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Review

Novel insecticidal toxins from nematode-symbiotic bacteria

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Abstract. The current strategy of using transgenic crops expressing insecticidal protein toxins is placing increasing emphasis on the discovery of novel toxins, beyond those already derived from the bacterium *Bacillus thuringiensis*. Here we review the cloning of four insecticidal toxin complex (*tc*) encoding genes from a different bacterium *Photorhabdus luminescens* and of similar gene sequences from *Xenorhabdus nematophilus*. Both these bacteria occupy the gut of entomopathogenic nematodes and are released into the insect upon invasion by the nematode. In the insect the bacteria presumably secrete these insecticidal toxins, as well as a range of other antimicrobials, to establish the insect cadaver as a monocultural breeding ground for both bacteria and nematodes. In this review, the protein biochemistry and structure of the *tc* encoding loci are discussed in relation to their observed toxicity and histopathology. These toxins may prove useful as alternatives to those derived from *B. thuringiensis* for deployment in insect-resistant transgenic plants.

Key words. Insecticidal protein toxins; *Bacillus thuringiensis*; *Photorhabdus luminescens*; *Xenorhabdus nematophilus*.

Introduction

Current strategies for the control of insect pests in agriculture rely increasingly on the deployment of transgenic crops expressing insecticidal protein toxins. To date, such transgenic crops contain almost exclusively δ-endotoxins derived from the bacterium *Bacillus thuringiensis*, also termed Bt. Although Bt endotoxins occur in a variety of forms, toxic to a range of insects and noninsects (e.g. nematodes) [1, 2], the current widespread deployment of a limited number of active ingredients has prompted concerns over the rapid evolution of insect resistance to Bt [3–5]. Further, although recommended strategies are in place for the planting of nontransgenic refugia for susceptible insects [5], and the use of mixtures ('pyramiding' more than one toxin in a transgenic plant) or alternations (alternating the use of one transgenic active ingredient with another, or with conventional insecticides) are also under review, such strategies are hampered by the current lack of viable alternative insecticidal toxins for transgenic deployment.

In this context, entomophagous nematodes have long been used as biological control agents, and the insecticidal bacteria that they carry have also been studied in detail [6]. However, until recently we have had little idea of the active ingredients that these bacteria use to attack insects and of the genes that encode them. The purpose of this article is therefore to review recent progress in the purification and cloning of such toxins from two bacteria symbiotic with entomopathogenic nematodes: *Photorhabdus luminescens* and *Xenorhabdus nematophilus*.

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The *Photorhabdus*/*Xenorhabdus* life cycle

Both *P. luminescens* and *X. nematophilus* are insect pathogenic bacteria which are symbiotic with insect pathogenic nematodes [6, 7]. This 'symbiosis of pathogens' is designed to infect and kill insects and to turn their cadavers into a 'factory' for both bacterial and nematode reproduction (fig. 1). In this process infective juvenile nematodes actively invade insects and then release bacteria (normally harbored in their guts) directly into the open circulatory system (haemocoel) of the insect. Here both the bacteria and the nematodes undergo numerous rounds of replication, ultimately resulting in the release of a large number of infective juveniles from the dead insect. Presumably the role of the bacteria in this relationship is not only to kill the insect host but also to turn the insect cadaver into a ready source of nutrients for both bacterial and nematode growth. The nematodes therefore benefit from their association with the bacteria by gaining access to a dead insect and a readily accessible source of nutrients.

Purification and cloning of the Tc toxin complexes

If cultured away from the insect host, *P. luminescens* secretes a wide variety of compounds into extracellular growth media, including toxins, proteases, lipases and lipopolysaccharides [6]. Although a considerable amount of research has been done on these compounds as potential virulence factors, their direct toxicity (via oral ingestion or injection), and more important, their potential role in the normal infection process, is far from clear. To clarify the potential role of toxins and/or proteases in bacterial virulence, we have purified both classes of proteins from the bacterial supernatant in order to characterize their activities and to clone the genes which encode them.

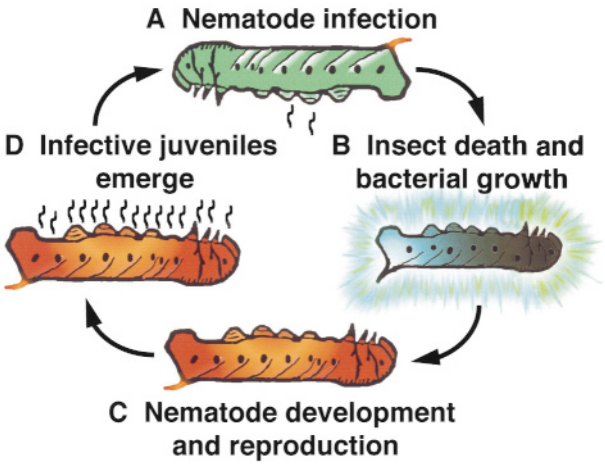

Figure 1. The *Photorhabdus luminescens* life cycle. *P. luminescens* bacteria are carried in the guts of nematodes that invade insects. (*A*) The insect is invaded by infective juvenile nematodes. (*B*) During the infection process the infected insect begins to glow due to light generated by the *P. luminescens* bacteria (from which is takes its specific name but whose biological significance is unclear). (*C*) As the infection continues, the insect is killed and becomes a monocultural breeding ground for rounds of both bacterial and nematode reproduction. (*D*) At the end of the infection process numerous infective juveniles are released from the cadaver to colonize new hosts.

The oral activity of the *P. luminescens* culture supernatant is associated with a high molecular weight fraction of the broth. We [8, 9], and others [10], have purified the active toxin components from this high molecular weight fraction. In our procedure several high molecular weight toxin complexes (Tcs) were purified by a series of column chromatograhic steps [8], with final separation by high-performance liquid chromatography (HPLC) [9]. This final HPLC step resolves the high molecular weight toxin fraction into four distinct complexes termed A, B, C and D or Tca, Tcb, Tcc and Tcd (fig. 2A), of which Tca and Tcd show oral toxicity to insects (fig. 2B). Purification of Tca shows that its oral LD_{50} (lethal dose causing 50% mortality) against *Manduca sexta* is 875 ng/cm^2 (fig. 2C), which is in the same range as some Bt endotoxins [11]. Importantly, larval weight gain is also greatly reduced at much lower doses (down to 40 ng/cm^2). Each of these toxin complexes runs as either a single or dimeric complex on a native gel but is resolved into a range of different polypeptides (ranging from 30 to 200 kDa) on a denaturing SDS gel [8, 9]. To characterize each of these different polypeptides, we derived N-terminal protein sequences from the major protein fragments of each of the individual toxin complexes. Then, to provide tools for the cloning of the genes encoding each of these complexes, we raised both a mono- and a polyclonal antibody against a mixture of the toxin complexes.

Screening of a genomic library with both antisera revealed a range of immunoreactive clones which derived from four different *toxin complex* (*tc*) encoding loci, termed *tca*, *tcb*, *tcc* and *tcd* [9]. These four loci have two different types of genomic organization (fig. 3). Both *tca* and *tcc* are operons with three different open reading frames (ORFs) transcribed in the same direction (e.g. *tcaA*, *tcaB* and *tcaC*) with a shorter terminal ORF running in the opposite orientation (*tcaZ*), whereas *tcbA* and *tcdA* are both single long ORFs of approximately 6 kb which are clearly homologs of one another as determined by their predicted amino acid sequences. Comparison of the predicted amino acid sequences of the different *tc* genes with the N-terminal sequences derived from the purified proteins revealed that the proteins recovered from the broth are extensively proteolytically cleaved [9]. Thus, for example, the single long polypeptides encoded by *tcbA* and *tcdA* are cleaved at

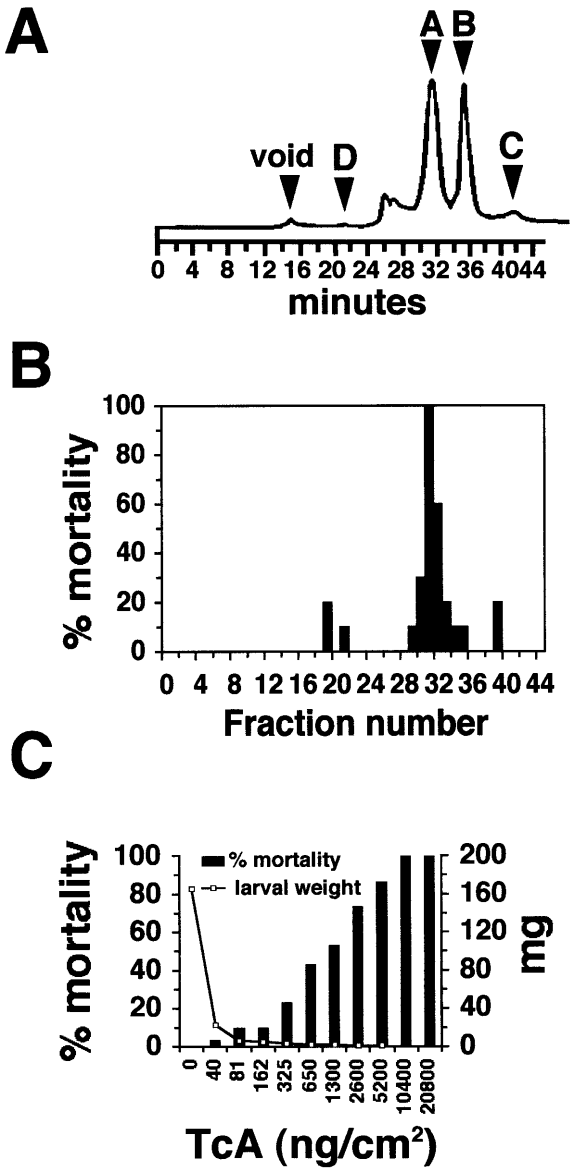

Figure 2. Purification and bioassay of the four different Tc toxin complexes. (*A*) HPLC separation of the toxin complexes. The final HPLC step resolves the high molecular weight toxin fraction into four components A, B, C and D (termed Tca, Tcb, Tcc and Tcd). (*B*) Oral bioassay of the different peaks with *Manduca sexta*. Peaks A, C and D correlate with peaks of toxicity to *M. sexta*. (*C*) Effect of pure Tca on both mortality and weight gain of *M. sexta*. Purified peak A (Tca) has an LD_{50} of ~ 50 ng/cm^2; however, much lower doses almost abolish insect weight gain.

both their N- and C-termini to yield three fragments, e.g. TcbAi, TcbAii and TcbAiii. Similarly, some of the polypeptides encoded by the different *tca* and *tcc* ORFs are also found cleaved in the bacterial culture supernatant. The potential role in this cleavage of extracellular proteases, also found in the culture supernatant, is discussed below.

Genetic knockout of the *tc* genes eliminates oral activity

In order to assess the role of the *tc* genes in oral toxicity to insects, we chose to delete each of the loci in turn directly in the *P. luminescens* host strain W14 itself [9]. By inserting different antibiotic resistance cassettes into each of the different loci and selecting for a successful knockout event, we were able to generate individual

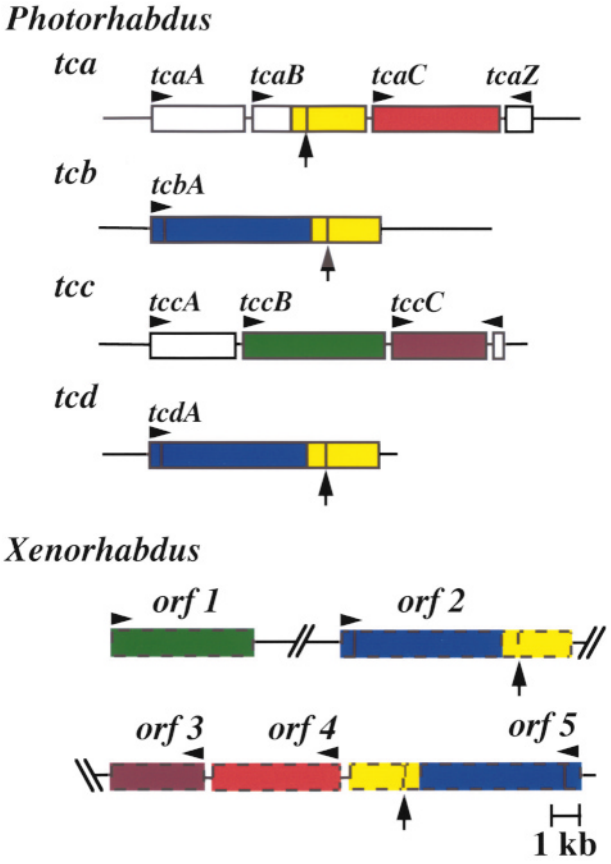

Figure 3. Genomic organization of the ORFs that encode the Tc toxin complexes from *Photorhabdus* and *Xenorhabdus*. In *Photorhabdus* the four *tc* loci (*tca*, *tcb*, *tcc* and *tcd*) display two types of genomic organization, whereby *tca* and *tcc* are operons and *tcb* and *tcd* are single long ORFs. To date we have no evidence that these are linked or closely associated in the *P. luminescens* genome. In contrast, in *Xenorhabdus* the five Tc-encoding ORFs are tightly linked within the same 40-kb piece of DNA (note that the *Xenorhabdus* ORFs are derived from unfinished sequence as deposited in the patent [16] and that therefore the assignation of the ORFs themselves is only putative). The figure is color-coded to illustrate the similarities within and between species. Thus, in *Photorhabdus*, *tcb* and *tcd* are clearly homologs of one another, and they also share a high level of predicted amino acid similarity with *orf 2* and *orf 5* of *Xenorhabdus*. The remaining ORFs in *Xenorhabdus* also share high levels of similarity with different components of the *Photorhabdus tc* genes (namely *orf 1* with *tccB*, *orf 3* with *tccC* and *orf 4* with *tcaC*). The arrows indicate a predicted point of proteolytic cleavage shared in ORFs of both species and around which there is a higher level of percentage amino acid indentity (see fig. 4).

strains deficient in either of the four loci, termed *tca*$^-$, *tcb*$^-$, *tcc*$^-$ and *tcd*$^-$. Deletion of either *tca* or *tcd* greatly reduced the oral activity of W14 against our model insect *Manduca sexta*. However, the deletion of both loci from the same strain, namely *tca*$^-$/*tcd*$^-$, eliminated oral toxicity altogether [9]. This suggests that both Tca and Tcd are oral lepidopteran toxins in their own right and that their combined effect contributes most of the secreted oral activity observed from strain W14.

The Tc toxins share little homology with known sequences

Searches of both nucleotide and protein databases revealed little overall homology of the *tc* sequences with other known sequences [9]. Thus, while the large size of both the *tcb* and *tcd* ORFs is reminiscent of the large *Clostridium* toxins [12], overall they show a low similarity (17% identity to both toxins A and B) in predicted amino acid sequence. However, short stretches of predicted amino acid sequence from both *tcaC* and *tccA* show similarity to the *Salmonella* plasmid virulence (*spv*) genes *spvA* and *spvB*. Interestingly, in *Salmonella* the *spv* genes correspond to small ORFs, whereas in *Photorhabdus* the *spv* homologs are fused as part of longer polypeptides. More importantly, this homology may suggest a biological function for these polypeptides in *P. luminescens*. In *Salmonella*, deletion of the *spvB* gene results in hinderance of the ability of the bacteria to persist in monocyte-derived macrophages [13]. This role in *Salmonella* may therefore infer that *Photorhabdus* uses the *spv* homologs as a method of persisting in, or overcoming attack by, insect hemocytes (the main basis of the cellular immune system in insects).

Although the predicted amino acid sequences from the *tc* genes share little homology with gene sequences currently deposited in the databases, they do show high levels of identity to each other [9]. This similarity is particularly pronounced around the regions of predicted cleavage (fig. 4). More specifically, percentage amino acid identities are high as the cleavage site is approached (53% in a 151-amino acid region), but immediately around the site this similarity disappears. This may infer either that protease cleavage is effected on a structural feature shared by all these dissimilar sequences (e.g. a 'loop') or that each sequence is in fact cleaved by its own sequence-specific protease. Whatever the method of cleavage, this sequence similarity does point to the suggested biological importance of proteolytic cleavage in Tc toxin processing (see below for a discussion of *Xenorhabdus*).

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TcaB  SNPVDNFSGPYGIYLWEIFFHIPFLVTVRMQTEQRYEDADTWYKYIFRSAGYRDANGQLI
TcbA  SEPMD-FSGANALYFWELFYYTPMMMAHRLLQEQNFDAANHWFRYVWSPSGY-IVDGKIA
TcdA  SEPMD-FSGANSLYFWELFYYTPMLVAQRLLHEQNFDEANRWLKYVWSPSGY-IVHGQIQ
Orf-2 -------ETKNQFIFTNDADHDSGMTQQGIVK--NIKKYKRGIQVVS----------MQ
Orf-5 -------ETKQQFVLINDADHDSGMTQQGIVK--NIKKYKGFLNVSIA----------

TcaB  MDGSKPRYWNVMPLQLDTAWDTTQPATTDPDVIAMADPMHYKLAIFLHTLDLLIARGDSA
TcbA  I-----YHWNVRPLEEDTSWNAQQLDSTDPDAVAQDDPMHYKVATFMATLDLLMARGDAA
TcdA  N-----YQWNVRPLLEDTSWNSDPLDSVDPDAVAQHDPMHYKVSTFMRTLDLLIARGDHA
Orf-2 D-----YYWNVRPLEEDTSWNANPLDSVDPDAVAQHDPMHYKVATFMKMLDLLITRGDSA
Orf-5 ----------------TGYSA-PMDFNSASALYY---WECSITPRMRLLDQLILRGDMA

TcaB  YRQLERDTLVEAKMYYIQAQQLLGPRPDIHTTNTWPNPTLSKEAGAIATPTFLSSPEVMT
TcbA  YRQLERDTLAEAKMWYTQALNLLGDEPQVMLSTTWANPTLGNAASKTTQQVRQQVLTQLR
TcdA  YRQLERDTLNEAKMWYMQALHLLGDKPYLPLSTTWSDPRLDRAADITTQNAHDSAIVALR
Orf-2 YRQLERDTLNEAKMWYVQALTLLGDEPYFSLDNDWSEPRLEEAASQTMRHHYQHKMLQLR
Orf-5 YRELTRDALNEAKMWYVRAFELLGDEPEDYGSQQWAAPSVWVAGNHTVQAGYQQDLTALD

TcaB  FAAWLSAGD---TANIGDGDFLPPYND-VLLGYWDKLELRLYNLRHNLSLDGQPLNLPLY
TcbA  LNSRVKTPL-LGTANSLTALFLPQENS-KLKGYWRTLAQRMFNLRHNLSIDGQPLSLPLY
TcdA  QNIPTPAPLSLRSANTLTDLFLPQINE-VMMNYWQTLAQRVYNLRHNLSIDGQPLYLPIY
Orf-2 QRAALPTKRRANFVNSLTALFLPQINK-KLQGYWQTLTQRLYNLRHNLTIDGQPLSLSLY
Orf-5 NGEGCTQPR---NANSLVVWSCRNITRNQPITGKPAFAPGMTGNRYRWRIT---------

TcaB  ATPVDPKTLQRQQAGGDGTGSSPAGGQGSVQGWRYPLLVERARSAVSLLTQPGNSLQTTL
TcbA  AKPADPKALLSAAVSASQGGA--DLPKAPLTIHRFPQMLEGARGLVNQLIQPGSSLLGYS
TcdA  ATPADPKALLSAAVATSQGGG--KLPESFMSLWRFPHMLENARGMVSQLTQPGSTLQNII
Orf-2 ATPADPSMLLSAAITASQGGG--DLPHAVMPMYRFPVILENAKWGVSQLIQPGNTLLSIT
Orf-5 -RAYDPKALLTSMVQPSQGGS--AVLPGTLSLYRFPVMLERARNLVAQLTQPGTSLLSMA

TcaB  EHQDNEKMTILLQTQQEAILKHQHDIQQNNLKGLQHSLTALQASRDGDTLRQKHYSDLI
TcbA  ERQDAEAMSQLLQTQASELILTSIRMQDNQLAELDSEKTALQVSLAGVQQRFDSYSQLY
TcdA  ERQDAEALNALLQNQAAELILTNLSIQDKTIEELDAEKTVLEKSKAGAQSRFDSYGKLY
Orf-2 ERQDAEALAEILQTQGSELALQSIKMQDKVMAEIDADKLALQESRHGAQSRFDSFNTLY
Orf-5 EHDDADELTTLLLQQGMELATQSIRIQQRTVDEVDADIAVLAESRRSAQNRLEKYQQLY
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Figure 4. Alignment of predicted amino acid sequences from *Photorhabdus* (TcaB, TcbA and TcdA) and *Xenorhabdus* (Orf2 and Orf5) showing the predicted proteolytic cleavage site (arrow). Note that the level of identity is high on both sides of the predicted cleavage site but falls away immediately adjacent to the site. This may infer either that the putative protease recognizes the immediate region as a structural loop or that a variety of different proteases cleave each sequence specifically (see text for discussion).

The potential role of extracellular proteases

Given the models of proteolytic cleavage and/or activation provided by other bacterial toxins, notably the required cleavage of some Bt toxins at both their N and C termini [11], we have been investigating the presence of extracellular proteases in the bacterial supernatant. Further, given the initial confusion over the identity of active toxins from *P. luminescens*, we wanted to prove that the observed oral toxicity was associated with the Tc toxins and not with the proteases themselves. We therefore purified protease-containing fractions from the culture broth and again separated these with a final HPLC step [14]. Three distinct protease fractions were recovered from the broth, one consisting of a single species of 55 kDa and two of several putatively related species of $\sim$40 kDa. All of these proteolytic fractions clearly separate from the oral insecticidal activity associated with the high molecular weight Tc proteins. These fractions also showed no effect on insect weight gain following injection into the hemocoel.

These results are difficult to compare with those of other investigators due to differences in the strains used, the phase variants examined and also their cul-

ture conditions [14]. It is clear that several different proteolytic species are secreted into the broth by *P. luminescens*, but their biological role is still unclear. This leaves open several alternative investigations for the potential role of these proteases. First, given the model of the cleavage and activation of some Bt toxins, we can investigate the ability of the different protease fractions to cleave and/or activate the Tc toxins. Second, we should still bear in mind the possibility that one or another of these proteases is involved in general proteolytic degradation of the insect cadaver and/or specific attacks on proteinaceous components of the insect immune system (such as cecropin and diptericin), as previously suggested by others [15].

The relationship between the *Photorhabdus* and *Xenorhabdus* toxins

Independent investigation of the high molecular weight toxins produced by another bacterium, *Xenorhabdus nematophilus*, has also resulted in the cloning of genes encoding similar toxin complexes [16]. In *Xenorhabdus*, the genes were cloned via a functional assay of a recombinant genomic cosmid DNA library for oral activity against insects. In this approach individual cosmids ($\sim$40 kb) were screened directly against caterpillar larvae. Sequencing and analysis of a toxic cosmid revealed several ORFs with extensive homology to the *tc* genes of *Photorhabdus*. However, the genomic organization of the *Xenorhabdus* ORFs differs markedly from that of similar ORFs in *Photorhabdus*. To illustrate this point, we have color-coded similar ORFs in the accompanying diagram (fig. 3). Thus, the *Xenorhabdus* cosmid contains two *tcb*/*tcd*-like ORFs, each transcribed in opposite directions (*orf 2* and *orf 5* in fig. 2), and then a collection of further individual ORFs similar to *tccB*, *tccC* and *tcaC* of *Photorhabdus*. The similarity of *orf 2* and *orf 5* to *tcb*/*tcd* homologs is also confirmed by the conservation of the similar high level of amino acid identity around the predicted proteolytic C-terminal cleavage site (fig. 4). In conclusion, the functional significance of this difference in genomic organization is at present unclear. However, what is clear is that the ORFs contained in the *Xenorhabdus* cosmid are sufficient for recombinant oral activity in *E. coli*, suggesting that they are functional homologs of the *tc* loci found in *Photorhabdus*.

Histopathology of Tc action on the insect gut

We are unaware currently of the precise mode of action of any of the *tc*-encoded toxin components. We have, however, been studying the histopathology of one of the complexes, Tca, on the insect midgut [17]. In these studies purified Tca was fed to *M. sexta* caterpillars, and their midguts were then sectioned at different time points after ingestion. Whether injected, or orally consumed, Tca seems to have a specific action on the midgut, and no other structures in the insect appear to be affected. Following ingestion, the midgut epithelium rapidly disintegrates by blebbing into the central lumen. Interestingly, similar midgut specific effects are seen when Tca is injected. Here the cells appear to round up and detach from the epithelium despite the fact that the toxin has been presented from the other side of the epithelium.

Although some of this general histopathology (e.g. accelerated blebbing of the midgut epithelium) is shared by other gut active compunds such as the δ-endotoxins and vegetative insecticidal proteins (Vip's) of Bt, and also the enzyme cholesterol oxidase [18–23], it is important to recognize that these effects are probably significantly downstream from the immediate mode of action of these different components. This communality of histopathology therefore probably does not immediately infer a similar initial mode of action (e.g. pore formation for Bt endotoxins versus membrane lipid attack for cholesterol oxidase). It is also important to bear in mind that we assume the Tc toxin is normally released by the bacteria *within* the hemacoel. Therefore, the actual oral toxicity of any of the toxin complexes themselves is perhaps itself unexpected. Further, the observation that Tca can destroy the insect midgut epithelium from either side suggests that the interaction of the toxin with the epithelial cells may be relatively nonspecific, i.e. either not directly receptor-mediated or mediated by a receptor(s) present on both sides of the epithelia. The mode of action of the Tc toxins and their potential interactions with the midgut epithelium are currently under investigation.

Summary

The Tc toxins represent an exciting new class of insecticidal toxins and may represent one of a limited number of viable candidate genes for codeployment with those from Bt. Such codeployment of toxins in transgenic plants is important to delay the evolution of resistance to one or another component. The Tc toxins are high molecular weight complexes encoded by loci of two distinct genomic organizations in *Photorhabdus*. However, this genomic organization is clearly not conserved in *Xenorhabdus* where similar genetic elements are found but in a different order. The toxins can readily be purified and have specific effects on the insect midgut. Future studies will focus upon the biological significance of the proteolytic toxin component cleavages observed in secreted Tc polypeptides and also on the likely mode of action of this interesting new group of toxins.

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