

# Insecticidal Toxins from the *Photorhabdus* and *Xenorhabdus* Bacteria

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**Abstract:** Insect pathogens are an excellent source of novel insecticidal agents with proven toxicity. In particular, bacteria from the genera *Photorhabdus* and *Xenorhabdus* are proving to be a genomic goldmine, encoding a multitude of insecticidal toxins. Some are highly specific in their target species, whilst others are more generalist, but all are of potential use in crop protection against insect pests. These astounding bacterial species are also turning out to be equipped to produce a vast range of anti-microbial compounds which could be of use to medical science. This review will cover the current knowledge of the lifecycles of the two genera and the potential role of the toxins in their biology, before a more in depth exploration of some of the best studied toxins and their potential use in agriculture.

**Keywords:** *Photorhabdus*, *Xenorhabdus*, Insecticidal, Toxin complex, Mcf, Nematode.

## 1. PHOTORHABDUS AND XENORHABDUS TAXONOMY

*Photorhabdus* and *Xenorhabdus* species are motile Gram-negative bacteria of the family *Enterobacteriaceae*. A major identifiable characteristic of these species is their ability to form symbiotic relationships with specific entomopathogenic nematodes or EPNs. In fact, they are rarely isolated in the absence of the nematode host. Due to these similarities, *Photorhabdus luminescens* was originally classified as a member of the *Xenorhabdus* genus (*X. luminescens*). However distinct differences in phenotypic traits, DNA sequence and nematode symbiont led to this species being classified in a new genus, *Photorhabdus*. All members of the *Photorhabdus* genus can be identified by their mutualistic relationship with nematodes from the family *Heterorhabditidae*, whilst *Xenorhabdus* species are mutualists of nematodes from the family *Steinernematidae*. The *Photorhabdus* genus currently consists of three species, *P. luminescens*, *P. temperata* and *P. asymbiotica*. The *P. luminescens* and *P. temperata* species have been recently split into subspecies due to DNA-DNA relatedness and 16S rDNA branching [1]. *P. asymbiotica* has been isolated from human infections in both North America and Australia. The genome sequence of a North American isolate has recently been described [2] and an Australian strain is currently being sequenced in our laboratory. It is highly likely that more species will be added to this genus as they are identified as has been the case for *Xenorhabdus*. After the reclassification of *X. luminescens*, the *Xenorhabdus* genus consisted of five species: *X. beddingii*, *X. bovienii*, *X. japonicus*, *X. nematophilus* and *X. poinarii*. Since then a total of fifteen new species have been identified from collections of *Steinernematidae* nematodes based on 16S rRNA gene

sequencing, molecular typing and phenotypic characterization. These are *X. budapestensis*, *X. ehlersii*, *X. innexi*, *X. szentirmaii*, *X. indica*, *X. cabanillasii*, *X. doucetiae*, *X. griffinae*, *X. hominickii*, *X. koppenhoferi*, *X. kozodoii*, *X. mauleonii*, *X. miraniensis*, *X. romanii*, and *X. stockiae* [3-5]. This increase in the number of described species is likely to grow as nematodes from around the world are collected and their symbiotic bacteria identified.

### 1.1. *Photorhabdus* and *Xenorhabdus* Lifecycles

The lifecycles of both *Photorhabdus* and *Xenorhabdus* are similar, revolving around the free-living infective form of their specific nematode, termed the infective juvenile (IJ), which acts as a vector for transferring the bacteria from host to host. The major similarities and differences between the lifecycles are represented in Fig. (1). Simplistically, the IJ nematodes actively seek out their prey, usually insect larvae within the soil. Upon finding a suitable host, the nematode physically penetrates the insect and migrates to the hemocoel. Here the bacteria, residing in the gut of the IJ, are transferred directly into the hemocoel where they are able to evade the insect's immune response and kill the insect using a variety of toxic mechanisms, before reducing its carcass to a nutrient soup. The bacteria feed off this soup and the nematodes then feed off the bacteria. During this time the nematodes undergo one to three rounds of sexual replication until food supplies become low, whereupon bacteria recolonise the nematode progeny and these new infective juveniles and their respective symbiotic bacteria emerge from the host in their thousands. Due to the nature of this life-cycle *Photorhabdus* and *Xenorhabdus* species have the potential to be pathogenic to a wide variety of insect hosts, indeed several insect species from a variety of insect orders including Lepidoptera, Coleoptera, Hymenoptera and Dictyoptera are susceptible [6, 7]. Some nematode-bacterial symbionts have also been shown to be pathogenic to non-insect species such as isopods, however these are a reproductive dead-end as no infective juveniles were

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other sites of infection are usually associated with a rapid darkening due to the synthesis of insoluble melanin which clumps around the bacteria causing them to become immobile and isolating them from nutrients. This melanization is initiated through the phenoloxidase cascade. Here prophenoloxidase, a proenzyme found in the hemolymph, is activated by proteolytic cleavage to form phenoloxidase which initiates melanin synthesis, melanotic encapsulation and the release of microbicidal reactive intermediates [22, 23]. Similar immune responses also target the nematodes, but their effects and the nematode immune evasion responses will not be discussed here.

### 1.1.3. Evasion of the Humoral Response

Despite the ability of the insect immune system to recognise and respond to infection by *Photorhabdus* and *Xenorhabdus*, the bacteria ultimately win the battle through counteracting specific responses. The humoral response centres around the production of anti-microbials peptides/proteins. *Xenorhabdus* species are able to specifically inhibit the host expression of anti-microbials such as lysozyme and cecropins [24-26]. Bacterial proteases which target the anti-microbials have also been implicated in evasion of the insect immune response. In *Photorhabdus* a serralysin-type protease, PrtA, has been found to target proteins with immune related function in *in vitro* assays [27, 28]. In *Xenorhabdus*, the flagella master switch regulator, FlhDC, regulates the expression of the haemolysins Xh1A and  $\alpha$ -*Xenorhabdolysin* (also known as C1) as well as the lipase XlpA and the metalloprotease PrtA [24, 29-33]. Thus mutants in *flhDC* show decreased virulence in insect models. In *Xenorhabdus*, expression of *flhDC* is regulated by the LysR-type transcription factor LrhA, which itself is regulated by the global regulator Lrp [34, 35]. Thus there is a hierarchy of regulation of these important virulence factors, indicating a strict control of expression. Expression of FlhDC can also be regulated by a second flagella regulator, FliZ, which directly regulates the expression of the haemolysin Xh1A and the XaxAB toxin [36]. Interestingly a prior infection of *Manduca sexta* caterpillars with non-pathogenic *Escherichia coli* elicits a temporary immunity against infection by *P. luminescens* [37]. This is due to a temporary up-regulation of microbial recognition proteins and proteins with anti-bacterial properties which overwhelms the bacteria. This indicates that *Photorhabdus* are not inherently resistant to anti-microbials. However, despite this there are several *Photorhabdus* genes (*phoP* and *pbgPE*) that have been implicated in virulence and resistance to the anti-microbial peptides [38, 39].

### 1.1.4. Evasion of the Cellular Response

Evasion of the cellular arm of the immune response requires mechanisms which specifically target the insect hemocytes. Interestingly, a feature of *Photorhabdus* infection is the lack of melanisation usually associated with a successful immune response [40]. This is despite specific activation of the melanisation cascade by a secreted *Photorhabdus* protease, PrtS, which can be found in culture supernatants [41]. In order to prevent the melanisation response, both *Photorhabdus* and *Xenorhabdus* appear to specifically inhibit phospholipase A<sub>2</sub> (PLA<sub>2</sub>) resulting in inhibition of the eicosanoid pathway which controls

hemocyte aggregation and nodulation by activation of the prophenoloxidase cascade [42-45]. In *Xenorhabdus*, LPS has been previously linked to inhibition of this cascade [46]. Recently two further systems for the inhibition of phenoloxidase have been discovered in *Photorhabdus* ssp. Although exact mechanisms are as yet unknown, the anti-microbial 3,5,-dihydroxy-4-isopropyl-stilbene has been shown to be an inhibitor of phenoloxidase, whilst a cosmid based screen has identified that the *MalPQT* operon from both *P. luminescens* and *P. asymbiotica* bestows PLA<sub>2</sub> inhibitory properties upon non-pathogenic *E. coli* [47, 48].

*Photorhabdus* ssp. encode a dedicated Type III secretion system (T3SS) which can suppress phagocytosis and nodule formation by injection of effectors, such as LopT and SctC, directly into hemocyte cells [49, 50]. A Type III effector protein with 23% and 26% identity to Cif from enteropathogenic *Escherichia coli* (EPEC) has recently been identified in the genomes of *P. luminescens* and *P. asymbiotica* [51, 52]. The *E. coli* Cif protein causes irreversible G<sub>1</sub>/S and G<sub>2</sub>/M cell cycle arrests in cultured mammalian cell lines due to the inhibition of cyclin-dependent kinase inhibitor degradation [53]. Interestingly the T3SS from EPEC is unable able to transport Cif proteins from either *Photorhabdus* species, however by using lipid-based delivery systems it has been shown that they are both also able to arrest the cell cycle of the cultured human HeLa cell line [51]. This indicates that both *P. luminescens* and *P. asymbiotica* may utilise this effector to prevent phagocytosis by hemocytes. However *P. asymbiotica* has been found replicating within hemocytes from infected larvae of the lepidopteran *Mythimna unipuncta* and indicating that it does not be completely inhibit phagocytosis in this species [54]. The genome sequence of *P. asymbiotica* strain ATCC43949 has revealed the loss of the LopT effector protein and this may explain why it is less effective at suppressing phagocytosis by hemocytes [2]. ATCC43949 also encodes a second Type III secretion system (T3SS2) which may be involved in promoting intracellular survival in a manner analogous to the spi2 T3SS of *Salmonella enterica* serovar Typhimurium. Interestingly it has been recently reported that injection of *P. asymbiotica* into *Drosophila* embryos results, not in infection of the hemocytes, but in a hemocyte freezing phenotype. This is associated with a significant rearrangement of the cytoskeleton mediated by endocytic uptake of the bacterial toxin Mef1 [55].

*Xenorhabdus* species lack a Type III secretion system and associated effectors however they do encode several other cytotoxic strategies in order to evade the cellular response. Hemocytes infected with *X. nematophila* have been shown to follow a typical apoptotic process indicating specific activation of this process [56]. Three phenethylamide compounds, purified from the culture broth of *X. nematophila* have demonstrable cytotoxicity towards hemocytes and may induce apoptosis by activating cytochrome c-dependent caspase-3 [57, 58]. In addition a type I fimbrial protein, MrxA, has been shown to be toxic to cultured hemocytes and is able to form pores in cell membranes [59, 60]. Bacteria unable to produce this protein showed reduced hemocyte toxicity in the larval hemocoel during early stage of infection and delayed larval mortality [60].

### 1.1.5. Insect Toxicity and Bioconversion

Having successfully evaded the onslaught of the insect immune system the bacteria systematically begin to first kill the insect and then bioconvert its tissues. The insecticidal toxins produced by both species are a focus of this review and will be covered in depth in subsequent sections. Studies in a *M. sexta* model have revealed the pathology of *Photorhabdus* infection [61]. Initially the bacteria colonise the anterior of the midgut and subsequently spread along its length. Colonising bacteria can be visualised occupying folds between the extracellular matrix and basal side of the midgut epithelium. From there *Photorhabdus* begins to destroy the midgut epithelium via secretion of toxins. This destruction leads to a cessation in feeding behaviour in the insect and ultimately results in starvation. Prior to insect death, bacteria are mainly found in the hemoceol or associated with the gut. After death there is a large shift in the distribution of bacteria within the insect host as bacteria spread to every available tissue in the cadaver and begin bioconversion to generate a ready nutrient source for bacterial replication, which also results in plenty of bacteria for the replicating nematodes to feed upon [40].

Studies in the lepidopteran *Spodoptera littoralis* and the orthopteran *Locusta migratoria* have elucidated the stages of insect infection by *Xenorhabdus* [15]. GFP-labelled bacteria were shown to initially grow within the hemolymph. Bacteria then migrated to the anterior region of the midgut where they associated with the connective tissues surrounding the muscle fibres and tracheae. In the *L. migratoria* model, bacteria mainly colonised the connective tissue surrounding the specialised hematopoietic organs. Interestingly no fluorescent bacteria were seen in contact with or within hemocytes at any stage during infection. Once again, after death the bacteria are able to colonise all tissues of the cadaver and begin bioconversion.

During bioconversion, the production of a variety of small molecule antibiotics by *Photorhabdus* and *Xenorhabdus* ssp. are secreted and protect the cadaver from contaminating organisms during its bioconversion. *Xenorhabdus* has been reported to produce a whole range of antimicrobials based on secondary metabolites and linear and cyclic peptides, such as benzylideneacetone, nematophin, xenocoumacins 1 and 2, xenortides A and B, xenematide, and the bicornutins A, B and C [62-67]. *P. luminescens* strains have been reported to produce the antimicrobials 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3-diol, 3,5-dihydroxy-4-isopropyl-stilbene and the  $\beta$ -lactam carbapenem as well as a whole host of small organic compounds synthesised by large polyketide synthetase genes [67-71]. Not only does the cadaver need protecting from bacterial and fungal opportunists, but also from foraging insects. Both *Photorhabdus* and *Xenorhabdus* have been shown to be able to repel ants by the production of as yet unidentified chemicals [72, 73].

### 1.1.6. Nematode Recolonisation

Bioconversion of the insect cadaver is critical for nematode reproduction and the formation of new infective juveniles. *Heterorhabditis* will only grow when *Photorhabdus* are present in high cell density and have entered their post-exponential phase [74]. Indeed the

nematode will only respond to the presence of its cognate bacterial partner (or a close relative) and the specific interactions which allow bacterial retention in the nematode gut only occur with this partner [75, 76]. This growth and development has found to be tightly regulated to bacterial "food signals" which in *Photorhabdus* these are proposed to be a biosynthetic product of a phosphopantetheinyl transferase, NrgA [77, 78]. The recolonisation of infective juveniles by *Photorhabdus* and *Xenorhabdus* was initially thought to occur via ingestion of bacteria along with the bioconverted insect tissues. However it has since been shown to be far more complicated. *Heterorhabditis* nematodes will replicate through three generations before producing infective juveniles indicating a strict control of development. These infective juveniles start life as eggs which develop inside the maternal body cavity instead of being laid [16]. Internal development of the larvae ultimately results in matricide, a process known as *endotokia matricida*. At this stage symbiotic bacteria grow within the maternal intestinal lumen and invade rectal gland cells where they replicate within vacuoles before being released into the body cavity. Here they invade the pharyngeal intestinal valve cells of the developing infective juvenile and subsequently colonise the intestinal lumen [16]. Similarly, just prior to emergence of *S. carpocapsae*, the specialised vesicle of infective juveniles are colonised by a small number of *X. nematophila*. The source of the bacteria has not been investigated, but it is possible that *S. carpocapsae* also undergo *endotokia matricida* and acquire their symbiotic bacteria via this mechanism. Colonising *Xenorhabdus* adhere to an untethered cluster of anucleate spherical bodies within the vesicle termed the intravesicular structure (IVS). Treatment with insect hemolymph induces anal release of the IVS spherical bodies indicating that they may act not only as the site of colonisation but also as the bacterial delivery mechanism [79]. Colonisation is specific, with even closely-related *Xenorhabdus* species unable to colonise the *S. carpocapsae* vesicles indicating differences in specific ligand-binding [15]. Once the infective juvenile stage has fully developed the colonising *Xenorhabdus* bacteria multiply to fill the vesicle [80]. The type 1 fimbrial protein, MrxA, has recently been implicated in *S. carpocapsae* colonisation [60] as have ten other candidates which were identified by transposon mutagenesis of *X. nematophila* [81]. Three of these genes are part of a single locus which encodes an inner membrane protein, NilA, an outer membrane protein, NilB, and an outer membrane lipoprotein which is orientated towards the periplasm, NilC [81, 82]. Further studies have identified two synergistic repressors of *nil* expression, NilR and the global regulator Lrp which induces expression of virulence genes during insect infection [82, 83]. The current working model is that the *nil* locus encodes a putative adhesion complex which is depressed by unknown signals immediately prior to recolonisation [84]. These depression signals probably act as a switch between infection and colonisation state of the bacteria by serving to repress the Lrp activated genes which encode toxins, proteases etc. required for virulence in the insect.

### 1.1.7. Human Infection by *P. asymbiotica*

Little is known about the pathogenicity of *P. asymbiotica* towards humans or its ability to evade the human immune

system. It is likely that factors which affect the insect immune system may also have immunomodulatory properties in humans. *P. asymbiotica* causes a local infection of soft tissue, with the formation of subcutaneous nodules and abscesses which can spread to multiple sites after initial infection [9, 10, 85]. The bacterium can survive as a facultative intracellular pathogen, replicating within human macrophages which may be the vehicle for bacterial spread. Interestingly the Australian, but not US, *P. asymbiotica* isolates are also able to invade non-phagocytic human cell lines [54]. Analysis of the *P. asymbiotica* genome has revealed the presence of a second Type III secretion system (T3SS2) and an orthologue of the *Salmonella* effector SopB, both of which may be important in human infection and intracellular survival [2]. In the case of the human pathogen *Salmonella typhimurium*, the *spi1* T3SS is involved in the direct injection of effector proteins, including SopB, into phagocytic cells in order to promote bacterial uptake. SopB can persist in the host cell for up to 12 hours and is involved in controlling the maturation of the *Salmonella* containing vacuole (SCV) [86]. A second T3SS, *spi2*, is then activated in order to deliver effectors which are required for intracellular bacterial persistence [87]. Thus the presence of two T3SSs may be an indication of species which are adapted to intracellular survival. The ability to survive and replicate inside host phagocytic cells allows the evasion of many aspects of host immunity and promotes bacterial spread. Indeed it is likely that this ability has allowed *P. asymbiotica* to evolve into a human pathogen and may explain the spread of the symptomatic abscesses to multiple sites.

## 2. AN ARSENAL OF INSECTICIDAL TOXINS

Evasion of the insect immune system allows *Photorhabdus* and *Xenorhabdus* to multiply within the host and migrate to their specific colonisation sites. At this critical point in their infective stage both species then rely upon rapid host toxicity before bioconversion can commence. They achieve this by the release of a multitude of toxins and toxic secondary metabolites which they hold within their arsenal. Indeed, when the genome sequence of *P. luminescens* strain TT01 was published it was remarked that it encoded more toxins than any other known bacterial genome [88]. This has resulted in these species being studied and extensively patented as a resource for insecticidal toxins. Interestingly, although the human-pathogenic *P. asymbiotica* has lost many of the known insecticidal toxins from its genome it is more effective at killing model insect hosts than the insect specialists *P. luminescens* or *P. temperata* [2]. This section will review the most well studied toxins from these genera and their potential applications in the bio-control of crop pests.

### 2.1. The Makes Caterpillars Floppy Toxins

The *Mcf1* gene of *P. luminescens* was initially discovered during screening of a cosmid library generated from the genomic DNA of strain W14. Injection of a fifth instar *M. sexta* larvae with approximately  $2 \times 10^7$  *E. coli* transformed with one of the cosmids caused loss of body turgour and death of the caterpillar. End sequencing revealed that the cosmid containing a 33kb insert, whilst insertional mutagenesis pinpointed a single 8.8kb ORF as being

responsible. This highly insecticidal protein was subsequently named Makes caterpillars floppy (*Mcf*) after the effects it had on the insect larvae [89]. Random end-sequencing of the same cosmid library revealed a second *Mcf*-like ORF (*mcf2*) which also caused loss of body turgour when injected into *M. sexta* larvae [90]. The two encoded proteins are 77.5% identical across the majority of their length (~2000 amino acids) only differing in their N-terminal regions. *Mcf1* contains a long 900 amino acid N-terminal region with no similarity to other proteins in the database. *Mcf2* contains a shorter 300 amino acid N-terminal region which contains a domain with similarity to several Type III secreted proteins. This region was previously termed HMRA-like due to its 40% identity to the C-terminus of the plant avirulence protein *HmrA* from *P. syringae* [90]. We now refer to this as a HopA1-like region, as the *P. syringae* protein has been renamed HopA1. *P. asymbiotica* appears to have lost *Mcf2* as the insecticidal regions of its genome are downsized yet has retained *Mcf1* [2]. Surprisingly *mcf1* is located in a different part of the genome in *P. asymbiotica* indicating that it is highly mobile or has been acquired independently. *Mcf1* of *P. luminescens* strain K122 is also located in yet another different region which adds weight to the fact that this gene is mobile within the genome [2].

Genome sequencing has revealed the presence of other *Mcf*-like proteins in other bacterial species. *P. fluorescens* encodes a *Mcf1*-like protein, with 73.5% identity across the entire protein, which has been termed *FitD* [91]. Similarly *Mcf2*-like proteins have been identified in several *Providencia* ssp. and in *Vibrio harveyi* and *V. campbellii* (although this has been mis-annotated as a DNA ligase). Analysis of the adjacent coding sequences in these species reveals very little similarity indicating that the *Mcf* proteins are functional as stand-alone proteins. However, three putative Type I secretion system proteins (*MptB*, *MptD* and *MptE*) similar to known RTX toxin transporters are located immediately upstream of *mcf2* in *P. luminescens*. These are not found associated with *Mcf1* in *P. luminescens* but are present immediately upstream of the *P. fluorescens fitD* ORF and have been termed *fitA*, *fitB* and *fitC* in this species [91]. The mechanisms by which *Mcf1*-like and *Mcf2*-like proteins are secreted are still not understood. It is possible that these Type I secretion proteins are involved in the secretion of *Mcf2* and *FitD*, however *Mcf2* also contains an N-terminal domain similar to that of Type III secreted effectors. Also associated with the *fitD* locus of *P. fluorescens* are a TolC-family outer membrane efflux protein (*fitE*), two response-regulators (*fitF*, *fitH*) and a LysR-like regulator (*fitG*). Paralogues of *fitE-H* are not present in *Photorhabdus* and may be involved in the specific regulation of *fitD* in *P. fluorescens*.

Comparisons with other known proteins in the databases reveal that the ~2000 amino acid region of similarity between *Mcf1* and *Mcf2* contains a BH3-like domain, two domains found in RTX-like toxins and a large domain found in the *Clostridium difficile* binary toxins (Fig. 2). In the Clostridial toxins this domain is predicted to be involved in their translocation due to the presence of a hydrophobic region with putative membrane spanning regions [92]. Translocation of *Mcf1* into the host cell cytoplasm is an essential part of its mode of action. Inhibition of endosomal acidification with Bafilomycin A1 results in a lack of



## 2.2. The “Toxin Complexes”

The high molecular weight insecticidal “Toxin complexes” were first identified in *P. luminescens* strain W14 and subsequently in *X. nematophila* [6, 98, 99]. Since then they have been identified in a wide range of bacterial species of diverse origin, some with no known association with insects. The initial discovery of the oral toxicity of culture supernatants from *P. luminescens* to *Manduca sexta* larvae led to the identification of the first insecticidal toxin from an entomopathogenic bacterium [6]. The toxic activity of culture supernatants was associated with the high molecular weight fraction and was heat labile indicating a large proteinaceous toxin. Native gel analysis of this high molecular weight fraction revealed three separate proteins with varying degrees of toxicity. SDS-PAGE analysis of these proteins revealed that the active toxin was a complex of several protein subunits ranging in size. Subsequent purification of the active protein complex by various chromatographic steps including high performance liquid chromatography (HPLC) revealed the presence of four distinct protein “Toxin complexes” which were termed Tca, Tcb, Tcc and Tcd [98]. All show injectable toxicity to *M. sexta*, whilst two of the complexes, Tca and Tcd, also show oral toxicity. Subsequent cloning of the loci encoding these Toxin complexes (Tc’s) further revealed their highly similar yet distinct natures.

### 2.2.1. Genetics of the “Toxin Complexes”

Cloning and sequencing of the Tca, Tcb, Tcc and Tcd loci revealed a high degree of similarity between loci. The individual genes within these loci can be placed into three major groups, the *tcdA*-like or [A], the *tcaC*-like or [B] and the *tccC*-like or [C] [100]. These groupings suggest similar roles of their encoded proteins within the assembled Toxin complex (Tc). Initial work demonstrated only Tca and Tcd to be orally active, however Tcc and Tcb are toxic when injected directly into the hemocoel of insect larvae. Subsequent work demonstrated that for full toxicity a representative of [A] [B] and [C] was required [101]. Fig. (2) shows a comparison of the four originally identified *tc* loci, from *P. luminescens* strain W14, along with those identified from the genome sequences of *P. luminescens* strain TT01, *P. asymbiotica* strain ATCC43949 and the single *tc* locus from *X. nematophila* strain ATCC19061. In the *Photorhabdus* loci the [A] and [B] subunit encoding genes are named according to their locus, whilst the [C] subunit encoding genes are all designated *tccC* due to the extremely high level of identity between them. Sequence alignment of *P. asymbiotica* compared to *P. luminescens* suggests that *P. asymbiotica* is evolving away from insect hosts as it has lost many of its insecticidal genes [2]. This is extremely evident in the *tc* genes, where all four loci (*tca*, *tcb*, *tcc* and *tcd*) are significantly affected by gene loss and pseudogene formation. This has also resulted in a loss of oral toxicity of bacterial cells and culture supernatants towards *M. sexta* larvae [2].

The *tca* locus of *P. luminescens* strain W14 consists of three open reading frames, *tcaA*, *tcaB*, and *tcaC*. The [A] subunit of this particular Tc consists of two proteins encoded by *tcaA* and *tcaB*. These have been truncated in strain TT01 due to a putative fusion/deletion event leaving only gene

remnants, whilst in *P. asymbiotica* only the truncated *tcaB* is present in the genome. The [B] subunit is encoded in all three genomes by *tcaC*. The [C] subunit, although required for the mature complex, is not encoded in this locus in either of the *P. luminescens* strains. In these strains this subunit is acquired from the *tcc* locus. In *P. asymbiotica* the gene encoding this [C] subunit, *tccC*, appears to have migrated to become associated with the rest of the locus. The fourth open reading frame which has been associated with the *tca* locus, *tcaZ*, is encoded in the opposite orientation to the other genes indicating that it may be independently regulated. No function has yet been described for this protein and it is not purified along with the other subunits as part of the mature complex.

The *tcb* locus consists of a single [A] gene, *tcbA*, which is not known to associate with any other subunits. This locus has been lost in strain TT01, and only a minor gene remnant is present in the *P. asymbiotica* genome. The *tcc* locus consists of an [A] encoded by *tccA* and *tccB* and a [C] encoded by *tccC*. In strain TT01 the region containing *tccA* and *tccB* has been duplicated twice, resulting in three copies in different parts of the genome, yet only one copy of *tccC*. This locus has been lost entirely by *P. asymbiotica* with the exception of *tccC* which has relocated to associate with the *tca* locus. The *tcd* locus is by far the largest of the four *tc* loci. In the two *P. luminescens* strains this locus consists of four [A] genes, *tcdA1-tcdA4*, two [B] genes, *tcdB1-tcdB2*, and four [C] genes, *tccC2-tccC5*. In *P. asymbiotica* this has been reduced to two [A] genes and a single [B] gene with two [C] gene fragments. It is not known if this replication of *tcd* coding sequences is important in pathogenicity. In stark contrast *X. nematophila* encodes only a single *tc* locus encoding all three subunits with two [A] genes, *xptA1* and *xptA2*, a single [B] gene, *xptC*, and a single [C] gene, *xptB*. These show the greatest levels of identity to the *P. luminescens* genes *tcdA*, *tcaC* and *tccC* respectively

Since their discovery in *Photorhabdus* and *Xenorhabdus*, orthologues of *tc* encoding genes have been identified in a wide variety of Gram-negative bacteria and have even been reported in the Gram-positive *Paenibacillus*. Interestingly, this a bacteria which is also known to associate with Infective Juvenile *Heterorhabditid* nematodes [102]. The surge in genome sequencing has led to the mapping of entire loci from many of these species. Fig. (3) shows the current knowledge of *tc* loci from the non-entomopathogenic species. Many of these loci show signs of horizontal acquisition and are associated with transposase-like or bacteriophage-like genes. For example, the *tc* locus of *Serratia entomophila* is found on a plasmid, pADAP, and encodes the three subunits, all of which are required for full toxicity of the complex [103]. These have been termed *sepA* (*tcdA*-like), *sepB* (*tcdB*-like) and *sepC* (*tccC*-like). Related *Serratia* species *S. liquefaciens* and *S. proteamaculans* along with *Yersinia frederiksenii* also contain plasmids which encode *tc* loci with high similarity to the *sep* locus, yet on different plasmid backbones [104]. This indicates that the *tc* loci are indeed highly mobile and can be transferred between species. This may explain the presence of *tc* loci not only in a diverse range of species associated with insects such as *Pseudomonas entomophila*, but also in bacterial species such as the plant pathogens *P. syringae*, *P. fluorescens* and *Erwinia pyrifoliae*, the human-pathogenic *Yersinia* and the





depending on their trophisms. During the course of our work on the *Yersinia* Tc's we noticed that the Tca toxin of *P. luminescens* also demonstrated toxicity to some mammalian tissue culture cell lines [109]. This indicates that the mechanism of action for insecticidal and mammalian toxicity must be similar and that something as simple as differences in receptor expression are likely to determine the susceptibility of host cell types.

Little is known about the regulation of gene expression of *tc*'s in any bacterial species aside from its temperature dependence. The Insecticidal *tc*'s have been shown to be preferentially expressed at low temperatures (<15°C), whilst the mammalian specific *tc*'s appear to be expressed at higher temperatures (30-37°C). Along with phage-like genes previously mentioned, the *Yersinia tc* loci also share a common LysR-like DNA regulator (now termed *tcaR2*) upstream of the [A] gene. Expression of the *tcaR2* DNA regulator has been shown to be responsible for upregulation of expression of the *tcaA*, *tcaB* and *tcaC* genes in *Y. pestis* and therefore may be involved in temperature dependent regulation of *tc* expression in all *Yersinia* spp. [110]. However orthologous regulators are not found associated with *tc* loci in non-*Yersinia* species. A genomic island present in some strains of *B. pseudomallei* encodes a ToxR orthologue which may be involved in regulation of the two *tc* encoding operons associated with it. The *tc* loci in other species have no obvious associated regulators, however the lipoprotein immediately upstream of the *tc* genes in *S. baltica* is known to be regulated by a ToxR orthologue encoded elsewhere in the genome. Thus ToxR may also play a role in *tc* regulation in both *B. pseudomallei* and *S. baltica* and possibly other species as well.

### 2.2.2. The "Toxin Complex" Proteins

Despite being cloned and characterised over 10 years ago, the proteins encoded by the *tc* loci still remain somewhat of an enigma and their role in *Photorhabdus/Xenorhabdus* biology is still far from clear. They are all large proteins (>1MDa) with very little homology to any other proteins in the ever-growing databases. Indeed any similarities to known proteins are weak and generally not universal across all the toxin subgroups. For example, the N-terminus of TccA from *P. luminescens* is similar to the N-terminus of *Salmonella* plasmid virulence A (SpvA), however this is not shared by any of the other *Photorhabdus* [A] subunits. The two [B] subunits (TcaC and TcdB) both share an N-terminal region with ~40% sequence identity to the N-terminus of SpvB. The only group of proteins with any real level of similarity with other known proteins are the [C] subunit proteins. These form a family of "core-extension" proteins similar to *rhs* elements [111, 112]. They are composed of a 600 amino acid 'extended core' (25-74% identity between the individual TccC proteins) followed by a highly conserved core region of 65 amino acids (>75% identity) and a C-terminal tail which can be of varying length and shows <13% identity between the individual proteins.

The Tc's of *Photorhabdus* spp. appear to be very promiscuous in their activity, with demonstrable toxicity towards a wide variety of insect species. In contrast the Tc's of *Serratia* spp are highly specific in their activity, only demonstrating toxicity towards the New Zealand grass grub

*Costelytra zealandica* [113]. In *X. nematophila* the two separate [A] genes have been shown to be responsible different host species specificity within the Tc's. The XptA1 protein confers specificity towards *Pieris brassicae* and *Pieris rapae*, whilst the XptA2 protein confers specificity towards *Heliothis virescens* [99]. This indicates that these [A] subunits must interact with some kind of specific receptor in order for the complex to cause toxicity. Interestingly the Tc proteins of *Y. pestis* and *Y. pseudotuberculosis* share approximately 90% identity at the amino acid level yet demonstrate differential specificities towards cultured mammalian cells [109]. By swapping individual subunits we have since determined that the region responsible for this difference in specificity between the *Yersinia* Tc's is also located in the [A] subunit (Hares *et al.*, unpublished data). Only by solving the crystal structure of this subunit and performing site directed mutagenesis of target residues will we be able to fully determine the residues responsible for receptor binding and species specificity.

Cloning and expression of the subunits of the *P. luminescens* W14 *tcd* locus revealed that the [A] subunit itself possesses a low level of toxicity which is 'potentiated' by [BC], a complex formed by [B] and [C] when expressed together in the same *E. coli* cytoplasm [101, 112]. The [BC] complex itself does show mild oral toxicity towards *M. sexta* whilst the [B] and [C] subunits individually are not orally toxic. In *Photorhabdus*, this potentiation of [A] by [BC] occurs irrespective of the source of the subunits as the *tca* [A] subunit can be potentiated by the [BC] subunits of the *tcd* locus. Potentiation has since been demonstrated for the Tc's of *X. nematophila*, *Y. pestis* and *Y. pseudotuberculosis* [99, 109]. Interestingly the [BC] subunit of the non-insecticidal *Y. pseudotuberculosis* and *Y. pestis* Tc's are not able to potentiate the insecticidal activity of the *P. luminescens tca* [A] subunit against *M. sexta* (Hares *et al.* unpublished data). This indicates that the subunits from these species are 'incompatible' and unable to form an active protein complex. One theory is that specific interactions between [A] and [BC] cause conformational changes in [A] and thus increasing its toxicity however we cannot determine this without proper structural data. Other possibilities are that [BC] is required for optimal translocation of [A] into the host cell cytoplasm, or that [BC] also binds to specific receptors on the host and somehow adapts the host cell to make it more susceptible to the toxic effects of [A].

The role played by [C] in the complex is an important one as its presence is required for oral toxicity of Tca and Tcd. Furthermore it has been shown that it must be expressed in the same cytoplasm as [B] in order to potentiate the toxicity of [A] indicating that the [BC] complex forms during or immediately after translation. There was some debate as to whether the [C] subunit is actually present in the mature complex. It was not purified along with the other subunits during the initial characterisation of the Tc's and when viewed by transmission electron microscopy the complex appears unchanged in the absence of [C] [6, 112]. However, we are able to visualise [BC] by 2D gel electrophoresis when it is expressed in *E. coli* and subsequent MALDI-TOF analysis of spots reveals that both proteins are present in a stable complex (Hares *et al.* unpublished data). The presence of [C]-like genes in many genomes which do not encode *tc* loci indicates that this



types rather than whole organisms was by Bowen *et al.* [98] who showed that ingestion of purified *P. luminescens* Tca by *M. sexta* led to complete destruction of the midgut epithelium leading to cessation of feeding and eventual starvation of the insect host. This was followed by a more comprehensive study into the histopathological effects of Tca on *M. sexta* [115]. Within 3 hours of administration, ingestion of Tca resulted in apical swelling of the columnar cells in the epithelium of the anterior midgut and blebbing of vesicles into the gut lumen. By six hours the blebs contained nuclei and large vacuoles and the lumen contains debris from both columnar and goblet cells. By twelve hours the destruction is essentially complete and the basal membrane is exposed. This pathology progressed within the midgut from anterior to posterior yet had no effects on undifferentiated regenerative cells. Injection of Tca directly into the hemocoel produced similar effects on the midgut epithelium, but to a lesser extent and over a greater time period. It is likely that Tc expression only occurs once *P. luminescens* has colonised the space between the extracellular matrix on the basal side of the midgut epithelium. Thus the toxic effects are likely to be as rapid and effective as those associated with ingestion of the toxin. The fact that there are no effects on undifferentiated regenerative cells and no pathological effects on any other tissues indicates a definite specificity to the toxin. This is presumably due to the expression of a specific receptor on the target cells and differential receptor expression between the anterior and posterior regions of the midgut could also explain the differences in toxic effects along the length of the midgut.

Similarities to the midgut epithelium toxicity can be seen with the mammalian-specific Tca toxin of *Y. pseudotuberculosis* which is toxic to the human gut epithelial cell line Caco-2 [109]. Topical application of *Y. pseudotuberculosis* to Caco-2 cells results in a variety of toxic phenotypes. These mainly seem to be due to aberrant distortions of the cell cytoskeleton and include membrane ruffling, the production of large vacuoles within the cells and even cell fusion resulting in multi-nucleation and nuclear fragmentation (Fig. 6). A phenotype consisting of heavily condensed actin appears to be the final result of toxicity as the cells eventually round up and die. Nuclear fragmentation and condensed actin are a sign of apoptosis and TUNEL staining confirmed that this process occurs in a high percentage of intoxicated cells. Topical application of purified Tc's from *Y. entomophagous* to Caco-2 cells also gives identical toxic effects (Hares *et al.* unpublished).

However, despite its high level of similarity to the Tca toxin of *Y. pseudotuberculosis*, the Tca toxin of *Y. pestis* shows no toxicity towards Caco-2 cells. Yet it is toxic to the murine fibroblast cell line NIH3T3, which are also susceptible to the Tca of *P. luminescens* but not the Tca toxin of *Y. pseudotuberculosis* [109]. Thus the Tca toxins of the two *Yersinia* species have evolved different specificities since their divergence less than 20,000 years ago. These toxic effects are reminiscent of the bacterial toxins VacA, which causes the formation of large cytoplasmic vacuoles, and cytotoxic necrotising factor (CNF), which constitutively activates RhoGTPases resulting in abnormal actin polymerisation. VacA is a pore forming toxin which produces small anion-selective, voltage-dependent channels

in the plasma membrane of cells. These are then endocytosed which leads to acid activation and permeabilisation of the endosomes eventually resulting in vacuolation. The "Toxin complexes" have often been thought of as being possible pore forming toxins. Liu *et al.* [116] reported that a *P. luminescens* Tca-like toxin, which they termed PL toxin, caused channel formation in the midguts of *M. sexta*. Furthermore they also reported that PL toxin also permeabilised unilamellar lipid vesicles (LUVs) of *Manduca sexta* in a pH-dependent manner. Indeed the hollow open-ended cage-like structure of the [A] tetramer from *X. nematophila* would seem an ideal candidate for the pore when inserted through cell membranes. However, in the structural paper Lee *et al.* [114] reports that purified XptA1 binds specifically to brush border membrane vesicles (BBMV) from *P. brassicae* and to Sf21 cells but does not form pores in the membranes. Thus it seems that the [A] tetramer alone cannot form pores whilst a mature complex is able to. Thus a putative role for [BC] could be to aid the insertion of [A] into membranes which would explain its 'potentiation' of the toxicity of [A].

Mammalian expression vectors have been used to transiently express the individual Tc subunits in tissue culture cells in order to gain insight into their mode of action. The Tca proteins of *Y. pseudotuberculosis* have been expressed in Caco-2 cells, whilst the Tca proteins of *Y. pestis* and the Tca and Tcd proteins of *P. luminescens* have all been expressed in NIH3T3 cells [101, 109]. The TcaA and TcaB proteins from all three species, along with two constructs representing the large N-terminal domain and the small C-terminal domain of *P. luminescens* TcdA, revealed a cytoplasmic localisation. Cells expressing TcaA from *P. luminescens* showed no toxic effects, whilst membrane ruffling, actin condensation and nuclear fragmentation were seen in cells expressing TcaA from the two *Yersinia* species. Actin condensation was evident in cells expressing TcaB from *P. luminescens* or *Y. pestis*, whilst expression of the TcaB from *Y. pseudotuberculosis* resulted in actin condensation and multi-nucleation. Expression of the large N-terminal region of TcdA from *P. luminescens* also resulted in actin condensation, whilst the small C-terminal region had no effect. These results confirm that the [A] subunit is toxic in its own right and demonstrate that the toxicity results from more than one domain in the subunit. More surprising was the toxicity demonstrated by the [B] subunits when expressed in the absence of [C]. The N-terminal region of *P. luminescens* TcdB1 localised in the cell nucleus, as did the TcaC protein of *Y. pseudotuberculosis*. Interestingly the TcaC protein of *Y. pestis* associated with the nuclear membrane but did not appear to enter the nucleus. This nuclear association may be due to the presence of the SpvB-like domain at the N-terminus of these proteins. Despite its nuclear localisation the N-terminal region of TcdB1 showed no toxic effects, whilst expression of the cytoplasmic C-terminal region resulted in actin condensation. Expression of the TcaC proteins of *Y. pseudotuberculosis* resulted in large vacuole formation and nuclear fragmentation. Thus the [B] subunits are also toxic in their own right and the potentiation of [A] by [BC] may be due to additional toxicity rather than [BC] assisting the entry of [A] into the host cell. No toxicity was observed with expression of the [C] subunit, TccC, from either *P. luminescens* or *Y. pestis*. This furthers the theory



**Fig. (6).** Toxicity of the *Y. pseudotuberculosis* “Toxin complex” to Caco-2 cells after topical application. Cellular effects include actin condensation (ac), membrane ruffling (mr), multi-nucleation (mn), nuclear fragmentation (nf) and vacuolation (v) of topically treated cells

both 37°C and 30°C [105,109]. The pYV-encoded Type III secretion system cannot be responsible for secretion at both temperatures and therefore there must be another mechanism for secretion. Both *Y. pseudotuberculosis* and *Y. pestis* encode a second Type III secretion system on their chromosomes. These are little understood systems and are the conditions for their expression are not known and therefore they may be responsible for secretion at the lower temperature, however Gendlina *et al.* [110] state that there is unpublished evidence that this system is inactive at 30°C. Further weight to the hypothesis that these proteins are not Type III secreted comes from our demonstration that the Tc’s of *P. luminescens* and *Y. pseudotuberculosis* and the individual toxin subunits [A] and [BC] are toxic when applied topically onto cultured cells, revealing that they do not need to be injected directly into the cell via a Type III mechanism in order to cause toxicity [109]. This, together with their multi-subunit nature and the lack of a Type III secretion in some “Toxin complex” encoding species indicates that they must be secreted via a different mechanism. It is possible that the “Toxin complex” proteins of *Y. pestis* may have evolved the ability to be Type III secreted in order to direct their toxicity towards specific cell types such as mammalian phagocytes, however this cannot be a general mechanism.

### 2.3. A host of other Insecticidal Toxins

The genome sequences of *X. nematophila*, *P. luminescens* and *P. symbiotica* have revealed a variety of other previously unknown insecticidal toxins due to their similarity to other known proteins. The TT01 genome sequence revealed two loci with high similarity to the Juvenile Hormone Esterase (JHE) of *Leptinotarsa decemlineata*. JHEs are involved in regulating metamorphosis by inactivating the juvenile hormones which maintain the larval stage. These have since been renamed as the “*Photorhabdus* insect-related” binary toxins, PirAB, and are present in both *P. luminescens* and *P. asymbiotica*. The two PirAB toxins encoded by *P. luminescens* TT01 possess oral toxicity towards insects including three mosquito

species, *Aedes aegypti*, *Culex pipiens* and *Anopheles gambiae* and the Lepidopteran *Plutella xylostella* [88]. This is the first observed oral toxicity towards mosquitoes by a bacterial toxin and was originally proposed to be due to inappropriate activation of the endocrine machinery by the esterase-like proteins. However these proteins have since been shown to lack the necessary esterase activity and therefore the mechanism for this potent insecticidal toxicity therefore remains unclear [117]. A different binary toxin identified in *X. nematophila*, *Xenorhabdus*  $\alpha$ -*xenorhabdolysin* (XaxAB), has also been shown to have cytotoxic effects on insect hemocytes and even mammalian cells [31, 118]. As yet no mechanism of action has been determined, however orthologues of XaxAB are also present in the entomopathogens *Photorhabdus* and *Pseudomonas entomophila*, in the mammalian pathogens *Yersinia enterocolitica* and *Proteus mirabilis*, and in the plant pathogen *Pseudomonas syringae*. Many of these species also encode *tc* loci indicating that a large amount of genetic transfer occurs between these species, presumably within a shared environmental niche. The sequencing of the genomes of *Photorhabdus* and *Xenorhabdus* species also revealed orthologues of insecticidal toxins which have previously been identified in other species. An example of this is the “*Photorhabdus* virulence cassettes” (PVCs) which are homologous to a prophage-like locus on the pADAP plasmid of *S. entomophila* [119]. The pADAP locus has been associated with anti-feeding effects which are separate to the effects due to the “Toxin complex” which is also encoded on the same plasmid [120]. These loci consist of approximately 15 phage-like genes immediately upstream of a varying number of effector coding sequences. These effectors vary between loci and encode proteins with similarity to known toxins such as LopT, Cif, PhxA and domains of CNF and MCF. The PVC phage-like proteins form a structure similar to R-type pyocins with a sheathed needle structure which presumably delivers the effector payload directly into target cells [119]. Recombinant expression of various PVC loci from *P. asymbiotica* and *P. luminescens* demonstrated that they have differing toxicities towards *G. mellonella*. *E. coli*

expressing many of the PVC loci are toxic to insect hemocytes, with different effector proteins showing different toxic effects, thus allowing persistence of recombinant bacteria in *G. mellonella* larvae. In *P. luminescens* strain TT01, four coding sequences and four pseudogenes have been identified as being highly similar to the *Vibrio cholerae* RtxA toxin which causes actin cross-linking and depolymerisation [121]. These were found in two loci in the genome adjacent to a specific RTX secretion system [88]. Other RTX toxins were also identified from the TT01 genome sequence as were proteins similar to the  $\delta$ -endotoxin from *Bacillus thuringiensis* and the heat stable cytotoxic enterotoxin from *Aeromonas hydrophila*. Another relatively uncharacterised toxin is the aptly named *Photorhabdus* insecticidal toxin (Pit) which was identified due to its 30% amino acid sequence similarity to part of the insecticidal crystal protein of *B. thuringiensis* [122]. Oral administration of purified Pit protein results in an inhibition of growth with the larvae of *Spodoptera litura* and *H. armigera*, but did not kill the insects; however the injectable activity of the toxin was much greater. In *Xenorhabdus* the type 1 fimbrial protein, MrxA, recently implicated in nematode colonisation was previously identified as a putative pore-forming toxin which targets hemocytes thus preventing phagocytosis [59, 60]. The same group also identified an insecticidal pilin subunit associated with outer membrane vesicles (OMVs) which appear to bleb from *X. nematophilus* [123, 124]. Recombinant pilin subunit caused cultured hemocytes to agglutinate and release the cytoplasmic enzyme lactate dehydrogenase. It also showed oral toxicity to the larvae of *Helicoverpa armigera*, causing damage to the midgut epithelial membrane [124]. Another insecticidal protein associated with the OMVs has recently been identified as a GroEL homologue [125]. This is an orally toxic protein with toxicity associated with all three domains and a chitin-binding region located in the apical domain.

Whole genome screening approaches have begun to reveal previously unidentified insecticidal toxins. Cui *et al.* screened a *X. nematophila* genomic cosmid library for clones demonstrating insecticidal activity and identified five insecticidal toxins which are currently labelled A-E. These were submitted to GenBank in 2002 (accession no. AAM77224-AAM77228) yet remain unpublished. A more in depth study was the Rapid Virulence Annotation technique performed by Waterfield *et al.* [126]. A genomic cosmid library of the *P. asymbiotica* ATCC43949 strain was assayed for toxicity to the insect larvae of *M. sexta* and *G. mellonella* as well as the nematode *C. elegans*, the protozoan *Acanthamoeba polyphaga* and the murine macrophage cell line J774. Species-specific toxic regions were identified by ascertaining the regions of minimal overlap between positive clones. Global alignment of all the identified regions was then used to further characterise them into those which were generally toxic and those with high specificity. RVA identified a number of polyketide synthesis (PKS) and non-ribosomal peptide synthesis (NRPS) enzyme complexes proving that many of these uncharacterised secondary metabolites possess toxic activities at very low concentrations. The RVA screen also identified several loci encoding putative *pdl*-like class III lipases along with a tightly-linked negative regulator, *orf54*. These were specifically toxic to insects and/or *C. elegans*. Two type VI

secretion systems were identified and demonstrated to possess insecticidal activity with one of them showing activity in all assays. Putative Type IV secreted VgrG-like proteins were also identified as having insecticidal activity even in the absence of the secretion machinery. Interestingly, not all cosmids transferring insecticidal activity to *E. coli* encoded specific toxins. The RVA screen also identified a high affinity potassium pump which allowed *E. coli* to survive hemocyte phagocytosis and resulted in a moribund phenotype in *M. sexta*.

### 3. TOXINS AS BIO-CONTROL AGENTS

The identification of novel insecticidal toxins in the entomopathogenic bacteria has led to a whole host of patent applications as each new one is discovered. These patents are all based on one premise - that these toxins will one day become commercially viable as bio-control agents against crop pests. Due to the secret nature of the industry it is impossible to determine exactly how far many of these potential pest control agents have progressed towards use in the field, however there have been several publications from independent research groups reporting promise in this field. The PirAB binary toxins have been extensively patented due to their oral toxicity to mosquito larvae. This makes them an ideal candidate for control of malaria and other vector-borne diseases. Recently recombinant PirAB toxin from *P. asymbiotica* has been demonstrated to be an effective larvicide against the Dengue vectors *Aedes aegypti* and *Aedes albopictus* when expressed in *E. coli* and introduced into their blood meal [127]. Crucially for a potential control agent the toxins specifically killed the mosquito larvae but did not affect *Mesocyclops thermocyclopoides* a common predator and biological control agent of the first-stage larvae of *Aedes* mosquitoes. However the issue of how these toxins can be safely delivered in the wild has yet to be addressed.

The "Toxin complexes" are a major candidate for replacement of the Bt toxins in transgenic crops. These have been heavily patented by Dow Agrosiences and are a focus of their research. In 2003 they reported that the TcdA protein can be expressed in *Arabidopsis thaliana* to sufficient levels as to be toxic when ingested by *M. sexta* and inhibit growth of the southern corn rootworm, *Diabrotica undecimpunctata howardi* [128]. The addition of the 5' and 3' untranslated regions of the tobacco osmotin (*osm*) gene was used in order to achieve high levels of expression and this transgene was able to survive for at least five generations in all progeny. Since then no other reports of transgenic plants expressing the "Toxin complexes" have been forthcoming. Interestingly a different method of pest control by the "Toxin complexes" has been reported, namely the control of termites using a transgenic indigenous gut bacterium [129]. This ingenious scheme involves generating an *Enterobacter cloacae* strain expressing both TcdA and TcdB which is then fed to the termites. Transfer of the bacteria from infected to uninfected termites occurs readily within a colony indicating that the toxins can be passed on before they mediate their toxic effects. However not all infected termites are killed by the toxin-expressing bacteria and thus they can only be used to control numbers rather than wipe out colonies. The reasons for this are unclear. A danger of this approach is that by exposing termites to sub-lethal doses of the toxin there is the possibility that toxin resistance can evolve. Thus rather than

purify or express specific toxins to which resistance can be evolved, some groups are looking into the possibilities of using culture supernatants of *Photorhabdus* and *Xenorhabdus*, or even whole bacteria, as bio-control agents [130, 131].

So what makes a good bio-control agent? Essentially it all boils down to two aspects of the agent and its mode of administration, Efficacy and Specificity. First and foremost it has to prevent crop damage by either efficiently killing or deterring the insect pests. For toxins this usually means that it must be orally active and highly toxic to a wide range of potential pests. Current methods of administration are either expression of the toxin, or toxic subunit/domain, in transgenic crops or coating them onto the crops in a stable form. These are technologically challenging procedures and thus may be a major stumbling block for many potential agents. For example, the orally active "Toxin complexes" consist of large protein subunits which are intrinsically difficult to express transgenically in crop plants. At present only individual proteins, which have limited toxicity, can be expressed and thus the full potential of the "Toxin complexes" cannot be fulfilled unless the proteins can be trimmed down to smaller active domains or the transgenic technology improves to allow all subunits to be expressed. Orally active toxins are ingested by the insect pests during feeding on the crop which allows for a certain amount of specificity as only insects actually feeding on the crop will ingest the toxin directly. However the toxins would still need to be specifically active towards their target species so as not to harm non-pest species. This includes bystander insect species, predatory species which feed off the intoxicated pests and the end user by whom the crop is intended to be consumed. With the orally active toxins, bystander insect toxicity is likely to be low therefore generalist insect-specific toxins are likely to be excellent candidates. However there must be rigorous testing to ensure that the toxins are insect specific and will not harm other organisms. The toxic effects of Mcf are non-specific and extremely potent against insect and mammalian cells alike [89, 90, 93, 94]. Worryingly the insecticidal "Toxin complexes" of *P. luminescens* have also been shown to be toxic to cultured mammalian cells [101, 109]. Indeed, in species such as the human pathogenic *Yersinia*, these toxins have adapted to be fully mammalian specific. Thus before any toxins can be utilised for crop protection we need to fully investigate their mode of action and characterise any regions responsible for host specificity. It is also imperative that we characterise the mode of action of any potential crop protection agent in order to predict mechanisms of resistance and monitor for them in wild insect populations.

#### 4. CONCLUSIONS

The recent sequencing of several *Photorhabdus* [2, 88] and *Xenorhabdus* (Forst *et al.*, personal communication) genomes threatens to expand the ever increasing list of toxins from these two bacterial genera. This increasing list provides two challenges. First, how do we identify which toxins are active against which hosts or targets? This problem has been recently addressed by the development of the Rapid Virulence Annotation (RVA) approach. RVA involves mass parallel screening of genomic libraries of bacteria for gain of toxicity, or persistence within,

invertebrate hosts such as insects, nematodes and amoebae [126]. This approach has recently been used against the genome of a North American isolate of *P. asymbiotica* and has provided functional data for many known toxins as well as identifying many more new candidate toxins for use in crop protection. The power of this approach is that several organisms are screened in parallel allowing both for the independent verification of hits (new toxins) and for a first idea of their relative toxicity to different hosts e.g are they specific in their activity towards nematodes (potential nematicides) or do they kill all invertebrates tested (like Mcf1). Secondly, and perhaps most importantly, RVA provides a growing list of potential virulence factors with unknown roles in the biology of *Photorhabdus* or *Xenorhabdus*. Assigning biological roles to these candidate virulence factors is now a major challenge.

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