Received: 10 November 2019 | Revised: 11 March 2020 | Accepted: 12 March 2020

DOI: 10.1002/arch.21673

REVIEW ARTICLE

Archives of INSECT BIOCHEMISTRY AND PHYSIOLOGY WILEY

How do toxins from *Bacillus thuringiensis* kill insects? An evolutionary perspective

David G. Heckel

Department of Entomology, Max Planck Institute for Chemical Ecology, Jena, Germany

Correspondence
David G. Heckel, Department of Entomology, Max Planck Institute for Chemical Ecology, Hans-Knoell-Str. 8, Jena D-07745, Germany.
Email: heckel@ice.mpg.de

Funding information
Max-Planck-Gesellschaft

Abstract

Three-domain Cry toxins from the bacterium *Bacillus thuringiensis* (Bt) are increasingly used in agriculture to replace chemical insecticides in pest control. Most chemical insecticides kill pest insects swiftly, but are also toxic to beneficial insects and other species in the agroecosystem. Cry toxins enjoy the advantages of high selectivity and the possibility of the application by sprays or transgenic plants. However, these benefits are offset by the limited host range and the evolution of resistance to Bt toxins by insect pests. Understanding how Bt toxins kill insects will help to understand the nature of both problems. The recent realization that ABC transporters play a central role in the killing mechanism will play an important role in devising solutions.

KEYWORDS

ABC transporter, *Bacillus thuringiensis*, cadherin, evolution, resistance

1 | INTRODUCTION

The discovery of bacteria in dead insects has led to one of the oldest commercialized insecticides as well as the newest application in pest-resistant transgenic plants (Bravo, Likitvivatanavong, Gill, & Soberón, 2011). The same bacterium, named "*Bacillus sotto*" by Ishiwata and "*Bacillus thuringiensis*" (Bt) by Berliner was developed into a commercial preparation "Sporéine" in 1938 (Sanchis, 2011). When cultures of Bt sporulate, they produce large

parasporal proteinaceous crystalline inclusions (Cry toxins) which kill susceptible insects that eat them. Several other commercial preparations were developed, but these were eventually eclipsed by DDT and other chemical insecticides. Bt re-emerged as a significant crop protectant with the development of transgenic cotton, maize, and soybean. Cotton transformed with the Cry1Ac gene was developed by Monsanto and released in the United States and Australia in the late 1990s. Bt cotton enabled reduction of the ineffective and environmentally damaging chemical sprays by 50–80%, while providing good protection against some highly susceptible lepidopteran pests. In 2017, 103 million hectares of transgenic Bt crops were grown worldwide (ISAAA, 2017).

The most widely used Bt toxins are the 3-domain Cry toxins, so-called on the basis of their common structure. Examples include Cry1Ac active against certain Lepidoptera, Cry2Ab targetting some Lepidoptera but also active on Diptera, and the coleopteran-active Cry3Aa. Not all members of this structural family are officially designated as toxins, for example, certain parasporins have no known animal targets, although some are toxic to human cancer cells (Krishnan et al., 2017). Bt also makes other types of insecticidal proteins; for example, the VIP proteins with different domain structures and modes of action. The Bt nomenclature committee is currently revising the classification to apportion different protein folds into differently named families (Crickmore, 2017).

For our purposes, the point is that the 3-domain Cry toxins are cytotoxic, not to all cells but to certain cells of certain insect species. They are so potent that they are useful in pest control. How do they work? We could pose the question at two levels: (a) How do Cry toxins kill insect cells? At least some cell death is required for pest control, even if the insect is not killed. (b) What eventually kills the pest insect? Effective control need not require pest mortality, only reduction of feeding damage. But operationally the effectiveness of the toxin is measured by its killing potency, for example, the median lethal concentration, LC50. We would also want to know why some other pests, as well as beneficial insects, are not killed.

Are the answers to these questions important? At one level, possibly not. If Bt toxins keep working, and if we are not interested in expanding their range, answering these questions may satisfy our curiosity but will not have any practical value. However, Bt toxins will not keep working forever, and expanding their use to other pest targets would have great benefits in further reducing the use of chemical insecticides. Answering these questions will be beneficial for the sustainable and increasing use of Cry toxins in agriculture.

The second question deserves more attention than we can give it here, but it is important to point out that there was a controversy in the past as to whether Cry toxins themselves were sufficient to kill insects. A widely cited study claimed that infection with other bacteria was necessary, because prior feeding with antibiotics could protect gypsy moth larvae from Bt (Broderick, Raffa, & Handelsman, 2006). Subsequent studies criticized the experimental design and provided evidence that toxin with or without Bt spores could kill insects regardless of midgut microbiota, and that some bacteria were actually protective (Broderick et al., 2009; Johnston & Crickmore, 2009; Raymond et al., 2009). A comprehensive review drove home the point that Bt itself is a *bona fide* pathogen, with many evolutionary adaptations to pathogenesis (Raymond, Johnston, Nielsen-LeRoux, Lereclus, & Crickmore, 2010). Insects feeding on crops, whether transgenic or not, have bacteria in their midguts and keeping these bacteria in check with the immune system undoubtedly improves insect survivorship in the field. Experimentally weakening the immune response by silencing immune genes can increase bacterial populations, hastening mortality after exposure to Cry toxin (Caccia et al., 2016). However, an enhanced immune response is unlikely to provide as much protection against Cry-induced mortality as resistance-causing mutations in Cry-interacting host proteins, to be described below.

The immediate challenge is the evolution of resistance to Cry toxins in target pest insects. The widespread use of Bt sprays quickly resulted in resistance in the diamondback moth, *Plutella xylostella*, a species that had already evolved resistance to most classes of chemical insecticides (Shelton et al., 1993; Tabashnik, Schwartz, Finson, & Johnson, 1992). The first deployment of Cry toxins in transgenic plants was done more cautiously. A concerted resistance management plan for Bt cotton was devised and implemented in the United States and Australia. This "high dose/refuge" strategy (Roush, Fitt, Forrester, & Daly, 1998) was based on generic population models that predicted conditions that would minimize the selection pressure for resistance. In agroecosystems where assumptions of these models have been satisfied, resistance has been effectively delayed. When more assumptions

are violated, especially in the developing world where several factors make resistance management difficult to implement, resistance is accelerating (Tabashnik, Brevault, & Carrière, 2013). The latest data indicate a surge in insect resistance to transgenic crops (Tabashnik & Carrière, 2017). The biggest threat to Cry toxin sustainability is yet to emerge, but can be predicted from the toxin mode of action: mutations with no fitness costs in the molecular targets.

A longer-term challenge is the rational design of Cry toxins to target a wider range of pest insects, especially sucking insects such as aphids and whiteflies. Most attempts to do this, such as mutagenesis, domain-swapping, directed evolution, or fusion to lectin domains, are not informed by recent advances in the Cry toxin mode of action. A detailed understanding of how Cry toxins kill susceptible species will be required to modify them to kill refractory species.

Our thesis is that Cry toxins are ancient weapons, used by Bt to overcome insect hosts to convert their biomass into more bacteria. Cry toxins are encoded in groups on plasmids, giving them the flexibility and versatility of a Swiss army knife. This enables them to counter changes in their primary targets in insect cells, which are also very ancient. There is good reason to believe that Cry toxins and their targets in the midguts of insects have coevolved for hundreds of millions of years. This is because the primary killing mechanism is fundamentally the same for all Cry toxins and their insect targets, but with variations on a theme that has unfolded during evolution. Current-day examples of the evolution of Bt resistance can inform the coevolutionary interaction, and vice-versa.

2 | CRY TOXIN MODE OF ACTION

As currently understood, there are several steps in the mode of action of 3-domain Cry toxins after ingestion by insect larvae (Bravo, Gill, & Soberón, 2007; Ferré & Van Rie, 2002). The crystalline protein associated with the spore, or the protein produced by the plant, dissolves in the insect midgut, releasing the protoxin which may be as large as 135 kDa. The insect's own digestive proteases cleave the protoxin down to a protease-resistant core, the active toxin of ~65 kDa. The toxin binds to membrane-bound proteins on the surface of the midgut epithelial cells. Eventually monomers of the toxin form oligomers, either in solution or after having inserted into the lipid bilayer. Membrane-spanning alpha-helix hairpins of the oligomers create a small pore (10–20 Å) in the membrane. These pores enable cations to flow into the cell, and water follows, possibly through aquaporins, causing the cells to swell and lyse. This is the so-called “colloid-osmotic lysis” mechanism (Knowles & Ellar, 1987). Minor damage might be healed by the insect, but major damage destroys the midgut epithelium, resulting in rapid cessation of feeding and eventual death of the insect.

The discovery that there were specific “receptors,” that is, sites of specific, competitive Cry toxin binding in insect midgut membranes was a major advance (Van Rie, Jansens, Höfte, Degheele, & Van Mellaert, 1989). Specific binding to these receptors was found to be necessary, but not sufficient for toxicity. Analysis of competitive binding curves could even provide an estimate of the number of different binding sites and their cross-reaction with different toxins (Ferré & Van Rie, 2002). Eventually, in 1994 the first Cry-binding insect proteins were discovered: aminopeptidase N (APN) and alkaline phosphatase (Sangadala, Walters, English, & Adang, 1994). Cry1Ab immobilized on an affinity column was used to trap a GPI-anchored APN from the midgut of the tobacco hornworm *Manduca sexta* (Knight, Knowles, & Ellar, 1995). A GPI (glycosylphosphatidylinositol) linkage joins an amino acid near the C-terminal of a protein directly to a lipid inserted in the bilayer. Other GPI-anchored Cry-binding APNs were found, but there was little evidence that these were altered in most Bt-resistant insect strains. A different approach, using co-immunoprecipation with antibodies to the toxin, isolated a protein named BT-R1 from *M. sexta* with 12 extracellular cadherin domains (Vadlamudi, Ji, & Bulla, 1993). Instead of being GPI-anchored, this protein had a single transmembrane domain and a small intracellular domain. Studies on the homologous protein BtR175 in the domesticated silkworm showed that when it was expressed in cultured insect cells (Sf9 cells), it was bound by labeled Cry1Aa toxin (Nagamatsu, Koike, Sasaki, Yoshimoto, & Furukawa, 1999).

A number of other Cry toxin binding proteins have also been discovered using different techniques, some with an effect on resistance.

3 | DISCOVERY OF CRY TOXIN RESISTANCE GENES

The first resistance-conferring mutation found was in the homologous 12-cadherin-domain protein HevCaLP of the tobacco budworm *Heliothis virescens* (Gahan, Gould, & Heckel, 2001). A transposable element insertion caused a frameshift truncating the protein before the transmembrane domain, and the absence of this protein from the epithelial membrane was sufficient to confer high levels of resistance to Cry1Aa, Cry1Ab, and Cry1Ac. Inactivating mutations in the same protein were soon found in other species with Bt resistance (Morin et al., 2003; Xu, Yu, & Wu, 2005). A clever modification of the Cry1A toxins was devised to overcome this type of resistance. Binding of a toxin monomer to the cadherin ectodomain facilitates its N-terminal α1-helix being clipped off by an unknown protease, resulting in monomers that could more efficiently form the "prepore" oligomer in solution (Gómez, Sánchez, Miranda, Bravo, & Soberón, 2002). The ingenious step was to design a modified toxin lacking the α1-helix sequence (Soberón et al., 2007). The so-called Cry1AMod toxins, therefore, could efficiently form pre-pores without the requirement of cadherin binding: a rational antiresistant strategy from detailed studies of the mode of action. The Cry1AMod toxins are also effective on some other resistance mechanisms as well, for reasons not fully understood (Tabashnik et al., 2011).

The second gene with resistance-causing mutations was revealed by positional cloning. In *H. virescens*, a mutation was found in ABCC2, member 2 of the C subfamily of ABC transporters (Gahan, Pauchet, Vogel, & Heckel, 2010). A deletion in the second exon caused a frameshift, preventing the expression of the full-length protein. An independent positional cloning effort in the domesticated silkworm showed that a single tyrosine insertion in an extracellular loop of ABCC2 allowing expression of a full-length protein (not a frameshift) conferred resistance to Cry1Ab in that species (Atsumi et al., 2012). This was confirmed by germ-line transformation: a single transgenic copy of the susceptible ABCC2 gene expressed in the resistant strain conferred susceptibility to Cry1Ab. Subsequent studies in Cry1A-resistant and Cry1F-resistant strains of other Lepidoptera have also found inactivating mutations of ABCC2 (Banerjee et al., 2017; Xiao et al., 2014). These mutations typically cause even greater resistance than inactivation of the cadherin. Expression of wild-type ABCC2 proteins in Sf9 cells made them susceptible to pore formation by Cry1A toxins (Bretschneider, Heckel, & Pauchet, 2016; Tanaka et al., 2013).

The discovery that ABC transporters are involved in the Cry toxin mode of action was completely unexpected. ABC transporters had never been identified in any of the previous binding studies. It is significant that the two pioneering publications used positional cloning—a genetic approach to identify the gene responsible for a given phenotype, independent of any assumptions about biochemical or physiological mechanisms. In hindsight, it can be seen that a protein with very small loops for toxin binding projecting into the lumen, with transmembrane domains buried in the membrane and large cytosolic nucleotide-binding domains isolated from the toxin, would not have been easily detected by affinity column purification, two-dimensional gel electrophoresis, or coimmunoprecipitation. Also unexpected was the finding that different Cry proteins target different ABC transporters. The deletion of ABCA2 of the cotton bollworm confers extremely high resistance to Cry2Ab, one of the additional proteins expressed in transgenic cotton to avoid resistance (Tay et al., 2015). Moreover, a frameshift in the gene encoding ABCB1, a P-glycoprotein, confers resistance to the Cry3Aa toxin in a leaf beetle (Pauchet, Bretschneider, Augustin, & Heckel, 2016). Knocking out the right ABC transporter can make a susceptible species highly resistant to a given Cry toxin. This implies that instead of using unrelated modes of action, the different three-domain Cry toxins target different members of the same superfamily of proteins in a fundamentally similar manner.

To rationalize this common mode of action, it was hypothesized that the three-domain Cry proteins exploit a common feature of all ABC transporters, the so-called ATP-switch mechanism (Heckel, 2012). ABC transporters move small molecules across the lipid bilayer of cells. A small cavity surrounded by the transmembrane domains is

alternatively open to the cytoplasmic side (below) and the lumenal side (above) of the membrane during the transport cycle, driven by ATP binding, hydrolysis and ADP release (Figure 1). It was hypothesized that when the cavity is transiently open to the outside facing the lumen, the prepore inserts into it and is pulled into the lipid bilayer (Figure 2). Evidence for this transient interaction will be challenging to obtain, and there is currently no alternative model for pore insertion of Cry toxins.

Fitting this hypothesis into the "sequential binding" model (Bravo et al., 2011), the binding steps are presumed to occur in a specific order. First, toxin monomers bind to APNs (Garczynski & Adang, 1995), alkaline phosphatases (Perera, Willis, Adang, & Jurat-Fuentes, 2009), polycalins (Hossain et al., 2004), glycoconjugates (Valaitis, Jenkins, Lee, Dean, & Garner, 2001), and other proteins, either to amino acid residues or glycosyl groups (Jurat-Fuentes & Adang, 2004). This reversible binding increases the toxin concentration at the membrane surface. Then toxin monomers bind to the cadherin, accelerating the cleavage of the N-terminal α1-helix which enables oligomer "prepore" formation in solution (Gómez et al., 2002). Binding to the cadherin may also be reversible, but each α1-helix can only be cleaved once. There is likely some background cleavage of the α1-helix because Cry toxins can still kill insects lacking a functional 12-cadherin domain protein. Moreover, ABCC2 of diamondback moth expressed in transgenic *Drosophila* makes them susceptible to Cry1Ac, even though *Drosophila* lacks the ortholog of the 12-cadherin domain protein (Stevens, Song, Bruning, Choo, & Baxter, 2017). The last step in the sequence is the irreversible insertion of the prepore into the membrane, facilitated by possibly reversible interactions with the transmembrane domains of the ABC transporter.

Recently the specialized water channels called aquaporins have been found to promote colloid-osmotic lysis. Knowles and Ellar (1987) were vague about whether water entered through the Cry toxin pore itself, but water also enters through aquaporins to counter the ionic imbalance caused by cation flux through the pore. In Sf9 cells expressing ABCC2, chemical inhibitors of aquaporin inhibited swelling, and overexpression of aquaporins in the same cells promoted swelling (Endo, Azuma, Adegawa, Kikuta, & Sato, 2017).

4 | SYNERGISM IN THE MODE OF ACTION

Studies in which *Bombyx* ABCC2 and BtR175 are expressed in Sf9 cells have shown strong synergism. Pore formation by Cry1Ab is weak when the cadherin BtR175 is expressed alone, strong when ABCC2 is expressed alone, and very strong when both are expressed together (Tanaka et al., 2013). Similar results are obtained with Cry1Ac and the ABCC2 and cadherin HevCaLP of *H. virescens* (Bretschneider et al., 2016). Significantly, this strong synergism is also seen in voltage-clamp experiments with *Xenopus* oocytes expressing the *Bombyx* proteins (Tanaka, Endo, Adegawa, Kikuta, & Sato, 2016). This method currently offers the only direct assay of pore function in a living system, that is, cation flow through the open pore. These experiments can explain why larvae of the double knockout strain YHD3 of *H. virescens* are so much more resistant than the YFO strain with the cadherin knockout or the YEE strain with the ABCC2 knockout (Gahan et al., 2010).

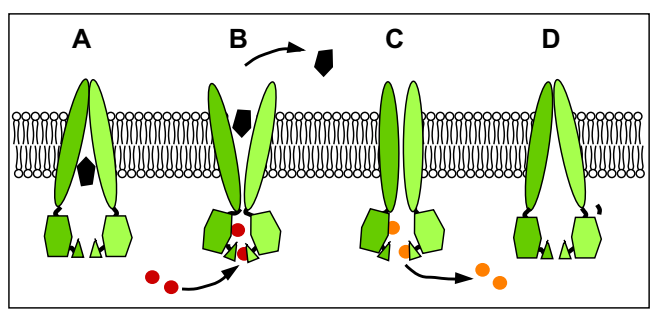

FIGURE 1 ATP-switch mechanism of substrate transport by ABC transporters. Midgut lumen is above, cytoplasm below. Black polygon: transport substrate. Red dots denote ATP and Orange dots denote ADP. ADP, adenosine diphosphate; ATP, adenosine triphosphate

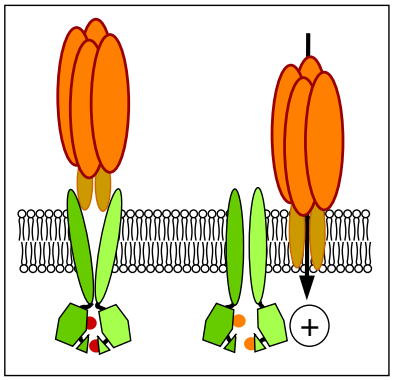

FIGURE 2 The hypothesized mechanism of Cry pore insertion following transient interaction with transmembrane domains of ABC transporter

In opposition to the colloid-osmotic lysis model, publications from the group that originally discovered the cadherin BT-R1 have put forth a different cell death pathway, the "signal transduction" model. This is often presented as a competing theory in review papers by other authors (Soberón, Gill, & Bravo, 2009). This interpretation needs to be re-evaluated in light of recent findings. The signal transduction hypothesis results from experiments in which the 12-cadherin domain protein from *Manduca sexta*, Bt-R1, was expressed in a cell line derived from *Trichoplusia ni* (High Five cells, H5 cells) and exposed to Cry1Ab toxin. The first publication (X. Zhang, Candas, Griko, Rose-Young, & Bulla, 2005) states "Toxin oligomers integrated into cell membrane do not produce lytic pores and do not kill the cells whereas monomeric Cry1Ab toxin specifically binds to BT-R1, activating an Mg2+-dependent cellular signaling pathway that leads to necrotic cell death." The mechanism put forth in the second paper (X. B. Zhang, Candas, Griko, Taussig, & Bulla, 2006) is that "the Cry1Ab toxin binds specifically to BT-R1, stimulating G protein and adenylate cylase, which brings about the accumulation of cyclic AMP and activation of protein kinase A (PKA) .... Activated PKA alters downstream effectors that, in turn, actually dismantle the cell by destabilizing both the cytoskeleton and ion channels in the cell membrane." Although the second publication stops short of stating that pores are absent, it "argues against the previously postulated lytic-pore formation model and explains why the toxin–receptor interaction is prerequisite to cytotoxic action."

This hypothesis was published 4 years before the discovery that mutations in ABC transporters cause high-level Bt resistance (Atsumi et al., 2012; Gahan et al., 2010) and 11 years before the discovery that aquaporins play a role in colloid-osmotic lysis in *B. mori* (Endo et al., 2017). An examination of the transcriptome of H5 cells shows that they do not naturally express ABCC2 or any of the three homologs of *B. mori* aquaporins previously investigated (Endo et al., 2017; Table 1). Sf9 cells do naturally express one of these aquaporins (AQP3), which may contribute to the Cry1Ac-induced swelling that is universally observed when Sf9 cells are transfected to express ABCC2 (Endo et al., 2017). Recently both ABCC2 and cadherins have been experimentally expressed in H5 cells. Cry1Ac toxin induced swelling in H5 cells expressing *Helicoverpa armigera* ABCC2, which was enhanced by co-expression of cadherin from *H. armigera* but not cadherin from *Spodoptera littoralis* (Ma et al., 2019). Establishing how much water enters through aquaporins versus the pore itself would clarify the interpretation of these experiments.

Therefore the phenomena described in these publications (X. Zhang et al., 2005; X. B. Zhang et al., 2006) should not be interpreted as arguing against colloid-osmotic lysis as claimed by the authors, but instead, as a possible series of events when colloid-osmotic lysis cannot occur because ABCC2 is absent and aquaporins are scarce. These proteins are naturally present in the midgut cells of all Lepidoptera targetted by Cry1A toxins, therefore, the signal transduction mechanism is irrelevant for these species. It might be relevant to ABCC2 knockout mutants of *H. virescens* (Gahan et al., 2010) and *Spodoptera frugiperda* (Banerjee et al., 2017). But because these mutants lacking ABCC2 are resistant to the Cry1A toxins, the signal transduction death pathway, if it occurs, is much less potent than colloid-osmotic lysis. Moreover, an independent effort to test this mechanism in CF1 cells did not provide

TABLE 1 Relative expression of aquaporins, housekeeping genes and Bt targets in Sf9 cells from *Spodoptera frugiperda* and H5 cells from *Trichoplusia ni*

Gene	Sf9 RPKM	Sf9 reads	H5 RPKM	H5 reads
AQP1	0.2	398	n.d.	6
AQP2	n.d.	0	n.d.	2
AQP3	35.3	15,743	n.d.	20
LDH	228.2	110,961	163.9	49,767
eIF4A	28.2	15,061	7.1	600
RpL5	1,605.40	342,697	1,837.5	130,759
cadherin	n.d.	9	n.d.	8
ABCC2	n.d.	8	n.d.	0
Total reads		23,763,328		23,788,969

Note: Illumina reads were downloaded from GenBank SRA Archives SRR5892097 (Sf9 cells, Shu et al., 2017) and SRP068276 (H5 cells, Yu et al., 2016). CLC Genomics Workbench was used for assembly, mapping, and RPKM expression calculations.

Abbreviations: AQP, aquaporin; eIF4A, eukaryotic initiation Factor 4A; LDH, lactate dehydrogenase; n.d., not enough reads to assemble a contig for RPKM calculations; RPKM, reads per kilobase of transcript per million mapped reads, a normalized index of transcript expression; RpL5, ribosomal protein L5.

support; Cry1Ab induced apoptosis in those cells instead (Portugal, Muñóz-Garay, Martínez de Castro, Soberón, & Bravo, 2017). Finally, the signal transduction killing mechanism has not been shown to occur in larvae of any species to date.

Another aspect of the interaction of the 12-cadherin domain protein and ABCC2 *in vivo* was shown in a recent study (Wang, Kain, & Wang, 2018). A naturally occurring mutation affecting ABCC2 confers 600-fold resistance to Cry1Ac in the GLEN-Cry1Ac strain of the cabbage looper, *Trichoplusia ni*. The 12-cadherin domain protein from *T. ni*, TnCAD, is not strongly bound by native Cry1Ac, and accordingly knocking out TnCAD with CRISPR/Cas9 does not confer Cry1Ac resistance on the susceptible Cornell strain (unlike the naturally occurring cadherin mutation in *H. virescens*). Nor does the TnCAD knockout significantly affect the potency of Cry1Ac on the resistant GLEN-Cry1Ac strain. In an ingenious directed evolution approach, Cry1Ac was experimentally modified to produce Cry1Ac-A01s, which bound TnCAD much more strongly than did Cry1Ac (Badran et al., 2016). Cry1Ac-A01s is about ten times more toxic to susceptible Cornell strain *T. ni* than is Cry1Ac. The CRISPR/Cas9 knockout of TnCAD is about 3.5-fold resistant to Cry1Ac-A01s, a small but significant effect, unlike the response of the knockout to Cry1Ac. Cry1Ac-A01s is also toxic to the GLEN-Cry1Ac strain with its naturally mutated ABCC2, which exhibits only about 8-fold resistance (compared with its 600-fold resistance to Cry1Ac). But when the TnCAD knockout is combined with the ABCC2 mutation, the double mutant strain is more than 3,700-fold resistant to Cry1Ac-A01s. The authors interpreted the results as evidence for two independent pathways of toxicity, one involving TnCAD and the other involving ABCC2 (Wang et al., 2018).

However, the results are also consistent with the sequential binding and pore insertion model. The pathways are sequential, not independent, but TnCAD binding makes a greater proportionate contribution to Cry1Ac-A01s toxicity than to Cry1Ac toxicity. Cry1Ac, which does not bind strongly to TnCAD, is activated to form oligomers by the alternate mechanism that occurs in the cadherin-deficient YFO strain of *H. virescens*. Therefore, knocking out TnCAD has little effect on Cry1Ac oligomer formation, but has a larger effect on Cry1Ac-A01s oligomer formation due to the latter's affinity for TnCAD. This affinity strengthens the synergistic interaction of TnCAD with ABCC2, making the relative contribution of the ABCC2 mutant to Cry1Ac-A01s resistance less than to Cry1Ac resistance. When both TnCAD and ABCC2 are knocked out, Cry1Ac-A01s loses most of its potency, just as Cry1Ac does on

the double mutant YHD3 strain of *H. virescens*. More work on Cry1Ac-like toxins with intermediate affinities to TnCAD would be useful in testing this interpretation.

Most researchers have ignored the possibility that the protoxin may play a role in Bt toxicity. However, side-by-side comparisons of activated Cry1Ab or Cry1Ac with the corresponding protoxin show that the latter is more toxic for several insects, including some Bt-resistant strains (Tabashnik et al., 2015). There is evidence that activated Cry1Ab and its protoxin make different prepore structures (Gómez et al., 2014). However, since C-terminal protoxin domains have been shown to bind to cadherin fragments (Fabrick & Tabashnik, 2007; Gómez et al., 2014) and to GPI-anchored APN and ALP (Peña-Cardeña et al., 2018), more research is needed to determine whether they merely enhance the sequential-binding pathway of activated toxins, or kill insects via a different, secondary toxic pathway as claimed (Tabashnik et al., 2015).

5 | LONG- AND SHORT-TERM TRENDS IN EVOLUTION

So far there is no known naturally occurring toxin with the same sequence as Cry1Ac-A01s, or the same high affinity for TnCAD. Cry1Ac binds to the cadherin of other noctuid moths, why not to *T. ni*? Did Cry1Ac bind to the cadherin of the common ancestor of *H. virescens* and *T. ni*? If not, then in the course of evolution, the cadherin of *H. virescens* must have evolved to bind to Cry1Ac: not an evolutionary progression that would be favored by natural selection on *H. virescens*. If Cry1Ac did bind to the common ancestor, then the cadherin of *T. ni* must have evolved to avoid binding to Cry1Ac. Could this be a natural example of the gradual evolution of Cry1Ac resistance by changes in the protein sequence of the TnCAD of *T. ni*? Although not conferring absolute resistance, these changes do reduce the susceptibility to Cry1Ac, by reducing TnCAD binding, and would be favored by natural selection. Site-directed mutagenesis experiments on the cadherin have shown that certain amino acid substitutions can also reduce or eliminate Cry1Ac binding to the cadherin of *H. virescens* (Xie et al., 2005). We must now face the prospect of such substitutions arising in field populations of *H. virescens* consuming Bt cotton. Up to now, resistant cadherin alleles recovered from field populations by the F_2 screen have completely knocked out protein expression (Morin et al., 2003; Xu et al., 2005), a change that comes with a high fitness cost in the absence of toxin selection. Do mutations that merely reduce Cry1Ac binding to the cadherin incur any fitness cost in the absence of Bt selection? If not, such mutations threaten the continued use of Cry1A toxins for insect control.

Resistant ABCC2 alleles found in laboratory-selected strains or field populations are also mostly knockouts of the protein, due to frameshifts or splicing aberrations. Loss of transporter function is believed to incur a fitness cost in the absence of toxins. One significant exception is the Cry1Ab-resistant allele of ABCC2 in *B. mori*. The allele found in the resistant strain encoded a full-length protein differing from the susceptible sequence by amino acid substitutions and one tyrosine insertion at position 233 in an extracellular loop (Atsumi et al., 2012). Mutagenesis showed that the tyrosine insertion was necessary and sufficient to make ABCC2-expressing Sf9 cells resistant to Cry1Ab (Tanaka et al., 2016). The resistant allele is not rare: it is fixed in predominantly elite Japanese and Chinese strains, while the susceptible allele is more common in tropical strains. It is unlikely that the tyrosine insertion affects the transport capability of the ABCC2 since it is far from the membrane-spanning regions that interact with the transport substrate. An analogous situation is seen in comparing the ABCC2 proteins of *S. frugiperda and S. litura*. Glutamine in position 125 of the *S. frugiperda* protein conferred greater susceptibility to Cry1Ac than the glutamate in the same position of the *S. litura* protein (Liu et al., 2018). Resistance-causing mutations with little or no fitness costs are those most likely to increase due to continuous use of Cry toxins in sprays or transgenic crops.

Another threat to the sustainability of Bt are semidominant mutations, which confer some resistance in the heterozygote. A key assumption of the high-dose/refuge resistance management strategy is that resistance alleles are recessive so that heterozygotes are killed as effectively as homozygous susceptible genotypes. However, a cadherin mutant with a truncated C-terminal intracellular domain (H. N. Zhang, Wu, Yang, Tabashnik, & Wu, 2012),

and an amino acid substitution in a tetraspanin (Jin et al., 2018) both show some degree of dominance in resistance. The connection with pore formation is not understood and should be a high priority for future study.

6 | CONCLUSIONS

The preponderance of evidence to date points to the formation of membrane pores as the primary cause of cytotoxicity of the three-domain Cry toxins in insects. ABC transporters are emerging as the most important proteins that Cry toxins interact with to form pores. The existence of three different ABC transporters, each a member of a different ABC subfamily, that specifically interact with three different Cry toxins, each a member of a different Cry family, suggests that this is an evolutionarily ancient association. A special member of the cadherin superfamily containing 12-cadherin domains in Lepidoptera is also significantly involved, with suggestions that similar cadherins in beetles and mosquitoes might also be important. Mutations in either ABC transporters or cadherins are the most potent resistance mechanisms discovered so far. Although most such mutations have the drastic effect of deleting the protein, a few can provide resistance with only minor structural changes. These are the mutations that have survived over evolutionary time to give rise to differences among insect species in the host ranges of Cry toxins. And these are the mutations that must be understood if modern agriculture is to adapt to the inevitable rise of Bt resistance in insect pests.

ACKNOWLEDGMENTS

This study is based on a contribution to the symposium "The multiple layers of host-pathogen interactions" at the 2019 Society for Invertebrate Pathology meeting in Valencia. I thank the organizers, Umut Toprak and Salvador Herrero, and many participants for discussions, and two reviewers for useful comments. I thank Heiko Vogel for the transcriptome assemblies and expression analysis. The Max-Planck-Gesellschaft provided financial support.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

David G. Heckel http://orcid.org/0000-0001-8991-2150

REFERENCES

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., … Noda, H. (2012). Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, E1591–E1598.

Badran, A. H., Guzov, V. M., Huai, Q., Kemp, M. M., Vishwanath, P., Kain, W., … Liu, D. R. (2016). Continuous evolution of *Bacillus thuringiensis* toxins overcomes insect resistance. *Nature*, *533*, 58–63.

Banerjee, R., Hasler, J., Meagher, R., Nagoshi, R., Hietala, L., Huang, F., … Jurat-Fuentes, J. L. (2017). Mechanism and DNA-based detection of field-evolved resistance to transgenic Bt corn in fall armyworm (*Spodoptera frugiperda*). *Scientific Reports*, *7*, Article no. 10877.

Bravo, A., Gill, S. S., & Soberón, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, *49*, 423–435.

Bravo, A., Likitvivatanavong, S., Gill, S. S., & Soberón, M. (2011). *Bacillus thuringiensis*: A story of a successful bioinsecticide. *Insect Biochemistry and Molecular Biology*, *41*, 423–431.

Bretschneider, A., Heckel, D. G., & Pauchet, Y. (2016). Three toxins, two receptors, one mechanism: Mode of action of Cry1A toxins from *Bacillus thuringiensis* in *Heliothis virescens*. *Insect Biochemistry and Molecular Biology*, *76*, 109–117.

Broderick, N. A., Raffa, K. F., & Handelsman, J. (2006). Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 15196–15199.

Broderick, N. A., Robinson, C. J., McMahon, M. D., Holt, J., Handelsman, J., & Raffa, K. F. (2009). Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of Lepidoptera. *BMC Biology*, *7*, 11.

Caccia, S., Di Lelio, I., La Storia, A., Marinelli, A., Varricchio, P., Franzetti, E., … Pennacchio, F. (2016). Midgut microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism. *Proceedings of the National Academy of Sciences of the United States of America*, *113*, 9486–9491.

Crickmore, N. (2017). *Bacillus thuringiensis* toxin classification. In L. M. Fiuza, R. A. Polanczyk & N. Crickmore (Eds.), Bacillus thuringiensis *and* Lysinibacillus sphaericus: *Characterization and use in the field of biocontrol* (pp. 41–52). Cham: Springer.

Endo, H., Azuma, M., Adegawa, S., Kikuta, S., & Sato, R. (2017). Water influx via aquaporin directly determines necrotic cell death induced by the *Bacillus thuringiensis* Cry toxin. *FEBS Letters*, *591*, 56–64.

Fabrick, J. A., & Tabashnik, B. E. (2007). Binding of *Bacillus thuringiensis* toxin Cry1Ac to multiple sites of cadherin in pink bollworm. *Insect Biochemistry and Molecular Biology*, *37*, 97–106.

Ferré, J., & Van Rie, J. (2002). Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annual Review of Entomology*, *47*, 501–533.

Gahan, L. J., Gould, F., & Heckel, D. G. (2001). Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science*, *293*, 857–860.

Gahan, L. J., Pauchet, Y., Vogel, H., & Heckel, D. G. (2010). An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. *PLOS Genetics*, *6*, e1001248.

Garczynski, S. F., & Adang, M. J. (1995). *Bacillus thuringiensis* CryIA(c) delta endotoxin binding aminopeptidase in the *Manduca sexta* midgut has a glycosyl-phosphatidylinositol anchor. *Insect Biochemistry and Molecular Biology*, *25*, 409–415.

Gómez, I., Sánchez, J., Miranda, R., Bravo, A., & Soberón, M. (2002). Cadherin-like receptor binding facilitates proteolytic cleavage of helix alpha-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. *FEBS Letters*, *513*, 242–246.

Gómez, I., Sánchez, J., Muñoz-Garay, C., Matus, V., Gill, S. S., Soberón, M., & Bravo, A. (2014). *Bacillus thuringiensis* Cry1A toxins are versatile proteins with multiple modes of action: Two distinct pre-pores are involved in toxicity. *Biochemical Journal*, *459*, 383–396.

Heckel, D. G. (2012). Learning the ABCs of Bt: ABC transporters and insect resistance to *Bacillus thuringiensis* provide clues to a crucial step in toxin mode of action. *Pesticide Biochemistry and Physiology*, *104*, 103–110.

Hossain, D. M., Shitomi, Y., Moriyama, K., Higuchi, M., Hayakawa, T., Mitsui, T., … Hori, H. (2004). Characterization of a novel plasma membrane protein, expressed in the midgut epithelia of *Bombyx mori*, that binds to Cry1A toxins. *Applied and Environmental Microbiology*, *70*, 4604–4612.

ISAAA. (2017). *Global status of commercialized of biotech/GM crops in 2017: Biotech crop adoption surges as economic benefits accumulate in 22 years*. Brief 53. Ithaca, NY: International Service for the Aquisition of Agri-biotech Applications.

Jin, L., Wang, J., Guan, F., Zhang, J. P., Yu, S., Liu, S. Y., … Wu, Y. D. (2018). Dominant point mutation in a tetraspanin gene associated with field-evolved resistance of cotton bollworm to transgenic Bt cotton. *Proceedings of the National Academy of Sciences of the United States of America*, *115*, 11760–11765.

Johnston, P. R., & Crickmore, N. (2009). Gut bacteria are not required for the insecticidal activity of *Bacillus thuringiensis* toward the tobacco hornworm, *Manduca sexta*. *Applied and Environmental Microbiology*, *75*, 5094–5099.

Jurat-Fuentes, J. L., & Adang, M. J. (2004). Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. *European Journal of Biochemistry*, *271*, 3127–3135.

Knight, P. J. K., Knowles, B. H., & Ellar, D. J. (1995). Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. *Journal of Biological Chemistry*, *270*, 17765–17770.

Knowles, B. H., & Ellar, D. J. (1987). Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* delta-endotoxins with different insect specificity. *Biochimica et Biophysica Acta*, *924*, 509–518.

Krishnan, V., Domanska, B., Elhigazi, A., Afolabi, F., West, M. J., & Crickmore, N. (2017). The human cancer cell active toxin Cry41Aa from *Bacillus thuringiensis* acts like its insecticidal counterparts. *Biochemical Journal*, *474*, 1591–1602.

Liu, L. L., Chen, Z. W., Yang, Y. C., Xiao, Y. T., Liu, C. X., Ma, Y. M., … Liu, K. Y. (2018). A single amino acid polymorphism in ABCC2 loop 1 is responsible for differential toxicity of *Bacillus thuringiensis* Cry1Ac toxin in different *Spodoptera* (Noctuidae) species. *Insect Biochemistry and Molecular Biology*, *100*, 59–65.

Ma, Y. M., Zhang, J. F., Xiao, Y. T., Yang, Y. C., Liu, C. X., Peng, R., … Liu, K. Y. (2019). The Cadherin Cry1Ac Binding-Region is Necessary for the Cooperative Effect with ABCC2 Transporter Enhancing Insecticidal Activity of *Bacillus thuringiensis* Cry1Ac Toxin. *Toxins*. Advance online publication. *11*, e538. https://doi.org/10.3390/toxins11090538

Morin, S., Biggs, R. W., Sisterson, M. S., Shriver, L., Ellers-Kirk, C., Higginson, D., … Tabashnik, B. E. (2003). Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 5004–5009.

Nagamatsu, Y., Koike, T., Sasaki, K., Yoshimoto, A., & Furukawa, Y. (1999). The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin. *FEBS Letters*, *460*, 385–390.

Pauchet, Y., Bretschneider, A., Augustin, S., & Heckel, D. G. (2016). A P-glycoprotein is linked to resistance to the *Bacillus thuringiensis* Cry3Aa toxin in a leaf beetle. *Toxins*, *8*, 362.

Perera, O. P., Willis, J. D., Adang, M. J., & Jurat-Fuentes, J. L. (2009). Cloning and characterization of the Cry1Ac-binding alkaline phosphatase (HvALP) from *Heliothis virescens*. *Insect Biochemistry and Molecular Biology*, *39*, 294–302.

Peña-Cardeña, A., Grande, R., Sánchez, J., Tabashnik, B. E., Bravo, A., Soberón, M., & Gómez, I. (2018). The C-terminal protoxin region of *Bacillus thuringiensis* Cry1Ab toxin has a functional role in binding to GPI-anchored receptors in the insect midgut. *Journal of Biological Chemistry*, *293*, 20263–20272.

Portugal, L., Muñóz-Garay, C., Martínez de Castro, D. L., Soberón, M., & Bravo, A. (2017). Toxicity of Cry1A toxins from *Bacillus thuringiensis* to CF1 cells does not involve activation of adenylate cyclase/PKA signaling pathway. *Insect Biochemistry and Molecular Biology*, *80*, 21–31.

Raymond, B., Johnston, P. R., Nielsen-LeRoux, C., Lereclus, D., & Crickmore, N. (2010). *Bacillus thuringiensis*: An impotent pathogen? *Trends in Microbiology*, *18*, 189–194.

Raymond, B., Johnston, P. R., Wright, D. J., Ellis, R. J., Crickmore, N., & Bonsall, M. B. (2009). A mid-gut microbiota is not required for the pathogenicity of *Bacillus thuringiensis* to diamondback moth larvae. *Environmental Microbiology*, *11*, 2556–2563.

Roush, R. T., Fitt, G. P., Forrester, N. W., & Daly, J. C. (1998). Resistance management for insecticidal transgenic crops: Theory and practice. In M. P. Zalucki, R. Drew, & G. G. White (Eds.), *Pest management - future challenges*, *Proceedings* (Vols. 1 and 2, pp. 247–257), Brisbane: University of Queensland Printery.

Sanchis, V. (2011). From microbial sprays to insect-resistant transgenic plants: History of the biopesticide *Bacillus thuringiensis*. A review. *Agronomy for Sustainable Development*, *31*, 217–231.

Sangadala, S., Walters, F. S., English, L. H., & Adang, M. J. (1994). A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and (Rb+- K+)-Rb-86 efflux in vitro. *Journal of Biological Chemistry*, *269*, 10088–10092.

Shelton, A. M., Robertson, J. L., Tang, J. D., Perez, C., Eigenbrode, S. D., Preisler, H. K., … Cooley, R. J. (1993). Resistance of diamondback moth (Lepidoptera, Plutellidae) to *Bacillus thuringiensis* subspecies in the field. *Journal of Economic Entomology*, *86*, 697–705.

Shu, B. S., Zhang, J. J., Sethuraman, V., Cui, G. F., Yi, X., & Zhong, G. H. (2017). Transcriptome analysis of *Spodoptera frugiperda* Sf9 cells reveals putative apoptosis-related genes and a preliminary apoptosis mechanism induced by azadirachtin. *Scientific Reports*. Advance online publication. *7*. https://doi.org/10.1038/s41598-017-12713-9

Soberón, M., Gill, S. S., & Bravo, A. (2009). Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells? *Cellular and Molecular Life Sciences*, *66*, 1337–1349.

Soberón, M., Pardo-López, L., López, I., Gómez, I., Tabashnik, B. E., & Bravo, A. (2007). Engineering modified Bt toxins to counter insect resistance. *Science*, *318*, 1640–1642.

Stevens, T., Song, S. S., Bruning, J. B., Choo, A., & Baxter, S. W. (2017). Expressing a moth ABCC2 gene in transgenic *Drosophila* causes susceptibility to Bt Cry1Ac without requiring a cadherin-like protein receptor. *Insect Biochemistry and Molecular Biology*, *80*, 61–70.

Tabashnik, B. E., Brevault, T., & Carrière, Y. (2013). Insect resistance to Bt crops: Lessons from the first billion acres. *Nature Biotechnology*, *31*, 510–521.

Tabashnik, B. E., & Carrière, Y. (2017). Surge in insect resistance to transgenic crops and prospects for sustainability. *Nature Biotechnology*, *35*, 926–935.

Tabashnik, B. E., Huang, F. N., Ghimire, M. N., Leonard, B. R., Siegfried, B. D., Rangasamy, M., … Soberón, M. (2011). Efficacy of genetically modified Bt toxins against insects with different genetic mechanisms of resistance. *Nature Biotechnology*, *29*, 1128–U1198.

Tabashnik, B. E., Schwartz, J. M., Finson, N., & Johnson, M. W. (1992). Inheritance of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera, Plutellidae). *Journal of Economic Entomology*, *85*, 1046–1055.

Tabashnik, B. E., Zhang, M., Fabrick, J. A., Wu, Y. D., Gao, M. J., Huang, F. N., … Li, X. C. (2015). Dual mode of action of Bt proteins: Protoxin efficacy against resistant insects. *Scientific Reports*, *5*, 15107.

Tanaka, S., Endo, H., Adegawa, S., Kikuta, S., & Sato, R. (2016). Functional characterization of *Bacillus thuringiensis* Cry toxin receptors explains resistance in insects. *FEBS Journal*, *283*, 4474–4490.

Tanaka, S., Miyamoto, K., Noda, H., Endo, H., Kikuta, S., & Sato, R. (2016). Single amino acid insertions in extracellular loop 2 of *Bombyx mori* ABCC2 disrupt its receptor function for *Bacillus thuringiensis* Cry1Ab and Cry1Ac but not Cry1Aa toxins. *Peptides*, *78*, 99–108.

Tanaka, S., Miyamoto, K., Noda, H., Jurat-Fuentes, J. L., Yoshizawa, Y., Endo, H., & Sato, R. (2013). The ATP-binding cassette transporter subfamily C member 2 in *Bombyx mori* larvae is a functional receptor for Cry toxins from *Bacillus thuringiensis*. *FEBS Journal*, *280*, 1782–1794.

Tay, W. T., Mahon, R. J., Heckel, D. G., Walsh, T. K., Downes, S., James, W. J., … Gordon, K. H. J. (2015). Insect resistance to *Bacillus thuringiensis* toxin Cry2Ab is conferred by mutations in an ABC transporter subfamily A protein. *PLOS Genetics*, *11*, e1005534.

Vadlamudi, R. K., Ji, T. H., & Bulla, L. A. (1993). A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp Berliner. *Journal of Biological Chemistry*, *268*, 12334–12340.

Valaitis, A. P., Jenkins, J. L., Lee, M. K., Dean, D. H., & Garner, K. J. (2001). Isolation and partial characterization of gypsy moth BTR-270, an anionic brush border membrane glycoconjugate that binds *Bacillus thuringiensis* Cry1A toxins with high affinity. *Archives of Insect Biochemistry and Physiology*, *46*, 186–200.

Van Rie, J., Jansens, S., Höfte, H., Degheele, D., & Van Mellaert, H. (1989). Specificity of *Bacillus thuringiensis* delta-endotoxins—Importance of specific receptors on the brush-border membrane of the midgut of target insects. *European Journal of Biochemistry*, *186*, 239–247.

Wang, S. H., Kain, W., & Wang, P. (2018). *Bacillus thuringiensis* Cry1A toxins exert toxicity by multiple pathways in insects. *Insect Biochemistry and Molecular Biology*, *102*, 59–66.

Xiao, Y. T., Zhang, T., Liu, C. X., Heckel, D. G., Li, X. C., Tabashnik, B. E., & Wu, K. M. (2014). Mis-splicing of the ABCC2 gene linked with Bt toxin resistance in *Helicoverpa armigera*. *Scientific Reports*, *4*, 6184.

Xie, R. Y., Zhuang, M. B., Ross, L. S., Gomez, I., Oltean, D. I., Bravo, A., … Gill, S. S. (2005). Single amino acid mutations in the cadherin receptor from *Heliothis virescens* affect its toxin binding ability to Cry1A toxins. *Journal of Biological Chemistry*, *280*, 8416–8425.

Xu, X. J., Yu, L. Y., & Wu, Y. D. (2005). Disruption of a cadherin gene associated with resistance to Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. *Applied and Environmental Microbiology*, *71*, 948–954.

Yu, K., Yu, Y., Tang, X. Y., Chen, H. M., Xiao, J. Y., & Su, X. D. (2016). Transcriptome analyses of insect cells to facilitate baculovirus-insect expression. *Protein & Cell*, *7*, 373–382.

Zhang, H. N., Wu, S. W., Yang, Y. H., Tabashnik, B. E., & Wu, Y. D. (2012). Non-recessive Bt toxin resistance conferred by an intracellular cadherin mutation in field-selected populations of cotton bollworm. *PLOS One*, *7*, e53418.

Zhang, X., Candas, M., Griko, N. B., Rose-Young, L., & Bulla, L. A. (2005). Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R-1 expressed in insect cells. *Cell Death and Differentiation*, *12*, 1407–1416.

Zhang, X. B., Candas, M., Griko, N. B., Taussig, R., & Bulla, L. A. (2006). A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 9897–9902.

How to cite this article: Heckel DG. How do toxins from *Bacillus thuringiensis* kill insects? An evolutionary perspective. *Arch. Insect Biochem. Physiol.* 2020;104:e21673. https://doi.org/10.1002/arch.21673