1-T6SS might be unable to eliminate *V. cholerae* if a debris of dead cells were to stand between the two competitor species.

To understand whether *P. aeruginosa* was locally protecting *E. coli* from *V. cholerae* T6SS attacks, we used fluorescence microscopy to observe the spatial organisation of the multispecies population. Our observations showed that *V. cholerae* cells initially aggregated into clusters (Figure 5.4 A and B), and as the community grows during incubation, each species segregated into subpopulations within the general population rather than remaining well-mixed (Figure 5.4 C and D). This has been reported before when *V. cholerae* was part of a microbial community, whereby T6SS-wielding strains undergo phase separation compared to their T6SS-deficient counterparts [234,310]. However, we observed that species segregation occurred regardless of *P. aeruginosa* H1-T6SS activity within the multispecies population.

Our observations also revealed the occurrence of a debris of dead cells at the boundary where species meet (Figure 5.4 E). This suggests that "corpse barrier effect" might also play an important role in the protection of *E. coli* from T6SS attacks. Collectively our observations suggest that *P. aeruginosa* T6SS+ protects *E. coli* from T6SS attacks by eliminating aggressors at the boundary between competitor species. These localised competitions create a debris of aggressor corpses that prevent *P. aeruginosa* T6SS+ for continuing killing, enabling aggressors that are not in contact with *P. aeruginosa* T6SS+ to grow unhindered.

It is important to note that, *V. cholerae* T6SS+ does not efficiently kill *P. aeruginosa* T6SS- (results presented in Chapter 2). Therefore, when *P. aeruginosa* T6SS- is part of the multispecies population, *V. cholerae* T6SS will more efficiently eliminate *E. coli* than *P. aeruginosa* T6SS- (Figure 5.2). Hence why there might be a stark reduction in the recovery of *E. coli* compared to *P. aeruginosa* T6SS- after a 2 hour incubation period (Figure 5.2). On this occasion it is possible that a debris of dead *E. coli* cells accumulates between competitors, further preventing *V. cholerae* from efficiently killing *P. aeruginosa* T6SS-.

However, our microscopy analysis examined only static population dynamics at two distinct time-points. In our experimental setup, it was challenging to microscopically analyse multispecies communities after extended incubation periods due to drying of the agarose pad and the lack of nutrient availability. Therefore, future experiments should incorporate real-time dynamic observations using techniques such as microfluidics microscopy, which allows precise control over the input of nutrients, longer observational periods and higher resolution [319]. Using microfluidics microscopy could enable a more detailed analysis of the spatiotemporal organisation of our multispecies community over extended periods.

Previous studies have shown that *V. cholerae* T6SS effectors play distinct roles in triggering *P. aeruginosa* H1-T6SS retaliation and *E. coli* elimination [186,247]. However, there is a debate as to whether a specific *V. cholerae* effector is responsible for eliciting *P. aeruginosa* H1-T6SS retaliation. A competition between *P. aeruginosa* and different *V. cholerae* effector mutants showed that mutants with lipase effector TseL could trigger *P. aeruginosa* H1-T6SS retaliation [186]. Moreover, when individual *V. cholerae* effectors were expressed in the periplasm of *P. aeruginosa*, the expression of TseL resulted in the strongest H1-T6SS retaliation, suggesting this effector as a key player for H1-T6SS retaliation [186]. To the contrary, another study has shown that *P. aeruginosa* H1-T6SS significantly killed a *V. cholerae* TseL

mutant, which suggests that H1-T6SS retaliation is not triggered specifically by TseL [247]. It isimportant to notice that both studies used different *V. cholerae* strains, which could explain this differential response by *P. aeruginosa* H1-T6SS.

Because previous observations are contradictory and might reflect differential experimental setups, we aimed to elucidate whether *P. aeruginosa* H1-T6SS is able to retaliate against a specific *V. cholerae* effector under our specific experimental conditions. To do this, we employed *V. cholerae* single effector mutant strains as T6SS aggressors in our multispecies competitions assay. We observed that *P. aeruginosa* T6SS+ could protect *E. coli* regardless of which effectors were being delivered by *V. cholerae* T6SS (Figure 5.5 C and D). However, this effect was not due to the total elimination of *V. cholerae* by *P. aeruginosa* H1-T6SS (Figure 5.5 A and B). Since it was unclear whether an individual *V. cholerae* effector is the key player in killing *E. coli* or eliciting *P. aeruginosa* H1-T6SS retaliation, we explored this by performing pairwise competitions between each intervening species. We observed that *V. cholerae* effectors are equally required for extensive *E. coli* elimination (Figure 5.6), but dispensable for eliciting *P. aeruginosa* H1-T6SS retaliation (Figure 5.7). These observations support a previous report on pairwise competitions between these species [247], and also our observations resulting from the multispecies competition assays (Figure 5.5). Although *V. cholerae* single effector mutant strains are less harmful to *E. coli*, this effect is undetectable in a multispecies population where *P. aeruginosa* H1-T6SS is able to eliminate *V. cholerae*, despite which effectors are being secreted by the T6SS-agressor species. There are some limitations to this study which could be clarified by further experimental work. It would be valuable to perform this analysis with a larger initial population of *P. aeruginosa*, as our findings indicate that this is the amount required for H1-T6SS-dependent protection of *E. coli* (Figure 5.2).

We then questioned whether *P. aeruginosa* H1-T6SS protects *E. coli* from other T6SS-aggressor species. Unlike *V. cholerae* T6SS, *A. baylyi* T6SS significantly eliminates T6SS-deficient *P. aeruginosa*. We thus asked whether this species-specific antagonism could differentially shape the dynamics of a multispecies community. To understand this, we replaced the T6SS-aggressor species in the multispecies competition assay with *A. baylyi*. We observed that when *P. aeruginosa* T6SS+ was a small part of the initial population (5%), *E. coli* protection was significantly higher after 4h of incubation (Figure 5.8 C). However, this protective effect was not due to full elimination of *A. baylyi* by *P. aeruginosa* H1-T6SS (Figure

5.8 A). On the other hand, when *P. aeruginosa* T6SS+ increased in the population (25%), *E. coli* protection (Figure 5.8 D) was due to *A. baylyi* elimination in a H1-T6SS-dependent manner (Figure 5.8 B). Interestingly, unlike what we observed for *V. cholerae* (Figure 5.3), when increasing *A. baylyi* in the initial population, *P. aeruginosa* H1-T6SS was unable to protect *E. coli* from being eliminated (Figures 5.8 I and J).

It is important to note that these effects were observed after 4h of co-incubation rather than after 2h, as previously seen for competitions with *V. cholerae*. This could be explained by the fact that *A. baylyi* grows slower than *V. cholerae*. The generation time for *V. cholerae* is around 20 minutes whilst that of *A. baylyi* is twice as long (40 minutes). As such, *A. baylyi* cells require twice as much time as *V. cholerae* to reach the same population size. This was reflected in an increased survival of *E. coli*, as *P. aeruginosa* H1-T6SS might be triggered by greater amounts of the T6SS-aggressors in the population (Figure 5.8).

Our previous observations allowed us to delineate a model of how *P. aeruginosa* retaliatory H1-T6SS influences the dynamics of a multispecies community consisting of a T6SS-aggressor species and a T6SS-sensitive species (Figure 5.9). *P. aeruginosa* efficiently protects T6SS-sensitive *E. coli* in a H1-T6SS-dependent manner when it constitutes a large part of the multispecies population thorough almost total elimination of aggressors. However, as *P. aeruginosa* T6SS+ decreases in the population, *E. coli* protection is not solely due to mass elimination of T6SS-aggressors. Instead, *P. aeruginosa* confers *E. coli* physical protection from the attacks of T6SS-aggressors. *P. aeruginosa* H1-T6SS continues on killing aggressors, though perhaps less efficiently because of the accumulation of dead cell debris at the boundary between competitor species (Figure 5.9). Furthermore, aggressors that do not directly contact *P. aeruginosa* T6SS+ will continue to grow unimpeded. This behaviour has been observed before, whereby cells within a cluster are protected from exogenous T6SS attacks [234,309]. In these “bacterial battles”, *P. aeruginosa* acts as a paladin, deploying its weaponry to not only to defend itself but also to safeguard vulnerable *E. coli* bystanders from T6SS-aggressors.

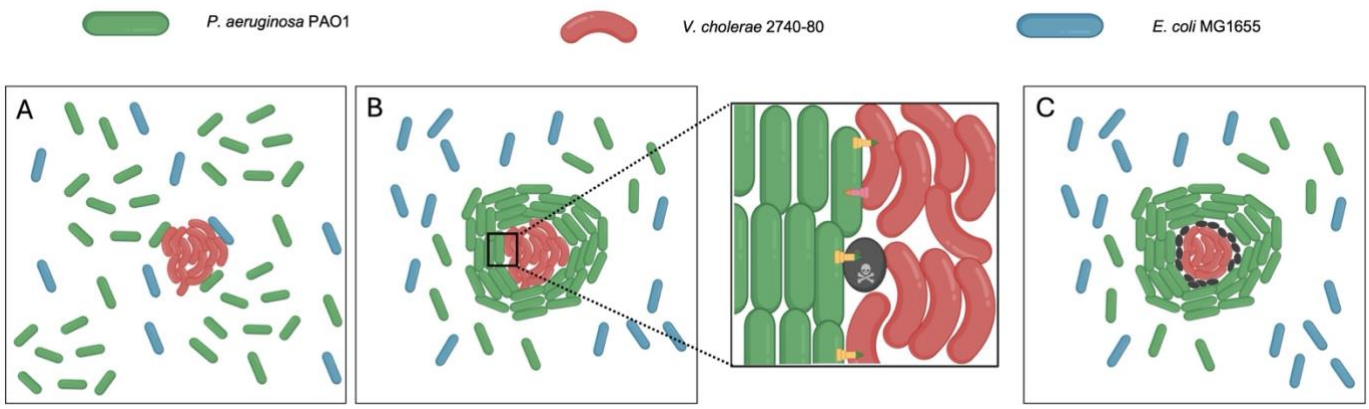

Figure 5.9 Proposed model for the influence of *P. aeruginosa* retaliatory H1-T6SS in populations dynamics of a multispecies community. The model represents a multispecies community consisting T6SS-aggressor *V. cholerae*, T6SS-sensitive *E. coli* and T6SS-retaliator *P. aeruginosa*. A) *V. cholerae* cells initially form aggregates whereas *P. aeruginosa* and *E. coli* remain well dispersed in the population. When *E. coli* cells directly contact *V. cholerae* cells they are eliminated by *V. cholerae* T6SS, whereas *P. aeruginosa* H1-T6SS is able to counterattack and eliminate *V. cholerae* instead. B) *P. aeruginosa* cells surround *V. cholerae* cell clusters limiting their action as a T6SS-aggressor against *E. coli*. A "bacterial battle" occurs at the boundary between T6SS-aggressor and T6SS-retaliator, whereby *P. aeruginosa* H1-T6SS eliminates *V. cholerae* cells. C) Cell debris resulting of the elimination of *V. cholerae* will form a barrier between *V. cholerae* and *P. aeruginosa* – "corpse barrier effect". However, *V. cholerae* cells that are not in direct contact with *P. aeruginosa* cells, and protected by this barrier will continue to grow unimpeded. In summary, *P. aeruginosa* protects *E. coli* from *V. cholerae* T6SS attacks by directly eliminating aggressors in a H1-T6SS dependent manner and by forming a physical barrier that prevents aggressors from contacting prey.

Importantly, this model reflects our observations from species enumeration and microscopic visualisation of multispecies communities, where *V. cholerae* acts as the T6SS aggressor. However, our previous findings with *A. baylyi* as the T6SS aggressor suggest that *P. aeruginosa* H1-T6SS-mediated protective effect depends on both the T6SS aggressor species and their proportion within a multispecies community. Future experiments could explore the effects of different aggressor and bystander species to determine how species-specificity influences the population dynamics of a multispecies community.

Our study provides a foundation for further investigations. Here, we examined population dynamics in a multispecies community composed of species with distinct T6SS behaviours. Future research could leverage this approach by using species that co-colonise the human body to better elucidate how population dynamics mediated by the T6SS may impact host colonisation and pathogenesis. Moreover, studying the population dynamics of clinically relevant species or clinical isolates that co-infect the same bodily compartment could further advance our understanding of the complexity of polymicrobial infections. Additionally, *in vivo* studies could provide a more accurate representation of bacterial interactions in

complex environments, where biotic factors shape microbial behaviour. Previous *in vivo* models have already proven valuable for studying microbial community dynamics [320].

In conclusion, the effects of the T6SS in microbial communities have been extensively studied, albeit only in pairwise combinations. To our knowledge, this is the first study investigating how T6SS shapes population dynamics within a multispecies community. In particular, how *P. aeruginosa* retaliatory H1-T6SS influences species-specific interactions and the spatial organisation and structure of a multispecies population involving three different species. This knowledge can help us understand how bacterial populations establish, persist, and compete within diverse environments, including host-associated microbiomes and polymicrobial infections. Ultimately, our findings can contribute to a deeper understanding of bacterial ecology, with potential implications for infection prevention and control.

6 Conclusion and future perspectives

The Type 6 Secretion System (T6SS) is a contact-dependent mechanism capable of secreting toxic effectors into neighbouring cells [54,70]. As discussed in previous Chapters, many bacterial species employ their T6SS in interbacterial competition, significantly impacting the population dynamics of microbial communities. The T6SS has been comprehensively studied for the past eighteen years, offering the scientific community a deep knowledge of its structure and function.

P. aeruginosa encodes three distinct T6SS apparatus, one of which (H1-T6SS) acts in a retaliatory or defensive manner, assembling and firing in the presence of exogenous T6SS activity [99]. Upon being targeted by *V. cholerae* T6SS, *P. aeruginosa* promptly assembles H1-T6SS and retaliates. This counterattack is powerful and has lethal consequences for the competitor without causing collateral damage [99]. *P. aeruginosa* H1-T6SS has been continuously studied for its species-specific retaliatory behaviour. Previous reports showed that the complete removal of *A. baylyi* T6SS effectors still elicited H1-T6SS retaliation by *P. aeruginosa* [137,247]. On the other hand, the removal of all *A. tumefaciens* and *V. cholerae* T6SS effectors abolished this retaliatory effect [144,247]. The T6SS assembly and firing is energetically consuming for a cell, and thus *P. aeruginosa* might conserve energy by only firing H1-T6SS against specific species instead of indiscriminately.

Interestingly, disarming this retaliatory behaviour by inactivating H1-T6SS does not increase the susceptibility of *P. aeruginosa* to *V. cholerae* or *A. baylyi* T6SS attacks. This was first observed after competition assays between *V. cholerae* WT and a *P. aeruginosa* H1-T6SS mutant [99]. We have thus questioned whether this observed effect was because predator and prey species were competing in equal proportions. Upon increasing the predator-to-prey ratio in competition assays between these species, we observed an interesting phenomenon whereby *P. aeruginosa* H1-T6SS mutant strain was significantly affected by *A. baylyi* T6SS but not by *V. cholerae* T6SS. Increasing the number of predators has extended the opportunity for T6SS-wielding aggressors directly contact their prey. However, the increase in points of contact has not further sensitised *P. aeruginosa* to the lethal effects of *V. cholerae* T6SS. *P. aeruginosa* species-specific resistance to T6SS attacks is a novel contribution to the field and what strongly motivated this project.

Given that each species delivers a unique set of T6SS effectors, we hypothesised whether *P. aeruginosa* was selectively resistant to *V. cholerae* effectors. While *V. cholerae* T6SS effectors showed no toxicity when expressed in the cytosol of *P. aeruginosa*, their expression in the cell's periplasm was significantly toxic. This observation aligns with the fact that *V. cholerae* effectors target cell wall components [103,169,171]. Previous studies have shown that the T6SS can deliver effectors targeting the periplasm, either by direct delivery into the periplasmic space or by being translocated from the cytosol [122,124]. It is important to note that plasmid expression might result in greater expression of effectors than T6SS-mediated effector delivery. Therefore, it is possible that *V. cholerae* effectors are delivered into *P. aeruginosa*, but their toxic effects are undetectable. Our study has some limitations, as we have not confirmed whether toxic effect was lost by complementary expression of the cognate immunity protein along with each effector in the periplasm. If toxicity was abolished by expression of the effector-immunity pair in *P. aeruginosa* it would indicate that toxicity was due to the effector and further strengthen our findings.

Our results suggest that *P. aeruginosa* is not selectively resistant to *V. cholerae* T6SS effectors. However, proper localisation of effectors into the periplasm appears crucial for toxicity. Collectively, these observations point to multiple potential explanations. *V. cholerae* T6SS might directly deliver effectors into the periplasm of *P. aeruginosa*. However, this can happen either in insufficient amounts to exert an extensive toxic effect, or *P. aeruginosa* might possess an unknown mechanism to specifically neutralise *V. cholerae* effectors. Conversely, *V. cholerae* T6SS might directly deliver effectors into the cytosol of *P. aeruginosa*, yet effectors are unable to be translocated into the periplasm and exert their toxic effect. As such, we questioned whether *V. cholerae* T6SS was able to directly deliver effectors into the cytosol of *P. aeruginosa*. To investigate this, we engineered fluorescence- and antibiotic-based reporter systems to detect T6SS-mediated cytosolic delivery of *V. cholerae* effectors. However, despite numerous attempts, we were unable to ascertain whether *V. cholerae* T6SS can directly deliver effectors into the cytosol of *P. aeruginosa*. These results were surprising since heterologous protein delivery has been achieved before, albeit with modest success rates [268].

Previous studies have shown that *V. cholerae* delivers effectors into the cytosol of sister cells, which can then be translocated into the periplasm [122,123]. However, others have shown that *P. aeruginosa* effectors are delivered into the periplasm of target cells and can then be

translocated into the cytosol [124–126]. These observations suggest that different T6SS-wielding species deliver effectors into different compartments of the target cell. However, the mechanism by which *V. cholerae* T6SS interacts specifically with *P. aeruginosa* remains unclear: whether it penetrates the cell wall and delivers effectors into the cytosol, or it punctures the outer membrane and directly delivers effectors into the periplasmic space.

It is entirely possible that *V. cholerae* T6SS is unable to puncture and deliver effectors into *P. aeruginosa*. Therefore, we questioned whether *P. aeruginosa* species-specific resistance was due to physical protection provided by the cell wall. A previous study reported that deleting a gene responsible for EPS production sensitised *V. cholerae* cells to T6SS-mediated killing by wild-type sister cells [194]. At the single-cell level, EPS does not prevent *V. cholerae* from firing its own T6SS but rather acts as a physical barrier to exogenous T6SS attacks. Our observation showed that *P. aeruginosa* mutant strains lacking genes that encode for important EPS components were not more susceptible to *V. cholerae* T6SS attacks. This suggests that, unlike *V. cholerae*, *P. aeruginosa* EPS might not play a role in the protection against exogenous T6SS attacks. In *P. aeruginosa*, the expression of EPS genes can be influenced by temperature [293]. As such, we explored whether *P. aeruginosa* species-specific resistance was influenced by the temperature at which cell cultures had grown overnight. Interestingly, we observed that *P. aeruginosa* cells that had grown at a higher overnight temperature were more resistant to *A. baylyi* T6SS attacks, yet this temperature-dependent resistance was not observed in *V. cholerae*. These observations reiterate how *P. aeruginosa* resistance to T6SS attacks is species-specific. Additionally, we investigated whether the OM increased the susceptibility of *P. aeruginosa* to exogenous T6SS attacks. We tested this by exposing *P. aeruginosa* mutants lacking genes responsible for lipid transport systems to *V. cholerae* T6SS. However, our resulted showed that *P. aeruginosa* mutant strains were not increasingly susceptible to *V. cholerae* T6SS attacks.

Lastly, we investigated how *P. aeruginosa* retaliatory H1-T6SS shapes the population dynamics in a multispecies community. This community consisted of an aggressor that deploys T6SS indiscriminately, and a bystander that lacks a T6SS and is vulnerable to T6SS damage. We observed that, in this multispecies community, *P. aeruginosa* H1-T6SS can protect *E. coli* bystanders from *V. cholerae* aggressors. This H1-T6SS-mediated protection is granted by mass elimination of *V. cholerae* when *P. aeruginosa* is a major constituent of the population.

However, when *P. aeruginosa* makes up a moderate portion of the population, protection of *E. coli* occurs through a physical barrier, whereby *P. aeruginosa* prevents direct contact between aggressors and bystanders. These observations were also reflected when *V. cholerae* amounts increased within the population by 100 times, indicating that *P. aeruginosa* H1-T6SS effectively protects *E. coli* even when outnumbered. Moreover, we demonstrate that protection is independent of which effectors are being secreted by *V. cholerae* T6SS, suggesting that *P. aeruginosa* H1-T6SS is not triggered by a specific effector, unlike what has been previously reported [186]. Additionally, we show that *P. aeruginosa* H1-T6SS is also able to protect *E. coli* against the attacks of another T6SS-aggressor, *A. baylyi*. However, this protective effect is only observed when *P. aeruginosa* is not greatly outnumbered by *A. baylyi*.

Our findings represent a significant step forward in understanding species-specific T6SS-mediated behaviours. Here, we demonstrated that *P. aeruginosa* is specifically more resistant to *V. cholerae* T6SS attacks compared to another species. Notably, *P. aeruginosa* is not selectively resistant to *V. cholerae* effectors, though effectors need proper periplasmic localisation to exert a toxic effect. Additionally, our research indicates that disrupting *P. aeruginosa* exopolysaccharide production or lipid transport systems might not influence *P. aeruginosa* resistance to *V. cholerae* T6SS attacks. Lastly, we present the first report on how the retaliatory H1-T6SS of *P. aeruginosa* influences the spatial organisation and population dynamics of a multispecies community.

Collectively, our data unveil novel insights into T6SS interactions between heterologous species, opening the field for further investigations into interbacterial competition.

Ongoing research in the field continues to reveal new insights into the T6SS, and our study may contribute to new avenues of exploration. It is entirely possible that *P. aeruginosa* has species-specific mechanisms that prevent incurring damage caused by *V. cholerae* T6SS attacks. We investigated the role of specific genes encoding for proteins in the formation of *P. aeruginosa* OM. However, *P. aeruginosa* has a complex OM composed of many other proteins that can participate in this species-specific protection [321]. Additionally, because the OM of *P. aeruginosa* has low permeability, it could also hinder *V. cholerae* T6SS puncturing. Further studies could investigate whether permeabilising the OM, by pre-emptively exposing the cells to membrane-disrupting antibiotics, would make *P. aeruginosa* more susceptible to

V. cholerae T6SS. Lastly, in *P. aeruginosa* several key regulons control virulence, metabolism and environmental adaptation [212]. Therefore, these regulatory pathways may be involved in the species-specific resistance of *P. aeruginosa* to the effects of specific T6SS aggressors, like the one observed for *V. cholerae*.

7 Materials and Methods

7.1 Bacterial strains and growth conditions

Details of bacterial strains can be found in each corresponding Chapter. For cultures in liquid media, *E. coli*, *V. cholerae* and *P. aeruginosa* were grown in Lysogeny broth (LB) Lennox, and *A. baylyi* was grown in LB Lennox or Terrific broth (Growth media details can be found on Table 7.1). For cultures on solid media, bacteria were grown in LB agar. Media were supplemented when appropriate with supplements described in Table 7.2. Unless otherwise stated, bacteria were grown at 37^{o}C under orbital shaking at 200 rpm.

Table 7.1 Growth media

Growth media	Composition
LB Lennox formulation	Tryptone 10 g/L, Yeast extract 5g/L, NaCl 5g/L
Terrific broth	Tryptone 12 g/L, Yeast extract 24g/L
LB agar	Tryptone 10 g/L, Yeast extract 5g/L, NaCl 10g/L, Agar 15g/L

Table 7.2 Supplements

Supplement	Final concentration
Arabinose	0.002/0.02/0.1/0.2/0.4% (v/v)
Carbenicillin	100 μg/ml
Chloramphenicol	5-15 μg/ml
Gentamicin	10-30 μg/ml
Glucose	0.2% (v/v)
Irgasan	20 μg/ml
IPTG	0.1 mM
Kanamycin	50 μg/ml
Streptomycin	50 μg/ml
Sucrose	10%

7.2 Bacterial competition assays

7.2.1 Pairwise competitions

Bacterial competition assays were performed as described before [122]. Overnight cultures were diluted 1:100 (for *V. cholerae* and *P. aeruginosa*) or 1:30 (for *A. baylyi*) in fresh liquid media and incubated at 37°C shaking at 200 rpm, until mid-log phase was reached (OD_{600}=0.5-0.8 for *V. cholerae* and *P. aeruginosa*; OD_{600}=0.9-1.2 for *A. baylyi*). A 1 ml of each culture was centrifuged at 5,000g for 5 min and the pellet was resuspended in Phosphate-buffered saline (PBS) to concentrate to an OD_{600}=10. Predators and prey were mixed at defined ratios, spotted on LB agar and incubated at 37°C for 2 hours. After incubation, agar plugs were removed using a wide-bore pipette tip and resuspended in 1 ml of LB. Serial dilutions were made in LB and spotted on LB plates supplemented with the appropriate antibiotics to individual select for recovered predators and prey in colony forming units per millilitre (CFU/ml).

7.2.2 Multispecies competitions

Bacterial competition assays were based on previous methods of pairwise competition assays and modified appropriately. Growth conditions were performed as before. *V. cholerae* or *A. baylyi* were firstly mixed with *E. coli* at pre-defined ratios of 1:1 or 100:1, and then mixed with defined amounts of *P. aeruginosa* (5, 10, 25, 35, 50 or 75%). Bacterial suspensions were spotted on LB agar and incubated at 37°C for either 2, 4, 8 or 21 hours. After incubation, agar plugs were removed using a wide-bore pipette tip and resuspended in 1 ml of LB. Serial dilutions were spotted on LB agar with the appropriate antibiotics to select and enumerate CFUs of each recovered species.

7.3 Molecular Biology techniques

7.3.1 DNA amplification

For genomic DNA amplification, genomic DNA was obtained with GenElute bacterial genomic DNA (Sigma) following the manufacturer's directions. For plasmid DNA amplification, DNA was obtained with Nucleospin Plasmid DNA (Macherey-Nagel) following the manufacturer's directions. DNA was amplified using Q5 High-fidelity DNA polymerase (New England Biolabs, NEB). A list of the oligonucleotides used for gene amplification can be found in each corresponding Chapter.

7.3.2 Restriction digestion and ligation

To clone genes of interest into vectors, restriction enzyme were used for DNA digestion at 37^{o}C for 1 hour (NEB). DNA ligations were performed using Instant Sticky-end Ligase Master-Mix (NEB) following the manufacturer's instructions. Plasmids used for cloning and constructs can be found on each corresponding Chapter.

7.3.3 Bacterial transformation

E. coli strains NEB 10-beta (New England Biolabs, NEB) or One Shot PIR1 (ThermoFisher scientific) were used for cloning. Commercial chemically competent *E. coli* were transformed according to the manufacturer's directions. Constructs were cloned into *E. coli* and recovered in SOC (Super Optimal broth with Catabolite repression) at 37^{o}C for 1 hour shaking at 200 rpm. Bacterial suspensions were plated in LB agar with the appropriate supplements to select for the construct and incubated at 37^{o}C overnight. Colonies were selected and cultured in LB following which cultures were miniprepped using Nucleospin Plasmid DNA (Macherey-Nagel) and constructs were verified by Sanger sequencing (Eurofins genomics).

E. coli SM10 λ*pir* (lab collection) or MFD *pir* (lab collection) were made electrocompetent and used as donor strains for bacterial conjugation. *E. coli* cultures were incubated until early exponential phase was reached (OD_{600}=0.3), incubated in ice for 30 minutes and centrifuged at 4,000g rpm for 15 min at 4^{o}C. Supernatants were discarded, pellets were washed with 10%

glycerol (ice cold) and centrifuged at 4,000g for 15 min at 4°C. This process was repeated three times. The pellets were finally resuspended in a small volume of 10% glycerol (50-100μl) and transferred into electroporation cuvettes (0.1cm gap, Biorad). Cells were electroporated using a Gene Pulser XCell Electroporator at 1.8 kV (Biorad), recovered in SOC and incubated for 1h before plating in LB agar with the appropriate supplements for selection.

7.3.4 Bacterial conjugation

Donors containing a conjugative plasmid and recipients were grown on LB agar at 37°C overnight. Colonies were scraped from agar plates and swirled together onto a new pre-warmed LB agar plate. After 2 hours of incubation at 37°C, bacteria were recovered and suspended in LB, serially diluted and plated in LB with the appropriate antibiotics to select for colonies that had received the conjugative plasmid.

7.3.5 Periplasmic localisation of effectors

The Sec-secretion signal of *E. coli* DsbA was fused to the N-terminus of each effector gene in order to localise effectors to the periplasm of *P. aeruginosa* (Figure S 2.2). In our laboratory collection we had an existent construct with the DsbA Sec-secretion signal (MKKIWLALAGLVLAFSASA|AQYED) fused to sfGFP by a flexible linker (LEGPAG). The sec-sfGFP fusion was amplified, cloned into vector pPGA and its sequence confirmed by Sanger sequencing (Eurofins genomics). Each effector gene was amplified with restriction enzyme sites to clone into the newly created construct (pPGA-sec-sfGFP) replacing the existing *sfGFP*. Cloning procedures were the same as for genes as previously described. All constructs were verified by Sanger Sequencing (Eurofins genomics). Details for gene amplification and construct engineering can be found in Chapter 2 (Table 2.1).

7.3.6 *P. aeruginosa* mutagenesis

P. aeruginosa deletion mutants were constructed by modifying a previously described method [322]. Briefly, around 900 bp of the flanking regions of the target gene were amplified and overlapped using polymerase chain reaction (PCR) and cloned into suicide vector pEXG2

in *E. coli* NEB 10-beta. Each construct was transformed into *E. coli* SM10 λpir by electroporation and cells were plated onto LB agar supplemented with 20 µg/ml of gentamicin to select for cells that had received the pEXG2 plasmid. Donor *E. coli* SM10 λpir containing pEXG2 was conjugated with recipient *P. aeruginosa* PAO1 through biparental mating as previously described. Transconjugant colonies were picked and suspended in LB and incubated for 2 hours at 37°C shaking at 200 rpm. An aliquot of each bacterial suspension was streaked onto LB agar supplemented with 10% sucrose and plates were incubated at 30°C until colonies appeared. Individual colonies were picked and a colony PCR was perform to detect the deletion of the target gene. PCR products were extracted from 1% agarose gel, cleaned with Nucleospin Gel and PCR clean-up (Macherey-Nagel) and gene deletion was confirmed by Sanger sequencing (Eurofins genomics).

7.4 Reporter-based assays

7.4.1 FLP reporter chromosomal integration in *P. aeruginosa*

P. aeruginosa tssB1 (PA0083) was replaced by the FLP reporter to simultaneously inactivate H1-T6SS and allow chromosomal expression of the FLP reporter. The method used has been previously described.

7.4.2 FLP delivery assay

Donor and recipient cell cultures were grown overnight, diluted 1:100 in fresh LB and incubated at 37°C with 200 rpm agitation for 2 hours with 0.2% arabinose. A 1 ml of each culture was centrifuged at 5,000g for 5 min and the pellet resuspended in LB to concentrate to an OD_{600}=10. Donors and recipients were mixed at 10:1 ratio, spotted on pre-warmed LB agar with 0.2% arabinose and incubated at 37°C for 3 hours. After incubation, agar plugs were removed using a wide-bore pipette tip and resuspended in 1 ml of LB. Serial dilutions were plated on LB agar with antibiotics to select for recipients only. Plates were incubated and 37°C overnight and imaged with a ChemiDoc MP Imaging System (Biorad) using Alexa488 channel (532/28) to detect green fluorescence, and Rhodamine channel (602/50) to detect red fluorescence.

7.4.3 Reporter spontaneous recombination assay

Recipient cell cultures were grown overnight, diluted 1:100 in fresh LB and incubated at 37ºC with 200 rpm agitation for no longer than 2.5 hours. After incubation, cultures were vortexed, serially diluted and plated on LB agar with antibiotics to select for naïve or recombined colonies. Plates were incubated and 37ºC overnight and CFUs enumerated.

7.4.4 Cre endogenous expression

Donor and recipient cell cultures were grown overnight, diluted 1:100 in fresh LB and incubated at 37ºC with 200 rpm agitation for no longer than 2.5 hours. A 1 ml of each culture was centrifuged at 5,000g for 5 min and the pellet resuspended in LB to concentrate to an OD_{600}=10. Donors and recipients were mixed at a 2:1 ratio, spotted on pre-warmed LB agar with DAP (Diaminopimelic acid) and incubated at 37ºC for 2 hours. After incubation, agar plugs were removed using a wide-bore pipette tip and resuspended in 1 ml of LB. Serial dilutions were spotted on LB agar with antibiotics to select for transconjugant colonies and incubated at 37ºC overnight. Two individual colonies were picked from transconjugant plates into 1 ml of LB with 0.02% or 0.2% arabinose, and incubated at 37ºC for 3 hours with 200 rpm agitation. After incubation, bacterial suspensions were serially diluted and plated in LB agar with antibiotics to select for total (transconjugants), naïve and recombined colonies. Plates were incubated and 37ºC overnight and CFUs enumerate.

7.4.5 T6SS-mediated Cre delivery assay

Donor and recipient cultures grown overnight were diluted 1:100 in fresh LB and incubated at 37ºC with 200 rpm agitation for 3 hours with 0.4% arabinose added in the last hour. A 1 ml of each culture was centrifuged at 5,000 g for 5 min and the pellet resuspended in LB to concentrate to an OD_{600}=10. Donors and recipients were mixed at 10:1 ratio, spotted on pre-warmed LB agar with 0.2% arabinose and incubated at 37ºC for 2 hours. After incubation, agar plugs were removed using a wide-bore pipette tip and resuspended in 1 ml of LB. Serial dilutions were spotted on LB agar with antibiotics to select for naïve or recombined recipients. Plates were incubated and 37ºC overnight and CFUs counted.

7.5 Protein assays

7.5.1 Sample preparation for protein assays

Overnight cultures were diluted 1:100 into fresh LB with different concentrations of arabinose (0.002, 0.02, 0.1 and 0.4%) to induce gene expression. Cultures were incubated and then centrifuged at 5,000g for 5min and pellets were resuspended in PBS to concentrate to an OD_{600}=10. Suspensions were spotted on pre-warmed LB agar with arabinose at different concentrations and incubated at 37°C for 2 hours. After incubation, agar plugs were removed using a wide-bore pipette tip and resuspended in 1 ml of LB. Bacterial suspensions were vortexed for 1 minute and incubated at 37°C for 30 minutes. Following incubation, suspensions were centrifuged at 5,000g for 5 minutes and pellets and supernatants were collected. Pellets were washed twice in PBS, resuspended in NuPAGE LDS (Lithium dodecyl sulphate) Sample Buffer (Invitrogen) containing Coomassie blue and Phenol red dyes, and incubated at 100°C for 10 minutes.

Supernatants were passed through a 0.22µm filter, following which Trichloroacetic acid (TCA) solution was added to a final concentration of 20% and placed on ice for 1 hour. After incubation, supernatants were centrifuged at 20,000 g for 10 minutes at 4°C, and the resultant pellets were resuspended in ice-cold Acetone. Suspensions were then vortexed for 1 minute and centrifuged at 20,000 g for 10 minutes at 4°C. Supernatants were discarded and pellets air dried before being resuspended in LDS Sample Buffer.

7.5.2 SDS-PAGE, Coomassie protein assay and Western blotting

Pellet and supernatant samples, previously suspended in LDS Sample Buffer, were loaded onto a pre-cast 4–12% Bolt Bis-Tris Plus mini protein gel (ThermoFisher Scientific) and separated at 185V for 30 minutes by electrophoresis (Bio-rad) using Bolt MES SDS running buffer (ThermoFisher Scientific). Gels were either treated for Coomassie protein assay or Western blotting.

For Coomassie protein assays, polyacrylamide gels were stained with Instant Blue Coomassie protein stain (Abcam) for 15 minutes at room temperature under orbital shaking at 60rpm.

For Western blotting assays, HA (Human influenza hemagglutinin) was introduced for the detection of protein fusions. The HA tag was fused to the C-terminus of *V. cholerae* VgrG3 and PAAR2 by firstly cloning VgrG3- and PAAR2-FLP fusions into a vector containing HA. Polyacrylamide gels were transferred to a Polyvinylidene difluoride (PVDF) membrane (ThermoFisher scientific) using a tank transfer system (Invitrogen) with Bolt Transfer buffer (ThermoFisher Scientific) + 10% methanol. Transfers were performed at 20V for 1 hour. The blots were then blocked in 5% skimmed milk dissolved in PBST (PBS with 1% Tween-20) for 1 hour at room temperature and shaking at 60 rpm. The blots were incubated for 1h at room temperature with the primary antibody (HA Tag rabbit mAb, Cell Signaling) diluted 1:2 in blocking buffer. Following incubation, the blots were incubated for 1h at room temperature with the secondary antibody (Goat Anti-rabbit IgG Starbright Blue 700, Bio-Rad) diluted 1:2 in blocking buffer. After membrane blocking and between antibody incubations, blots were washed with PBST three times for 5 minutes shaking at 60 rpm. Visualisation of the blots was done using a ChemiDoc MP Imaging System (Bio-rad).

7.6 Fluorescence microscopy and microscopy analysis

Cultures were prepared as previously described. For the microscopic analysis of bacterial cells containing fluorescence genes in Chapter 2 and 3 (including the FRT or lox-reporters), 1μl of bacterial suspension was spotted onto glass slides containing an LB agar pad before imaging.

For the microscopic analysis of multispecies communities, 0.1mM of IPTG or 0.2% arabinose were added during culture exponential growth to induce gene expression in *P. aeruginosa* and *E. coli*, respectively. Multispecies bacterial suspensions were prepared as described before and 1μl of each suspension was spotted onto a glass slide containing 1.5% agarose (Fisher Scientific) in M9 minimal media (Sigma Adrich) + 0.4% casamino acids (Formedium). Once the spot was completely absorbed onto the agarose, slides were either immediately imaged or incubated at 37^{o}C for 2 hours without the coverslip to allow oxygenation. Three biological replicates were performed for each experiment.

Microscopic visualisation was performed using a Nikon ECLIPSE Ti2 inverted microscope with a CoolED pE4000 illuminator and a Zyla 4.2 Megapixel Camera. Images were recorded using Nikon Elements software and analysed using Fiji (ImageJ)[323].

7.7 Statistical analysis, graphs and images

Statistical analysis and graphs were done using Excel Version 16.94 for macOS and GraphPad Prism 10 Version 10.4.1 for macOS. All figures are original and were created using Microsoft PowerPoint Version 16.93.2 for macOS and Biorender.com.

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