Bt Research 2024, Vol.15, No.2, 76-86
http://microbescipublisher.com/index.php/bt

Research Report Open Access

Characterization of Plasmid-encoded Toxins in *Bacillus thuringiensis*

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Bt Research, 2024, Vol.15, No.2 doi: 10.5376/bt.2024.15.0008
Received: 05 Feb., 2024
Accepted: 18 Mar., 2024
Published: 06 Apr., 2024

Preferred citation for this article:
Dong Z.Y., and Wu Z.Q., 2024, Characterization of Plasmid-encoded Toxins in *Bacillus thuringiensis*, Bt Research, 15(2): 76-86 (doi: 10.5376/bt.2024.15.0008)

Abstract *Bacillus thuringiensis* (Bt) is a bacterium widely used in pest management due to its production of plasmid-encoded toxins. These toxins play a crucial role in Bt's insecticidal properties. This study aims to provide a comprehensive overview of plasmid-encoded toxins in Bt, their genetic and molecular mechanisms, functional analyses, methods of characterization, ecological implications, and applications in pest management. The study begins by exploring the types and characteristics of plasmids in Bt, highlighting their role in Bt genetics and their diversity among different Bt strains. It then delves into the various plasmid-encoded toxins, including Cry, Cyt, Vip, and other toxins, explaining their gene structure, regulation, and mechanisms of horizontal gene transfer. The functional analysis section examines the insecticidal activity, specificity, and mechanisms of toxicity of these toxins. Methods for characterizing these toxins, such as genomic sequencing, proteomic analysis, functional assays, and bioinformatics approaches, are also discussed. The ecological and evolutionary implications of plasmid-encoded toxins, including their role in Bt evolution, impact on host range and virulence, and environmental persistence, are considered. Finally, the study discusses the applications of Bt toxins in pest management, focusing on Bt-based biopesticides, transgenic crops expressing Bt toxins, and strategies to combat resistance. This study underscores the importance of plasmid-encoded toxins in Bt, summarizing key findings and highlighting their significance in pest management. Future research should focus on advancing our understanding of these toxins to enhance their efficacy and sustainability in agricultural practices.

Keywords *Bacillus thuringiensis*; Plasmid-encoded toxins; Cry toxins; Pest management; Horizontal gene transfer

1 Introduction

Bacillus thuringiensis (Bt) is a Gram-positive, soil-dwelling bacterium that has been extensively studied for its insecticidal properties. Bt produces crystal (Cry) and cytolytic (Cyt) proteins during sporulation, which are toxic to a wide range of insect pests. These proteins have been utilized in biopesticides and genetically modified crops to control agricultural pests, reducing the reliance on chemical insecticides and promoting environmentally friendly pest management strategies (Pérez et al., 2005; Pardo-López et al., 2013; Liang et al., 2022).

The insecticidal properties of Bt are largely attributed to the Cry and Cyt proteins, many of which are encoded by plasmids. These plasmid-encoded toxins are crucial for the bacterium's pathogenicity and effectiveness as a biocontrol agent. For instance, the Cry11Aa and Cyt1Aa proteins produced by *Bacillus thuringiensis* subsp. *israelensis* are known to synergize, enhancing their toxicity against mosquito larvae and overcoming insect resistance (Pérez et al., 2005). Additionally, novel Cry proteins such as Cry31Aa and Cry78Ba1 have been identified, showing high nematicidal and insecticidal activities, respectively, and offering potential for the control of specific pests like rice planthopper and plant parasitic nematodes (Cao et al., 2020; Liang et al., 2022). Understanding the molecular mechanisms and interactions of these toxins is essential for developing new strategies to combat insect resistance and improve the efficacy of Bt-based biopesticides (Pardo-López et al., 2013; Guo et al., 2019).

The study is to provide a comprehensive overview of the current knowledge on plasmid-encoded toxins in *Bacillus thuringiensis*. This includes elucidating the molecular mechanisms of action and synergism of key Cry and Cyt proteins. Additionally, the study explore the genetic and structural basis of insect resistance to these toxins. Another goal is to highlight recent advancements in the identification and characterization of novel Cry

proteins with potential applications in pest management. The study also discusses the implications of these findings for the development of more effective and sustainable biopesticides. By synthesizing information from multiple studies, this study aims to enhance our understanding of Bt toxins and their role in pest control, ultimately contributing to the advancement of agricultural biotechnology and integrated pest management practices.

2 Overview of Plasmids in Bt

2.1 Types and characteristics of plasmids

Bacillus thuringiensis (Bt) harbors a variety of plasmids that play crucial roles in its insecticidal properties. These plasmids vary significantly in size and genetic content. For instance, the plasmid pTAND672-2 from Bt serovar *israelensis* is a 144 kb integrative and conjugative element (ICE) that carries mosquitocidal toxin genes, conjugation genes, and recombinase-encoding genes (Geng et al., 2023). Another example is the mega plasmid poh1 in Bt ATCC 10 792, which is 584,623 bps in size and contains genes responsible for antimicrobial, insecticidal, and antibiotic resistance activities (Chelliah et al., 2019). Additionally, the plasmid pH3-180 in the H3 strain of Bt carries a wide repertoire of mobile genetic elements and novel Cry proteins (Fayad et al., 2020).

2.2 Role of plasmids in Bt genetics

Plasmids in Bt are pivotal for its genetic diversity and adaptability. They often carry genes encoding insecticidal toxins, such as Cry and Cyt proteins, which are essential for Bt's biopesticide properties. For example, the plasmid pIS56-63 in Bt subsp. thuringiensis strain IS5056 harbors the *cry1Ab21* gene, which encodes a delta-endotoxin highly toxic to Trichoplusia ni larvae (Tanapongpipat et al., 2003; Murawska et al., 2014). Plasmids also facilitate horizontal gene transfer, enhancing the genetic variability and adaptability of Bt. The pTAND672-2 plasmid, for instance, can transfer between different bacterial species, contributing to the spread of mosquitocidal toxin genes (Figure 1) (Geng et al., 2023).

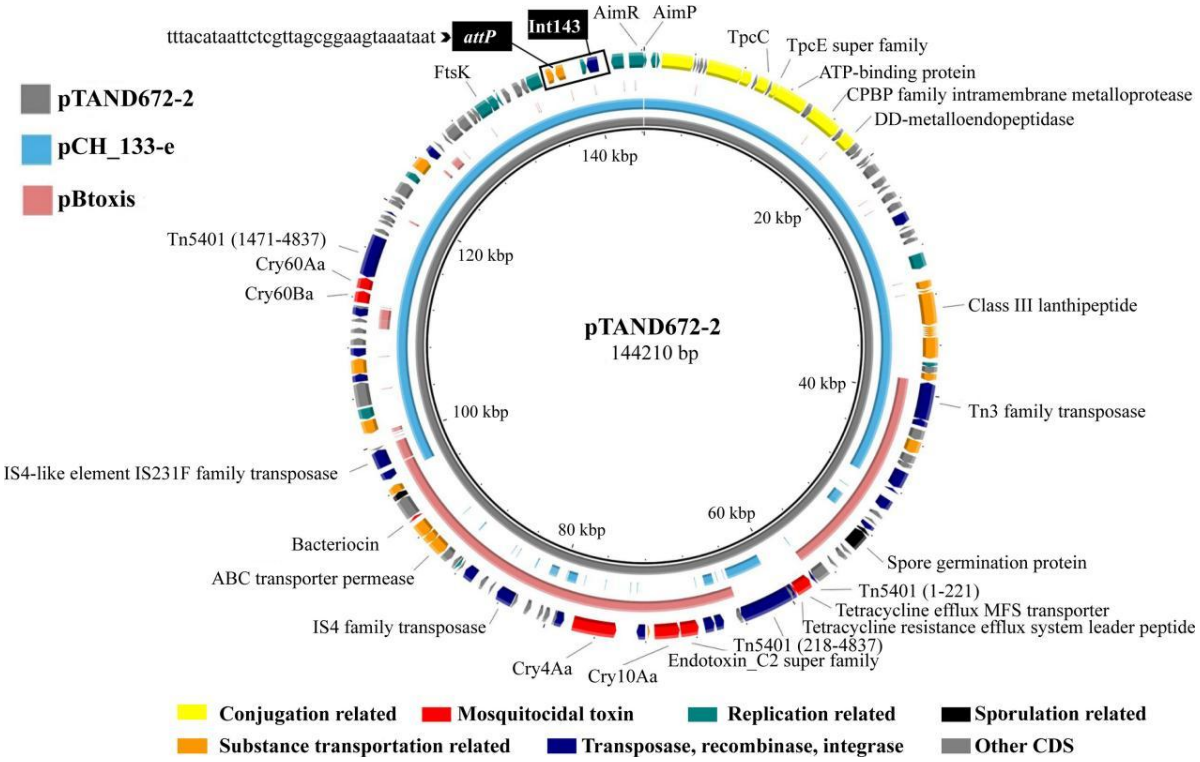

Figure 1 Circular map comparison among pTAND672-2, pCH_133-e, and pBtoxis (Adopted from Geng et al., 2023)

Image caption: pCH_133-e and pBtoxis display 77% and 43% coverage to pTAND672-2, respectively, with 100% identity. No plasmid replication/partition genes were predicted in the pBtoxis-like region in pTAND672-2. The overlap regions among the three plasmids contain several IS elements (Adopted from Geng et al., 2023)

2.3 Plasmid diversity in Bt strains

The diversity of plasmids in Bt strains is extensive, with each strain potentially harboring multiple plasmids of varying sizes and genetic compositions. The Bt isolate T414, for example, contains a chromosome and 15 different types of plasmids, each carrying various insecticidal genes such as cry1Aa, cry1Ab, cry1Ac, and vip3Aa (Reyaz et al., 2019). Similarly, the Bt strain Bt-UNVM_94 has a plasmid that encodes Cry7Ga2 and Mpp2Aa3 toxins, providing dual insecticidal activity against lepidopteran and coleopteran pests (Peralta et al., 2021). This diversity is further exemplified by the presence of cryptic plasmids in Bt strain YBT-1520, which contain putative toxin-antitoxin systems that contribute to plasmid stability and maintenance (Liu et al., 2008).

3 Types of Plasmid-encoded Toxins

Bacillus thuringiensis (Bt) is renowned for its production of various plasmid-encoded toxins that are pivotal in its role as a bioinsecticide. These toxins are categorized based on their structure and target specificity. The primary types of plasmid-encoded toxins include Cry, Cyt, Vip, and other less common toxins.

3.1 Cry toxins

Cry toxins are the most extensively studied and utilized group of Bt toxins. These proteins form parasporal crystals during sporulation and exhibit insecticidal activity by binding to specific receptors in the insect midgut, leading to cell lysis and death. Cry toxins are highly diverse, with numerous subtypes targeting different insect orders. Cry1A toxins bind specifically to cadherin receptors in the midgut of Lepidoptera, such as Manduca sexta, and are crucial for the entomopathogenicity of Bt (Dorsch et al., 2002). Cry4Aa and Cry4Ba toxins are mosquito-active toxins that form pores in the target membranes, with specific residues like His180 playing a critical role in their biotoxicity (Bourchookarn et al., 2021). The Cry34Ab1/Cry35Ab1 binary toxin is a novel binary toxin specific for the western corn rootworm, forming ion channels in lipid membranes and demonstrating a unique mode of action (Masson et al., 2004). Cry19B toxin, a newly identified class of delta-endotoxin from Bt serovar higo, shows specificity towards Culex pipiens molestus.

3.2 Cyt toxins

Cyt toxins, such as Cyt1Aa and Cyt2Ba, are another important group of Bt toxins. These proteins synergize with Cry toxins, enhancing their insecticidal activity and overcoming resistance mechanisms. Cyt1Aa toxin functions as a membrane-bound receptor for Cry11Aa, enhancing its binding and toxicity. This synergistic interaction is crucial for the effectiveness of Bt subsp. *israelensis* against mosquito larvae (Pérez et al., 2005). Another example of synergy is the coexpression of CytA and CryIVD genes in Bt, which results in parasporal inclusions that are highly toxic to mosquito larvae, with CytA enhancing the toxicity of CryIVD.

3.3 Vip toxins

Vip (Vegetative Insecticidal Proteins) toxins are secreted during the vegetative growth phase of Bt and have a different mode of action compared to Cry and Cyt toxins. They target a broader range of insect pests and are valuable in integrated pest management strategies. Vip3A toxins are effective against a wide range of Lepidoptera and are used in transgenic crops to provide protection against pests that are resistant to Cry toxins (Figure 2) (Pardo-López et al., 2013).

3.4 Other plasmid-encoded toxins

In addition to Cry, Cyt, and Vip toxins, Bt produces other plasmid-encoded toxins that contribute to its insecticidal properties. Chitinase and cellulase, enzymes encoded on large plasmids, enhance the bioactive properties of Bt strains by degrading the insect cuticle and facilitating toxin penetration (Fayad et al., 2020). Bacitracin, an antibiotic produced by some Bt strains, also contributes to their overall bioactivity and potential use in biocontro (Fayad et al., 2020).

In summary, the diverse array of plasmid-encoded toxins in *Bacillus thuringiensis*, including Cry, Cyt, Vip, and other bioactive molecules, underscores its versatility and effectiveness as a bioinsecticide. Understanding the specific roles and interactions of these toxins is crucial for developing new strategies to combat insect resistance and enhance the efficacy of Bt-based products.

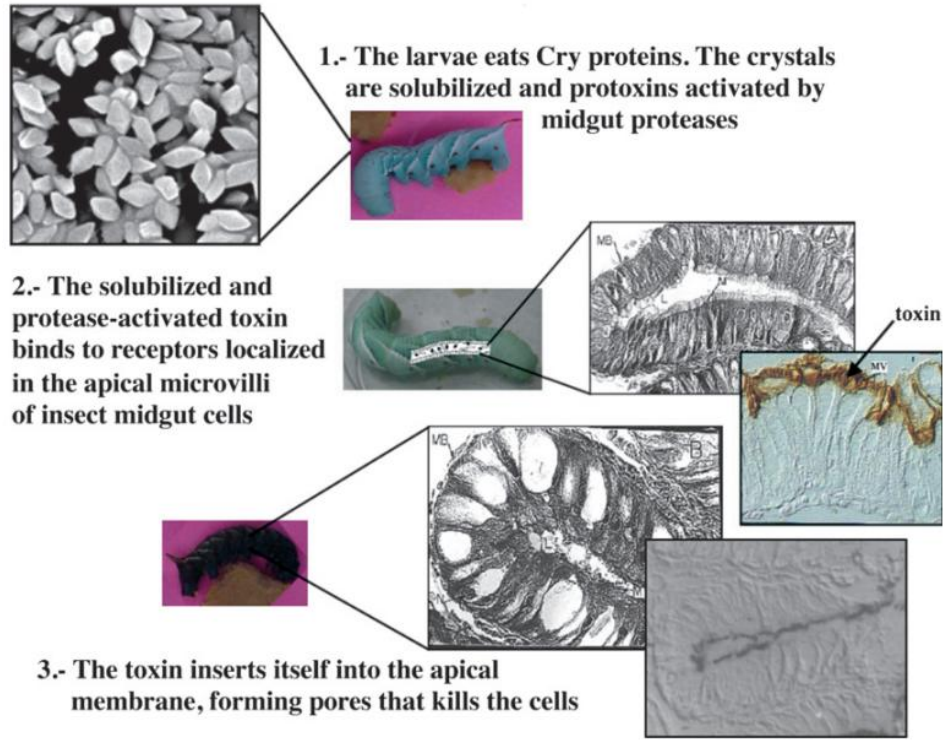

Figure 2 Schematic representation of the mechanism of action of 3d-Cry toxins in Lepidoptera at the cellular level, showing the immunolocalization of Cry toxin during intoxication (Adopted from Pardo-López et al., 2013)

Image caption: It illustrates the cellular mechanism of action of 3d-Cry toxins in Lepidoptera. The process begins with the ingestion of Cry proteins by larvae, leading to the solubilization and activation of protoxins by midgut proteases. The activated toxin binds to receptors located in the apical microvilli of insect midgut cells. Subsequently, the toxin inserts itself into the apical membrane, forming pores that result in cell death. The schematic includes immunolocalization images of Cry toxin within the insect midgut cells, highlighting the progression from ingestion to cellular intoxication (Adopted from Pardo-López et al., 2013)

4 Genetic and Molecular Mechanisms

4.1 Gene structure and organization

The gene structure and organization of plasmid-encoded toxins in *Bacillus thuringiensis* (Bt) are complex and diverse. For instance, the plasmid pTAND672-2 from *B. thuringiensis* serovar *israelensis* carries mosquitocidal toxin genes along with genes for conjugation and recombination, forming a novel integrative and conjugative element (ICE) (Geng et al., 2023). This plasmid integrates into the chromosome of *Lysinibacillus sphaericus* through site-specific recombination mediated by a tyrosine integrase, Int143 (Geng et al., 2023). Another example is the plasmid pH3-180 in the novel Bt strain H3, which contains 11 novel Cry toxin genes organized in an orf1-gap-orf2 structure, highlighting the dynamic nature of toxin gene organization in Bt plasmids (Fayad et al., 2020). Additionally, the large plasmid pBtoxis in *B. thuringiensis* subsp. *israelensis* encodes all insecticidal toxins and includes 125 potential coding sequences, many of which are involved in gene regulation and physiological processes (Stein et al., 2006; Zhang et al., 2017).

4.2 Regulation of toxin gene expression

The regulation of toxin gene expression in *Bacillus thuringiensis* involves multiple mechanisms. The transcription of cry genes, which encode crystal proteins responsible for insecticidal activity, can be dependent or independent of sporulation (Lereclus et al., 2000). The pleiotropic regulator PlcR activates the transcription of various genes encoding extracellular proteins, including toxins, and its expression is controlled by the transition state regulator SpoOA (Lereclus et al., 2000). In the case of the plasmid pBtoxis, transcriptional analysis revealed that 29 out of 40 surveyed coding sequences were transcribed, including those with similarities to known transcriptional regulators, suggesting a complex regulatory network influencing toxin gene expression (Stein et al., 2006).

4.3 Mechanisms of horizontal gene transfer

Horizontal gene transfer (HGT) plays a crucial role in the dissemination of toxin genes among *Bacillus thuringiensis* and related species. The plasmid pTAND672-2 is a prime example, as it can transfer horizontally from *B. thuringiensis* serovar *israelensis* to *Lysinibacillus sphaericus*, where it integrates into the chromosome via site-specific recombination (Geng et al., 2023). This plasmid and others like it within the Bacillus cereus group share a similar genetic backbone and exhibit conjugative capabilities, facilitating the spread of toxin genes across different bacterial species (Geng et al., 2023). The presence of mobile genetic elements, such as insertion sequences and transposable elements, in plasmids like pH3-180 further underscores the importance of HGT in the evolution and adaptation of Bt strains (Fayad et al., 2020).

5 Functional Analysis of Toxins

5.1 Insecticidal activity

Bacillus thuringiensis (Bt) produces a variety of Cry and Cyt toxins that exhibit potent insecticidal activity against a broad range of insect pests. The insecticidal activity of these toxins is primarily determined by their ability to bind to specific receptors on the midgut epithelial cells of target insects, leading to cell lysis and death. For instance, the Cry1Ca toxin has been shown to be highly effective against Spodoptera exigua larvae, with specific mutations in domains II and III significantly reducing its toxicity, thereby highlighting the importance of these domains in determining insect specificity (Herrero et al., 2004). Additionally, the Cry4Ba toxin exploits specific residues in its C-terminal domain to interact with target receptors, such as the *Aedes aegypti* membrane-bound alkaline phosphatase, which is crucial for its insecticidal activity (Thammasittirong et al., 2021).

5.2 Specificity to target insects

The specificity of Bt toxins to target insects is largely influenced by the presence of high-affinity binding sites on the brush border membrane of the insect midgut. For example, the Bt2-toxin and Bt4412-toxin exhibit high-affinity binding to the midgut vesicles of Manduca sexta and Pieris brassicae, respectively, correlating with their toxicity to these species. Similarly, the Cry8Ea1 toxin is specifically toxic to the underground larvae of *Holotrichia parallela*, with its mode of action involving the formation of transmembrane pores upon binding to midgut receptors (Guo et al., 2009). The Cry1Ca toxin's specificity towards S. exigua is also determined by its ability to form oligomeric structures upon activation, a process that is disrupted in mutants with altered domain II and III, thereby reducing their binding affinity and toxicity (Herrero et al., 2004).

5.3 Mechanisms of toxicity

The mechanisms of toxicity of Bt toxins involve a series of interactions with midgut proteins that facilitate the formation of oligomeric structures and their insertion into the membrane, leading to pore formation and cell lysis. The Cry toxins, for instance, undergo proteolytic activation to form active toxins that bind to specific receptors on the midgut epithelial cells, initiating the formation of lethal transmembrane pores (Pardo-López et al., 2013). The Cry4Ba toxin's interaction with the *Aedes aegypti* membrane-bound alkaline phosphatase via its C-terminal domain is a critical step in mediating larval toxicity (Thammasittirong et al., 2021). Additionally, the Cry1Ca toxin's ability to form oligomeric structures upon activation is essential for its insecticidal activity, with mutations in domain II and III affecting this process and thereby altering its specificity and toxicity (Herrero et al., 2004). The coexistence of *Cry9* and *Vip3a* genes in the same plasmid also suggests a synergistic mechanism of toxicity, enhancing the overall insecticidal efficacy and delaying resistance development in target pests (Wang et al., 2020).

6 Methods for Characterizing Plasmid-encoded Toxins

6.1 Genomic sequencing

Genomic sequencing is a fundamental method for characterizing plasmid-encoded toxins in *Bacillus thuringiensis* (Bt). Whole-genome sequencing allows for the identification and annotation of genes responsible for toxin production. For instance, the genome sequencing of Bt isolate T414 revealed the presence of multiple plasmids

harboring parasporal crystal protein genes such as *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1IAa*, *cry2Aa*, *cry2Ab,* and *cyt1*, as well as vegetative insecticidal protein gene *vip3Aa* (Reyaz et al., 2019). Similarly, the sequencing of the pBtoxis plasmid in Bt subsp. *israelensis* identified 125 potential coding sequences, many of which are involved in toxin production and gene regulation (Stein et al., 2006). Another study on a novel Bt strain H3 identified 11 novel Cry proteins through whole-genome sequencing, highlighting the dynamic nature of toxin-carrying plasmids (Fayad et al., 2020).

6.2 Proteomic analysis

Proteomic analysis complements genomic sequencing by identifying and characterizing the proteins expressed by the toxin genes. Techniques such as SDS-PAGE and MALDI-TOF/TOF are used to analyze the protein composition of Bt strains. For example, SDS-PAGE analysis of the spore-crystal mixture of Bt isolate T414 showed the presence of two major protein bands, 130 and 65 kDa, corresponding to the Cry and Cyt toxins (Reyaz et al., 2019). In another study, MALDI-TOF/TOF was used to confirm the identity of Cry8 proteins in the parasporal body of Bt strain FCC 7, which was toxic against various lepidopterans and coleopterans (Lazarte et al., 2021). These proteomic techniques are crucial for verifying the expression and functionality of the identified toxin genes.

6.3 Functional assays

Functional assays are essential for determining the bioactivity of the identified toxins. These assays involve testing the toxicity of the Bt strains or their purified toxins against target insect species. For instance, the novel Bt strain H3 was tested for its mosquitocidal activity, showing a unique killing profile with higher toxicity against *Aedes albopictus* and *Anopheles gambiae* compared to *Culex pipiens* (Fayad et al., 2020). Similarly, the toxicity of Bt strain FCC 7 was evaluated against the cotton boll weevil, *Anthonomus grandis*, confirming its insecticidal properties (Lazarte et al., 2021). These assays provide practical insights into the effectiveness of the toxins and their potential applications in pest control.

6.4 Bioinformatics approaches

Bioinformatics approaches are employed to analyze and predict the functions of the genes and proteins identified through genomic and proteomic methods. Tools such as NCBI BLAST, RAST, and BtToxin_scanner are used to annotate genomes and identify toxin genes. For example, the genome of Bt strain BLB406 was analyzed using BtToxin_scanner, revealing a unique combination of cry and vip genes that contribute to its larvicidal activity against *Aedes aegypti* (Zghal et al., 2018). Additionally, bioinformatics analysis of the pGI1 plasmid in Bt H1.1 identified a toxin-antitoxin system, tasA-tasB, which is widely distributed among various microorganisms (Fico and Mahillon, 2006). These computational tools are invaluable for understanding the genetic and functional diversity of Bt toxins.

7 Ecological and Evolutionary Implications

7.1 Role in Bt evolution

The evolution of *Bacillus thuringiensis* (Bt) is significantly influenced by the presence of plasmid-encoded toxins. These toxins, particularly the Cry and Cyt proteins, play a crucial role in the pathogen's adaptation and specialization to various hosts. The coevolution between Bt and its hosts, such as nematodes and insects, drives the selection of virulent strains with high toxin gene copy numbers, as demonstrated by the fixation of the BT-679 genotype in coevolutionary experiments (Argôlo-Filho and Loguercio, 2013; Masri et al., 2015). Additionally, the acquisition and maintenance of plasmids carrying these toxin genes are essential for Bt's pathogenicity and host specificity, facilitating rapid adaptation to new ecological niches (Figure 3) (Masri et al., 2015; Zheng et al., 2017).

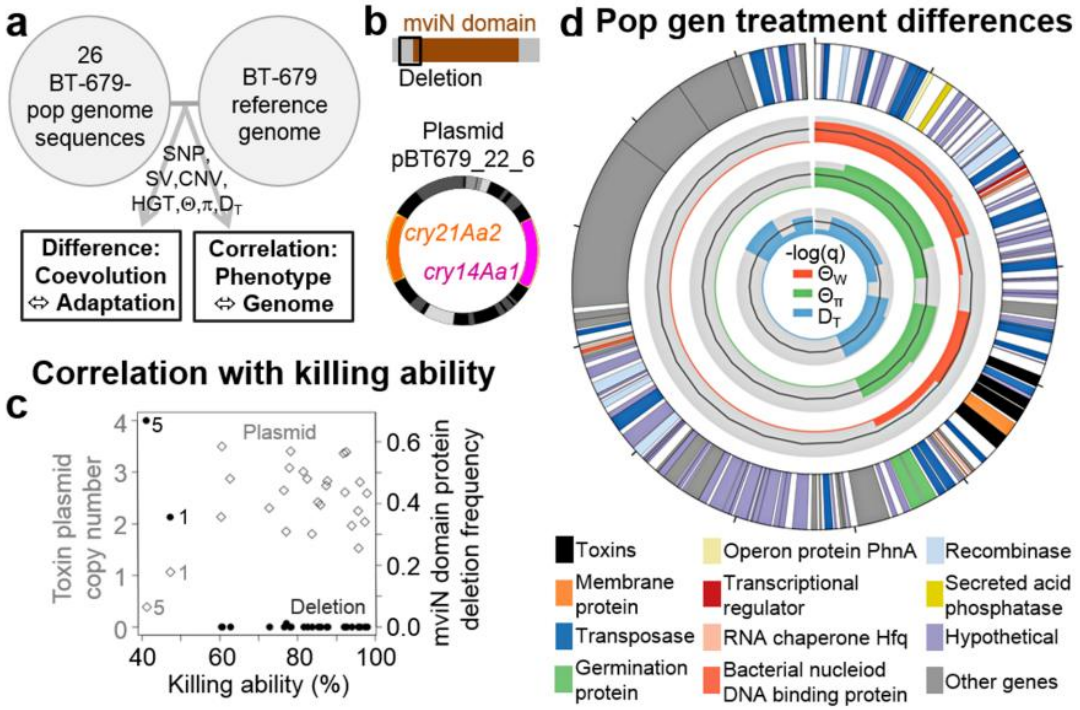

Figure 3 Fine-scale genomics and functional analysis demonstrate importance of nematocidal toxins and other genetic elements during adaptation (Adopted from Masri et al., 2015)

Image caption: A, Workflow: Genomic variation of BT-679 populations was contrasted between treatments or correlated with phenotypic variation. B, mviN gene deletion and plasmid with cry toxins. C, Pathogen killing ability correlates negatively with mviN deletion frequency (left axis, filled circles) and positively with toxin plasmid copy number (right axis, open diamonds). The two most deviating values in all three considered traits were recorded for the same two populations (coevolved populations one and five, both from transfer 20, as indicated adjacent to the measured values), strongly indicating a link between reduced plasmid copy number, increased deletion frequency, and loss of virulence. D, Significant variation among the evolution treatments in population genomic statistics for the plasmid Bti_GWDALJX04I0LJH_51-405_fm319.5 (its structure is given in the outer circle) (Adopted from Masri et al., 2015)

7.2 Impact on host range and virulence

Plasmid-encoded toxins significantly expand Bt's host range and enhance its virulence. The Cry toxins, for instance, are responsible for the bacterium's ability to infect a wide variety of invertebrates, including insects and nematodes (Stein et al., 2006; Malovichko et al., 2019). The presence of multiple virulence factors on plasmids allows Bt to overcome host resistance and adapt to different insect orders, thereby broadening its host range (Zheng et al., 2017). Moreover, the instability of certain toxin-encoding plasmids, such as BTI_23p, highlights the dynamic nature of Bt's virulence factors, which can be lost and reacquired, influencing the bacterium's pathogenicity (Sheppard et al., 2016; Tetreau, 2021).

7.3 Environmental persistence and spread

The environmental persistence and spread of Bt are closely linked to the stability and mobility of its plasmid-encoded toxins. Studies have shown that Cry proteins from transgenic crops expressing Bt toxins can persist in soil for varying durations, affecting non-target organisms and potentially leading to ecological imbalances (Clark et al., 2005). The ability of Bt to maintain low levels of unstable plasmids, such as BTI_23p, in the absence of a host suggests a mechanism for long-term environmental persistence, allowing the bacterium to remain viable and infectious in diverse environments (Sheppard et al., 2016). Furthermore, the horizontal transfer of plasmids among Bt populations facilitates the spread of virulence traits, contributing to the bacterium's ecological success and adaptability (Méric et al., 2018).

8 Applications in Pest Management

8.1 Development of Bt-based biopesticides

Bacillus thuringiensis (Bt) has been extensively utilized in the development of biopesticides due to its potent insecticidal properties. Bt-based biopesticides are particularly effective against a wide range of insect pests, including those that affect economically important crops. For instance, Bt toxins have been shown to cause significant mortality in pests such as the diamondback moth, *Plutella xylostella*, by targeting specific midgut receptors (Guo et al., 2019). Additionally, Bt-based biopesticides have been employed to manage pests like the castor semilooper, Achaea janata, which is a major threat to castor crops (Dhania et al., 2019). The use of Bt biopesticides not only reduces the reliance on chemical insecticides but also minimizes environmental impact and promotes sustainable agricultural practices.

8.2 Transgenic crops expressing Bt toxins

Transgenic crops expressing Bt toxins have revolutionized pest management by providing an effective and environmentally friendly alternative to chemical pesticides. These crops, such as Bt corn and Bt cotton, express Cry proteins that are toxic to specific insect pests. For example, transgenic tobacco plants expressing the Cry2Aa2 protein have demonstrated high levels of resistance against both susceptible and Bt-resistant insect strains, including the tobacco budworm and cotton bollworm. Similarly, transgenic potato plants expressing hybrid Bt toxins have shown resistance to pests from different insect orders, such as the Colorado potato beetle and the European corn borer (Naimov et al., 2002). The widespread adoption of Bt crops has significantly reduced crop losses and pesticide usage, contributing to increased agricultural productivity and sustainability.

8.3 Strategies to combat resistance

The evolution of resistance in insect pests poses a significant challenge to the long-term efficacy of Bt-based pest management strategies. To address this issue, several approaches have been developed to delay or overcome resistance. One such strategy involves the use of CRISPR/Cas9-mediated genome editing to study and manipulate resistance genes in pests. For instance, knockout strains of the diamondback moth with mutations in the PxABCC2 and PxABCC3 genes have been created to understand the genetic basis of resistance to Cry1Ac toxin (Guo et al., 2019). Another approach is the development of transgenic plants expressing multiple Bt toxins or hybrid toxins with different modes of action, which can reduce the likelihood of resistance development (Naimov et al., 2002). Additionally, understanding the molecular mechanisms of Bt toxin action and resistance, such as the role of ABC transporters and other midgut proteins, can inform the design of novel toxins and resistance management strategies (Heckel, 2012; Pardo-López et al., 2013; Coates et al., 2016). These efforts are crucial for maintaining the effectiveness of Bt-based pest management and ensuring the sustainability of agricultural practices.

9 Concluding Remarks

The characterization of plasmid-encoded toxins in *Bacillus thuringiensis* (Bt) has revealed significant insights into the genetic and functional diversity of these elements. Several studies have identified novel Cry toxins and their unique genetic organizations, such as the orf1-gap-orf2 structure found in the H3 strain, which carries 11 novel Cry proteins on its plasmid pH3-180. The transcriptional analysis of the pBtoxis plasmid in Bt subsp. *israelensis* has shown that many of its coding sequences are actively transcribed, including those involved in gene regulation and physiological processes. Additionally, the horizontal transfer of toxin-encoding plasmids, such as pTAND672-2, has been demonstrated, highlighting the role of mobile genetic elements in the diversity and adaptability of mosquitocidal bacteria. Furthermore, the construction of recombinant plasmids combining genes from different Bt subspecies has broadened the host range and enhanced the larvicidal activity against various mosquito species.

Plasmid-encoded toxins in *Bacillus thuringiensis* are crucial for the bacterium's insecticidal properties, which are widely utilized in biopesticides. These toxins, particularly the Cry and Cyt proteins, target specific insect larvae, making Bt a valuable tool in integrated pest management. The presence of multiple plasmids carrying diverse

toxin genes, as seen in strains like H3 and ATCC 10792, enhances the bacterium's ability to combat a broad spectrum of insect pests. The ability of these plasmids to transfer horizontally between species, as demonstrated by pTAND672-2, further underscores their evolutionary significance and potential for genetic engineering to develop more effective biopesticides. Additionally, the discovery of toxin-antitoxin systems on these plasmids, such as the KyAB and tasAB systems, provides insights into plasmid stability and maintenance, which are essential for the long-term efficacy of Bt-based products.

Future research should focus on several key areas to further our understanding and application of plasmid-encoded toxins in *Bacillus thuringiensis*. Genomic and proteomic characterization efforts should continue, as sequencing and analyzing the genomes and proteomes of various Bt strains will help identify novel toxins and their genetic organizations. This can lead to the discovery of new biopesticidal agents with enhanced efficacy and specificity. Investigating the mechanisms underlying the horizontal transfer of toxin-encoding plasmids, such as pTAND672-2, can provide insights into the spread and evolution of these elements. Understanding these processes can aid in developing strategies to prevent the emergence of resistance in target insect populations. Detailed studies on the function and regulation of toxin-antitoxin systems, like KyAB and tasAB, are also crucial. These studies can reveal their roles in plasmid stability and bacterial survival, and this knowledge can be leveraged to design more stable and effective Bt strains for biopesticide production. Recombinant plasmid development should be pursued by constructing recombinant plasmids that combine genes from different Bt subspecies or other bacteria. This approach can create strains with broader host ranges and improved insecticidal properties, leading to the development of next-generation biopesticides that are more effective and environmentally friendly. By addressing these areas, researchers can enhance the utility of *Bacillus thuringiensis* as a biocontrol agent and contribute to sustainable agricultural practices.

Acknowledgments

The authors are quite grateful to two anonymous peer experts for their feedback on the manuscript of this study.

Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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