Interactions of the emerging human pathogen

*Photorhabdus* with the human immune system and the

novel injectosomes it utilises

by

Max Laurens Addison

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*Supervisors:* Prof Nicholas R. Waterfield and John E. Connolly

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Finally I would like to thank my two supervisors, Nick Waterfeild and John Connally for being sources of inspiration and guidance throughout my project.
Declaration

The work in this thesis is submitted to the University of Warwick in support of my application for a PhD in Molecular Biomedicine. This is an entirely novel work of research by the authors and has not previously been submitted for any other degree or published, as of writing, in any journal. A significant portion of this work was conducted while in Singapore at the A*star research institute, with the work done here also being of the authors own.
Abstract

*Photorhabdus* is a genus of entomopathogenic luminescent bacteria which is used as a bioweapon by its nematode symbiont. In its normal lifecycle it carried in the gut of the insect pathogenic nematodes, *Heterorhabditis*, and released when the nematode invades the body of a host insect. *Photorhabdus* then produces a large range of toxins to kill, sterilise and bio-covert the insect host into the perfect habitat for the nematode.

While primarily an insect pathogen, certain species of *Photorhabdus* found in Australia and North America, have been shown to be capable of infecting humans, where they cause large lesions across the body and suppression of the immune system. Recently species which were originally thought not to be human infective, have also caused disease, giving the possibility of an emerging new emerging pathogen.

Here the interactions between these human infective species and the mammalian immune system have been investigated, to get deeper understanding into how the bacteria evade the host and establish an infection. It was found that geographically distinct sub-species differ greatly in their behaviour towards the immune system, even when closely related phylogenetically.

The role of the unique *Photorhabdus* toxin delivery system, known as PVCs was also examined. These act as targeted Nano-syringes, a mix between a T4 bacteriophage and the T6SS, capable of delivering a wide range of toxins to eukaryotic cells. It was found that different PVC operons were activated in the presence of varying *Photorhabdus* hosts. The system by which effectors are loaded into the PVCs was also discovered, seemingly being linked to a long N-terminal tail, dubbed the “Leader sequence”. These leader sequences were found to be promiscuous in their ability to load, even loading non-Photorhabdus proteins such as Cre recombinase. This opens the possibility in the future of using PVCs as a targeted protein delivery system.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PVC</td>
<td>Photorhabdus Virulence Cassette</td>
</tr>
<tr>
<td>MAC</td>
<td>Metamorphosis-Associated Contractile Structure</td>
</tr>
<tr>
<td>AAA+ ATPase</td>
<td>ATPases Associated with diverse cellular Activities</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ssDNA</td>
<td>single stranded Deoxyribonucleic acid</td>
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<td>gDNA</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>IJ</td>
<td>Infective Juvenile</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>TRL</td>
<td>Temperature Restriction Locus</td>
</tr>
<tr>
<td>MCF</td>
<td>Make Caterpillars Floppy</td>
</tr>
<tr>
<td>T[x]SS</td>
<td>Type [number] Secretion System</td>
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<tr>
<td>Bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Patterns</td>
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<td>Natural Killer</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>CIS</td>
<td>Contractile Injection Systems</td>
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<td>Abbreviation</td>
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<td>-----------</td>
</tr>
<tr>
<td>eCIS</td>
<td>Extracellular Contractile Injection Systems</td>
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<tr>
<td>PAAR</td>
<td>Proline-Alanine-Alanine-aRginine</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>O/N</td>
<td>Over Night</td>
</tr>
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<td>Electron Microscopy</td>
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<td>Transmission Electron Microscopy</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
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<td>RT-PCR</td>
<td>Reverse Transcription PCR</td>
</tr>
<tr>
<td>nm</td>
<td>Nano-Metre</td>
</tr>
<tr>
<td>µm</td>
<td>Micro-Metre</td>
</tr>
<tr>
<td>mm</td>
<td>Milli-Metre</td>
</tr>
<tr>
<td>MDa</td>
<td>Mega-Dalton</td>
</tr>
<tr>
<td>aa</td>
<td>Amino Acids</td>
</tr>
<tr>
<td>PenStrep</td>
<td>Penicillin-Streptomycin</td>
</tr>
<tr>
<td>W/V</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>SH buffer</td>
<td>Sucrose-HEPES</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>TM buffer</td>
<td>Tris-Magnesium Chloride buffer</td>
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<tr>
<td>CV</td>
<td>Column Volume</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>MQ</td>
<td>Milli-q Water</td>
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Chapter 1

Introduction

Throughout my PhD the main focus was on the bacteria *Photorhabdus*, a worldwide insect pathogen and nematode symbiont. It is a fascinating species of bacteria for a variety of reasons, such as being the only terrestrial bacteria to bio-luminesce, although the reason for this is still poorly understood. One theory is that it wards off scavengers that may try to consume the cadavers of the insects that *Photorhabdus* infects.

Due to its unique biology, *Photorhabdus* and its bio-products have found several uses. Firstly, it is currently used alongside its host nematode, as a bio-insecticide to kill crop pests, such as the wax moth (Keskes et al, 2021). Secondly, its version of luciferase, the protein behind its ability to luminesce, has found use in research as a marker for imaging (Gregor et al, 2018).

However, the research in this thesis focused not on these aspects, but on two major parts of the *Photorhabdus* lifecycle and biology. First, the ability of some species of *Photorhabdus* to infect humans, and why some can and cannot be human pathogenic. It was also elucidated as to how *Photorhabdus* changes its strategies depending on if it is infecting mammalian or insect hosts, mainly by investigating how it changes its behaviour at either mammalian (37°C) or insect (28°C) simulated body temperatures.

The second focus of my research was on the newly discovered toxin delivery system in *Photorhabdus*, the PVCs [Photorhabdus Virulence Cassettes], which are a secreted injectosome that can inject proteins directly into specific cell types distant from the bacteria. Little is known about this system, so this project tried to understand what the functions of some PVCs may be in the *Photorhabdus* lifecycle and how the injected effectors are chosen to be loaded into the PVC. During this research I also investigated ways in which the PVCs could be used for delivery of proteins in both research and therapeutic settings.
1.1 A partnership between insect pathogenic bacteria and nematodes.

*Photorhabdus* is a Gram-Negative, obligate symbiont and bio-weapon of the entomopathogenic nematodes *Heterorhabditis*. *Photorhabdus* produces a range of toxins that kill and sterilize the nematode’s host insect allowing the nematode to invade and consume the host. Strangely it was perhaps first reported, albeit unknowingly, during the American civil war for being the cause of reports that certain wounds of soldiers left on the battlefield would glow a mysterious pale blue known at the time as the “Angels Glow”. It was believed that the *Photorhabdus* would out compete other bacteria in the wound preventing more dangerous infections. Due to the large amount of toxins they produce, they may possibly also give us a treasure trove of new anti-bacterial, anti-fungal compounds and other beneficial natural products. However, before we can discuss the bacteria itself though, we first have to understand the symbiotic nematode partner it is intrinsically tied to.

1.2 The parasite: *Heterorhabditis*

*Heterorhabditis* is part of the order *Rhabditida* which consists mainly of microvore nematodes, and all generally have part of their life cycle living in soil. Many *Rhabditida* are parasites by nature with *Heterorhabditis* itself being a very effective parasite of numerous insect larvae such as; *Popillia japonica*, *Conotrachelus nenuphar* and *Otiorthynchus sulcatus*. *Heterorhabditis* and some other *Rhabditida* insect parasites such as *Steinernema* are commercially relevant (Lacey et al, 2012), with increasing use as a bioinsecticides to kill crops and garden pests (Rezaei et al, 2015). The nematode’s parasitic ability is reliant on its symbiosis with *Photorhabdus*, which is the active agent in killing of the host and is key in progression of certain stages of the nematode’s lifecycle. Indeed, the nematode cannot survive or develop without the bacterium. The nematodes carry the *Photorhabdus* inside their guts while in the soil, then release them after invading the insect, making the nematode itself more a delivery system for the...
Photorhabdus rather than the actual cause of death of the insects. In fact, while the nematodes are often referred to as parasites of insects, they do not actually largely feed on insect tissue. Instead, the nematode mainly feeds on the growing Photorhabdus population, which are breaking down and bio-converting the insect tissues into more bacteria that act as feed stock (Koppenhofer et al, 2007).

Due to this, the symbiosis between the two is highly controlled and conserved to the point where each species/subspecies of Photorhabdus can only form a symbiotic relationship with a certain cognate species/subspecies of nematode (Emelianoff et al, 2008; Ferreira et al, 2014). While it was thought that only the Photorhabdus contributed to the disabling and killing of the insect host, a recent study has shown that Heterorhabditis itself has a role in immunosuppression of the host. Where in the early stages of infection, they secrete proteins that suppress the Imd pathway which is an insect immune response pathway linked to production of antibacterial compounds and activation of immune cells (Kenney et al, 2019; De Gregorio et al, 2002).
**Fig 1.1 | Life cycle of the *Heterorhabditis* nematode.**  

**A** - Infective juvenile’s (IJ) live in the soil searching for host insects, neither eating nor reproducing. **B** - Once a host is found the IJ enters the body cavity by burrowing through the cuticle. **C** - Once inside the host the IJs regurgitate *Photorhabdus* from their gut, which sets up an infection, killing the host. **D** - IJ’s develop into adults and asexually reproduce for a few generations. **E** - When the insect carcass has been fully consumed some adults undergo endotokia matricida, where eggs hatch inside the adult. The internally hatched juveniles eat the mother from the inside, at the same time taking *Photorhabdus* into their guts initiating a new round of symbiosis. **F** - Once the mother is fully consumed the juveniles emerge, further developing into full IJs. The new IJs then burrow out of the insect carcass and search for new hosts.

### 1.2.1 *Heterorhabditis* life cycle

The *Heterorhabditis* lifecycle consists of an Infective juvenile (IJ) stage living in the soil which seeks new hosts, and a hermaphroditic adult stage which reproduces inside insect larvae (**Fig 1.1**). Infective juveniles travel through the soil until they find a host insect larva, at which point they penetrate its body cavity either by burrowing through the cuticle, or through natural openings, such as the spiracles (Poinar *et al.*, 1990). Approximately 30 mins post-invasion of the host haemocoel, the IJ releases...
around 100 *Photorhabdus* bacteria from its gut through regurgitation (Ciche *et al.*, 2003), which set up an infection that quickly spreads throughout the host insect, killing and sterilizing it for the nematode. It is in these first 30 minutes where the importance of the nematodes own immune suppression can be seen, as pre-*Photorhabdus* release, is usually the point at which the host immune system will kill the invading nematodes. For instance, compared to a common laboratory model target of *Heterorhabditis*, the greater wax moth *Galleria mellonella* and the Colorado potato beetle *Leptinotarsa decemlineata* can encapsulate and kill invading IJs in within 15 minutes, thus evading *Photorhabdus* infection (Ebrahimi *et al.*, 2011).

Post-*Photorhabdus* release, the IJs develop into hermaphrodite adults that feed upon the *Photorhabdus*, which by this point has colonised the insect cadaver. Both species go through several rounds of reproduction inside the cadaver, typically 2-4 generations of nematodes, during which the *Photorhabdus* produces a range of secondary metabolites to assist and control the development and replication of the nematodes (Tobias *et al.*, 2017).

Once the insect carcass has been fully consumed, *Photorhabdus* re-initiates symbiosis and new IJ development. While this process is poorly understood it appears to involve; the bacteria forming a biofilm adhered to the maternal adult nematode’s posterior gut. From here the bacteria then travel to and invade the rectal gland cells, which allows release into the body cavity (Ciche *et al.*, 2008). At a currently unconfirmed stage during this process the *Photorhabdus* induces endotokia matricida, a process where egg laying is prevented and so hatch inside an adult nematode allowing the larvae feed on the parent before emerging (Chen & Caswell-Chen, 2003). It is during this process that the Photorhabdus re-colonize the new IJs. Endotokia matricida was first noted in *C. elegans* as a response to starvation, leading to development of dauers, which are of a similar developmental state to IJs (Chen & Caswell-Chen, 2003). Endotokia in *Heterorhabditis* has been noted as being necessary for re-symbiosis and IJ formation. Once IJs have hatched inside the mother, *Photorhabdus* seems to invade their pharyngeal intestinal valve cells and migrate towards the gut of the IJ which is then colonised (Ciche *et al.*, 2008). The new infective juveniles then emerge from the mother and the insect carcass to seek out new hosts. It is possible that at this point the blue light of the bioluminescence is used to drive the new IJs from the insect cadaver as they are adverse to blue light.
1.2.2 The model nematode: *Caenorhabditis elegans*

Closely related to *Heterorhabditis*, another of the *Rhabditida* nematodes, is the model organism *Caenorhabditis elegans*. While *C. elegans* is free living in the soil, it shares many similarities to *Heterorhabditis*, including reproduction through hermaphrodites, and feeding on bacteria. Unlike *Heterorhabditis* it does not normally undergo endotokia matricida, except in long term starvation stress conditions where it is more commonly referred to in the literature as “Bagging”. Due to the extensive knowledge of *C. elegans* biology, being the only organism to have a complete connectome, and their relative ease to grow, they make an excellent model organism for the study of nematodes and bacterial infections (Gravato-Nobre & Hodgkin, 2005; Sifri et al, 2005).

1.3 The symbiont: *Photorhabdus*

Originally the *Photorhabdus* genus was split into three main species; *P.luminescens*, *P.temperata* and *P.asymbiotica* (Fig 1.2). Recently, there have been attempts to reclassify the various species and sub-species as to better distinguish them from one another and elevate many of the sub-species to species level (Machado et al, 2018). However, for the purposes of this thesis I will be sticking to the older form of classification using *P.luminescens*, *P.temperata* and *P.asymbiotica*. The existence of these groups was what led to the formation of some the hypotheses and experimental designs, as to which strains may be acting similarly.

All three of the *Photorhabdus* species can be found worldwide, with multiple sub-species having been identified for each (Thanwisai et al, 2012; Mandadi et al, 2015). Despite a fairly large number of *Photorhabdus* strains having now been identified, they have never been directly isolated from the soil. Instead, most isolates come from the host nematode IJs which were in the soil. This indicates again the fact that *Photorhabdus* is intrinsically linked to its host *Heterorhabditis* and cannot replicate
successfully without it. However, despite this, it is possible to grow in normal LB [Lysogeny Broth] in lab conditions.

Due to its dependence on the host nematode in the environment, *Photorhabdus* is in a niche unlike many bacteria where it is intrinsically geographically locked with potentially very little opportunity for mixing of distant species, as both would need to be infecting the same insect. In cases where multiple infections are initiated in the lab, one or the other predominates and kills the other. Because of this it can be assumed that an isolate from a certain region will be unique to that region, as mutations would not be able to disseminate over large distances easily, again having to rely on the host nematode for dispersion.

Most species of *Photorhabdus* are limited to growth below 34°C, seemingly most comfortable at the environmental temperature of 28°C which is roughly simulates insect body temperature (Fischer-Le Saux et al., 1999), although of course in the environment temperatures will fluctuate. Laboratory experiments have shown that, at least for one strain, P. luminescens TT01, that when grown above this temperature the bacteria appear to first go into growth arrest then eventually die. This growth temperature restriction seems to be due to the presence of a three gene operon called the Temperature Restriction Locus [TRL] (Hapeshi et al., 2020). Strangely in this case, presence of the TRL is what restricts growth at higher temperatures, suggesting an evolutionary advantage for most *Photorhabdus* species to be unable to grow above insect body temperature. This actually makes sense as *Photorhabdus* has a clear need to maintain symbiosis with *Heterorhabditis*, which is solely insect pathogenic and cannot grow above 34°C, so this restriction may help maintain the symbiosis. The presence of the TRL, and subsequent temperature restriction appears to mainly fall along species lines, with both *P.luminescens* and *P.temperata* being unable to grow at the higher, mammalian body temperatures, while the *P.asymbiotica* which lack the TRL can (Mulley et al., 2015). However, there have been some observations of subspecies and strains that differentiate from the norm. One clade of *P.luminescens* is missing the TRL and as such is able to grow at 37°C. While the two European genospecies strains of *P.asymbiotica* grow very poorly at this temperature (Mulley et al., 2015), although like the other *P. asymbiotica* are also lacking TRL. These variants have only been recently discovered so their diversity and range are currently still
under study. Nevertheless, both these variants are important to the work in this thesis so will be covered in more detail later.

Fig 1.2 | Subclades of the various *Photorhabdus* species.

Tree lengths are not drawn to scale and for illustrative purposes. Displayed are the approximate thermotolerances and known potential hosts of archetypal strains of *Photorhabdus* within each species. Data on thermotolerances is from the paper by Mulley *et al.*, 2015 (22) and own experience working with strains in our lab. *While published data on the HIT and JUN strains showed inability to grow at 37°C, during this project it was found that given extended growth times, of 48 hours compared to 24 hours, they would regularly reach higher ODs and eventually reach stationary phase.

1.3.1 *Photorhabdus luminescens* and *temperata*: Insect pathogens

*P. luminescens* is the best studied of all the species of *Photorhabdus*, with the majority of research being focused on it. In fact, strain TT01, and the mutant DJC, are the main laboratory strains used for study of *Photorhabdus*. Phenotypically *P. luminescens* and *P. temperata* are quite similar, although compared to *P. luminescens*, *P. temperata* is still not well characterised. Both are reported as being exclusively insect pathogenic, both cannot for the most part grow above 34°C and they have similar growth rates and colony morphologies. One of the main differences between the two species are the
locations they have been isolated (Fig 1.3, A), with *P. luminescens* being isolated warmer climates while *P. temperata* is found in more temperate locations nearer the poles. These differences could be down to different their host nematode species which are likely to be restrained by geological conditions. However, this seeming difference in climate preference maybe simply due to low amounts of isolates having been collected, with *P. temperata* also having been isolated from warmer regions, such as Turkey.

*P. luminescens* and *P. temperata* are also the main species being used for bio-pesticides, with extensive research also being carried out on their use for control of disease carrying insects such as mosquitos (da Silva *et al*, 2020). On the other hand, *P. asymbiotica* hopefully is not used as a bio pesticide, for reasons that will be covered shortly.

### 1.3.2 Human infective *Photorhabdus: Asymbiotica*

*P. asymbiotica* differs greatly from its sister species in two ways, firstly most strains seem to be able to grow at 37°C (Mulley *et al*, 2015), and secondly has been the cause of multiple human infections being dubbed an emerging human pathogen (Costa *et al*, 2009; Wilkinson *et al*, 2009). While like the other species, strains have been isolated worldwide, so far human infections have only been reported in Australia, continental North America and one from Nepal (Farmer *et al*, 1989; Pujol & Bliska, 2005). In these cases, many of the infections were clustered in areas of those countries, Texas in North America and Victoria in Australia and strains of *Photorhabdus* were isolated from lesions and blood of infected patients. These areas have sandy soils, and infections seem to coincide with warm, wet weather, which may stimulate nematode activity or perhaps insect host availability.

The initial site of infection in these cases were usually located at the extremities, such as the hands and feet, and in people who had been working outdoors with exposure to soil. There was also a trend of insect bites around the infected area. The likely scenario here is that an open wound, from an insect bite, was exposed to soil containing either *Photorhabdus* or host nematodes which were then transferred into the wound setting up the infection. It is also possible that the nematode pierced the skin itself, initiating the *Photorhabdus* infection, as the infective juveniles of some
related nematodes to *Heterorhabditis*, such as *Strongyloides stercoralis* have been shown to be able to burrow through human skin (Gang *et al.*, 2020). Nevertheless, a *Heterorhabditis* nematode has never been isolated from one of the human infections, suggesting that if they do attempt burrow through human skin they do not get very far.

While the number of recognised human infections may seem very low, it should be noted that until as recently as 2017, *Photorhabdus* was not included in the standard databases used by many medical professionals to identify infections. This led to it being commonly mistaken for other bacteria when tested in a lab setting, for instance a VITEK 2 Gram-negative identification card was shown to misidentify it as *Pseudomonas fluorescens* (Weissfeld *et al.*, 2005). This leaves the main way to identify *Photorhabdus* infections as 16S ribosomal RNA sequencing, which is not always possible due to time constraints, taking 48-72 hours (Boyles and Wasserman, 2015). These factors, along with the ease most *Photorhabdus* infections can be treated, with a fairly standard antibiotic course, means that *Photorhabdus* infections likely go underdiagnosed. This may be why we have only had reports of infections in the USA and Australia, as other places where *P. asymbiotica* has been found, such as rural Thailand, may not have the resources or infrastructure to properly identify infections. This indicates that *Photorhabdus* human infections maybe much more widespread than first thought.

This idea is backed up by a study from Japan which was looking at the human antibody titres against the V-antigen of various Gram-negative bacteria, including *P. luminescens* (Kinoshita *et al.*, 2020). V-antigens (Virulence associated proteins) were first discovered in *Yersinia pestis*, with homologs since found in many different pathogenic Gram-negative bacteria, and are associated with the toxic effects of Type 3 secretion systems. Kinoshita *et al.*, found that many of the serums they tested reacted to the presence of the *P. luminescens* V-antigen (LssV), indicating immune system exposure to *Photorhabdus* is not uncommon.

The disease caused by *Photorhabdus* infection, has become known as “Photorhabdosis”. A typical human infection starts as a large skin lesion, presumably at the site of initial infection, followed by a high fever. If untreated secondary lesions appear on other parts of the body, confirming bacteraemia. In some cases bacteria were found in the respiratory system and the heart causing endocarditis, confirming
the ability of *Photorhabdus* to disseminate throughout body. It has been hypothesised
they may achieve this by hijacking immune cells and essentially “hitchhiking” via the
lymphatic system. This facultative intracellular infection behaviour is seen in the
phylogenetically closely related *Yersinia pestis*, which are carried inside phagocytes
around the body (St John *et al.*, 2014). Some bacteria can also display this behaviour
without invading the host cell, as in the case of *Streptococcus pyogenes* which remains
extracellular during its traversal of the lymphatic system (Siggins *et al.*, 2020).

While previous research shows that *Photorhabdus* is capable of surviving in human
serum (Sifri *et al.*, 2005), little research has been done into the cellular host-pathogen
interactions of *P. asymbiotica* with humans. One of the few studies showed that
*P.asymbiotica* is a facultative intercellular pathogen, able to survive within
macrophages and induce apoptosis through the activation of caspases (16). Interestingly the North American and Australian clinical isolates acted differently,
despite both causing similar disease in humans. The Australian strains were found to
be more heavily phagocytosed, than the North American strains. Also, while the
Australian strains were observed invading non-phagocytic cells, (in this study Hela
cells), the North American strains did not (Gravato-Nobre & Hodgkin, 2005). This
indicates that *P.asymbioitca* strains from different geographical locations have
evolved independent strategies for overcoming the mammalian immune system.

As mentioned at the beginning of this Chapter *P.asymbiotica* strains can grow at
higher temperatures then the other species. This is the case for most strains that have
been identified, apart from two nematode isolates found in Europe HIT and JUN
which had very poor growth at above 34°C (Mulley *et al.*, 2015). However, from
research conducted in our lab, they do seem capable of growing at 37°C, unlike most
*P.luminescens* and *P.temperata* however their growth is far slower and more
inconsistent than the other *P.asymbiotica* strains.

1.3.3 *P. luminescens* Texas strain: An emerging human pathogenic
lineage

Until recently only *P.asymbiotica* strains had been reported to cause human infections,
being believed to be the sole mammalian pathogenic lineage of *Photorhabdus*, mainly
due to the other species being believed to not be able to grow at mammalian host
body temperature. However, in 2018, an abandoned neonate was admitted to the Texas Children’s Hospital, Houston with an hypothermia, lowered white blood cell count, bacteria in blood samples and lesions of the thoracic area of their spine (Dutta et al., 2018). The bacteria was later identified as a *P.luminescens* strain, which had the unique ability among the known *P.luminescens* to grow above 34°C, much like the *P. asymbiotica*. This was the first reported case of a non-asymbiotica *Photorhabdus* species causing a human infection. The neonate was found in rather unique circumstances which would have been ideal for exposure to *Photorhabdus*. Doctors theorised that at time of admission the new-born baby only 6 hours old and had been abandoned outside on the ground under an air-conditioning unit, with multiple insect bites. These conditions, warm and moist, plus the presence of insects would be perfect for the host nematodes of *Photorhabdus*. In fact, a researcher later went back to the area where the neonate was found approximately one year later and managed to isolate nematodes from the soil containing the same strain of *P.luminescens* isolated from the neonate (Personal communication).

Very little is known about this strain of *Photorhabdus* and what differences from its brethren *P.luminescens*, allow for it to infect humans. We have subsequently genome sequenced the clinical and nematode isolates and we note is that its ability to grow above 34°C is likely due to the absence of the TRL.
Fig 1.3 | *Photorhabdus* isolates from around the world.

A- The locations at which various *Photorhabdus* species were first isolated. This is not a comprehensive map of all isolates of *Photorhabdus* there have been. It should be noted that while *P. luminescens* spp. and *P. temperata* spp. were all isolated from nematodes found in soil samples, *P. asymbiotica* spp. have also been isolated from infected patients in clinical settings.

B- Locations of known *Photorhabdus asymbiotica* infections. So far cases have only been seen in Australia and the USA.

### 1.3.4 *Photorhabdus* toxins

In order to fulfill its role as the nematode’s bioweapon *Photorhabdus* produces a large range of toxins and small molecule “natural products” that target a wide scope of organisms. Firstly, to kill the insect multiple insecticidal toxins are made examples being the large, toxin delivery injectosome Tc toxins (Yang and Waterfield, 2013) and
the Make Caterpillars Floppy [MCF] toxins which induce apoptosis (Dowling et al., 2004). In fact, MCF is so effective at killing insects, that the addition of this one toxin allows previously non-insect infective E.coli to be able to kill and colonise caterpillars (Daborn et al., 2002).

A very important role *Photorhabdus* also has is in shutting down the insect immune system. In order to do this, it appears to use a range of toxins that target immune cells. One instance of this is the utilisation of its Type 3 Secretion System [T3SS] to target insect phagocyte-like cells, such as plasmatocytes. One of the toxins that is delivered by the T3SS is LopT, which is a homolog of YopT from *Yersinia*. *Yersinia* is also an insect pathogen, with some strains that can also infect humans. It uses its T3SS to deliver YopT to immune cells, which prevents phagocytosis (Shao *et al.*, 2002). The T3SS delivered LopT in *Photorhabdus* can also also prevent phagocytosis (Brugirard-Ricaud *et al.*, 2005). Strangely, in the *P.asymbiotica* T3SS the LopT toxin gene is absent, having been replaced by a homolog of the *Pseudomonas aeruginosa* ExoU toxin. ExoU is a phospholipase which causes acute lung injury in *Pseudomonas* infections (Pankhaniya *et al.*, 2004). Despite this, *P.asymbiotica* does however, have a LopT homolog associated with one of its PVC operons, suggesting this maybe its preferred delivery system for this toxin, possibly in this case also to human macrophages as well. While I will describe the PVC system later in more detail, in brief they are a mobile injectosome complex, which bind and inject specifically into target cells. It should be noted that *P.asymbiotica* strains also seem to have a second T3SS operon not found in the other species. The possession of a second T3SS is common in some other species such as certain *Salmonella* strains, in which it is used to secrete effectors once they have established an intracellular infection (Pezoa *et al.*, 2014).

After killing the insect host, a range of anti-bacterial compounds are also produced to stop other bacteria and fungi from colonising the energy rich insect carcass, such as the broad range antibiotic stilbene (Park *et al.*, 2017). Despite the large amount of anti-microbial products produced by *Photorhabdus*, certain other bacteria can also colonise the insect carcass (Wollenberg *et al.*, 2016). Recently it was observed that some bacteria can even co-colonise in the nematode gut, such as *Ochrobactrum*, which can co-exist with *Photorhabdus*, and certain strains may have evolved resistance to some of its toxins (Aujoulat *et al.*, 2019). Alongside the anti-bacterial compounds, red pigments such as anthroquinone and luciferase are produced in large quantities.
(Clarke, 2008), believed to function to ward off potential scavengers of the dead insect.

Finally, there are symbiosis related compounds which control host nematode growth, development and prevent feeding by non-symbiont nematodes (Tobias et al, 2017). One example is again stilbene which causes recovery of infective juveniles and acts on Photorhabdus itself, supressing growth in a stringent response like manner (Park et al, 2017).

Within this large number of natural products, some have been shown to have possible biomedical applications, against various pathogenic organisms. A secondary metabolite known as Photorhabdus-derived leishmanicidal toxin [PLT], was shown to be lethal to Leishmania amazonensis promastigotes, by causing mitochondrial membrane depolarization (Antonello et al, 2018).

Interestingly in addition to having differing T3SS, it was found in a genome comparison of the human infective P.asymbiotica strains and P.luminescens, that P.asymbiotica is missing a range of insect toxicity genes, resulting in a genome approximately 600,000 base pairs [bp] smaller. These include genes related to the Tc toxins and Mcf toxins. Despite this P.asymbiotica does not seem to be any less capable of infecting and killing insects (Wilkinson et al, 2009).

## 1.4 *Photorhabdus* Host immunity

One of *Photorhabdus’* main roles during infection is to overcome the host’s immune system, allowing the nematode to establish itself. This applies both in mammalian and insect infections, and many of the virulence factors produced by *Photorhabdus* likely play roles in both types of infection. Here I will talk about the Insect and mammalian immune systems and how they differ and conform.

### 1.4.1 Insect Immunity

The insect immune system while not as complex as mammalian immune systems, being solely innate, is still able to fight off invasions from a range of pathogens such
as fungi, bacteria and multicellular parasites. The immune system of insects varies greatly in both function, and nomenclature, between different genera. However, the immune systems of Lepidoptera, one of the main targets of Heterorhabditis, and the common model organism for studying insect immunity Drosophila melanogaster share many similarities. From study of Drosophila melanogaster, two types of immune response to pathogens have been identified, the humoral and cellular.

The humoral response consists of the release of various cytotoxic compounds in response to the detection of bacterial invasion into cells. One of the main mediators of the humoral response is the Toll receptor, which has a mammalian homolog the Toll-like receptors [TLRs]. Toll receptors detect invasion of bacteria into cells through the production of a cysteine-knot protein called Spätzle (Govind 2008). Activation of the Toll receptor causes a signalling cascade to initiate the activation of transcription factors and production of antimicrobial peptides (Valanne et al., 2011). The other main pathway involved in the humoral response is the Imd pathway. This pathway recognises extracellular peptidoglycan, a common component of bacterial cell walls, upon which it activates genes related to antimicrobial peptide production (Kleino & Silverman, 2014). A third important humoral response is the Phenol Oxidase response, which is serine protease cascade, leading to the activation of the Phenol Oxidase enzyme on the surface of the pathogen, encasing it in melanin and isolating it.

The cellular response on the other hand consists of immune cells, known as haemocytes, circulating within the haemolymph which respond immediately upon recognition of an invading pathogen. This means that the cellular response is the one of the key immune responses to Photorhabdus infection. As the humoral response can take a few hours to initiate post-infection, which would be too slow when dealing with a Heterorhabditis and Photorhabdus infection. A number of hemocytes make up the cellular response including granular cells, crystal cells, and plasmatocytes (Vlisidou & Wood, 2015), of these the best understood and studied in Drosophila are; crystal cells, plasmatocytes, and lamellocytes (Parsons & Foley, 2016).
1.4.2 Plasmatocytes

Plasmatocytes are the most abundant haemocyte, making up 95% in Drosophila adults (Honti et al., 2014). They are small and long-lived cells, capable of forming pillia. Their main function in Drosophila is to act as the professional phagocyte, although some insect species use granular haemocytes instead (Rosales et al., 2011). Much like in mammals the plasmatocytes recognise bacteria through surface receptors capable of detecting either Pathogen Associated Molecular Patterns [PAMPs] or opsonins, phagocytosis enhancing molecules created by the humoral response (Browne et al., 2013; Ratcliffe & Rowley, 1984). Once recognised they engulf and kill the bacteria inside the phagosome. Alongside phagocytosis, haemocytes also undergo nodulation for when overwhelming infections are present. Nodulation involves the surrounding of bacteria with haemocytes which aggregate together to form a shell around the site of infection. This also involves the PO cascade, forming a shell is then melanised “nodule” trapping the infection within, which can be bacterial, fungal or viral in nature (Satyavathi et al., 2014).

1.4.3 Lamellocytes

For larger invasive pathogens, such as parasites and nematodes, hemocytes form a capsule around the invader, in a process known as encapsulation. This process is mediated by another type of hemocyte known as a lamellocyte. Lamellocytes, are long flat cells mainly found in the larval stages. When an invading parasite is detected, lamellocytes bind to it in layers forming a capsule, which is later melanised by crystal cells. (Satyavathi et al., 2014, Ratcliffe & Gagen, 1977). After encapsulation the pathogen is either killed by cytotoxic products or asphyxia (Carton et al., 2009).

1.4.4 Crystal cells

Crystal cells mainly support the humoral response and other immune cells through activation of melanisation (Meister & Lagueux, 2003). Melanisation plays a role in both encapsulation and nodule formation, as well as wound healing. Crystal cells are large cells with granules containing ProPhenoloxidase, which is activated to Phenoloxidase and released from the cells, initiating the melanisation process.
Of these processes, it is likely that the *Heterorhabditis* produced compounds made early in infection, before the release of *Photorhabdus*, work to slow down and evade the lamellocyte mediated encapsulation. Then once *Photorhabdus* is released the bacteria’s various toxins and natural products kill and shut down the entire immune system, particularly the plasmatocytes, allowing both it and its symbiont nematode to parasitize and kill their host unperturbed. One *Photorhabdus* compound that acts to block the PO cascade is Rhabduscin, which also block the alternative pathway of the human complement cascade, suggesting it may also be relevant to human infection.

1.4.5 Human immune system

Compared to insect’s the human immune system is more complex having not only an innate response, but also an adaptive response as well. However, many parts of the immune system are only relevant to certain pathogens, and so to keep this chapter focused I will be only talking about the parts relevant to a *Photorhabdus* infection. The most likely way that *Photorhabdus* gets into the body is either though a skin wound or being delivered by the nematode burrowing through human skin. In both these cases its first interaction is going to be with skin resident immune cells, and then later on in the infection circulating blood cells.

It is likely that human infective *Photorhabdus* has adapted many of its pathogenicity factors normally used for insects into use against humans. In fact studies have shown that toxins from non-human infective *Photorhabdus*, such as MCF toxin, can still cause apoptosis in mammalian cells (Dowling *et al.*, 2004). This indicates that there would likely not have to be many changes to the catalytic parts of toxins and instead just their delivery systems. We can see this in many other insect-mammalian pathogens such as *Yersinia*, which is closely related to *Photorhabdus* (Heermann & Fuchs, 2008).

1.4.6 Innate immunity against *Photorhabdus*

The innate immune system of humans has many functionally homologous systems to that of insect immunity, in fact some of our understanding of human immunity comes from the study of insects such as the toll-like receptors. As with insect
immunity, the innate systems are quick to activate and respond to pathogens, unlike adaptive immune systems. Thus, due to being the first immune cells to react to *Photorhabdus* infection, and their similar functions to insect immune cells, the innate immune cells are probably what *Photorhabdus* is most capable of dealing with.

### 1.4.7 Dendritic cells

Dendritic cells are a type of phagocyte that is normally present in tissues with exposure to the outside environment, such as skin. Unlike the professional phagocytes such as macrophages their main role is not in clearance of pathogens, but instead in antigen presentation to T and B cells causing activation of these cells and acting as an early alert system to bacteria and viruses (Nagl *et al.*, 2009). They start as immature dendritic cells and sample the environment using TLRs (Banchereau & Steinman, 1998). After detecting a foreign antigen either from the environment, engulfing live bacteria, or contact with infected cells, they mature and migrate to the lymph nodes (Banchereau *et al.*, 2000; Albert *et al.*, 1998). Once at the lymph nodes, the mature dendritic cells activate naïve T-cells, allowing the adaptive immune system to start targeting the pathogen.

Due to how effective and essential the adaptive immune system is in clearing bacterial infections, many bacteria attempt to avoid alerting T and B cells for as long as possible. This is very clearly seen in some gut pathogens such as *Yersinia* and *Salmonella*, which have devised a range of methods to prevent Dendritic cell activation of T-cells (Bedoui *et al.*, 2010).

### 1.4.8 Professional phagocytes

Professional phagocytes have a number of roles in immunity and maintenance of tissues, though their main functions are engulfment of dead or dying cells, and pathogens. There are three main types of professional phagocyte: neutrophils, macrophages and monocytes.

Neutrophils are normally the first phagocytes to arrive at the site of an infection, being highly motile and numerous. Their main role is to clear and contain bacterial infections early on, before the adaptive immune system can activate. They have three
main methods of combating bacteria. First, is phagocytosis where they engulf bacteria, then secrete reactive oxygen species and hydrolytic enzymes into the phagosome, killing any bacteria. Secondly, they release anti-microbials, such as defensins, in a process known as de-granulation (Xu & Lu, 2020). Finally, they release web like structures composed of chromatin and serine proteases known as neutrophil extracellular traps (Brinkmann et al, 2004). These trap and kill large groups of bacteria, preventing the infection from spreading. The mammalian neutrophils are probably the most homologous to the insect plasmatocyte, both mainly having a role in pathogen engulfment and killing, with neutrophils having little interaction with the adaptive immune system.

Conversely the other main professional phagocyte, macrophages have many roles outside of just killing bacteria. Macrophages arrive at the site of infection much later compared to dendritic cells and neutrophils. Once there they both phagocytose bacteria and engulf dead or dying neutrophils to clear them and mediate inflammation. However, unlike neutrophils, macrophages also act as antigen presenting cells, displaying antigens from digested bacteria. Their main role in this case is to present antigens to activate T-helper cells, causing them to multiply, this is done both at the site of infection and in the lymph nodes (Gaudino & Kumar, 2019).

Monocytes are blood resident phagocytes which can differentiate into macrophages and dendritic cells. While their main role is to replenish tissue resident macrophage populations, they can phagocytose and present antigens themselves (Döring et al, 2015).

While phagocytosis is a very effective way of dealing with bacteria, some bacteria have found ways to circumvent killing by of phagocytosis and even use it to their advantage. The easiest way to prevent being killed by phagocytes is to simply avoid it altogether. Bacteria have evolved many strategies for this from preventing opsonization and recognition (Rooijakkers et al, 2005; Kuipers et al, 2016), to using toxins to prevent the cytoskeleton rearrangement needed for engulfment (Jank et al, 2007; Higgs & Pollard, 2000). The other way is for the bacteria to allow itself to be engulfed and captured into a phagosome, but then prevent maturation of the phagosome or escape it (Ehrt & Schnappinger, 2009; Jamwal et al, 2016). This method has the added benefit of allowing the bacteria to gain access to the inside of
phagocytes, from where they can influence host behaviour and be protected from the immune system (Mitchell et al, 2016).

1.4.9 Natural Killer cells

Natural Killer [NK], cells are innate immune cells whose main role is detection and killing of virus infected and cancerous cells. However, research has shown that they can also play a role in dealing with bacterial infections. It would appear from studies in mice that the cytokine Interferon-gamma [IFN-γ] produced by NK cells is a key cytokine in clearing of bacterial infections (Spörri et al, 2006). IFN-γ regulates many different aspects of the immune system, specifically it increases antigen expression of macrophages, primes them against infections and increases migration of leukocytes (Schroder et al, 2004). They also seem to be able to induce apoptosis in bacterial infected macrophages and even directly target and kill intercellular bacteria (Brill et al, 2001).

1.4.10 T-cells

T-cells are a major component of the adaptive immune system, which is slow to activate, but when fully activated can quickly clear infections. For this reason, for many bacteria, it is vital to either prevent activation or establish a very large infection before activation. T-cells are very complex and for brevity will only be focusing on their more general functions, however they can be roughly grouped into two groups, Cytotoxic T-cells, and T-helper cells.

Cytotoxic T-cells function similarly to NK-cells causing the death of infected or cancerous cells, but unlike NK-cells are primed to recognise specific pathogen antigens. One of the anti-bacterial compounds they produce to kill bacteria are be granulysin, which alongside perforin lyses infected cells and kills the bacteria within by interfering with the bacterial membrane (Silva & Lowrie, 2000; Stenger et al, 1998; Woodworth et al, 2008).

Helper T-cells on the other hand mainly serve to activate and modulate the response of other immune system cells, such as B-cells and cytotoxic T-cells. The only time they
seem to directly interact with bacteria is in maintenance of the commensal gut microbiota (Sorini et al., 2018).

### 1.4.11 B-cells

B-cells are the other important part of the adaptative immune system, and once activated through a combination of antigen detection and T-helper cells, which themselves are activated by dendritic cells and macrophages, start producing antibodies. Antibodies are a very important part of the immune system allowing for a diverse range of functions from, neutralisation of bacterial toxins to optimization of bacteria allowing fast and effective killing and clearing of infections. However, some bacteria can actually invade and hide in B-cells preventing clearing (Nothelfer et al., 2014). Some bacteria, such as *Brucella abortus*, can even manipulate B-cells into becoming immunosuppressive, to such a point where mice without B-cells were better at combating these infections (Goenka et al., 2011). Other bacteria prevent activation of B-cells, such as *Yersinia pseudotuberculosis* instead prevents activation of T and B-cells using one of its Yop toxins YopH (Yao et al., 1999).

### 1.4.12 Immune response to the nematode

As none of the human cases of Photorhabdosis have been reported to have a co-infection with the nematode, it can be assumed that it cannot establish an infection in human tissue and is killed by the immune system. The main mediators of anti-helminth immunity in humans are Dendritic cells, Eosinophils and B-cell production of Immunoglobulin-E [IgE]. In this case the immune response is likely similar to that in *Ancylostoma duodenale* (Hookworm) infections, as both are roundworms that enter the body through penetration of the skin.

It would not be surprising if *Heterorhabditis* cannot establish an infection in humans, due to its very aggressive form of infection that prioritises quick host death, at odds with most successful human pathogenic helminths. Looking at widespread, highly infectious human pathogenic helminths, such as *Schistosomes, Onchocerca volvulus* and *Trichuris trichiura*, there is a noticeable trend towards long-term, chronic illnesses that can last upwards of 20-30 years before causing host death (McSorley & Maizels, 2012). In fact, much of the pathogenicity of many of these species do not come directly from
virulence factors released from the helminths, but from the host’s own immune response. This may even be triggered not by the helminth but by the common intracellular symbiont Wolbachia (Taylor et al., 2000), which many of these parasites require for reproduction (Tamarozzi et al., 2011). Although it should be noted that unlike Photorhabdus, Wolbachia does not itself seem to be human pathogenic. There are even theories that due to the problems that an intense immune response towards the parasites can cause, that the human immune system has adapted to mediate inflammation and immune cell activation to helminth infections (Maizels & McSorley, 2016).

1.5 Bacterial secretion systems

Having such a range of toxins, also known as effectors, Photorhabdus like many bacterial pathogens, needs a way to translocate these from its cytoplasm and into the host. This is a particular problem for Gram negative bacteria as they have both an inner and outer membrane, which secreted proteins must cross. In order to achieve this, bacteria have developed a number of secretion systems ranging from simple gated pores to large multimeric protein structures that transverse both the bacteria and host membranes. While most of these secretion systems seem to play a role in pathogenesis and sometimes symbiosis, some seem to be solely used for non-infective purposes, such as the Type 8 Secretion System [T8SS] which is involved in the exporting part of the extracellular matrix known as Curli (Bhoite et al., 2019; Chapman et al., 2002).

Bacterial secretion systems can be split into two groups, ones that release toxins straight into the extracellular milieu, such as the Type 1, 2 or 5 secretion systems, from which they disperse until they find a target cell. While the other group directly inject the effectors into the target, this group includes the Type 3, Type 4 and Type 6 secretion systems.
1.5.1 Non-injection secretion systems

Of the secretion systems that release into the extracellular milieu, the T2SS and T5SS require the effector to first be transported into the periplasm between the inner and outer membrane by the Sec pathway. The Sec pathway consists of a simple motor protein and protein targeting component that recognises an N-terminal signalling tag. Depending on this tag the proteins are then either passed to the T2SS/T5SS or remain in the periplasm (Hartl et al., 1990). Unlike the T5SS, the T2SS secretion system transports folded proteins, however the Sec pathway is only capable of transporting unfolded proteins, and thus these proteins must be folded in the periplasm (Korotkov et al., 2012). Unlike the T2SS and T5SS, the T1SS does not require the Sec pathway, being able to transport effectors straight from the cytoplasm to outside the cell, although it can only transport unfolded proteins (Thomas et al., 2014).

One of the more common types of toxin which are released into the extracellular milieu, are the AB toxins. The AB toxins are a large diverse group of toxins which include many well-known and extremely potent toxins such as; Diphtheria toxin, Anthrax toxin, Botulism toxin and Cholera toxin. Despite their wide range of targets and effects the AB toxins share a common strategy for entering a cell. Firstly, their effects act within the cell meaning they need a way to enter from the extracellular milieu. In order to achieve this, they are comprised of two domains, the catalytic domain which acts as the effector and a binding domain, which translocates the catalytic domain into the cell. When a target cell is encountered the binding domain will bind to a surface protein, causing the AB toxin to undergo a conformational change which moves the catalytic domain through the plasma membrane and inside the target cell. The binding domain is then cleaved from the catalytic domain allowing it to induce the desired effects within the cell (Odumosu et al., 2010). Often this conformational change and cleaving also activating the catalytic domain, which is not toxic in its native form.

While the AB toxins can cross membranes themselves, most of the other toxins are not capable of this. Thus, these have to be directly injected from the bacterial cell into the host to have their cytotoxic effects. This encompasses the second group of secretion systems, the “injection” systems under which PVCs fall. Currently the best understood systems are the Type 3 and Type 4 secretion systems. While their functions are ultimately very similar, the mechanism they employ, and their
evolutionary origins are quite different. With again two main classes appearing, the non-contractile and contractile systems.

### 1.5.2 Type 3 Secretion System

Firstly, the Type 3 Secretion System (T3SS) is a non-contractile system widely spread throughout gram negative bacteria, used exclusively to inject effectors into eukaryotes ranging from human to plant cells. Many important bacterial human pathogens have T3SSs that are essential for their human pathogenesis, including *Shigella* (Deane et al., 2008), *Salmonella* (Johnson et al., 2018) and *Yersina pestis* (Plano et al., 2013).

The structure of the T3SS is highly conserved and the high structural homology of its component proteins suggests it has the same evolutionary origins as the flagellar motor and flagellar export system. In fact the export apparatus for flagellar proteins, sometimes confusingly called the Type 3 Secretion Apparatus, and T3SS are essentially the same (Erhardt et al., 2010). Due to its similarities to the flagellar system and large improvements in structural electron microscopy the structure of the T3SS is now well understood. Much like the flagellar basal body, it consists of a series of ring proteins, surrounding a hollow rod which is assembled from the cell membrane outwards. At the end of the rod, when fully assembled, is a translocation pore formed of two proteins. The rod components and effectors are exported by an export apparatus at the base (Lombardi et al., 2019).

Despite our detailed structural knowledge of the T3SS, it is still not known how it translocate effectors into the target cell. Some theories suggest that a pore forming complex at the end of the rod binds to the target membrane, forming a tube through which effectors can travel. It is also not currently known how the T3SS detects the target to initiate secretion.

One example of proteins that are injected into the host through the T3SS are the *Yersinia* outer protein (Yop) effectors, from *Yersinia pestis*. While *in vitro* *Y.pestis* seems capable of injecting the Yops into a variety of cell types, *in vivo* it mainly targets immune cells macrophages (Köberle et al., 2009). There are six Yop proteins that are translocated into target cells, which seem to have two main roles. YopH, YopE, YopT
and YpkA cause host cytoskeleton rearrangement (Iriarte et al, 1998), while YopJ (Ma & Ma, 2016) and YopM (Chung et al, 2016) interfere with host cell signalling supressing inflammation and innate immunity. Interestingly, *Phototubus* seems to have a number of Yop-like effectors in its genome, which it may have received from *Yersinia* through horizontal transfer.

### 1.5.3 Type 4 Secretion System

As the T3SS has evolutionary homology to the flagellar system, the pathogenic version of Type 4 secretion system (T4SS) has its homology to the pilus system, which also has a Type 4 secretion apparatus. The pilus system is used for DNA conjugation between bacteria, allowing for horizontal gene transfer and at some point, diversified to be used as an injection system into eukaryotes. Due to this evolutionary history, uniquely among the bacterial secretion systems, the T4SS can transfer both proteins and DNA. The T4SS is also found across many lineages, being found in Gram negative, Gram positive and even some archaea (Guglielmini et al, 2014).

When used as a non-pathogenic system, it is used for transfer of DNA between bacteria, in the process known as conjugation, allowing for horizontal gene transfer. As well as transferring DNA between cells, the T4SS can also be used to excrete and take up DNA from the environment which is an important part of some cells, such as *Helicobacter pylori*, ability to undergo transformation (Hofreuter et al, 2001).

There are two main pathogenic functions of the T4SS which have so far been identified and seems to target only eukaryotes. Firstly, some plant pathogenic bacteria, such as *Agrobacterium tumefaciens*, use a modified version of this T4SS to transfer tumour causing ssDNA to target plant cells (Zupan et al, 2007). Secondly, the T4SS can be used, much like the T3SS to transport proteins between cells, such as in *Legionella pneumophila* from which around 300 potential effectors have been identified that are transported by the T4SS (Hubber et al, 2010).

Interestingly in some cases the T4SS transports the toxins into the extracellular millu instead of directly into a cell. One such case is one of the main toxins, of the causative agent of whooping cough, *Bordetella pertussis*’ (Walden et al, 2010), pertussis toxin, an AB toxin. When released from the cell it binds to sialic-acid containing glycoproteins
causing endocytosis, which allows the toxin to eventually disseminate from the endoplasmic reticulum.

The T4SS shows us how diverse the uses of many of these secretion systems can be, playing roles that are both pathogenic and non-pathogenic.

![Fig.1.4 | Secretion systems of Gram-negative bacteria.](image)
The Red circle indicates an effector/toxin which would be loaded into the secretion system, while the grey heptagon is an adaptor protein used by the T6SS.

### 1.6 Contractile secretion systems

Out of the secretion systems found within nature, those with some of the most fascinating mechanisms are the contractile injection systems (CISs). These are a group of diverse macromolecular devices that use a contractile sheath coupled to a tube with a spike like domain to inject either DNA such as Type 4 [T4] bacteriophage, or Proteins such as T6SS.

#### 1.6.1 Type 6 Secretion System

Like many of the CISs, the Type 6 secretion system [T6SS] appears to have its origins from bacteriophages with studies of some of its proteins, e.g., VgrG and HCP,
showing to be structurally and functionally homologous to those in bacteriophage T4 (Pukatzki et al, 2007). Much like the T4 bacteriophage, the T6SS consists of a long repeated protein unit needle like tube (Hcp) with a spike at the end (VgrG+ PAAR domain protein), surrounded by a sheath also comprised of repeating protein units (TssBC). This forms the syringe like apparatus which is anchored into the bacterial membrane by a baseplate-membrane spanning complex. The tip of the needle sits within the membrane spanning complex and from here the rest of the syringe is assembled downwards into the bacterial cell, often spanning the entire length of the bacteria. The proteins to be injected are either packaged within the tube or attached to the spike. When ready to fire, the sheath contracts causing the needle like tube to be forced out the bacterial cell and through the adjacent target cell’s membrane. The tube then dissociates releasing the toxins (Zoued et al, 2014).

The T6SS has a range of functions, targeting both eukaryotes and other bacteria. Many pathogenic bacteria use the T6SS to inject toxins into the host either while sitting on the surface of the cell, or through endo/phagosomes. For instance, Francisella tularensis, the causative organism of the disease tularemia has been shown to use its T6SS to escape phagosomes, allowing infection of macrophages (Clemens et al, 2018). F.tularensis‘s eukaryotic effectors are so potent at establishing an infection, that in the most infective strain the 50% Lethal Dose [LD50] in mice was found to be only 1-4 organisms (Bell et al, 1955). The antibacterial use of the T6SS is well documented in many bacterial species, often with competitor bacteria firing at each other with constantly reloading T6SS while competing for dominance. Sometimes they will hit their sister cells, however they will not kill them, instead it has been observed that this acts as a way of both cell signalling and potential recycling of the T6SS components between related cells (Gallique et al, 2017).

While the T6SS is probably the best studied contractile system in bacteria, there exists a group of contractile syringes which are not membrane bound, instead being released outside of the cell to attack the targets. These have been referred to as extracellular Contractile Injection Systems [eCIS] (Chen et al, 2019).
1.6.3 Tailocins

The most common type of eCIS are the Tailocins, also known as R-type pyocins as they were first identified in *Pseudomonas aeruginosa* (Michel-Briand *et al.*, 2002). The common nomenclature now used for new versions is to call them after the bacteria with -cin suffix added. The tailocins are a subtype of bacteriocins, which are bacteria made anti-bacterial proteins, that act as simple pore forming devices normally used to kill closely related competitor bacteria. Bacteriocins have been known about and studied for many decades, being first properly characterised in *E. coli* in 1946 (Gratia & Fredericq, 1946). They are found in various different bacterial species, having been reported in; *E.coli* (Gratia & Fredericq, 1946), *P. aeruginosa* (Michel-Briand *et al.*, 2002), *Enterobacter cloacae* (Oudega *et al.*, 1979), *Serratia marcescens* (Fuller & Horton, 1950), as to name a few. Bacteriocins, and conversely the tailocins, are mainly for interspecies bacterial competition, so have to be highly specific, normally only targeting single sub-species.

Unlike the other eCISs, tailocins do not deliver any effectors. Instead, when a target cell is found, they bind using bacteriophage like tail fibres, and puncture the bacterial cell membrane, forming a pore that eventually leads to cell death (Carim *et al.*, 2021).

1.6.4 Protein delivery Extracellular Contractile Injection Systems

Less well characterized are the eCISs that are capable of delivering proteins. While sequence analysis has revealed a wide range of bacterial species with eCIS like operons (Geller *et al.*, 2021), many of these seem to be incomplete, missing key genes. So far only three examples have been studied which have been confirmed to be capable of making a functional protein delivery eCIS’s. The *Serratia entomophila* AfP (Anti-feeding Prophage), which causes the larvae of *C. zealandica* to cease feeding (Rybakova *et al.*, 2013). *Pseudoalteromonas luteoviolacea’s* metamorphosis-associated contractile structure [MAC], which causes its symbiont *Hydroides elegans* to undergo metamorphosis (Ericson *et al.*, 2019). Interestingly unlike the other known eCIS’s the MACs seem to form large arrays where many MACs are joined at the base (Shikuma *et al.*, 2014). The final example, being the only bacteria so far found with multiple eCIS operons, are the PVCs from *Photorhabdus*. 
1.7 PVCs: Headless bacteriophages

PVCs are large multi-protein structures produced by *Photorhabdus*, morphologically similar to a headless T4 phage particle and a non-membrane associated T6SS (Fig 1.5, A). Their main function in *Photorhabdus* is to inject various effectors into eukaryotic cells, either to control their symbiont nematode development or to kill and overcome the host insect immune system. Unlike other secretion/injection systems seen in bacteria, PVCs are released from the bacterial cell into the extra-cellular milieu, where they can act independently on their targets. After being released from the bacteria they selectively bind to target cells using bacteriophage like tail fibres and inject effectors into target.

Fig 1.5 | Structure and genetics of the *Photorhabdus* PVC system.

A – Comparison of the structure of PVCs and the T6SS, with functionally similar proteins being the same colour. Some proteins do not have a homolog such as the T6SS membrane complex TssJLM, which has had no identifiable homolog in the PVC. OM – Outer membrane, IM – Inner membrane B – Typical PVC operon structure. The structural genes are always found first and in a set order, followed by a series of effector genes.
1.7.1 PVC Structure

Most PVCs have the same basic structure consisting of 16 proteins arranged into an operon (Vlisidou et al., 2019). These form: a base plate (PVC 9, 11, 12), Proline-Alanine-Alanine-aRginine [PAAR]-like spike domain to pierce the cell membrane (PVC 8), outer sheath (PVC 2,3,4), inner tube (PVC 1,5,7), tail terminator cap protein (PVC 16) and tail binding fibres (PVC 13) (Fig 1.5, B). The only exception to this general description found so far is PVC_LopT, found in all studied Photorhabdus species, which is missing the gene for tail fibres. Thus, it is thought that this PVC may not be specific and instead inject into any cell it encounters.

The general structure of the PVC is similar to that of the T6SS mixed with a bacteriophage. The main body/tail of the PVC consists of an inner tube, made of stacked repeating hexamer rings of PVC1. This grows from the baseplate where a ring of PVC7 and a ring of PVC5 initiate growth. This is surrounded by a sheath made of rings of PVC2, which binds to the baseplate through PVC3. Both the tube and sheath are capped by the tail terminator PVC16. PVC14, the tape measure protein, runs along the length of the tube and is theorised to control how long the tail grows. The tail is anchored to a baseplate consisting of dimers of PVC11-12 arranged into wedges radially around a trimer of PVC8, which makes the main body of the spike. At its tip the spike protein has PVC10, a PAAR domain protein, which is used to puncture the target cell. The tail and baseplate are held together by interactions between PVC7 and the PVC11-12 dimer. Finally, the tail fibres are attached to the baseplate and dock into PVC3 (Jiang et al., 2019).

Mechanically PVCs operate much like the other CIS. In the case of the PVCs it is hypothesized that; the PVCs are created within the cells, at which point the effectors are loaded. Then either through deliberate lysis of the cell or through a secretion pathway the PVCs are released from the Photorhabdus into their surroundings. unknown a target cell is encountered the tail fibres bind causing a conformational change to occur in the sheath, forcing the spike and tube to thrust into the target, piercing the cell membrane. The tube then dissociates, releasing the effectors into the target cell.
In the T6SS, effectors are loaded onto either the PAAR spike domain, or the Hcp inner tube, by chaperones known as adaptor proteins. Different adaptor proteins have varying levels of affinity for PAAR/Hcp, so when multiple T6SS effectors are being produced they will compete for loading, leading to effectors with the higher affinity adaptor proteins being loaded more often (Unterweger et al., 2017). The PVCs conversely seem to lack adaptor proteins, so the mechanism by which how effectors are loaded is still unknown.

### 1.7.2 PVC diversity

The genes for PVCs are arranged into a conserved layout in operons under one promotor with the structural genes first in a specific order followed by a region of possible effectors at the 3’ end (Fig 1.5, B). Each Photorhabdus strain encode a range of different PVCs, with most species having around six different operons each, likely with different functions.

The homology of PVC operons within different species of Photorhabdus was investigated using the best studied strains from each of the main species’ P.luminescens, P.temperata and P.asymbiotica. (Fig 1.6). It appears that the PVCs are more related by type rather than species of origin with each operon corresponding to a different PVC type. The types identified so far are Unit1, Unit2, Unit3, Unit4, Lop’T, Pnf, Cif, Cer and LumT. While each PVC_type forms its own grouping, overall, most of the PVC_types are generally very similar to each other, indicating a high conservation pressure to keep the general structure the same. This makes sense as due to the complexity of the PVC protein interactions small changes could lead to losses in function. However, Pnf and LumT diverge from the other PVCs in terms of sequence identity. PVC_LumT encodes additional genes which may mean it is mammalian adapted and, which explains why its operons cluster away from the other PVCs, but Pnf does not seem to have any extra or missing genes. Both of these PVC types are only found in the human infective strains of P.asymbiotica, suggesting that they have changed over time to better adapt to mammalian infection. Also PVC_Pnf is the most closely related PVC type to the eCIS homolog found in Xenorhabdus, meaning this could be an ancestral form of PVCs, or was obtained from Xenorhabdus through horizontal gene transfer.
Table 1.1 | Presence of known PVCs in various strains of *Photorhabdus*.

It should be noted that some strains may have extra PVCs in their genome that have not been found yet. Green boxes indicate the presence of that PVC operon type in the strains genome.

<table>
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<tr>
<th>SPECIES</th>
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<th>UNIT2</th>
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<th>LOPT</th>
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Closely related *Photorhabdus* species have the same types of PVC, as would be expected, and from what is seen so far from identified species, this seems to split on the species level. Most species have 4-6 PVC operons, although it is not known if the ones with less simply have not had all the operons found yet. All *Photorhabdus* species have the PVC_LopT and PVC_Cif types, suggesting these are required for survival regardless of host. PVC_Unit4 also seems to be present in most of the studied species.

The *P.luminescens*, has two PVCs not found regularly in the other species being Unit1 and Unit2. Unit1 displays a lot of sequence homology to Unit3, meaning it could be a second copy of this operon, however in these strains that have both Unit3 and Unit1, the effectors associated with each operon differ.
The *P. asymbiotica* on the other hand do not have Unit1-3, instead having the Pnf and LumT operons. Considering that *P. asymbiotica* are capable of infecting mammals these two PVCs are likely involved in overcoming the mammalian immune system, as it is more complex than the insect immune system. This is already experimentally confirmed in the case of PVC_Pnf, as studies have shown it can target and kill mammalian cells (Vlisidou *et al*, 2019). These two PVC operons are also found in some *P. temperata* strains. However, not much is known about the *P. temperata* strains lifecycles so it is unknown if they can infect mammals.

The European strains of *P. asymbiotica*, JUN and HIT, strangely have an extra unique PVC operon, known as Cer. Little is known about this PVC operon, or these strains, though it does appear as mentioned earlier that unlike the other *P. asymbiotica* they have difficulty growing at 37°C. It should be noted at this point that HIT and JUN, while appear to be closely related to the clinical strains, are actually phylogenetically distinct so we should not expect them to behave in exactly same way. Interestingly, while all the PVC operons of JUN are complete, in the HIT genome they are all seemingly truncated at the PVC12 gene. While this could be a sequencing artefact, it would seem strange for them all to truncate at the same gene in every operon if this was the case. Looking for genes downstream of PVC12, in these cases shows no obvious Open Reading Frames [ORFs] that have any homology to known PVC structural genes. Since these operons lack so many of the downstream PVC genes including the cap and tape measure proteins, it would seem that HIT is the only *Photorhabdus* strain identified so far that is lacking functional PVCs, if the genomic sequencing is accurate. However, as it was isolated from soil nematodes, this does not seem to affect its ability to symbiose.
Fig. 1.6 | Phylogenetic tree comparing the relatedness of various PVC operons.

Tree was constructed from concatenated genes of the PVC structural proteins, (PVC 1-16), or their homologs.

1.7.3 Other bacteria with eCIS

There have been a few studies looking for the presence of eCIS genes and operons in other bacteria, although the majority have not been experimentally confirmed. Geller et al, 2020; identified the presence of putative eCIS operons in around 2% of the prokaryotic species they analysed, though they were only looking for a minimum of three eCIS associated genes so it is unknown how many of the operons are complete or active (Geller et al, 2021). They also looked at the correlation between the presence...
of eCIS and the lifestyle of prokaryotes, finding that eCIS’ were enriched in environmental and symbionts, but had a decreased frequency in pathogens. Also, in the eukaryotic associated bacteria they have evaluated, those associated with the microbiome of human tissues also showed a reduced presence in eCIS’. However, the lifestyle factors were based on the metadata from the database that was used, Integrated Microbial Genomes, of which some entries are either incomplete or in some cases the labels are misleading. The presence of eCIS’ does not seem to be homogenous throughout all strains or species of any one genus, instead being restricted to certain phylogenetic clades. For instance, while all the Photorhabdus genomes contained eCIS operons, another genus that is rich in eCIS’ was Chitinophaga, with 89% of the genomes analysed containing an eCIS genome. Like Photorhabdus, Chitinophaga is a symbiont, but of the plant associated fungus Fusarium keratoplasticum (Shaffer et al, 2017). However; unlike Photorhabdus it does not assist in pathogenicity but instead seems to play a role in nutrient acquisition (Shaffer et al, 2017). If Chitinophaga’s eCIS’ are active then they may play a role in either killing competitor bacteria, or like the MACs control the host development somehow.

1.8 Protein delivery into cells

There is a long history of biomolecular machines being co-opted for use in medicine. The extracellular contractile injection systems have the potential for many different uses in medicine. While only starting to come to prominence in the West, bacteriophages, phage therapy, have a long history of use previously in the Soviet Union and still are currently in Eastern Europe. For instance one use that gained prominence was, lytic bacteriophages, such as T6, which were studied as a novel antibacterials, but then fell out of favour with the discovery of antibiotics (Rohwer & Segall, 2015). However, with the recent increase in antibiotic resistance to a wide range of antibiotic types (Aslam et al, 2018), and the lack of new antibiotics being discovered there has been a resurgence of interest in them, particularly in the West (Furfaro et al, 2018). They have also been studied for use in treating cancer (Eriksson et al, 2009) and even delivering proteins.
PVCs and other eCISs might also have potential not in targeting bacteria but instead targeting eukaryotes. Due to the ability of PVCs to deliver proteins to specific cells, there are a range of uses they could find in both medicine and research.

### 1.8.1 Problems with gene therapy

A major hurdle for medical science currently is being able to get medically relevant proteins into cells *in vivo*. The ability to get a certain protein into a cell easily and specifically would revolutionize medicine, such as being able to deliver an enzyme that is missing due to a genetic abnormality, or a toxin to a cancer cell. While in a laboratory environment, DNA delivery via transfection has improved greatly over the years, the ability to deliver proteins still lags behind. This may make it seem favourable to use DNA transfection to get the target cells to produce the protein themselves, known as gene therapy, but this can have several problems *in vivo*. Firstly, DNA transfection efficiency varies widely between cell types, especially for stable transfections, with transfecting wild type cells being notoriously difficult. Secondly, some complex proteins, particularly from non-animal cells, may not fold correctly when expressed within a mammalian cell.

Since PVCs are a purpose-built protein delivery system by bacteria, which unlike most bacterial delivery systems are mobile, it could be possible to re-engineer them to deliver useful proteins to cells.

While there are now many technologies for delivering proteins into eukaryotic cells, the main focus of current research and the holy grail of sorts is the system that can be used in humans for effectors with medicinal purposes. Such a system would require several properties. Firstly it would need to be specific, as to only deliver effectors to the target cell type. Secondly, be able to deliver a range of effectors; from toxins to kill cancer cells, to gene editing tools such as CRISPR to name a few. Thirdly, it would also have to be highly stable in order to survive long enough to get to the target. Moreover, it would also have to be non-toxic to the cell, a problem many DNA delivery methods have. Finally, it would have to be feasible to make large quantities and not too prohibitively expensive.
So far a number of different solutions have been devised to try and combat the problem of protein delivery, however none of them have managed to solve all the problems presented. We can already rule out most of the bacterial secretion systems due to them being bound to the membranes and thus working version would not be able to be purified away from the cell.

1.8.2 Microinjection

Microinjection is the method of using a micron sized pipette to pierce the plasma membrane and deliver molecules to the cell. The needle is normally attached to a micromanipulator to allow for better control but is still a slow, finicky process that requires training. It has been used for many years for injection of DNA to create transgenic lines, such as with mosquito larvae (Jasinskiene et al., 2007). In more recent years this technique has been adapted for injection of proteins into cells as well, delivering molecules such as CRISPR (Chaverra-Rodriguez et al., 2018). However, there are some severe limitations to this technique. Firstly it only allows transfection of proteins into single cells at a time and can only be done to cells that can be reached by the micropipette, making it impractical in a medical context. Even simply as a tool for manipulation of cells in a research environment it leaves much to be desired. The main problem being that the injection causes significant disruption to the cell membrane, leading to cell survival rates of only around 50% in some cases (Lim et al., 2011).

1.8.3 Hijacking endocytosis

One proposed approach of delivering proteins in a medical scenario is using the mechanisms of the target cells directly to endocytose proteins. Commonly in these methods the protein is fused to or trapped within another molecule which induces endocytosis. There are a number of methods that induce endocytosis, one of the more promising being the use of nanoparticles. Nanoparticle methods, describe a range of inert, non-toxic nanomaterials which are used as scaffolds for multiple functional domains that facilitate, uptake by the target cell, cargo protein binding and
endosomal escape. A few of the nanomaterials that have been tested so far are, gold (Ghosh et al., 2008), silica (Tang & Cheng, 2013) and mesoporous silica (Du et al., 2016).

The main problem with these endocytic methods however is escaping from the endosome, as most endosome pathways will end with degradation. A few ways have been devised to escape the endosome such as GALA3 a synthetic amphipathic peptide derived from the part of the major envelope glycoprotein of HIV, HA2 (Li et al., 2020). The theory is that GALA3 fused to the cargo protein fuses to the membrane of endosome, forming a pore and disrupting the endosome, allowing the cargo to enter the cytosol and have its effect. This is still an emerging field of study however.

1.8.4 Direct delivery into the cytosol

As using endocytosis has some issues in protein delivery, methods that deliver the protein directly to the cytosol, without going through the cell’s trafficking system first are preferable.

One of the commercially available lipid-based methods of protein delivery is Bioporter (Genlantis). Bioporter works in a very similar fashion to DNA transfections, such as lipofectamine, where positively charged lipids form a complex with the protein of interest. This complex can then either fuse with the cell’s plasma membrane, releasing the proteins into the cytosol, or be endocytosed. It is unclear what the mechanisms leading to these two possibilities are, or if only one pathway is actually used. One major disadvantage of Bioporter is that it is not cell specific.

1.8.5 Advantages of PVCs for protein delivery

PVCs have the potential to overcome many of the weaknesses of the other methods currently used or proposed for protein delivery. Firstly, they inject directly into the cytosol meaning there is no need to add extra domains to effectors to facilitate escape from phagosomes/endosomes. Unlike most of the systems I have described here they are purpose built for specificity, with the different tail fibres of each operon possibly allowing for injection into a range of cell types. They also do not seem to be directly cytotoxic when injecting into cells, as empty syringes seem to have no effect.
(Unpublished data). Finally, they seem to be fairly stable for long periods of time (Unpublished data).

There are still some potential disadvantages to using PVCs. The main being that the PVC is a such a large protein structure, it is likely immunogenic. If this is the case then its uses could be very limited in whole organisms as it could initiate an immune response which could either disable the PVCs or cause life threatening inflammation. This possible effect is still being studied in the lab.
Fig 1.7 | Methods of protein delivery into eukaryotic cells.

A- PVCs. Loaded PVCs are exposed to cells and bind only to target cell type (I.). Binding of tail fibres causes conformational change causing contraction of sheath and the tail spike-inner tube complex to pierce the cell membrane, however does not seem to causes lasting damage (II.). The spike-inner tube sub units dissociate releasing the loaded protein into the cytoplasm (III.).

B- Lipid based methods. Vesicles containing proteins bind to cell membrane (I.). From here there are two possible pathways depending on proteins associated with vesicle: Either vesicles fuses fully with plasma membrane releasing proteins directly into cytosol (II a.), or vesicle is endocytosed (II b.). If vesicle is endocytosed, in order for effective protein delivery, there must be some kind of endosome escape protein associated to the protein/vesicle in which case protein escapes vesicle into cytosol (III b.). If protein is not able to escape vesicle
most likely it will be taken to the endosome for degeneration, failing in delivery. C-Nanoparticles. Proteins are fused to nanoparticle scaffold, with a number of functional domains. From here follows very similar pathway to lipid vesicles, either being endocytosed (II b.), or moving directly into the cytoplasm (II a.). D- Micro/Nano Injection. Needle and syringe are loaded with protein (I.). Normally using a micromanipulator, needle carefully pierces plasma membrane and protein is injected directly into cytosol (II.). Needle is removed, but in doing so causes damage to cell membrane that can potentially lead to cell death (III.).

1.9 Goals of this thesis

My research in this thesis revolved around *Photorhabdus* and how human infective and non-human infective species differ in their behaviour toward different cell types. I also wanted to study with the emergence of the new human infective *P. luminescens* Texas strain and if there were substantial differences in pathogenicity linked to strains isolated from American and Australian *P. asymbiotica* strains.

I also investigated the PVCs and their potential role in infection and pathogenicity, which is still very poorly understood. This enquiry separated into two parts. Firstly, I looked at what the targets for each PVC might be by testing what may activate transcription of them. Alongside this I also investigated the effector regions of each PVC, to determine which effectors may be linked to each operon type. This would not only give us an idea of the various toxins *Photorhabdus* has access to, but also give some insight into what the different PVC operons may be used for. During this investigation I discovered an effector of PVC_Unit4, which did not seem to show homology to any known toxin. Thus, I also investigated its effects on eukaryotic cells and the host nematodes.

The other part of this project involving PVCs, was trying to better understand how effectors are selected for loading into the PVC needle complex. As each PVC has multiple putative effectors in their effector region, it has been unknown up until now how one effector is chosen to be loaded over another. After finding the mechanism that selected effectors for loading, there were also attempts to change what was loaded into a PVC. The idea being that if we can change the payload of a PVC, it may have possible uses in medical settings as a cell specific delivery system.
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*Photorhabdus* interactions with the mammalian immune system.

Until recently only *P. asymbiotica* had been reported to cause human infections, and of them only the Australian and North American strains, being believed to be the sole mammalian pathogenic lineage of *Photorhabdus*. However, in 2018, a neonate was admitted to the Texas Children’s Hospital, Houston with an increased temperature, lowered white blood cell count, bacteria in blood samples and lesions of the thoracic area of their spine (Dutta *et al.*, 2018). The bacteria was later identified as *Photorhabdus luminescens*, which had the unique ability among the *P.luminescens* to grow above 34°C, much like the *P.asymbiotica*. This was the first reported case of a non-Asymbiotica *Photorhabdus* causing a human infection. The neonate was found in rather unique circumstances which would have been ideal for exposure to *Photorhabdus*. They had seemingly just been born, doctors theorised that at time of admission it was only 6 hours old and had been abandoned outside on the ground under an air-conditioning unit, with multiple insect bites. These conditions, warm and moist, plus the presence of insects would be perfect for the host nematodes of *Photorhabdus*. In fact, a researcher (Dr John Gerrard – Goldcoast hospital, Australia) later went back to the area where the neonate was found and managed to isolate nematodes from the soil containing the same strain of *Photorhabdus* isolated from the neonate. (Personal communication)

Very little is known about this strain of *Photorhabdus* and what differences from its brethren *P.luminescens*, allow for it to infect humans. The only difference of note is that this is the only *P.luminescens* that has been reported to grow above 34°C due to losing the TRL which is responsible for most *Photorhabdus* not being able to grow at high temperatures (Hapeshi *et al.*, 2020). The fact that some strains of *P.luminescens*
may be capable of evolving mammalian pathogenicity, simply by losing the TRL, brings up safety concerns with the common use of *Photorhabdus* and their nematodes as over the counter bio-insecticides. This makes understanding the human-infective strains ever more important.

While it has been a long time since the first recorded case of a *Photorhabdus* human infection, being in 1977 (Farmer et al, 1989) or possibly earlier if the angel glow theory is true, there has been little study on the emerging human pathogenic species of *Photorhabdus*. The only research done so far has been on *P.asymbiotica* confirming that it is both human pathogenic and is capable of invading cultured human immune cells (Costa *et al*, 2009). This research did bring up an interesting observation however, that the American and Australian strains of *P.asymbiotica* varied in their ability to invade human cells, despite both being human infective. This raises the interesting question of if geographically distinct strains of *Photorhabdus* differ in their strategies for evading/resisting the immune system response. So far, all the research on *P.asymbiotica* has only been done solely on the American and Australian strains, the two confirmed to be human infective. However, many new strains have been isolated around the world which are genetically distinct from each other and have not yet been associated with a human infection human infection. This could even raise the possibility that not all *P.asymbiotica* are capable of human infection, a strong possibility as the two Northern European isolates (HIT and JUN) seem to have trouble growing at $>34^\circ$C in a lab environment (Mulley *et al*, 2015). As for the newly discovered human infective *P.luminescens* spp Texas strain, it is a complete unknown, including how it differs from its brethren *P.luminescens*, and what allows enables it to infect humans. In this chapter I will detail our efforts to better understand the interactions of the various *Photorhabdus* species with human immune cells, so that we can start to define the characteristics and tropisms which allow for some species to move from insect to mammalian hosts.

To get a clearer understanding of how the geographically distinct strains of *Photorhabdus* differ in their strategies for evading/resisting the immune system response five geographically distant *P.asymbiotica* strains were included in this study *(Fig 2.1)* The North American *P. asymbiotica* subsp. *asymbiotica* strain ATCC43949, the Australian *P. asymbiotica* subsp. *Australis* strain (Kingscliff), the closely related Thai
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*Photorhabdus* and the immune system

*P. asymbiotica* subsp. *Australis* strain (PB68) and the two Northern European *P. asymbiotica* subsp, designated HIT and JUN. Kingscliff and ATCC43949 are clinical isolates from confirmed human infections, while the other three were isolated from soil dwelling nematodes, it being unknown if they can cause human infections. Alongside these *P. asymbiotica* strains, two *P. luminescens* strains were also studied; the human infective Texas stain and the non-human infective lab strain TT01-DJC.

In order to better understand how the behaviour and mechanisms of these strains compared, they were then tested for their ability to survive or avoid phagocytosis in the human macrophage model cell line, THP-1. We also decided to examine how they interact with a more natural and complete model of the human immune system. In order to do this, we studied the interactions of *P. asymbiotica* with human derived PBMCs [Peripheral blood mononuclear cells] from healthy human volunteers.

Here for the first time, we reveal how *P. asymbiotica*, selectively associates with certain immune cells while avoiding others. We also show that the immune cells infected varies depending on the geographical location the strain was isolated from. We also show that the Northern European genospecies strains of *P. asymbiotica*, HIT and JUN, lack the ability to survive within mammalian cells, indicating that unlike previous assumption, not all members of *P. asymbiotica* genospecies clade are capable of infecting mammals. We show a unique invasion phenotype from the *P. asymbiotica* subsp. *Australis* strains, Kingscliff and PB68, which were seen to invade the nucleus, the first time to our knowledge this has been observed in a Gammaproteobacteria. Finally, a possible toxin delivery system that may be involved in human infection was identified using reporter strains, in the form of PVC_Cif, which was upregulated in response to the presence of human phagocytes.
### Table 2.1 | Global distribution of Photorhabdus strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolated from</th>
<th>Source</th>
<th>Species</th>
<th>USA (Clinical)</th>
<th>Australia (Clinical)</th>
<th>Other</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATCC 43949</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td></td>
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<tr>
<td>ATCC 43949</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>ATCC 43949</td>
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<td>No</td>
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</table>

**Fig. 2.1** | Global distribution of Photorhabdus strains used in this study.
2.1 Interactions of *Photorhabdus* with Peripheral Blood Mononuclear Cells

While previous studies have investigated the interactions of two confirmed clinical isolates, Kingscliff and ATCC43949, with cultured mammalian cells the specific cell type tropisms of *P. asymbiotica* for human immune cells remains unknown. Firstly, all those studies were done using *Photorhabdus* cultures grown overnight at 28°C, before being added to the Mammalian cells, at 37°C for only 30 mins, potentially not giving enough time for the induction of mammalian relevant virulence factors. *Photorhabdus* has been shown in previous studies (Hapeshi *et al.*, 2020) to only activate certain genes when grown at human body temp (37°C) compared to a poikilothermic model insect-relevant body temp (28°C). This is common among bacteria that can use both insect and mammalian hosts, where certain virulence factors will only be expressed when grown at the relevant temperature. Therefore, we propose that the behaviour of *P.asymbiotica* is not fully captured when grown at a non-human body temperature prior to infection and study. With *P. asymbiotica* strains being able to grow at human body temperature, it is of interest to determine the temperature dependant behaviour during host cell interactions.

Secondly, these prior studies were all done on cultured THP-1 macrophages. Cultured cells while an invaluable research tool can often differ significantly from primary blood cells, particularly when it comes to immune responses (Tedesco *et al.*, 2018). It should also be noted that in the study the THP-1 cells were only differentiated into non-polarised macrophages, M0, which can differ in pathogen response to polarised M1 (Classically activated) and M2 (alternatively activated) macrophages (Orecchioni *et al.*, 2019). Also, while macrophages are a significant part of the immune system response to bacterial pathogens; there are many other cell types that are important at different stages of an infection.

A large part of the immune system response comes from the PBMCs which are round nucleated white blood cells found in circulating in the blood. The PBMCs consist of; lymphocytes (T-cells, B-cells and NK-cells), monocytes and dendritic cells. This gives them components representing both the early innate immune response, phagocytosis and antigen presentation by dendritic cells and tissue sequestered monocytes, and
the late adaptive immune response, through the activation of T and B cells and subsequent antibody production. PBMCs represent an excellent resource for studying the interactions of *P. asymbiotica* with a “natural” complete immune system model.

In order to determine which PBMC cell types, *P. asymbiotica* interacts with, either through adhesion or internalisation, constitutively GFP expressing strains of the; Australian (Kingscliff) and Thai (PB68) *P. asymbiotica*, the recently identified human infective *P. luminescens* (Texas) in addition to a *P. luminescens* non-clinical strain (TT01), as a negative control, were created. Attempts were made to create a GFP expressing strain of the American *P. asymbiotica* (ATCC43949) as well, but this did not prove possible, consistent with previous failed attempts to transform this strain with a range of plasmids. The GFP strains were grown at either 28°C or 37°C, then allowed to interact with freshly harvested PBMCs for 2 hours, before being analysed by flow cytometry. For control samples the bacteria were killed prior to addition to the PBMCs to distinguish between passive phagocytic ingestion and active virulence activity. The flow cytometry not only allowed for identification of the different PBMC cell types through detection of differentiating surface markers, but also which were infected with *Photorhabdus* by identifying the GFP signal associated with each cell (Fig.2).
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**A**

GFP *Photorhabdus*  
Grown at either human or insect body temperature

PBMCs  

Exposure to bacteria ~2h

Flow cytometry

**B**

- All cells
- PBMCs
- T-cells
- B-cells
- NK-cells
- Monocytes
- Dendritic

**C**

<table>
<thead>
<tr>
<th>CELL MARKER</th>
<th>FLORESCENT DYE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>Pacific Orange</td>
</tr>
<tr>
<td>CD3</td>
<td>AF-700</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>BV-786</td>
</tr>
<tr>
<td>CD56</td>
<td>PE-Cy7</td>
</tr>
<tr>
<td>CD19</td>
<td>APC</td>
</tr>
<tr>
<td>CD14</td>
<td>BV711</td>
</tr>
<tr>
<td>CD16</td>
<td>BV605</td>
</tr>
<tr>
<td>CD11C</td>
<td>BV421</td>
</tr>
<tr>
<td>CD123</td>
<td>PE</td>
</tr>
<tr>
<td>I/D</td>
<td>Near-IR</td>
</tr>
</tbody>
</table>

**D**

- + TT01
- + TT01 (GFP)

**E**

- T-cells
- Monocytes
- B-cells
- Dendritic
- NK-cells
Fig. 2.2 | Flow cytometry analysis of PBMC infection by Photorhabdus.
A- Human derived PBMCs were exposed to various strains of *Photorhabdus* expressing GFP for 2 hours, then analysed by flow cytometry to calculate the percentage of PBMCs infected. In theory GFP signal should be detected from PBMCs which have attached or internalised bacteria. The *Photorhabdus* strains were cultured first at either 28°C (simulated ambient insect body temperature) or 37°C (human core body temperature) prior to the infection. As controls *Photorhabdus* cultures of each strain were killed 1 hour before exposure to the PBMCs. B- Gating strategy for identifying the different PBMC cell types from the flow cytometry. This was achieved by staining the PBMCs with a panel of fluorophore conjugated antibodies, as seen in this table. D- Alongside detecting signals for each of the antibodies, once PBMC types was gated out, GFP signal from any internal or attached bacteria was also detected. A distinct population of the cells (in this case monocytes) can be seen to have a GFP signal (+TT01_GFP), which is not seen when PBMCs are infected with non-GFP expressing bacteria (TT01). E- Proportion on average of each cell type in the human derived PBMC samples. F- Percentage of each PBMC cell type that had a GFP signal, indicating infection with bacteria (3 experimental replicates for each sample). Four strains of *Photorhabdus* were tested; Texas – a recently identified human infective *P. luminescens* strain isolated from an abandoned neonate baby girl in Texas, PB68 – a *P. asymbiotica* subsp. *Australis* nematode isolate from Thailand, Kingscliff – a *P. asymbiotica* subsp. *Australis* isolate from a human infection in Australia, and TT01 the *P. luminescens* lab strain originally isolated from soil nematode. G- Comparison of the different strains in one graph for easier viewing. (n = 2-4, 2-way anova, ** = < 0.01) Note: Only relevant P-values have been shown due to the amount of statistical comparisons.

These findings indicate that the different *Photorhabdus* strains exhibit unique responses to the different PBMC cell types. Surprisingly, *P. asymbiotica* PB68 and *P. luminescens* TT01, showed very similar cell-type profile interactions (Fig.2.2 A,B). Both had low levels of association with any of the PBMCs cell types, ~0-30%. It should be noted that this was slightly higher when the bacteria were cultured at 28°C, though this result was not significant. There was no significant change in cell association when the bacteria had been killed prior to exposure to the PBMCs suggesting phagocytosis or passive cell surface binding. The dendritic cells on the other hand showed very high levels of bacterial association, 80-90% at 28°C. Importantly there was a significant drop for the pre-killed PB68, though the number of infected cells was still significantly higher than the other PBMCs cell types, the drop was much larger for the TT01 strain, dropping down to that of the other PBMC cell types.

The two confirmed human clinical isolates of *Photorhabdus* also varied in their apparent cell type tropisms. They showed very high levels of interaction with all PBMC cell types, including dendritic cells at both 28°C and 37°C, though was higher at 28°C nearly reaching 100% cells “infected”. When the Texas strain was killed prior to infection, there is a large drop in the amount of infected PBMCs, though the levels
of “infected” dendritic cells dropped less than other PBMC cell types in this relative control.

Finally, the Australian *P. asymbiotica* strain Kingscliff, which despite being a human clinical isolate, like the Texas *P. luminescens* strain, again showed a diverse pattern of cell tropism interaction with the PBMCs. Bacteria grown at 28°C generally showed low levels of cell association, ranging from 30-10% for each PBMC cell type. However, this increased significantly for Kingscliff when pre-cultured at 37°C, reaching levels of 60-100%. When 37°C cultured Kingscliff was killed prior to exposure to the PBMCs, association rates dropped to a similar level seen for 28°C cultured cells, ~40-10%. Unlike the other *Photorhabdus* strains, at both cultured temperatures, even if the bacteria were killed prior to addition to the PBMCs, the dendritic cell association level was significantly lower than the other PBMCs. This contrasts with the other *Photorhabdus* strains where the dendritic cells always had association levels either the same, or higher than the other PBMC cell types.

All the data points mentioned here are statistically significant, but due to the large number of comparisons only some of the relevant comparisons are shown on the above figure. The full list of comparisons has not been included in this thesis due to how large a data set it is, over 1000, if comparing every sample to each other to determine significance.
2.2 *P. asymbiotica* strains differ in their ability to invade mammalian cells

From the PMBC flow cytometry data, it can be seen that growth temperature effects how certain strains of *Photorhabdus* are able to associate with different cell types and that different *Photorhabdus* strains are selective about what mammalian cells it infects. It was also clear that the association with the different mammalian cell types is not homogeneous between the different *Photorhabdus* isolates.

To investigate if the bacteria are being internalised/invading and surviving inside the host cells we used gentamicin protection assays using three diverse cell lines. These being [phorbol 12-myristate 13-acetate] PMA differentiated THP-1 Macrophages-like cells, the Human Embryonic kidney cell line {HEK293T}, and Chinese hamster epithelial ovarian cell line [CHO] (Fig 2.3).

To clarify a point of issue first, due to us not knowing if the bacteria are actively invading the tested cell line, such as though *Listeria*’s zipper mechanism (Eierhoff et al, 2014) or, simply surviving phagocytosis, there will be some overlap of the use of the words invasion and internalisation to simply refer to the ability of the bacteria to get into and survive inside the host cell. While I recognise that these words have specific meanings in this context unfortunately due to our lack of understanding and range of cells used, knowing which word is appropriate in each situation if difficult to elucidate.

As previously reported, the American strain ATCC43949 exhibited low levels of cellular invasion/internalisation into THP-1 cells, which was also consistent with the findings with the non-phagocytic HEK and CHO cell lines. The Australian strain Kingscliff showed similar results to the previous reports in invasion of THP-1 cells, showing high levels of invasion. Interestingly, while high invasion levels were seen for the CHO cell line, this was not seen in the HEK293T cell line. Conversely, these observations were essentially opposite for the poorly characterised Thai strain PB68,
where low levels of invasion comparable to that of the American strain were recorded in THP-1 and CHO cell lines, while showing significantly increased levels of invasion in the HEK293T cells well above the other strains. Finally, perhaps unsurprisingly the uncharacterised Northern European \textit{P. asymbiotica} strains HIT and JUN, showed either no active invasion or were seemingly phagocytosed by the THP-1 cell line.

### 2.3 Temperature dependent effects on the ability of \textit{P. asymbiotica} to invade mammalian cells

As in described in the native human blood PBMC studies above, prior growth temperature appears to play a key role in how these \textit{Photorhabdus} strains interact with host cells. A previous study with strain ATCC43949 confirmed a big difference in gene expression and metabolic abilities at 28°C and 37°C. We therefore tested these strains in temperature dependant cell line infection studies (Fig 2.3).

The cell line invasion assays showed that growth temperature effects the ability for \textit{P. asymbiotica} to invade Mammalian cell lines in a manner similar to what we observed in the PMBC infection study.

Firstly, temperature did not seem to have any significant effect on ATCC43949’s ability to invade cells, even when grown at 37°C. However, growing Kingscliff at 37°C prior to exposure to the cells, significantly increased its ability to invade both HEK and THP-1 cells. Of note while the invasion rate of 28°C cultured Kingscliff into THP-1 cells was still significantly higher than the other \textit{P. asymbiotica} strains, for HEK cells the invasion rate remained similar to ATCC43949, that is only becoming significantly higher when grown at 37°C. Once again PB68 showed an opposite trend compared to that of Kingscliff, showing increased invasion when grown at 28°C, relative to 37°C.
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A

![Graph A](image1)

B

![Graph B](image2)

C

![Graph C](image3)

D

![Graph D](image4)

N=3
Fig.2.3 | Invasion and survival of *P. asymbiotica* species inside mammalian cells.

Gentamycin based invasion assays were performed on several different mammalian cell lines using the different bacterial isolates. Bacteria were grown at either 28°C or 37°C prior to assay. During the assay the bacteria were allowed to interact with the mammalian cell lines for 2 hours, after which gentamycin was added to kill any extracellular bacteria. The mammalian cells were subsequently lysed, and surviving (presumably intracellular) bacterial numbers assessed using colony forming count plating assays. The infection assays were done with; **A** – HEK 293T, **B**- CHO cells, and **C** – THP-1 cells that had been differentiated into macrophage like cells using PMA, prior to infection. **D** – Comparison of all the different invasion assays. (2way Anova, ** = < 0.01, **** = < 0.0001, n = 3 biological replicates)

### 2.4 Temperature dependant THP-1 derived macrophage internalisation / attachment of *P. asymbiotica* varies according to strain

While the PBMC flow cytometry findings revealed the tropism of the different *Photorhabdus* strains with the with the varied components of the PBMC cell type cohort, it does not allow us to predict if the bacteria are being internalised or simply associating with the cells surface. We submit that the gentamycin protection assays with the THP-1 cells on the other hand also do not give the full picture (as has been shown in the case of other pathogenic intracellular bacteria), such experiments only tell us if the bacteria are being internalised and surviving for a short while. Therefore, we decided to use the GFP expressing Kingscliff, PB68 and the *P. luminescens* Texas strain, to microscopically investigate the interaction between these phagocytic THP-1 derived macrophage cells and the *Photorhabdus*. Imaging with the human volunteer PBMCs was not an option due to COVID restrictions during this study.

From the infection assays in THP-1, it may have been expected that the HIT and JUN strains might either simply surface attach to the THP-1 cells or not interact at all. This was indeed the case with the JUN, strain, where at both temperatures the bacteria were not seen to associate with the THP-1, this may even suggest that they are actively avoiding the THP-1s (**Fig 2.4**). The HIT strain, however, was seen to attach to the THP-1 cells when grown at both temperatures, with possible invasion at 38°C though from the images it is not possible to confirm this (**Fig 2.4**).
Conversely, the infection assay data made it clear that both Kingscliff and PB68 were being internalised by the THP-1 cells at both culture temperatures, which was confirmed by the florescence microscopy studies (Fig 2.4). Only in the experiment where Kingscliff was grown at 37°C were the bacteria also seen attaching to the THP-1 cells, though the best way to confirm the incidence of attachment vs internalisation would be attachment assays, in a similar vein to the invasion. An interesting observation was that when Kingscliff was grown at 28°C, occasionally long bacteria could be seen crossing the THP-1 cell membrane, indicating that it is either invading or emerging from the cell (Fig 2.4). This would suggest an active bacterial mechanism for invasion rather than endocytosis by the THP-1 cell. It was not clear if the bacterial filaments were chains or a single long “hyphae”.

While there are examples of bacteria that can escape the phagosome after ingestion, others have adaptations which allow them to remain inside the vesicles and prevent their destruction. In previous studies with an Austrian isolate of *P. asymbiotica*, transmission electron microscopy was used to confirm that in the insect professional phagocytes (haemocytes), this strain could be observed within phagosomes for at least ~2 hours post infection. The light microscopy methods used in this study did not have the capacity to determine if they were enclosed in phagosome vesicles or free in the cytoplasm.
Before the experiment the THP-1 cells were seeded into 24 well plates on glass coverslips and differentiated into macrophage-like cells using PMA. THP-1 cells were stained with DAPI (blue) while the Kingscliff and PB68 strains were constitutively expressing GFP (green). Before the experiment the THP-1 cells were seeded into 24 well plates on glass coverslips and differentiated into macrophage-like cells using PMA. Before the experiment the THP-1 cells were seeded into 24 well plates on glass coverslips and differentiated into macrophage-like cells using PMA. Before the experiment the THP-1 cells were seeded into 24 well plates on glass coverslips and differentiated into macrophage-like cells using PMA. Before the experiment the THP-1 cells were seeded into 24 well plates on glass coverslips and differentiated into macrophage-like cells using PMA.
2.5 Internalisation of *P. asymbiotica* requires actin rearrangement

As it has been confirmed that certain *P. asymbiotica* species are capable of entering a range of cell types, we wanted to better understand the processes involved in cell entry. Many bacteria facilitate uptake and entry into host cells by manipulating host cell actin rearrangements. In non-phagocytic host cells this involves using either a zipper or trigger style mechanisms (O Cróinín *et al.*, 2012), while in phagocytic cells host medicated phagocytosis can be used to gain entry. Rearrangement of the host cell actin cytoskeleton and polymerisation of F-actin at the site of the bacterial attachment is a common feature of these processes. Cytochalasin-D is a fungal toxin that binds strongly to the end of actin filaments thus acting as a potent inhibitor of actin polymerisation and thus phagocytosis. In order to see if actin rearrangement is important for the entry of Photorhabdus into host cells, THP-1 cells were treated with Cytochalasin-D prior to infection with either Kingscliff or PB68, using the methods consistent with the previous infection assays.

Inhibition of actin polymerisation by Cytochalasin caused a significant reduction in both Kingscliff and PB68’s ability to invade. Perhaps not surprisingly, this differential effect was only seen when the bacteria were cultured at the temperature at which they had previously been shown to be most invasive at in the previous THP-1 invasion assays (**Fig 2.3**)

For example, the Kingscliff strain can invade THP-1 cells but only when cultured at 37°C prior to infection. When grown at 28°C, the addition of Cytochalasin-D made no difference to any observed ability to “invade” cells. However, it should be noted that in these tests the measurements were approaching the lower sensitivity limit for the CFU counts, being only 1.6x10^1. Strain PB68, on the other hand showed the converse of what was seen for the Kingscliff strain, with a significant reduction in invasion only at 27°C. Nevertheless, while we did still see a drop in invasion numbers in the 37°C samples with strain PB68 upon the addition of Cytochalasin-D, it did not appear to be significant.
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**Fig. 2.5**  
*Photorhabdus asymbiotica* subsp *Australis* strains, Kingscliff and PB68, with phagocytosis inhibited THP-1 cells.

THP-1 cells were activated by PMA 48 hours prior to experiment. Bacteria was grown at either 28°C or 37°C prior to infection and allowed to infect for 2 hours. Prior to infection assay phagocytosis inhibitor cytochalasin D was added to the THP-1 cells. (T-test, * = < 0.05, **** = < 0.0001, n = 3 biological replicates)

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### 2.6 Some *P. asymbiotica* strains can invade the nucleus of the cell

Many bacteria that exhibit an intracellular phase in their infection strategy typically localise to either host vesicles or remain free in the cytoplasm. However, for some pathogens it has been noted that they can be found within certain organelles of their host cell (Burgdorfer, *et al* 1968).

For both the PB68 and Kingscliff strains we observed that some of the bacteria had gained entry to the nucleus of the experimental host THP-1 cells. In such cases, it appeared that a chain of bacteria wrapped around the host’s nucleus, and in some
cases a portion of the chains penetrated into the nucleus. Nevertheless, despite this unique and surprising observation, this behaviour was only infrequently observed and typically only seen in a few cells per infection experiment we analysed microscopically. Thus, the biological relevance of these observations remain a matter of debate. In addition, in the cases where nuclear invasion was observed there were no obvious differences in cellular or nuclear morphology.
Fig. 2.6 – Kingscliff strain of *P. asymbiotica* subsp *Australis*, inside the nucleus of a THP-1 macrophage.

Kingscliff was grown at 37°C prior to infection and allowed to infect for 2 hours, before imaging. THP-1 cells were stained with DAPI (blue), while Kingscliff was constitutively
expressing GFP (green). **A**- florescent phase contrast image. **B**- DAPI and GFP floresence along ROI line across the cell seen in A, showing overlap of the two channels. **C**- 3D recreation of Z-stack confirming the bacteria were inside the nucleus.

**2.7 PB68 produces the injectosome PVC_Cif, in response to phagocytic cells when grown at ambient temperatures mimicking the insect host**

The PVCs are a unique type of toxin delivery system, consisting of a contractile needle fused to bacteriophage like tail fibres, which are loaded with effector proteins and released from the bacteria. This allows for injection of effectors (typically toxins) into specific cell targets, distal from the bacteria. The injectosome structural proteins and associated effector genes are organised into operons, of which each isolate has around 4-5 per genome. Thus, each *Photorhabdus* is capable of producing a range of PVCs that presumably differ in both target and certainly in delivered toxins.

The PVCs are known to play a role in *Photorhabdus* infection of insects, with certain variants demonstrated to target the innate immune system. Thus, we hypothesized that some may also have a role the infection of humans, again targeting the immune system. In order to study this, we created GFP reporter constructs for each PVC operon encoded in strain PB68. Each reporter strain carried a plasmid which fused the GFP gene to the first 5 amino acid codons of first structural gene (*Pvc1*) for each respective PVC operon reporter. These also encoded the 500 bp sequence upstream, which in theory should contain both the promoter and Shine-Dalgarno sequence for that operon. Thus, we propose that GFP expression on the plasmid should only be expressed when the respective PVC operon in the Photorhabdus’ genome is also being expressed.

The PVC reporters were first tested on the activated THP-1 cells using a method of infection similar to the infection assay performed earlier. However, instead of adding gentamycin to kill the external bacteria instead, after 2 hours the bacteria were removed from the THP-1 cells, washed, and resuspended in PBS, and mounted onto uniform thickness agarose pads for imaging by florescence microscopy using the same settings for each sample. Using the particle detection software in FUJI, the
Florescence microscopy images of the bacteria were analysed to determine the relative GFP signal per bacteria. Control samples were done in parallel with test samples in the same conditions, 37°C in RPMI buffer for 2 hours, but without the presence of the THP-1 cells. A threshold for determining activation of the operon was calculated using the highest fluorescent signal from a strain of PB68 containing the base plasmid used for creation of the reporter stains, pAGAG, missing a promotor for the GFP. All statistical tests for determining significant difference between the control and test sample for each PVC operon was done using only values above this lower threshold (Fig 2.7, A).

Without exposure to the THP-1 cells some operons already showed some activation, the most significant being the PVC_Cif operon, although the PVC_LopT operon also was observed to be expressed in a large number of bacteria within the population. This is in comparison to the Pnf and Unit4 operons where only one or two of the bacteria were expressing. When exposed to THP-1 cells both the PVC_LopT and PVC_Pnf operons of PB68 showed a large and significant increase in activation. The PVC_Unit4 and PVC_Cif operon remained the same, though it is currently unknown if it was perhaps the RPMI or some other environmental condition which caused the PVC_Cif activation.

As we had now shown that PVCs are differentially activated in response to cultured human immune cells, we next wanted to see what the responses were to primary human immune cells. Thus, the PVC reporter strains of *P. asymbiotica* PB68, were exposed to PBMCs from healthy human volunteers and analysed by flow cytometry as in the previous infection assays which used constitutively expressed GFP reporters (Chapter 2.1). A major difference in the experimental procedure here compared to the THP-1 experiment is that the flow cytometry would only be analysing bacteria associated with, attached or internalised, the PBMCs. While in the previous THP-1 assay only external bacteria were analysed.

Unlike with the THP-1 cells, most of the reporter strains did not display any significant increase in GFP expression (Fig 2.7, B). Even the PVC_LopT and the PVC_Pnf which from the previous THP-1 cell experiments would be expected to activate in the presence of the macrophages showed low to no GFP expression. The only exception was the PVC_Cif operon which showed a strong increase in GFP positive cells indicating operon activation. Interestingly, this increase only occurred
when the *Photorhabdus* had been previously cultured at 28°C, prior to infection, but not when grown at human body temperature, 37°C.
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PB68 (38°C) PVC activation with THP-1 cells

Cif  LopT  Pnf  Unit4

Relative fluorescence

PB68 Cif

PB68 Pnf

PB68 LopT

PB68 Unit4

T-cells  B-cells  Dendritic  NK-cells  Monocytes

B

N=3

N=500

pAGAG  Control  +THP-1  Control  +THP-1  Control  +THP-1  Control  +THP-1

0 2000 4000 6000 8000

PB68 (38°C) PVC activation with THP-1 cells

Cells with GFP signal (%)
**Fig 2.7** | Activation of *P. asymbiotica* PB68, PVC operons in response to human immune cells.

PVC operon activation reporters were created by fusing GFP to the first 5 amino acids of PVC1 from the respective PVC operon. Also included was 500bp upstream of the PVC1, which should include the promoter region for the operon. This construct was cloned into a plasmid, which was then transformed into *P. asymbiotica* PB68. Four reporters were made one for each of the PVCs in the PB68 genome: Cif, Pnf, LopT and Unit4.  

A - PB68 PVC reporter strains were exposed to THP-1 cells, activated with PMA, for 2 hours. The bacteria were then isolated and imaged using fluorescence microscopy. The average relative fluorescence for each bacteria was then analysed using FUJI.  

B - PB68 PVC reporter strains were exposed to and allowed to infect PBMCs as done in the previous flow infection assay (Fig.2). After infection, PBMCs were isolated from extracellular bacteria and analysed by flow cytometry to identify PBMC types and GFP signal from cell. Any GFP signal would be the result of fluorscening bacteria associated with the cell either internal or external. (A - Mann-Whitney test, B - 2-way Anova, ** = < 0.01, **** = < 0.0001)

### 2.8 *Photorhabdus* species have homologs to *Yersinia* invasion and attachment proteins

It has now been confirmed that the *Photorhabdus* species associated with human disease are capable of invading and/or attaching to mammalian cells. However, few mechanisms or associated proteins have yet been discovered or proposed. Examining the already annotated genomes of these *Photorhabdus* species there are no obvious proteins associated with invasion or attachment. Thus, we considered similar species of human pathogenic bacteria to identify any virulence factors that could have unidentified homologs in *Photorhabdus*. For this we first investigated *Yersinia*, which is closely related and has been shown to have many homologous virulence factors with *Photorhabdus*, including many PVC effectors. *Yersinia* has a number of adhesions that it expresses, adhesins being cell surface molecules linked to adhesion and invasion, the three key factors being Ail, YadA and invasin (Mikula et al, 2013).

*Yersinia’s* behaviour to host cells and expression of invasion related proteins changes as the infection progresses. On initial infection *Yersinia* actively invades host cells, expressing large amounts of invasin on its surface. Invasin binds to host cell receptors such as β1 integrin which causes host cell actin rearrangement which leads to the bacteria being endocytosed (Mikula et al, 2013). While invasin seems to be expressed
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constitutively even before exposure to host cells, YadA only starts being expressed when *Yersinia* is above 34°C, which often coincides with invasin repression (Mikula et al, 2013). YadA has a number of roles in infection, but the main purposes are to facilitate adhesion through collagen binding and block activation of the complement system (Mikula et al, 2013; Eitel & Dersch, 2002; Mühlenkamp et al, 2015). Ali also seems to have a role in blocking the complement system, though its main function appears to be binding to the extracellular matrix which is essential for *Yersinia* to deliver effectors though its T3SS (Mikula et al, 2013, Hinnebusch et al, 2011).

Homologs of some of these Yersina proteins were found in the both the *P.asymbiotica* HIT and Kingscliff, as well as the *P.luminescens* Texas and TT01. Both species seemed to have fairly similar amount of homologs to the Yersina proteins. Each had multiple Ali homologs with one gene that had a greater sequence identity followed by one or two more genes that showed a lower homology. The genes homologous to YadA and Invasin, had lower homologies and most strains only had one possible homolog.

While these homologs do not have very high sequence percentage identity it does give possible target genes for follow-up experiments and would be interesting to see if *Photorhabdus* loses the ability to adhere / internalise if some of these genes are knocked out.
Using NCBI blast, homologs of three Yersina adhesion related proteins were searched for in the genomes of the P. luminescens strains Texas and TT10, and the P. asymbiotica strains HIT and Kingscliff. In some cases multiple putative homologs were found which are labelled 1, 2 etc, while where no extra homologs are represented by an X. Amino acid percentage identity of the homologs was calculated using Clustal Omega.

Fig 2.8 | *Photorhabdus* homologs of *Yersinia* adhesion related genes.
2.9 The *P. luminescens* Texas strain upregulates adhesion genes and downregulates motility genes upon a change from insect to mammal body temperature.

Throughout this chapter we have shown multiple instances of *Photorhabdus* adapting its response to host cells depending on whether it was grown at simulated insect or human body temperature. This was seen most prominently in the *P.asymbiotica* strains, Kingscliff and PB68, with only slight responses to temperature change seen in the human infective *P.luminescens* Texas strain. As Texas showed so few temperature dependent behavioural changes, we wondered if temperature was having any effect on gene expression at all, especially genes related to pathogenicity. As these stains would likely see much greater differential gene expression which may be hard to interpret with no prior knowledge.

In order to achieve this total RNA was extracted from cultures of the Texas strain grown at either 28°C (Insect body temp) or 37°C (Human body temp) until they were at mid log phase, ~OD 0.5. These cultures were sub-cultured from the same starter culture grown O/N at 28°C grown from a single colony, reducing the chance of genetic differences between paired samples (Fig 2.9, A). Three biological repeats were done, using a freshly picked colony each time, leading to three RNA samples for each temperature. The RNA was processed to remove ribosomal RNA and analysed using the Agilent Bioanalyzer to make sure the samples were not degraded. Once samples were obtained that were of acceptable purity and quality, library preparation and sequencing was outsourced to the company Novogene. For each sample a read depth of 10 million, 150bp paired reads were generated, which were then analysed in house. In brief analysis involved alignment of RNA reads to the Texas reference genome using Hisat2 (Kim *et al.*, 2019), followed by counting of reads per gene by LiBiNorm (Dyer *et al.*, 2019). Finally, determination of differentially expressed genes between the two temperatures was done using the Deseq2 algorithm (Love *et al.*, 2014).
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**A**
- Texas O/N
- Grown to \( \sim \text{OD 0.5} \)
- Sequencing
- Alignments + counts

**B**
- PCA plot
- 28°C and 37°C

**C**
- Normalised counts (Log10)

**D**
- Expression of genes at 28°C vs 37°C
- 79 down genes
- 21 up genes
- \( p_{adj} = 0.06 \)
- Total genes = 4922

**E**
- Differentially expressed genes 28°C Vs 37°C
- Gene categories
  - Unknown
  - Adhesion
  - DNA binding
  - Metabolism
  - Motility
  - Prophage
  - Regulation
  - Rosetta
  - Transport/Scavenger
  - T2SS
  - T4SS

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Fig.2.9 | RNA-seq data showing differential gene expression of the *P. luminescens* Texas strain when growth temperature is increased from 28°C to 37°C.

A- O/N Texas culture grown from a single colony was split into two sub-cultures which were grown at either 28°C or 37°C until at OD ~0.5 around 4 hours growth. RNA was then extracted from the cultures and processed to remove rRNA. Processed RNA samples were library prepped and sequenced at a read depth of 10 million, 150bp paired reads. Reads were then aligned with Hisat2 and counted using LiBiNorm. This was repeated 3 times using a freshly picked colony, generating 3 biological repeats. B- PCA plot of RNA-seq samples. C- Evaluation of quality of RNA-seq data. Graph shows normalised counts generated for each gene for each RNA-seq sample, black line indicating median. None were found to be significantly different. D- Volcano plot of differential expression of genes at 28°C vs 37°C. Sample 1 was removed at temperature points from analysis due to being outliers in the PCA plot. Significantly differentially expressed genes are defined as those with a p adjusted value < 0.05, aka above the dotted line. These genes are marked in colour depending on if they are being upregulated at the higher temperature (pink) or downregulated (blue). 100 genes were found to be significantly differentially expressed. E- Heat map showing significantly differentiated genes in Texas upon change from 28°C to 37°C. Genes are ordered by category as to their rough function in the cell, unknown indicating that function is unknown. (N = 3 biological replicates)

The quality and reproducibility of the different samples was analysed using a PCA plot (Fig 2.9, B) and assessing at how the normalised counts compared between the samples (Fig 2.9, C). While the normalised counts were consistent throughout the samples, none being significantly different from each other, the PCA plot showed that replicate 1 at both temperatures, did not cluster with the other two replicates. This was possibly a result of that specific colony selected being “old”, although previous work on *Photorhabdus* has demonstrated a great deal of variation between colonies. For this reason we removed this replicate from the data set and proceeded with the analysis of any differentially expressed genes using only replicates 2 and 3.

It was found that 100 genes of the 4922 analysed from Texas were significantly differentially expressed (p adjusted value = 0.05) between the two growth temperatures (Fig 2.9, D). Of these 21 were upregulated and 79 were downregulated when the gene transcription findings from the two growth temperatures were compared between the simulated insect body temperature of 28°C to the mammalian body temperature 37°C. Homology searching was used to investigate the potential roles these genes may have in the cell. While many of the genes had known functions and homologs, some did not. For the genes of unknown function HHpred was used to find homologs and discern possible functions, a list of all these genes and their products can be found in supplementary (SuppFig 1). Interestingly many of these genes were found in series/ tandem in the genome, particularly the ones related to
motility and adhesion, which suggests they be single operons/regulons. Once the predicted functions of all these gene products were deduced, they were grouped by function and if expression was increasing or decreasing (Fig 2.9, E). It was found that the majority of downregulated genes were motility related, such as flagella biosynthesis, with the only motility related gene that saw an increase in expression being a hypothetical which showed homology to a quorum sensing regulator, mqsR. The other two “groups” of genes that decreased in expression were either scavenger molecules or prophage related genes. Of the 37°C more highly transcribed genes, many were a group of sequential hypothetical genes which showed homology to proteins involved in adhesion, the majority being homologous to adhesins from other pathogens. Alongside these there were some metabolism related genes that were upregulated. The most upregulated gene was another hypothetical, which showed good homology to the DNA binding protein, MvaT from Pseudomonas aeruginosa. In this bacterium, this protein is involved in arginine metabolism, pyocyanin synthesis, and suppression of prophage activation, which would correlate with the suppression of the prophage genes (Li et al, 2009).

2.10 Discussion - *P. asymbiotica*: a species that shows considerable variation in its response to mammalian host immune cells.

Due to the obligate association of *Photorhabdus* with symbiont host nematodes, their phylogenetic diversity is likely to be highly geographically constrained. While only two sub-species of *P. asymbiotica* have so far been identified from a limited number of clinical samples, other genospecies isolates have been found from environmental samples of infected insects. The reasons for the dearth of human isolates could be; poor identification rate, poor reporting of infections in the literature or that *P. asymbiotica* strains are indeed rare. There is also the possibility that human pathogenicity is not a trait shared by all *P. asymbiotica*’s and that, despite being closely related, the various strains interact with their hosts in very different ways. We speculate that a human host would represent a “dead end” for *Photorhabdus*, as it seems unlikely that it could re-associate with its mutualist nematode.
It remains possible that small wild mammals (or even birds) could provide productive hosts for *P. asymbiotica*. While this has not been observed, there have been no published studies, and dead small animals in the environment would be of little interest to medical or veterinary experts. If this does prove to be the case, with different available target hosts, it may explain some of the variation that has observed in *P. asymbiotica* infection strategies described here. Indeed, the America and Australia strains present differing clinical manifestations and previous studies have shown interact very differently with cultured cells. There is also the possibility that one may infect mammals while the other infects birds based on maximum growth temperatures of 37°C and 42°C which are typical core temperature of mammals and birds respectively.

In order to establish an infection, bacteria have to overcome their host’s immune system. One of the main mediators of immune response is phagocytosis, which both kills the bacteria and allows for presentation of antigens to other parts of the immune system and triggers various immune signalling pathways. Here we studied the interactions of *Photorhabdus* with two professional phagocytes, dendritic cells (as primary cells isolated from human blood) and macrophages (as primary undifferentiated monocytes from human blood and cultured THP-1 cells). There are roughly two methods that bacteria have evolved to overcome phagocytosis. The first is prevent phagocytosis from occurring, this is often achieved through the use of anti-phagocytic toxins, hiding surface antigens or direct killing of immune cells. The second method is survival following phagocytosis, this is usually achieved either by escaping the phagosome, or releasing toxins that preventing phagosome maturation. The data we have presented here shows that geographically distant *P. asymbiotica* strains seem to differ greatly in how they interact and react to the different mammalian host cells.

**2.10.1 Methodology limitations**

Before discussing the results, we present here and their possible implications in detailing the pathogenicity of *Photorhabdus* towards humans, it would be prudent to first discuss the limitations our methodology might have. Especially when being used as a model for human infections.
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Profiling the interactions with ex vivo PBMCs using flow cytometry, has elucidated many aspects of \textit{Photorhabdus} interactions with the human immune system. Nevertheless, caution should be used when interpreting the results. One such consideration is that even though gentamycin should kill any external bacteria; due to the long half-life of GFP, \textasciitilde26h (Corish and Tyler-Smith \textit{et al}, 1999), any strongly attached bacteria to the PBMCs, while dead, might still be detected. This means it is not possible using only the flow cytometry data to definitively determine if we observe invasion or attachment to the cell. Despite this we argue that the behaviour is likely to be similar to what we observe when we challenge cultured THP-1 macrophages.

If internalisation is indeed occurring (as our results do suggest), there is a question as to whether this is mediated by the bacterium itself (active invasion), or if they need to first be phagocytosed by cell dependant mechanisms. We can use an actin inhibitor such as Cytochalasin-D to inhibit phagocytosis, however many intracellular bacteria require actin rearrangement for their active invasion processes, which complicates interpretation of the findings. It is more likely phagocytosis is involved in internalisation in the case of the professional phagocytes, such as dendritic cells and monocytes. However, it does remain possible that non-professional lymphocytes (such as the NK-cells) could also phagocytose at low levels (Voigt \textit{et al}, 2014). From the bacterial strain angle, it is not possible to transform the USA clinical isolate model we use (the fully closed genome sequenced ATCC43949), so we could not tag them with GFP, thus leaving an unfortunate gap in our knowledge of how the Australian and North American strains are differently adapted to human infection. All of our invasion assays were also only done at one time point, mainly due to time constraints, so we do not know for how long the bacteria can survive inside the cells, or how long before the bacteria emerge. This would be an interesting experiment in the future based on our hypothesis that \textit{Photorhabdus} may deliberately “hide” from immune recognition in the privileged niche of the cytoplasm. Brief experiments looking at longer time points were in fact done, using a 6 hour incubation and infection time instead of the 2 hours reported here. This was only done using a non-fluorescent PB68 and due to time constraints was not repeated with other strains or reporters, also the experiment was only repeated once meaning it cannot be considered reliable data, although certainly the basis for hypothesis driven future work. Nevertheless, from
images taken from this single experiment, in some fields of view the bacteria seem to have been dividing and have taken up the entire cytoplasm of the host cell (SuppFig.2). This indicates the bacteria’s ability to scavenge resources from the host cytoplasm, and long-term survival inside of phagocytes.

The PMBCs used in this study were directly derived from human volunteers, and as such we would expect them to respond as they would in an actual infection. Nevertheless, there are some caveats to keep in mind. Firstly, while monocytes differentiate into macrophages upon receiving the correct signals, and some may have done given exposure to the bacteria, the majority may remain “inactivated. This would likely limit their phagocytic response to Photurhahbus.

### 2.10.2 HIT and JUN: the non-infective European strains

Whole genome sequencing of the HIT and JUN strains which were isolated from soil dwelling nematodes in northern Europe, suggested they are members of the *P. asymbiotica* species, albeit in their own separate sub-clade, as are the USA and Australian strains. However, unlike the other *P. asymbiotica* strains identified so far, they are temperature intolerant. In our experiments growth at 37°C was unreliable and very slow. The fact that these strains have yet to be associated with a human illness and they have such trouble growing at human body temperature, suggests that they are likely not adapted to be mammalian pathogens unlike the other *P. asymbiotica* strains. This hypothesis is supported by our invasion assay observations in which neither HIT nor JUN managed to produce any colonies, after infecting the differentiated THP-1 cells. This suggests that they are either avoiding phagocytosis completely or are simply being killed by the THP-1 cells. Microscopic imaging of the two strains with THP-1 cells suggests the former explanation for JUN and the latter for HIT. In these images we did not observe JUN interacting with the THP-1 cells at all, neither internalised nor attaching to their surface. As differentiated THP-1 cells should be actively phagocytic, this suggests that JUN can inhibit the process. As JUN is not attaching to the THP-1 cells, it would not be able to deliver any toxins through the T3SS. The T3SS is the one of the most common mechanisms that Gram-negative pathogens use to manipulate lymphocytes and prevent phagocytic destruction.
Thus, if JUN is preventing phagocytosis, it must be using other virulence mechanisms such as secreted toxins or capsule production. Conversely HIT is internalised yet we can recover no viable cells from colony count assays, suggesting they are being taken up and killed by the THP-1 cells. We speculate that HIT remains an exclusive insect pathogen, while JUN has some potential as a mammalian pathogen.

2.10.3 ATCC43949: The American strain

ATCC43949 is our representative strain of the North American clinical *P. asymbiotica*, isolates. Previously it was found that multiple North American strains of *P. asymbiotica* were weakly phagocytosed and did not seem to invade Hela cells unlike the Australian strains (Costa et al, 2009). Our data confirms these previous experiments, showing that ATCC43949 shows only weak internalisation into either phagocytic or non-phagocytic cells. We also expand on this previous research to show that unlike PB68 and Kingscliff, the ability to invade did not change in any of the cell lines that were tested, all showing very low to no internalisation. Also, this weak internalisation rate remains consistent whether ATCC43949 is previously grown at either 37°C or 28°C. This suggests that unlike PB68 and Kingscliff, which do show temperature related differences in their behaviour, that the North American strain does not have temperature related internalisation genes. These genes could be either those that prevent phagocytosis or genes that promote invasion. Taken together, it suggests that the ATCC43949 is primarily an extracellular pathogen. Previous work has shown that they cannot be killed by humoral factors in human serum which correlates with this hypothesis. Interestingly our microscopic analysis shows them attaching to the surface of THP-1 cells, which suggests they could be using their T3SS to deliver antiphagocytic effectors. As mentioned previously, *P. luminescens* spp. have been shown to use their T3SS to deliver the antiphagocytic toxin LopT. However, in the case of ATCC43949 the T3SS *lopT* gene is replaced by the cytotoxin gene *exoU*. This could suggest that rather than preventing phagocytosis, that they are simply killing the macrophages.

Much like the Australian strain, in the North American *P. asymbiotica* infections, secondary lesions formed distant from the primary site of infection. This is generally suggestive of a pathogen capable of dissemination through either the blood or
lymphatic system, often by hiding in immune cells. A good example of this infection strategy is the plague bacterium *Yersina pestis*, which is a facultative intracellular bacterium which can disseminate by being carried inside phagocytes in the lymphatic system. However, not all bacteria that disseminate in the blood travel inside of immune cells. For example, *Streptococcus pyogenes* attaches to the outside of immune cells as they travel to lymphatic nodes. It is therefore possible that this is how ATCC43949 disseminates in the body.

Further study into ATCC43949, is needed to investigate how it prevents phagocytosis and why it has evolved a method of immune evasion different from the related Australian strains.

### 2.10.4 Kingscliff: The Australian strain

Kingscliff is a *P. asymbiotica* subsp. *Australis* strain isolated from a human infection in New South Wales, Australia. The Australian *P. asymbiotica* can cause a serious disease if left untreated. Like all other *Photorhabdus* strains they are also vectored by a nematode symbiont, *Heterorhabditis gerrardi*. While still poorly understood, it was shown to be able to survive phagocytosis by macrophages, cause apoptosis and invade non-phagocytic cells. Here we have characterised Kingscliff’s interactions with human PBMCs and shown how prior growth at human or insect body temperature influences its behaviour.

The most notable finding of Kingscliff’s interactions with PBMCs was that compared to all the other cell types, the percentage of infected dendritic cells was always significantly lower. This is the opposite of what was seen for the other *Photorhabdus* species we tested, which always associated with dendritic cells at either the same level, or often higher, than with the other lymphocytes. We propose that high levels of dendritic cell association would be expected as they are highly phagocytic. This therefore suggests that Kingscliff is actively avoiding the dendritic cells. Dendritic cells are one of the main mediators of early immune response, being responsible for presenting antigens at the lymph-nodes to activate T and B cells. Thus, by avoiding them Kingscliff, may be delaying the immune response until it can establish a more robust infection.
The temperature of prior growth was also seen to play a role in how Kingscliff interacts with PBMCs. While Dendritic cell association was low for both temperatures, there was a significant increase in the interaction with all of other PBMC types when Kingscliff was grown at the human body temperature of 37°C compared to the simulated insect body temperature of 28°C. This suggests that pathogenicity genes involved in invasion/association are being expressed only at human body temperature, showing for the first time that a human infective *Photorhabdus* specifically changes behaviour in response to mammalian conditions.

Infecting PBMCs at higher levels is potentially a strategy used by Kingscliff to hide from the immune system by invading various cell types, and or to intimately associated in order to deliver toxins to kill/suppress immune cells. The microscopic imaging and invasion assays done with cultured THP-1 cells (Fig 2.2, Fig 2.4), indicated high levels of invasion, suggesting this is also what is happening during interaction with the *ex vivo* PBMCs. It should also be noted that Kingscliff was sometimes seen traversing a THP-1 cell membrane, suggesting the ability to enter or leave phagocytes through methods other than phagocytosis or cell lysis (Fig 2.4).

In conclusion, we show for the first time here that Kingscliff actively invades most PBMCs cell types while avoiding dendritic cells in a mammalian body temperature dependant manner. This suggests different gene regulons for pathogenicity are involved in insect and mammalian infections.

### 2.10.5 The *P. asymbiotica* strain from Thailand: PB68

*P. asymbiotica* strain PB68 is an isolate from Thailand, that is most closely related to Australian strains *P. asymbiotica* subsp. *Australis* (Tobias *et al.*, 2016), however, this is an environmental isolate discovered using insect soil baiting and was not isolated from a clinical sample. It has been classed as a *P. asymbiotica* genospecies based on temperature growth range, genome sequence and carriage of the hallmark plasmid. Here we showed that despite being very closely related at the genetic level, the Australian Kingscliff strain and PB68 differ greatly in their behaviour regarding interactions mammalian cells.

They do share some similarities, such as the ability to survive challenge by mammalian phagocytes, which suggests some adaptation to the mammalian immune
system. Nevertheless, one major difference between these strains can be seen in how they interact with phagocytes. PB68 exhibited reduced phagocytosis by cultured THP-1 macrophages compared to Kingscliff, though still significantly higher than with strain ATCC43949. It is possible that this may not necessarily reflect a higher uptake of Kingscliff, but rather that it can replicate once inside the cells at a higher rate than other P. asymbiotica. It may also be a consequence of an ability of Kingscliff to efficiently actively invade the THP-1 cells. However, microscopic imaging showed that both PB68 and Kingscliff cells were mainly inside cells with no discernible differences between the two.

In the non-phagocytic CHO cells, PB68 showed little to no invasion compared to Kingscliff which showed very high invasion levels. However, the opposite trend was seen for HEK cells, in which PB68 invaded at much higher rates than Kingscliff, which showed little to no invasion. HEK cells, were originally derived from the kidney of a human foetus and were long thought to have kidney epithelial cell like expression profiles. However, recent studies into HEK cell surface proteins and expression profiles, found the cell line to have elements most similar to adrenal cells with neuronal properties (Lin et al., 2014; Shaw et al., 2002). Either way it would seem unlikely for PB68 to selectively target kidney or adrenal cells. Further study is needed to uncover why Kingscliff and PB68 have these different, and opposite preferences in invasion.

The other major difference between the strains are the changes to pathogenicity in response to simulated human and insect body temperatures. While Kingscliff in every cell line invaded at higher rates when grown at 37°C, again the converse was true for PB68 which was more active when grown at 28°C, insect body temperature. This would be expected for a strain that is more adapted for an insect host than mammalian, and the differences likely represent the activation of pathogenicity factors when PB68 is at an insect body relevant temperature. This may also explain why no human infections have been reported with PB68, as it is only expressing pathogenicity factors required for an infection when below human body temperature, despite being able to grow at these higher temperatures. One example of this is the PVC_Cif operon, which is potentially involved in immune cell suppression, which was shown here to be activated in the presence of human phagocytes but only when PB68 had been previously grown at 28°C. However, we
do not know the exact function of PVC_Cif, or if it has a similar activation profile in the confirmed human pathogenic \( P.\ asymbiotica \) species. Thus, the PVC_Cif could be an exclusively insect pathogenic factor, while other PVC operons may be deployed in human infection.

Further findings that suggest PB68 may be typically restricted to insect hosts and rarely if ever infecting mammals, is that it infects PBMCs in a pattern similar to the fully insect restricted TT01 strain. In these two strains the only PBMC where a large percentage of bacteria were seen to associate was with the dendritic cells. We speculate that this is likely due to phagocytosis by the dendritic cells, which are one of the primary professional phagocytes important in early infection, particularly in tissues in contact with external environments. When activated dendritic cells migrate to secondary lymphoid organs, this means they may possibly be a potential dissemination route to other areas of the body, an ability that \( Photorhabdus \) exhibits during human infections.

### 2.10.6 The human infective \( P.\ luminescens \) strain: Texas

The Texas strain of \( P.\ luminescens \) is the only one of its species to have been found to grow at 37°C and the only non-asymbiotica found to infect humans. However, when it was first isolated it was from an immuno-compromised neonate, bringing some question as to whether it was comparable to human infective Asymbiotica cousins and if it would be able to infect immunocompetent individuals. Here we have shown for the first time the Texas strain is capable of invading human immune cells, though differs greatly in behaviour to the human infective \( P.\ asymbioticas \).

From the flow cytometry experiments we found that the Texas strain is by far the most aggressive of the strains investigated, in most cases nearly 100% of cells were infected, associating heavily with the PBMCs regardless of cell type or growth temperature. This is a notable difference from Kingscliff which was selective in both the cell types it associated with and was influenced heavily by growth temperature. This was pattern which was repeated with the THP-1 invasion assays where again, unlike with the \( P.\ asymbioticas \), growth temperature had no influence on Texas’s rate of invasion. In these invasion assays it was also seen that Texas did not invade THP-1 cells at as high as Kingscliff, despite associating with the comparable PBMCs,
dendritic cells and monocytes, at similar or higher rates then Kingscliff. While this could as mentioned previously be due to THP-1s not being a perfect homolog of any PBMC. It would seem more likely to hypothesise that in the PBMC experiment the majority of the Texas were not invading but adhering to the PBMCs, or at least in the case of the phagocytes. A distinction that the experimental design unfortunately could not make.

Temperature having little influence on Texas’s behaviour is to some extent backed up by the RNA-seq data, which showed a low amount of genes being differentially expressed when growth temperature is increased. However, of the genes that did see changes in expression two main groups jump out. The first were the upregulated adhesion related genes which were possibly an operon. These genes being upregulated would make some sense if our previous hypothesis that Texas has high levels of adhesion, as it these may be specialised for mammalian cells. While the flow cytometry data might at first seem to contradict this finding, as it would be expected that there would be an increase at 37°C, it should be noted that the almost 100% of PBMCs were already infected at 28°C. So even if Texas was adhering better this would not be observable. That experiment also gave no indication of number of bacteria per cell. Of course, these genes are all hypotheticals, so their function still needs to be confirmed. Adhesion assays of Texas with THP-1 cells would give us a better idea of what is happening. As then we would be able to both compare invasion and adhesion rates and see of there are any temperature related difference in number of bacteria adhering per cell. The other group of genes detected in the RNA-seq were a range of motility related genes which decreased when Texas was grown at 37°C. This is the opposite of many human pathogenic bacteria where flagellar production is linked to increased invasion and adhesion (Anjuwon-Foster & Tamayo, 2017; Haiko & Westerlund-Wikström, 2013), while in Texas it seemed to have no influence. As the RNA was extracted at Log phase, it is possible we are missing the differential expression of some genes which are only expressed during stationary phase. Although some papers have reported that while growth phase may effect antibiotic resistance (Pletnev et al, 2015), it does not seem to effect cell adhesion/invasion (Kusters et al, 1993). However, if Texas is already capable of infecting host cells without high motility, then deactivating flagella production could be advantageous as flagellin is highly pro-inflammatory (Ramos et al, 2004; Hajam et al, 2017). It would
be interesting in the future to compare the temperature related gene expression changes of Texas, with Kingscliff where cell invasion is highly influenced by temperature.

In conclusion the Texas strain while being able to grow at human body temperature does not show many changes in behaviour of expression when grown at this higher temperature. Texas overall seems to be an indiscriminately aggressive strain towards many cell types which may indicate a less refined and subtle approach to mammalian infection then what has evolved in the Asymbiotica strains. Either way, this confirms that some P.luminesecens strains are capable to infecting human cells. Which could have serious implications in the selection of strains being used as bio-insecticides alongside their host nematodes. Where up till now all non-P.asymbiotica have been considered non-hazardous for humans (Keskes et al, 2021; da Silva et al 2020; Shawer et al, 2018) with the only safety concerns being about bio-diversity (Mohan & Sabir, 2005).

2.10.7 PVC activation in PB68 as a response to human immune cells

As mentioned above when PB68 was exposed to human dendritic and monocytes, both types of phagocytes, the PVC_Cif operon was activated, but only when it had been grown at 28°C prior to the exposure. This suggests that this PVC is possibly anti-phagocytic in function. However, since it was only activated when the bacteria had been grown at insect body temperature and not human this suggests that in the normal lifecycle of PB68, where it is already questionable it is capable of infecting humans, this PVC is not utilised against mammalian cells.

Alongside testing the PVC operon activation with PBMCs, the activation of the PVCs was also tested when PB68 was exposed to cultured macrophages in the form of activated THP-1 cells. In this experiment only PB68 grown at human body temp 37°C was tested, with the insect body temperature 28°C not being, mainly due to time constraints. Here it was seen that like in the flow cytometry experiments the Cif operon showed no activation at 37°C. However, unlike the flow cytometry, LopT and Pnf operons were activated when the bacteria were exposed to the THP-1 cells, in both cases quite significantly. While this does seem to contradict some the data from the PBMC experiment, where at 37°C neither of these PVC operons were seen to be
activated, there are some significant differences in what these two experiments are investigating. Firstly, while activated THP-1 cells are very similar to macrophages found in human blood they are not a perfect replica and may produce different surface molecules and cytokines that the bacteria react to. Also, in PBMC fractions there are actually little to no fully activated macrophages, instead the precursors to macrophages, monocytes, are found which again would be displaying different surface molecules that may influence how the bacteria detect them. Also, the PBMC assay was using flow cytometry to analyse GFP signal directly from the PBMCs, this means that only bacteria that were associating with the PBMCs, attaching or internalised, would be detected, as the rest were washed off. Compare this to the THP-1 experiment where we isolated the bacteria from the THP-1 cells then imaged them, so only external bacteria would have been analysed.

Thus, it may not be prudent to directly compare the results of the PBMCs and THP-1 experiments. However, the apparent contradictions between these experiments may indicate that different PVC operons activate depending on if the bacteria are internal or external of the target cells. There would actually make a lot of sense as PVCs seem designed as “long distance” effector delivery systems, so it would make sense for them to be deactivated when inside or attached to the target cell, where systems like the T3SS or T6SS would be better suited. Considering this, it suggests there be an extra layer of sophistication to the use of the PVCs. Where external *Photorhabdus* when they first detect cytokines or other signalling molecules characteristic of their target, start producing certain PVCs, but after attachment/internalisation these are turned off and likely new pathogenicity factors are activated instead. This is quite common in bacterial pathogens where different pathogenicity factors are activated externally and internally.

Thus, from this data it could be suggested that when encountering professional phagocytes; dendritic cells, monocytes and macrophages, PB68 first starts producing the LopT and Pnf PVCs as an early “long range” attack. Then later in the infection upon attachment and internalisation into the phagocytes expression of these PVCs are stopped, and the Cif PVC starts being produced. This theory is likely being complicated by the fact that as hypothesised above (*Chapter 2.8.5*) PB68 is likely not specialised for mammalian infection. Thus, future work will have to look more into how the PVCs are being activated at the insect body temperature. Particularly
repeating the THP-1 experiment at insect body temperature and investigating PVC activation in response to insect phagocytes, such as S2 cells, may give us a clearer idea of the targets of each PVC. It will also be interesting to repeat these report experiments with other strains of Photorhabdus, especially the known human pathogens such as the \textit{P.\textit{luminescens}} Texas strain and the \textit{P.\textit{asymbiotica}} Kingscliff strain. In theory these two strains would likely show similar activation of PVCs to the PB68, though perhaps upregulating more at human body temperature, which is when they also invade best.

Of course, it should be remembered that the activation of the operon promotor and expression of the first structural gene PVC1, which is what our reporters simulate, does not necessarily mean that complete PVCs will end up being created. There are possibly secondary regulation mechanisms, such as termination regions, which allow for the expression of the operon to start from one signal but prevents full transcription unless a second signal is received. There is already some evidence for regulation occurring after transcription has begun in the PVC operons, as seen in a later chapter (\textit{Chapter 3, Fig.3.1 C}), where some PVC genes are transcribed in lesser amounts then others. However, even considering this, the data still represents the activation of pathogenicity factors in response to the changing environment of the bacteria.

\textbf{2.10.8 Photorhabdus invasion of the nucleus}

Kingscliff showed a unique invasion phenotype at 37°C, where bacteria were seen to invade the nucleus of THP-1 cells (\textit{Fig 2.6}). However, this was only observed occasionally, as an uncommon occurrence with an unknown trigger. While many bacteria live in the cytoplasm intercellularly, bacteria that invade the nucleus of their host cells are much rarer. Examples include the causative agent of spotted fever \textit{Rickettsia rickettsia} (Burgdorfer \textit{et al}, 1968) and \textit{Candidatus Berkiella cookevillensis} which was also seen in THP-1 nuclei (Chamberlain \textit{et al}, 2019). However there has never been a reported case of a Gamma-proteobacteria invading a mammalian nucleus. It is currently unknown as to the exact mechanisms of how these bacteria enter the nucleus, but the propulsion in the form of actin-tails that some of these bacteria use for movement and phagosome escape may play a role (Ogata \textit{et al}, 2006; Vadivelu \textit{et al}, 2017; Schulz \textit{et al}, 2015). Some bacteria, \textit{Euglena hemichromata}, have also
been shown to enter the nucleus through fusion of the endosome and nuclear membrane (Shin et al, 2003).

It may be advantageous for bacteria to invade the host nucleus for a couple of reasons. Firstly, it the nucleus is nutrient rich environment which is safe from some of the cell’s protective measures against intercellular pathogens. One example of this is Candidatus Endonucleobacter bathymodiolin which is believed to feed upon its host’s cells chromatin (Zielinski et al, 2009). It also allows for direct translocation of toxins into the nucleus, which can be used to regulate host behaviour and transcription.

Further study will be needed to confirm that Photorhabdus is actively seeking an internuclear niche. For example, transmission electron microscopy studies could reveal if the bacteria are in vacuoles and confirm nuclear localisation. It would also be of interest to determine a mechanism for the entry, as Photorhabdus has not been reported to produce the actin tails that are found in many internuclear bacteria.
Fig 2.10 | Summary of *Photorhabdus* species interactions with mammalian cells.

<table>
<thead>
<tr>
<th>bacterial growth at 37°C</th>
<th>bacterial growth at 28°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A dark green box indicates that at that temperature the strain of <em>Photorhabdus</em> invaded/attached to the corresponding mammalian cell.</td>
<td>A dark green box indicates that at that temperature the strain of <em>Photorhabdus</em> invaded/attached to the corresponding mammalian cell.</td>
</tr>
<tr>
<td>A line through the box indicates that no experiment has yet been done for that cell type <em>Photorhabdus</em> strain combination.</td>
<td>A line through the box indicates that no experiment has yet been done for that cell type <em>Photorhabdus</em> strain combination.</td>
</tr>
<tr>
<td>A dark green box indicates that at that temperature there was still invasion/attachment to the corresponding mammalian cell type it was at a level significantly lower than at the other temperature. Red box indicates that there was no significant invasion or attachment to the corresponding cell type.</td>
<td>A dark green box indicates that at that temperature there was still invasion/attachment to the corresponding mammalian cell. Light green indicates that at that temperature there was no significant invasion or attachment to the corresponding cell type.</td>
</tr>
<tr>
<td>43499</td>
<td>48241</td>
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<tr>
<td>ACT</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Jun</td>
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<td>THe1</td>
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</tbody>
</table>
2.11 Conclusions

Overall, here we have shown that despite being closely related, the various *P. asymbiotica* strains differ greatly in their responses to the human infections, suggesting differential adaptations. We also show that growth temperature plays an important part in the activation of pathogenicity factors relevant to some strains response to human immune cells. One such factor being the injectosome PVC_Cif which is seen to be a response to phagocytic cells. It can be concluded that not all the *P. asymbiotica* strains have the ability to infect human hosts, as the North European strains *P. asymbiotica* subsp. HIT and JUN are incapable of surviving phagocytosis. Although further study is needed to see if they are avoiding phagocytosis altogether. The human infective Australian *P. asymbiotica* strains seems to have adopted an aggressive invasive strategy in response to immune cells, which is not seen in the closely related PB68 Thai strain or the North American strain. We also showed for the first time a unique invasion phenotype in the *P. asymbiotica* subsp. *Australis* strains where the bacteria are capable of invading the nucleus, and that actin rearrangement is required for internalisation of *Photorhabdus* into THP-1 cells. Finally, we showed that the newly identified, first known human infective strain of *P.luminescens*, indiscriminately infects all human immune cells. We also showed that unlike its asymbiotica cousins, growth temperature has little effect on its pathogenicity and behaviour.
Fig 2.11 | Internalisation of various Photorhabdus strains when exposed to human PBMCs

Diagram showing the behaviour of various Photorhabdus strains when exposed to human PBMCs when the bacteria had been grown prior to exposure at either 28°C or 37°C. Solid arrow indicates that strain is internalised or adheres PBMC cell type, while dashed line indicates they do not. PBMCs are identified by colour of border as follow; Dendritic cells = grey, Macrophage = dark blue, NK-cell = yellow, B-cell = orange and T-cell = light blue. Images as top indicates if those strains are able to infect only insects or insects and humans.
Chapter 3

Synthesis and loading of PVCs

The vast majority of research that has been published on PVCs and similar extracellular injection systems, such as the MACs, is related to uncovering their structures and mechanics of injection (Jiang et al., 2019). Due to this we now have a good idea on the molecular structure of the needle complex and the mechanism that causes the needle to contract and inject its payload. While this is invaluable to better understanding the systems, one part that has been overlooked is how the payload, effector, is loaded and chosen.

3.1 Effector selection in PVCs

Getting a better understanding how loading of effectors works in PVCs is important for two reasons. Firstly, it would let us make more informed theories as to which effectors are being loaded into which PVCs, making it easier to unravel their targets, effects, and purpose in the *Photorhabdus* life cycle. The adaptor proteins which load effectors in the T6SS was used for this very purpose recently facilitating the discovery of a range of novel T6SS effectors (Liu et al., 2020). The other, and one of the main goals of my research, is that it may allow us to choose what is loaded into the PVCs, including proteins not native to *Photorhabdus*. This ability combined with knowledge of what cell type the PVC binds and successful purification methods, might allow for the use of PVCs as a selective drug delivery system.

One theory is that each PVC operon has one effector associated with it that is always loaded, being incorporated into the PVC structure simply due to being expressed at the same time. However, this would seem unlikely as most injection systems are designed to be versatile and inject a range of effectors into the target, the T3SS being
one such example (Ogawa et al, 2008). PVCs seem no different in this sense, as each operon has a range of putative effectors associated with it, making it unlikely that only a single one would ever be loaded. A more likely scenario is that each PVC is capable of loading a range of different effectors depending on the situation, however no published research presents a mechanism on how this could be achieved.

Due to the similarities between the PVC system and the T6SS, it has been theorised that they may use similar methods for selecting effectors to load. The T6SS also has a range of effectors linked to each operon encoding the secretion system. In this case each effector that is loaded has a corresponding adaptor protein that is expressed alongside it and is encoded in an adjacent locus in the genome (Unterweger et al, 2015). The adaptor proteins show a high degree of variance, being specific to a certain effector, and compete for binding to either the VgrG or Hcp of the T6SS complex. When an adaptor protein binds, it effectively “loads” its effector into that T6SS complex, which then goes on to be delivered into the target cell (Unterweger et al, 2015). As each adaptor protein has a different affinity for the VgrG/Hcp, some have a higher probability of binding than others and thus load their effectors more. However, the chance of loading is also dictated by how much of the adaptor-effector are being expressed, so even a low affinity adaptor protein can achieve high rates of loading if expressed at high levels. This leads to a dual control system of loading efficiency for various effectors depending on both their linked adaptor and how much they are expressed.

It was thought that the PVC effectors might also have adaptors. However, no adaptor-like proteins were found in or near the PVC operons. Thus, it was thought that the targeting sequence may be part of the effector.

The best characterised effector of the PVCs and one of the only to be shown to be loaded and injected is Pnf effector from P. asymbiotica. To look for possible targeting sequences, we compared the sequence and predicted structure, courtesy of Alphafold (Jumper et al, 2021), of Pnf and its homolog Cnf from E. coli (Fig 3.1 A, B). In E. coli Cnf is secreted from the cell and acts as an AB-style toxin. It contains 3 main domains, a cell binding domain for specific binding to target cells, a transmembrane domain which allows insertion into the target membrane and translocation of the final domain, the catalytic domain which causes the toxic effects. Pnf is highly truncated
compared to Cnf, missing both the cell binding and transmembrane domains. This makes perfect sense as it is injected by the PVCs, so only needs the catalytic domain. Interestingly however, when you compare the structures of the two proteins, Pnf can be seen to have a long-disordered N-terminal tail (highlighted in orange in the model), which emerges from the main body of the protein, comprising of the first 50 amino acids. This suggests that it may be having an effect separate from the main catalytic body of the protein, and tails like this have been previously shown to be involved in targeting (Hwang et al, 2004).

Thus, it was theorised that these first 50 amino acids of each effector proteins might act as an adaptor-like sequence, which is referred to as the “Leader Sequence”. In order to test this hypothesis however we needed a reliable and efficient way of both creating PVCs and expressing any effectors we would wish to load.

Fig 3.1 | Comparison of the sequence and structure of *E.coli* Cnf and the *Photorhabdus asymbiotica* PVC effector Pnf.

A- Alignment of protein sequence of the two versions of Cnf found in *E.coli* and the Pnf effector in *P.asymbiotica*. Red highlights areas of some similarity. The Pnf can be seen to align mainly to the catalytic region of the Cnfs. B- Predicted structures of Cnf and Pnf generated by alphafold. Pnf is lacking cell
binding and transmembrane domains of Cnf and N-terminal 50 amino acids forms a long disordered tail.

3.2 PVC_Unit4 overexpression and regulation

For our initial attempts to synthesise PVCs and investigate how effectors are loaded, we decided to use a *Photorhabdus* strain directly. While previous studies (6) have shown the ability to express PVC operons in *E.coli*, the PVCs produced are often in low numbers and when observed under EM show irregularities in PVC length and shape. Also Transmission Electron Microscopy [TEM] done in our lab with these. The irregularities in the *E.coli* produced PVCs were likely due to incorrect expression levels of certain genes in the PVC operon. Previous studies both published (Vlisidou *et al* 2019) and in our lab, have shown that not all PVC operon genes are evenly expressed (unpublished RNAseq data), suggesting a level of regulation beyond just the promoter of the operon. Production in a *Photorhabdus* strain would remove these possible variables and guarantee the best chance of proteins folding correctly and correct regulation of the various genes in the PVC operon.

The strain that was chosen was the fully sequenced lab strain, *Photorhabdus* *luminescens*. SubSp *laumondii* DJC. This stain was originally referred to as TT01, an isolate from Trinidad and Tobago (Will *et al*, 2002) which was used extensively in research. However, a rifampicin resistant mutant of the strain was developed which due to its superior applicability in research applications superseded the wild type. This new rifampicin mutant has since been reclassified to DJC due to research finding significant mutations, changes to bioluminescence, pigmentation, biofilm formation, haemolysis and growth compared to the wild type (Zamora-Lagos *et al*, 2018). Alongside the rifampicin resistance, which allows for genetic conjugation methods, this strain is also relatively easy to genetically manipulate, grows in lab conditions and is not human pathogenic unlike *P. asymbiotica*. The main disadvantage of this strain is that it cannot grow above 28°C, so has slow growth compared to *E. coli*.

To date the majority work on PVCs has been done using the PVC_Pnf operon from the human infective American strains *Photorhabdus. asymbiotica*. SubSp *asymbiotica*. While the DJC strain, like all *Photorhabdus*’ has multiple operons encoding different types of PVC, it lacks a homolog of the PVC_Pnf operon which is possibly a
mammalian targeting PVC. Therefore, the PVC operon chosen for the initial experiments was PVC_Unit4. PVC_Unit4 was chosen as it had a genetic layout typical of PVCs, unlike for instance PVC_LopT which is missing tail fibre proteins, and seemed to have only a few possible effectors linked to it (Fig 3.2 A).

It is theorised that *Photorhabdus* normally does not produce PVCs in large amounts unless needed, likely as it requires a heavy investment of resources and energy.

Previous studies using GFP promoter reporters indicated that a PVC operon is only expressed in a small proportion of the cell population (*Vlisidou et al., 2019*). This is likely due to it requiring a heavy investment of resources and energy, or that they are released by lysis as a sacrificial sub-population. Also, *Photorhabdus* cultures can in principle produce a variety of the different PVCs they encode, which would likely confound interpretation of the findings. Thus, to combat these problems an arabinose inducible AraBad promotor, was integrated into the DJC genome replacing the native promotor for the PVC_Unit4 operon by a previous lab member (Fig 3.2, B). This means with the addition of arabinose, the DJC strain could be induced to start expression of a large amounts of PVC_Unit4.

Sequencing confirmed that the replacement of the native promotor with the arabinose promotor had been successful, not shown here. Reverse Transcription PCR [RT_PCR] was used to confirm the arabinose induction did indeed induce transcription. The PVC_Unit4 production DJC strain (henceforth referred to DJC_Unit4) was grown overnight and sub-cultured at a 1:10 dilution in LB containing either arabinose or glucose, growth in glucose helps block some of the inherent “leakiness” of the arabinose promotor. RNA was extracted from samples of the bacteria grown for either 6 or 24 hours post addition of arabinose, and genomic DNA [gDNA] was removed. A sample of the gDNA was also purified from the bacteria to use as a control. The extracted RNA was then reverse transcribed nto complementary DNA [cDNA] and used as a template for a subsequent PCR reaction to detect the presence or absence of the structural genes of PVC_Unit4 (Fig 3.2, C).
Fig 3.2 | Production and purification of PVC_Unit4 from *Photorhabdus.luminoscens.DJC*.

A- Operon of PVC_Unit4 from DJC which was the PVC that was chosen to be overexpressed and used for subsequent experiments. B- In order to overexpress the PVC_Unit4 operon in DJC, the natural

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**Typical PVC operon**

<table>
<thead>
<tr>
<th>5,000</th>
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<th>15,000</th>
<th>20,000</th>
<th>25,000</th>
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<tbody>
<tr>
<td>Structural genes</td>
<td>Effector region</td>
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B - Operon of PVC_Unit4 from DJC which was the PVC that was chosen to be overexpressed and used for subsequent experiments. B - In order to overexpress the PVC_Unit4 operon in DJC, the natural

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D - Operon of PVC_Unit4 from DJC which was the PVC that was chosen to be overexpressed and used for subsequent experiments. B - In order to overexpress the PVC_Unit4 operon in DJC, the natural
promotor, which is found just upstream of PVC1, was removed and replaced an arabinose inducible promotor, AraBad. This edited strain was referred to after as DJC_Unit4.  

C. RNA was extracted from DJC_Unit4 grown with either glucose or arabinose, at 6 hours and 24 hours of growth. The RNA was then reverse transcribed into cDNA and used as the template for PCRs amplifying each individual PVC structural gene. The results of which were run on an agarose gel PVC1 to PVC16, left to right. gDNA was also extracted from the DJC_Unit4 strain to act as a positive control for the primers.  

D. TEM of PVCs purified from TT01-pCEP_Unit4. Photorhabdus TT01-pCEP_Unit4 was induced with 0.2% arabinose, the supernatant was then taken and purified through a mix of PEG precipitation and a CsCl gradient.  

E. Electron microscopy images showing examples of PVCs where the tail fibres are visible.  

When gDNA extracted from DJC_Unit4 was run it showed that the primers being used were capable of successful amplification of the PVC operon structural genes, Pvc_1-16 (Fig 3.2, C). The sequences for these primers may be seen in the methods section (Chapter 5, Fig 5.5). The AraBad promotor was capable of increasing expression of the operon genes, at 6 hours of growth when arabinose has been added. Interestingly, by 24 hours most of genes had stopped expression. Also, at both timepoints the expression of the genes was not homologous, instead it seems that the earlier genes are expressed at the highest levels, with expression decreasing along the operon. This indicates a level of secondary regulation within the PVC operon that had not been previously reported. Putative antitermination sequences have been identified within some of the operons suggesting a possible mechanism where transcription is sometimes terminated midway through expression of the operon, thus leading to the higher levels of expression of early PVC genes seen here. This would make sense as many of the proteins early in the operon make up the majority of the PVC structure, e.g the sheath (PVC_2,3,4) and tube (PVC_1,5), so it would make sense that more of these proteins would need to be made relative to other proteins such as the tube cap (PVC_16). Some of the genes seem to show nearly no expression at either timepoint, e.g PVC14, though this could simply suggest that it is only expressed very early or late during the PVC creation.  

Even with glucose added, which should supress the arabinose promotor, there was weak expression of some of the operon genes indicating some leakiness of the promotor However was not perceived as a problem as the main goal of this study is simply requires the over-expression of PVC_Unit4. However, the leakiness will have to be kept in mind for any future experiments that use the addition of glucose as a control for PVC-Unit4 production.
By 24 hours of growth most transcription had stopped in both the glucose and arabinose treated samples, apart from a few genes, with PVC_5 in particular still showing a high level of transcription. This could be due either to depletion of the arabinose, and thus no longer driving activation of the promotor, or some a secondary level of regulation preventing expression of PVC operon genes in response to large quantities of PVCs being produced. We propose that continued over-expression of PVCs would likely lead to cell death, either due to overuse of resources or protein aggregation.

### 3.3 Purification of PVCs

As we confirmed that the replaced promotor was capable of driving high levels of transcription of the PVC operon genes. We subsequently moved on to purification studies to confirm correct synthesis and assembly of the needle complexes.

Due to their similarities in structure to bacteriophages, previous studies had shown that PVCs could be purified by techniques commonly used for bacteriophage virus purification (Yang et al, 2006; Luong et al, 2020). In brief the purification process involved (Fig 3.3); induction of the PVC_Unit4 overproduction strain DJC_Unit4 with Arabinose and growth for 48h. Removal of cells from supernatant by centrifugation. Followed by concentration of extracted proteins by PEG precipitation, which preferably precipitates large protein complexes, allowing their separation from smaller proteins produced by the cells. The lower size limit of proteins precipitated can be decreased by increasing the concentration of PEG added to the lysate. The concentrated protein complexes were then further separated by size using a CsCl gradient and ultra-centrifugation, which produces a band within the gradient that should only contain the PVCs and similar sized complexes. Finally, the purified PVC mix was then passed through an Amicon column, which acts as a size based filter only keeping molecules above 10000 kDa, which both removes any remaining small molecules, concentrates the PVCs further, allowing for the resuspension of the PVCs in a tris-based buffer.
The main disadvantage of this method of purification is that because it mainly relies on size and weight to separate out the PVC_Unit4 similarly sized complexes can also be purified alongside, such as other PVCs encoded in the genome. This could confound interpretation of downstream tests. However, the overexpressed PVC_Unit4 should in theory outnumber the any of the “naturally” produced PVCs.

To determine if the DJC_Unit4 strain was indeed synthesising structurally intact PVCs, we imaged purified samples using transmission electron microscopy [TEM]. Purified samples of wild-type DJC supplemented with 0.2% w/v arabinose or glucose, and DJC_Unit4 supplemented with glucose showed few or no PVCs when imaged. Conversely, when DJC_Unit4 was induced with 0.2% arabinose PVCs could be easily observed in large numbers under electron microscopy (Fig 3.2, D). This indicates that despite the leakiness of the promotor shown earlier, that only when induced does the DJC_Unit4 strain reliably produce large amounts of this specific PVC. Since no PVC like structures could be seen in the wild-type DJC sample, this suggests that naturally PVCs, in these conditions at least, are at expressed at low levels. Thus, allowing us to be confident that in our purifications from the overexpression strain, the majority of PVCs are indeed PVC_Unit4.
Fig 3.3 | PVC purification from Photorhabdus pipeline.

The species of Photorhabdus which PVCs were purified from was the DJC, overexpression strain. Effector construct plasmids were added to some strains to control what is loaded. Photorhabdus secretes PVCs into the media meaning lysis of pellet is unneeded. TM buffer = 20mM Tris-HCL, 20mM MgCl₂.
3.3.1 PVC length varies between types

The length of the PVCs observed by EM were measured, with the mean length of the PVC_Unit4 being ~187nm (Fig 3.2, E, F). Interestingly this is longer than what has been reported for PVC_Pnf which had an average length of 117nm (Jiang et al, 2019). This suggests that the length of the tube and sheath may vary depending on the PVC type, though even within the PVC_Unit4s imaged there was variance. This difference in length could also be due to the PVC_Pnf in the previous study being made in E. coli instead of Photorhabdus. Several of the PVCs structures were seen to be vastly elongated compared to the average, with at least one example stretching to over 500nm long. This variation in length is most possibly caused by aberrant behaviour of the tape measure protein (PVC_14). It is currently unknown what effect length of the needle complex may have on the PVCs ability to function and deliver effectors. As some studies investigating the structure of loaded PVCs have shown that effectors seem to be packaged along the whole length of the tube (Wang et al, 2021), it is possible that longer PVCs are capable of delivering a higher quantity of effectors at once. There is likely a balancing act in length however as while longer PVCs might be able to deliver more effectors, they are also probably less mechanically stable. In T4 bacteriophages, which have a similar structure to PVCs, length is regulated in a similar way to what is thought to happen in PVCs using a tape measure protein, though little research has been done on aberrations from the normal length. While the tube length of the T6SS, which is also closely related to PVCs, is determined by the width of the cell stretching from one membrane to the opposite (Santin et al, 2019), this does not seem to be a determining factor in PVC length as the average Photorhabdus cell has a width of around 900nm, much longer than even the longest PVCs observed.

While we have now shown that we can overexpress and purify PVCs of a certain type, two unsolved problems remained. Firstly, is the problem of possible contaminants in the purified mixture. As the protein purification method relies on size exclusion, any proteins of similar sizes to the PVCs would also be purified alongside them. However, Due to the large size of the complete PVCs, around 1 MDa in weight, there is unlikely to be many other protein complexes of a similar size. The only likely contaminants would be R-type pyocins of which Photorhabdus has been shown to have at least one type (ffrench-Constant et al, 2003). On the TEM images
some small round non-PVC containments that can be seen. These are theorised to either be incomplete PVCs, possibly the baseplate which is assembled first and is very stable (Jiang et al, 2019), or lipopolysaccharide membrane vesicles. Secondly, while electron microscopy seems to be an effective way of confirming the induction of PVC synthesis, no accurate quantitative method has yet been found to enumerate concentrations in a purified sample. For instance, Bradford assays and protein absorbance (280nm), do not seem to reflect the abundance of PVCs very precisely as seen by EM. We could count them on EM and from this, assuming an even distribution across the EM grid, calculate PVCs per µl. However, this is both very time and cost intensive, so would not be ideal for long term use.

3.4 Leader sequences and Loading effectors into PVCs

We have shown that we can produce large amounts of PVCs from *Photorhabdus* and purify them to the point of removing most contaminants. This meant we now had a model we could use for testing how effectors were being loaded. As mentioned before, it was theorised that the PVCs used a similar system to T6SS adaptor proteins, but instead of being expressed separately the loading sequence, or “Leader sequence”, was fused directly onto the N-terminus of the effector.

If we look at the predicted structure, courtesy of Alphafold (Jumper et al, 2021), of the Pnf effector from *P.asymbiotica* (Fig 3.4 A,B). We can see that the part that is hypothesised to be the leader sequence (highlighted in orange in the model) forms a long, disordered, N-terminal tail which emerges from the main body of the protein. This already suggests that it is having an effect separate from the main catalytic body of the protein, and tails like this have been previously shown to be involved in targeting (Hwang et al, 2004).

3.4.1 Loading native effectors

In order to test the leader sequence theory, we started with the native effectors of the PVC systems, which should already have leader sequences and thus be loaded. If they were loaded then we could then remove the first 50 amino acids [aa], aka the leader sequence, and see if this effects their ability to be loaded. Three PVC effectors
were initially chosen for these experiments. From the PVC_Unit4 operon of DJC, the operon being used in the *Photorhabdus* based PVC synthesis, HvnA and PVC17 were chosen. Both were seen to have possible catalytic domains when analysed by HHpred, making them likely candidates for effectors. The other effector chosen was the well characterized effector Pnf from the *P. asymbiotica* operon PVC_Pnf, which was chosen as it has been shown to be associated and delivered by PVCs in previous experiments (Vlisidou *et al*., 2019; Yang *et al*., 2006; Wang *et al*., 2020). Testing if the Pnf effector was loaded would also show if the leader sequences are specific to the PVC types, in this case whether Pnf does load into PVC_Pnf but does not load into PVC_Unit4.

In order to achieve this the chosen effectors were separated from their putative leader sequences into the N-terminal 50aa (leader sequence) and the rest of the protein. From these spilt effectors a number of plasmid constructs were made expressing either; the PVC effectors with their first 50 N-terminal amino acids (the putative leader sequence) reattached, without their first 50 N-terminal amino acids, the first 50 N-terminal amino acids alone, and the effectors with the first 50 N-terminal amino acids of other effectors. The fusion of leader sequences and main body of the effectors introduced a 2 amino acid linker between them (Fig 3.4 D). Each of these protein constructs were also tagged at the C-terminus with a Myc epitope tag and put under the control of the AraBad promoter on a plasmid (Fig 3.4, C). This way when the plasmids were transformed into the PVC_Unit4 overexpression strain both the PVC and effector construct could be induced at the same time by the addition of arabinose. In theory due to the effector constructs being expressed at high levels, they should out compete any “natural” Unit4 effectors in the DJC genome being made, for loading into the PVCs.

*DJC_Unit4* strains transformed with any one of the above effector constructs were induced to produce the PVCs, and effector constructs, and then purified as done before (Fig 3.3) No further purification of the PVCs was needed beyond what was done previously, as any non-loaded effectors, due to being much smaller than the PVCs, should be removed during the purification process and thus not be detected. This way we could be confident that any effectors in the sample would have to been loaded into a PVC. To check for loading of the effector constructs the purified PVCs were broken apart by boiling in β-mecaptoethanol for 10 mins, the proteins were then
analysed by western blot using anti-Myc antibodies, thus only the effector constructs should be detected.

**A**

<table>
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<tr>
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</tr>
<tr>
<td>KVNVKVLSEDIVSSGKGNTVKAINSLKRL</td>
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**B**

**C**

- Promoter
- Arabinose
- Effector-Construct
- pBad

**D**

- Leader
- Linker
- Myc-Tag

**E**

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<th></th>
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<th>+Pnf leader</th>
<th>+PVC17 leader</th>
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**F**

- Photobacterium cell line
- PVC_Unit 4
- Effector
- Loaded PVCs
- Western blot
Fig 3.4 | Loading of PVC leader-effector fusions into PVC_Unit4.

The ability of various PVC effector leader sequences, the first 50aa, to load proteins into PVC_Unit4. **A** - Protein sequence of Pnf effector highlighting the leader sequence in orange. **B** - Predicted structure of Pnf generated by Alphafold showing the leader sequence (Orange). **C** - Design of effector construct plasmid, where effector constructs are under control of arabinose inducible promotor. Plasmids were transformed into TT01_Unit4 expression strain. **D** - Design of effector constructs. Leader sequences were separated from native effectors and N-terminal fused to main body of the leaderless effectors using a 2aa linker. A Myc-tag was also fused onto the C-terminus of the effector. **E** - Western blots of disassembled PVCs loaded with different leader-effector constructs and broken apart before being run on the protein gel, to release any proteins which may have been loaded. The effector constructs made for each of the 3 effectors HvnA, PVC17 and Pnf, were; the effector with a 2aa linker added between it and the leader (Whole protein). The effector without the first 50aa (No-Leader). The first 50aa of the effector only (Leader only). The effectors with their leader replaced with a different effector’s leader (+Pnf/PVC17/HvnA leader). All the effector constructs were tagged with a myc-tag at the C-terminus. A red line indicates that the corresponding effector was not tested. **F** - Production and loading of PVC, where Photorhabdus luminescens, will have the Leader-effector fusion on a plasmid and the PVC operon within the genome, both driven by an arabinose inducible promotor.

Only the Pnf and PVC17 leader sequences were shown to be able to load both their corresponding effector and other effectors, even ones from other PVC operons into the PVC_Unit4. However, when the leader sequence was removed the effector was not loaded (Fig 3.4 E). This confirms our hypothesis about the leader sequences acting like the adaptor proteins from the T6SS and are necessary and sufficient for allowing loading into the PVC. Interestingly the Pnf leader was also able to load effectors into the PVC_Unit4, despite Pnf being from in the P.asymbiotica PVC_Pnf operon, an operon not even found in the DJC strain. This shows that the leaders are promiscuous and likely capable of loading into any PVC. This would also suggest that the mechanism for loading and detecting leader sequences is conserved among the various operons. In this case it there may be a secondary regulation system that prevents effectors being loaded into the “wrong” PVC complex. One way this may be achieved is that, as with the adaptor proteins of the T6SS, different leader sequences have varying affinities for the different PVCs (Unterweger et al, 2017). Thus, effectors for a certain PVC will outcompete for loading those of other PVCs. However, in the case of the Pnf_Leader used here there was no noticeable difference in the size of the western blot bands when compared to proteins loaded with the PVC17_leader. Of course, these are not quantitative blots making such comparisons inaccurate. One way of testing if leader sequences do have differing affinities, could be to dual express both leader-sequences in the same cell and use quantitative blots.
to see if one is preferentially loaded over the other. The other way *Photorhabdus* could be preventing the loading of incorrect effectors, is through very selective expression of the operons, so that not more than one is expressed at any one time. This would be similar to what happens in T6SS, where effectors that are expressed at higher amounts are more often loaded.

While we knew from previous experiments that Pnf was an effector, PVC17’s function was unknown. Since we have now confirmed that PVC17 is loaded, we can confidently hypothesize that it is being used as an effector by the PVC_Unit4 system. Further studies in other chapters look at the possible function and targets of PVC17 (Chapter 4).

Since the HvnA N-terminus sequence was unable to load either HvnA or Pnf, we can conclude this is likely not a Leader sequence, and HvnA is not being loaded into PVC_Unit4. However, HvnA is shown to be expressed alongside the PVC_Unit4 genes in RNASeq data from previous work in the lab (Unpublished) suggesting it may be playing a supporting role to the function of PVC_Unit4, the possible functions are discussed further in Chapter 4.4. This also confirms for the first time that not all proteins encoded in the effector regions are capable of being loaded into the PVC.

### 3.4.2 Leader sequence diversity

We have now established experimentally the existence of at least two leader sequences and can hypothesise that any effector that is loaded has one. This leaves us with three known leader sequences, Pnf and PVC17, as shown here and the one that can be inferred from known effectors of previous studies: Pdp1 (*Wang* et al., 2021). Comparing these three leader sequences does not reveal any obvious homology, even between the two found in the same operon Pnf and Pdp1. Though it should be noted that their N-terminal 50aa share slightly more sequence similarity when compared to the other putative effectors in these two PVC operons However the similarity is still very low at below 40% (*Fig 3.5, B*). This alludes to the leader sequence’s ability to load proteins being linked to tertiary structural folding which is currently unknown.

Using the protein structure prediction software alphafold, as done previously with Pnf, we created the predicted structures of the three confirmed proteins with leader
sequences and of HvnA, which could be used as a control as we know it is not loaded (Fig 3.5, A). The leader sequences in all three known loaded effectors formed the same long disordered N-terminal tail seen previously with the Pnf leader. As stated previously N-terminal tails have been shown to play a role in targeting, and this disordered tail might be what is binding and allows loading of the effector. Notably while the N-terminal of HvnA also forms a disordered tail, it is much shorter than the others with the C-terminal end folding back on itself to form a β-sheet. This might be an indication of why it is not loaded, as it is not long enough to bind to whatever is loading the effectors. The predicted structures of two of other unknown proteins in the effector regions of each operon were created as well. Interestingly in these cases only PLT_1677 from the Unit4 operon seemed to have the disordered tail, although considering the rest of the structure which also seems very disordered compared to the known effectors which have globular bodies, this may not be indicative of a leader sequence.

Altogether this suggests that the characteristics of a leader sequence are a long disordered peptide tail which most likely binds to one of the PVC structural proteins for loading. The most likely candidate for binding of the leader sequence is PVC15, which seems to have two distinct domains, one with unknown function, and the other an AAA+ ATPase domain. AAA+ ATPases, are a family of ATPases which are involved in a diverse range of functions including protein translocation and remodelling (Erzberger et al, 2006). An AAA+ ATPase is found in the T4 bacteriophages which is involved in loading DNA into the head capsid (Hilbert et al, 2015). Thus it is hypothesised that PVC15 may be loading the effectors into the PVC, with the unknown domain potentially binding the leader sequence. Currently further research into this hypothesis is on-going in the lab.

One of the major hurdles with trying to predict leader sequences and compare known ones is that their length being 50 amino acids is only a hypothesis. It is possible that the functional part of the leader sequence could in fact be shorter.
3.4.3 Loading non-native effectors

We have shown that in order for a protein be loaded into a PVC it must have a leader sequence at the N-terminal and identified two functional leader sequences from Pnf and PVC17. We have also shown that by adding a leader sequence to a *Photorhabdus* protein which is not normally loaded, such as HvnA, we can force it to be loaded into the PVC. This, along with the fact that we can ‘load’ just the small Myc tag alone, indicates that novel proteins can be loaded into PVCs. However, it has not yet been shown if a sizeable protein, not native to *Photorhabdus* can be loaded.
For the initial test we would want a protein with a few set characteristics to give it the best chance of being loaded. First, it should be a non-bacterial protein ideally, to show that proteins from a wide range of species can be loaded. Next, it should be around the same size as the 

*Photorhabdus* effectors, as we do not yet know the upper limit on size for loading into the PVC, which has an inner diameter of 40 angstroms (Jiang *et al.*, 2019). Finally, it would also have to be a protein that has simple folding and has some activity that can be measured. This will allow to also see if the proteins being loaded are still active after loading and injection into the target. The two proteins that were chosen in the end were, Cre recombinase.

Cre recombinase is a small (38kDa) tyrosine recombinase isolated from P1 bacteriophage (Sternberg *et al.*, 1981) and is commonly used now as a gene editing tool in a wide range of organisms, particularly eukaryotes, (Sauer *et al.*, 1990) such as Mice (Sauer *et al.*, 1998), *Drosophila*, *C.elegans* and Zebrafish. Cre recombinase binds to and causes the recombination of LoxP sites. LoxP sites are short DNA sequences consisting of a variable asymmetrical 8bp sequence flanked on either side by fixed symmetric 13bp sequences, for instance the P1 wildtype LoxP sequence is: ATAACCTCGTATA -ATGTATGC – TATACGAAGTTAT (Hamilton *et al.*, 1984). As the middle 8bp sequence is non-symmetrical this gives LoxP sites a direction. When used for genetic manipulation generally two LoxP sites are located either side of the target DNA, known as the “floxed” sequence. If the two sites are going in the same direction, then the DNA between the sites will be cut out. While if the two sites are going in opposite directions the floxed sequence will instead be inverted (Branda *et al.*, 2004). This allows either for the removal of a gene entirely, or to flip a gene in and out of a promoter’s reading frame, effectively turning it on and off. Cre recombinase makes an excellent test protein for the PVC system as it is around the same size as many of the PVC effectors we have tested. It should assert its effect relatively quickly and in low amounts, which is very important as we still do not know the amount of protein the PVCs are capable of delivering to cells. It will also work in a range of both prokaryotic and eukaryotic cells as all that is required is the insertion of a piece of DNA containing the LoxP sites. Furthermore, in eukaryotic cells unlike some genetic manipulation tools, it can spontaneously cross the nuclear envelope (Will *et al.*, 2002).
Fig 3.6 | Loading and testing of Cre recombinase in the PVC_Unit4 system.

A – Predicted structure of Cre-recombinase with N-terminal leader sequence and C-terminal myc tag. B – Design of Leader::Cre fusion, where the Pnf leader was added to the N-terminal of the Cre and a Myc tag to the C-terminal. This construct was inserted into a plasmid with an arabinose inducible promotor, pBAD_Cre. C – Example of how Cre can be used to observe injection. In this case if Cre is introduced to a cell it will cause recombination of the LoxP sites.
excising the GFP gene. D—Western blot of Cre loaded PVC_Unit4, probed with α-Myc antibody showing a band at the correct size for the Cre. This indicates that the Leader::Cre fusion had been loaded. E—Amplicons from PCR across the LoxP sites region, as seen in C, in a plasmid. DNA was extracted from a strain of Photobacterium DJC transformed with a plasmid containing the floxed GFP [pET28_Flox_GFP], and the pBad_Cre.

A plasmid construct to allow expression of the Cre recombinase in our PVC_Unit4 overexpression strain was created in a similar way to how the afore mentioned effector constructs were made using the pBad plasmid, the construct being labelled pBad_Cre. The Cre was also tagged with a Myc tag at the C-terminus and at the N-terminus the Pnf leader was fused onto the Cre (Fig 3.6 A,B). Alongside the addition of the Pnf leader, the start codon was removed from the Cre and replaced with a 2aa linker, an NdeI (CATATG) site, which joined the Pnf leader and Cre. This was done to remove the chance that translation might occur from the Cre’s start codon rather than the start codon on the Pnf leader. The Pnf leader was chosen for the fusion, as it has already shown the ability to load effectors not native to it’s PVC system, such as the HvnA. This plasmid was then transformed into the DJC PVC_Unit4 overexpression strain, and the PVCs were induced and purified as before. To see if the Cre had been loaded, as done previously, the purified PVCs were broken apart using β-mercaptoethanol and run by western blot using an α-myc antibody to detect the Cre. It was found that the Cre could indeed be detected on the western blot, thus confirming that it is being loaded into the PVCs by the leader sequence (Fig 3.6 – D). This is a significant finding, as it is the first time a non-Photobacterium protein has been shown to be loaded into a PVC, showing we can load seemingly any protein into the PVCs. This opens up a wide range of possibilities for the uses of the PVC system in specific delivery of a large range of proteins to cells, either for therapeutic or research purposes.

While we had shown that we could load the Cre, we had not tested if it was active and properly folded. It is possible that the native PVC effectors are loaded in an unfolded state, then spontaneously refold upon injection, a trait many proteins may not possess. If the proteins loaded into the PVCs are in an unfolded or misfolded state, then this would severely limit the possible proteins the system could deliver.

As Cre is an enzymatic protein it is possible that the addition of the leader sequence might interfere with its folding and thus its ability to function. So to test for this possibility before testing occurred using the loaded Cre, we first tested if our
leader::Cre fusion was still active. The simple test devised for this was to express the leader::Cre fusion in *Photorhabdus* DJC containing a plasmid with the floxed GFP. As the leader::Cre fusion was under the control of an arabinose inducible promoter, in theory upon adding arabinose the GFP gene should be cut out and loss of GFP from the bacteria would be seen (Fig 3.6, C).

Unfortunately, the arabinose promotor is slightly leaky, which meant that as soon as the bacteria were transformed with the pBad_Cre they lost fluorescence, as obviously the small amount of Cre expressed cut out the GFP. However; while we were not able to perform our original assay and show a decrease in GFP signal overtime, this does still show that the leader::Cre fusion is active. This was later confirmed using a PCR which amplified across the LoxP sites, that showed a decrease in amplicon size corresponding to the GFP gene in bacteria which had been transformed with pBad_Cre (Fig 3.6, E).

Now that we had shown that the Cre is loaded and the addition of the leader sequence does not interfere with function, the next step would be to test if it has its effects when injected. The assay that was designed to test to see if the Cre was being injected and working correctly used the floxed GFP. A plasmid containing a GFP flanked by two loxP sites would be introduced into the target cell of the PVC. This way if the Cre was injected into the cell it would cause recombination of the two loxP sites cutting out the GFP gene, and this could then be detected by either a loss of fluorescence from the cell or by extracting the DNA and using PCR to see if the gene is still present.

Unfortunately, we do not know the exact cell types that the PVC_Unit4 injects into, making designing an assay difficult. From experiments detailed in later chapters (Chapter 4), our findings suggest that PVC_Unit4 can influence the behaviour of nematodes so were the possible target. However, we have yet to determined the exact cell or cells that are being targeted. We did attempt an experiment using a *C.elegans* strain which had a floxed GFP inserted into their genome where, in theory, if the Cre was injected into a cell that cell would stop fluorescing. However, due to both the complexity of using a whole organism and *C.elegans’* strong auto florescence none of these experiments yielded any interpretable results (more details in Chapter 4.3.2). This along with the time constraints of the PhD meant that no further work could be conducted by myself on this subject. However, this work has already being continued.
by others in the lab, and the work on the leader sequences has become part of the basis for a patent and new spin-out company.

3.5 Conclusions and future work

In this chapter I detailed the hypotheses related to the loading of effectors into the PVCs. I showed that unlike the T6SS PVCs do not use separately expressed adaptor proteins but instead loading occurs due to a certain sequence of the first 50 amino acids of the effectors which we refer to as “leader sequences”. The exact characteristics important to the function of a leader sequence remain unclear, however. Structural models suggest they all form long, disordered N-terminal regions, physically extending from the globular enzymic effector domains. With these models we may be able to use this information to better understand the crucial characteristics for the loading process and better identity the loaded effectors for each PVC operon by looking for the presence of these long tails.

I have also demonstrated that leader sequences can be swapped between effectors and yet remain functional for loading even between different PVC complexes and species of Photorhabdus. This suggests that the leader sequence loading system is highly conserved. We also hypothesize that the leaders could be interacting with the AAA+ ATPase, Pvc15, being the best conserved protein between PVC operons. These inter-PVC leader sequences do raise some questions as to how the Photorhabdus prevents loading of effectors from other operons. However, as already seen the operons are tightly controlled and it is possible that only one PVC operon is activated at a time.

One of the major findings of this chapter however must be the fact that non-Photorhabdus proteins can also be loaded by the fusion of a leader sequence onto the N-terminus. This opens up many possibilities for the selective injection of proteins depending on the specificity of the PVC operon, we already know that some PVCs target eukaryotic cells, and better understanding in the future of each PVC types target will allow for more applications of the use of the PVCs.

There are still many questions that must be answered about what can be loaded. What are the size limits? Can very hydrophobic/hydrophilic and proteins with low or high
isoelectric points? Do complex proteins retain function and folding once loaded and injected? Hopefully systems such as the Cre will help us answer these questions in the future.

Though this work we have also confirmed one of the effectors of PVC_Unit4, PVC17 contains a leader sequence and presumably has some role in the Photorhabdus lifecycle. This is discussed in more detail in the next chapter. Strangely HvnA despite being a very close homolog for an already existing protein, unlike PVC17 which is for the most part a mystery, does not seem to be loaded. However, we show in the next chapter that it might still have a role and maybe is instead secreted directly rather than being loaded into a PVC.
Chapter 4

PVC effectors and induction of endotokia matricida

In recent years research on the PVCs and similar systems has been increasing giving many insights into various aspects of this unique system. We now have a model for the structure and assembly of the PVC_Pnf system (Jiang et al., 2019). Additionally, there is evidence for selective injection of effectors into a range of cell types, such as insect and mammalian (Vlisidou et al., 2019; Yang et al., 2006). And a good idea of the use and effect of PVC_Pnf from P. asymbiotica. However, outside of Pnf and LopT very little is known about any of the other PVC effectors, or even the targets of the various PVCs. In this section I examine the effector regions of each PVC type, looking at the diversity of effectors across the different Photorhabdus species. I also show for the first time that PVC_Unit4 is a possible nematode specific PVC related to Photorhabdus-nematode symbiosis. This is supported by finding that one of the PVC_Unit4 effectors induces endotokia matricida in C.elegans, and effects mammalian cell growth and metabolism.

4.1 PVC operon types and their possible targets

As described in the introduction, each Photorhabdus has around 4-5 different PVC operon types which share homology of their structural genes across species. In other words two PVC operons of the same type are more homologous between different species that two PVC operons of different types within the same genome. From looking at the various sequenced genomes of different species of Photorhabdus, the PVC operons can be roughly separated into nine “types”; Unit1, Unit2, Unit3, Unit4, Pnf, LumT, LopT, Cif and Cer.
Apart from PVC_Pnf, so far, the targets of these PVCs are not very well understood. Even in the case of PVC_Pnf while it has been shown to inject into both murine macrophages and insect haemocytes (Vlisidou et al, 2019; Wang et al, 2021), how specific its target cells are is unknown. The only knowledge of the targets of PVCs outside of PVC_Pnf, is an experiment that showed PVC_LopT and PVC_LumT had insecticidal effects suggesting these PVCs can target insect cells (Yang et al, 2006).

It is possible to narrow down the possible targets of each PVC by first identifying the organism which it is being used against. As it is presumed that the PVCs are mainly an anti-eukaryotic delivery system, we can narrow down possible targets by looking at the eukaryote we know it interacts with during its lifecycle. All Photorhabdus species during their lifecycle will encounter both, nematodes, either their symbiont or occasionally other entomopathogenic species, and the host insect. While the temperature tolerant, mammalian infective P.asymbiotica and P.luminescens strains, will also likely encounter on a regular basis either mammals or possibly birds as secondary hosts. One way of better understanding the targets of the various PVCs is to look for when Photorhabdus starts producing them, as due to the resource demanding nature of the PVCs, constant production would be detrimental to the cells health, so are likely to only start production when exposed to organisms or conditions for which the PVC will be used.

4.1.1 PVC operon activate in response to host cells

In a previous chapter (Chapter 2, Fig 2.7) we showed that the PVC_Pnf and PVC_Cif operons were upregulated when the P.asymbiotica species PB68 was exposed to THP-1 cells, mammalian macrophages. This suggests that these PVCs are targeting the THP-1 cells, or at least mammalian cells in general, though of course further research is needed to prove this hypothesis. To get a better understanding of the possible targets of some of the PVCs, these same experiments using the PVC operon reporters were repeated, but this time exposing them to either C. elegans or S2 cells. C. elegans, is a nematode related to the Photorhabdus symbiont Heterorhabditis both being part of the Rhabditida order. While S2 cells, on the other hand are an insect cell line derived from Drosophila melanogaster embryos, thought to be derived from a macrophage-like lineage (Schneider, 1972). As before the PVC operons tested were Cif, Lopt, Unit4 and
Unit1 in *P.luminescens* TT01, and Cif, Lopt, Unit4 and Pnf in *P.asymbiotica* PB68. Control samples were done in parallel with test samples in the same conditions, Schneider’s media at 28°C for insect cell test and M9 buffer at 28°C for the nematode tests, and for the same amount of time, but without the presence of the nematodes or S2 cells. As before a threshold for determining activation of the operon was calculated from the highest florescent signal from a strain of the tested bacteria containing pAGAG, the vector used for creation of the reporter stains, missing a promoter for the GFP. All statistical tests for determining significant difference between the control and test sample for each PVC operon was done using only values above the threshold. More details on the experimental design can be found in the previous testing using THP-1 cells (Chapter 2, Fig 2.7).

**Fig.4.1 | Activation of PVC operons in the presence of either nematodes or insect cells.**

*Photorhabdus* species, PB68 and TT01, were transformed with either a reporter plasmid containing a GFP under the control of a PVC operon promoter, so when the bacteria expressed the PVC operon GFP would also be expressed, or they were transformed with a plasmid containing a promotorless GFP, pAGAG. The bacteria containing one of the reporter plasmids were then exposed to either *C.elegans* or S2 cells for 2 hours at 28°C, in either M9 buffer or Schneider’s media respectively. Control samples were treated the same way without the presence of the *C.elegans* or S2 cells. After the 2-hour incubation the bacteria were washed and resuspended in PBS and imaged by florescence microscopy and analysed using Image J to determine each cell’s relative GFP fluorescence. A threshold florescence value, the dotted line, above which bacteria were characterised as expressing the PVC operon was determined as any cell above the highest fluorescing pAGAG cell. (Mann-Whitney test, ** = < 0.01, *** = < 0.001, **** = < 0.0001)
When exposed to *C.elegans* nematodes, the most obvious activation of any operon is the PVC_U4 in both PB68 and TT01. This increase is significantly larger in TT01 where some cells are producing massively high amounts of GFP far higher than seen in any of the other samples, indicating strong and prolonged expression of the operon. The PVC_U4 operon was not seen to be upregulated in response to either the THP-1 cells or the S2 cells, suggesting it is specially targeted towards nematodes. The PVC_LopT operon also has an increase in expression in both the PB68 and TT01, though this is much slighter than what is seen in the Unit_4 operon. In PB68 both the PVC_Pnf and PVC_Cif operons also show slight but significant increases. In both these cases though, even without the nematodes there is a large amount of cells with activated operons so it would seem likely that these PVC operons are also being activated due to factor independent of nematodes, such as the M9 buffer. The PVC_LopT activation is more convincing, due to both species showing an increase, and the small number of cells in the control sample above the threshold. Interestingly, as the nematodes were not lysed prior to imaging of the bacteria this would mean that even the external bacteria simply have to be in the close vicinity to the *C.elegans* to start producing the U4 and LopT PVCs. This would indicate that *Photorhabdus* can detect a secreted cytokine from the nematodes. The fact that we see these responses even with *C.elegans*, which is a species of nematode that *Photorhabdus* is unlikely to encounter naturally and does not associate with, not being entomopathogenic, indicates that whatever they are detecting must be a generalised nematode molecule. Nematodes secrete a number of small signalling molecules, many of which are in response to bacteria, which are detected by both bacterial pathogens and commensals of the nematodes (Midha *et al* 2017; Liang *et al*, 2019).

When exposed to the S2 cells neither of the *Photorhabdus* species had any significant increase in PVC operon activation. This was even the case with PVC_Pnf which has been show previously to have insecticidal activity. There are a few reasons that this could be the case; firstly, and most simply, that S2 cells are either the wrong cell type or from the wrong species of insect, being isolated *Drosophila*. Most previous experiments looking at *Photorhabdus* oral toxicity and PVC toxicity have been done using *Galleria* which while both insects are not very closely related, not even being in the same order, *Diptera* and *Lepidoptera* respectively. Although some studies have
shown that Photorhabdus also does have some oral toxicity against *Aedes aegypti*, which are also *Diptera* (da Silva *et al.*, 2013), and even some *Drosophila* larvae (Shawer *et al.*, 2018), although neither of these studies looked at PVCs. Secondly, both PVC_Pnf and PVC_Cif seem to already be being expressed in a large amount of the control sample cells even without the addition of S2 cells. So it could be that the operon is already at maximum expression and even if the S2 cells are capable of causing activation, it is being masked by prior activation. As to what may be causing the activation of the PVC_Pnf operon, there are also a large number of cells activated when grown only in PBS as seen in the nematode control, while on the other hand the Pnf operon was not activated when grown in RPMI only, during the THP-1 experiments. So, it is possible that there may be something in the PBS and Schneider’s media causing the operon to activate. But there are few similarities between Schneider’s media and PBS in terms of ingredients, so it is more likely that activation of the operon is due to a physiological effect, such as a starvation response.

In summary, from the three reporter experiments, we show for the first-time differential activation of PVC operons against examples of the main organisms that *Photorhabdus* will encounter in its lifecycle. This confirms the previous theories that each PVC is specialised for use against certain organisms, rather then being a generalised delivery system like the T3SS. This data also gives insights into the targets of previously poorly studied PVCs. Firstly, PVC_Unit4 appears to be a nematode targeted PVC, only being upregulated in response to them. PVC_LopT shows slight upregulation in the presence of nematodes and a more significant increase with THP-1 cells, suggesting it may be targeting a range of organisms. PVC_Pnf responded strongly to the presence of THP-1, however it also seemed to activate when the bacteria was simply grown in either PBS or Schindler’s media, possibly indicating its activation is linked to stress responses. Of course, a generalised stress response activating the PVC could also be the case with the THP-1 cells as well. PVC_Cif was seemingly activated in all samples including controls, suggesting constitutive activation of the operon. This seems strange as this would surely be very metabolically taxing on the cells but does indicate that PVC_Cif is a generalised PVC, a theory backed up by the fact that like PVC_LopT it is found in all *Photorhabdus* species. Finally, PVC_Unit1 did not seem to be significantly expressed in any situation.
There are some limitations of these reporter construct studies, of course. Firstly, with the insect cells, as mentioned above they are from a different order to that of the natural prey of *Photorhabdus* and its nematodes. Thus, to get a better representation of the effect exposure to insects has on the activation of PVCs the experiments can be repeated with either *galleria* haemolymph or cultured sf9 cells, which are derived from the *Lepidopterain*, *Spodoptera frugiperda*, which is much closer related to *Galleria*. There is also the problem that expression of PVC operon, and PVC1, does not mean that functional PVCs are being made. It is possible that the operon is has a secondary regulation system, such as antitermination signalling (Santangelo *et al*, 2011), where the first few genes are expressed in response to a primary signal, but later ones essential to PVC formation require further signalling. In this case our reporter stains would only show activation by the primary signal and not secondary ones.

Future experiments can investigate the activation of more PVC types and the PVCs of more species of *Photorhabdus*. In particular it would be interesting to compare the *P.luminescens* Texas strain to the TT01 stain, and the *P.asymbiotica* Kingscliff to the PB68.

### 4.2 Diversity of effectors throughout the PVC

Each PVC type has the effector region located directly downstream of the structural genes, which seem to be under the expression of the same promotor as the structural genes. Within these regions are multiple putative effector like proteins, with many showing homology to known effectors of other bacterial pathogens. The exact length of these regions and number of effectors they contain seem to vary between PVC types and is currently poorly understood. What is known from studies of PVC_Pnf, that not just the first effector after the structural genes is loaded, as the Pnf effector is the 4th gene downstream. Also it has been recently shown that a second effector from the PVC_Pnf effector region is also sometimes loaded into the PVC, suggesting that multiple effectors are used (38).

Additionally, many of the open reading frames inside the effector regions seem to encode proteins with catalytic domains, indicating their function as a toxin. There are also some that seem to be proteins that would be unlikely to be loaded into the PVCs, such as tail fibre like proteins. In order to narrow down what proteins in the effector
regions are maybe being used by the PVCs, the effector regions of different species of Photorhabdus for each PVC type were compared to look for similar and recurring effectors. The idea being that if an effector is found in multiple species of Photorhabdus it would indicate that it is being evolutionary conserved and thus likely to be important for the PVCs function. While a number of Photorhabdus species have been sequenced, with this number increasing as time goes on, many of these assemblies are not complete making finding very long operons like the PVCs difficult. Thus, for this analysis 8 species were chosen which had well documented genomes. These were the P.luminescens strains TT01 and Texas, the P.asymbiotica strains Kingscliff, ATCC43949 and PB68, the P.temperata strains Khan and PTT, and finally the P.bodei strain Bodei.

There are two major problems with attempting to identify possible effectors associated with the PVCs solely from genomics. Firstly, the presence of a complete ORF does not mean that a protein is actually being expressed. There could be termination sequences that have not been identified stopping transcription before the ORF. The other problem is that identifying where the effector region stops, and what proteins downstream of the structural genes of the PVC are actually associated with that operon. One way of solving these issues could be RNAseq, which would show if the ORFs are being expressed, and if they are being expressed at the same time as the PVC operon would suggest they are linked.

Throughout all the PVC operons, there seems to be the common occurrence of a couple of likely non-effector proteins. One type of these are scavenger molecules, such as nitrilases and lectins. These may be associated with the PVCs as a method of scavenging nutrients after the PVC leads to death and lysis of the target. Another, are motile genetic elements such as transposases, which could be indicate how PVC operons are transferred and copied between species, or how some the effectors were obtained.

A list of the putative effectors found in the various PVC operons can be found below. Interestingly there were cases where effectors seemed to have been swapped between PVC operons in different species. For instance, an anthrax like toxin can be found in 3 different operons depending on the strain of Photorhabdus (Table 4.1).
Table 4.1 | Putative effectors found in the effector regions of Photorhabdus species PVC operons

<table>
<thead>
<tr>
<th>HOMOLOG (SPECIES)</th>
<th>SECRETION OF HOMOLOG</th>
<th>PVC</th>
<th>HOMOLOG TARGET</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANTHAX</td>
<td>AB Toxin</td>
<td>cAMP Unit1, Unit3, Pnf</td>
<td></td>
</tr>
<tr>
<td>DIPHTHERIA</td>
<td>AB toxin</td>
<td>EF-2 (Protein synthesis) Unit1, Pnf</td>
<td></td>
</tr>
<tr>
<td>GOGB</td>
<td>T3SS</td>
<td>SCF-complex degradation (Protein degradation) Unit1, Unit2, Unit4</td>
<td></td>
</tr>
<tr>
<td>SSEI</td>
<td>T3SS</td>
<td>G-proteins / cAMP production Unit2, LumT</td>
<td></td>
</tr>
<tr>
<td>RAVO</td>
<td>Unknown</td>
<td>Unknown Unit2, Unit3</td>
<td></td>
</tr>
<tr>
<td>VOPS</td>
<td>T3SS</td>
<td>Rho GTPases/ G-proteins (Inflammasome inactivation) Unit2, LopT</td>
<td></td>
</tr>
<tr>
<td>CHOL_A</td>
<td>AB Toxin</td>
<td>G-protein (Increased cAMP levels) Unit2, Cif, LopT</td>
<td></td>
</tr>
<tr>
<td>PVC17</td>
<td>N/A</td>
<td>N/A Unit4, Unit2</td>
<td></td>
</tr>
<tr>
<td>CIF</td>
<td>T3SS</td>
<td>Proteosome inhibition (Cell cycle arrest) Unit3, Cif</td>
<td></td>
</tr>
<tr>
<td>LOPT</td>
<td>T3SS</td>
<td>Rho-GTPases (Cytoskeleton and phagocytosis) LopT</td>
<td></td>
</tr>
<tr>
<td>RPOS</td>
<td>Not secreted</td>
<td>RpoS?? (Regulation of stress pathways) Cif</td>
<td></td>
</tr>
<tr>
<td>BOTOX</td>
<td>AB Toxin</td>
<td>SNAREs LopT</td>
<td></td>
</tr>
<tr>
<td>PNF</td>
<td>T3SS</td>
<td>Rho GTPases (Cytoskelton) Unit2, Unit3, Cif, Pnf</td>
<td></td>
</tr>
<tr>
<td>YENC2</td>
<td>Unknown</td>
<td>LopT</td>
<td></td>
</tr>
<tr>
<td>TOXA</td>
<td>Unknown</td>
<td>LopT</td>
<td></td>
</tr>
</tbody>
</table>

4.3 PVC17 / EmP – the Unit4 effector

Of the many novel effectors found within the PVC operons, one that held promise for study was PVC17, within the PVC_Unit4 operon. It is found in all known PVC_Unit4 operons and is highly conserved, with each species’ homolog showing a high level of
sequence identity, on average sharing ~88% sequence identity between the four known instances of PVC17. Its position in the operon also seems to be conserved, always being the first gene directly downstream of PVC16, the final structural gene. So far, the PVC_Unit4 operon has only been identified in four species, the *P.luminescens* TT01 and the three *P.asymbiotica* strains that have been studied; Kingscliff, ATCC43949 and PB68. Other non-*P.asymbiotica* that have been studied (HIT, JUN, Texas, PTT) have PVC_Unit2, a phylogenetically related operon to PVC_Unit4, which TT01 also has. However, a homolog of PVC17 has only been found in the PVC_Unit2 of *P.temperata* SubSp khanii, with a sequence identity of ~77% compared to the Unit4 PVC17s. Thus, it is possible that the other species of Photorhabdus have yet undiscovered homologs of PVC17 which are delivered though either other PVCs, or other secretion systems. This would not be completely unprecedented, as LopT can seemingly be delivered by both the T3SS and PVCs in *Photorhabdus*.

We know from the leader sequence experiments done in the previous chapter ([Chapter 3.4, Fig 3.4](#)) that the TT01 version of PVC17 has a leader sequence and is loaded into PVC_Unit4. Also, previous RNASeq analysis from TT01 showed that the PVC17 gene is expressed alongside the PVC_Unit4 operon. These two observations suggest that PVC17 is an effector that is currently being delivered by *Photorhabdus* for some purpose and is the most likely effector of the PVC_Unit4. One of my goals was discover the use, target and mechanism for this effector. As this would lead to a better understanding of the bacterial host interactions and with the possibility that it could have a novel use in science, industry or medicine.

### 4.3.1 PVC17 predicted structure and functional domains

The first step to discovering the function of a new effector, is to see if it has similarities to known proteins. The protein sequence of PVC17 was run through two protein structure prediction tools HHpred ([Zimmermann *et al*, 2004](#)) and Phyre2 ([Kelley *et al*, 2015](#)) to find any proteins of similar structure, as structure is inevitably linked to function. These tools look for remote homology in protein sequence from large databases of proteins with known structures. These homologous structures can then be used as a template for the structure of regions of the protein of interest, as even
distant homologues seem often to have a comparable tertiary structure (Kinch & Grishin, 2002). Even if sequence similarity is low, this can still indicate a possible function. (Pawłowski et al, 2000).
Table 4.2 | Predicted structural homologs of PVC17.

Possible structural homologs for PVC17 were predicted from its amino acid sequence using HHpred and Phyre2, both with standard settings. The predicted homolog proteins hits with a confidence above 50%, the recommended limit for considering a hit seriously, and a known function were collated in this table. Confidence refers to the probability that the protein is an actual homolog of the query sequence. Percentage ID on the other hand refers to the proportion of the query sequence that aligns to the possible homolog, this is only reported in PHYRE2.

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>DESCRIPTION</th>
<th>CONFIDENCE</th>
<th>%ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHYSRE 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PYOCIN S5</td>
<td>Antimicrobial protein that creates holes in membranes. (Pseudomonas aeruginosa)</td>
<td>61.4</td>
<td>24</td>
</tr>
<tr>
<td>METHYLMALONIC ACIDURIA TYPE A PROTEIN</td>
<td>Transports cobalamin into the mitochondria across the membrane.</td>
<td>61.2</td>
<td>13</td>
</tr>
<tr>
<td>HYDROLASE (T6SS)</td>
<td>Putative T6SS protein (E.coli)</td>
<td>59.3</td>
<td>18</td>
</tr>
<tr>
<td>MITOCHONDRIAL GLYCOPROTEIN MAM33-LIKE</td>
<td>Chaperones mito-ribosomal proteins. (Homo sapiens)</td>
<td>51.8</td>
<td>23</td>
</tr>
<tr>
<td>HHpred</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NUCLEAR PORE COMPLEX PROTEIN NUP155</td>
<td>Part of the nuclear pore complex (Homo sapiens)</td>
<td>90.92</td>
<td></td>
</tr>
<tr>
<td>COLICIN IA</td>
<td>Antimicrobial protein that creates holes in membranes. (E. coli)</td>
<td>85.58</td>
<td></td>
</tr>
<tr>
<td>COLICIN N</td>
<td>Antimicrobial protein that creates holes in membranes. (E. coli)</td>
<td>70.29</td>
<td></td>
</tr>
<tr>
<td>NUCLEAR PORE GLYCOPROTEIN P62</td>
<td>Part of the nuclear pore complex (Homo sapiens)</td>
<td>67.12</td>
<td></td>
</tr>
<tr>
<td>TAIL NEEDLE PROTEIN GP26</td>
<td>Role in penetration of host membrane and plugging created DNA channel. (Bacteriophage P22)</td>
<td>58.32</td>
<td></td>
</tr>
<tr>
<td>PHAGE PHI29 GP7 PROTEIN</td>
<td>DNA binding and phage maturation (Bacteriophage phi29)</td>
<td>56.16</td>
<td></td>
</tr>
</tbody>
</table>
With the HHpred and Phyre2 analysis there was no obvious single protein homolog of PVC17 detected, indicating that the structure is novel. Of the homologs found nearly all of them aligned closer to the C-terminus of PVC17, suggesting this may be the main catalytic region of the protein. Four main groups of proteins showed up; nuclear pore forming complexes, mitochondrial related proteins, phage needle proteins and bacteriocins, which are toxins used by bacteria to punch holes in the membranes of competitors (Lazdunski et al, 1988). All these proteins seem to have some function of membrane binding, suggesting that PVC17 interacts with membranes, this is supported by the PHYRE2 modelling that predicts transmembrane helices. Mapping this transmembrane region onto the PVC17 model generated using alphafold from the previous chapter (Chapter 3.4.2, Fig 3.5), indicates that the helices may be buried in the protein and thus unavailable for binding. However, there are a number of pore forming proteins which when in their water soluble state have a buried helix, which then “flips out” when forming a multimer or under changing conditions, e.g. acidity (Tanaka et al, 2015). The phage and colcin homologs suggest a possible function of PVC17 as these proteins are all involved in creating holes in membranes. In the case of the phages this is for translocation of DNA, while for colcins it is to cause membrane depolarisation leading to death of the cell. Pore forming toxins are one of the most common forms of bacterial toxins due to their simple function and effect, disrupting target homeostasis (Los et al, 2013). While most target the plasma membrane, some have been found to specially target phagosomes (Schnupf et al, 2007). Considering PVC17 is delivered into the host cytoplasm by the PVC, it is possible that it is creating pores in an organelle. However, pore forming toxins often require multiple copies to form a multimer to create the pore (37). While PVCs seem to be loaded with multiple copies of their effector (38), large multimer like pores would still likely require multiple PVC
injections, which would seem inefficient and reduce the likelihood of the intended effect in a target cell.

Fig 4.2 | Predicted structure and transmembrane domains of the putative Photorhabdus. luminescens TT01 effector, PVC17.

A- Predicted structure of PVC17 generated by alphafold. Predicted transmembrane helices are marked in black, with the cytoplasmic part of the protein in white and extracellular part in orange. B- Prediction of transmembrane, cytoplasmic and extracellular regions of PVC17, by Phyre2.

This brings us onto the nuclear pore forming complexes and mitochondrial related proteins which may indicate the target of PVC17. Both the nucleus and mitochondria are only found in eukaryotes, making it likely that PVC_Unit4 is targeting eukaryotic cells rather than bacterial. Also, since both of these organelles are internal it would give a reason why a possible membrane targeting protein needs to be delivered into the cell, unlike for instance colicins which target external membranes. It is interesting that while both software found a bacteriocin homolog and a eukaryotic organelle membrane related homolog, the organelles are different. This makes it more difficult to be certain of the target inside the cell but does allow us to narrow down the possible targets to membraned organelles. HHpred found multiple nuclear pore protein homologs, which are associated with translocation of various proteins in and out of the cell. It is also interesting that one of the other hits from HHpred was the protein Gp7 from the bacteriophage Phi29, which while of course not associated with the nucleus, does seem to bind DNA again suggesting a nuclear target for PVC17. In this theory, PVC17 may be creating holes in the nucleus thus preventing proper transcription and translation leading to eventual cell death. Some bacteria have been
found in target the nucleus of host cells in order to influence host cell behaviour (Ohara et al, 2004), however no reported cases of nuclear envelope pore forming toxins could be found.

PHYRE2 on the other hand detected homolog proteins related to a different organelle, the mitochondria. The two proteins were MAM33 and MMAA, both proteins associated with translocation across the mitochondrial membrane. This fits in with the bacteriocin homologs also detected, both suggesting that PVC17 may be binding to and forming pores in the mitochondrial membrane. This in theory would reduce the cells’ ability to create ATP, inhibiting all cell processes and eventually cause cell death. Unlike the nuclear envelope, some toxins have been discovered that target and create pores in the mitochondria outer membrane, such as the Cry toxins from Bacillus thuringiensis (García-Sáez et al, 2010; Peraro et al, 2016; Xu et al, 2014). Like PVC17 these proteins have homology to bacteriocins (Peraro et al, 2016). This makes sense as bacteriocins target bacterial membranes, and mitochondria share features with their theorised bacterial ancestors, such as the presence of an inner and outer membrane (Roger et al, 2017), with some theorizing that Rickettsiae are the closest ancestor (Emelyanov et al, 2001).

Overall while the data from the homology searches did not detect any very closely related proteins, it does suggest that PVC17 is a pore forming protein most likely targeting an organelle inside eukaryotic cells. Out of the two possible organelle targets it would seem most likely for them to be targeting the mitochondria. Due to both the discovery of bacterial proteins that target the mitochondria and the multiple homologies to colicins, a bacterial membrane targeting protein that could be easily reworked to target the bacteria like membranes of mitochondria. However, it has to be kept in mind that homology searches are not full proof and can often detect homologies that are not real, especially at the lower percentage IDs and probabilities many of the homologies found had. In fact, over the course of my PhD the supposed homologs detected by both software’s have changed dramatically. Although, it is of note that bacteriocins and some form of pore forming translocation domain have consistently been detected. The lack of obvious homologs though does suggest that PVC17 is a toxin that acts in a novel way, making research into it while harder also more interesting and valuable.
4.3.2 PVC17 causes endotokia matricida in *C. elegans*

While the homology search of PVC17, did give some indication as to its possible function and target inside cells. It did not give any information on type of cell it is being delivered to and thus the target of its respective PVC. This makes selecting the model organism to start testing on a daunting task, though we can be fairly confident the target is a eukaryote. This is complicated by the fact that *Photorhabdus* interacts with a number of organisms with vastly different biologies during its lifecycle, including nematodes, insects and sometimes mammals. Considering that PVC_Unit4 is found in TT01, a non-human infective strain, this leaves only two possible targets for this PVC; the host insect, or the nematode symbiont. There is already some insight into which it could be from the PVC operon reporters (Fig 4.1), which showed that PVC_Unit4 was highly expressed when Photorhabdus was exposed to nematodes, but not when exposed to insect cells. This indicates that PVC_Unit4 is most likely a nematode targeting PVC.

In order to test this theory, we exposed *C. elegans*, synchronised to adulthood, to the previously created purified PVC_Unit4 loaded with myc-tagged PVC17 (Chapter 3.4, Fig 3.4). The synchronisation to adulthood would allow us to control for how juveniles and adults may react differently and mean any juveniles that are seen must be developing. By using the PVC17::Myc overexpression plasmid alongside the arabinose controlled PVC_Unit4, we could guarantee that our purified PVC sample would be mainly loaded with PVC17, instead of any other effectors in the PVC_Unit4 operon.
Fig 4.3 | Effects of PVC_Unit4 loaded with PVC17 on *C. elegans* adults.

PVC_Unit4, loaded with PVC17, was added to adult synchronised liquid cultures of *C. elegans*, which were left overnight, then observed for presence of endotokia matricida. A– Percentage of Adult worms undergoing Endotokia matricida when exposed to PVC_Unit4 loaded with PVC17, or heat denatured PVC_Unit4 loaded with PVC17 (90°C, 10mins). B – Phase contrast image showing normal *C. elegans* adult with developing eggs soon to be laid and endotokic adult displaying larvae which have hatched internally.

When the adult *C. elegans* were exposed to the PVC17 loaded PVC_Unit4 the initial response was thrashing, and erratic movement followed by paralysis after a few hours. The adults could be seen to be still alive dispute the paralysis, as there was movement in pharynx. When the nematodes were checked the next day, roughly after 12 hours, the eggs inside them had hatched into larvae inside the adults, a process
known as endotokia matricida (Fig 4.3, B). The number of adults with hatched larvae were counted and when the PVCs were heat denatured prior to addition to the nematodes, there was a significant decrease in the amount of adults undergoing endotokia matricida (Fig 4.3, A).

Endotokia matricida is normally only seen in *C. elegans* in times of very heavy stress and starvation, where the parent itself is food for in the internally hatched larvae which then eat their way out. The starvation though has to last far longer than the 4 hours the worms were without food in this experiment (Chen *et al.*, 2003). However, in the *Photorhabdus* symbiont nematode *Heterorhabditis* endotokia matricida is a vital part of the worms’ lifecycle and larvae that are laid “normally” do not become infective juveniles as *Photorhabdus* does not re-symbiose with them (Ciche *et al.*, 2008; Johnigk & Ehlers, 1999). What exactly initiates the *Photorhabdus* nematodes to undergo endotokia matricida is currently unknown, some speculating that like in *C. elegans* it is due to starvation occurring from complete consumption of the insect cadaver. However, *Heterorhabditis* does not seem to share some the pheromones linked to dauer formation and endotokia found in *C. elegans* (Johnigk & Ehlers, 1999; Golden & and Riddle, 1984), and research suggests that endotokia is not linked to food supply and all adults reproduced by endotokia, even after normal egg laying (Johnigk & Ehlers, 1999). This leaves the question of how endotokia is being initiated and controlled in *Heterorhabditis* still unanswered. It can be presumed however, that *Photorhabdus* is influencing the development of infective juveniles to some extent in order to maintain symbiosis. Unfortunately, there has been little research into the development of infective juveniles in *Heterorhabditis* in the absence of the symbiotic bacteria.

This data suggests that PVC17 might have a role in inducing endotokia matricida in the nematodes, as a way for *Photorhabdus* to control when its symbiont leaves the insect carcass to search for new hosts. This would be very useful for *Photorhabdus*, as this way it can guarantee that it will be taken along with any infective juveniles and thus to any new insect hosts. However we do not know what cell type it is targeting or how it is causing the adult nematodes to become paralysed.

While the data indicates that the PVC17 loaded PVC_Unit4, is having some effect on the adults nematodes there are some limitations with this data so far. Firstly, empty PVC_Unit4, or PVC_Unit4 loaded with something else such as HvnA, has to be tested
to rule out the possibility that the PVC itself is causing the effects in the nematodes rather than what is being injected. Another way of showing that PVC17 has is the causative factor would be to deliver it through a different method than PVC to the nematodes. However, this itself would have many limitations due the lack of information on how specific the PVC_Unit4 is in injection, e.g., does it inject into only a specific cell type in the nematode. Thus, any method used to deliver PVC17 might be targeting the wrong cells, and thus wrong biological processes in the nematode. It is also possible that while either the PVC_Unit4 or PVC17 is inducing paralysis/death in the nematodes, the endotokia is a by-product of a non-traumatic death rather than a direct effect of the toxin. In order to test this, we would have to induce non-traumatic death in the adult nematodes, in roughly the same timeframe the PVC takes to cause death without starvation, which could activate endotokia pathways. Even if endotokia is not a direct result of toxin, but instead a downstream effect, this does not rule out the possibility that eventual endotokia activation is still the purpose of this PVC. Finally, we also have to test the effects of the PVC_Unit4 in the Photorhabdus host nematode, Heterorhabditis, as it may present completely different responses.

Knowing the target cells in the nematode of PVC_Unit4 and PVC17, would make solving a many of these limitations much easier. One way that was briefly attempted was to target PVC17, which is tagged with a myc tag, with florescent antibodies thus observing localisation of the effector by immunofluorescence. However, this ran into a series of problems itself, as immunofluorescence is difficult in nematodes, as in any whole organism. C.elegans has three major hurdles to precise fluorescent imaging. Firstly, they are very motile and react strongly to light, meaning they either must be killed or in some way paralysed. This was not too much of a problem due to the paralysis caused by PVC_Unit4/PVC17, and the nematodes could always be fixed if needed as live imaging was not necessarily. The next major problem was permeabilization of the nematodes tissues to allow the antibodies to infiltrate, which is blocked by the thick cuticle around each nematode. There are reliable methods for removing the cuticle and permeabilising nematode tissue that we were able to get working. However, these all are long process that often damaged the integrity of the nematode’s body structure, which could lead to degradation of PVC17 if injected before permeabilization, or the inability for PVC_Unit4 to bind if done after permeabilization. In fact, since the PVC_Unit4 are themselves unlikely to be able to
pass through the cuticle. It is possible that the PVCs are first being ingested before reaching their targets. This would seem likely as observations of re-symbiosis in the natural \textit{Photorhabdus-Heterorhabditis} lifecycle, see the bacteria in the adult nematode’s gut before endotokia and development of the infective juveniles take place. The final issue is that \textit{C.elegans} has very strong autofluorescence, especially if dead (Pincus \textit{et al}, 2016), which would likely mask any small signal from a few injected effectors. There are some methods of removing the autofluorescence using triple band filters (Teuscher \textit{et al}, 2018), but this are quite expensive and specialist equipment that we did not have access to.

4.3.3 PVC17 causes mild cell cycle arrest in mammalian cells

Due to the difficulties imposed by using \textit{C.elegans} as a model for better understanding PVC17, we instead elected to test the effects of the effector on cell lines. This would allow us to get a more in depth look at how the effector interacting with eukaryotic cells. However, nematode cell lines are rare and poorly characterized, so we decided to use human cell lines instead as they are both easy to grow, work with and are very well characterized. While human cells of course are not identical to nematode cells, much of the underlying cell biology past the surface proteins would likely be similar enough to be comparable. This would be especially true if the theory that PVC17 targets mitochondria is correct. Also we are just testing the direct effects of the toxin and not relying on the PVC for delivery, which we know is very cell specific due to its tail fibres.

PVC17 was first tested in a Human Embryonic Kidney line [HEK 293T] to investigate what toxic effects it may have on the cell line. In order to do this, cells were transfected with a eukaryotic expression plasmid containing PVC17 tagged with a Myc tag. These plasmids also carry GFP so transfected cells could be easily identified (Fig 4.4, A). Three controls were used throughout these experiments, the first being the plasmid with a random protein sequence in place of PVC17, which would show that any effects seen from the PVC17 plasmid are not just due to the strain of protein production on the cells. The second was the plasmid with the Pnf effector from \textit{Photorhabdus}, Pnf having been shown in previous studies to be toxic to Eukaryotic cells (Vlisidou \textit{et al}, 2019), so would make a good comparison. The final control were
cells that underwent the transfection protocol, in this case lipofectamine, but with no plasmid added. This would show that any effects seen were not just due to the strain of the transfection on the cells.

To check the plasmids were working and the HEK 293T cells were expressing the proteins, the cells were transfected and had whole protein extracted which was then run by western blot using an anti-Myc IgG. The resulting western blot showed bands for the expected sizes of the protein, confirming that the PVC17 and Pnf were being expressed (Fig 4.4, B). An extra higher band was seen in the PVC17 sample, though this is most likely to be a dimer or aggregate.

The first toxicity test of PVC17 was a MTS assay, which measures the activity of NAD (P) H-dependent dehydrogenase enzymes in metabolically active cells, by using substrate that goes from colourless to coloured. Thus, the more metabolically active cells the higher the absorbance, with low readings indicating cells are dead or dying. As expected, the control plasmid had no effect on the metabolism or number of cells when compared to the cells with no plasmid transfected (Fig 4.4, C). However, both the cells transfected with PVC17 and Pnf showed a significant decrease in metabolism, most likely through cell death, when compared to the control. However, the PVC17 caused less reduction in metabolism than the Pnf toxin.

This confirms the idea that PVC17 targets eukaryotic cells. Interestingly it is less toxic than Pnf, which is a specialised mammalian toxin, suggesting that either PVC17’s function is not to directly kill the cells or that mammalian cells are not the main target.

To get a better understanding of how PVC17 was affecting the HEK 293T cells, a cell adhesion assay was next performed using the excellence plate system. The excellence plate is lined with gold circuitry and can measure the amount of cell adhesion over time which is given as cell index, with a higher cell index indicating more adhered cells. This is measured by the plate and reader by measuring an electrical current going through the gold circuits at bottom of the plate. Adherent cells growing on the plate will disrupt these currents allowing an estimation of the amount of adhered cells. Thus, the trend of both cell growth and cell death can be seen, with most cell types detaching when they die. The HEK 293T cells were seeded onto the plate and grown for 24 hours first, which can be seen on the graph as the initial period of linear increase in cell index as the cells divide. At 24 hours the cells were taken out.
of the plate reader and transfected with either control, Pnf or PVC17 plasmids, or in the case of “No plasmid” just had the media changed. This took about an hour, which is why there is a slight jump in number of adhered cells when the plate is put back into the reader at 25 hours.

The cells with no transfected plasmid then continued growing and peaked at around 54 hours, 30 hours post transfection, at which point cells begun detaching (Fig 4.4, D). This decrease was likely due to cells beginning to die because of a lack of resources from a saturation of cell growth, being the type of growth curve expected when no new media is added.

All the cells transfected saw a decrease in cell attachment after transfection indicating that the transfection process itself was somewhat toxic. However, Pnf showed a significantly larger and steeper decrease over the others indicating that the Pnf toxin was causing increased numbers of cells to die and detach from the plate. This is what would be expected from looking at the MTS assay which also showed that the Pnf toxin has a strong effect of causing decreased metabolism and possible cell death.

Strangely however, the PVC17 toxin, which did cause a significant decrease in metabolism in the MTS assay, did not cause any increased detachment of cells compared to the control plasmid. It would be expected if PVC17 was causing cells to die that they would be rounding and detaching from the plate. So, when this data is taken with the MTS assay it suggests that the mechanism of action of PVC17, interferes with the cell’s ability to metabolise while not directly killing the cell outright. This would make some sense if the assumptions about how PVC17 works, and what it is used for, is correct. If it is in fact a toxin used by *Photorhabdus* to paralyse the nematode in order to induce infective juvenile development, being able to reduce the efficacy of mitochondria in muscles without directly killing the cells would be beneficial. This would allow for paralysis of the egg laying muscles, while keeping the parent nematode alive, as necrosis of adult cells could cause release of molecules that could damage to the developing larvae. Leading to endotokia matricida to take place which is required for proper development of infective juveniles. Thus through this method using PVC17 the *Photorhabdus* could control when its host moves onto the next stage of its lifecycle.
Fig 4.4 | Effects of the Photorhabdus PVC effectors PVC17 and Pnf on Hek293T cells.
A- Using pD659 as a backbone, plasmids for mammalian expression were created, containing either the sequence for P.asymbiotica Pnf (pD659_Pnf), or P.luminiscens PVC17 effector (pD659_PVC17), both tagged with a Myc-tag and codon optimised for mammalian expression. Alongside these a control plasmid was also created which contained a random sequence (pD659_Con). HEK293T cells, seeded at 2x10⁵, were either transfected with these plasmids, or underwent the transfection procedure but with no plasmid added. B- 24 hours post transfection, protein was extracted from transfected cells. The protein containing lysate was then run by SDS-PAGE and western blot, being probed with an anti-myc antibody. Alongside the cell lysate was run a sample of PVC17 loaded PVCs, to be used to confirm the presence of the PVC17 band. C- 20 hours post transfection, an MTS kit was used to detect the amount of cell proliferation, giving an indication of dead or dying cells. D- Excelligence assay measuring cell adhesion to a plate. Hek293T cells were grown on plate for 24 hours at which point they were transfected with the pD659 plasmids (Red line). Cell adhesion was then measured for a further 48h. E- A single slice confocal image of fixed HEK293T cells 24 hours post transfection with pD659_PVC17. Only the central cell in the image was successfully transfected, with the surrounding cells being un-transfected, this was checked by looking for expression of GFP. Cells were stained with DNA stain DAPI (Blue), Mitochondria stain MitoTracker Red CMXRos (Red) and an anti-myc IgG (Purple). (C- 1way Anova, ** = < 0.01, *** = < 0.001)

Considering PVC17 seems to be having a non-killing effect on mammalian cells, the next step was to try and find what it may be binding to. As from the structural similarity searches PVC17 seemed to have similar domains to mitochondrial associated proteins, this possibility was looked at using immuno-straining. Hek_293T cells expressing myc tagged PVC17 were stained with; anti-myc antibodies, a mitochondrial stain and DAPI nuclear stain. The cells were then imaged 16h post-transfection.

When expressed by the Hek_293T cells, PVC17 seems to be found throughout the cell not localising to any one area, forming large dots. While it can be seen overlapping with mitochondrial signal, the fact that it is spread all over the cell suggests they may not be the target. This signal is unlikely to be background, or unspecific binding as none can be seen in surrounding cells, which were not successfully transfected, as indicated by lack of GFP expression (not pictured here). It is possible that the signal is being masked by too much PVC17 being produced by the cell, leading to the formation of inclusion bodies which would explain the dots throughout the cell. Another explanation is that the dots represent vesicles, with PVC17 either binding to, or being packaged into them.

The possibility of PVC17 binding to vesicles would make some sense for what has been seen from it so far; that it does not directly kill cells expressing it but instead slows their metabolic processes, and causes paralysis is nematodes. Also, the
previous protein structure predictions of PVC17 indicate the presence of transmembrane domains (Fig 4.2, A). This means it could be inserting into vesicle membranes and interfering with vesicle docking and transport. One toxin that also causes paralysis and binds vesicles is Botulism toxin which in one paper showed spotty binding throughout the cell similar to what was seen with PVC17 (Matsumura et al., 2015). However, from all the structural predictions done, never were there any similarities to a Botulism toxin, or any related toxins, seen. Suggesting that if they are having similar effects on cells, the mechanism of action or binding sites are very different.

4.3.4 Conclusions on PVC17 and future work

Due to time limitations on the PhD project, I was not able to fully ascertain the mechanism or targets of PVC17. However, I believe we have presented enough evidence here to form the hypothesis that PVC17 is an effector of PVC_Unit4 and is used to influence the host nematode. From the assays done, I hypothesise that PVC17 is delivered to host nematode muscle cells by the PVC_Unit4 and causes a decrease in cell metabolism while not directly killing the cells, causing paralysis. This then induces endotokia matricida and development of infective juveniles, allowing *Photorhabdus* to initiate re-symbiosis. Exactly how PVC17 may be doing this is still unclear, but two hypothesis I have are that it is either interfering with mitochondria, perhaps putting holes in the outer membrane or binding to vesicles and thus preventing cell transport.

However, more research needs to be done before any sure-fire theories can be made. Currently I think the best course of action would be to find what PVC17 is binding to in the cells. Further microscopy work could be done using PVC17 delivered into the cells, such as by BioPorter, instead of the cell expressing it. This would make it easier to see localisation. Another way would be to do a pulldown on the transfected cells, to see what PVC17 is binding. This was briefly attempted but not much progress was made.

The other aspect of research that needs to be done is finding the target cell of the PVC_Unit4, currently there is work being done on this by others researching the binding targets of the tail fibres and their possible interacting partners. With some
promising work showing that the PVC_Unit4 tail fibres bound to muscle related proteins such as myosin (Unpublished as of writing).

### 4.4 HvnA – a mediator of nematode behaviour?

One of the *P.luminescens* TT01 PVC_Unit4 effectors that has been of interest due to showing some expression during pervious RNaseq experiments done in the lab, was a homolog of HvnA from *Vibrio fischeri*, a squid symbiont. In *V.fischeri* HvnA, and a similar protein HvnB, act as NADases and were originally theorized to be linked to signalling between the bacteria and its squid host (Reich *et al.*, 1996; Reich *et al.*, 1997; Stabb *et al.*, 2001). However, there has yet to be any conclusive evidence of this.

While the leader sequence and loading experiments in PVC_Unit4 showed that HvnA does not seem to be normally loaded into the PVC, the fact that it showed expression suggests it may have some use in *Photorhabdus*. It was theorised that, like the proposed use of HvnA in *Vibrio fischeri*, that it may have a role in signalling between *Photorhabdus* and its symbiont nematode. In order to see if HvnA would have any effect on nematode behaviour, a food choice assay was set up using the nematode feed bacteria OP50, wild type TT01 and a mutant with the HvnA gene knocked out. Food choice assays work by growing bacterial colonies equidistant from the centre of a NGM plate, then adding nematodes to the centre of plate and allowing them to move freely. After a set amount of time, preferably short enough that eggs are not laid and develop, the amount of nematodes in each bacterial colony is counted. Nematodes use both chemical and mechanistic signals, such as how easy a bacteria is to eat, to decide if they want to stay and eat the bacteria colony. They will keep moving towards and through bacterial colonies until they find one which they like and then remain there.
Fig 4.5 | *C. elegans* food preference assay between OP50, TT01 and TT01 with the HvnA gene deleted.

A- Food assay, 3 assays were done comparing each of the bacteria against each other. Bacterial colonies were grown at opposite ends of a plate and nematodes were added to the centre. After 5 hours the number of nematodes in each bacterial colony were counted. The choice index, (No of Nematodes in Bacteria colony_1 - No of Nematodes in Bacteria colony_2) / (Total nematodes), was then calculated. A positive choice index represents more nematodes going to choice 1, while a negative choice index represents more nematodes going to choice 2. B- Set up of food assay on NGM plate. (2-way Anova, ** = < 0.01)

As would be expected the nematodes preferred the feeding bacteria OP50 over either of the TT01. However, when the nematodes had a choice between the TT01 with HvnA and the TT01 with the HvnA knocked out, there was an obvious preference for the TT01 missing the HvnA gene.

This first shows that the HvnA gene is being expressed in this situation, due to the loss of the gene having an effect, something it would not be expected to see if the HvnA was not expressed. It also suggests that the expression of HvnA somehow discourages feeding by *C. elegans*. This discouragement could either be mechanical or sensorial, for instance it could be that HvnA is either toxic or “bad tasting” and so
nematodes do not want to stay to feed more after eating some. Or HvnA could be a signalling molecule that discourages the *C. elegans* from ever even going near the bacteria.

HvnA being used to discourage feeding would make sense, as *Photorhabdus* wants to prevent nematodes that are not its symbiont from eating it or coming near the insect carcass. However, we do not know yet if this feeding discouragement also affects the host nematode or not. If it does then HvnA might also be a way of *Photorhabdus* preventing the host nematode from eating too much of the *Photorhabdus* biofilms when it is still establishing an infection in the host insect.

It is interesting to note that we have confirmed in a previous chapter that HvnA is not likely to be loaded into the PVC ([Chapter 3, Fig 3.4](#)). Thus, HvnA is most likely being either secreted, or is kept with the bacteria thus only released if eaten by a nematode. It also indicates that HvnA is expressed possibly independently of the PVC operon, suggesting this may be the case with other PVC operon effector regions.

Overall, this preliminary work on HvnA shows that there is a lot of complexity in what each PVC operons effector regions might do and when they may be expressed. It also opens up an avenue of research into discovering how *Photorhabdus* both regulates its own and other nematodes during its life cycle.
Chapter 5

Conclusions and Future perspectives

Throughout this thesis I have covered many aspects of *Photorhabdus* and how it infects its various hosts. This is one of the things that makes *Photorhabdus* such a fascinating bacteria to study, the fact that throughout its lifecycle it interacts with so many different organisms. From controlling the nematode symbiont and discouraging foreign nematodes, to infecting it’s hosts both insect and human, and finally stopping opportunistic bacteria and fungi from feeding upon its killed prey. It is probably because of all these different hosts and competitors that *Photorhabdus* has evolved such as complex and diverse system of secondary metabolites and toxins. Which is also what makes its such an excellent source of new pest and pathogen killing molecules.

Here I have shown how *Photorhabdus* interacts with some of these, and some insight to different strategies it has evolved for these diverse hosts. Interestingly we have seen that there is no universal strategy employed by *Photorhabdus*, with even strains of the same species differing. This is most clearly seen in the human pathogenic strains, which we now know are not limited to only *Photorhabdus asymbiotica*. The newly discovered human infective *P.luminescens* strain being highly aggressive against all mammalian cells. This is at odds with its *P.asymbiotica* cousins which seem to pick and choose which cells to invade, with the Australian strain seeming to avoid dendritic cells which play a very important role in early host detection. Yet both of these can seemingly infect humans suggesting that mammalian pathogenicity has evolved multiple times in the *Photorhabdus* lineage.

This diversity in approach to potential hosts even applies to stains within the *P.asymbiotica* themselves. It was known that the Australian and American strains acted very differently in response to macrophages, a difference we confirmed here.
But it would seem all the geographically isolated \textit{P.\textit{asymbiotica}} differ in their approach to mammalian cells. Even very closely related strains such as the Thai strain and Australian strains, where the Thai strain preferably infected hamster cells, and evaded all human immune cells, in contract to the Australian’s obvious preference for mammalian cells. This is not also forgetting the European stains which seem to be completely at odds with other \textit{P.\textit{asymbiotica}} by not being able to survive phagocytosis by macrophages and struggling to grow at the higher mammalian body temperatures its cousins are well known for being fine in. This all just goes to show that this genus is incredibly diverse and making assumptions, such as some species may or may not be able to infect humans, is both erroneous and potentially dangerous.

During this thesis I also investigated the novel delivery system of Photorhabdus the PVCs. Until now there had been little research into what the possible uses of the various different PVCs might be. But from the research I have done here we can see that they are activated in response to different organisms suggesting that each PVC operon has been adapted for use at different stages of the \textit{Photorhabdus} lifecycle.

One PVC I studied in depth was PVC\_\textit{Unit4} which we now know likely targets nematodes and causes endotokia matricida, an important stage of the nematode \textit{Photorhabdus} lifecycle. While further study is required to elucidate the exact targets of this PVC, and how its delivered effector PVC17 functions, the research presented here will hopefully lead to us having a better understanding of the complex symbiosis between the bacteria and its host. This may be of particular importance in the future for improving production of \textit{Heterorhabditis} \textit{Injective} Juveniles for use as bio-pesticides.

Finally I also illuminated our understanding of how effectors are loaded into the PVCs, a topic previously hypothesized upon but poorly understood. It would appear that the loading is the result of a N-terminal long disordered 50aa tail, we have dubbed the leader sequence. I also showed that these leader sequences can be switched between proteins to load proteins that are not native to the system. This finding will be vital for the future study of PVCs two reasons. Firstly it will allow us to get a better understanding of what maybe being loaded into each different PVC type, and what biological role in infection they may play. Secondary, and perhaps most importantly, it also brings us one step closer to be able to reengineer the PVCs as a new type of delivery system. One that it both cell specific and can deliver proteins.
directly into the cytosol. With a company already having been set up to bring this vision to life, hopefully we will in the future see PVCs being used not to hinder and cause harm to humans, but instead to help and cure them.

Alongside the scientific work I have performed, I also learned a lot about how to approach scientific research and if I was to start this PhD and project from the beginning again now, there would be many things I would change in my approaches with the benefit of hindsight.

Firstly, as a major part of my project involved analysis of DNA and genetic modification using previously created tools, I would always recommend checking plasmids and sequences personally. This is not to say do not trust your colleagues, but mistakes and mutations occur especially if a plasmid has been passed around for a long time. Thus, for both your own peace of mind and troubleshooting that will inevitably occur it is always best to sequence a new plasmid or strain, especially with how cheap and fast sequencing services are now. This allows a better understanding of what you are working with and to control for factors that may not have been obvious in the first instance.

This also applies to confirming for yourself information which may be understood to be true for most strains of your organism but has not been confirmed directly within any scientific literature. There are many cases where an initial researcher in the field made an observation which while never rigorously confirmed was passed down by word of mouth to eventually become gospel. By blinding believing statements without scientific proof, you not only train yourself to ignore fundamental scientific principles, but can also lead to overlooking interesting and fruitful lines of inquiry. It often does not take long to confirm or refute such apocryphal accounts as they are often formed when a field is in its infancy and scientific tools were not as developed. While these findings may not be ground breaking or even publishable, they will be of great use for both yourself and others in the field. Which brings me onto the next point, that not everything done during your PhD has to be publishable. While it would be wonderful for all your data and experiments to be impactful enough for publication, often unfortunately many lines of enquiry just don’t lead anywhere. However, during a PhD is one of the few times where you will be under the least pressure to only follow up on the things that are guaranteed to be publishable. A PhD is the perfect time in your career to simply be able to do scientific research for the
interests and fun of it and I would recommend any PhD student to while think about the publishability of results not make it in the only end goal of the experiments you design. As with the word of mouth, apocryphal stories, small experiments that others do not have the time to do may lead to opening up many new avenues of research. Thus it is a good idea to be a little risky in what experiments you plan even if it may not lead anywhere.

As well as learning about doing research and a PhD in general I also become very familiar with *Photorhabdus* and its many peculiarities, some which may trip up new researchers. As with many non-model organisms, partially those isolated directly from infections, working with *Photorhabdus* can be difficult and time consuming. One of the best examples of this was the American *P.asymbiotica* strain, ATCC 43939, which proved impossible to genetically modify despite numerous attempts to transform it using chemical, mechanical, electrical and conjugation methods. This brings up interesting discussions for future students working with *Photorhabdus* as to which strains to focus their research on. While it would be easiest to simply do all experiments in one or two lab strains, most likely the DJC *Photorhabdus.luminescens* and Kingscliff *Photorhabdus.asymbiotica* as the respective non-human and human infective models. This is not necessarily an appropriate response, as my research in this thesis has shown that each strain, even when very closely genetically related, does not act similar in an infection setting. Thus if investigating the human infective strains I think the best course of action is to always compare one of the Australian strains to one of the American strains, leaving out the European strains of *P.asymbiotica* as they do not seem to be clinically relevant. There are a number of isolated American *P.asymbiotica* strains other than the ATCC 43949, I personally did not use these as I had already gathered much of the initial data using the ATCC 43949 and would not have had time to redo all the experiments. However, for any students working with the American *P.asymbiotica* strains in the future I would defiantly recommend testing out some of the other isolates to see both how similar they are and if any are genetically amenable. This could potentially open up many new experiments for looking at the ways the Australian and American strains have diverged in their ability to infect mammals and insects.

Another thing to remember about *Photorhabdus* is that it displays phase-variance which while not heavily researched in *Photorhabdus*, this could lead to changes its
responses to host cells like with many other bacteria. This is most clearly seen when grown on an agar plate where different coloured colonies can been seen. Depending on the species and strain the colonies can range from pale to brownish-red, with some strains showing greater colour variation then others. Some strains also show a tendency for paler colonies to get darker over time though often there are some dark colonies from the start. This just goes to show how variable even clones grown in the same media can be with Photorhabdus and while these changes may have no effect on behaviour within an experiment, but it would still be a pertinent variable to control for. The easiest way to do this would be to choose the same colour and age of colony from a plate for liquid growth each time. Though if future students wish to further study into the nature of the Photorhabdus phase variance then there may be some interesting findings that will help the entire field.

There are many interesting avenues of study into Photorhabdus for future students and researchers. Possibly the most interesting is the host immune side and finding out what factors allow a strain of Photorhabdus to become “human infective”. In particular I am sure many fascinating discoveries will come out of the research into the newly discovered human infective variant of *P.luminesces* from Texas. Other research will hopefully go into the various effectors that *Photorhabdus* produces, as it is possible that some could have uses especially as novel insecticides.
Chapter 6

Materials and Methodology

All materials and methods used during the studies found in this thesis have been collected into this section as to make for easier reading. Where appropriate in other chapters brief descriptions of methodology have been repeated to provide clarity and context.

Table 6.1 | Bacterial and Cell lines used in this study.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>SOURCE</th>
<th>FEATURES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BACTERIA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5A (<em>ESCHERICHIA COLI</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5A_GFP</td>
<td>This Study</td>
<td>pBam7 (Constitutive GFP expression)</td>
</tr>
<tr>
<td>DH5A_LOXGFP</td>
<td>This Study</td>
<td>pET-28_LoxGFP (Plasmid with floxed GFP)</td>
</tr>
<tr>
<td>TT01 (DJC) (<em>P.LUMINESCENS SUBSP LAUMONDII</em>)</td>
<td>David Clarke Lab</td>
<td>Lab strain of Photorhabdus. (Isolated from soil nematode in Trinidad and Tobago)</td>
</tr>
<tr>
<td>TT01_GFP (<em>P.LUMINESCENS SUBSP LAUMONDII</em>)</td>
<td>This study</td>
<td>Lab strain of Photorhabdus. Growth range: &lt;30°C</td>
</tr>
<tr>
<td>TT01_UNIT4 (<em>P.LUMINESCENS SUBSP LAUMONDII</em>)</td>
<td>Waterfield lab</td>
<td>pCEP_Unit4 (PVC Unit4 operon structural genes under Arabinose inducible promoter)</td>
</tr>
<tr>
<td>TT01_UNIT4 [PBAD 17]</td>
<td>Waterfield lab</td>
<td>pCEP_Unit4</td>
</tr>
<tr>
<td>----------------------</td>
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<td>------------</td>
</tr>
<tr>
<td><em>(P. LUMINESCENS SUBSP LAUMONDII)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT01_UNIT4 [PBAD 8]</td>
<td>Waterfield lab</td>
<td>pCEP_Unit4</td>
</tr>
<tr>
<td><em>(P. LUMINESCENS SUBSP LAUMONDII)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT01_UNIT4 [PBAD 1+10]</td>
<td>Waterfield lab</td>
<td>pCEP_Unit4</td>
</tr>
<tr>
<td><em>(P. LUMINESCENS SUBSP LAUMONDII)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT01_UNIT4 [PBAD 9]</td>
<td>Waterfield lab</td>
<td>pCEP_Unit4</td>
</tr>
<tr>
<td><em>(P. LUMINESCENS SUBSP LAUMONDII)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT01_UNIT4 [PBAD 9+3]</td>
<td>Waterfield lab</td>
<td>pCEP_Unit4</td>
</tr>
<tr>
<td><em>(P. LUMINESCENS SUBSP LAUMONDII)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT01_UNIT4 [PBAD CRE]</td>
<td>This study</td>
<td>pCEP_Unit4</td>
</tr>
<tr>
<td><em>(P. LUMINESCENS SUBSP LAUMONDII)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT01_UNIT4 [PBAD YFAST]</td>
<td>This study</td>
<td>pCEP_Unit4</td>
</tr>
<tr>
<td><em>(P. LUMINESCENS SUBSP LAUMONDII)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT01_UNIT4_REPORTER</td>
<td>Waterfield lab</td>
<td>pAGAG_TT01_Unit4</td>
</tr>
<tr>
<td><em>(P. LUMINESCENS SUBSP LAUMONDII)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT01_CIF_REPORTER</td>
<td>Waterfield lab</td>
<td>pAGAG_TT01_Cif</td>
</tr>
</tbody>
</table>
## Materials and Methodology

<table>
<thead>
<tr>
<th><strong>(P. LUMINESCENS SUBSP. LAUMONDII)</strong></th>
<th>(GFP based expression reporter for activation of PVC_Cif operon)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TT01_LOPT_REPORTER</strong></td>
<td>Waterfield lab</td>
</tr>
<tr>
<td>(P. LUMINESCENS SUBSP. LAUMONDII)</td>
<td>(GFP based expression reporter for activation of PVC_LopT operon)</td>
</tr>
<tr>
<td><strong>TT01_UNIT1_REPORTER</strong></td>
<td>Waterfield lab</td>
</tr>
<tr>
<td>(P. LUMINESCENS SUBSP. LAUMONDII)</td>
<td>(GFP based expression reporter for activation of PVC_Unit1 operon)</td>
</tr>
<tr>
<td><strong>TEXAS</strong></td>
<td>Lisa Owens Lab</td>
</tr>
<tr>
<td>(P. LUMINESCENS)</td>
<td>(Clinical isolate from Human neonate in Texas, USA)</td>
</tr>
<tr>
<td><strong>TEXAS_GFP</strong></td>
<td>As above</td>
</tr>
<tr>
<td>(P. LUMINESCENS)</td>
<td>Growth range: &gt;30°C</td>
</tr>
<tr>
<td><strong>JUN</strong></td>
<td>(Isolated from soil nematode in the Netherlands)</td>
</tr>
<tr>
<td>(P. ASYMBIOTICA SUBSP.)</td>
<td>Growth range: &gt;30°C but limited.</td>
</tr>
<tr>
<td><strong>HIT</strong></td>
<td>(Isolated from soil nematode in Sweden)</td>
</tr>
<tr>
<td>(P. ASYMBIOTICA SUBSP.)</td>
<td></td>
</tr>
<tr>
<td><strong>PB68</strong></td>
<td>(Isolated from soil nematode in Thailand)</td>
</tr>
<tr>
<td>(P. ASYMBIOTICA SUBSP. AUSTRALIS)</td>
<td></td>
</tr>
<tr>
<td><strong>PB68_GFP</strong></td>
<td>Waterfield lab</td>
</tr>
<tr>
<td>(P. ASYMBIOTICA SUBSP. AUSTRALIS)</td>
<td>pBam7</td>
</tr>
<tr>
<td><strong>KINGSCLIFF</strong></td>
<td><strong>(P. ASYMBIOTICA SUBSP AUSTRALIS)</strong></td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td><strong>KINGSCLIFF_GFP</strong></td>
<td><strong>(P. ASYMBIOTICA SUBSP AUSTRALIS)</strong></td>
</tr>
<tr>
<td><strong>ATCC 43949</strong></td>
<td><strong>(P. ASYMBIOTICA SUBSP ASYMBIOTICA)</strong></td>
</tr>
</tbody>
</table>

**EUKARYOTIC CELL LINES**

<table>
<thead>
<tr>
<th><strong>HEK 293T</strong></th>
<th><strong>(HOMO SAPIENS)</strong></th>
<th>ATCC Human cell line isolated from embryonic kidney.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHO</strong></td>
<td><strong>(CRICETULUS GRISEUS)</strong></td>
<td>ATCC Chinese hamster cell line isolated from ovaries.</td>
</tr>
<tr>
<td><strong>THP-1</strong></td>
<td><strong>(HOMO SAPIENS)</strong></td>
<td>ATCC Human monocyte cell line. Can be differentiated into macrophages with addition of PMA.</td>
</tr>
<tr>
<td><strong>S2</strong></td>
<td><strong>(DROSOPHILA MELANOGASTER)</strong></td>
<td>ATCC Insect cell line derived from Drosophila embryo.</td>
</tr>
</tbody>
</table>

**NEMATODE LINES**

| **N2** | **(CAENORHABDITIS ELEGANS)** | Pires da Silva Lab WT lab strain of C.elegans. Isolated from mushroom compost near Bristol, England by L.N. Staniland. |
6.1 Bacterial protocols

6.1.1 Bacterial culturing

Routine growth of bacterial strains was carried out in standard lysogeny broth (LB), with shaking at ~180rpm unless otherwise stated. *E. coli* cultures were grown at 37°C, while *Photorhabdus* were grown at either 28°C, or 37°C, as required for the experiment. It should be noted that *P. luminescens* were normally grown 28°C, due to having growth arrest above 34°C. However, for some experiments a sub-culture of *P. luminescens* would be incubated at 37°C, and while the bacteria remained viable, no further growth would occur. For growth on solid media, LB was supplemented with 1.5% agar, 0.1% sodium pyruvate and any relevant antibiotics; plates were incubated in the dark.

6.1.2 Antibiotics

Various antibiotics were used during these studies for selection of transformed strains, the concentrations of which can be found in the table below (Table 6.2). It was observed that the *P. asymbiotica* strains had a natural resistance to ampicillin, so this was avoided for use a selective agent in plasmids where possible.

<table>
<thead>
<tr>
<th>USE</th>
<th>SUPPLEMENT</th>
<th>WORKING CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANTIBIOTICS</strong></td>
<td>Gentamycin</td>
<td>10 µg/mL-¹</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>100 µg/mL-¹</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>25 µg/mL-¹</td>
</tr>
<tr>
<td></td>
<td>Kanamycin</td>
<td>25 µg/mL-¹</td>
</tr>
<tr>
<td></td>
<td>PenStrep</td>
<td>50 U/mL-¹</td>
</tr>
<tr>
<td><strong>PROMOTOR</strong></td>
<td>Arabinose</td>
<td>0.2% (w/v)</td>
</tr>
<tr>
<td><strong>CONTROL</strong></td>
<td>Glucose</td>
<td>0.2% (w/v)</td>
</tr>
</tbody>
</table>
6.1.3 Transformations

All transformations of *E.coli* were done using the DH5a strain which was made chemically component to allow for introduction of plasmids through heat shock. Most of the transformations into *E.coli* were simply for the creation and replication of plasmids, due to its efficiency of plasmid uptake. *Photorhabdus* on the other hand does not seem to readily produce chemically competent cells. Thus they were transformed using electroporation instead.

6.1.4 Electroporation of Photorhabdus

It has been found that unlike some cells, *Photorhabdus* loses competency when frozen, thus electrically competent cells must be created on the same day as the transformation. On the day of the transformation, 100ml of LB would be sub-cultured with 4ml of *Photorhabdus* grown overnight. This would be left to grow, as per normal *Photorhabdus* growth conditions, till ∼OD 0.2 (roughly 4 hours) then be placed on ice for 90 mins. The bacteria would then be pelleted at 4000xg, 10mins, 4°C then resuspended in 100ml of ice-cold SH buffer (5% [wt/vol] sucrose, 100 mM HEPES). The bacteria were the pelleted and resuspended in increasing smaller volumes of SH buffer; 50ml, 1.6ml, before being finally resuspended in 160µl of SH buffer. To pre-chilled 2mm Electroporation cuvettes, 40µl of the cells were added. While still on ice 4 µl of DNA was added to the cells and electroporation was carried out using the following parameters: 2.5 kV, 25 µF, and 200 Ω. After electroporation 1 mL of LB was quickly added to the bacteria and incubated in normal *Photorhabdus* growth conditions for 1h, after which they were plated onto LB plates with appropriate antibiotics.

6.1.5 CFU/OD growth curves

To calculate the CFU [Colony Forming Units] for a certain OD [Optical Density] of *Photorhabdus*, the bacteria were diluted 1/100 from overnight cultures. Every hour the OD was measured, and a mL of the culture was taken and serially diluted from
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1/10 to 1/10000. Six 10ul drops of these dilutions were then pipetted onto a LB agar plate, divided into four. These were left dry and then incubated overnight at 28°C. The next day the number of colonies per drop were counted and the CFU calculated.

6.1.6 RNA purification

Before processing of samples, the work area and equipment was cleaned using RNA-ZAP (Thermo-Fisher). RNA to be used for both sequencing and RT-PCR [Reverse Transcription-PCR], was purified from overnight cultures of *P. luminescens* sub-cultured into liquid LB medium and grown at either 28°C or 37°C. Cultures were harvested after 24 hours by centrifugation and the pellets stored at −80°C until further processing. Total RNA was extracted from the pellets using the MiRNeasy Mini Kit (Qiagen) with slight modifications to the lysis of the bacteria. The pellets were resuspended in 1 mL of quizol lysis reagent, vortexed for 3 mins and incubated at room temperature for 5 mins. After the incubation 200ul of 100% chloroform was added and incubated for a further 3 mins, before being centrifuged at 12000xg , 15 mins, 4°C. This produced a biphasic mixture and the top, clear, aqueous phase was taken. From here on the manufacturer’s specifications were followed with a final elution volume of 2x 40 µL.

The sample was then treated with Turbo-DNase (Ambion) for 50 mins, plus the addition of 2 µL the RNase inhibitor Superase.in (Thermo-scientific). Finally, the RNA sample was cleaned of left-over enzymes and buffers using the ReliaPrep RNA Clean-up and Concentration kit (Promega).

The RNA samples were then either used for RT-PCR or were sent to Novogene for RNA sequencing.

6.1.7 RT-PCR

RNA samples that were to be used for identification of qualitative gene expression rather than relative expression were converted to cDNA using ThermoFisher SuperScript™ IV Reverse Transcriptase kit, as per manufacturer’s instructions. The cDNA generated was then used for PCR as detailed in the Q5 polymerase section.
6.1.8 RNA Sequencing

Sequencing of RNA samples and library construction was done by Novogene. For each sample 10 million 150bp paired reads were done.

6.1.9 RNA_SEQ data analysis

RNA_seq libraries of raw reads were received from Novogene as plain fasta files. All further analysis of the data was done in house by us. Processing of data was done on university of Warwick linux server clusters.

Raw reads were first aligned to our *Photorhabdus luminescens* Texas strain reference genome, for which an index file had been created using Hisat2, using Hisat2 with standard settings, which generated the alignment files in .sam format. The .sam alignment files were then converted to .bam files and indexed using Samtools. The number of reads associated with each gene in the .bam alignment files were then counted using LiBiNorm with standard settings using the annotation file we created for the Texas strain genome. A basic version for the code used can be found below (Fig 5.1)

```
#!/bin/bash
hisat2 -x <Reference genome> -1 <Raw Read file in Fatsta format> -S <Output sam file>
samtools view -bS <Sam file from Hisat2> | samtools sort - <Output Bam file>
samtools index <Bam alignment file>
LiBiNorm count -c <Output file name and directory> -z -r pos -i gene_id -s reverse <Bam alignment file>
<Genome gene annotation file>
```

Fig 6.1 | .Sh script used for processing raw RNA-seq reads into gene count .txt files.

Script was written in bash and run on a Unix system. <> indicates where the path to the indicated file would be placed.

Further processing and analysis of the data was done in R from this point on. The DESeq package in R was used to do the default analysis of the raw counts, producing log fold changes between the two conditions, and calculating P-values. This output
was then used to generate PCA and volcano plots for accessing replicate clustering, and the final heatmap showing differentially expressed genes.

6.1.10 PVC reporter quantification

Overnight cultures of the *Photorhabdus* PVC reporters grown at either 28°C or 37°C, were added to PBS with or without *C.elegans*. The bacteria and nematodes were left together for around 2 hours at which point the nematodes were separated from the bacteria by centrifugation. The bacteria were then deposited on uniform agarose pads and imaged at 100x magnification, using set microscope settings.

Quantification was done using Image J. Reporter images were split into separate channels, then the phase contrast channel was made binary, and edges were detected using Image J. Individual cells were identified using the analyse particle function and the mean GFP signal across the cell was measured and recorded (Fig 6.2).
Fig 6.2 | Image J macro used to quantify per cell, PVC reporter GFP expression.

6.1.11 Electron Microscope Negative staining

Protein samples, mainly purified PVCs, in TM buffer (20mM Tris-HCL, 20mM MgCl₂), were directly used for EM Negative staining, while bacterial cells were first
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fixed in 0.4% Glutaraldehyde, for 15 mins. Around 5uL of sample was placed onto a
glow discharged, formvar/carbon mesh grid and incubated for 1 min, before excess
liquid was blotted off. The grids were then stained with ~5ul 2% uranyl acetate, for 1
min, 4 times each time blotting away excess liquid. Samples were then visualised by
transmission electron microscopy using the Jeol 2100Plus TEM. Length of PVCs was
measured using the Image J software.

6.1.12 PVC purification

A large proportion of this project was focused on the PVCs produced by *Photorhabdus*. Thus, in order to study them a way of reliably producing, purifying and editing their
payloads had to be devised. To this extent, a dual plasmid system of ruse in
Photorhabdus was used. The first plasmid contains the PVC operon structural genes,
PVC1-PVC16, while the second contains a leader-protein of interest fusion tagged
with a Myc tag. Both of these plasmids are under the control of the AraBAD arabinose
inducible promoter, allowing both to be induced at the same time.

6.1.13 Harvesting PVCs from *Photorhabdus*

*Photorhabdus* cultures containing an arabinose inducible PVC operon and/or plasmid
with an arabinose inducible leader-effector-tag construct, were grown overnight then
the next day sub-cultured 1:100 in 600ml LB and grown for 4 hours. The production
of the PVC_Unit4 was then induced by addition of 0.2% Arabinose and the cultures
were grown for a further 24h. The next day, the cultures were centrifuged at 8000xg
for 20mins at 4°C, to the pellet cells, the supernatant was taken and 0.25 U/ml of
DNAsel was added and incubated for 40 mins, at room temp. After this a PEG
precipitation was done, by adding 0.5M NaCl and 80g/L of PEG6000 to the
supernatant, then incubating O/N, while stirring at ~4°C. Once the PEG was fully
dissolved, the solution was spun at 8000xg, 20 mins and the pellet was resuspended
in 5mL of Tm Buffer (20mM Tris-HCL, 20mM MgCl₂), then left shaking at room
temperature for 2h. After the incubation the solution is spun at 13000xg, 10 mins and
the supernatant can be stored at 4°C. Further purification as done using a Caesium
Chloride gradient.
6.1.14 Caesium Chloride purification

Once PVCs had been harvested from cell cultures, the first major purification step is a CsCl gradient. The gradient was set up in a 14ml transparent ultracentrifuge tube, using first 2ml of 1.7 density CsCl solution, then 3ml of 1.5 density, then 3ml of 1.45 density being careful not to disturb the previous layer when adding the next on top. Finally the PVC solution was added to the top of the gradient, around 5ml. The gradient was spun at 35,000rpm, 2 hours, 4°C. After centrifugation there should be a single “halo”, normally the PVCs would be located in 1 ml samples above or in the halo. The 1 ml samples from the caesium chloride gradient were then dialysed into TM buffer through a 100k Amicon column.

6.1.15 Monolithic column purification

The final stage of purification was done using a CIMmultus™ QA-1 (2um) Advanced Composite Column (BiaSeparations). Before loading the sample was dialysed into Loading buffer (20mM Tris-HCl, 8mM MgSO₄). The column is first equalized before loading the sample, this was done by passing 5 Column Volumes (CVs) of 20% ethanol through, followed by 5 CVs of distilled water, 10 CVs of Loading buffer, 10 CVs of Elution buffer (20mM Tris-HCl, 8mM MgSO₄, 3M NaCl, pH 7.4), and finally 10 CVs of Loading buffer. The sample was then loaded onto the column, being allowed to pass through multiple times for around 20 mins/ 10 CVs, before being fully passed through and the flow through being disposed of. The column was washed with 15ml of Wash buffer (20mM Tris-HCl, 8mM MgSO₄, 1.5M NaCl, pH 7.4), with the flow through being collected in case any protein was eluted. Finally the protein was eluted by passing through ~15ml of Elution buffer, with the eluent being collected and stored at 4°C. To store the column, it was washed with 10 CVs of elution buffer, followed by 10 CVs of water and 10 CVs of 20% Ethanol being careful to make sure the column did not run dry as it was disconnected.
6.1.16 SDS-PAGE

Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis was used routinely to estimate the presence and size of purified proteins. Premade gels acquired from Biorad, specifically Biorad TGX Mini-protean 4-15%, were used in all cases and were run as per the manufacturer’s specifications. Briefly SDS-PAGE gels would be set up in the Biorad Mini-PROTEAN Tetra Cell cassette with SDS-PAGE running buffer added to both the tank and the cassette. Samples were denatured prior to loading using a 3:1 ratio of Biorad Laemmli Sample Buffer, with β-mercaptoethanol added at a sample buffer: β-mercaptoethanol 9:1 ratio just before use, and boiled in the sample buffer for 10 mins. After denaturing samples were loaded into the SDS-PAGE gel and run at 200V for around 35 mins, or until dye had reached end of gel. Gels were removed from the cassette and either used straight for western blotting or stained. Staining was performed by shaking overnight in a Coomassie blue solution, after which the gels were observed using the Biorad Chemdoc imaging system.

- SDS-PAGE Running buffer (in 5L MQ water)
  - 15g Tris
  - 72g Glycine
  - 5g SDS

6.1.17 Western blot

Gels were run as described above and after electrophoresis were washed in running buffer briefly. The protein bands were transferred to activated polyvinylidene fluoride (PVDF) membranes via the Biorad TransBlot Turbo electroblotter, using the 7 minute turbo protocol. Washing, antibody binding was done using the Pierce Fast Western Blot kit from Thermo according to the manufacturer’s instructions.

Blots were stained with mouse anti-Myc tag antibodies obtained from Abcam for all experiments using Myc-tagged proteins. Secondary antibody was an anti-mouse conjugated to HRP (HorseRadish Peroxidase), which allowed chemiluminescent visualisation of stained proteins with the addition of luminol. Blots were again visualised using the Biorad Chemdoc imaging system, with auto-exposure settings.
6.2 Eukaryotic cell protocols

6.2.1 Maintenance of cultured mammalian and insect cells

Maintenance of Mammalian cells was done at 37°C with a humidified atmosphere of 10% CO₂ with no shaking. HEK 293T and CHO cells were grown in DMEM media supplemented with 10% FBS, L-glutamine and non-essential amino acids. While THP-1 cells were grown in RPMI supplemented with 10% FBS, L-glutamine and non-essential amino acids. Cells were generally split at around 70-80% confluence.

Insectile Drosophila melanogaster derived S2 cells were maintained at 28°C in normal atmospheric conditions with no shaking. The cells were grown in Schneider’s media with media being replaced every 3 days, and cells split at around 80% confluency.

6.2.2 Transfection

All transfections were done in HEK 293T cells using plasmids optimised for lipid-based transfection. The plasmids had a GFP reporter alongside the protein of interest, which was expressed to allow identification of transfected cells. Cells were seeded and grown for 24 hours, at 37°C. After 24 hours of growth, the media on the cells was changed to Opti-mem and the cell were allowed to recover for 30 mins, before being transfected with the plasmids PD_Con, PD_17 or PD_Pnf using Lipofectamine 3000 [ThemoFisher], following the protocol set out by the manufacturer. Cells were checked for GFP expression at roughly 12, 24 and 48 hours after transfection. Experiments using the transfected cells aimed to have at least 60% of the cells expressing GFP, and thus the protein of interest.

6.2.3 HEK 293T Protein Extraction

Hek293T cells were seeded in a 10cm cell culture dish and transfected as described above. ~24 hours post transfection the cells were then harvested using a cell scraper
and pelleted at 1500xg for 5mins at 4°C. The cells were washed in ice-cold PBS and then resuspended in ice-cold lysis buffer at 3x the volume of the pellet and pipetted until the pellet was fully lysed, around 5-10 mins. The lysed cells were incubated on ice for ~30 mins, before being centrifuged at 15,000xg for 10 mins at 4°C. Supernatant was transferred to a new tube and samples were stored at -80°C.

### 6.2.4 MTS assay

HEK 293T cells were seeded into a 96 well plate at 2x10^5 cells and Transfected using Lipofectamine 3000, as described above. 20 Hours post-transfection the cells were measured for viability using an MTS assay [Abcam] following the manufacturer’s protocol.

### 6.2.5 Excelligence assay (Cell adhesion)

HEK 293T cells were seeded into an Excelligence 96 well plate at 1.5x10^4 cells. These cells were grown and transfected as in the MTS assay, but during growth phases were docked into the Excelligence plate reader. Once transfected, the cells were left docked into the Excelligence plate reader for 24 hours with a reading taken every 1 hour.

### 6.2.6 Infection assays

Mammalian cells were seeded at 2x10^5 onto sterilized glass coverslips in 24-well plates, in 500ul of their respective cell media without antibiotics and left to adhere overnight at 37°C with a humidified atmosphere of 10% CO₂. In the case of THP-1 cells, 100nM of PMA was added after seeding 48 hours prior to the addition of bacteria to induce activation and adherence. Once the cells had fully adhered, bacteria grown to mid-log phase (OD 0.4-0.6) and resuspended in the cell media, were added to each well at a MOI of 1:50 (~1x10^7). Bacteria infection of cells was allowed to carry out for 2 hours, at 37°C with a humidified atmosphere of 10% CO₂. Afterwards 200μg/ml of gentamicin was added to each well for 1h to kill any non-internalised bacteria. After incubation with the gentamycin, the media was removed and the cells were washed 3x with PBS, finally being resuspended in 100ul of 1% Triton-X-100 (in
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PBS) for 10mins at room temperature, to lyse the cells and release any internalised bacteria. 900µL of LB was then added and the cells were homogenised by pipetting. CFU counts were then done for each well as described above in the CFU/OD assay.

For infection assays of the Insect S2 cells, the same procedure was carried out as above, but all incubation and growth steps of the cells was done at 28°C in normal atmospheric conditions.

6.2.7 Inhibition of phagocytosis

THP-1 cells were seeded and activated as specified above for an infection assay, but 2 hours prior to addition of bacteria, 1µg/mL of Cytochalasin D was added to the THP-1 cells. Afterwards the infection assay was carried out as normal.

6.2.8 Imaging of infected eukaryotic cells

Cells were grown and infected as with the infection assays above, but after the gentamycin incubation and washing steps, the cells were not lysed. Instead, the coverslips with the attached cells were transferred to new wells and fixed with 2% PFA (Paraformaldehyde) for 30 mins. After fixation, the cells were washed 3x with PBS and then had DNA stained using 300nM of DAPI for 5 mins. After staining, cells were again washed 3x with PBS and the coverslips were placed cells down onto a glass slide and sealed with clear nail varnish. Cells were then imaged using a Leica DMi8 fluorescence Microscope.

6.2.9 Generation of 3D model of imaged cells

Inbuilt microscope “Software” was used to generate estimated 3D models of cells using a Z-stack of images. Around 100 images, in the z plane were used to generate each 3D image.
6.2.10 PBMC extraction and purification

Fresh whole human blood was diluted at a 1:1 ratio with PBS-EDTA and layered onto 12.5ml of ficoll medium in a 50 ml tube, making sure the blood and ficoll layer to not mix. The tube containing the ficoll/blood layers was spun at 400xg, room temperature for 30 mins, with acceleration and deceleration set at the minimum. There should now be a layer of PBMCs between the plasma and ficoll, which was carefully removed being sure to not take any of the other layers. The PBMCs were washed in PBS-EDTA and spun again at 300xg, room temperature, for 5 mins. The supernatant was removed and the pellet containing the PBMCs was resuspended in 20mL PBS-EDTA. The cells were then counted using a haemocytometer. PBMCs were then either used straight away or resuspended in RPMI at a dilution of around 20 million cells per mL for freezing. PBMCs for freezing after being resuspended had 2X freezing media added at 1:1 ratio and were aliquoted into cryotubes. The tubes were then either stored at -80°C in Mr. Frosty freezing containers or in liquid nitrogen for long-term storage.

- 2X Freezing media
  - 20% DMSO in Foetal Bovine serum (FBS).

6.2.11 Flow cytometry of infected PBMCs

PBMCs were defrosted at 37°C and resuspended in RPMI supplemented with 10% FBS and no antibiotics. Around 1x10⁶ PBMCs were seeded in wells for each condition/bacteria including wells for the controls of no bacteria and unstained PBMCs. Bacteria, grown to mid log phase at either 28°C or 37°C, washed with PBS and resuspended in RPMI were added to the PBMCs at a MOI of 1:50. For the dead bacteria conditions, aliquots of the bacterial cultures were taken and killed with a mixture of 10ul/ml penstrep and 2ul/ml chloramphenicol for 1 hour prior to washing and addition to PBMCs. The bacteria and PBMCs were incubated together for 2 hours at 37°C, after which 200µg/mL of gentamycin was added for a further 1-hour incubation. After the gentamycin incubation the PBMCs were washed 3x in RPMI, then resuspended in 200ul of ice cold BSB and transferred to a 96-well V-bottom plate.
PBMCs were spun in the plate at 400xg for 5 mins, then resuspended in 50µL of near-IR L/D stain (in PBS) and incubated in the dark on ice for 10 mins. PBMCs were washed in staining buffer and then resuspended in 25µL of antibody cocktail (Table 4.1) and incubated in the dark on ice for 30 mins. PBMCs were then washed in 125µL BSB, then 150µL PBS and finally resuspended in 200µL Staining buffer.

Stained PBMC samples were analysed by flow cytometry using a Cytek Aurora, with compensations having been previously generated for each antibody-fluorescence channel. The GFP signal from the bacteria was detected using the FITC channel on the instrument, with compensations for this channel being generated using PBMCs stained with a GFP conjugated antibody. This antibody was not included in the final experimental panel.

**Table 6.3 | Antibodies used for identification of PBMCs in flow cytometry.**

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<tr>
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<td>L/D</td>
<td>Near-IR</td>
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### 6.3 Nematode protocols

#### 6.3.1 Maintenance of Nematode strains

The lab C.elegans strain N2 was used for all experiments, stocks were maintained at room temperature on Nematode Growth Media (NGM) plates (15g Agar, 2.4g NaCl, 2.72g KH2PO4, 0.8ml 1M CaCl2, 0.8ml 1M Cholesterol, 1M MgSO4, 1L water) seeded
with 100ul O/N, E.coli strain OP50. Worms are transferred to new plates from old plates with a metal wire, or by taking a chunk of the old agar, containing nematodes, and placing on a new seeded plate.

### 6.3.2 Synchronization of Nematodes

To obtain synchronized cultures, where all nematodes are at the same developmental stage, plates with a large amount of gravid adults were washed with 1mL M9 buffer to collect nematodes. The nematodes, in a 15ml tube, were pelleted at 400xg for 2 mins and washed with 15 mL of M9 buffer three times. After the final pelleting, Bleaching solution (2.75 mL water, 1.25 mL 1M Sodium Hydroxide, 1 mL 4% Sodium Hypochlorite) was added and the nematodes were viscously shaken for ~8 mins, or until no intact adults could be seen. The bleaching reaction was stopped by filling the tube with M9 buffer, followed by 3 washes with 15 ml M9 buffer, spinning at 400xg for 1 min. Eggs were pelleted after the final wash by spinning at 400xg for 5 mins, these were finally resuspended in 1 mL of M9 buffer and allowed to hatch O/N with slight shaking. The hatched larvae were then transferred to a new NGM plates seeded with OP50 bacteria.

### 6.3.3 Toxin effect assay

Nematodes were washed off growth plates with 1ml of M9 buffer [3g KH2PO4, 6g Na2HPO4, 5g NaCl, 1ml 1M MgSO4, in 1L of MQ water] and transferred to a 1.5 mL Eppendorf tube. The nematodes were spun at 200xg for 1 min, the supernatant was removed and the nematodes were washed with M9 buffer 3 times before being resuspended in 1.5ml M9 buffer. 300ul aliquots of the nematodes were added to wells in a 24 well plate, then 30ul of PVC purifications or buffer was added to nematodes, and effects were observed using a dissecting microscope at 1, 2, 6, 12 and 24 hours post.
6.3.4 Food choice assay

Nematodes were collected and washed as described above. NGM plates were seeded with 20ul of O/N cultures of the control bacteria and the test bacteria on either side of the plate, these were grown O/N at 28°C. Around 100 nematodes were transferred to the centre of the food choice plate between the two bacterial colonies, and left at room temperature for 3 hours. After 3 hours the plates were placed on ice for a short time to slow nematode movement and using a dissection microscope the number of nematodes in either bacterial colony were counted.

6.4 Molecular Techniques

All DNA, plasmids and primers were stored at -20 °C and kept on ice when in use. RNA was stored at -80 °C and separated into aliquots, to prevent repeated freeze-thawing.

6.4.1 List of plasmids

Table 6.4 Plasmids used in this study.

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<thead>
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<th>SOURCE</th>
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<tr>
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<th>Effector Construct</th>
<th>Description</th>
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<td><strong>PAGAG_TT01_CIF</strong></td>
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<tr>
<td><strong>PBAM7</strong></td>
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**EFFECTOR CONSTRUCTS**

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<th>Construct</th>
<th>Description</th>
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<td>Leaderless HvnA::Myc</td>
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<tr>
<td>PBAD CRE</td>
<td>Cre::Myc</td>
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</table>
6.4.2 Purification of genomic DNA

Bacterial genomic DNA (gDNA) was isolated using the Qiagen “Blood and Tissue” extraction kit, with the following the manufacturers recommendations, with a few changes: 5 mL of overnight culture was set up in appropriate conditions and centrifuged at 8000 xg. From here the protocol proceeds directly to the manufacturers step 2 and follows the standard procedure until elution. Elution was conducted in 2 x 17.5 µL washes in H2O.

6.4.3 Purification of Plasmids

Plasmids were purified from bacterial strains using the Qiagen Miniprep Spin Kit as per the manufacturer’s instructions. 5 mL overnight cultures were used, and the final elution was conducted in 2x 20 µL washes using molecular grade water.
6.4.4 Taq and colony PCR

When sequence reliability was not a concern such as during colony PCR, or for very small amplicons Taq polymerase was used. PCR parameters and relative volumes of reagents were done per manufacturers recommendations.

For rapid screening of possible transformants, a small amount of individual bacterial colonies were taken from plates and boiled in 50 µL water at 95°C for 5 mins. This was then used as the template for the colony PCR.

6.4.5 Q5 polymerase PCR

For all situations when sequence fidelity was of importance, such as cloning and amplification of genes from gDNA, the high-fidelity enzyme Q5 was used, obtained from New England Bio-labs.

As with the Taq polymerase PCR parameters and relative volumes of reagents were done per manufacturers recommendations.

6.4.6 DNA clean-up after PCR, restriction digest and Gel electrophoresis

After PCR, restriction digestion and Gel electrophoresis, it was required to clean up the sample to remove any leftover buffers or enzymes that could be contaminating the DNA. This was achieved by using the GE Healthcare PCR DNA and Gel Band Purification Kit, as per the manufacturers instructions.

6.4.7 Agarose gel electrophoresis

Quantification and identification of DNA fragment sizes was done using Agarose gel electrophoresis. 1% gels (w/v) were added to 1x Tris-Acetate-EDTA (TAE) buffer and the mixture was microwaved until the agarose had melted. The solution was allowed to cool slightly then SYBER-safe gel stain was added at a dilution of 1:10000.
The agarose was poured into a mould and left to set for around 20 mins after which the gel was loaded with the sample, alongside the GeneRuler 1kb Plus Ladder from Thermo-Fisher, and the gel was run at 110 V for around 40-45 mins. Visualization of the gel was done using the Bio-Rad ChemiDoc MP Imaging System.

- 50X Stock TAE Buffer (pH 8.2-8.4):
  - 2 M Tris Base (C4H11NO3)
  - 57.1 mL Glacial Acetic acid (CH3COOH)
  - 50 mM EDTA (C10H16N2O8)

### 6.4.8 Gel extraction

Gel extraction was used to isolate DNA fragments from mixed fragment populations for further use later, such as after PCR, or restriction digest. Bands were visualised using a blue light trans-illuminator and excised from the gel with a scalpel. The excised band was then processed using the PCR clean-up kit as specified in Section 5.4.6

### 6.4.9 Restriction digest

Where possible all cutting out of DNA fragments was done using two different restriction enzymes, this was to increase the efficiency of the final ligation by guaranteeing the fragment went into the plasmid the correct way. All restriction digestion enzymes were acquired from NEB, and were used according to the manufacturer’s instructions, using the buffer which gave the highest combined efficiency of the two enzymes. Digestions were left for 3 hours at 37°C.

### 6.4.10 Ligation

Ligation were executed using T4 DNA ligase from NEB as specified by the manufacturer at either room temperature for 1 hour or overnight at 4°C. A 1:3 insert:vector ratio was used, with the exact molar concentrations being calculated
using the NEB’s own ligation calculator. After ligation, the enzymes were heat denatured and the reaction was used directly in transformations.

6.4.11 Primers

All primers used in these experiments were ordered from IDT.

Table 6.5 | Primers used in this study.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE</th>
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<td>GFP_LOXP_FW</td>
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### PVC OPERON EXPRESSION

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6.5 Miscellaneous protocols

6.5.1 Statistical tests

All statistical tests along with P-values used are found in the figure legends of the appropriate figure. Unless otherwise stated all tests were done in prism using standard settings.

6.5.2 Protein homolog detection

Detection of possible homologs for proteins of unknown function was done using HHpred from the MPI bioinformatics toolkit using standard settings.

6.5.3 Protein identity calculation

The shared amino acid identity between protein homologs was calculated using the Clustal Omega software from EMBL-EBI using standard settings.

6.5.4 Protein structure prediction models

Predicted structural models for proteins were generated from amino acid sequences using the AlphaFold Colab google notebook (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb) using default settings.

PVC15_RV  CTGCTGTTGTGGAGTATTT
PVC16_FW  TCCAGCAACCCGTTCATTATTA
PVC16_RV  CTGCCAGAGCTTGATTGATTTG
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Supplementary figures

**SuppFig 1 | List of differentially expressed genes when growth temperature of \textit{P. asymbiotica} PB68 is increased from, 28°C to 37°C.**

Data was generated from RNA_seq using RNA extracted from \textit{P. asymbiotica} PB68 grown at either 28°C to 37°C. RNA was extracted when cultures were at mid-log phase, roughly OD 0.5. RNA samples were library prepped and sequenced at a read depth of 10 million, 150bp paired reads. Reads were then aligned with Hisat2 and counted using LiBiNorm. This was repeated 3 times using a freshly picked colony, generating 3 biological repeats. Differentially expressed genes were those with a P-adjusted value (PADJ) of <0.05. Differentially expressed genes had their locus tag and gene product recorded (Product). In the case of hypothetical genes with no known function, they were analysed using HHpred to look for homologs, with the best match being recorded (in brackets). Differentially expressed genes were then grouped by the general role they play in the cell (Category). For more information see \textit{Fig 2.9, Section 2.9.}

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SuppFig 2 | Example of *P. symbiotica* PB68 division inside THP-1 cells after longer incubation time at 28°C.

As with other infection assays shown in the main text, bacteria were grown prior to assay at 28°C and then added to differentiated THP-1 cells. However, in this case longer infection and incubation time was used, 6h instead of 2h. This lead to an example of where the bacteria has started to divide inside the THP-1 cells that they have entered.