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4 Dissecting the immune response to the entomopathogen 5 *Photorhabdus*

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Bacterial pathogens either 'hide' from, or modulate, the host's immune response to ensure their survival. *Photorhabdus* are potent insect pathogenic bacteria vectored by entomopathogenic nematodes which form a useful tool for probing the molecular basis of immunity. During the course of infection, *Photorhabdus* multiplies rapidly within the insect, producing a range of toxins that inhibit phagocytosis of the invading bacteria and eventually kill the insect host. We have recently established *Photorhabdus* bacteria as a tool to investigate immune recognition and defense mechanisms in model hosts such as *Manduca* and *Drosophila*. Such studies set the scene for examining gene-for-gene interactions between pathogen virulence factors and host immune genes and ultimately for understanding how some *Photorhabdus* species have made the leap to becoming human pathogens.

The insect pathogen *Photorhabdus*

Photorhabdus is a genus of entompathogenic bacteria within the family *Enterobacteriaceae*. In addition to being a highly virulent pathogen of insects, *Photorhabdus* also maintains a mutualistic relationship with nematodes from the family *Heterorhabditidiae* [1]. Phylogenetic analyses have defined three species of *Photorhabdus*, *P. luminescens*, *P. temperata* and *P. asymbiotica* whilst more than 12 species of nematodes have been described [2,3]. The relationship between the bacteria and the nematode is highly specific and nematodes will only maintain mutualistic associations with their cognate bacteria or very closely related strains. The genome sequences of two species of *Photorhabdus*, *P. luminescens* TT01 and *P. asymbiotica* ATCC43949 have recently been published facilitating a molecular insight into the complex nature of the *Photorhabdus* lifestyle [4,5]. In addition a program to produce a high quality draft sequence of the genome of the nematode partner of *P. luminescens* TT01, *H. bacteriophora*, is currently underway and this will enable a more complete understanding

1 of the molecular contributions of the nematode to this mutualistic association [6]. Here we
2 review recent advances in our understanding of how an insect pathogen overcomes the insect
3 immune system. We also examine the hypothesis that insect pathogens hone their skills on
4 invertebrate hosts before attempting the transition to the infection of vertebrates.
5

6 Life cycle of *Photorhabdus*

7 *Photorhabdus* is found in the gut of a specialized stage of the *Heterorhabditis* nematode
8 called the infective juvenile (IJ), a non-feeding stage that is morphologically and functionally
9 analogous to the dauer juvenile of the free-living model nematode *Caenorhabditis elegans*
10 [6,7]. In *C. elegans* the dauer juvenile is an alternative, developmentally arrested diapause
11 stage that forms as a response to adverse environmental conditions [8]. However, the
12 *Heterorhabditis* IJ is an obligate part of the nematode life cycle that is required for infection
13 of insect larval hosts living in the soil (Figure 1). As well as acting as a vehicle for insect
14 infection the IJ is also thought to act as a vector in the environment allowing dormant bacteria
15 to persist in the IJ gut away from their insect hosts. Susceptible insect larvae are generally
16 soft cuticled, soil dwelling larvae from the orders Coleoptera (beetles), Lepidoptera (moth
17 and butterflies) and Diptera (flies). The IJ actively seeks out and infects the insect through
18 natural openings (e.g. mouth, anus, and spiracles) or directly through the soft cuticle of the
19 insect larvae using a buccal tooth-like appendage. Once inside the insect, the IJ responds to
20 an unidentified signal and regurgitates *Photorhabdus* into the hemolymph where the bacteria
21 begin to divide and rapidly proliferate [9]. After 2-3 days of bacterial growth, the insects
22 succumb to septicemia with the concomitant conversion of the internal organs and tissues of
23 the insect into bacterial biomass. This bioconversion is facilitated by the production of a wide
24 range of bacterial toxins and hydrolytic enzymes [7,10]. At the same time that the bacteria are
25 replicating in the insect, the IJ exits diapause and develops into an adult hermaphrodite

nematode in a process called IJ recovery. The nematode feeds on the bacterial biomass and nematode growth and development has an obligate requirement for the presence of a high density of *Photorhabdus* bacteria. The adult hermaphrodite lays eggs that hatch and develop through 4 juvenile stages into adult nematodes. Nematode reproduction continues for 2-3 generations until conditions within the insect cadaver deteriorate to such an extent (due to an increasing nematode population and the decreased availability of bacteria as food) that the developing generation of juvenile nematodes are stimulated to enter diapause and form IJs that eventually emerge from the insect cadaver into the soil in search of new hosts. Remarkably a single IJ entering an insect larvae will result in the production of >100,000 IJs over a time scale of 2-3 weeks. This extremely efficient symbiosis not only provides a fascinating model system for studying bacteria-host interactions (Box 1), but also has previously led to the development of the *Photorhabdus-Heterorhabditis* complex as a commercial biopesticide (e.g. [11]).

Transmission of *Photorhabdus*

The gut of each IJ is initially colonized by 1-2 bacterial cells that replicate to produce a mature bacterial population of approximately 100 cfu (colony forming units) per IJ [12]. Colonization (or transmission) is a complex process and it was originally assumed that the IJs would be colonized horizontally by bacteria coming directly from the insect cadaver. However it now appears that transmission to the IJ is dependent on the bacteria first infecting the adult hermaphrodite [12]. Whilst the nematodes are feeding on *Photorhabdus*, some bacteria escape crushing by the pharynx (this is suggested to be an adaptation by *Heterorhabditis* to the symbiosis) and these bacteria enter the gut of the hermaphrodite where they invade specific cells called rectal gland cells (Figure 2). The bacteria replicate in these cells before colonizing the IJ nematode that also develops within the adult hermaphrodite, in

a process called *endotokia matricida* [12]. Recent genetic data has identified several bacterial genes that are involved in the transmission process [13,14]. Many of these genes are predicted to be involved in lipopolysaccharide (LPS) biogenesis (e.g. *galE* and *galU*) implicating an important role for the bacterial surface in colonization of the IJ. Interestingly, mutations in these genes also affect virulence against insect larvae suggesting a significant genetic overlap in the requirements for colonization of the nematode (i.e. transmission) and colonization of the insect (i.e. virulence) [13,14].

Insect immune responses to bacterial pathogens

Insects have a multilayered immune system consisting of several defensive mechanisms that parallel many aspects of the vertebrate innate immune system [15]. The first line of defense involves the barrier epithelial response, which can fight against invading microorganisms by producing local antimicrobial peptides (AMPs) and reactive oxygen species (ROS). Activation of the innate immune system upon microbial recognition results in the induction of highly efficient humoral and cellular responses. Humoral responses are characterized by the transcriptional activation of numerous genes encoding AMPs targeted against bacteria and fungi [16]. The cellular arm of insect immunity is mediated by the activity of circulating hemocytes, which kill microbes directly through phagocytosis, the formation of hemocyte aggregates, nodulation and via encapsulation, or indirectly by releasing systemic signals [17]. An important component of both arms of the insect immune system is the phenoloxidase (PO) response or 'cascade', which deposits melanin at the site of an immune reaction and leads to the release of microbiocidal reactive intermediates [18]. Although the fat body and hemocytes (functionally equivalent to the mammalian liver and macrophages) are the major contributors of systemic reactions, epithelial responses in the gut are also crucial in combating bacterial infections. These immune defenses include regulation of the native

microbiota in the insect gut through AMPs [19], and cytotoxic effects employed by high concentrations of nitric oxide (NO), which also plays a signaling role by controlling innate immune responses to bacteria and parasites [20].

Pathogens can either hide from the host immune system by avoiding detection because they lack immune elicitors on their cell surfaces, or they can suppress the immune response [20]. In this respect it is notable that *Photorhabdus* has evolved multiple virulence factors and strategies that can impair both humoral and cellular insect immune responses and to kill the host, which we discuss below.

***Photorhabdus* and immune recognition**

To recognize invading pathogens insects use pattern recognition proteins (PRPs) that bind conserved pathogen associated molecular pattern (PAMP) molecules produced by microorganisms [22]. Three main PRPs, hemolin, immulectin-2 (IML-2) and peptidoglycan recognition protein (PGRP) have been studied in the insect model *Manduca*. Hemolin is a PRP exclusive to Lepidoptera which can bind both hemocytes and bacteria and is also implicated in opsonization or in 'trapping' bacteria in nodules formed by hemocytes [23]. IML-2 is crucial in protecting insects against Gram-negative bacteria and binds to serine proteases in plasma, which participate in activating pro-phenoloxidase (PPO) to active PO (described below) [23]. Finally, PGRPs also recognize peptidoglycan found in bacterial cell walls [24]. These three genes are expressed at low levels in unchallenged insects but are induced rapidly by infection of *Manduca* with *Photorhabdus*. Further, RNA interference (RNAi)-mediated knockdown of any of these genes results in increased susceptibility to *Photorhabdus* [25]. Interestingly, silencing of IML-2 prevented normal activation of PO, which was linked to the impaired ability of the insect to encapsulate the bacteria [24]. It now remains to be investigated whether the protective effects of hemolin and PGRP mediate

downstream signaling events that initiate the direct expression of antibacterial effectors. It would also be interesting to measure immune recognition gene expression upon *Photorhabdus* infection in *Drosophila,* as a previous study did not look at the transcription of any genes involved in the recognition of pathogens but merely at the antimicrobial peptide effectors released upon pathogen recognition [26]. These studies elegantly demonstrate how RNAi can be used to dissect the interactions between *Photorhabdus* and the insect immune system at the level of individual genes.

***Photorhabdus* and humoral responses**

As a consequence of the activation of signaling pathways dependent on immune recognition, the invading pathogens are either restricted or destroyed by antimicrobial effectors [27]. Although in insects antimicrobial reactions also include cell-mediated responses, the best known effectors of the insect immune system are AMPs, which are secreted into the hemolymph [28]. Following *Photorhabdus* challenge and initial detection of the bacteria by PRPs, certain genes encoding different antibacterial peptides (attacin, cecropin, lebocin, lysozyme and moricin) have high levels of gene transcription in the *Manduca* fat body, which implies that the antibacterial responses of the insect host are not only deployed but exert an important (although ultimately ineffective) defense against infection by these pathogens [29]. Interestingly, older *Manduca* larvae are less able to induce the transcription of PRP and AMP mRNA suggesting a critical role for host age or developmental stage in bacterial immune challenge [29].

Photorhabdus can also be used to probe signaling pathways and several other features of host-pathogen interactions (Box 2). It has been shown that the *Manduca* immune system can be efficiently primed by prior infection with non-pathogenic bacteria so that immunity to infection by *Photorhabdus* is subsequently enhanced [30]. Using systemic RNAi to prevent

the upregulation of single immune genes in *Manduca* revealed that the effects of individually silencing any one PRP gene were greater than silencing any one AMP gene. This was because knockdown of any one PRP was able to block translation and secretion of multiple AMPs in the hemolymph leading to lower *Photorhabdus* growth and slower death of the insect host. This also suggests that *Photorhabdus* is sensitive to the action of AMPs. However, the AMP response may not be equally important in all *Photorhabdus*-insect interactions. Experiments in *Drosophila* showed that four AMP genes (*Metchnikowin*, *Drocomycin*, *Attacin* and *Diptericin*) were expressed upon *Photorhabdus* infection in wild type flies [26]. However, mutants with defects in their Toll and Imd signaling pathways (pathways within which binding of pathogens to PRPs results in transcriptional activation of the AMP genes [2]) and wild type flies died at similar rates following infection. This suggests that in *Drosophila* other signaling pathways, perhaps those involving cellular immune mechanisms, may be more important in defense against *Photorhabdus*. These studies begin to show how the combination of *Drosophila* genetics with the genetic manipulation of the entomopathogenic bacterium can increase our understanding of host-pathogen interactions in a gene-for-gene fashion.

***Photorhabdus* and cellular responses**

Following release of *Photorhabdus* into the hemolymph by the IJ nematode, the first response of the insect immune system is to phagocytose or encapsulate the invading bacteria [31]. Indeed, *Photorhabdus* infection in *Manduca* promotes the appearance of hemocytes with an extreme 'spreading' ability [32]. The presence of these spreading cells in response to *Photorhabdus*, as well as to other pathogens, is not a pathological effect of infection but a discrete reaction of the insect immune system. Evidence also suggests that these spreading cells might play a role in nodule formation. One type of cytokine involved in the hemocyte

spreading process is the Plasmatocyte Spreading Peptide or PSP. The PSP precursor protein, proPSP, is expressed in the fat body, but not hemocytes, in response to *Photorhabdus* challenge and RNAi-mediated knockdown of proPSP in both the fat body and hemolymph plasma significantly increases susceptibility to *Photorhabdus* and leads to a reduction in overall cellular immune response [33]. However, despite the fact that mRNA levels of *Hemolin*, *PGRP*, and several AMPs but not *IML-2* are increased in *Manduca* hemocytes during *Photorhabdus* infection [28], the role of these proteins in modulating hemocytic defenses is uncharacterized.

Previous work has also shown that upon *Photorhabdus* injection into caterpillars, the bacteria grow rapidly and they are able to persist within *Manduca* while the number, viability and phagocytic competence of hemocytes are dramatically decreased. Interestingly, *in vitro* incubation of hemocytes with *Photorhabdus* supernatants causes distinct changes in the actin cytoskeleton morphology of different hemocyte cell types [34]. Additionally, *Photorhabdus* can inhibit phospholipase A2 that catalyzes the first step of eicosanoid biosynthesis, which is important for hemocyte nodulation [35]. Together these results show that *Photorhabdus* evades the cellular immune response at least in part by killing hemocytes and by employing mechanisms that suppress key cellular insect immune functions. They also begin to describe how such a few bacterial cells can be used to modulate and eventually overcome the complexity and robustness of the insect immune system.

Avoiding phagocytosis by the insect hemocytes

There are several lines of evidence that *Photorhabdus* resists phagocytosis by insect hemocytes. Originally, a heat-stable anti-phagocytic moiety was documented in the supernatant of *P. luminescens* strain W14. This factor is produced during infection and cell-free hemolymph recovered from an infected caterpillar retained the anti-phagocytic activity

1 [36]. More recent advances have shown that *Photorhabdus* is also equipped with a number of
2 toxins and effectors, each capable of efficiently inhibiting phagocytosis (Figure 3).
3 Type III secretion systems (TTSSs) are found in numerous bacterial pathogens where they
4 deliver effector molecules to modulate the behavior of host cells. TTSSs deliver their effector
5 proteins directly into the cytosol of host cells and can either facilitate the uptake of bacteria or
6 prevent their phagocytosis. The LopT effector protein, encoded by the *P. luminescens* TT01
7 and W14 TTSS is homologous to YopT from *Yersinia* and inhibits phagocytosis [31,37]. A
8 LopT-like open reading frame is also found as a 'payload' region on one of the numerous
9 *Photorhabdus* virulence cassettes (PVCs). The PVCs are phage-like elements homologous to
10 the anti-feeding prophage from another insect pathogenic bacterium *Serratia entomophila*
11 [38]. The PVCs encode a structure similar to the R-type pyocins and might act as a delivery
12 system carrying various toxic payloads to target cells [39]. *P. luminescens* TT01 and *P.*
13 *asymbiotica* ATCC43949 contain numerous loci encoding PVCs with very different putative
14 effector proteins with homology to regions of: toxin A in *Clostridium difficile* (Mcf), YopT in
15 *Yersinia entercolitica* (LopT), the active domain of Cytotoxic Necrotizing Factor 1 or CNF1
16 from *Escherichia coli* and others, which have no obvious similarities and possibly represent
17 novel effectors [40]. Recombinant *E. coli* expressing PVCs are toxic to the Wax moth
18 *Galleria mellonella* when injected, and dramatically rearrange the actin cytoskeleton of
19 recovered hemocytes. Given the number and variety of the PVCs, they could confer toxicity
20 to different groups of insects, or in the case of *P. asymbiotica*, against mammals [39].
21 Makes caterpillars floppy 1 (Mcf1) is a toxin that destroys both the insect hemocytes and
22 the midgut by apoptosis [41]. Recently Mcf1 has also been observed to rapidly 'freeze'
23 *Drosophila* embryonic hemocytes, preventing their motility and ability to phagocytose
24 bacteria [42]. Several genetic mutants of *Drosophila* modulate this Mcf1 mediated response
25 showing that endocytosis of Mcf1 is necessary for 'freezing' and cytoskeletal rearrangement.

1 Similarly, signaling mutants of the small Rho GTPase, Rac, also modulate Mcf1 mediated
2 effects on *Drosophila* hemocytes, indicating a requirement for toxin internalization and early
3 Mcf1-mediated activity on the cytoskeleton. Studies are underway to determine whether the
4 anti-phagocytic ability of Mcf is upstream of apoptosis and is the eventual cause of
5 programmed cell death in hemocytes, or whether inhibition of phagocytosis and apoptosis are
6 separate phenomena.
7 Recently, combinations of Toxin complexes (Tc's) encoded by the *tcd* pathogenicity
8 island: TcdA1, TcdB2 and TccC3 or TccC5 have also been shown to cause alterations in the
9 actin cytoskeleton in Wax moth (*Galleria*) hemocytes inhibiting phagocytosis. It was
10 revealed that the components responsible for this activity were TccC3 and TccC5; with
11 TccC3 causing ADP-ribosylation of actin and TccC5 causing ADP-ribosylation of the Rho
12 GTPases RhoA and Rac resulting in their activation [43]. Both TccC3 and TccC5 enter the
13 cytosol via TcdA1 (observed to be an *in vivo* pore former) where they act together to disrupt
14 the actin cytoskeleton. TccC3 and TccC5 are active on both lepidopteran and human cells
15 suggesting that *Photorhabdus* may be able to use these effectors against both mammalian and
16 insect hosts and supporting the hypothesis that the Tc toxins may be virulence factors in
17 pathogens of man. A novel actin-targeting mono-ADP-ribosyltransferase (mART) toxin,
18 Photox, has also recently been discovered in *P. luminescens*, which inhibits the
19 polymerization of actin filaments [44]. This activity would have profound effects on the
20 cytoskeleton of the target cell, possibly resulting in the inhibition of phagocytosis as seen
21 with TccC3 the actin-targeting ADP-ribosylating Tc. The mechanism of entry of Photox into
22 the host cell and role in insect infection remains to be elucidated. However, the presence of a
23 neighboring gene encoding VgrG, which is involved in type VI secretion could indicate
24 delivery of Photox via this route. This section emphasises how *Drosophila* can be used as a

model to dissect the insect immune system and how we can use a model pathogen, *Photorhabdus*, to probe the system further.

***Photorhabdus* interaction with the PO cascade**

Phenoloxidase (PO) is an important component of the immune defences of most arthropods [45]. The enzyme is normally present as an inactive precursor, prophenoxidase (PPO). Activation is due to a serine protease cascade, which is initiated upon recognition of invading microorganisms, leading to proteolytic cleavage of PPO that is present in the hemolymph plasma, thus causing the synthesis of the pigment melanin and the production of melanotic nodules around microbes, thereby isolating the pathogens [45]. A characteristic of insects infected by *Photorhabdus* and other nematode-associated entomopathogenic bacteria is that their hemolymph does not melanize (blacken) upon bleeding, which implies an interaction between *Photorhabdus* and the PO cascade [31,35]. A major secreted product of *Photorhabdus* both *in vitro* and *in vivo* is a hydroxystilbene compound (ST) that inhibits the growth of microbial competitors in the dead insect [46]. ST is not only a potent inhibitor of activated insect PO, but PO inhibition also results in decreased host resistance to *Photorhabdus* [47]. Thus, PO inhibition during insect infection appears to be a specific adaptation of this bacterium to its pathogenic lifestyle. These results also denote a gene-for-gene interaction between ST production in the bacterium and PO synthesis in the insect host.

More recently, *in vitro* screens of *Photorhabdus* cosmid libraries led to the identification of overlapping cosmid clones that suppressed previously activated *Manduca* PO, reduced nodule formation, persisted longer within insects and showed increased pathogenicity towards *Manduca* larvae [48]. Finally, it has been reported that the metalloprotase PrtS isolated from *Photorhabdus* supernatants induces a melanization response upon injection in *Manduca* [49], which is in contrast with the suppression of melanization that is observed

during *Photorhabdus* infection. It is therefore possible that secretion of a PO inhibitor could counteract the activation response to PrtS or that PO activation and inhibition are temporally separated. Another possibility is that the role of PrtS is to attack proteins involved in cell adhesion and thereby block nodule formation around the proliferating bacteria, rather than interfering directly with PO activation.

Bacteria from several species of both *Photorhabdus* and *Xenorhabdus* have also been found to suppress melanization and nodule formation through inhibition of the enzyme phospholipase A2, blocking synthesis of the local eicosanoid signals that co-ordinate nodule formation and associated local PPO activation [35]. The chemical responsible for this inhibition has not been identified, however.

These results indicate a complex pattern of manipulation of phenoloxidase-based host defenses by the pathogen, implying that such defenses are likely important in protecting the insect host.

Photorhabdus destruction of the _Manduca_ gut

Probably the major group of toxins responsible for activity against the insect gut is the Tcs. The mature native toxin complex produced by *Photorhabdus* is a high molecular weight gut active toxin that is lethal to four orders of insects (Lepidoptera, Coleoptera, Hymenoptera and Dictyoptera) when injected into the hemolymph or orally ingested [50]. The mature complex consists of subunit Tcs: Tca, Tcb, Tcc and Tcd [50]. Tca is responsible for most oral activity against *M. sexta*, having a median lethal dose of 875 ng/cm^2 of artificial diet, and causing significant weight reduction at 40 ng/cm^2 [51]. Ingestion or injection of purified Tca alone causes an effect on the gut histopathology similar to that observed following *Photorhabdus* infection [52] (Figure 3).

1 The oral activity of the Tcs is unexpected given the route of attack via the cuticle and
2 hemocoel of the host larvae by the entomopathogenic nematodes vectoring *Photorhabdus* and
3 Tcs are now thought to be derived from ancestral *tc* genes acquired by *Photorhabdus* and are
4 normally employed as active toxins on the hemocoel side of the gut [53]. Oral toxicity to
5 insects has been achieved by cloning toxin complex component A (*tcdA*) from *P. luminescens*
6 strain W14 into *Arabidopsis* creating a transgenic plant capable of killing first instar *M. sexta*
7 [54]. Expression *tcdA* in recombinant *E. coli* produces oral toxicity at high expression levels,
8 but to reconstitute full oral toxicity components B and C (encoded by *tcdB* and *tccC*) are
9 needed [53].

10 Structural and biophysical studies have been carried out on *Xenorhabdus nematophila*
11 PMF1296 toxin complex component XptA1, equivalent to *P. luminescens* TcdA1 [55]. This
12 work has suggested a mechanism of action for XptA1 where it binds to the cell membrane
13 forming a structure with a central cavity and complexes with partner components XptB1 and
14 XptC1 producing the mature insecticidal toxin [55]. The structure of XptA1 was shown to be
15 a 1.15 MDa tetramer with a cage-like structure. Importantly, the same structure is found in
16 both alkaline and neutral pH environments, indicating that the toxin's structure can survive in
17 the highly alkaline midgut of the Lepidoptera host.

18 Other toxins with gut activity are the Mcf1 and Mcf2 toxins (Figure 3). Originally Mcf1
19 was discovered due to its ability to destroy the insect midgut rapidly via apoptosis, resulting
20 in a loss of body turgor at 12 h and insect death 24 h following injection with *E. coli*
21 expressing the toxin [41]. The Mcf1 homolog, Mcf2, is also known to induce insect death in a
22 similar manner, but this toxin's mode of action is still unknown. Differences in homology in
23 the putative active N-terminal regions of Mcf1 and 2 suggest the possibility of multiple
24 modes of action. This section emphasises the level of functional redundancy that has already
25 been found in *Photorhabdus* virulence factors. A growing list of virulence factor encoding

1 genes found in sequenced *Photorhabdus* genomes suggests that still further toxic actions

2 contributing to the suppression or evasion of host cellular immunity await discovery.

3

4 **Immune responses in the gut**

5 Following direct injection of *Photorhabdus* into the body cavity and successful suppression

6 of insect immune defenses, the pathogen grows excessively in the hemolymph and midgut,

7 and then subsequently colonizes the fat body and the remaining tissues of the cadaver. This is

8 considered a strategy that the bacteria employ in order to stop insect feeding and to avoid

9 attacks by hemocytes patrolling the hemocoel [1]. Midgut colonization in *Manduca* has been

10 found to be associated with occupation of a specific niche next to the basal lamina of the

11 extracellular matrix that surrounds the midgut epithelium itself [36]. It is unknown whether

12 *Photorhabdus* elicits a local immune response at the site of infection by provoking the

13 synthesis of AMPs in the midgut epithelium, as is the case with other pathogens [21], and if

14 so whether the bacteria can fight this immune reaction by protecting themselves from the

15 harmful effects of antibacterial peptides, or by degrading these effectors.

16 Finally, in relation to oral ingestion of *Photorhabdus* (a route not normally found in nature

17 as the bacteria are vectored directly into the insect hemocoel) it was recently shown that the

18 bacteria stimulate the expression of nitric oxide synthesis (NOS), an important component of

19 the insect immune system [56], exclusively in the gut of *Manduca* and that the induced NOS

20 expression plays an important, yet ultimately unsuccessful role in defending the insects

21 against the pathogen through the production of NO [57]. Preventing NOS induction in orally

22 infected insects by systemic RNAi or pharmacological manipulation reduces NO levels in the

23 gut and promotes crossing of the bacteria into the hemolymph through the gut wall, thereby

24 decreasing the survival of NOS deprived caterpillars. These results highlight two important

25 points. First, NOS is a major signaling component of the insect innate immune system by

contributing to pathogen resistance and, second, according to the location of the experimental infection, different organs play distinct roles in the response to *Photorhabdus*.

Making the leap to humans

Photorhabdus asymbiotica has been isolated from clinical infections in humans and is an emerging human pathogen [58]. The majority of clinical isolates recovered are currently from Australia and America. Invertebrates have previously been realized as a potential source of emerging human pathogens. In the case of *P. asymbiotica* it is hypothesized that following acquisition of key plasmids by insect pathogenic *Photorhabdus, P. asymbiotica* emerged equipped to cause human infection [59]. The genome sequence of *P. asymbiotica* strain ATCC43949, originally isolated from a human infection in North America, has recently been completed. Comparative genomics of this emerging human pathogen with the insect pathogenic *P. luminescens* strain TT01 reveals that *P. asymbiotica* ATCC43949 has a smaller genome than TT01 and has acquired a plasmid related to pMT1 from *Yersinia pestis,* the causative agent of the bubonic plague [5]. The *P. asymbiotica* ATCC43949 genome has a reduced diversity of insecticidal toxin encoding genes including those of the Tcs, Mcf and the PVCs. Despite all the toxin gene absences, *P. asymbiotica* ATCC43949 is in fact more lethal to insect hosts than *P. luminescens* TT01 or *P. temperata* K122 [29]. Many of the anti-insect virulence factors do indeed remain in the genome of *P. asymbiotica* ATCC43949 suggesting either that they are also active on mammalian immune systems or that *P. asymbiotica* cycles through insects and only infects man on an irregular or accidental basis.

P. asymbiotica is able to survive within and induce apoptosis in mammalian macrophages [60]. The acquired plasmid, *pAU1*, and additional virulence factors, including homologues of known effectors which facilitate intracellular persistence (e.g. SopB), are thought to hold the key to equipping *P. asymbiotica* ATCC43949 for activity against man. Draft sequencing of

1 the highly virulent Australian *P. asymbiotica* Kingscliff isolate has revealed some interesting
2 differences from the American sequenced strain. A key finding is the presence of an
3 additional plasmid, *pPAA3*. This plasmid bears similarity to *pCRY* from *Yersinia pestis*. It is
4 hypothesized that the presence of *pPAA3* could account for the increased virulence of
5 Australian isolates [61]. This illustrates the remarkable ability of bacteria to acquire the
6 necessary virulence factors from the 'pathosphere', the net pool of virulence factor encoding
7 genes that can be swapped between different bacterial species. This means that bacteria that
8 have learnt to overcome the insect immune system may acquire additional virulence factors
9 that allow them to extend their range to humans. The identification of the genes facilitating
10 this host switch is therefore a key area of current research.
11
12 **Concluding remarks and future perspectives**
13 We have reviewed recent advances in the study of the insect pathogen *Photorhabdus* and its
14 interaction with the immune system. The most striking conclusion is that originally
15 *Photorhabdus* was assumed to only interact with the immune system of its insect host but
16 now with stunning new details on the complex association with its partner nematode, it is
17 apparent that subtle interactions with the immune system of the nematode are also likely to
18 play a critical role in the *Photorhabdus* life cycle (Box 3). In the case of *P. asymbiotica* this
19 interaction is now further extended to three hosts: the vector nematode, the host insect and
20 man. An Australian isolate of *P. asymbiotica* [61] was recently sample sequenced in order to
21 understand why isolates from Australia appear more pathogenic to man than those from the
22 USA. We therefore believe that detailed comparative genomics between different
23 *Photorhabdus* strains with different life cycles, informed by functional analysis with Rapid
24 Virulence Annotation [62], will allow us to begin to understand how this insect pathogen has
25 made the leap to humans.

1

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Figure legends

Figure 1. The life cycle of the *Photorhabdus-Heterorhabditis* complex. The *Heterorhabditis* IJ is the only free-living stage of the nematode and all stages of nematode reproduction and development (including the larval molts L1, L2, L3 and L4) take place within the insect cadaver in the presence of high titers of *Photorhabdus* bacteria (see text for details).

Figure 2. *Photorhabdus* in the rectal gland cells of an adult hermaphrodite *Heterorhabditis* nematode. The adult nematode was incubated with green fluorescent protein (GFP)-labeled *P. luminescens* TT01 for 5 days before being transferred to non-GFP-labeled *P. luminescens* TT01 cells for a further 1-2 days. The nematodes were then mounted on agar pads (with levamisole added as an anesthetic) and analyzed using a Zeiss LSM 5 Exciter microscope. GFP-labeled bacteria can be clearly seen colonizing the rectal gland cells found near the anus (indicated by an 'a') of the adult hermaphrodite nematode. Images courtesy of Catherine Easom and David Clarke, University College Cork, Ireland.

Figure 3. Interactions of *Photorhabdus* toxins with the insect immune system. This diagram of an insect larva shows the known immune system targets of *Photorhabdus* toxins. Toxin complex components TccC3 and TccC5 from the Tcd pathogenicity island act to ADP-ribosylate either actin or Rho GTPases respectively, blocking the ability of hemocytes to phagocytose. The PVCs destroy insect hemocytes, but the mechanism is currently unknown. Toxin complex Tca causes midgut destruction, interestingly the Tca pathogenicity island does not contain a TccC homolog. One of the toxins, Mcf1, has a three-fold activity on the insect immune system acting to block phagocytosis (mechanism unknown at present), and destroy hemocytes and the midgut epithelium via apoptosis. A newly discovered *Photorhabdus* toxin Photox is known to ADP-ribosylate actin and might putatively be capable of interfering with

phagocytosis, although the properties of this toxin in insect infection have yet to be
confirmed.

Box 1. Advantages of using *Photorhabdus* and insect models for studying host immune responses

- Unlike many animals associated with bacterial symbionts, *Heterorhabditis* nematodes are viable in the absence of *Photorhabdus*. Thus, each partner of the symbiotic/pathogenic relationship can be separated and studied in isolation or in combination, thus enabling pathogenesis and symbiosis to be studied individually or together.
- All three players of the interaction (*Drosophila* or *Manduca – Heterorhabditis – Photorhabdus*) can be genetically manipulated. RNAi in the insect and nematode combined with bacterial genetics can be readily applied to investigate the molecular basis of symbiosis and parasitism and interaction with immune mechanisms.
- The genomes of *Photorhabdus luninescens* (strain TT01), *Photorhabdus asymbiotica* (strain ATCC43949) and twelve *Drosophila* species have been sequenced[15,16], and the genome of *Heterorhabditis bacteriophora* is currently being sequenced.
- *Drosophila* and *Manduca* are cheap and have a short life cycle, they are easy to handle and rear in the laboratory, and their size facilitates artificial infections and extraction of hemolymph or hemocytes.
- *Photorhabdus* vector, *Heterorhabditis* nematodes, are of growing interest as potential models for human parasitic nematodes and as biocontrol agents for insect pests and disease vectors. They also offer useful features as model organisms, including small size, short generation time, hermaphroditism, and *in vitro* culturing, and they are closely related to *Caenorhabditis elegans* and some mammalian parasitic nematodes.

Box 2. What can we learn from *Photorhabdus*-insect host interactions?

Research on *Photorhabdus* interactions with insect host immune responses will undoubtedly enlighten our understanding of the molecular processes that distinguish beneficial and harmful interactions. In particular:

1. It will shed light on the mechanisms used to fight bacterial infections in other holometabolous insects that have dramatic repercussions on human life as agricultural pests or as vectors of diseases.
2. Characterization of the pristine host defense against *Photorhabdus* infection in insect models may reveal novel evolutionary conserved immune pathways in mammals.
3. Original immune responses to *Photorhabdus* found in host insect models may guide the development of new antibacterial therapeutics.
4. Given that *Photorhabdus* is a member of the Enterobacteriaceae, it is likely that this research will also contribute to similar studies with important mammalian pathogens such as *E. coli* and *Salmonella* spp.
5. It could have considerable potential in biological control and agriculture. Since entomopathogenic bacteria represent an alternative to chemicals for insect pest control, it is fundamental to understand the basis of the infectivity of nematode-bacteria complexes and the interaction with the insect immune system.
6. It is well established that vertebrates possess many beneficial associations with bacteria, which have been shown to provide both nutritional and defensive advantages. Research that uses invertebrate models can be used to elucidate the role of such bacteria in human and animal health.
7. *Photorhabdus* maintains a species-specific interaction with its cognate nematode host, yet can kill an extensive range of insect species, making it an attractive model for understanding the molecular basis of host range and the role of host immune function.

8. *Photorhabdus* is a vectored pathogen and so it can provide insights into the transition from one host environment to another and how host immune mechanisms regulate the transmission.
9. Finally, this research will potentially uncover mechanisms that ensure persistence and transmission of bacteria and might lead to the development of strategies for blocking the dissemination of pathogens and thus preventing infectious diseases.

Box 3. Questions for future research

- How exactly *Photorhabdus* interacts with either the nematode or the insect host?
- Which *Photorhabdus* genes are important for the transition between nematodes and insects and how the bacteria sense the host-to-vector transition?
- What is the genetic overlap between *Photorhabdus* pathogenicity and symbiosis?
- What is the mechanism of *Photorhabdus* detection by the insect immune response?
- How insects combat *Photorhabdus* and *Heterorhabditis* infections and which are the similarities/differences between these immune responses?
- Are these immune mechanisms common between different insect hosts?
- How the bacteria cope with the *Heterorhabditis* immune system?
- What is the minimum number of *Photorhabdus* cells that allows nematodes to grow and infect insects efficiently?
- How *Photorhabdus* contributes to *Heterorhabditis* development and reproduction?
- What is the range of secondary metabolites the bacteria produce to protect their host from competitor microbes?

Figure 1

Figure 1

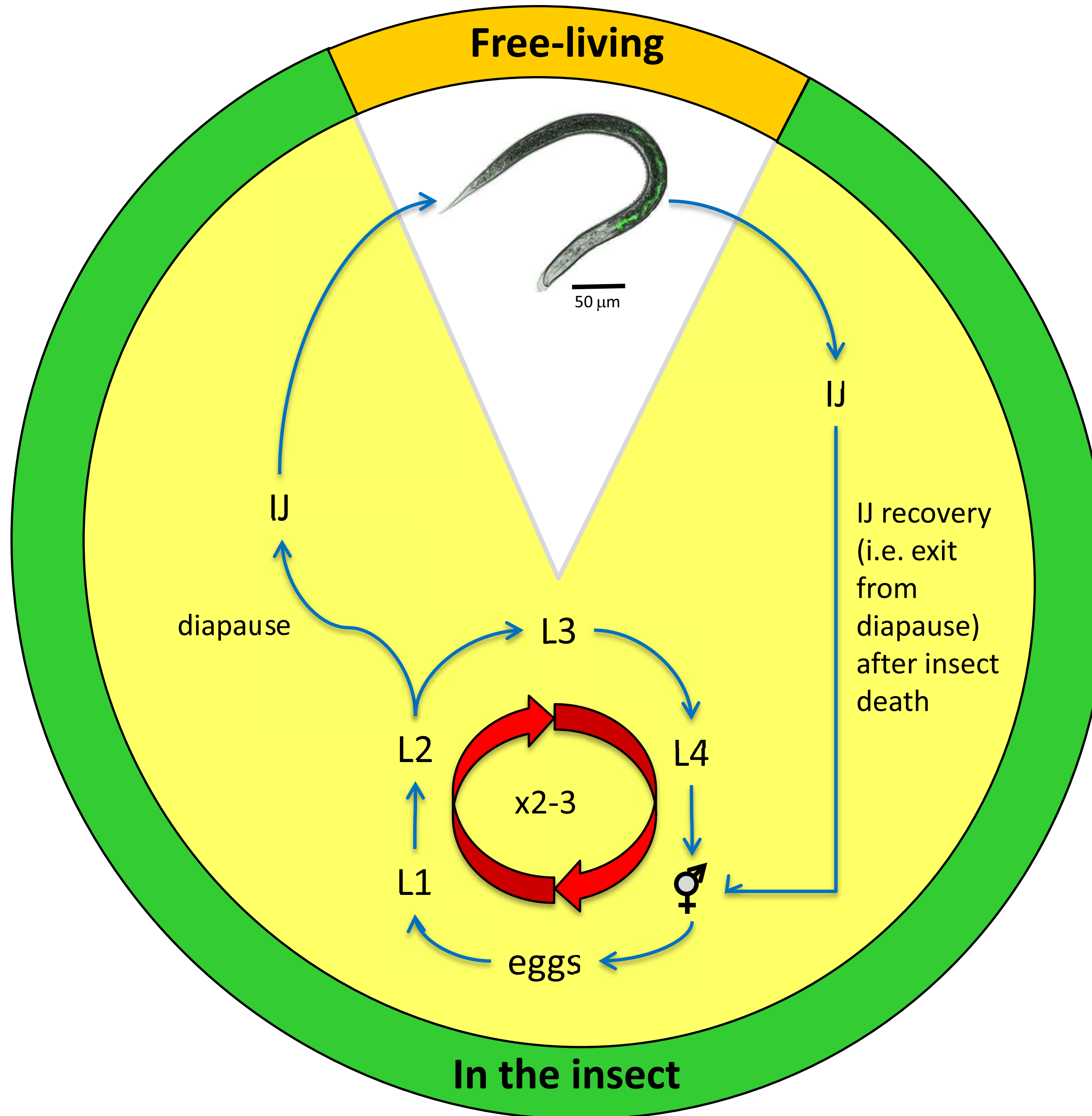

Figure 2

Figure 2

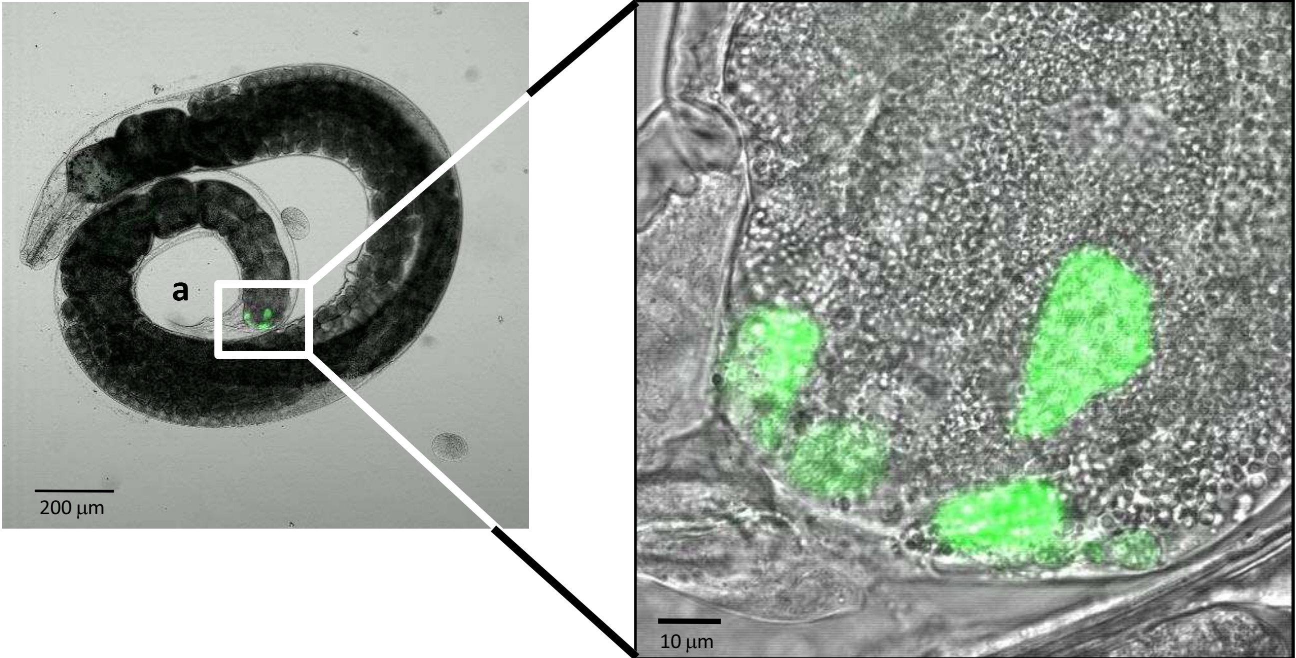

Figure 3

Figure 3