How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world

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Bacillus thuringiensis **is a bacterium of great agronomic and scientific interest. Together the subspecies of this bacterium colonize and kill a large variety of host insects and even nematodes, but each strain does so with a high degree of specificity. This is mainly determined by the arsenal of crystal proteins that the bacterium produces during sporulation. Here we describe the properties of these toxin proteins and the current knowledge of the basis for their specificity. Assessment of phylogenetic relationships of the three domains of the active toxin and experimental results indicate how sequence divergence in combination with domain swapping by homologous recombination might have caused this extensive range of specificities.**

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Bacillus thuringiensis (Bt) is an endospore-forming bacterium characterized by the presence of a protein crystal within the cytoplasm of the sporulating cell (Fig. 1). The proteins within this crystal are toxic to insects, which explains the extensive use of Bt as a biological insecticide. The ecology of Bt remains unclear, this ubiquitous bacterium has been isolated from soil, stored grain, insect cadavers and the phylloplane (plant surfaces), and it is probably best described as an opportunist pathogen[1]. By synthesizing the crystal during sporulation, the bacterium can be thought of as providing for its future. A dead insect will provide sufficient nutrients to allow germination of the dormant spore. Despite the actual, or presumed, presence of various pathogenicity factors (summarized in Box 1), Bt does not have a significant history of mammalian pathogenicity, and research has concentrated on the insecticidal nature of the crystal proteins (Cry and Cyt proteins; also called δ-endotoxins). To date (January 2001), 89 different genes encoding crystal proteins have been cloned from Bt and two other species (a full list with further links can be found at http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/).

In this article, we will focus on the Cry proteins only. A given strain will normally synthesize between one and five of these toxins packaged into a single, or multiple, crystals. Thousands of strains are kept in collections all over the world, and a multitude of gene combinations exists, although certain combinations of genes appear to be more common than others. The genes encoding the crystal proteins are found (often clustered) on transmissible plasmids and flanking transposable elements, explaining how they can spread easily within the species[1]. Conjugation between different strains has been observed both in a soil environment and within insects[2].

Individual Cry toxins have a defined spectrum of insecticidal activity, usually restricted to a few species within one particular order of insects. To date, toxins for insect species in the orders Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), Coleoptera (beetles and weevils) and Hymenoptera (wasps and bees) have been identified. A small minority of crystal toxins shows activity against non-insect species such as nematodes[3]. A few toxins have an activity spectrum that spans two or three insect orders – most notably Cry1Ba, which is active against larvae of moths, flies and beetles[4]. The combination of toxins within a given strain, therefore, defines the activity spectrum of that strain. Besides their long-term use as a biological insecticide in the form of sprays of spore–crystal mixtures, individual Cry toxins have been expressed in transgenic plants to render crops resistant to insect pests. Since 1996, transgenic maize, cotton and potato carrying a *cry* gene have taken large crop market shares world wide, particularly in the US[5].

The structural diversity of Cry toxins

Currently, the crystal toxins are classified on the basis of amino acid sequence homology, where each protoxin acquired a name consisting of the mnemonic Cry (or Cyt) and four hierarchical ranks consisting of numbers, capital letters, lower case letters and numbers (e.g. Cry25Aa1), depending on its place in a phylogenetic tree. Thus, proteins with less then 45% sequence identity differ in primary rank (Cry1, Cry2, etc.), and 78% and 95% identity constitute the borders for secondary and tertiary rank, respectively. This system replaces the old nomenclature using roman numerals[6].

Alignment of the Cry toxins reveals the presence of five conserved sequence blocks common to a large majority of the proteins. Figure 2a shows the presence or absence of each of these blocks in subgroups of the toxin family. Also apparent from this figure is the diversity in length between the different protoxins; in particular, one large group contains protoxins that are approximately twice as long as the majority of the rest. The C-terminal extension found in the longer protoxins is not part of the active toxin (it is digested by proteases in the

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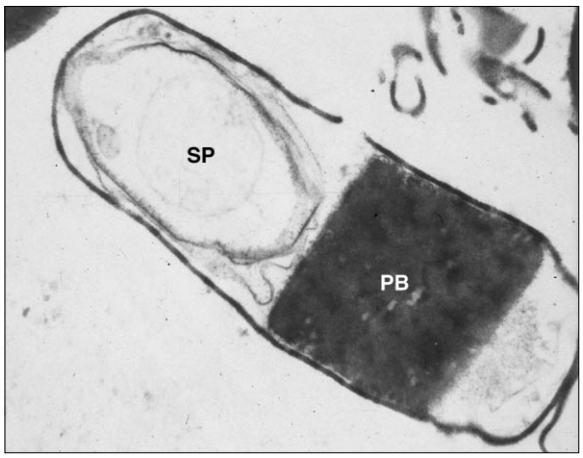


Fig. 1. Transmission electron micrograph of a sporulating *Bacillus thuringiensis* (Bt) cell. δ-Endotoxins are produced as regularly shaped crystals (PB; protein body) – hence the name crystal (Cry) proteins – next to a spore (SP). The vegetative cell wall will eventually break to release the spore and crystal. The cell shown is approximately 2 μm long.

insect gut, see Fig. 2a), but it is believed to have a role in the formation of the crystal[1]. The three-dimensional structures of three activated forms of Bt toxins, a Cry1 (Ref. 7), a Cry2 and a Cry3 (Ref. 8), have been solved by X-ray crystallography and are remarkably similar, each consisting of three domains (Fig. 2b). The N-terminal domain I consists of seven α-helices (six amphipathic helices around a central core helix). Domain II is a so-called 'β-prism', with three-fold symmetry consisting of three β-sheets having a 'Greek key' conformation. The C-terminal domain III consists of two antiparallel β-sheets in a 'jelly-roll' formation. Domain I is involved in membrane insertion and pore formation. Domains II and III are both involved in receptor recognition and binding. Additionally, a role for domain III in pore function has been found.

Box 1. The armory of an insect pathogen

Besides the Cry proteins, which are the focus of this review, cytolysins (Cyt toxins), which act by a different mechanism, are also found within the crystal. Bt produces various virulence factors other than the crystal proteins, including secreted insecticidal protein toxins, α-exotoxins, β-exotoxins, hemolysins, enterotoxins, chitinases and phospholipases[a]. The spore itself contributes to pathogenicity, often synergizing the activity of the crystal proteins[b]. All of these factors might have a role in insect pathogenesis under natural conditions, helping the bacterium to develop in the dead or diseased insect larvae, but the exact contribution of each factor is often unknown. Although the Cry proteins are commonly referred to as 'Bt toxins', a few Cry proteins were found in *Bacillus popilliae* and in *Clostridium bifermentans*[c].

References

a Hansen, B.M. and Salamitou, S. (2000) Virulence of *Bacillus thuringiensis*. In *Entomopathogenic Bacteria: From Laboratory to Field Application* (Charles, J. *et al.*, eds), pp. 41–64, Kluwer Academic Publishers

b Johnson, D. *et al.* (1998) Spore coat protein synergizes *Bacillus thuringiensis* crystal toxicity for the Indianmeal moth (*Plodia interpunctella*). *Curr. Microbiol.* 36, 278–282

c Schnepf, E. *et al.* (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* 62, 775–806

Mode of action and structure–function relationships

The toxin exerts its pathological effect by forming lytic pores in the cell membrane of insect midgut epithelial cells by the proposed mechanism outlined in Fig. 3. Each step of this mechanism could modulate activity against a particular insect and therefore the overall specificity of a toxin. Solubilization of protoxins (i.e. the protein as it appears in crystals, before the activation by gut proteases) with an extended C-terminus depends on the highly alkaline gut pH of Lepidoptera and Diptera. Some of the toxins with potential activity against Coleoptera are only toxic after *in vitro* solubilization, probably because the protoxin is insoluble at the neutral to weakly acidic gut pH of Coleoptera; for example, Cry1Ba for some species[9] and Cry7Aa (which additionally also requires *in vitro* activation)[10]. Differences in proteolytic activity between target insects can also lead to changes in specificity[9,11]. Again, large differences in gut physiology among the different insect orders might have a role in this aspect: the main digestive proteases of Lepidoptera and Diptera are serine proteases, whereas those of Coleoptera are mainly cysteine and aspartic proteases[12].

By far the most studied determinant of toxin specificity is receptor binding. Interactions of toxins with specific high-affinity binding sites on the gut epithelium, where different toxins recognize different or (partially) overlapping sites, have been demonstrated in many insects[13]. The crucial role of this receptor binding for toxicity is emphasized by the observation that insects selected for resistance to a Cry toxin often have no or reduced binding capacity for that toxin[1]. Domain II, especially the highly variable loops in its apex (Fig. 2b), is involved in specific receptor binding as shown by mutagenesis studies[1]. More recent is the recognition of the role of domain III in receptor binding and thus in insect specificity[14–16]. Most notably, domain III of Cry1Ac has lectin-like properties such that it binds the sugar *N*-acetylgalactosamine on the putative receptors[17,18]. Two such Cry toxin receptors from various insects have been identified and studied so far, but the debate on whether these are functional receptors leading to pore formation is still on going. Aminopeptidase N (APN), a ubiquitous gut enzyme attached to the gut epithelial cell membranes by a glycosyl-phosphatidylinositol (GPI)-anchor has been studied *in vitro* in some detail[19–21].

The observation that domain II and domain III can each function separately in a two-step mechanism of binding of Cry1Ac to an APN (Ref. 22) could help to explain why novel combinations of domains II and III can have a changed specificity (see below). Many studies, demonstrating that APN is important in toxin binding exist, and some reconstitution studies link toxin binding to APN with functional pore formation in artificial membranes[23]. However, several attempts to increase toxin sensitivity in cultured insect cells by transforming them with the appropriate APN-encoding genes have failed so far. Also, some toxin-gene mutations that have a profound effect on binding

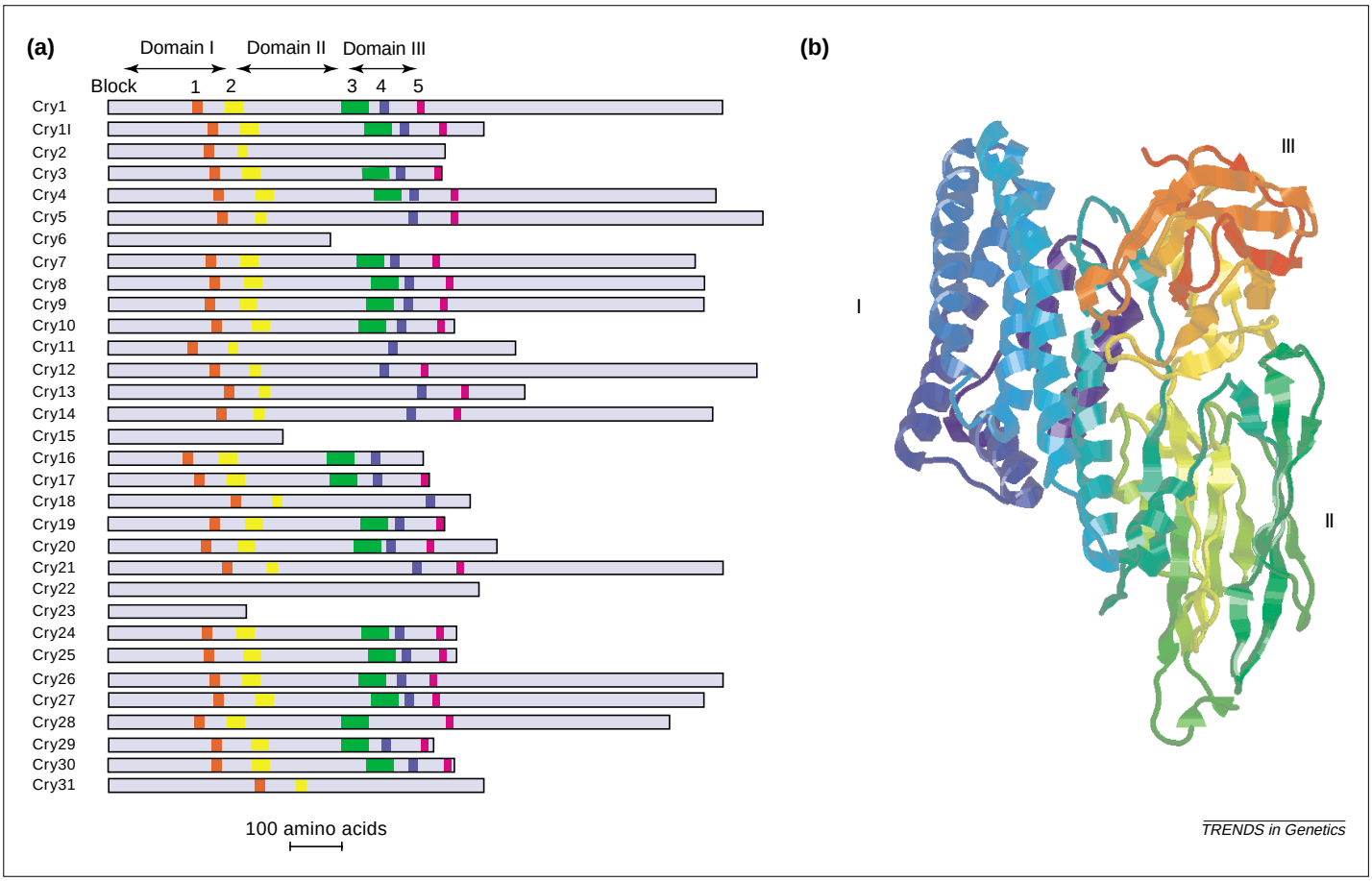


Fig. 2. Primary and tertiary structure of Cry toxins. (a) Relative lengths of Cry protoxins and position of the five conserved blocks, if present. More details on these conserved blocks, as well as the identification of three more blocks in the C-terminal ends of the longer protoxins, can be found elsewhere[1]. The positions of the three domains of the activated toxin are indicated for Cry1 and vary with the positions of blocks 2 and 3 for the other toxins. The remainder of the protoxin, consisting of short N-terminal part (20–40 amino acids) preceding the first domain and the C-terminal part following the third domain in the longer protoxins, is digested away by gut proteases during the activation process. (b) Three-dimensional structure of an activated toxin, Cry1Aa (Ref. 7). The toxin has three structural domains. Domain I (blue) is involved in membrane insertion and pore formation. Domain II (green) and domain III (yellow-red) are involved in receptor recognition and binding. Conserved block 1 is in the central helix of domain I, block 2 is at the domain I–II interface, block 3 is at the boundary between domains II and III, block 4 is in the central β-strand of domain III and block 5 is at the end of domain III.

of the toxin to purified APN have little effect on *in vivo* toxicity[18,24]. These observations make the status of APN as a functional *in vivo* receptor unsure. The other putative receptor, a cadherin-like protein[25] seems to be a better candidate because its expression on the surface of insect cells renders previously resistant cells sensitive to the toxin[26].

Following binding, at least part of domain I inserts into the membrane in an oligomer to form an aqueous pore with other toxin molecules. Domain I resembles other pore-forming or membrane-translocation domains of bacterial toxins[27], and, by analogy to those, membrane entry might start by insertion of a hydrophobic two-helix hairpin. In an umbrella-like model, based on mutational and biophysical studies, α-helices four and five of several toxin molecules in an oligomer make up the pore, with the rest of the protein spreading over the membrane surface[28,29]. There is also evidence that domain III is involved in pore function[1].

Phylogenetic relationships of Cry sequences

Cry proteins were originally classified on the basis of the insect order to which the species they affect belong[30]. As the number and variety of Cry proteins grew, this classification could no longer be maintained, and it was abandoned for the current system based on amino acid sequence similarity[6]. However, all parts of the active toxin and the protoxin-specific C-terminal parts (if present) contribute to the overall similarity that determines classification. Separate phylogenetic analysis of the three domains of the active toxin is more likely to yield interesting insights into how the extensive variety in structure and specificity came into existence[31,32]. Such an analysis is shown in Fig. 4, with the insect-order specificity shown as color-coded branches. The role of the protoxin-specific part in the evolutionary development of insect specificity should not be discarded however, as the presence of a C-terminal extension might affect activity as mentioned above for Cry1Ba and Cry7Aa.

A brief comparison of the trees for domains I and II shows that their overall structure is very similar, having identical main branches that correspond to the current classification of the protoxins; that is, the nearest neighbors of Cry1Aa are the other Cry1A toxins, the nearest neighbor of Cry1Ca is Cry1Cb, and so on. Domains I and II of Cry9Aa behave differently; they don't cluster with the other Cry9 toxins. The classification of Cry9Aa is therefore primarily based on its homology with Cry9Ea in

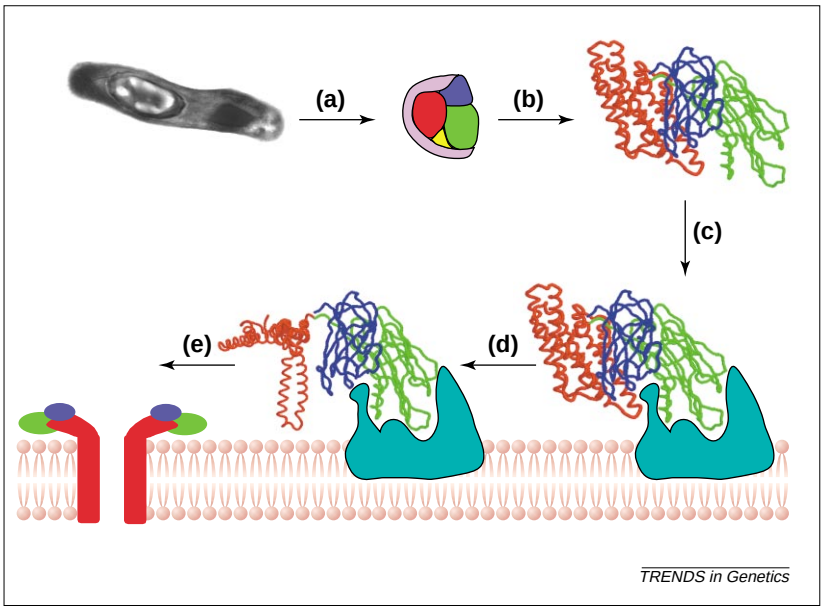


Fig. 3. Mode of action of Cry toxins. (a) After ingestion by the insect, crystals dissolve in the gut juice. (b) Gut proteases subsequently clip off the C-terminal extension in the longer Cry proteins (purple) as well as a small N-terminal fragment (yellow). (c) The resulting 'activated' toxin (i.e. the structure depicted in Fig. 2b) binds to receptors on the epithelial cell membrane, a process in which both domain II and domain III are involved. (d) Structural rearrangement of domain I might follow allowing a two-helix hairpin to insert into the membrane. (e) Inserted toxins form pores probably as oligomers, but the architecture of the pore is still unknown.

domain III and with all Cry9 proteins in the protoxin-specific part[31,32], suggesting that Cry9Aa evolved independently from the other Cry9 proteins but obtained a Cry9-specific C-terminal extension more recently, perhaps by a recombination event.

Domains I and II

The domain I and domain II trees show a correlation between the clustering patterns and the overall insect-order specificity, as was noticed previously[31]. This might not have been expected for domain I, as it is not generally thought to be a major determinant of insect-species specificity. This correlation might reflect other factors that affect insect-order specificity, such as target membrane composition, pH or involvement of pest-specific proteases. An example of possible host-gut pH involvement in toxin co-evolution was suggested by Grochulski *et al.*, who noted that for basic amino acids in Cry1 sequences there is a distinct bias for arginine over lysine[7]. This bias is lacking in Coleoptera-specific toxins. The very high pK_a of arginine compared with lysine might be required for maintaining positive charge even at the high pH of Lepidoptera guts. We find the same bias for arginine over lysine in all Lepidoptera-active toxins (Cry1, Cry2 and Cry9) except, curiously, in the Cry1I toxins. Alternatively, structural constraints might have prevented domains I and II evolving independently, thus linking domain I to the specificity-determining domain II.

Although there is clustering according to insect-order activity, it is also clear that comparatively unrelated clusters have similar activities, suggesting that activity in a particular order might have developed along different evolutionary paths. For example, four clusters in the domain I and II trees have Lepidoptera-specific activity. The largest cluster, containing, among others, the Cry1A toxins, is exclusively active against Lepidoptera. Surprisingly, the remaining Cry1B, Cry1I and Cry1K toxins are in a separate cluster, relatively distant from the Cry1A cluster, and at least some of its members also have activity against Coleoptera. One might speculate that the neutral to slightly acidic pH of Coleoptera guts, as opposed to the highly alkaline gut pH of Lepidoptera, could partially or wholly explain this different position. This is another example of how the classification based on the overall protoxin sequence could conceal a diverging evolutionary history. Cry2 toxins and Cry9 toxins (except Cry9Aa) form two other Lepidoptera-active clusters (Cry2Aa also has Diptera-specific activity). Also the Coleoptera-active toxins are found in different clusters, one comprising the Cry3, Cry7 and Cry8 toxins. The proximity of the recently reported Cry26 and Cry28 toxins to this cluster suggests they might be toxic to Coleoptera.

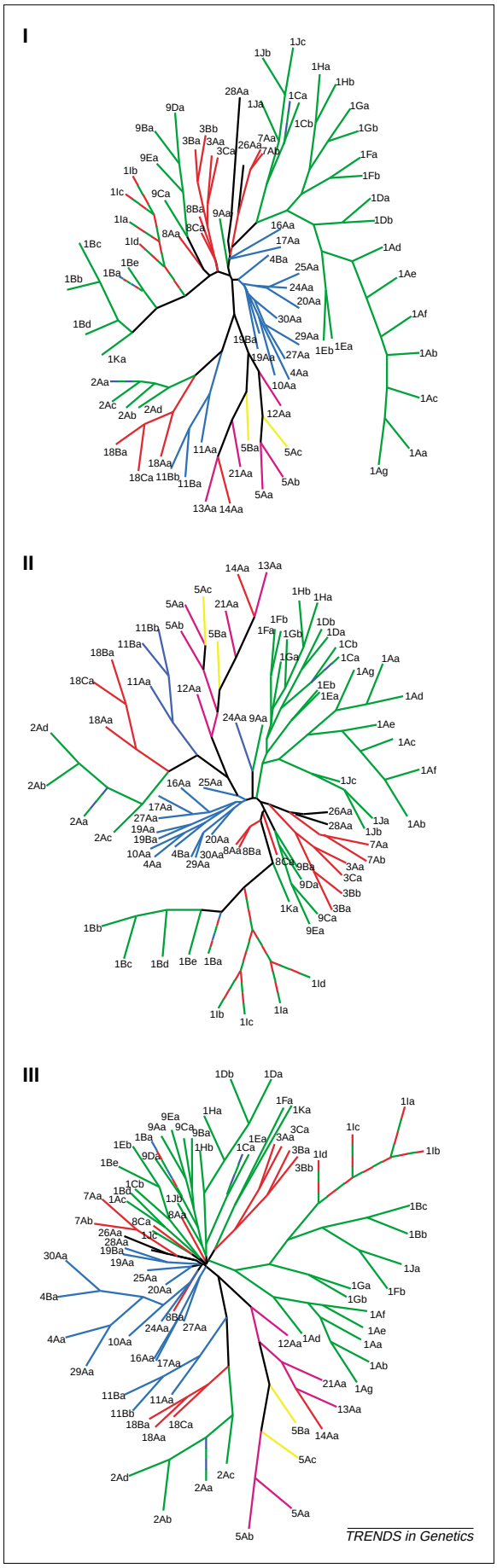
Another Coleoptera-active cluster is that comprising the Cry18 toxins of *Bacillus popilliae*, which is on a branch together with Lepidoptera- and Diptera-specific sub-branches. Interestingly, the Scarab beetles, against which Cry18 toxins are active, have an alkaline gut pH like the Lepidoptera and Diptera, and they might lack gut cysteine proteases. However, other Coleoptera (against which Cry3 and Cry7 toxins are active, as are Cry1Ba and Cry1I toxins) have a neutral to slightly acidic pH, which might again explain this divergence[12].

Diptera-specific toxins are also found in two or three different clusters. Nematode- and Hymenoptera-specific toxins (Cry5, Cry12, Cry13 and Cry21) are arranged together in a single branch. An exception is Cry14, a Coleoptera-specific toxin that is found in this group.

Domain III

The topology of the phylogenetic tree of domain III (Fig. 4) is very different from the topology of the domain I and domain II trees. The domains III from the Coleoptera-specific toxins are distributed along the tree in different branches, and only the domains III of Cry3 toxins are arranged in a single branch. By contrast, the domains III from the Nematode- and Hymenoptera-specific toxins are arranged together in a single branch, as are their domains I and II. The phylogenetic analysis of the three domains of this group of proteins indicates that they have co-evolved as a separate group that is relatively far from the rest of the Cry protein family. It will be worthwhile to determine whether Cry14A toxin, also in this group, has any activity against nematodes, because this toxin has only been described as toxic to *Diabrotica* spp., which are

Fig. 4. Phylogenetic relationships of the separate domains. Unrooted phylogenetic trees of domains I, II and III of 79 known subgroups of Cry proteins obtained by the parsimony method. Trees were constructed basically as described earlier[31], except that toxin alignments were made using DbClustal[45], and updated with Cry protein sequences that were released since 1997. Cry6, Cry15, Cry22 and Cry23 sequences were not included because they do not show similarities with the rest of the Cry protein family (see also Fig. 2a). Shown are consensus trees resulting from 100 analyses using the bootstrapping tool and the CONSENSE program. Branches are color-coded according to the insect order-specificity of the toxins, as far as is known: red, Coleoptera specific; green, Lepidoptera specific; blue, Diptera specific; magenta, nematode specific; yellow, Hymenoptera specific.



Coleoptera[33]. Similarly, the distribution of the three domains of the Cry2, Cry11 and Cry18 toxin group suggest that they have evolved as a separate group, also far from the rest of the Cry protein family.

The domains III from the Lepidoptera-specific toxins are arranged in two main branches. Domains III from Cry1A toxins are arranged in a single branch with the exception of Cry1Ac, which clearly evolved from a different origin, similar to that of Cry1Bd. In this branch, we find the domains III of Cry proteins with double specificity (e.g. Cry1I is Lepidoptera and Coleoptera specific) and some Cry1B sequences, together with Cry1Ja, Cry1Fb and Cry1G. Thus, the Cry1I and Cry1B toxins have domains I and II that share higher similarity with the Coleoptera-specific toxins, whereas their domains III are more related to the Lepidoptera-active toxins. This could suggest a reason why these toxins have dual insect-order specificity.

Domain swapping as a putative mechanism for evolution of new specificities

Although it seems that domains I and II have co-evolved, experimentation shows that this is not necessarily owing to structural constraints because domain I swapping between different toxins can yield biologically active toxins[34–37]. Such experiments show that exchange of domain I can affect the size of the membrane pores formed and can negatively affect toxicity against an insect. To our knowledge, they have not yet revealed a role for domain I in determining specificity at the insect-species level[37].

Although domains I and II, and in some cases domain III as well, seem to have co-evolved towards certain specificities, there are also strong indications that part of the variation in toxin structure and specificity is a result of domain III swapping by homologous recombination, especially among Cry1 toxins. Cry1Ca and Cry1Cb toxins have domains I and II that are so similar that they can be considered as variants of the same protein. The same is true for the Cry1Ea and Cry1Eb toxins. However, it is clear that domain III from Cry1Ca and Cry1Ea have a common origin, whereas domain III from Cry1Cb and Cry1Eb are variants of the same molecule, closely related to domain III of Cry1Be. These data suggest that a reciprocal swap between Cry1C and Cry1E progenitors might have occurred. Similarly, Cry1Fa and Cry1Fb have identical domains I and II.

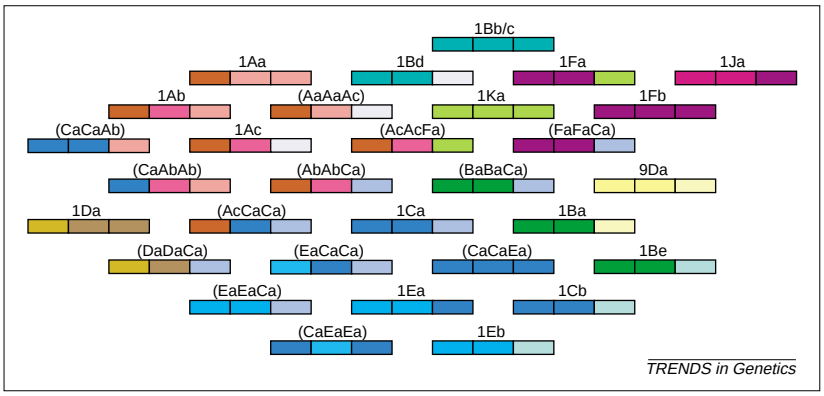


Fig. 5. Domain swapping as a mechanism for generating diversity. Domain sequence similarities among some of the natural class Cry1 toxins and artificial Cry1 hybrids or chimeras, demonstrating how several natural toxins might have arisen by 'domain swapping' between different parents. Domains with identical colors in different natural toxins are closest neighbors in the phylogenetic trees of Fig. 4. Toxins with domain composition in parentheses are hybrid toxins made by either *in vivo* recombination or restriction fragment exchange[35,37–42]. Domain III of Cry1Ca is a similar color to that of Cry1Ea to show that although they are closely related, they have distinct effects on toxin specificity[38]. We speculate, therefore, that reciprocal 'domain III swapping' between Cry1Cb and Cry1Ea gave rise to Cry1Eb and an ancestor of Cry1Ca much like the artificial hybrid (CaCaEa), which then evolved into Cry1Ca proper.

However, domain III of Cry1Fa is clustered with that of Cry1Ka and domain III of Cry1Fb with that of Cry1Ja. Also, Cry1Ba has domains I and II closely related to those of the rest of the Cry1B toxins, but its domain III is clustered with that of Cry9Da. Cry1D toxins have a domain I related to those of the Cry1A proteins, a domain II similar to that of the Cry1C proteins and domains III that are clustered with those of the Cry1H toxins. Cry1Ac domain III is not related to domain III of any other Cry1A toxin, but almost identical to that of Cry1Bd.

The above observations tempted us to speculate that domain III shuffling is a mechanism for generating new specificities in Nature. Results of laboratory experiments involving domain swapping by homologous recombination or restriction-fragment exchange support this assumption[34,36,38–43]. For example, several Cry1 toxins (e.g. Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ea) with low or no activity against beet armyworm (*Spodoptera exigua*) become active when their domain III is replaced by that of Cry1Ca (Ref. 41). Alternatively, hybrids of Cry1Ac and Cry1Fa have a wider target spectrum than either of the parental toxins from which they were derived[42]. Figure 5 shows the relative similarities between different members of the Cry1 subfamily, as well as of hybrid toxins generated by experimentation, revealing where domain shuffling might have (in Nature), or has (in the laboratory), occurred. It should be noted that even if domain III similarity is strong, small differences (which might be indicating a sequence divergence after a recombination event) might still strongly affect specificity. For example, although Cry1Ca and Cry1Ea domains III are nearest neighbors in the phylogenetic tree, these domains are sufficiently divergent to explain a difference in activity spectrum of the two toxins. Cry1Ca domain III confers beet armyworm activity when transferred to several Cry1 toxins including Cry1Ea, whereas Cry1Ea domain III does not[38,44]. Indeed small changes to a toxin by *in vitro* modification have resulted in the production of mutants with enhanced activity as a result of increased binding or proteolytic stability[1].

Conclusion

We are still far from understanding exactly what determines the insect specificity of a particular δ-endotoxin from *Bacillus thuringiensis*. This is mainly owing to the shortage of available cloned or purified receptors for different toxins that could allow the detailed study of their interaction at the molecular level. Also, we do not yet fully understand the role of factors like gut pH, activating proteases and target membrane composition that might affect the broader insect-order specificity of the main classes of toxins. Study of the phylogenetic relationships, however, suggests how the overwhelming diversity and specificity might have come into existence – by sequence divergence and by domain swapping through homologous recombination. This insight has led, and will lead, by experimentation, to a better understanding of the basis of specificity and the practical application of improved toxins in agriculture.

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References

1 Schnepf, E. *et al*. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* 62, 775–806
2 Thomas, D. *et al*. (2001) Plasmid transfer between *Bacillus thuringiensis* subsp. *israelensis* strains in laboratory culture, river water, and Dipteran larvae. *Appl. Environ. Microbiol.* 67, 330–338
3 Marroquin, L.D. *et al*. (2000) *Bacillus thuringiensis* (Bt) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. *Genetics* 155, 1693–1699
4 Zhong, C.H. *et al*. (2000) Characterization of a *Bacillus thuringiensis* delta-endotoxin which is toxic to insects in three orders. *J. Invertebr. Pathol.* 76, 131–139
5 de Maagd, R.A. *et al*. (1999) *Bacillus thuringiensis* toxin mediated insect resistance in plants. *Trends Plant Sci.* 4, 9–13
6 Crickmore, N. *et al*. (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* 62, 807–813
7 Grochulski, P. *et al*. (1995) *Bacillus thuringiensis* CryIA(a) insecticidal toxin – crystal structure and channel formation. *J. Mol. Biol.* 254, 447–464
8 Li, J. *et al*. (1991) Crystal structure of insectidicidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. *Nature* 353, 815–821
9 Bradley, D. *et al*. (1995) The insectidal CryIB crystal protein of *Bacillus thuringiensis* ssp. *thuringiensis* has dual specificity to Coleopteran and Lepidopteran larvae. *J. Invertebr. Pathol.* 65, 162–173
10 Lambert, B. *et al*. (1992) Novel *Bacillus thuringiensis* insecticidal crystal protein with a silent activity against coleopteran larvae. *Appl. Environ. Microbiol.* 58, 2536–2542
11 Haider, M.Z. *et al*. (1986) Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal delta-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. *Eur. J. Biochem.* 156, 531–540
12 Terra, W. and Ferreira, C. (1994) Insect digestive enzymes: properties, compartmentalization and function. *Comp. Biochem. Physiol.* 109B, 1–62
13 Hofmann, C. *et al*. (1988) Specificity of *Bacillus thuringiensis* delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. *Proc. Natl. Acad. Sci. U. S. A.* 85, 7844–7848
14 Aronson, A.I. *et al*. (1995) Mutagenesis of specificity and toxicity regions of a *Bacillus thuringiensis* protoxin gene. *J. Biol. Chem.* 177, 4059–4065
15 de Maagd, R.A. *et al*. (1996) Different domains of *Bacillus thuringiensis* delta-endotoxins can bind to insect midgut membrane proteins on ligand blots. *Appl. Environ. Microbiol.* 62, 2753–2757
16 Lee, M.K. *et al*. (1995) Domain III exchanges of *Bacillus thuringiensis* Cry1A toxins affect binding to different Gyspy Moth midgut receptors. *Biochem. Biophys. Res. Commun.* 216, 306–312

17 de Maagd, R.A. *et al*. (1999) Domain III of the *Bacillus thuringiensis* delta-endotoxin Cry1Ac is involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. *Mol. Microbiol.* 31, 463–471
18 Burton, S.L. *et al*. (1999) N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. *J. Mol. Biol.* 287, 1011–1022
19 Knight, P.J.K. *et al*. (1994) The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. *Mol. Microbiol.* 11, 429–436
20 Garczynski, S.F. and Adang, M.J. (1995) *Bacillus thuringiensis* CryIA(c) delta-endotoxin binding aminopeptidase N in the *Manduca sexta* midgut has glycosyl-phosphatidylinositol anchor. *Insect Biochem. Mol. Biol.* 25, 409–415
21 Gill, S.S. *et al*. (1995) Identification, isolation, and cloning of a *Bacillus thuringiensis* Cry1Ac toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. *J. Biol. Chem.* 270, 27277–27282
22 Jenkins, J.L. *et al*. (2000) Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gypsy moth aminopeptidase N receptor. *J. Biol. Chem.* 275, 14423–14431
23 Luo, K. *et al*. (1997) The *Heliothis virescens* 170 kDa aminopeptidase functions as 'receptor A' by mediating specific *Bacillus thuringiensis* Cry1A delta-endotoxin binding and pore formation. *Insect Biochem. Mol. Biol.* 27, 735–743
24 Jenkins, J.L. *et al*. (1999) Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. *FEBS Lett.* 462, 373–376
25 Vadlamudi, R.K. *et al*. (1995) Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. *J. Biol. Chem.* 270, 5490–5494
26 Nagamatsu, Y. *et al*. (1999) The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin. *FEBS Lett.* 460, 385–390
27 Parker, M. and Pattus, F. (1993) Rendering a membrane protein soluble in water: a common packing motif in bacterial protein toxins. *Trends Biochem. Sci.* 18, 391–395
28 Gazit, E. *et al*. (1998) The structure and organization within the membrane of the helices composing the pore-forming domain of *Bacillus thuringiensis* delta-endotoxin are consistent with an 'umbrella-like' structure of the pore. *Proc. Natl. Acad. Sci. U. S. A.* 95, 12289–12294
29 Masson, L. *et al*. (1999) Helix 4 of the *Bacillus thuringiensis* Cry1Aa toxin lines the lumen of the ion channel. *J. Biol. Chem.* 274, 31996–32000
30 Höfte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53, 242–255
31 Bravo, A. (1997) Phylogenetic relationships of *Bacillus thuringiensis* delta-endotoxin family proteins and their functional domains. *J. Bacteriol.* 179, 2793–2801
32 Crickmore, N. (2000) The diversity of *Bacillus thuringiensis* delta-endotoxins. In *Entomopathogenic Bacteria: from Laboratory to Field Application* (Charles, J. *et al*.), pp. 65–79, Kluwer Academic Publishers
33 Payne, J. and Narva, K. (1994) Process for controlling corn rootworm larvae. Patent WO94/16079
34 Widner, W.R. and Whiteley, H.R. (1990) Location of the Dipteran specificity region in a Lepidopteran-Dipteran crystal protein from *Bacillus thuringiensis*. *J. Bacteriol.* 172, 2826–2832
35 Honée, G. *et al*. (1991) The C-terminal domain of the toxic fragment of a *Bacillus thuringiensis* crystal protein determines receptor binding. *Mol. Microbiol.* 5, 2799–2806
36 Masson, L. *et al*. (1994) Specificity domain localization of *Bacillus thuringiensis* insecticidal toxins is highly dependent on the bioassay system. *Mol. Microbiol.* 14, 851–860
37 Rang, C. *et al*. (1999) Interaction between functional domains of *Bacillus thuringiensis* insecticidal crystal proteins. *Appl. Environ. Microbiol.* 65, 2918–2925
38 Bosch, D. *et al*. (1994) Recombinant *Bacillus thuringiensis* crystal proteins with new properties: possibilities for resistance management. *Biotechnology* 12, 915–918
39 Ge, A.Z. *et al*. (1991) Functional domains of *Bacillus thuringiensis* insecticidal crystal proteins. *J. Biol. Chem.* 266, 17954–17958
40 de Maagd, R.A. *et al*. (1996) Domain III substitution in *Bacillus thuringiensis* delta-endotoxin CryIA(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. *Appl. Environ. Microbiol.* 62, 1537–1543
41 de Maagd, R.A. *et al*. (2000) *Bacillus thuringiensis* delta-endotoxin Cry1C domain III can function as a specificity determinant for *Spodoptera exigua* in different, but not all, Cry1-Cry1C hybrids. *Appl. Environ. Microbiol.* 66, 1559–1563
42 Malvar, T. and Gilmer, A. (1998) Broad-spectrum delta-endotoxins. Patent WO 98/22595
43 Schnepf, H.E. *et al*. (1990) Specificity-determining regions of a Lepidopteran-specific insecticidal protein produced by *Bacillus thuringiensis*. *J. Biol. Chem.* 265, 20923–20930
44 de Maagd, R.A. *et al*. (1999) Identification of *Bacillus thuringiensis* delta-endotoxin Cry1C domain III amino acid residues involved in insect specificity. *Appl. Environ. Microbiol.* 65, 4369–4374
45 Thompson, J. *et al*. (2000) DbClustal: rapid and reliable global multiple alignments of protein sequences detected by database searches. *Nucleic Acids Res.* 28, 2919–2926

What regulates mitochondrial DNA copy number in animal cells?

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The study of the control of mitochondrial DNA copy number spans several decades and has identified many factors involved in the replication of the mitochondrial genome. However, the mechanisms involved in the regulation of this process are still obscure, particularly in animal cells. During the past decade, however, the identification of human diseases associated with drastically reduced levels of mtDNA caused renewed interest in this topic. Here, I will discuss recent work that sheds some light on how animal cells might maintain and control mtDNA levels.

Although I focus this article on recent advances in mtDNA copy-number control in animal cells, it is useful to make a brief comparison with the better-understood system in *Saccharomyces cerevisiae*. A more detailed comparison between these two systems can be found elsewhere[1]. However, it is important to bear in mind that there could be important differences in the genetic control and mechanisms of mtDNA maintenance between yeast and animal cells. As in animal cells, mtDNA replication in the budding yeast depends on a DNA polymerase, RNA polymerase, single-stranded-DNA-binding proteins (mtSSB) and RNA processing enzymes. In this system, however, additional protein factors that influence mtDNA maintenance or recombination have been identified. In a haploid yeast cell, the ~80-kb mitochondrial genomes are organized in 10–20 distinct nucleoids (protein–DNA complexes), which are spherical or ovoid, measuring 0.3–0.6 μm in

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