Photorhabdus asymbiotica as an Insect and Human Pathogen

Alexia Hapeshi and Nick R. Waterfield

Abstract *Photorhabdus asymbiotica* is a species of bacterium that is pathogenic to humans whilst retaining the ability to infect insect hosts. Currently, there are two recognised subspecies, *P. asymbiotica* subsp. *asymbiotica* and *P. asymbiotica* subsp. *australis* with strains isolated from various locations in the USA, Australia, Thailand, Nepal and Europe. Like other species of *Photorhabdus*, *P. asymbiotica* subsp. *australis* was shown to form a symbiotic relationship with a *Heterorhabditis* nematode. In contrast to most strains of *Photorhabdus luminescens*, *P. asymbiotica* can grow at 37 °C and this is a defining factor in its ability to cause human disease. Insights into other adaptations it has undergone that have enabled host switching to occur have come from whole genome sequencing and transcriptomic studies. *P. asymbiotica* has a smaller genome compared to *P. luminenscens* with a lower diversity of insecticidal toxins. However, it has acquired plasmids and several pathogenicity islands in its genome. These encode genes with similarity to effectors or systems found in other known human pathogens such as *Salmonella* and *Yersinia* and are therefore likely to contribute to human pathogenicity. Of crucial importance to virulence is the fact that *P. asymbiotica* undergoes a large metabolic shift at the human host temperature.

Contents

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1 Introduction

Photorhabdus asymbiotica was originally described in 1989 after being isolated from a patient with a leg ulcer in the USA. This was followed by a search of the records of the Centres for Disease Control and Prevention that revealed three more previously reported cases (Farmer et al. 1989). There are 15 cases reported in the literature and they involve soft tissue infections that are often multifocal, whilst in five cases there was also an associated bacteraemia (see also the current review of clinical cases by John Gerrard in this volume). All of the reported cases occurred in North America and Australia and were generally associated with outdoor activities in warm weather conditions (Gerrard et al. 2004). The existence of additional isolates from Thailand and Nepal (Gerrard et al. 2011; Thanwisai et al. 2012) indicates a widespread geographical distribution of the species. However, epidemiological information on *P. asymbiotica* is likely to be incomplete since infections are probably misdiagnosed, as commonly used automated bacterial identification systems misidentify this pathogen (Gerrard et al. 2003). The fact that the majority of cases reported originate from neighbouring locations may therefore simply reflect the increased awareness of clinicians in those areas regarding the possibility of infection with this unexpected pathogen (Weissfeld et al. 2005). Alternatively, it could point to the existence of specific localised pools of closely related strains, or their symbiont nematodes, that have acquired adaptations enabling them to infect human hosts. So far there are two recognised subspecies of *P. asymbiotica*, *P. asymbiotica* subsp. *asymbiotica*, found in the USA and *P. asymbiotica* subsp. *australis*, which includes the Australian isolates (Gerrard et al. 2006; Peat et al. 2010) and the isolate from Thailand (Thanwisai et al. 2012).

Since the bacteria were first identified from clinical isolates, it was originally believed they did not form the normal symbiotic relationship with the nematode vector. They were therefore originally described as being ‘a-symbiotic’ as the species name suggests. Eventually however, a nematode belonging to the genus *Heterorhabditis* was found to be associated with the clinical *P. asymbiotica* Kingscliff isolate from Australia (Gerrard et al. 2006). The nematode is most closely related to *H. indica*, but is classified as a distinct species, *H. gerrardi* (Plichta et al. 2009). In addition, certain bacterial strains isolated from soil isolates of Japanese *Heterhorhabditis* nematodes are phylogenetically most related to *P. asymbiotica* species (Kuwata et al. 2008). It should be pointed out, however, that *Heterhorhabditid* nematodes vectoring any North American isolates have not yet been identified.

Nevertheless, *Photorhabdus* species have never been found to occur free-living in the soil, even though at least some can grow in soil under laboratory conditions (Bleakley and Chen 1999). The mechanism of transmission to humans also remains unknown. In the case of infection with *P. asymbiotica* strain Kingscliff a preexisting skin injury was reported (Gerrard et al. 2006). However, in other incidences there was no known prior skin lesion (Gerrard et al. 2011). The most likely hypothesis is that the nematode vector penetrates human skin and delivers the *Photorhabdus* subcutaneously enabling establishment of the infection. Interestingly, it remains a formal, but unproven, possibility that as yet unrecognised mammalian or bird species could provide natural hosts for these particular strains and their associated nematodes.

Importantly, all North American and Australian *P. asymbiotica* isolates tested have the ability to grow at temperatures of 37 °C or above. Interestingly, the strains isolated from Australia are able to tolerate even higher temperatures than those isolated from North America. Amongst all the *P. asymbiotica* genospecies so far examined, the only strains not able to grow at temperatures over 34 °C are two strains isolated from Northern Europe (*P. asymbiotica* strain HIT and *P. asymbiotica* strain JUN) (Mulley et al. 2015). These observations suggest that adaptation of the *P. asymbiotica* 'genospecies' to elevated temperatures may have been influenced by geographical location and climatic conditions. While most strains of *P. luminescens* tested cannot grow at elevated temperatures there are examples of some that can (Fischer-Le Saux et al. 1999). It is not known if these would be capable of causing human infection given the opportunity. However, as no clinical cases have been ascribed to any member of the genus other than *P. asymbiotica*, we suggest it implies certain adaptations are required that are not found in the *P. luminescens* genospecies.

2 Insights from the *P. asymbiotica* Genome

Genomic data is an invaluable source of information enabling the identification of factors that have allowed host switching to occur. Currently, a complete genome of a USA isolate (ATCC 43949) (Wilkinson et al. 2009) and two draft genomes of Australian isolates, *P. asymbiotica* Kingscliff (Wilkinson et al. 2010) and *P. asymbiotica* subsp. *australis* DSM 17609 (our unpublished data) are available. Comparison between these and the genome sequences of *P. luminescens* strains has revealed a smaller genome size for *P. asymbiotica* with a concurrent acquisition of novel elements. The genome of *P. asymbiotica* ATCC 43949 is 5,064,808 bp (Wilkinson et al. 2009) compared to that of *P. luminescens* TTO1 at 5,688,987 bp (Duchaud et al. 2003). The *P. asymbiotica* strains harbour extra-chromosomal elements, with the USA strain carrying one plasmid, pPAU1, which bears some homology to the pMT1 plasmid from *Yersinia pestis*. The presence of the latter is necessary for *Y. pestis* to be able to colonise its flea vector. The Australian *P. asymbiotica* strain also carries a pPAU1 homologue (pPAA1), but in addition

also carries a second plasmid element, pPAA3. This bears similarity to pCRY from *Yersinia pestis* 91001 and encodes a Type IV pilus system, which may be involved in either plasmid conjugation or, alternatively as a Type IV toxin secretion system. No plasmids have yet been identified from either *P. luminescens* or *P. temperata* isolates despite an early report of plasmid isolation from *P. luminescens* MU2 (Bondi et al. 1999). Interestingly our unpublished Illumina whole genome sequencing of the HIT and JUN isolates has confirmed that these strains also contain sequences homologous to the pPAU1 plasmid. In the case of pPAJ from *P. asymbiotica* strain JUN, we can also confirm that this exists as a closed circle independent of the chromosome. Interestingly in this pPAJ plasmid an alternative origin of replication has inserted into the normal pPAU1 *repA* gene, although the significance of this, if any, is not known.

Even though the exact function of the *P. asymbiotica* plasmids has not been determined, our previous attempts to cure pPAU1 failed. Furthermore, *P. luminescens* TT01 transformed with pPAU1, genetically tagged with an antibiotic resistance marker, becomes non-viable after only two or three subcultures. This suggests there is genetic cross talk between plasmid and chromosome and that the presence of these plasmids is somehow required for normal growth of *P. asymbiotica*. Furthermore, comparisons of whole genome phylogeny of *P. asymbiotica* isolates with the whole plasmid sequence phylogeny shows the same tree topology suggesting that acquisition of the pPAU1-like plasmid was ancestral to the speciation of *P. asymbiotica* within the genus.

Other differences noted between the genomes of *P. asymbiotica* and *P. luminescens* are in Type III secretion systems (T3SS). T3SS are used by pathogens to introduce their own protein effectors into host cells. *P. luminescens* possesses one T3SS with at least one tightly linked effector, LopT that is homologous to the *Yersinia pestis* YopT. YopT is a serine protease that causes disruption of the host cell cytoskeleton (Shao et al. 2002). As is the case with *Yersinia*, *lopT* is found in an operon with *slcT*, which encodes a putative LopT chaperone, so it is likely that the function of the two proteins in *Photorhabdus* is similar to that of the *Yersinia* homologues. Indeed previous work by Brugirard-Ricaud et al. confirmed the role of LopT in preventing phagocytosis of *P. luminescens* by insect hemocytes (Brugirard-Ricaud et al. 2005). At the corresponding T3SS locus in *P. asymbiotica*, however, LopT is absent and instead the T3SS operon encodes a homologue of ExoU from *Pseudomonas aeruginosa*. ExoU is a known phospholipase (Sato et al. 2003) that is the cause of acute lung injury leading to sepsis (Pankhaniya et al. 2004). Additionally, *P. asymbiotica* appears to have acquired a second T3SS structural operon, related to a system from clinical isolates of *Vibrio parahaemolyticus* (Park et al. 2004). Moreover, at a separate genomic location, and likely exported by the T3SS, *P. asymbiotica* also encodes a homologue of *sopB*. SopB is T3SS effector of *Salmonella enterica* serovar Typhimurium and is involved in the maturation of the *Salmonella*-containing vacuole via recruitment of Rab5 (Mallo et al. 2008). Finally, *P. asymbiotica* contains a homologue of *vopS* from *V. parahaemolyticus*, which is another T3SS effector, which modifies small Rho-family GTPases by AMPylation.

Photorhabdus Virulence Cassettes (PVCs) are discrete operons encoding proteins similar to phage tail and Type 6 Secretion System components. They encode macromolecular phage tail-like structures superficially similar to R-type pyocins (Yang et al. 2006). Unlike pyocins however, which target other bacterial cells, the PVCs appear to be used as a mechanism for delivery of *Photorhabdus* effectors into eukaryotic cells (Yang et al. 2006). Each cassette consists of typically 16 genes that encode the structural components of the PVC 'needle complex', followed by one or more genes encoded at the 3′ end of the operon that are homologues of typical T3SS-like toxin effectors. Indeed transient expression of several example effectors in mammalian cells confirmed profound effects of the cell cytoskeleton (Yang et al. 2006). The genome of *P. luminescens* contains six PVC loci, four of which are arranged in tandem (PVC units 1–4). Different putative effectors are associated with each unique PVC cassette. The genome of *P. asymbiotica* encodes only five PVCs (Fig. 1). In particular, PVC units 1–3 are absent from the genome and only the apparently ancestral PVCunit4 is conserved in the same syntenic locus. Interestingly this PVCunit4 encodes effectors distinct from those found on the *P. luminescens* equivalent. The *P. asymbiotica* PVCunit4 encodes two effectors that are homologous to *gogB* from *Salmonella enterica* serovar Typhimurium and *Escherichia coli* and thus may play a role in the mammalian infection process. In contrast, at the equivalent *P. luminescens* locus there are two genes related to

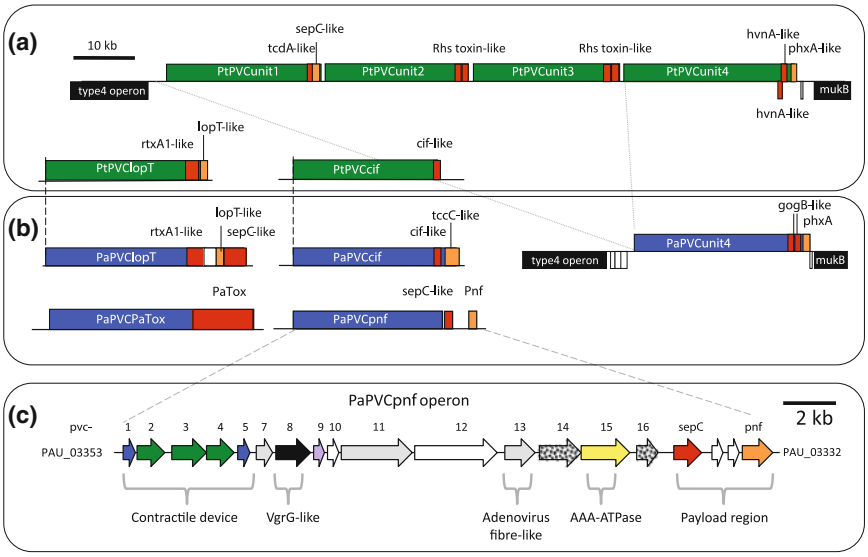

Fig. 1 The *Photorhabdus* virulence cassettes (PVCs). PVCs found in the genome of *P. luminescens* are shown in (**a**) and PVCs found in *P. asymbiotica* 43949 are shown in (**b**). *Dashed lines* between (**a**) and (**b**) show conserved elements, whilst dotted lines indicate the genomic region of *P. luminescens* TT01 that is absent from the corresponding locus of the *P. asymbiotica* 43949 genome. A more detailed schematic representation of the *P. asymbiotica* PVCpnf operon is shown in (**c**)

halovibrin from *Vibrio fischeri*. Halovibrins are secreted proteins that may facilitate the symbiotic relationship between *V. fisheri* and the Bobtail squid (Stabb et al. 2001). As such, it is tempting to speculate that this particular PVC locus might be performing a similar symbiosis role between the bacteria and the *Heterorhabditis* nematode. It should be noted that an analogous, and at least partially homologous, PVC-like 'injectosome' needle complex system is employed by the bacterium *Pseudoalteromonas luteoviolacea* which it uses to control the metamorphosis of the marine worm *Hydroides elegans* (Shikuma et al. 2014). This suggests that the PVC-like elements may be an ancient and a general mechanism for controlling many aspects of interaction with invertebrate hosts, including their development.

Two more PVC elements, PVClopT and PVCcif are conserved between the two species. However, *P. asymbiotica* also contains two additional unique PVC elements: PVCPaTox and PVCpnf. The latter is toxic to insects by injection (and their hemocytes) when heterologously expressed from a cosmid clone in *E. coli* (Yang et al. 2006). The Pnf effector is homologous to the cytotoxic necrotizing factor (CNF1) from *E. coli* (Fabbri et al. 2010). Interestingly, while a PVC operon is present in the equivalent locus in the genome of the Australian strain *P. asymbiotica* Kingscliff, a gene coding for an alternative effector has replaced *pnf*. This highlights the variability and flexibility in these novel delivery systems (Wilkinson et al. 2010). Moreover, the effector of the PVCPaTox element, encoded by gene PAU_02230, has been identified as a *P. asymbiotica* toxin and is termed PaTox (Jank et al. 2013). PaTox has some similarity to the *Salmonella* virulence factor SseI. SseI is an effector of the *Salmonella* pathogenicity island 2 T3SSS that is thought to inhibit migration of immune cells and in particular antigen presenting cells (McLaughlin et al. 2009). The gene encoding PaTox was identified separately following a search in the *P. asymbiotica* genome for the presence of motifs found in the glycosylating toxins of *Clostridium* and *Legionella* (Jank et al. 2013) (see also the Chapter by Andrea Dolwing in this volume).

Another well-known toxin system first discovered in *Photorhabdus* are ABC-type 'Toxin Complexes' encoded by *tc* genes (see also the Chapter by Klaus Aktories in this volume), some of which are responsible for oral toxicity to insects. The toxins are composed of TcA, TcB and TcC subunits. The TcA subunit contains the host cell receptor binding component and forms a homo-oligomeric injection complex which is used to inject the TcB-TcC subunits into the host cell cytoplasm (Gatsogiannis et al. 2013). Different TcC homologues possess different C-terminal domains and at least one of which has been shown to function as an ADP-ribosyltransferase. The genes encoding these were initially seen to be located on four distinct pathogenicity islands in strain *P. luminescens* W14, from which the toxins were first identified and purified (Bowen and Ensign 1998). These pathogenicity islands were named, *tca*, *tcb*, *tcc* and *tcd*. Since genome sequences have become available it is possible to see significant variation in *tc* toxin gene complement and organisation, even between different *P. luminescens* strains. For example, in strain TT01 the *tca* island has undergone deletion of most of *tcaA* and *tcaB* and the *tcb* island has also been mostly deleted. However, a comparison with

the *P. asymbiotica* genome reveals even greater differences in *tc* gene complement. For example, in addition to an independent *tcaAB* deletion event, in *P. asymbiotica* an additional *tccC* homologue is encoded on the *tca* island. Furthermore, the *tcd* island is much reduced with four of the equivalent *P. luminescens* genes missing (Wilkinson et al. 2009). These differences are observed in both the American and the Australian sequenced *P. asymbiotica* isolates (Wilkinson et al. 2010). Indeed comparative genomics suggests that the *P. asymbiotica tcd* island is likely to represent a smaller more ancestral state and that the *P. luminescens* strains have acquired additional homologues.

The genome of *P. asymbiotica* also shows a reduced complement of other toxin homologues compared to *P. luminescens*. For example, while *P. luminescens* possesses two homologues of the large Makes Caterpillars Floppy toxin (*mcf1* and *mcf2*), *P. asymbiotica* only carries the *mcf1* homologue. Mcf1 possesses a BH3-like N-terminal domain which induces apoptosis in hemocytes and the insect midgut epithelium (Daborn et al. 2002). Mcf2 on the other hand, has an N-terminal domain that shows similarity to HrmA (Waterfield et al. 2003), a T3SS effector of *Pseudomonas syringae*. Transfection of this N-terminal domain into mammalian NIH3T3 cells resulted in cell death (Waterfield et al. 2003). Expression of either Mcf1 or Mcf2 confers virulence properties to *E. coli* and enables the bacteria to kill insects (Daborn et al. 2002; Waterfield et al. 2003). An additional example can be found with the *pirAB* toxin genes. Unlike *P. luminescens*, which has two loci encoding PirAB toxins, *P. asymbiotica* only encodes one such locus, equivalent to the *plu4093-plu4092* genes of TT01. The PirAB toxins have strong insecticidal activity against Diptera and Lepidoptera (Waterfield et al. 2005; Ahantarig et al. 2009). A crystal structure of the *Vibrio parahaemolyticus* PirA and PirB homologues has revealed similar topology to the *Bacillus* pore-forming Cry toxin, suggesting a similar mode of action (Lee et al. 2015). Finally, *P. asymbiotica* 43949 is missing four haemolysin-encoding loci, which are present in *P. luminescens*. It must be noted, however, that a comparison between the genomes of the Australian *P. asymbiotica* Kingscliff isolate and *P. asymbiotica* 43949 does reveal that the former contains several haemolysin or haemagglutinin genes that are not found in the USA isolate (Wilkinson et al. 2010). Whether these are employed during the infection in the insect or in the mammalian infection process is still unknown.

The differences discussed above in the toxin compliments of *P. asymbiotica* 43949 and *P. luminescens* TT01 are illustrated in a functional genomics comparison between the two species. This follows the results of a screen whereby parts of the two genomes were cloned into cosmids and introduced into *E. coli*. The resulting *E. coli* clones were then tested for toxicity against insects, macrophages, amoebas or nematodes (Waterfield et al. 2008). As can be seen in Fig. 2, a comparison of the genetic regions of the two bacteria, which are capable of conferring toxicity, shows that a number of toxins found in *P. luminescens* TT01 are absent from *P. asymbiotica* 43949, whilst the latter has acquired novel elements. Additionally, there are parts of the genome that are conserved between the two species that do not seem to encode toxic products in *P. luminenscens*, but do so in *P. asymbiotica*. This

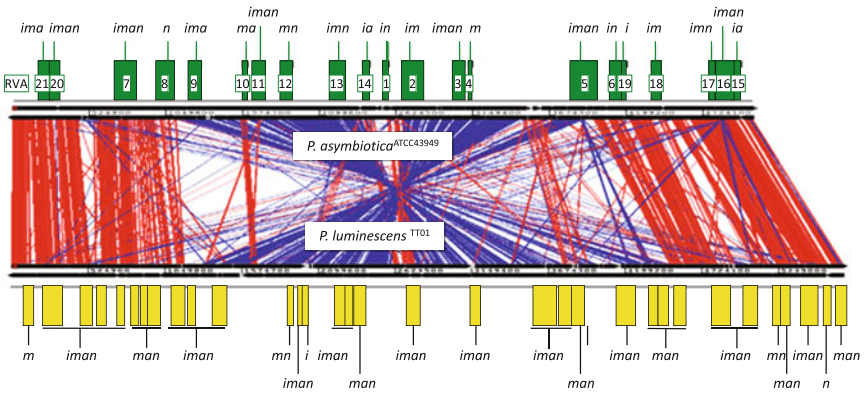

Fig. 2 A comparison of the rapid virulence annotation (*RVA*) functional genomics screens of *P. asymbiotica* 43949 and *P. luminescens* TT01. The image represents an artemis comparison tool alignment of the two genomes. *Boxes above* and *below* the genomes represent regions of the genome containing genes that elaborate toxic products in *E. coli* cosmid clones. *RVA* gain of toxicity screens were performed against *Manduca sexta* insects (*i*), Mammalian macrophages (*m*), *Acanthamoeba polyphaga* amoeba (*a*) and *C. elegans* nematodes (*n*). The *RVA* numbers at the top refer to the relevant regions presented in the supplementary data in (Waterfield et al. 2008), where the *P. asymbiotica RVA* screen was described. The *P. luminescens RVA* data is unpublished

may reflect specific adaptations in *P. asymbiotica* that facilitate infection of the human host.

Genetic differences between *P. asymbiotica* and the other species of the genus involving genes that encode non-toxin products may also be of significance to its life style and its ability to infect humans. One such example is the LPS biosynthesis locus whereby there is a large region that is different between *P. asymbiotica* 43949 and *P. luminescens* TT01. In particular, a 17 kb region of *P. luminescens* TT01 has been replaced by a region of 18 kb that contains putative O-antigen synthesis genes. Elucidation of the structure of the *O*-polysaccharide of various *P. asymbiotica* strains has revealed that there are some subtle differences between Australian and American isolates since there are higher levels of *O*-acetylation in the American strains. However, in general the structure resembles those of other important phylogenetically distant pathogens, such as *Francisella tularensis* or *Shigella dysenteriae* type 7 (Kondakova et al. 2011). Thus, the differences observed between *P. asymbiotica* and *P. luminescens* in this respect may reflect an additional adaptation of the former to its new host.

3 *P. asymbiotica* as an Insect Pathogen

Despite the overall lower diversity of insecticidal toxin genes in the *P. asymbiotica* genome, the ability of the bacterium to infect model insects is unaffected. Indeed, *P. asymbiotica* is more pathogenic against model insect hosts than the other

members of the genus. Eleftherianos and coworkers observed that using a dose of approximately 100 bacteria to infect *Manduca sexta* larvae, the LT_{50} of *P. asymbiotica* 43949 is 30 h, whilst that of *P. luminescens* is 56 h (Eleftherianos et al. 2006). Nevertheless, it cannot be ruled out that as a result of the lower diversity of toxin genes in *P. asymbiotica* the bacterium is unable to infect as large a range of insect host species as *P. luminescens*. To our knowledge, the virulence of *P. asymbiotica* against a range of insect hosts has not been tested.

Interestingly, *P. asymbiotica* becomes unable to cause a successful infection in *Manduca sexta* larvae when the infected insects are incubated at 37 °C (Mulley et al. 2015). This suggests that the bacteria are not naturally found in an insect host at this temperature and that they have adapted other mechanisms that are better suited for an infection of mammalian hosts. The apparent increased virulence of *P. asymbiotica* at the lower temperature of 28 °C could be a result of the presence of additional virulence factors that the species has acquired. For example, the PaTox is not found in other *Photorhabdus* species and could contribute to virulence against insects. The toxin consists of a deamidase and a glycosyltransferase domain. The activity of the deamidase domain constitutively activates heterotrimeric G proteins, whilst the glycosyltranferase domain modifies small Rho-family GTPases in their GTP-bound active state. Overall, PaTox activity results in actin cytoskeleton disassembly and thus inhibition of phagocytosis. Pa Tox is active against both insect cells and mammalian cells (Jank et al. 2013). Similarly, the PVCpnf that is specific to *P. asymbiotica* is another potent insect virulence factor. Injection of recombinant PVCpnf into *Galleria melonella* larvae is lethal and hemocytes recovered from the larvae show severe actin cytoskeleton rearrangement (Yang et al. 2006).

It is important to note here that *P. asymbiotica* can be found within insect hemocytes, unlike *P. luminescens* which remains extracellular (Brugirard-Ricaud et al. 2005; Costa et al. 2009; Vlisidou et al. 2010). The rate of uptake of *P. asymbiotica* by hemocytes appears to depend on the subspecies, since Australian isolates seem to be taken up more readily than USA isolates (Wilkinson et al. 2009; Costa et al. 2009). The ability of *P. asymbiotica* to survive in cells means that the pathogen may have alternative uses for factors that are present across the genus. For example, the KdpD/KdpE two component system of *P. asymbiotica* contributes to survival of the bacteria within insect hemocytes (Vlisidou et al. 2010). Vlisidou and coworkers showed that it alone could enable nonpathogenic *E. coli* to resist phagocytic killing by increasing expression of the native high affinity potassium pump Kdp (Vlisidou et al. 2010). How exactly this interferes with the hemocyte ability to kill the phagocytosed bacteria is not clear, but *E. coli* was able to persist long enough to result in the death of injected *Manduca sexta* larvae (Vlisidou et al. 2010). A KdpD/KdpE system is also present in *P. luminescens* but it is not clear whether this would provide an advantage to the bacteria during the infection if they are not found within host cells. Moreover, the ability of *P. asymbiotica* to enter and survive within the host cells implies that toxin molecules synthesised by the bacteria might become released directly into the host cell cytosol without the need for a delivery mechanism. This could allow for example the *P. asymbiotica* Tca BC to

act as a functional toxin despite the lack of the pore-forming TcA subunit, as there is no need for transport of the toxin across the host cell membrane. On the other hand, even though *P. asymbiotica* virulence against model insect hosts appears to be undiminished, oral toxicity of the bacteria is attenuated due to the lack of certain *tc* genes, required for full oral toxicity.

4 The Human Host

4.1 Overcoming Host Defences

Many of the immune-defence strategies that *Photorhabdus* must overcome in an insect have parallels in a human host. In fact many aspects of innate immunity at both a mechanistic and even genetic level are conserved between many invertebrates studied and humans (Browne et al. 2013; Buchmann 2014; Kimbrell and Beutler 2001). These could be either part of the humoral response, such as the presence of antimicrobial peptides and production of lysozyme, or the cellular innate immune response, which involves phagocytic attack by hemocytes in the insect or macrophages in the human host. Thus, it may be envisaged that the mechanisms employed by the bacteria to evade the response of the insect can be adapted to address these similar issues encountered in the human host. In support of this notion, it was recently demonstrated that both *P. luminescens* and *P. asymbiotica* can survive and grow in the presence of human serum (Mulley et al. 2015). This suggests that the adaptations that allow *Photorhabdus* to overcome killing by components of the insect serum, provide sufficient protection against human serum. Importantly though, unlike *P. luminescens*, *P. asymbiotica* is also able to survive in the presence of both pig and rabbit serum. Similarly, the aglycon precursor of rhabducin, a known inhibitor of the insect phenol oxidase cascade (Crawford et al. 2012) was also shown to inhibit the human alternative complement pathway (Mulley et al. 2015). Additionally, RNAseq analysis of *P. asymbiotica* revealed the up-regulation of *isnAB* transcription for the synthesis genes of rhabducin at 37 °C, compared to at 28 °C (Mulley et al. 2015). Even though there was no detectable increased secretion of free rhabducin itself (Mulley et al. 2015), most of the molecule is normally found on the cell surface (Crawford et al. 2012). Another *Photorhabdus* secondary metabolite whose levels actually increase at 37 °C and thus may be relevant in the human infection process is iso-propyl-stilbene (IPS). IPS is produced by both *P. luminescens* and *P. asymbiotica* and is an antibiotic compound with multiple other activities that is also required for symbiosis (Joyce et al. 2008; Li et al. 1995) (see also the Chapters by David Clarke and Helge Bode on secondary metabolism also in this volume). Interestingly, IPS also seems to have immunomodulatory properties (Zang et al. 2016) so it could be another example of a factor present in the *Photorhabdus* genome that is being repurposed by *P. asymbiotica* during a human infection. Finally, the secreted metalloprotease PrtA, which is found in both clinical and nonclinical strains of *Photorhabdus* might be involved in defence against the

immune system in the human host. This is supported by the identification of several PrtA-sensitive proteins with immune related function, following exposure of insect haemolymph to PrtA digestion in vitro (Felföldi et al. 2009). It remains to be confirmed whether these proteins are PrtA targets in vivo.

Bacterial pathogens can either remain outside host cells or survive and replicate inside them. Furthermore, some that are facultative intracellular pathogens can switch between both strategies. Experiments in vitro using both murine and human macrophage-like cells have shown the *P. asymbiotica* can enter these phagocytic cells (Wilkinson et al. 2009; Costa et al. 2009). Costa et al. (2009), observed that *P. asymbiotica* could actually survive and replicate inside human macrophage-like THP-1 cells. It is tempting to speculate that the ability to survive intracellularly is at least in part mediated by the *P. asymbiotica* SopB homologue discussed previously. Additionally, Australian but not American isolates are able to invade the non-phagocytic HeLa cells (Costa et al. 2009). It is possible that the increased ability of the Australian isolates to invade host cells is due to the presence of the Type 4 secretion system encoded by the second plasmid (pPAA3) found in these strains. This may enable the bacteria to gain access to specific sites in the host thus allowing for a more disseminated infection to take place. Indeed, we may contemplate that the bacteraemic dissemination of *P. asymbiotica* around the bodies of patients with Photorhabdosis is facilitated by the invasion of macrophage, neutrophils or other circulating white blood cells. Invasion into host cells can not only provide a niche sheltered from certain aspects of immunity, but can also grant access to certain nutrients, promoting persistence in the host.

Similarly to insect cells, following infection with *P. asymbiotica*, mammalian macrophage-like cell lines, as well as primary human macrophages later show signs of apoptosis. The effect is more pronounced with the Australian isolates as infection with the American isolates results in apoptosis being detected much later and at a lower rate. This appears to be specific to macrophage-like cells, as after infection of HeLa no signs of programmed cell death were observed (Costa et al. 2009). Apoptosis induction occurs after addition of bacteria to cells but does not require uptake of the pathogen. Additionally, it can be triggered by the addition of bacterial culture supernatant in the absence of bacteria. This indicates that the virulence factor responsible for host cell apoptosis is secreted. The identity of this virulence factor remains elusive and it is possible that there are multiple molecules exerting these effects, but one likely candidate is Mcf1. Even though Mcf1 was previously shown to cause apoptosis in HeLa cells (Dowling et al. 2004), which was not observed with *P. asymbiotica*, *mcf1* becomes up-regulated when the bacteria are grown at 37 °C, compared to expression at the normal growth temperature of 28 °C (Mulley et al. 2015). More clues come from the findings of a recent study performed with *E. coli* clones carrying cosmids that contain different regions of the *P. asymbiotica* genome (Dowling and Hodgson 2014). The effect of administration of *E. coli* lysates on macrophages was closely monitored. Here, a synergistic effect was observed between Mcf1 and the product of a nonribosomal peptide synthase (NRPS) cluster (PAU03356-PAU03357) located upstream of *mcf1*. Whilst

administration of Mcf1 alone resulted in condensation of the cytoskeleton, co-administration with the NRPS product led to the formation of cytoskeletal protrusions and actin ruffles (Dowling and Hodgson 2014). This indicates that such NRPS secondary metabolites may also play a role in pathogenicity of *P. asymbiotica* as is known for several other bacterial species.

The transcription of certain other putative virulence factors of *P. asymbiotica* was shown to be up-regulated at 37 °C. These include, PVCunit4 and its associated effectors, insect toxin *pirB*, an operon containing a putative invasin (PAU_02531-37) and a *sepC* toxin-like gene (PAU_0214) (Mulley et al. 2015). On the contrary, the gene encoding the insect toxin XaxA is one of the few virulence genes that are down-regulated at 37 °C. This suggests that this factor may be specific for insect cells or would otherwise cause a problem in a human infection. Finally, the expression of the second T3SS and its effectors appears to be unaffected, however it is likely that direct contact with host cells might be a requirement for up-regulation. It should be noted that the published RNAseq work looked at changes in gene transcription in relation to temperature alone.

4.2 Metabolic Adaptations

The nutrients available in the human host niche are likely to be distinct from those in the insect host. Therefore, in addition to the deployment of toxins and other immune effector defence mechanisms, pathogens must be able to adjust their metabolism to ensure optimal nutrient acquisition and assimilation. Temperature can be sensed by many bacteria, and used as a signal to determine entry into a mammalian host. The bacteria can then respond accordingly, changing the expression of not only virulence but also metabolic genes. To measure these responses a set of transcriptomic, metabolomic and proteomic assays were performed with *P. asymbiotica* grown at the environmental temperature of 28 °C or the mammalian host temperature of 37 °C (Mulley et al. 2015). These experiments revealed that the metabolic abilities of *P. asymbiotica* undergo a large shift when grown at the elevated temperature. For example, genes encoding products involved in carbohydrate uptake and metabolism are down-regulated at 37 °C. The decrease in transcript levels of genes in these pathways is in accordance with a reduction of protein levels and the ability to metabolise these compounds in culture. Overall, *P. asymbiotica* becomes restricted in the substrates it can use as a source of carbon at 37 °C and this could reflect their availability, or lack thereof, in the new host. Examples of carbon sources it can still utilise at 37 °C are glycyl-L-proline, a product of collagen degradation and hence possibly readily available in mammals and *N*-acetyl-D-glucosamine, a component of connective tissue in both mammals and insects. On the other hand *P. asymbiotica* can use L-serine as a carbon source at both temperatures, whilst it relies on only a few amino acids and peptides to provide a source of nitrogen at 37 °C. In accordance with this, several genes encoding peptidases and amino acid and peptide transporters become up-regulated at 37 °C.

This suggests that the bacteria may rely on the degradation of host proteins and the uptake of peptides and amino acids to be used as nutrient sources in the human. Again, the secreted metalloprotease PrtA might play a role in this. The gene encoding PrtA becomes highly up-regulated at 37 °C and it has been previously suggested that the function of the protease is to support nutrient acquisition even in the insect infection since its levels rise late in the infection process (Silva et al. 2002; Daborn et al. 2001). It is tempting to speculate that a requirement for degrading host proteins at 37 °C might lead to the clinical symptoms of tissue necrosis and ulceration seen in *P. asymbiotica* infection.

Furthermore, it has been suggested that depleting certain nutrients, such as specific amino acids, from the host can have adverse effects on host cell homeostasis or immune function. This phenomenon has been coined 'nutritional virulence' and several examples have been seen in well-studied human pathogens (Abu Kwaik and Bumann 2013). For example, reduction in host cell serine levels would deprive host cells of a source for NADPH generation (Fan et al. 2014), whilst depletion of glutamine, which is required for immune cell function, is known to dampen immune responses (Calder and Yaqoob 1999). Glutamine is used as a source of energy by fast dividing cells such as lymphocytes and is also needed for the synthesis of other amino acids and nucleotides. Additionally, it is required for the synthesis of glutathione, which is important for protection against reactive oxygen species. Many of these pathways are connected to the conversion of glutamine into glutamate (Newsholme et al. 2003). Glutamine deprivation would therefore be detrimental to immune cells not only because it would signify a loss of energy source, but also because of lowered resistance to oxidative damage. This phenomenon has been observed in *Helicobacter pylori*, which converts both extracellular glutamine and glutathione into glutamate that it then takes up and uses mainly as an energy source (Shibayama et al. 2007). This seems to be a major contributing factor to the pathology of the bacterium (De Bruyne et al. 2016). Glutamine and glutamate are, together with tyrosine, the only amino acids *P. asymbiotica* can use as a nitrogen source at 37 °C (Mulley et al. 2015). It is therefore possible that the *P. asymbiotica* metabolism might result in glutamine depletion in host cells and have similar associated effects.

Additionally, *P. asymbiotica* is able to use purines as carbon sources and xanthine as a nitrogen source at 37 °C, whilst at the same time it down-regulates the de novo purine biosynthesis pathway. This suggests that purines are either abundant or readily available in the host and this could be particularly relevant in an intracellular setting. Indeed some pathogens like *H. pylori*, *Chlamydia trachomatis* or *Ricketsia prowazekii*, which lack either parts of or the entire purine biosynthesis pathway, need to take up nucleotides from the host (Audia and Winkler 2006; Liechti and Goldberg 2012; Tjaden et al. 1999). Additionally, xanthine depletion may also be interfering with the host immune response as xanthine is a substrate of xanthine oxidoreductase (XO) whereby it is converted into uric acid with the associated release of hydrogen peroxide (Crane et al. 2013; Martin et al. 2004). Moreover, in hypoxic conditions XO can use xanthine as a reducing agent to generate nitric oxide

(Godber et al. 2000). Thus, the ability of *Photorhabdus* to utilise xanthine as a carbon source may not only be providing the bacteria with necessary nutrients, but also acting as yet another defence strategy against the immune system.

Iron restriction is used by many animal hosts as an immune-defence strategy to limit the growth of microorganisms (Ganz and Nemeth 2015; Schaible and Kaufmann 2004). Thus, bacterial pathogens often have mechanisms to overcome this restriction (Schaible and Kaufmann 2004; Fischbach et al. 2006). In accordance with this, *P. asymbiotica* up-regulates the iron compound ABC transporter (PAU_03286), the outer membrane siderophore receptor CjrC and a bacterioferritin co-migratory protein Bcp (Mulley et al. 2015). Interestingly, the strategies employed by the human body to limit iron availability to bacteria will have different effects depending on whether the pathogens are extracellular or intracellular. So, while the sequestration of ferric ions or siderophore-bound iron will limit its availability to most pathogens, inhibition of iron release into the plasma via the degradation of ferroportin leads to a rise in its concentration inside cells like macrophages. This could lead to increased iron availability for pathogens that can survive inside these cells and so additional strategies are required to ensure that iron is excluded from intracellular sites where microbes reside, such as phagosomes (Ganz and Nemeth 2015; Schaible and Kaufmann 2004). Thus, the ability of *P. asymbiotica* to act as a facultative intracellular pathogen may be enabling it to overcome yet another human defence mechanism.

5 Conclusions

The shift of *P. asymbiotica* to a new host seems to have been facilitated by three general mechanisms. The first involves making use of virulence mechanisms already present and which are used by all *Photorhabdus* species when they infect an insect host. These could be toxins or molecules that provide defence against the host immune system. The second mechanism involves the acquisition of additional virulence factors that are appropriate for the mammalian host infection strategy. For example, specific 'toxin' effectors, which exert their actions inside host cells, like ExoU or PaTox. It can be envisaged, however, that without the aforementioned preexisting adaptations the newly acquired virulence factors would not be sufficient to confer full pathogenicity to the bacteria. Finally, *P. asymbiotica* appears to be able to adapt its metabolism to best utilise the new niche. It is possible that the changes that are observed in its metabolism upon shift to 37 °C do not exactly reflect what occurs when the bacteria are found in a human host as additional signals might be present during an infection. Even so, similar experiments with other pathogens have shown a good correlation between such in vitro data and transcriptomic data from infected cells (Bent et al. 2015; Ren and Prescott 2003). Moreover, temperature has been shown to increase expression of known virulence genes in a variety of pathogens (Konkel and Tilly 2000), sometimes controlling translation directly by

altering the structure of messenger RNA molecules (Guijarro et al. 2015). Whether the metabolic shift seen with *P. asymbiotica* 37 °C is simply a result of differential availability of nutrients in the new environment, poor adaptation of certain bacterial enzymes and pathways or rather a specific strategy to use nutritional virulence to simultaneously manipulate the host, is an interesting question to consider. Finally, the inability of *P. asymbiotica* to infect insects at 37 °C despite an up-regulation of virulence genes, suggests that the changes it undergoes at this elevated temperature constitute specific adaptations to the mammalian host. We argue this failure is most likely due to the large restriction in metabolic abilities.

An analysis of *P. asymbiotica* genome sequences, in addition to temperature dependant differential transcriptomic, proteomic and metabolic studies have shed light on the characteristics that allow the members of the species to infect humans. Nevertheless, several important questions regarding their adaption to human pathogenicity remain unanswered. One obvious question that arises from comparison of the *P. asymbiotica* genome with that of the other *Photorhabdus* species is the role of the plasmid observed in *P. asymbiotica*. The fact that the European isolates HIT and JUN are unable to grow at elevated temperatures and also seem to contain a pPAU1-like plasmid, but in much lower copy numbers, and at least in one case with an altered origin of replication, hints at a role in the transition to human pathogenicity. Nevertheless, transcriptomics has not shown any significant difference in pPAU1 plasmid gene expression levels between 28 and 37 °C (Mulley et al. 2015), though the possibility that additional signals are required for gene up-regulation cannot be overlooked. One hypothesis for its function is that the plasmid may be introducing an additional level of transcriptional control over chromosomal genes. Such crosstalk between chromosome and plasmid has been observed in some other bacterial pathogens (Chitlaru et al. 2006; Zhu et al. 2010; Letek et al. 2010). This may allow better integration of the horizontally acquired genes enabling utilisation of a new niche. Furthermore, if such regulatory rewiring has occurred, it could explain why the *P. asymbiotica* ATCC43949 pPAU1 plasmid could not be cured even in laboratory conditions.

Other important questions involve the mode of transmission to humans and the presence, if any, of a mammalian host reservoir. The working hypothesis is that the nematode vector may penetrate human skin and release the bacteria; however, this has not been demonstrated so far. Certain nematodes such *Strongyloides stercoralis* or the hookworm *Ancylostoma duodenale* are known to possess this ability (Hotez et al. 2004), but this is not thought to be the case for *Heterorhabditis*. We have, however, observed that *Heterorabditis* nematodes can penetrate rat dermis ex vivo (unpublished results). Since human body temperature does not allow *Heterorhabditis* to survive (Sunanda 2009), such an infection would result in a dead-end for the nematode. Even so, the temperature on the surface of human skin at the extremities is well below 37 °C and this could allow introduction of the bacteria by the nematode. Finally, the possibility that a mammal with a lower body temperature, such as a marsupial, or poikilothermic animals like reptiles act as the reservoir for *P. asymbiotica* cannot be excluded.

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