Bacillus toyonensis biovar Thuringiensis: A novel entomopathogen with insecticidal activity against lepidopteran and coleopteran pests.

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Abstract

The *Bacillus cereus* group includes eight species: *Bacillus anthracis*, *B. cereus*, *Bacillus cytotoxicus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis* and *Bacillus toyonensis*, which are highly related at the phylogenetic level. In this work, we performed the isolation and characterization of a novel *Bacillus* sp. strain exhibiting parasporal crystals with insecticidal activity that was initially classified as a *Bacillus thuringiensis* strain. Its genome encoded three genes showing homology to known pesticidal proteins from *B. thuringiensis* and *Lysinibacillus sphaericus* proteins: Cry7Ga1 (a crystal protein), a Mpp2Aa3 (ETX/Mtx2 family) homolog and a mosquitocidal-like protein (NPP1). However, since its genome sequence shared >98% ANI with several *Bacillus toyonensis* genomes, the strain has subsequently been renamed as *Bacillus toyonensis* biovar Thuringiensis and designated Bto-UNVM_94. Bioassays demonstrated that this novel strain exhibited toxicity against *Cydia pomonella* (Lepidoptera: Tortricidae) and *Anthonomus grandis* (Coleoptera: Curculionidae), a low toxicity against *Aedes aegypti* (Diptera: Culicidae) and *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) whereas no toxicity was shown to the free-living nematode *Panagrellus redivivus* (Rhabditida: Panagrolaimidae).

Keywords: *Bacillus cereus* group, entomopathogenic bacteria, δ-endotoxins, *Bacillus toyonensis*, biovar Thuringiensis.

1. Introduction

The *Bacillus cereus* Group (BcG) is a composite of eight recognized species including *Bacillus anthracis*, *B. cereus sensu stricto*, *Bacillus cytotoxicus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis* and the recently included species, *B. toyonensis,* which has been used for several years as a probiotic supplement in animal nutrition (Jiménez et al., 2013). While the members of the BcG exhibit notable biological differences, their phylogenetic relationship, sharing highly conserved 16S rRNA gene sequences and genomes, makes genomic identification methods tricky and controversial.

B. thuringiensis was considered to be the unique species of the BcG bearing plasmids harboring one or more δ-endotoxin genes encoding invertebrate-active proteins and, in consequence, was thought for several decades as the sole species of the group capable of producing parasporal crystalline inclusions composed of various crystal proteins (previously grouped together as "Cry" proteins but now subdivided by structural families) and cytolytic (Cyt) proteins (Crickmore et al., 2020). Nevertheless, these plasmids have proven to be horizontally transferred among different species of the BcG (e.g. *B. cereus*, *B. anthracis* and *B. thuringiensis*) (Meric et al., 2018) and it has been suggested that some *B. thuringiensis* serovarieties may show the low temperature growth characteristics indicating that they are, in fact, *B. weihenstephanensis* strains (Soufiane and Cote, 2010).

Next-generation Sequencing (NGS) technology has facilitated the fast genome sequencing of bacteria and other organisms in a cost-effective way, delivering thousands of genomic sequences from strains that were previously mis-classified by using phenotypic methods (Parks et al., 2018). A recent example is a strain exhibiting mosquitocidal activity (Berón and Salerno, 2006) that was initially mis-identified and named *B. thuringiensis* FCC41 and later renamed as *Bacillus wiedmannii* biovar Thuringiensis, by using a combination of genome-genome sequence comparisons and phylogenetic methods, which demonstrated the presence of plasmids encoding δ-endotoxin proteins responsible

for a *B. thuringiensis*-like phenotype (presence of parasporal crystals and insecticidal activity) (Lazarte et al., 2018). Taking this into account, the *biovar* term was proposed within a new taxonomic nomenclature in order to denote isolates with interesting phenotypic characteristics where briefly, the first letter of the biovar name is capitalized and the name is not italicized (e.g. *B. toyonensis* biovar Thuringiensis) (Carroll et al., 2020).

δ-endotoxins are the best characterized group of insecticidal proteins from *B. thuringiensis*, synthesized during the sporulation phase and showing toxicity against a wide range of invertebrates (Schnepf et al., 1998; van Frankenhuyzen, 2009; van Frankenhuyzen, 2013). Crystal proteins may be classified into several distinct groups according to their homology and molecular structure (Crickmore et al., 2020). The largest group is composed of three-domain Cry proteins followed by Mpp (including Mpp2-formerly Mtx2) and Tpp (including Tpp1/Tpp2 formerly BinA/BinB) proteins typically produced by *Lysinibacillus sphaericus* (Berry, 2012) and other structural families. Three-domain Cry proteins show toxicity against insects in the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera but also against some nematodes (Palma et al., 2014; van Frankenhuyzen, 2009; van Frankenhuyzen, 2013). These proteins exhibit two different sizes of ~70 and ~130 kDa, generating smaller protease stable fragments upon proteolytic cleavage to produce their active forms (Jurat-Fuentes and Crickmore, 2017; Palma et al., 2014). *L. sphaericus* Tpp1/Tpp2, Mpp and Mtx proteins are toxic against some dipteran larvae (Berry, 2012) whereas Tpp and Mpp proteins from *B. thuringiensis* may be toxic for coleopteran, dipteran and hemipteran species.

Since *B. thuringiensis*-based biopesticides were first commercialized in France around 1940 (de Maagd, 2015), the identification of genes encoding novel insecticidal proteins spanning wider ranges of insect orders has been continuously increasing (Hernández-Rodríguez et al., 2009; Palma et al., 2013; Porcar and Juárez-Pérez, 2003). However, most of the *B. thuringiensis* strains and the insecticidal proteins used are known to be highly active against lepidopteran pests (Dominguez-Arrizabalaga et al., 2020). Strains showing toxic activity against coleopterans are limited and amongst the proteins they may encode, some produce three-domain Cry7 proteins, which have been described

to be toxic against some coleopteran (e.g. Cry7A) or lepidopteran (e.g. Cry7B) larvae (van Frankenhuyzen, 2009). Specifically, Cry7Aa was described to be toxic against species of *Cyla* (Coleoptera: Brentidae) and *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) whereas Cry7Ab was toxic against *Henosepilachna vigintiomaculata* (Coleoptera: Coccinellidae) and *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae) (Dominguez-Arrizabalaga et al., 2020).

The finding of novel bacteria producing different insecticidal proteins opens the possibility of discovering additional tools for controlling different insect pests but also, for delaying insect resistance (Peralta and Palma, 2017). In this work, we report for the first time the genome sequence and insecticidal characterization of a novel *Bacillus* strain proposed to be classified as *B. toyonensis* biovar Thuringiensis and designated Bto-UNVM_94, which was isolated from Cululú (Santa Fe province, Argentina) and showed toxicity against *Cydia pomonella* (Lepidoptera: Tortricidae) and *Anthonomus grandis* (Coleoptera: Curculionidae).

2. Materials and methods

2.1. Strain isolation and characterization

The soil samples were obtained with a tubular soil sampler from a native forest area at Cululú (Santa Fe province, Argentina) as a composite of 5 random sub-samples and consisted in total of ~20 g of soil. After collection, the sample was stored at 4 ºC in zip-lock bags until axenic isolation was performed. With this purpose, 3 g of the soil sample was homogenized into 10 ml of sterile distilled water. The soil suspension was later mixed by 1 min. vortexing and incubated at 80 ºC for 30 min. The sample was then subjected to five ten-fold dilutions and 50 µl from 10^{-3} to 10^{-5} dilutions were plated onto nutrient agar plates (0.5% Peptone, 0.3% beef extract, 0.5% NaCl and 1.5% agar) using a Drigalsky spatula. Inoculated plates were incubated at 28 ºC for 48-72 h. Bacterial colonies exhibiting a *B. thuringiensis*-like phenotype (matt-white colonies with uneven borders) were then sub-cultured for axenic isolation (purification) of the bacterium with a 5 µl inoculating loop and incubated as described before, until sporulation was produced. Each sporulated culture was then heat fixed onto a glass microscope slide and stained with a Coomassie blue solution (0,133 % Coomassie

Blue stain in 50 % acetic acid) (Ammons et al., 2002). The identification of *B. thuringiensis*-like parasporal crystals was performed using a light microscope and later confirmed by Scanning Electron Microscopy (SEM). The axenic sporulated colonies were stored at our bacterial collection in 15% glycerol and −80ºC. The composition of parasporal crystals was determined by SDS-PAGE following the procedure described by Pérez et al. (2017). *B. thuringiensis* svar. *kurstaki* HD-1, *B. thuringiensis* svar. *morrisoni tenebrionis* DSM2803 and *B. thuringiensis* svar. *israelensis* HD-567 were used as reference strains **(**Perez et al., 2017**)**.

2.2 DNA purification and sequencing

Purified total DNA (including chromosome and plasmids) was obtained using the Wizard genomic DNA purification kit (Promega), following the manufacturer's instructions for the isolation of DNA from Gram-positive bacteria. Total DNA was electrophoresed in 1% agarose gels stained with SYBR Safe (Thermo Fisher Scientific) and quantified using PICODROP (PICO 100 µl Spectrophotometer). The purified DNA was then used to construct a pooled Illumina library and sequenced at Stabvida (Portugal) by using high-throughput Illumina sequencing technology with a genomic coverage of 1000× (Caballero et al., 2018).

2.3 Genome assembly and analysis

The raw Illumina reads obtained were first trimmed and assembled into contigs by using Geneious R11 (www.geneious.com), with the *de novo* assembly tool and default parameters. The resultant contigs were then analysed with BLAST (Altschul et al., 1990) using a customized non-redundant insecticidal protein database (Caballero et al., 2018). Genome annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline (2018 release) although it was also annotated with RAST (Aziz et al., 2008). Multiple sequence alignments, phylogenetic trees and conserved domain searches were performed using suitable tools included in Geneious R11 (Drummond et al., 2021).

Phylogenetic relationships among different *Bacillus* species were analysed using a modification of the Drewnowska and Swiecicka (2013) method by including the *gyrB* (DNA gyrase, subunit B) gene

sequence into the concatenation along with seven more genes, namely: *glpF* (glycerol uptake facilitator protein), *gmk* (putative guanylate kinase), *ilvD* (dihydroxy-acid dehydratase), *pta* (phosphate acetyltransferase), *pur* (Phosphoribosyl aminoimidazole carboxamide formyltransferase), *pycA* (pyruvate carboxylase), and *tpi* (triosephosphate isomerase). Concatenated gene sequences were obtained using the *concatenate* tool included in Geneious R11 and accounted for 11,718 nucleotides. Multiple sequence alignments of each single gene and concatenated-gene sequences were obtained with Muscle (Edgar, 2004) and edited manually. The phylogenetic tree was constructed using the Neighbor-Joining method (NJ) with 1,000 bootstrap replicates for determining branch quality.

Percentages of average Nucleotide Identity (ANI) among related genomes were calculated using the Enveomics ANI calculator tool (http://enve-omics.ce.gatech.edu/ani/index) where values among genomes of the same species are typically found to be above 95% (Varghese et al., 2015).

This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under the accession number QGLX00000000. The version described in this paper is the first version, QGLX01000000. The sequences of the putative insecticidal proteins, namely Mpp, Cry7Ga-like and the mosquitocidal-like protein have been submitted to GenBank under the accession numbers OK001675, OK001676 and OK001677, respectively.

2.4. Insect and nematode bioassays

The insecticidal activity of Bto-UNVM_94 strain was qualitatively evaluated on four insect species -second instars of *Alphitobius diaperinus* P. (Coleoptera: Tenebrionidae), early fourth instars of *Aedes aegypti* (Diptera: Culicidae), neonates of *Anthonomus grandis* B. (Coleoptera: Curculionidae) and *Cydia pomonella* L. (Lepidoptera: Tortricidae), plus the free-living nematode *Panagrellus redivivus* L. (Rhabditida: Panagrolaimidae). Coleopteran and lepidopteran larvae as well nematodes were obtained from colonies reared in IMYZA-INTA laboratories at 29 ºC on artificial diets specific for each organism (Barrett and Butterworth, 1984; Perez et al., 2017). Eggs of *A. aegypti* were provided by Dra. María Micieli (Center for Parasitological and Vector Studies, CEPAVE,

Argentina) and the obtained larvae reared at 28°C under a 14:10 h (light:dark) photoperiod in plastic containers with dechlorinated water.

Powders of spore-crystal complexes were prepared as previously described (Sauka et al, 2010). One hundred µl of highly concentrated spore stock suspension of Bto-UNVM_94 strain was inoculated into 100 ml of sporulation BM broth (5 g glucose; 2.5 g K_2HPO_4; 1 g KH_2PO4; 2.5 g NaCl; 0.25 g $MgSO_4 \cdot 7H_2O$; 0.1 g $MnSO_4 \cdot H_2O$; 2.5 g starch and 4 g yeast extract, in a total volume of 1 l of distilled water, pH 7.2), at 340 rpm and 30°C, until complete autolysis was observed. Spore-crystal complexes were obtained by centrifugation at 12,000 *g* and 4 ºC for 15 min, then pellets were freeze-dried.

Each spore-crystal mixtures (final concentration of 5-1000 µg/ml) was later incorporated into polypropylene conical tubes containing the corresponding artificial diet (maintained at 60ºC) for *A. diaperinus*, *A. grandis* and *C. pomonella* and poured into each well of a 24-well plate (Nunc 143982) (Perez et al., 2017) or into plastic cups containing dechlorinated water for *A. aegypti* (Ibarra et al., 2003). Twenty-four coleopteran and lepidopteran (two replicates) and 25 dipteran larvae (three replicates) were used for each assay. .

For *P. redivivus*, approximately 4000 nematodes were exposed to a Bto-UNVM_94 culture that had been grown to confluence on sporulation BM agar (BM broth supplemented with 2 g agar per litre) plates (90 × 15 mm) at 29 ºC for 72 h before the assay.

Mortality was registered after 15 days at 29°C in *A. diaperinus* and *A. grandis*, five days at 29ºC in *C. pomonella*, and one day at 29ºC in *A. aegypti* and *P. redivivus* bioassays. Larvae were considered dead if they failed to respond to gentle probing.

Distilled water without crystal-spore mixtures was added to the natural mortality controls. Schneider-Orelli's formula was used in insect bioassays to calculate corrected mortality in comparison to the untreated control. The InfoStat software (Universidad Nacional de Córdoba, version 2014) was used for the statistical analysis, and the statistical significance was set at $p<0.05$.

2.5. PCR-based prediction and detection of β-exotoxin production.

In order to predict whether the Bto-UNVM_94 strain is able to produce type I β-exotoxin, a qualitative PCR-based method for the detection of the *thuE* gene was carried out, as previously described by Sauka et al. (2014). *B. thuringiensis* INTA H48-5 and *B. thuringiensis* svar. *thuringiensis* HD-2 were used as positive controls. In addition, the β-exotoxin synthesis capability of strain Bto-UNVM_94 was also screened by quantifying the number of emerged *Musca domestica* (Diptera: Muscidae) adults after treatment in triplicate as described previously (Sauka et al., 2014). *B. thuringiensis* svar. *thuringiensis* HD-2 was used as a positive control for β-exotoxin production (Supplementary Table S1).

3. Results

3.1 Strain isolation

As described above, new bacterial isolates were prepared from soil samples. One of the colonies identified isolated from Cululú soil sample from Santa Fe province, Argentina, exhibited the typical *B. thuringiensis*-colony morphology on nutrient agar (matt white colour, flat, dry and with uneven borders) and also showed the presence of Coomassie-blue stained parasporal crystals under the light microscope, which were later confirmed as bipyramidal crystals by SEM examination and SDS-PAGE analysis (Figure 1).

3.2 Bioassays

Mixed spore-crystal suspensions of the strain exhibited toxicity and showed 54.2% and 77.8% mortality (mean corrected mortality) against *C. pomonella* and *A. grandis*, respectively. Lower % mortality were also detected for *A. aegypti* and *A. diaperinus* larvae whereas no mortality was observed for the free-living nematode *P. redivivus* (Table 1).

3.3 Sequencing and characterisation

Genome sequencing produced >23 million Illumina raw reads that were trimmed and assembled into 43 contigs totaling 6,136,970 bp, with a G+C content of 34,9% and containing 6,223

predicted protein-coding genes (CDSs) plus 86 RNAs, consistent within the range of other sequences *Bacillus* sp. genomes (Makuwa and Serepa-Dlamini, 2019).

Phylogenetic analysis using eight concatenated housekeeping genes (*glpF*, *gmk*, *gyrB*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi*) showed that the strain forms a monophyletic group closely related to *B. cereus* Rock1-3 (Acc. No. CM000728), type strain *B. toyonensis* BCT-7112 (Acc. No. CP006863) (Jimenez et al., 2013) and to *B. thuringiensis* MC28 (Acc. No. CP003687) (Figure 2). This result was also consistent with that obtained after we performed the phylogenetic analysis by using the method proposed by Drewnowska and Swiecicka (2013), however, the inclusion of *gyrB* gene sequences improved tree robustness by increasing bootstrap support values at several nodes of the phylogenetic tree (data not shown).

In addition, the average nucleotide identity (%ANI) values of this strain were calculated and compared with 49 additional genomic sequences including genomes from *B. thuringiensis* and *B. cereus* plus eight genomes from different *B. toyonensis* strains that have successfully passed the taxonomy check analysis from GenBank (Table 2). Strain Bto-UNVM_94 shared the highest % ANI values (>95 %) with the eight *B. toyonensis* genomes but also with *B. cereus* Rock 1-3 and *B. thuringiensis* MC28 genomes. These two last species have failed to pass the taxonomy check from GenBank and were therefore assumed in this study, as being potentially mis-identified *B. toyonensis* strains. As a result of the above analyses (and the fact that the morphology of the colony is also consistent with the *B. toyonensis type* strain BCT-7112 phenotype (Jiménez et al., 2013), our strain was designated as a *B. toyonensis* strain and named *B. toyonensis* biovar Thuringiensis Bto-UNVM_94.

3.4 Genes encoding putative pesticidal proteins

Bto-UNVM_94 harbors one CDS (coding sequence) encoding a protein with 92% pairwise identity with the crystal protein Cry7Ga1 plus another CDS encoding a protein exhibiting 97% pairwise identity with an as-yet unnamed Mpp (ETX/Mtx2) family putative pore forming protein

from *B. thuringiensis* (GenBank Accession Number WP_065212007). This CDS also showed 42% pairwise similarity with Beta pore-forming pesticidal protein Mpp2Aa3 using Best Match Finder at the Bacterial Pesticidal Protein Resource Center database (Crickmore et al., 2020) and 53% pairwise identity with sequence 101 from US Patent 10793610 (Acc. No. QPY81898). We also found a third CDS showing 95% pairwise identity with a putative mosquitocidal protein, although no mosquitocidal activity has been yet reported for this protein from *B. cereus* Rock 1-3 (GenBank Acc. No. EEL19614), one of the strains that we suggest should be reassigned to *B. toyonensis.* This CDS also showed 65% pairwise identity with sequence 94 from US patent 8461421 (Acc. No. AGP18048) for which no insecticidal activity was described in the patent.

The >23 million Illumina reads were also filtered by using the *map to reference* tool from Geneious R11 using the *B. toyonensis* BCT-7112 sequence as the reference genome. The tool was set to separate unmapped reads for *de novo* assembly, interpreting the new contig sequences, as if they came from plasmid DNA. Later, custom BLASTx searches were performed using the Cry7Ga-like protein, the Mpp2 homolog protein and the mosquitocidal-like protein showing the location of such encoded genes in these draft-assembled plasmid sequences (data not shown). These results strongly suggest the presence of extrachromosomal DNA in the genomic sequence of the Bto-UNVM_94 strain.

In addition and consistent with the lack of teratological effects of strain Bto-UNVM_94 to *M. domestica*, no *β*-exotoxin (thuringiensin) synthesis genes were also identified in the genomic DNA and PCR amplification of the type I *β*-exotoxin *thuE* gene produced no amplification from this strain (Supplementary material, Figure S1).

4 Discussion

4.1 Pesticidal proteins

A number of putative pesticidal proteins are encoded by Bto-UNVM_94. The Mpp family sequence (Acc. no. OK001675) represents a protein of *ca* 33 kDa with a putative signal peptide (residues 1 to 33) and an aerolysin-like ETX_MTX2 pore forming domain (InterPro ID IPR004991)

277 from residues 84 to 290 making it a clear member of the Mpp group (Crickmore et al., 2020) that
278 would represent a new subclass of the family if activity against an invertebrate target is demonstrated.
279 The potential toxin gene encoding a protein related to a database entry annotated as mosquitocidal,
280 encodes a predicted protein of molecular weight of *ca* 57 kDa (Acc. no. OK001677). Conserved
281 domain search with InterProScan showed a putative signal peptide sequence from residues 1 to 19,
282 an NPP1 necrosis inducing protein conserved domain (InterPro ID IPR008701) from residues 50 to
283 253 and a RicinB_lectin_2 (InterPro ID IPR000772) conserved domain from residues 302 to 391.
284 Lectin-like domains are common in invertebrate-active pesticidal proteins and may have roles in
285 interactions with target cells. The SDS PAGE analysis of this preparation, (Figure 1 C) shows no
286 evident protein bands of *ca* 33 and 57 kDa that would correspond to the predicted Mpp or the
287 mosquitocidal-like protein, suggesting that these proteins may be synthesized by vegetative cells and
288 secreted (consistent with the presence of predicted signal sequences).
289 The predicted Cry7Ga-like protein from strain Bto-UNVM_94 (Acc. no. OK001676) has a
290 molecular weight of *ca* 128 kDa, which is consistent with the SDS-PAGE analysis of spore-crystal
291 mixtures, which showed a main band corresponding to a size of *ca* 130 kDa (Figure 1 C). However,
292 its expression level is lower than those shown by reference *B. thuringiensis* strains (Figure 1 C),
293 allowing us to hypothesize that strain Bto-UNVM_94 is not as efficient as *B. thuringiensis* for the
294 expression and crystallization of the encoded Cry7Ga-like protein. The crystals seen in our strain are
295 bipyramidal, which is also consistent with the reported morphology of the Cry7Aa1 crystals produced
296 by *B. thuringiensis* strain BTS137J (Lambert et al., 1992). As the insect toxicity that we have
297 demonstrated was achieved using a spore crystal mix from strain Bto-UNVM_94 it is possible that
298 the Cry7Ga-like protein is not the only responsible for the overall activity shown.
299 Previous studies demonstrated that Vip3A proteins possess a signal peptide and are secreted,
300 remaining adhered to spore and crystals and therefore, contribute to the overall toxicity of *B.*
301 *thuringiensis* (Donovan et al., 2001; Wang et al., 2020). A possibility here is that remanent fractions
302 from potentially secreted proteins (the Mpp homolog and the mosquitocidal-like protein) of our strain

could follow the same behaviour and be responsible of the low, but exhibited toxicity, against *A. aegypti.* For this reason they are also expected to be non-evident in the SDS-PAGE gel analysis. However, additional studies are needed to effectively test these hypotheses .

As mentioned previously, the insecticidal activity of Cry7 proteins has only been reported for a few members of this family. For example, Cry7Aa1 was reported for its insecticidal activity against coleopteran larvae of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), *Cylas brunneus* and *Cylas puncticollis* (Coleoptera: Brentidae) whereas toxicity was lacking against coleopteran larvae of *Diabrotica undecimpunctata*, *Anoplophora glabrupenius* and *A. grandis.* The Cry7Aa1 protein also lacked toxicity against lepidopteran larvae of *Heliothis virescens*, *Spodoptera littoralis* and *Manduca sexta* (Lambert et al 1992; Ekobu et al 2010). The Cry7Aa2 protein was recently reported to be toxic for *L. decemlineata* (Domínguez-Arrizabalaga 2019). Cry7Ab3 was described to be active against the spotted potato ladybeetle, *Henosepilachna vigintioctomaculata* (Coleoptera: Coccinellidae). In contrast to the coleopteran activity reported for the above Cry7A variants, Cry7Ba showed activity against the lepidopteran *Plutella xylostella* larvae (Lepidoptera: Plutellidae) whereas Cry7Ca1 has been reported to be toxic for adults of *Locusta migratoria manilensis* (Orthoptera: Acrididae) (Song et al., 2008; van Frankenhuyzen, 2009; Wu et al., 2011). To our knowledge, there is not currently any information available concerning the insecticidal activity and the host range for Cry7G homologs but our results suggests that the Cry7G-like protein described here is likely to be responsible for the toxicity shown by spore-crystal preparations against both lepidopteran and coleopteran pests. This would be the first indication of the insecticidal activity of a Cry7G protein, although confirmation of this property will require separate cloning and quantitative testing of this protein in isolation since we cannot rule out other factors from the strain in its activity (e.g. the Mpp homolog and the mosquitocidal-like protein).

Our study is also the first demonstration of insecticidal activity in a *B. toyonensis* strain (designated Bto-UNVM_94), a species that has been used for several years as probiotic supplement in animal nutrition (Jiménez et al., 2013). This strain is also lacking the production of β-exotoxin,

which is also a requirement for *B. thuringiensis* formulations in Europe and other countries (e.g. US and Canada) (Palma et al., 2014). We further speculate that some strains such as *B. cereus* Rock 1-3 and *B. thuringiensis* MC28 may be mis-classified *B. toyonensis* strains as genomic sequences have often received taxonomic mis-classifications as a result of using mainly phenotypic characteristics in assigning their identities (Federhen et al., 2016). Transfer of plasmids, on which insecticidal proteins are encoded in *B. thuringiensis*, between *B. cereus* group strains is well known and the discovery of toxin-coding plasmids in our strain is consistent with movement of these toxins within the group.

To date, *B. thuringiensis* remains as the most successfully exploited bacterium for the biological control of insect pests in agriculture but based in our new data, other member of the *B. cereus* group including *B. toyonensis* deserve to be explored to identify novel insecticidal activities.

Acknowledgements

This work was supported by grants PIC-UNVM 2017-2019 from Universidad Nacional de Villa María, PIP-2017-2019 GI from CONICET, 2019-PD-E4-I069-001 from INTA and PICT-2017-0087 from Ministerio de Ciencia, Tecnología e Innovación (MinCyT) Argentina.

References

Altschul, S. F., et al., 1990. Basic local alignment search tool. J Mol Biol 215**,** 403-410.

Ammons, D., et al., 2002. Usefulness of staining parasporal bodies when screening for *Bacillus thuringiensis*. J Invertebr Pathol. 79**,** 203-4.

Aziz, R. K., et al., 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics. 9**,** 1-15.

Barrett, J., Butterworth, P. E., 1984. Acetaldehyde formation by mitochondria from the free-living nematode *Panagrellus redivivus*. Biochem J. 221**,** 535-40.

Berón, C. M., Salerno, G. L., 2006. Characterization of *Bacillus thuringiensis* isolates from Argentina that are potentially useful in insect pest control. BioControl. 51**,** 779–794.

Berry, C., 2012. The bacterium, *Lysinibacillus sphaericus*, as an insect pathogen. J Invertebr Pathol. 109**,** 1-10.

Caballero, J., et al., 2018. Draft genome sequence of *Bacillus cereus* CITVM-11.1, a strain exhibiting interesting antifungal activities. J Mol Microbiol Biotechnol. 28**,** 47-51.

Carroll, L. M., et al., 2020. Proposal of a Taxonomic Nomenclature for the *Bacillus cereus* Group Which Reconciles Genomic Definitions of Bacterial Species with Clinical and Industrial Phenotypes. mBio. 11.

Crickmore, N., et al., 2020. A structure-based nomenclature for *Bacillus thuringiensis* and other bacteria-derived pesticidal proteins. J Invertebr Pathol. 107438.

de Maagd, R., 2015. *Bacillus thuringiensis*-Based Products for Insect Pest Control. *In:* Lugtenberg B. (eds) Principles of Plant-Microbe Interactions. Springer, Cham.

Dominguez-Arrizabalaga, M., et al., 2020. Insecticidal Activity of *Bacillus thuringiensis* Proteins Against Coleopteran Pests. Toxins (Basel). 12.

Donovan WP, Donovan JC & Engleman JT (2001) Gene knockout demonstrates that vip3A contributes to the pathogenesis of *Bacillus thuringiensis* toward *Agrotis ipsilon* and *Spodoptera exigua*. J Invertebr Pathol 78: 45-51.

Drewnowska, J. M., Swiecicka, I., 2013. Eco-genetic structure of *Bacillus cereus* sensu lato populations from different environments in northeastern Poland. PLoS One. 8**,** e80175.

Drummond, A. J., et al., 2021. Geneious R11. [Online.]. http://www.geneious.com/.

Edgar, R. C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 5**,** 113.

Federhen, S., et al., 2016. Meeting report: GenBank microbial genomic taxonomy workshop (12–13 May, 2015). Stand Genom Sci 11**,** 15.

Hernández-Rodríguez, C. S., et al., 2009. Screening and identification of *vip* genes in *Bacillus thuringiensis strains*. J Appl Microbiol. 107**,** 219-25.

Ibarra, J., et al., 2003. Diversity of *Bacillus thuringiensis* strains from Latin America with insecticidal activity against different mosquito species. Appl Environ Microbiol. 69, 5269-74.

Jiménez, G., et al., 2013. Description of *Bacillus toyonensis* sp. nov., a novel species of the *Bacillus cereus* group, and pairwise genome comparisons of the species of the group by means of ANI calculations. Syst Appl Microbiol. 36**,** 383-91.

Jurat-Fuentes, J. L., Crickmore, N., 2017. Specificity determinants for Cry insecticidal proteins: Insights from their mode of action. J Invertebr Pathol. 142**,** 5-10.

Lambert, B., et al., 1992. Novel *Bacillus thuringiensis* insecticidal crystal protein with a silent activity against coleopteran larvae. Appl Environ Microbiol. 58**,** 2536-42.

Lazarte, J. N., et al., 2018. *Bacillus wiedmannii* biovar thuringiensis: A Specialized Mosquitocidal Pathogen with Plasmids from Diverse Origins. Genome Biol Evol. 10**,** 2823-2833.

Makuwa, S. C., Serepa-Dlamini, M. H., 2019. Data on draft genome sequence of *Bacillus* sp. strain MHSD28, a bacterial endophyte isolated from *Dicoma anomala*. Data Brief. 26**,** 104524.

Meric, G., et al., 2018. Lineage-specific plasmid acquisition and the evolution of specialized pathogens in *Bacillus thuringiensis* and the *Bacillus cereus group*. Mol Ecol. 27**,** 1524-1540.

Palma, L., et al., 2014. *Bacillus thuringiensis* toxins: an overview of their biocidal activity. Toxins (Basel). 6**,** 3296-325.

Palma, L., et al., 2013. Screening of *vip* genes from a Spanish *Bacillus thuringiensis* collection and characterization of two novel Vip3 proteins highly-toxic to five lepidopteran crop pests. Biol control. 66**,** 141-149.

Parks, D. H., et al., 2018. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. Nat Biotechnol. 36**,** 996-1004.

Peralta, C., Palma, L., 2017. Is the insect world overcoming the efficacy of *Bacillus thuringiensis*? Toxins (Basel). 9**,** 1-5.

Perez, M. P., et al., 2017. Selection of *Bacillus thuringiensis* strains toxic to cotton boll weevil (*Anthonomus grandis*, Coleoptera: Curculionidae) larvae. Rev Argent Microbiol. 49**,** 264-272.

Porcar, M., Juárez-Pérez, V., 2003. PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol Rev. 26**,** 419-32.

406 Sauka, D. H., et al., 2014. PCR-based prediction of type I beta-exotoxin production in *Bacillus*
407 *thuringiensis* strains. J Invertebr Pathol. 122**,** 28-31.
408 Schnepf, E., et al., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol
409 Rev. 62**,** 775-806.
410 Song, L., et al., 2008. Specific activity of a *Bacillus thuringiensis* strain against *Locusta migratoria*
411 manilensis. J Invertebr Pathol. 98**,** 169-76.
412 Soufiane, B., Cote, J. C., 2010. *Bacillus thuringiensis* serovars *bolivia*, *vazensis* and *navarrensis* meet
413 the description of *Bacillus weihenstephanensis*. Curr Microbiol. 60**,** 343-9.
414 van Frankenhuyzen, K., 2009. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J
415 Invertebr Pathol. 101**,** 1-16.
416 van Frankenhuyzen, K., 2013. Cross-order and cross-phylum activity of *Bacillus thuringiensis*
417 pesticidal proteins. J Invertebr Pathol. 114**,** 76-85.
418 Varghese, N. J., et al., 2015. Microbial species delineation using whole genome sequences. Nucleic
419 Acids Res. 43**,** 6761-71.
420 Wang Z, Gan C, Wang J, Bravo A, Soberon M, Yang Q & Zhang J (2021) Nutrient conditions
421 determine the localization of *Bacillus thuringiensis* Vip3Aa protein in the mother cell compartment.
422 Microb Biotechnol 14: 551-560.
423 Wu, Y., et al., 2011. Novel *Bacillus thuringiensis* delta-endotoxin active against *Locusta migratoria*
424 manilensis. Appl Environ Microbiol. 77**,** 3227-33.
425

426 **Tables**

427

428 **Table 1**

Invertebrate tested	Spore-crystal complex (μg/ml)	Mortality (%)[2]
C. pomonella (Lepidoptera: Tortricidae)	5	54.2±17.7
A. aegypti (Diptera: Culicidae)	125	20.0±4.0
A. diaperinus (Coleoptera: Tenebrionidae)	1000	11.6±2.9
A. grandis (Coleoptera: Curculionidae)	1000	77.8±12.6
P. redivivus (Rhabditida: Panagrolaimidae)	ND	1.3±1.2

429 [1] No determinated; [2]Mean±SD

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433 **Table 2**

Species	Acc. No	%ANI	GenBank taxonomy check[a]
B. toyonensis BCT-7112 (type strain)	CP006863	99.5	OK
B. toyonensis P18	CP064875	98.7	OK
B. toyonensis HuB4-10	AHEE01000001	99.4	OK
B. toyonensis RUTrin4	VZK01000001	99.5	OK
B. toyonensis BacAer BTH38.1	MSAB01000001	98.5	OK
B. toyonensis SFC 500-1E	JAAONV010000001	99.8	OK
B. toyonensis G25-77	LDGO01000001.1	99.4	OK
B. toyonensis RM9	WBOP00000000	99.5	OK
B. cereus Rock 1-3	CM000728	99.3	Failed
B. thuringiensis MC28	CP003687	98.7	Failed

434 [a]GenBank species identification confirmed by %ANI.
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Table Legends

Table 1. Toxic activity of spore-crystal suspensions of the Bto-UNVM_94 strain against species of different invertebrate orders.

Table 2. %ANI of the four strains forming the monophyletic group including Bto-UNVM_94 strain obtained by genome-genome comparisons.

447 **Figures**

448 **Figure 1**

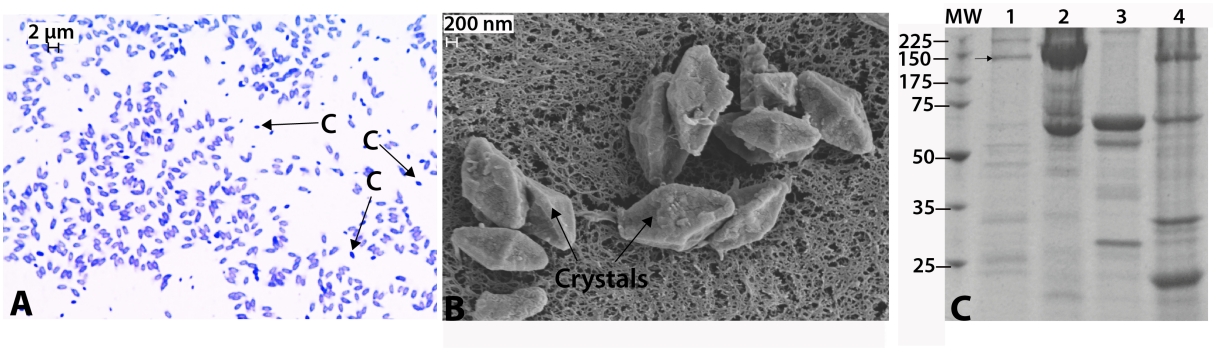

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Figure 2

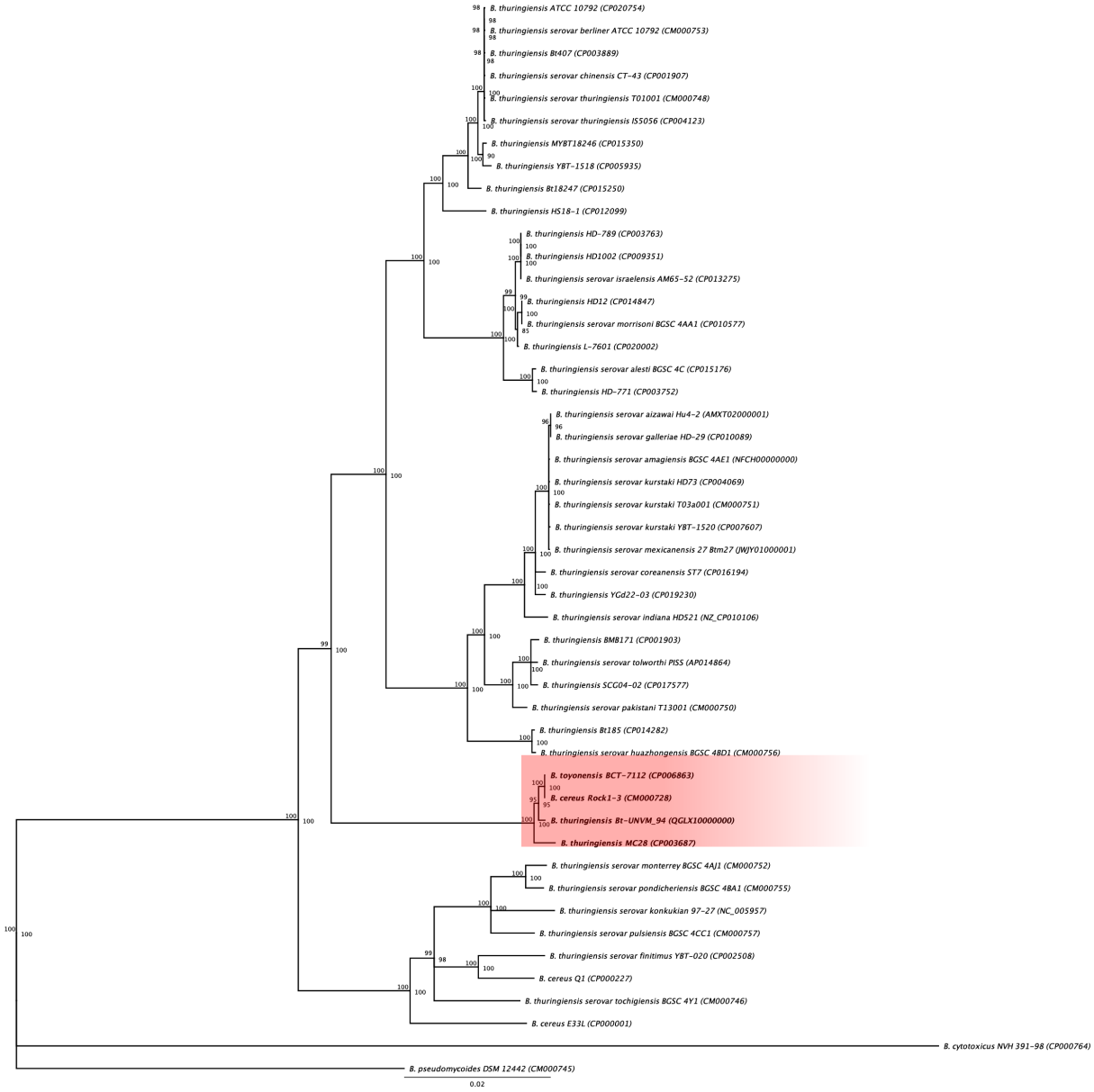

B. thuringiensis ATCC 10792 (CP020754)
B. thuringiensis serovar berliner ATCC 10792 (CM000753)
B. thuringiensis Bt407 (CP003889)
B. thuringiensis serovar chinensis CT-43 (CP001907)
B. thuringiensis serovar thuringiensis T01001 (CM000748)
B. thuringiensis serovar thuringiensis IS5056 (CP004123)
B. thuringiensis MYBT18246 (CP015350)
B. thuringiensis YBT-1518 (CP005935)
B. thuringiensis Bt18247 (CP015250)
B. thuringiensis HS18-1 (CP012099)
B. thuringiensis HD-789 (CP003763)
B. thuringiensis HD1002 (CP009351)
B. thuringiensis serovar israelensis AM65-52 (CP013275)
B. thuringiensis HD12 (CP014847)
B. thuringiensis serovar morrisoni BGSC 4AA1 (CP010577)
B. thuringiensis L-7601 (CP020002)
B. thuringiensis serovar alesti BGSC 4C (CP015176)
B. thuringiensis HD-771 (CP003752)
B. thuringiensis serovar aizawai Hu4-2 (AMXT02000001)
B. thuringiensis serovar galleriae HD-29 (CP010089)
B. thuringiensis serovar amagiensis BGSC 4AE1 (NFCH00000000)
B. thuringiensis serovar kurstaki HD73 (CP004069)
B. thuringiensis serovar kurstaki T03a001 (CM000751)
B. thuringiensis serovar kurstaki YBT-1520 (CP007607)
B. thuringiensis serovar mexicanensis 27 Btm27 (JMJY01000001)
B. thuringiensis serovar coreanensis ST7 (CP016194)
B. thuringiensis YCd22-03 (CP019230)
B. thuringiensis serovar indiana HD521 (NZ_CP010106)
B. thuringiensis BM8171 (CP001903)
B. thuringiensis serovar tolworthi PISS (AP014864)
B. thuringiensis SCG04-02 (CP017577)
B. thuringiensis serovar pakistani T13001 (CM000750)
B. thuringiensis Bt185 (CP014282)
B. thuringiensis serovar huazhongensis BGSC 4BD1 (CM000756)
B. toyonensis BCT-7112 (CP006863)
B. cereus Rock1-3 (CM000728)
B. thuringiensis Bt-UNVM_94 (QGLX10000000)
B. thuringiensis MC28 (CP003687)
B. thuringiensis serovar monterrey BGSC 4AJ1 (CM000752)
B. thuringiensis serovar pondicheriensis BGSC 4BA1 (CM000755)
B. thuringiensis serovar konkukian 97-27 (NC_005957)
B. thuringiensis serovar pulsiensis BGSC 4CC1 (CM000757)
B. thuringiensis serovar finitimus YBT-020 (CP002508)
B. cereus Q1 (CP000227)
B. thuringiensis serovar tochigiensis BGSC 4Y1 (CM000746)
B. cereus E33L (CP000001)
B. cytotoxicus NVH 391-98 (CP000764)
B. pseudomycoides DSM 12442 (CM000745)
0.02

Figure Legends

Figure 1. Morphological characterization of spore-crystal mixtures from strain Bto-UNVM_94. A) Parasporal crystals were stained with Coomassie brilliant blue (1000 ×) (Ammons et al., 2002). B) Scanning electron microscopy (SEM) of sporulated strain Bto-UNVM_94 showing a group of bipyramidal parasporal crystals and spore. C) SDS-PAGE analysis of spore-crystal mixtures from Bto-UNVM_94 and reference strains. MW: molecular weight marker, lane 1: strain Bto-UNVM_94; lane 2: *B. thuringiensis* svar. *kurstaki* strain HD-1; lane 3: *B. thuringiensis* svar. *morrisoni* strain *tenebrionis* DSM2803 and lane 4: *B. thuringiensis* svar. *israelensis* strain HD-567. Black arrow marks Cry7Ga-like protein band.

Figure 2. Genetic relationship of Bto-UNVM_94 with other *B. cereus*, *B. thuringiensis* and *B. toyonensis* strains by using the Drewnowska and Swiecicka multigene approach (Drewnowska and Swiecicka, 2013) but including *gyrB* (DNA gyrase, subunit B). The tree was constructed using the Neighbor-Joining method implemented in Geneious R11 (Drummond et al., 2021) and based on the concatenation of eight housekeeping genes (*glpF*, *gmk*, *gyrB*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi*) totaling 11,718 nucleotides. A 1000 bootstrap resampling was used and consensus support values higher than 50% are shown at each node. A monophyletic group includes the Bto-UNVM_94 genome with other highly related strains (red shaded) namely, *B. toyonensis* BCT-7112, *B. cereus* Rock 1-3 and *B. thuringiensis* MC28. *B. cytotoxicus* was used as outgroup.

Supplementary Material

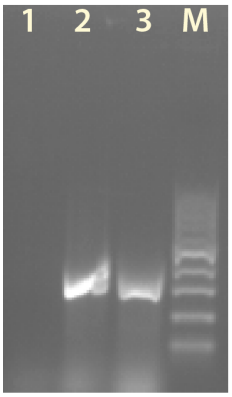
Supplementary Table S1.

	Number of emerged larvae		
Treatment	1	2	3
B. thuringiensis Bto-UNVM_94	10	10	10
B. thuringiensis svar. *thuringiensis* HD-2	0	0	0
Sterile distilled water	10	10	10

Supplementary Table legends.

Supplementary Table S1. Qualitative detection of *β*-exotoxin with *M. domestica* third instar larvae. Each strain was analyzed by triplicate. A strain is considered to produce *β*-exotoxin when over 80% of adults did not emerge. *B. toyonensis* Bto-UNVM_94 strain showed no changes in adults of *M. domestica* emergence when used in bioassays intended to detect *β*-exotoxin production.

Supplementary Figure S1



Supplementary Figure legends

Supplementary Figure S1. Agarose gel electrophoresis analysis on 1.0% agarose gel of DNA sequences amplified by PCR assay by using primers that targeted *thuE* genes (Sauka et al., 2014). Amplification product 406 bp. Lanes: 1, Bto-UNVM_94; 2, *B. thuringiensis* INTA H48-5; 3*, B. thuringiensis* svar. *thuringiensis* HD-2. M, molecular weight marker (100 bp Inbio Highway-Argentina).