Bacillus toyonensis biovar Thuringiensis: an overlooked entomopathogen?

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Abstract

Horizontal gene transfer (HGT) significantly influences prokaryotic genome evolution. *Bacillus cereus* and *Bacillus thuringiensis* are nearly identical genetically, except for *B. thuringiensis* producing parasporal crystals. The genes for these crystals, along with other insecticidal proteins (e.g., Vip3), are located on megaplasmids and can be horizontally transferred. Recently, Sauka et al. (2022) reported a *Bacillus toyonensis* strain that produces parasporal crystals with dual insecticidal activity. This strain was classified as *Bacillus toyonensis* biovar Thuringiensis (NCBI: txid2923195) following Carroll et al.'s (2020) nomenclature. Misclassified *B. toyonensis* strains, previously identified as *B. thuringiensis* (e.g., strain MC28), encode *cry* and *cyt* genes toxic to lepidopterous and dipterous insects. Advances in genome sequencing and bioinformatics tools now reduce misidentifications, enabling accurate reclassification in databases like GenBank.

Keywords: *Bacillus thuringiensis*, insecticidal proteins, insect pests, biological control; insect pests.

Bacillus thuringiensis is a gram-positive, spore-forming bacterium known for producing various proteins with specific biocidal activity against invertebrates. This characteristic has made *B.*

thuringiensis one of the most studied and widely used biological control agents for sustainable pest management in agriculture (targeting insects and nematodes) and mosquito control [1]. The repertoire of pesticidal proteins includes parasporal crystal proteins such as Cry, TPP, and Cyt (δ-endotoxins), produced during the stationary growth phase, and secreted proteins produced during vegetative growth, including binary Vpb1/Vpa2 (formerly Vip1/Vip2), Vip3, and Vpb4 (formerly Vip4) [2, 3].

Other gram-positive, spore-forming bacteria also produce biocidal proteins. For example, *Lysinibacillus sphaericus* produces Tpp proteins (formerly BinA/BinB proteins) [4], and *Brevibacillus laterosporus* produces Cry proteins, both of which exhibit toxicity against mosquitoes [5].

In 2022, Sauka et al. isolated and preliminarily classified a *B. thuringiensis* strain with parasporal crystals and insecticidal activity against *Cydia pomonella* (Lepidoptera) and *Anthonomus grandis* (Coleoptera) [6]. However, with an average nucleotide identity (ANI) of 98.6% against *Bacillus toyonensis*, this novel strain was officially named *B. toyonensis* biovar Thuringiensis strain Bto_UNVM-94 [6], following the nomenclature system proposed by Carroll et al. (2020) [7]. Another similar case, is that reported for *Bacillus wiedmannii* biovar thuringiensis, a strain harboring genes encoding for parasporal crystal proteins with mosquitocidal activity [8].

B. toyonensis was first described in 2013 by Jimenez et al. as member of the *Bacillus cereus* group, which includes *B. cereus*, *B. thuringiensis*, and other related pathogens. This species has been used as a probiotic in the swine, poultry, cattle, rabbit, and aquaculture industries for over 30 years.

However, by using a combination of genome-genome hybridization analysis with TYGS server [9] and % ANI calculations with FastANI v0.1.3 [10], we have detected the potential misclassification of two *Bacillus* species stored at GenBank database [11]. Strains *B. thuringiensis* MC28 (RefSeq GCF_000300475.1) [12] and *B. cereus* Rock 1-3 (RefSeq GCF 000161155.1) [13] are clustered in the same branch along with type strain *B. toyonensis* P18 (RefSeq GCF_016605985.1) (Figure 1).

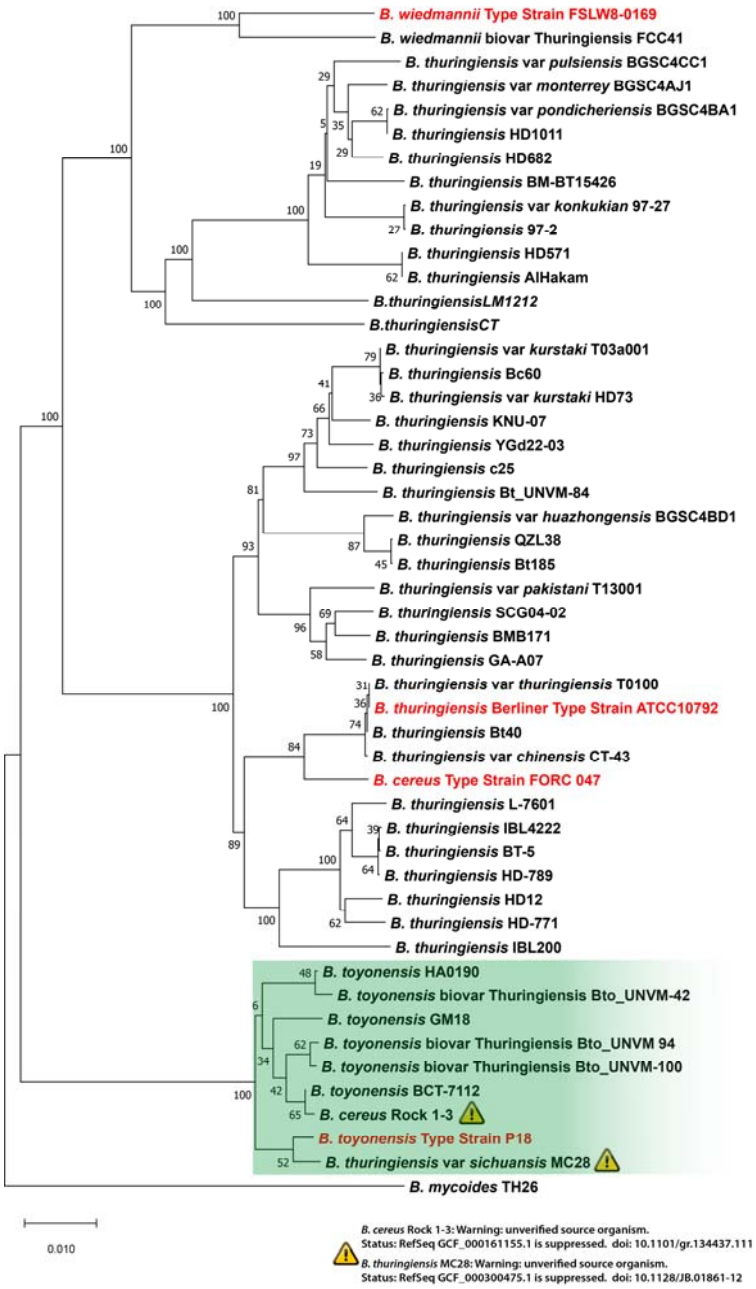

Figure 1. Genome BLAST Distance Phylogeny (GBDP) [14] based on genome data (whole-genome sequence-based) using TYGS server. The *B. toyonensis* cluster is highlighted in green color. The exclamation marks indicate potentially misclassified *B. toyonensis* strains.

In addition, estimated % ANI of these strains, strongly suggests the need of performing a reclassification of these *Bacillus* species by following the novel nomenclature system proposed by Carroll et al., (2020). We also added in the calculations the genomic sequences coming from novel *Bacillus* strains Bto_UNVM-42 and Bto_UNVM-100 (Table 1).

Table 1. % ANI calculation estimated by comparison with type strain *B. toyonensis* P18, showing the reclassification proposal following the nomenclature system by Carroll et al., (2020). A 95-96% ANI is generally accepted as the species boundary [15], therefore, genomes that share 95-96% ANI or higher may be considered as belonging to the same species.

Species	Strain	Acc. Number	% ANI	New species proposal
B. thuringiensis	MC28	CP003687	99.5	*B. toyonensis* biovar Thuringiensis
B. cereus	Rock 1-3	ACMG00000000	98.7	*B. toyonensis*
Bacillus sp.	Bto_UNVM-42	JAYEPS000000000	98.2	*B. toyonensis* biovar Thuringiensis
B. toyonensis[a]	Bto_UNVM-94	NZ_QGLX00000000	98.6	*B. toyonensis* biovar Thuringiensis
Bacillus sp.	Bto_UNVM-100	JAYGOI000000000	98.6	*B. toyonensis* biovar Thuringiensis
B. toyonensis	BCT-7112	CP006863	98.7	*B. toyonensis*
B. toyonensis	GM18	JADNYS000000000	98.5	*B. toyonensis* biovar Thuringiensis

[a]This strain has been already named as *B. toyonensis* biovar Thuringiensis (NCBI:txid2923195).

Strain MC28 can form Bt-like parasporal crystals and harbor several genes encoding for δ-endotoxins (Cry and Cyt), showing high toxicity against lepidopteran and dipteran insects [12]. However, this *B. toyonensis* strain has gone unnoticed as it was misclassified as *B. thuringiensis* MC28. We hypothesize here the existence of horizontal gene transfer (HGT) events. HGT is one of the most significative source of evolution that continuously causes major changes into prokaryotic genomes and has been previously described withing the *Bacillus* genus [16]. One consistent example of HGT within the *Bacillus cereus* group is the transfer of genes encoding insecticidal proteins, such as the parasporal crystal proteins (Cry and Cyt), which confer toxicity against insect pests. These genes are often located on mobile genetic elements, such as plasmids or transposons, facilitating their transfer between bacterial strains and species. Several studies have documented the occurrence of HGT events involving insecticidal genes within the *B. cereus* group. For instance, Liu et al. (2015) investigated the genomic diversity of *B. thuringiensis* strains and identified multiple instances of HGT-mediated acquisition of insecticidal genes from other *Bacillus* species [17]. Similarly, Geng et al. (2023) demonstrated HGT of the 144-kb

plasmid pTAND672-2, carrying mosquitocidal toxin genes, through conjugation experiments, between *B. thuringiensis* serovar *israelensis* and *L. sphaericus* [18].

Overall, HGT serves as a key mechanism driving genetic diversity and adaptation within the *Bacillus cereus* group, particularly in the context of insecticidal properties. Understanding the dynamics of HGT in this group is essential for elucidating its evolutionary history and developing strategies for microbial control and biotechnological applications [16].

In addition, the *B. cereus* strain Rock 1-3 has been also potentially misclassified and should be renamed as *B. toyonensis* biovar Thuringiensis.

In the case of *Bacillus* sp. strains Bto_UNVM-42 and Bto_UNVM-100, the same hypothesis involving HGT events may apply. Strain Bto_UNVM-42 and Bto_UNVM-100 have been isolated from provinces of Jujuy and Córdoba (Argentina), respectively, and they were able to produce parasporal crystals with their genomic sequences harboring several genes with potential insecticidal activity (Table 2), however, both TYGS server and estimation of % ANI against type strain *B. toyonensis* P18 showed these strains as belonging to *B. toyonensis* species, therefore, following the nomenclature proposed by Carroll et al., (2020) we propose to name these strains as belonging to *B. thuringiensis* biovar Thuringiensis species.

Following the same approach, strain GM18 has been correctly named as *B. toyonensis.* This strain is able to produce parasporal crystals, despite their morphology has not been described, and exhibited biocidal activity against human-cancer cells [19] (Table 2).

Table 2. Putative insecticidal gene content of *B. toyonensis* strains.

Strain	Homolog Proteins	Potential targets	Parasporal crystals	References
MC28	Cry4C, Cry30F, Cry53A, Cry54Aa/Ab, Cry68A, Cry69Aa1/Aa2, Cry70B, Cyt1D, Cyt2A	Lepidoptera and Diptera	Spherical	[12]
Bto_UNVM-42	Cyt1, Cry1Cb, Cry3A, Cry32F, Cry32K, Cry32P, Cry32Q, Cry32Ua	Lepidoptera, Coloeptera and Diptera	Bypiramidal	This work
Bto_UNVM-94	Cry7G, Mpp	Lepidoptera and Coleoptera	Bypiramidal	[6]
Bto_UNVM-100	Cry7B, Cry7G, Cry73A, Mpp2A, Xpp22A	Lepidoptera and Coleoptera	Bypiramidal	This work
GM18	Xpp22Ab2, App4Aa1, Tpp78Aa1, Vpb1Ab1, Mpp75Aa1, Vpb1Ea2	Human-cancer cells	Produced and not specified	[19]

Based on our phylogenetic analyses, the *B. toyonensis* biovar Thuringiensis strains described here show behavior very similar to *B. thuringiensis.* These strains not only produce parasporal crystals but also exhibit biocidal activity against insects and human cancer cells. However, it would not be surprising if, like *B. thuringiensis*, non-insecticidal isolates comprise over 90% of the natural populations in soils and on phylloplanes [20]. In this wok, our findings suggest that HGT events from *B. thuringiensis* to *B. toyonensis* have likely occurred in that direction. However, further research is needed to fully understand this genetic phenomenon. Such understanding could be harnessed through conjugation to develop new species and strains that are valuable for sustainable pest management in agriculture.

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