1   ***Bacillus toyonensis* biovar Thuringiensis: A novel entomopathogen with insecticidal activity**

2   **against lepidopteran and coleopteran pests.**

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28   **Abstract**

29   The *Bacillus cereus* group includes eight species: *Bacillus anthracis*, *B. cereus*, *Bacillus*

30   *cytotoxicus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, *Bacillus*

31   *weihenstephanensis* and *Bacillus toyonensis*, which are highly related at the phylogenetic level. In

32   this work, we performed the isolation and characterization of a novel *Bacillus* sp. strain exhibiting

33   parasporal crystals with insecticidal activity that was initially classified as a *Bacillus thuringiensis*

34   strain. Its genome encoded three genes showing homology to known pesticidal proteins from *B.*

35   *thuringiensis* and *Lysinibacillus sphaericus* proteins: Cry7Ga1 (a crystal protein), a Mpp2Aa3

36   (ETX/Mtx2 family) homolog and a mosquitocidal-like protein (NPP1). However, since its genome

37   sequence shared >98% ANI with several *Bacillus toyonensis* genomes, the strain has subsequently

38   been renamed as *Bacillus toyonensis* biovar Thuringiensis and designated Bto-UNVM_94. Bioassays

39   demonstrated that this novel strain exhibited toxicity against *Cydia pomonella* (Lepidoptera:

40   Tortricidae) and *Anthonomus grandis* (Coleoptera: Curculionidae), a low toxicity against *Aedes*

41   *aegypti* (Diptera: Culicidae) and *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) whereas no

42   toxicity was shown to the free-living nematode *Panagrellus redivivus* (Rhabditida: Panagrolaimidae).

43   **Keywords:** *Bacillus cereus* group, entomopathogenic bacteria, δ-endotoxins, *Bacillus toyonensis*,

44   biovar Thuringiensis.

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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

73   for a *B. thuringiensis*-like phenotype (presence of parasporal crystals and insecticidal activity)

74   (Lazarte et al., 2018). Taking this into account, the *biovar* term was proposed within a new taxonomic

75   nomenclature in order to denote isolates with interesting phenotypic characteristics where briefly, the

76   first letter of the biovar name is capitalized and the name is not italicized (e.g. *B. toyonensis* biovar

77   Thuringiensis) (Carroll et al., 2020).

78   δ-endotoxins are the best characterized group of insecticidal proteins from *B. thuringiensis*,

79   synthesized during the sporulation phase and showing toxicity against a wide range of invertebrates

80   (Schnepf et al., 1998; van Frankenhuyzen, 2009; van Frankenhuyzen, 2013). Crystal proteins may be

81   classified into several distinct groups according to their homology and molecular structure

82   (Crickmore et al., 2020). The largest group is composed of three-domain Cry proteins followed by

83   Mpp (including Mpp2-formerly Mtx2) and Tpp (including Tpp1/Tpp2 formerly BinA/BinB) proteins

84   typically produced by *Lysinibacillus sphaericus* (Berry, 2012) and other structural families. Three-

85   domain Cry proteins show toxicity against insects in the orders Lepidoptera, Diptera, Coleoptera,

86   Hymenoptera but also against some nematodes (Palma et al., 2014; van Frankenhuyzen, 2009; van

87   Frankenhuyzen, 2013). These proteins exhibit two different sizes of ~70 and ~130 kDa, generating

88   smaller protease stable fragments upon proteolytic cleavage to produce their active forms (Jurat-

89   Fuentes and Crickmore, 2017; Palma et al., 2014). *L. sphaericus* Tpp1/Tpp2, Mpp and Mtx proteins

90   are toxic against some dipteran larvae (Berry, 2012) whereas Tpp and Mpp proteins from *B.*

91   *thuringiensis* may be toxic for coleopteran, dipteran and hemipteran species.

92   Since *B. thuringiensis*-based biopesticides were first commercialized in France around 1940 (de

93   Maagd, 2015), the identification of genes encoding novel insecticidal proteins spanning wider ranges

94   of insect orders has been continuously increasing (Hernández-Rodríguez et al., 2009; Palma et al.,

95   2013; Porcar and Juárez-Pérez, 2003). However, most of the *B. thuringiensis* strains and the

96   insecticidal proteins used are known to be highly active against lepidopteran pests (Dominguez-

97   Arrizabalaga et al., 2020). Strains showing toxic activity against coleopterans are limited and amongst

98   the proteins they may encode, some produce three-domain Cry7 proteins, which have been described

99 to be toxic against some coleopteran (e.g. Cry7A) or lepidopteran (e.g. Cry7B) larvae (van

100 Frankenhuyzen, 2009). Specifically, Cry7Aa was described to be toxic against species of *Cyla*

101 (Coleoptera: Brentidae) and *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) whereas

102 Cry7Ab was toxic against *Henosepilachna vigintiomaculata* (Coleoptera: Coccinellidae) and

103 *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae) (Dominguez-Arrizabalaga et al., 2020).

104   The finding of novel bacteria producing different insecticidal proteins opens the possibility of

105 discovering additional tools for controlling different insect pests but also, for delaying insect

106 resistance (Peralta and Palma, 2017). In this work, we report for the first time the genome sequence

107 and insecticidal characterization of a novel *Bacillus* strain proposed to be classified as *B. toyonensis*

108 biovar Thuringiensis and designated Bto-UNVM_94, which was isolated from Cululú (Santa Fe

109 province, Argentina) and showed toxicity against *Cydia pomonella* (Lepidoptera: Tortricidae) and

110 *Anthonomus grandis* (Coleoptera: Curculionidae).

111 **2.   Materials and methods**

112 **2.1. Strain isolation and characterization**

113   The soil samples were obtained with a tubular soil sampler from a native forest area at Cululú

114 (Santa Fe province, Argentina) as a composite of 5 random sub-samples and consisted in total of ~20

115 g of soil. After collection, the sample was stored at 4 ºC in zip-lock bags until axenic isolation was

116 performed. With this purpose, 3 g of the soil sample was homogenized into 10 ml of sterile distilled

117 water. The soil suspension was later mixed by 1 min. vortexing and incubated at 80 ºC for 30 min.

118 The sample was then subjected to five ten-fold dilutions and 50 µl from 10^{-3} to 10^{-5} dilutions were

119 plated onto nutrient agar plates (0.5% Peptone, 0.3% beef extract, 0.5% NaCl and 1.5% agar) using

120 a Drigalsky spatula. Inoculated plates were incubated at 28 ºC for 48-72 h. Bacterial colonies

121 exhibiting a *B. thuringiensis*-like phenotype (matt-white colonies with uneven borders) were then

122 sub-cultured for axenic isolation (purification) of the bacterium with a 5 µl inoculating loop and

123 incubated as described before, until sporulation was produced. Each sporulated culture was then heat

124 fixed onto a glass microscope slide and stained with a Coomassie blue solution (0,133 % Coomassie

125   Blue stain in 50 % acetic acid) (Ammons et al., 2002). The identification of *B. thuringiensis*-like

126   parasporal crystals was performed using a light microscope and later confirmed by Scanning Electron

127   Microscopy (SEM). The axenic sporulated colonies were stored at our bacterial collection in 15%

128   glycerol and −80ºC. The composition of parasporal crystals was determined by SDS-PAGE

129   following the procedure described by Pérez et al. (2017). *B. thuringiensis* svar. *kurstaki* HD-1, *B.*

130   *thuringiensis* svar. *morrisoni tenebrionis* DSM2803 and *B. thuringiensis* svar. *israelensis* HD-567

131   were used as reference strains **(**Perez et al., 2017**)**.

132   **2.2 DNA purification and sequencing**

133   Purified total DNA (including chromosome and plasmids) was obtained using the Wizard

134   genomic DNA purification kit (Promega), following the manufacturer's instructions for the isolation

135   of DNA from Gram-positive bacteria. Total DNA was electrophoresed in 1% agarose gels stained

136   with SYBR Safe (Thermo Fisher Scientific) and quantified using PICODROP (PICO 100 μl

137   Spectrophotometer). The purified DNA was then used to construct a pooled Illumina library and

138   sequenced at Stabvida (Portugal) by using high-throughput Illumina sequencing technology with a

139   genomic coverage of 1000× (Caballero et al., 2018).

140   **2.3 Genome assembly and analysis**

141   The raw Illumina reads obtained were first trimmed and assembled into contigs by using Geneious

142   R11 (www.geneious.com), with the *de novo* assembly tool and default parameters. The resultant

143   contigs were then analysed with BLAST (Altschul et al., 1990) using a customized non-redundant

144   insecticidal protein database (Caballero et al., 2018). Genome annotation was performed with the

145   NCBI Prokaryotic Genome Annotation Pipeline (2018 release) although it was also annotated with

146   RAST (Aziz et al., 2008). Multiple sequence alignments, phylogenetic trees and conserved domain

147   searches were performed using suitable tools included in Geneious R11 (Drummond et al., 2021).

148   Phylogenetic relationships among different *Bacillus* species were analysed using a modification of

149   the Drewnowska and Swiecicka (2013) method by including the *gyrB* (DNA gyrase, subunit B) gene

150 sequence into the concatenation along with seven more genes, namely: *glpF* (glycerol uptake

151 facilitator protein), *gmk* (putative guanylate kinase), *ilvD* (dihydroxy-acid dehydratase), *pta*

152 (phosphate acetyltransferase), *pur* (Phosphoribosyl aminoimidazole carboxamide formyltransferase),

153 *pycA* (pyruvate carboxylase), and *tpi* (triosephosphate isomerase). Concatenated gene sequences were

154 obtained using the *concatenate* tool included in Geneious R11 and accounted for 11,718 nucleotides.

155 Multiple sequence alignments of each single gene and concatenated-gene sequences were obtained

156 with Muscle (Edgar, 2004) and edited manually. The phylogenetic tree was constructed using the

157 Neighbor-Joining method (NJ) with 1,000 bootstrap replicates for determining branch quality.

158 Percentages of average Nucleotide Identity (ANI) among related genomes were calculated

159 using the Enveomics ANI calculator tool (http://enve-omics.ce.gatech.edu/ani/index) where

160 values among genomes of the same species are typically found to be above 95% (Varghese et al.,

161 2015).

162 This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under the

163 accession number QGLX00000000. The version described in this paper is the first version,

164 QGLX01000000. The sequences of the putative insecticidal proteins, namely Mpp, Cry7Ga-like and

165 the mosquitocidal-like protein have been submitted to GenBank under the accession numbers

166 OK001675, OK001676 and OK001677, respectively.

167 **2.4. Insect and nematode bioassays**

168 The insecticidal activity of Bto-UNVM_94 strain was qualitatively evaluated on four insect

169 species -second instars of *Alphitobius diaperinus* P. (Coleoptera: Tenebrionidae), early fourth instars

170 of *Aedes aegypti* (Diptera: Culicidae), neonates of *Anthonomus grandis* B. (Coleoptera:

171 Curculionidae) and *Cydia pomonella* L. (Lepidoptera: Tortricidae), plus the free-living nematode

172 *Panagrellus redivivus* L. (Rhabditida: Panagrolaimidae). Coleopteran and lepidopteran larvae as well

173 nematodes were obtained from colonies reared in IMYZA-INTA laboratories at 29 ºC on artificial

174 diets specific for each organism (Barrett and Butterworth, 1984; Perez et al., 2017). Eggs of *A. aegypti*

175 were provided by Dra. María Micieli (Center for Parasitological and Vector Studies, CEPAVE,

176   Argentina) and the obtained larvae reared at 28°C under a 14:10 h (light:dark) photoperiod in plastic

177   containers with dechlorinated water.

178   Powders of spore-crystal complexes were prepared as previously described (Sauka et al,

179   2010). One hundred µl of highly concentrated spore stock suspension of Bto-UNVM_94 strain was

180   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;

181   0.25 g $MgSO_4·7H_2O$; 0.1 g $MnSO_4·H_2O$; 2.5 g starch and 4 g yeast extract, in a total volume of 1 l of

182   distilled water, pH 7.2), at 340 rpm and 30°C, until complete autolysis was observed. Spore-crystal

183   complexes were obtained by centrifugation at 12,000 g and 4 ºC for 15 min, then pellets were freeze-

184   dried.

185   Each spore-crystal mixtures (final concentration of 5-1000 µg/ml) was later incorporated

186   into polypropylene conical tubes containing the corresponding artificial diet (maintained at 60ºC) for

187   *A. diaperinus*, *A. grandis* and *C. pomonella* and poured into each well of a 24-well plate (Nunc

188   143982) (Perez et al., 2017) or into plastic cups containing dechlorinated water for *A. aegypti* (Ibarra

189   et al., 2003). Twenty-four coleopteran and lepidopteran (two replicates) and 25 dipteran larvae (three

190   replicates) were used for each assay. .

191   For *P. redivivus*, approximately 4000 nematodes were exposed to a Bto-UNVM_94 culture

192   that had been grown to confluence on sporulation BM agar (BM broth supplemented with 2 g agar

193   per litre) plates (90 × 15 mm) at 29 ºC for 72 h before the assay.

194   Mortality was registered after 15 days at 29°C in *A. diaperinus* and *A. grandis*, five days at

195   29ºC in *C. pomonella*, and one day at 29ºC in *A. aegypti* and *P. redivivus* bioassays. Larvae were

196   considered dead if they failed to respond to gentle probing.

197   Distilled water without crystal-spore mixtures was added to the natural mortality controls.

198   Schneider-Orelli's formula was used in insect bioassays to calculate corrected mortality in comparison

199   to the untreated control. The InfoStat software (Universidad Nacional de Córdoba, version 2014) was

200   used for the statistical analysis, and the statistical significance was set at $p<0.05$.

201   **2.5. PCR-based prediction and detection of *β*-exotoxin production.**

202   In order to predict whether the Bto-UNVM_94 strain is able to produce type I *β*-exotoxin, a

203   qualitative PCR-based method for the detection of the *thuE* gene was carried out, as previously

204   described by Sauka et al. (2014). *B. thuringiensis* INTA H48-5 and *B. thuringiensis* svar.

205   *thuringiensis* HD-2 were used as positive controls. In addition, the *β*-exotoxin synthesis capability of

206   strain Bto-UNVM_94 was also screened by quantifying the number of emerged *Musca domestica*

207   (Diptera: Muscidae) adults after treatment in triplicate as described previously (Sauka et al., 2014).

208   *B. thuringiensis* svar. *thuringiensis* HD-2 was used as a positive control for *β*-exotoxin production

209   (Supplementary Table S1).

210   **3. Results**

211   **3.1 Strain isolation**

212   As described above, new bacterial isolates were prepared from soil samples. One of the

213   colonies identified isolated from Cululú soil sample from Santa Fe province, Argentina, exhibited the

214   typical *B. thuringiensis*-colony morphology on nutrient agar (matt white colour, flat, dry and with

215   uneven borders) and also showed the presence of Coomassie-blue stained parasporal crystals under

216   the light microscope, which were later confirmed as bipyramidal crystals by SEM examination and

217   SDS-PAGE analysis (Figure 1).

218   **3.2 Bioassays**

219   Mixed spore-crystal suspensions of the strain exhibited toxicity and showed 54.2% and

220   77.8% mortality (mean corrected mortality) against *C. pomonella* and *A. grandis*, respectively. Lower

221   % mortality were also detected for *A. aegypti* and *A. diaperinus* larvae whereas no mortality was

222   observed for the free-living nematode *P. redivivus* (Table 1).

223   **3.3 Sequencing and characterisation**

224   Genome sequencing produced >23 million Illumina raw reads that were trimmed and

225   assembled into 43 contigs totaling 6,136,970 bp, with a G+C content of 34,9% and containing 6,223

226   predicted protein-coding genes (CDSs) plus 86 RNAs, consistent within the range of other sequences

227   *Bacillus* sp. genomes (Makuwa and Serepa-Dlamini, 2019).

228   Phylogenetic analysis using eight concatenated housekeeping genes (*glpF*, *gmk*, *gyrB*, *ilvD*,

229   *pta*, *pur*, *pycA*, and *tpi*) showed that the strain forms a monophyletic group closely related to *B. cereus*

230   Rock1-3 (Acc. No. CM000728), type strain *B. toyonensis* BCT-7112 (Acc. No. CP006863) (Jimenez

231   et al., 2013) and to *B. thuringiensis* MC28 (Acc. No. CP003687) (Figure 2). This result was also

232   consistent with that obtained after we performed the phylogenetic analysis by using the method

233   proposed by Drewnowska and Swiecicka (2013), however, the inclusion of *gyrB* gene sequences

234   improved tree robustness by increasing bootstrap support values at several nodes of the phylogenetic

235   tree (data not shown).

236   In addition, the average nucleotide identity (%ANI) values of this strain were calculated and

237   compared with 49 additional genomic sequences including genomes from *B. thuringiensis* and *B.*

238   *cereus* plus eight genomes from different *B. toyonensis* strains that have successfully passed the

239   taxonomy check analysis from GenBank (Table 2). Strain Bto-UNVM_94 shared the highest % ANI

240   values (>95 %) with the eight *B. toyonensis* genomes but also with *B. cereus* Rock 1-3 and *B.*

241   *thuringiensis* MC28 genomes. These two last species have failed to pass the taxonomy check from

242   GenBank and were therefore assumed in this study, as being potentially mis-identified *B. toyonensis*

243   strains. As a result of the above analyses (and the fact that the morphology of the colony is also

244   consistent with the *B. toyonensis type* strain BCT-7112 phenotype (Jiménez et al., 2013), our strain

245   was designated as a *B. toyonensis* strain and named *B. toyonensis* biovar Thuringiensis Bto-

246   UNVM_94.

247   **3.4 Genes encoding putative pesticidal proteins**

248   Bto-UNVM_94 harbors one CDS (coding sequence) encoding a protein with 92% pairwise

249   identity with the crystal protein Cry7Ga1 plus another CDS encoding a protein exhibiting 97%

250   pairwise identity with an as-yet unnamed Mpp (ETX/Mtx2) family putative pore forming protein

251 from *B. thuringiensis* (GenBank Accession Number WP_065212007). This CDS also showed 42%

252 pairwise similarity with Beta pore-forming pesticidal protein Mpp2Aa3 using Best Match Finder at

253 the Bacterial Pesticidal Protein Resource Center database (Crickmore et al., 2020) and 53% pairwise

254 identity with sequence 101 from US Patent 10793610 (Acc. No. QPY81898). We also found a third

255 CDS showing 95% pairwise identity with a putative mosquitocidal protein, although no

256 mosquitocidal activity has been yet reported for this protein from *B. cereus* Rock 1-3 (GenBank Acc.

257 No. EEL19614), one of the strains that we suggest should be reassigned to *B. toyonensis.* This CDS

258 also showed 65% pairwise identity with sequence 94 from US patent 8461421 (Acc. No. AGP18048)

259 for which no insecticidal activity was described in the patent.

260   The >23 million Illumina reads were also filtered by using the *map to reference* tool from

261 Geneious R11 using the *B. toyonensis* BCT-7112 sequence as the reference genome. The tool was set

262 to separate unmapped reads for *de novo* assembly, interpreting the new contig sequences, as if they

263 came from plasmid DNA. Later, custom BLASTx searches were performed using the Cry7Ga-like

264 protein, the Mpp2 homolog protein and the mosquitocidal-like protein showing the location of such

265 encoded genes in these draft-assembled plasmid sequences (data not shown). These results strongly

266 suggest the presence of extrachromosomal DNA in the genomic sequence of the Bto-UNVM_94

267 strain.

268   In addition and consistent with the lack of teratological effects of strain Bto-UNVM_94 to

269 *M. domestica*, no *β*-exotoxin (thuringiensin) synthesis genes were also identified in the genomic DNA

270 and PCR amplification of the type I *β*-exotoxin *thuE* gene produced no amplification from this strain

271 (Supplementary material, Figure S1).

272 **4 Discussion**

273 **4.1 Pesticidal proteins**

274   A number of putative pesticidal proteins are encoded by Bto-UNVM_94. The Mpp family

275 sequence (Acc. no. OK001675) represents a protein of *ca* 33 kDa with a putative signal peptide

276 (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

303   could follow the same behaviour and be responsible of the low, but exhibited toxicity, against *A.*

304   *aegypti.* For this reason they are also expected to be non-evident in the SDS-PAGE gel analysis.

305   However, additional studies are needed to effectively test these hypotheses .

306   As mentioned previously, the insecticidal activity of Cry7 proteins has only been reported for a

307   few members of this family. For example, Cry7Aa1 was reported for its insecticidal activity against

308   coleopteran larvae of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), *Cylas brunneus* and

309   *Cylas puncticollis* (Coleoptera: Brentidae) whereas toxicity was lacking against coleopteran larvae of

310   *Diabrotica undecimpunctata*, *Anoplophora glabrupenius* and *A. grandis.* The Cry7Aa1 protein also

311   lacked toxicity against lepidopteran larvae of *Heliothis virescens*, *Spodoptera littoralis* and *Manduca*

312   *sexta* (Lambert et al 1992; Ekobu et al 2010). The Cry7Aa2 protein was recently reported to be toxic

313   for *L. decemlineata* (Domínguez-Arrizabalaga 2019). Cry7Ab3 was described to be active against the

314   spotted potato ladybeetle, *Henosepilachna vigintioctomaculata* (Coleoptera: Coccinellidae). In

315   contrast to the coleopteran activity reported for the above Cry7A variants, Cry7Ba showed activity

316   against the lepidopteran *Plutella xylostella* larvae (Lepidoptera: Plutellidae) whereas Cry7Ca1 has

317   been reported to be toxic for adults of *Locusta migratoria manilensis* (Orthoptera: Acrididae) (Song

318   et al., 2008; van Frankenhuyzen, 2009; Wu et al., 2011). To our knowledge, there is not currently any

319   information available concerning the insecticidal activity and the host range for Cry7G homologs but

320   our results suggests that the Cry7G-like protein described here is likely to be responsible for the

321   toxicity shown by spore-crystal preparations against both lepidopteran and coleopteran pests. This

322   would be the first indication of the insecticidal activity of a Cry7G protein, although confirmation of

323   this property will require separate cloning and quantitative testing of this protein in isolation since we

324   cannot rule out other factors from the strain in its activity (e.g. the Mpp homolog and the

325   mosquitocidal-like protein).

326   Our study is also the first demonstration of insecticidal activity in a *B. toyonensis* strain

327   (designated Bto-UNVM_94), a species that has been used for several years as probiotic supplement

328   in animal nutrition (Jiménez et al., 2013). This strain is also lacking the production of β-exotoxin,

329 which is also a requirement for *B. thuringiensis* formulations in Europe and other countries (e.g. US

330 and Canada) (Palma et al., 2014). We further speculate that some strains such as *B. cereus* Rock 1-3

331 and *B. thuringiensis* MC28 may be mis-classified *B. toyonensis* strains as genomic sequences have

332 often received taxonomic mis-classifications as a result of using mainly phenotypic characteristics in

333 assigning their identities (Federhen et al., 2016). Transfer of plasmids, on which insecticidal proteins

334 are encoded in *B. thuringiensis*, between *B. cereus* group strains is well known and the discovery of

335 toxin-coding plasmids in our strain is consistent with movement of these toxins within the group.

336   To date, *B. thuringiensis* remains as the most successfully exploited bacterium for the biological

337 control of insect pests in agriculture but based in our new data, other member of the *B. cereus* group

338 including *B. toyonensis* deserve to be explored to identify novel insecticidal activities.

339 **Acknowledgements**

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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
430
431
432

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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439   **Table Legends**

440

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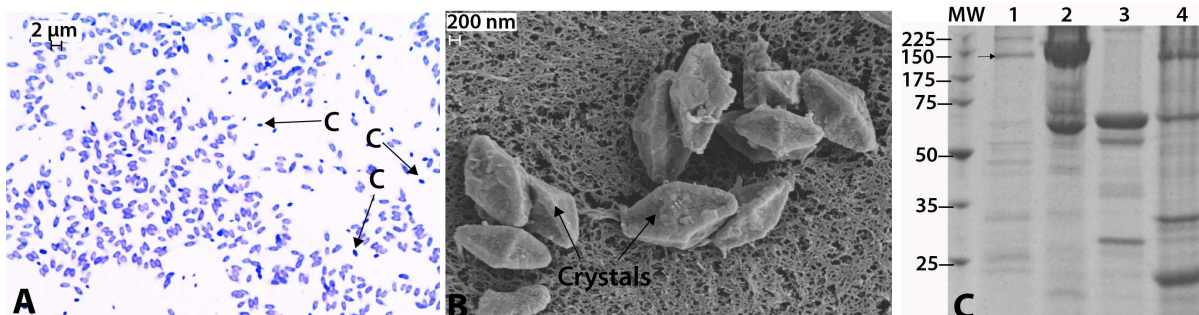
442   different invertebrate orders.

443   **Table 2.** %ANI of the four strains forming the monophyletic group including Bto-UNVM_94 strain

444   obtained by genome-genome comparisons.

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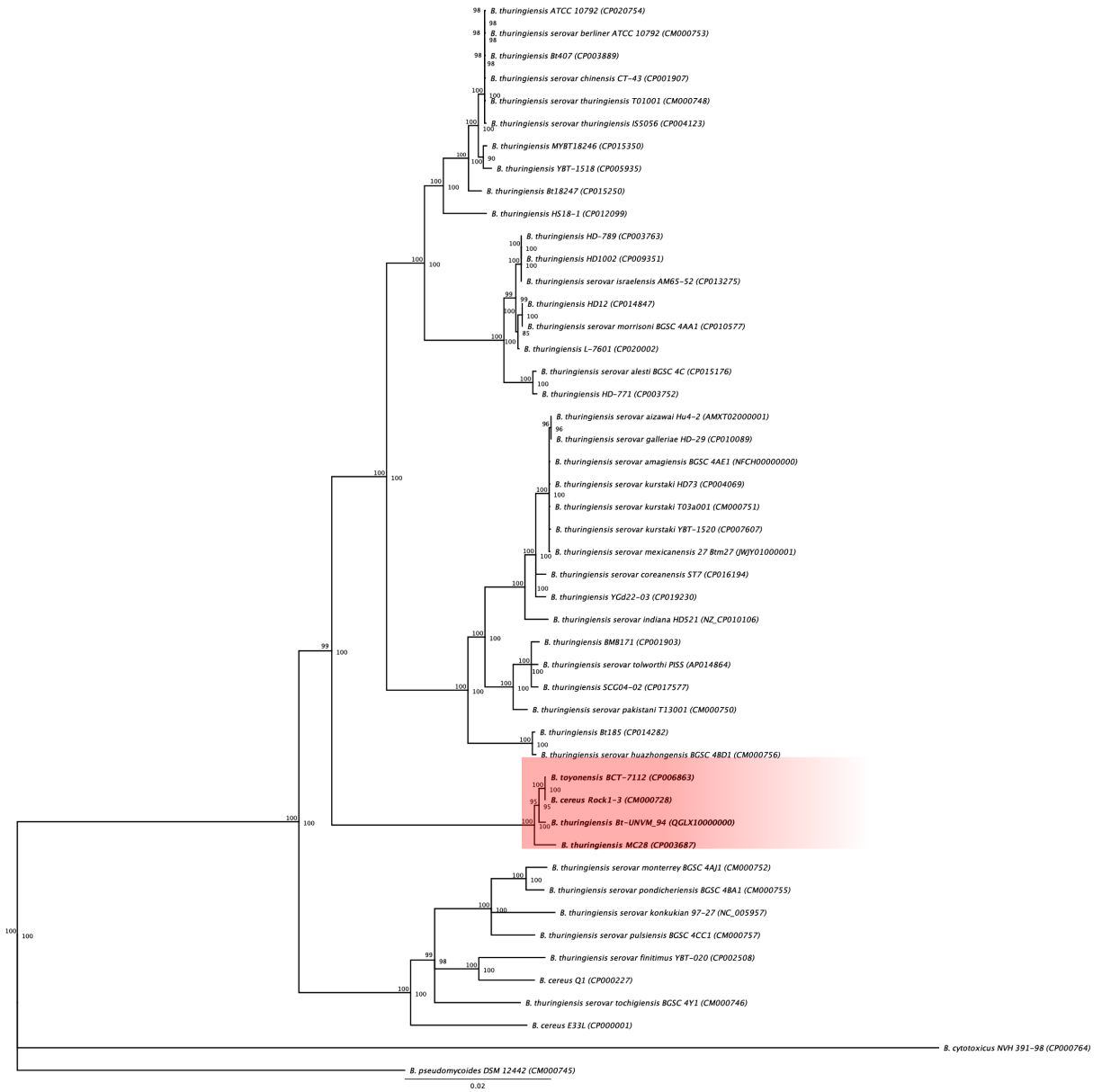447   **Figures**

448   **Figure 1**

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452   **Figure 2**

Figure Legends

461 **Figure 1.** Morphological characterization of spore-crystal mixtures from strain Bto-UNVM_94.

462 A) Parasporal crystals were stained with Coomassie brilliant blue (1000 ×) (Ammons et al.,

463 2002). B) Scanning electron microscopy (SEM) of sporulated strain Bto-UNVM_94 showing a

464 group of bipyramidal parasporal crystals and spore. C) SDS-PAGE analysis of spore-crystal

465 mixtures from Bto-UNVM_94 and reference strains. MW: molecular weight marker, lane 1: strain

466 Bto-UNVM_94; lane 2: *B. thuringiensis* svar. *kurstaki* strain HD-1; lane 3: *B. thuringiensis* svar.

467 *morrisoni* strain *tenebrionis* DSM2803 and lane 4: *B. thuringiensis* svar. *israelensis* strain HD-

468 567. Black arrow marks Cry7Ga-like protein band.

469 **Figure 2.** Genetic relationship of Bto-UNVM_94 with other *B. cereus*, *B. thuringiensis* and *B.*

470 *toyonensis* strains by using the Drewnowska and Swiecicka multigene approach (Drewnowska

471 and Swiecicka, 2013) but including *gyrB* (DNA gyrase, subunit B). The tree was constructed

472 using the Neighbor-Joining method implemented in Geneious R11 (Drummond et al., 2021)

473 and based on the concatenation of eight housekeeping genes (*glpF*, *gmk*, *gyrB*, *ilvD*, *pta*, *pur*,

474 *pycA*, and *tpi*) totaling 11,718 nucleotides. A 1000 bootstrap resampling was used and consensus

475 support values higher than 50% are shown at each node. A monophyletic group includes the Bto-

476 UNVM_94 genome with other highly related strains (red shaded) namely, *B. toyonensis* BCT-

477 7112, *B. cereus* Rock 1-3 and *B. thuringiensis* MC28. *B. cytotoxicus* was used as outgroup.

478 **Supplementary Material**
479
480 **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

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482

483   **Supplementary Table legends.**

484

485   **Supplementary Table S1.** Qualitative detection of *β*-exotoxin with *M. domestica* third instar larvae.

486   Each strain was analyzed by triplicate. A strain is considered to produce *β*-exotoxin when over 80% of

487   adults did not emerge. *B. toyonensis* Bto-UNVM_94 strain showed no changes in adults of *M. domestica*
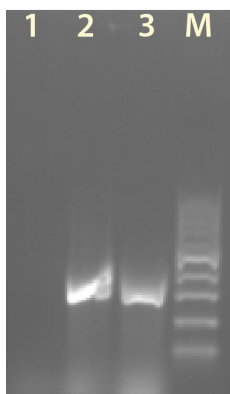
488   emergence when used in bioassays intended to detect *β*-exotoxin production.

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Supplementary Figure S1

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500 **Supplementary Figure legends**

501 **Supplementary Figure S1.** Agarose gel electrophoresis analysis on 1.0% agarose gel of DNA

502 sequences amplified by PCR assay by using primers that targeted *thuE* genes (Sauka et al., 2014).

503 Amplification product 406 bp. Lanes: 1, Bto-UNVM_94; 2, *B. thuringiensis* INTA H48-5; 3*, B.*

504 *thuringiensis* svar. *thuringiensis* HD-2. M, molecular weight marker (100 bp Inbio Highway-

505 Argentina).

506