

# Identifying Anti-host Effectors in *Photorhabdus*

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**Abstract** The death of the insect host is an essential part of the life cycle of *Photorhabdus*, and as a result, this bacterium comes equipped with a dazzlingly large array of toxins and virulence factors that ensure rapid insect death. Elucidation of the key players in insect infection and mortality has therefore proved difficult using traditional microbiological techniques such as individual gene knockouts due to the high level of functional redundancy displayed by *Photorhabdus* virulence factors. Thus, knockout of any individual toxin gene may serve to delay time to death but not to render the bacteria avirulent due to the continued presence of an array of other toxins and virulence factors in the single-gene mutant. This functional redundancy had led to the necessary development of an array of techniques and new model systems for identifying and dissecting apart the action of anti-insect effectors produced by *Photorhabdus*. These have been pivotal in both the identification of new toxins and virulence factors and in ascribing functions to them. These techniques have gone on to prove valuable in pathogenic bacteria other than *Photorhabdus* and are likely to be useful in many others.

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## 1 Introduction

*Photorhabdus* bacteria have a complex multi-host lifestyle in which they must switch from being a nematode mutualist to a potent insect pathogen. Insect death is a critical part of the life cycle and as such *Photorhabdus* produces a staggering array of anti-host effectors, those characterized so far include toxins, adhesins, enzymes, secondary metabolites and secretion systems amongst others (see also Chapter by Hapeshi and Waterfield in this volume). The discovery and elucidation of the anti-host effectors are not only important in understanding how *Photorhabdus* bacteria may interact with and infect insect and mammalian hosts but it also makes them an attractive source for novel insecticides and drugs. However, the discovery and understanding of such anti-host effectors via classical microbiology (single-gene knockouts) are exacerbated due to the issue of functional redundancy. Mutating a single toxin gene in *Photorhabdus* often results in very little or indeed no attenuation in pathogenicity, as there are a host of other toxins and effectors that compensate and insure death of the host following infection with a single-gene knockout. This has led to the development of several novel and innovative screening assays designed to circumvent functional redundancy and identify anti-host effectors on a genome-wide scale *in vivo*. A number of bioinformatic-based *in silico* techniques have also identified toxins and novel secretions systems from *Photorhabdus* that have biological activity. This chapter will review these approaches and discuss future directions for this research.

## 2 A Forward Genetics Approach

Single-gene knockouts in predicted toxins are often ineffectual in significantly reducing the pathogenicity of *Photorhabdus* due to a high level of functional redundancy. In order to circumvent this problem and elucidate the masked action of individual anti-insect effectors, a simple ‘forward genetic’ (gain of function) approach and the use of a model insect system for screening were developed. This original approach initially involved generating a cosmid library of *P. luminescens* subsp. *akkhurstii* strain W14 in *Escherichia coli* with each cosmid clone in *E. coli* containing an insert of ~40 kb of *Photorhabdus* genomic DNA. Cosmids were arrayed into 96-well micro-plates in order to give ~10X genomic coverage. Single

*E. coli* clones carrying individual cosmids were then injected separately into caterpillars of the tobacco hornworm, *Manduca sexta*. Laboratory strain *E. coli* is normally cleared successfully by the *M. sexta* immune system; however, one in every 300 of the cosmids injected caused the recipient caterpillar to lose turgor 12 h post-injection and subsequent insect death occurred at 24 h (Daborn et al. 2002). Insertional (transposon) mutagenesis of the associated cosmid revealed that a single 8.8 kb gene was responsible for this phenotype that was then termed *makes caterpillars floppy* or the *mcf* gene. The encoded Mcf protein toxin (324 kDa) facilitates the persistence of *E. coli* in the insect haemocoel and causes massive cell death via apoptosis of both haemocytes and midgut cells resulting in insect mortality.

Random end sequencing of the *P. luminescens* W14 cosmid library used in this screen identified a cosmid containing sequence which assembled to that of *mcf* but displayed clear differences at the nucleotide level. This newly identified homologue was named *makes caterpillars floppy 2* or *mcf2*, and correspondingly, *mcf* was renamed *mcf1*. *E. coli* expressing the Mcf2 toxin display similar insect toxicity to Lepidopteran larvae as Mcf1 (Waterfield et al. 2003).

The *mcf2* gene predicts another large (262 kDa) protein, and like Mcf1, amino acids 1015–1548 of Mcf2 are 39% similar and 20% identical to amino acids 867–1368 of *C. difficile* toxin B, but Mcf2 lacks the similarity to the C-terminus of an RTX-like toxin from *Actinobacillus pleuropneumoniae* carried by the C-terminus of Mcf1. Since the original description of the Mcf1 gene, a new entry in GenBank, shows that both Mcf1 and Mcf2 also show a second region of homology to an RTX-like cytotoxin (gene VVA1030) from *Vibrio vulnificus* strain YJ016, amino acids 695–914 of Mcf2 are 30% identical and 48% similar to amino acids 3250–3496 of VVA1030. The predicted N-terminal domain of Mcf2 is shorter than that of Mcf1 and lacks a BH3-like domain. Instead, the N-terminus carries a region with significant similarity (54% similarity and 40% identity) to the C-terminus of the plant avirulence protein HrmA from *P. syringae* pv. *syringae*. Further examination of unfinished genome sequences shows that Mcf1 and Mcf2 are part of a family of toxins including two undescribed homologues from *Pseudomonas fluorescens* strain Pf01, ORFs 4315 and 4316, which are 28% identical and 46% similar to each other. Domains containing *mcf* sequence are also present in the MARTX toxin family (Fullner Satchell 2007; Roig et al. 2011).

## 2.1 Rapid Virulence Annotation

One species of *Photorhabdus*, termed *P. asymbiotica*, is a pathogen of both insects and mammals. Originally isolated from a human infection, and with no nematode present, it was initially presumed to be literally ‘asymbiotic’, hence the name ‘*asymbiotica*’. However, a nematode vector for *P. asymbiotica* has since been found (Gerrard et al. 2006). For a detailed description of *P. asymbiotica*, see chapters by John Gerrard also in this volume. Analysis of *P. asymbiotica* therefore

provides a unique opportunity to identify and explore virulence factors which are specific for either insect or mammalian hosts, or indeed active against both (see also Chapter by Hapeshi and Waterfield in this volume).

The success of the forward genetic approach in revealing toxins otherwise disguised due to functional redundancy led to the pioneering Rapid Virulence Annotation (RVA) approach developed by Waterfield et al. (2008). In this approach, parallel assays were used to find effectors in a cosmid library of *P. asymbiotica* ATCC43949 with toxicity against a range of taxa: insects (*M. sexta* and *Galleria mellonella*), amoebae (*Acanthamoeba polyphaga*), nematodes (*Caenorhabditis elegans*) and mouse macrophages (J774-2 murine cell line). This approach allows us to detect effectors with species specific or broad-spectrum activity of individual clones that confer a gain of toxicity (GOT) to the library strain *E. coli* expressing them. Assembling the end sequences of clones that confer toxicity onto the *P. asymbiotica* genome is used to identify the virulence-related regions. The multiplicity of genome coverage within the library (~10X) means that when the end sequences of the positive cosmids are aligned, they generate overlapping clusters (minimum of two overlapping cosmids) allowing the identification of the minimum genomic region of interest (ROI) carrying the candidate effector ORFs denoted as RVA regions (Fig. 1). Twenty-one RVA regions were identified in the *P. asymbiotica* genome, and these included the re-identification of known virulence factors such as the toxin Mcf1. These assays revealed activity of Mcf1 against both *C. elegans* and amoebae, in addition to its known activity against insects and mammalian tissue culture cells, indicating a broad mechanism of action (Daborn et al. 2002; Dowling et al. 2004).

The other twenty RVA regions contained gene clusters and predicted functions related to: Type III secretion, hemolysins, hemagglutinins, *Photorhabdus* Virulence Cassettes (PVCs), production of secondary metabolite small molecules (non-ribosomal peptide and polyketide synthetases), Type VI secretion, lipases, enterotoxins, fimbrial operons, rtxA toxin homologues and several containing genes of unknown function.

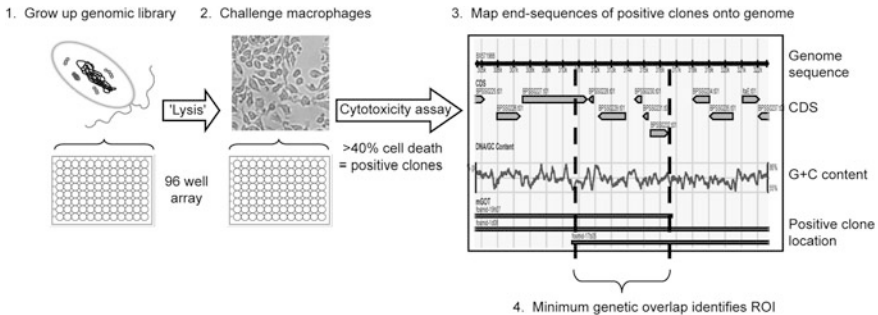
## 2.2 Secondary Metabolites as Virulence Factors

*Photorhabdus* contains a large number of gene clusters associated with the synthesis of secondary metabolites, the functions of many of which are unknown. A third of the RVA clusters identified contain polyketide synthetases (PKS) and/ or non-ribosomal peptide synthetases (NRPS) gene clusters, including a yersiniabactin-like compound, a virulence factor of *Yersinia pestis* causative agent of the black plague (Bearden et al. 1997). The large number of RVA regions carrying these clusters provides a strong link between certain secondary metabolites and a previously unknown role in *Photorhabdus* virulence. For more detailed references on secondary metabolites, see also chapters by Helge Bode and David Clarke in this volume.

### 2.3 Host Specificity

Several regions encoding putative lipase genes (*pdl*) were also identified in these RVA screens. These regions were active against insects and nematodes but not on murine macrophages, suggestive of host specificity. However, the majority of RVA regions active against macrophages were also active against insects indicating that factors active against the insect are also capable of effect on mammalian hosts. Fine scale mapping using insertion (transposon) mutagenesis is used on positive cosmids to directly identify the genes responsible for the phenotype. The mutants can then be rescreened in the desired assay to look for clones displaying a loss of function. These clones can then be sequenced in order to identify the gene/s responsible for the activity. Cloning the candidate gene into *E. coli* and retesting this clone in the requisite assay are then carried out in order to confirm activity.

The unbiased nature of this approach allows for the identification of genes that would not otherwise be identified as virulence factors. Insertional mutagenesis of cosmids from RVA17 that were toxic to insects, nematodes and macrophages revealed that virulence was linked to expression of a *kdp* operon encoding a Kdp potassium pump. Kdp systems are involved in potassium homeostasis perhaps making this an unexpected candidate virulence factor. However, follow-up studies have revealed that this KdpD/KdpE two-component system enables library strain *E. coli* to persist within insect haemocytes, eventually leading to mortality and causes a dramatic increase in phagocytic activity in murine macrophages (Vlisidou et al. 2010; Dowling and Hodgson 2014).



**Fig. 1** Workflow of a typical RVA-type screen for anti-phagocyte effectors. A genomic library of the strain of *Photorhabdus* of interest is made in cosmids or fosmids and transformed into library strain *E. coli*. Individual clones are arrayed in a 96-well plate format to achieve ~10X coverage of the genome and are then end-sequenced. Library plates are grown up, and crude lysate preparations of these are applied to phagocytes (macrophages or haemocytes) similarly arrayed in a 96-well plate. These are co-incubated for the desired length of time before assaying for cytotoxicity. Clones causing >40% cell death are called as 'positive', and the end sequences for these are assembled onto the genome sequence of the relevant *Photorhabdus* strain. Any genomic region with two or more overlapping clones mapped across it is designated a region of interest for further follow-up

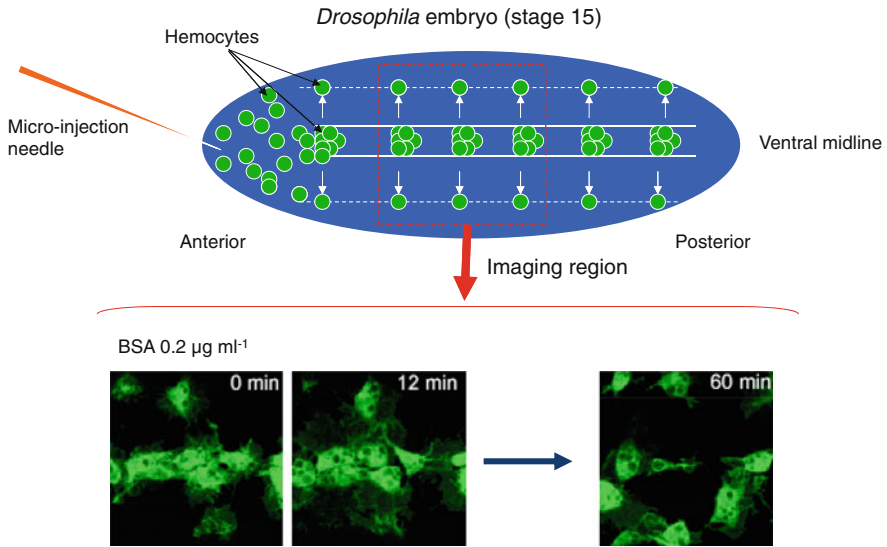
This approach has further untapped potential, and the capacity for detection of effectors can easily be expanded with screens and assays designed to look for effectors, not simply involved in toxicity, but those involved in other pathogenicity traits such as adherence, invasion and intracellular survival.

### 3 The *Drosophila* Embryo System

Once an effector is identified, it is then necessary to establish its function and mode of action in infection. *Drosophila* are a well-established powerful genetic model organism used to study microbial infections. In vivo studies are largely based on end-point analysis and a system in which infection can be studied in real time is therefore extremely useful for a continuous view of infection. The *Drosophila* embryo system, previously established as a model for studying development and wound repair, has also been developed as a tool which allows visualization of the early stages of infection and toxin action in real time using confocal time-lapse microscopy (Wood et al. 2006; Vlisidou et al. 2009). By using a combination of *Drosophila* genetics to look at different host genetic backgrounds and time-lapse confocal microscopy to follow bacterial infection in real time, it is possible to see how different mutant bacteria (or recombinant *E. coli* armed with *Photorhabdus* toxins) perform against different host genetic backgrounds. Micro-injection is used to introduce a fluorescently labelled microbe of interest, or purified toxin, into a recombinant *Drosophila* embryo with haemocytes expressing a fluorescent protein such as GFP. Embryonic haemocytes are motile macrophage-like cells that migrate throughout the developing embryo following stereotypical routes to disperse from their point of origin to eventually distribute themselves equally throughout the insect (Fig. 2) (Wood et al. 2006; Wood and Jacinto 2007). These haemocytes are dynamic, phagocytically competent and able to recognize and engulf non-pathogenic *E. coli*. When injected with the *P. asymbiotica* ATCC43949, the embryonic haemocytes are observed to undergo rapid paralysis or ‘freezing’. This phenotype could be reproduced by injection *E. coli* expressing Mcf1 or by injection of the purified toxin.

*Drosophila* genetics was then used to screen for mechanism of action. Mcf1 had previously been identified as requiring internalization via endocytosis in order to exert its pro-apoptotic activity (Dowling et al. 2007), and by using a mutant deficient in (clathrin-dependent) endocytic machinery (*shibire<sup>ts1</sup>* mutant), we were able to confirm that these haemocytes were unaffected by Mcf1 and did not freeze, meaning that internalization was also necessary for this phenotype.

The rapid effect on the hemocyte cytoskeleton led to the hypothesis that Mcf1 may be acting on a pre-existing eukaryotic molecular switch involved in cytoskeletal dynamics within the cell such as the Rho GTPases. Micro-injection of *Drosophila* mutants expressing dominant negative or constitutively active small GTPase Rac revealed that these haemocytes also failed to freeze in response to Mcf1. This indicates a need for the presence of wild-type Rac in order for the



**Fig. 2** The *Drosophila* embryo system. Stage 15 *Drosophila* embryos with haemocytes expressing a fluorescent protein such as GFP are immobilized on a microscope slide prior to anterior micro-injection with the bacteria or purified protein of interest. Z-stack time-lapse confocal microscopy is used to image the activity of the haemocytes post-injection in order to monitor their subsequent behaviour phagocytosis and observe the progress of the infection in real time

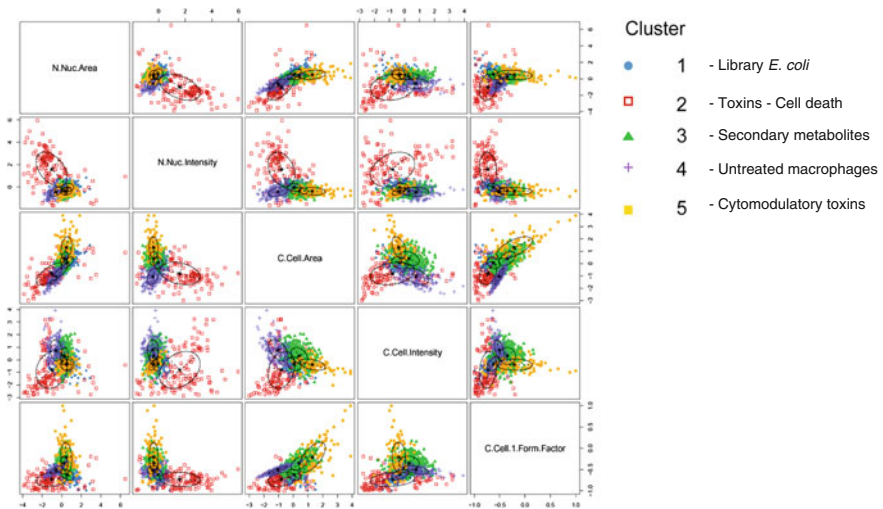
freezing process to occur. Several pathogenic bacteria produce toxins that inactivate Rho GTPases in order to avoid phagocytosis, known as RhoGAPs (Aktories 2011; Popoff 2014). However, whether Mcf1 is acting as a RhoGAP remains to be confirmed. This screening method is a powerful tool for identifying effector mode of action *in vivo*, and *Drosophila* genetic mutants present a valuable method of clearly dissecting many aspects of function in order to elucidate toxin mode of action and likely role in infection. Using *Drosophila* genetic mutants, we were able to determine that Mcf1 required internalization in order to exert activity and that the freezing phenotype was likely caused by Mcf1 acting on Rac.

## 4 Immune Cell Phenotype Screen

Although a cytotoxicity screen can identify effectors, it does not point us in the direction of how it is exerting effect on host cells. Typically visual analysis methods, such as fluorescence microscopy, are used to explore bacterial effector interaction with host cells. The original RVA screen for anti-macrophage factors from *P. asymbiotica* produced a wealth of candidates requiring further investigation to identify function (23 regions of interest covered by over 80 cosmids). Follow-up of this number of putative effectors would be very labour-intensive and

time-consuming to follow up on a case by case basis. In order to address this, we developed a novel genome scale morphology-based analysis method to functionally group the bacterial effectors (Dowling and Hodgson 2014).

Numerous functions and putative mechanisms of action can be characterized based on cell morphology, e.g. apoptosis (cellular and nuclear shrinkage followed by fragmentation and formation of apoptotic bodies), necrosis (cell swelling, leaky membranes), pyroptosis (cell swelling and significant cell size increase, rupture, nuclear condensation but integrity remains) and alteration in actin cytoskeleton, such as formation of stress fibres or depolymerisation, indicating activity on Rho GTPases (Gruenheid and Finlay 2003; Duprez et al. 2009; Lamkanfi and Dixit 2010; Aktories 2011). The targets of many bacterial pathogens effectors are the actin cytoskeleton and the nuclei where they manipulate or usurp normal cellular function or induce cell death to their advantage in establishing infection. The analysis uses high-content image analysis to visualize and quantify the morphological alterations induced in macrophages in response to treatment with preparations from the cosmid library clones. Phenotypes induced by the individual clones were quantified by measuring the morphology, staining intensity, and spatial attributes of the cellular cytoskeleton and nuclei. Application of statistical multivariate analysis to multiple cellular measures obtained from the treated macrophages revealed that we could group macrophage response based on phenotype. Five significantly distinct phenotype clusters linked to effector function were discovered (Fig. 3). Two of the clusters described control library *E. coli* lysate-treated



**Fig. 3** Typical output from a multivariate cluster analysis screen. Combining high-throughput high-content analysis microscopy as typically employed in pharmaceutical drug discovery. Plots are being made using morphometric measures in order to reveal and group distinct phenotypic responses from cells treated with individual *Photorhabdus* cosmid library clones. Here, five clusters in phenotype space are based on measures of two nuclear parameters: area and intensity and three cell parameters: area, intensity and form factor



macrophages and untreated macrophages, representing the normal cellular morphology. Three of the clusters describe alterations in cell morphology that link effectors with putative function. Cluster 2 describes nuclear and cytoplasmic condensation and collapse of the actin cytoskeleton. This cluster groups clones from regions of interest that include known toxins: Mcf1, toxin complexes (Tcs), XaxAB, along with putative RtxA-like toxins and Type VI secretion system amongst others. The grouping of Mcf1 into this cluster acts here as a 'phenotypic hallmark' indicating that the other candidate effectors in this group are likely to be highly cytotoxic, possibly pro-apoptotic toxins also. The cellular characteristics induced by effectors described by Cluster 3 are increased nuclear and cell size. The dominant predicted function of candidate effectors grouped into this cluster is linked to bacterial adhesion to the host cell and intracellular survival, including adhesins and hemagglutinins. However, Cluster 3 also contains candidates associated with synthesis of secondary metabolites, indicating that small molecules may play an important role in usurping macrophage function during *Photorhabdus* infection.

Interestingly, during the infection cycle of *P. asymbiotica*, both evasion of phagocytosis and intracellular survival play an important role (Costa et al. 2010). In order to ascribe phagocytic function to phenotype cluster, an additional screen was developed to detect alterations in macrophage phagocytosis in response to treatment with positive library clone preparations. Importantly, this screen found that pro-phagocytic and anti-phagocytic effectors belong to different clusters. Pro-phagocytic candidates belong exclusively to Cluster 3, and anti-phagocytic candidates are found in clusters 2 and 5. The most significant decrease in phagocytosis was caused by treatment with preparations containing PNF (*Photorhabdus* necrosis factor) (ROI 19, Cluster 5) and Mcf1 (ROI 20, Cluster 2). The most significant increase in phagocytosis is linked to ROI 14 that encodes a Kdp potassium transport system and a MACPF-domain perforin-like protein. As mentioned previously, the Kdp system from *P. asymbiotica* has been linked with intracellular survival in haemocytes (Vlisidou et al. 2010).

Potassium is considered to be one of the key regulators of cell response to pore formation. The cytosolic drop in K<sup>+</sup> normally associated with pore forming is associated with a number of different effects: autophagy, translation arrest, and the activation of MAPK pathways and proteolytic cascades (Bischofberger et al. 2012). Potassium is also important in the maturation of the phagolysosome important for killing engulfed microbes. The effectors contained within this ROI may play a role in the intracellular life stage of *P. asymbiotica*, but this is yet to be confirmed.

However, the mechanism by which the Kdp system and/or perforin is facilitating intracellular survival or promoting phagocytosis remains unclear.

Intriguingly, several cosmids were identified that contained *mcf1* and a tightly linked downstream NRPS, whereas others contained only *mcf1* or the downstream NRPS, indicating that NRPS gene product has a cytotoxic activity of its own. Treatment of macrophages with a combination of lysate preparations from two cosmids containing *mcf1* alone or NRPS alone caused increased cytotoxicity and dramatic cytoskeletal alterations compared to the activity of lysates from cosmids

carrying just one or the other. Further, injection of cosmids expressing both into *Galleria mellonella* larvae caused higher mortality than injection of cosmids carrying either *mcf1* or the NRPS alone.

## 5 *In Silico* Screens

Bioinformatic tools and motif searching have also been used successfully to reveal novel toxins in *Photorhabdus* taking advantage of the complete genome sequences available for several strains.

### 5.1 *Photox—A Toxin Unique to P. luminescens*

A novel mono-ADP-ribosyltransferase targeting toxin or ‘mART’ toxin was identified from *Photorhabdus luminescens* TT01 using a bioinformatic approach involving searching the genomic threading database using a shared core structure sequence identity (SCOP code) designed to identify new putative mART toxins (Visschedyk et al. 2010). As sequence identity between mARTs is low, the technique looks at both the primary amino acid sequence in order to identify key catalytic regions and the predicted fold pattern. A gene encoding a predicted protein of 45.9 kDa, *plu0822*, with significant identity to known mART toxins was discovered. Purified Photox possesses relatively high ADP-ribosyltransferase activity, and it specifically targets Arg<sup>177</sup> of actin preventing actin polymerization. Expression of Photox in yeast cells caused a severe defect in growth indicating high toxicity of the protein. Photox has a predicted 185 aa ‘disordered’ N-terminal region of unknown function, with no homologues identified using BLAST. It is hypothesized that this N-terminal region may potentially be involved in entry into the host cell. However, the precise role of this toxin during *Photorhabdus* infection currently remains unclear.

### 5.2 *PaTox—A Toxin Unique to P. asymbiotica*

Bio-active domain-focused bioinformatic analysis has been used successfully in several cases to identify novel *Photorhabdus* effectors. The *P. asymbiotica* toxin PaTox was discovered by using basic local alignment search tool (BLAST) analysis to look for sequence motifs (DxD) from known glycosylating toxins within the genome (Jank et al. 2013). A gene PAU\_02230 unique to *P. asymbiotica* was found encoding a putative protein toxin of ~3 kb with a potential C-terminal glycosyltransferase domain, and further analysis revealed a downstream region with 68% sequence similarity with the *Salmonella* virulence factor Sse1, whose function is

unknown. The putative toxin was named *P. asymbiotica* toxin 'PaTox'. Injection of full-length recombinant purified PaTox protein into *G. mellonella* larvae resulted in 100% insect mortality within ~3.5 days. Disruption of the DxD motif resulted in >60% of survival. Further, delivery of the glycosyltransferase domain into J774 murine macrophages and HeLa cells using anthrax protective antigen (PA) resulted in phagocytic block and breakdown of the actin cytoskeleton. Again, disruption of the DxD motif attenuated these activities proving the essential role of this domain in cytotoxicity. PaTox targets the small GTPase Rho, a target for several known bacterial effectors and toxins. Additionally, the SseI-like domain deamidates heterotrimeric G proteins as discovered upon testing a PaTox mutant deficient in DxD but containing SseI on HeLa cells the significant formation of stress fibres was observed indicating activation of RhoA. Therefore, PaTox is an AB-like toxin with two C-terminal catalytic domains and an N-terminal domain that promotes receptor translocation and enters host cells via endosomes. Indeed, tyrosine GlcNAcylation is detected during *P. asymbiotica* infection of insects but not during *P. luminescens* infection; PaTox is unique to *P. asymbiotica* and is likely responsible for this phenotype. However, again the exact role of this toxin in the infection process in human/ mammalian infection has yet to be elucidated.

### 5.3 *The Photorhabdus Virulence Cassettes*

Analysis of the complete genome sequences of *P. asymbiotica* ATCC43949 and *P. luminescens* TT01 revealed a noticeably significant number of prophage-like loci throughout the genomes of both species. These were initially recognized due to their similarity to a prophage-like locus in the pADAP plasmid from the insect pathogen *Serratia entomophila*. The pADAP plasmid is responsible for causing 'amber disease' in New Zealand grass grubs caused by homologues of the *tcs* (*sepA*, *B* and *C*); alongside this, the prophage-like locus is responsible for a separate anti-feeding effect highlighting such prophage-like loci as playing a role in pathogenesis (Hurst et al. 2004). The pADAP prophage-like locus is divided into two regions, one contains 18 putative ORFs predicting high similarity to phage tail and base plate proteins linked to a second containing a predicted putative effector protein thought to be responsible for the anti-feeding phenotype. The prophage-like loci from *Photorhabdus* were identified as they have the same genomic organization as the pADAP prophage-like locus, with a conserved phage-like unit but with numerous different putative effector domains, termed the *Photorhabdus* Virulence Cassettes (PVCs) (Yang et al. 2006). Analysis of the putative effectors revealed similarity to sections of known toxins including Mcf1, the active site of cytotoxic necrosis factor CNF1 from *E. coli* (Pnf) amongst others; further, several have no similarity to known toxins and possibly represent novel effectors. In order to test, toxicity towards insects was tested by injecting *E. coli* carrying PVC-expressing cosmid clones into *Galleria mellonella*. All PVCs from *P. asymbiotica* were found to cause high levels of mortality, whereas intriguingly mortality was much less

pronounced in those injected with *E. coli* expressing PVC carrying cosmids from *P. luminescens* (Yang et al. 2006). The PVCs represent a very interesting novel group of effector delivery systems with much to be elucidated about how they secrete and target and their effectors into host cells.

## 6 Future Approaches

In the quest to understand both *Photorhabdus* biology and discover novel anti-host effectors with the potential for use in agriculture, there are several technological advances and experimental avenues of particular interest. The variety of screening efforts to date has identified a wealth of effector proteins and molecules from *Photorhabdus*, the detailed activity of many of which remain to be elucidated. Transcriptomic analysis of *Photorhabdus* gene expression using RNA-Seq represents a very promising approach for understanding gene expression in response to certain environmental conditions, and some headway has been made using this approach in vitro (Mulley et al. 2015). Looking at gene expression in vivo as a function of host infection will be a valuable tool in understanding not only the anti-host effectors involved in usurping insect immunity, but also when they are deployed over the course of infection.

Finally, target host, infection biology or mode-of-action-focused extensions of library screens would prove a valuable opportunity to both discover further effectors and also understand how they are acting on hosts and host cells. Library assays could focus on activity against different insect hosts or specificity towards different tissues, e.g. haemocytes or midgut, or on conferring the gain or loss of different virulence phenotypes onto library *E. coli* towards mammalian or insect host cells. Examples of such assays could include the following: gain of adhesion, gain of intracellular survival and persistence and gain or loss of phagocytosis. More ‘mode-of-action’-based approaches could look at mechanisms of cell death (e.g. apoptosis, necrosis, pyroptosis), alterations to the cell cycle or effects on subcellular targets including the actin cytoskeleton, Golgi apparatus and mitochondria.

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