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Toxicity of a *Bacillus thuringiensis israelensis*-like strain against *Spodoptera frugiperda*

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Abstract *Bacillus thuringiensis* (Bt) Berliner is a promising agent for microbial control of agriculturally and medically important insects. This study aimed at searching for Bt strains encoding Cry proteins that act more efficiently against fall armyworm. Thirty Bt strains were isolated from soil samples in Pernambuco State and evaluated through bioassays. Among these, strain I4A7 was the most efficient against the fall armyworm, *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae), and thus it was characterized by biochemical sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and molecular (polymerase chain reaction (PCR) and sequencing reaction) methods. The protein pattern of this strain on a SDS–PAGE was similar to that of *B. thuringiensis israelensis* (Bti). Moreover, I4A7 *cry* DNA sequence showed high identity (99–100%) to genes *cry4Aa*, *4Ba*, *10Aa*, *11Aa*, *cyt1Aa* and *cyt2B* from Bti. The toxicity of the newly isolated Bti-like strain upon *S. frugiperda* should be considered as this strain might be used in combination with other Bt strains, such as *B. thuringiensis* var. *kurstaki* (Btk).

Keywords *Bacillus thuringiensis* · Biochemical characterization · δ-Endotoxins · Fall armyworm · Lepidoptera · Noctuidae · Molecular characterization

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Introduction

Bacillus thuringiensis (Bt) is the most widely used biopesticide in the world. The toxic property of Bt is due to the production of crystalline proteins during its sporulation phase called δ-endotoxins. The ingestion of these toxins by susceptible insects causes starvation and septicemia, with subsequent death (Schnepf et al. 1998). A number of Bt strains have been isolated and, depending on the type of endotoxins they contain, have shown specific toxicity towards specific insect orders. Endotoxins comprise two multigene families: Cry

proteins, which act by attaching to specific receptors in the insect midgut, and Cyt proteins, which act non-specifically.

The majority of Cyt proteins that have been characterized are encoded by Bt strains specific against Diptera insects. The most common of these proteins is produced by *B. thuringiensis* var. *israelensis* (Bti). Bti has been widely explored commercially for the control of mosquitoes. The insecticide activity of this strain is derived from a complex interaction of four inclusion proteins: Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa. The finding that Cyt1A interacts synergistically with other Cry proteins pointed that it could suppress the onset of resistance to cry4A, Cry4B and Cry11A toxins in dipteran pests (Wirth et al. 1998).

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a destructive pest of maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.) and rice (*Oryza sativa* L.) throughout the western hemisphere (Sparks 1979). Chemical control is the main method of its control as *Spodoptera* species are weakly susceptible to most of the known endotoxins. In addition, literature on the susceptibility of *S. frugiperda* to Cry protein family is limited. Currently, only Cry proteins 1C, 1D and 1E (Uribe et al. 2003) encoded by the lepidopteran specific strains *B. thuringiensis* var. *kurstaki* (Btk) and *B. thuringiensis* var. *aizawai* are employed for its control. Thus, the continuous search for Bt strains containing crystal proteins toxic to *S. frugiperda* is undoubtedly important. The aim of this study was to search for Bt strains encoding Cry proteins that act more efficiently against *S. frugiperda*. As a result, a typical Bti-like strain encoding the proteins Cry4Aa, Cry4Ba, Cry11Aa, Cyt1Aa and Cyt2Aa was found to be toxic against *S. frugiperda*. To our knowledge this is the first report of a Bti-like strain toxic against *S. frugiperda*.

Material and methods

Bacillus thuringiensis strains

Thirty strains were used from a collection of entomopathogenic *Bacillus* isolates of CPqAM/FIOCRUZ and from Biotecnologica Industria e Comercio Ltda-Bioticom (Recife-PE, Brazil). These isolates were obtained from soil samples from different areas of Recife metropolitan region and stored as dry spores at 4°C. The standard strains used in bioassays were Btk EG7841 (recovered from Crymax®, Certis, USA), Btk HD73, 407$^+$ (both kindly supplied by Dr. O. Arantes, UEL, Londrina, PR, Brazil), and Bti IPS-82.

Insect rearing

The insects used in the bioassays came from the CNPA/Embrapa laboratory of entomology, where the mass culture of fall armyworm is performed. The insects were reared according to Parra (1986) on artificial diet based on beans, germ wheat and agar, in a controlled room under the following conditions: 26 ± 1°C, 70 ± 10% relative humidity and a light:dark period of 12:12 h.

Culture conditions and production of spore–crystal biomass

Firstly, for single-dose bioassay all the strains were grown in a medium specific for Bt sporulation, composed (for 1,000 mL) by 7.5 g of Bactopepton, 100 mL of 0.5 M KH_2PO_4, 10 mL of solution 1 (0.615 g of $MgSO_4{\cdot}7H_2O$, 0.009 g of $MnSO_4{\cdot}1H_2O$, 0.07 g of $ZnSO_4{\cdot}7H_2O$, final volume 50 mL), 10 mL of solution 2 (0.18 g of $FeSO_2{\cdot}7H_2O$, 0.15 mL of H_2SO_4, final volume 50 mL), 10 mL of solution 3 (0.735 g of $CaCl_2$, final volume 50 mL), glucose solution at a final concentration of 1% and 10 mg mL^{-1} of Penicillin G. The cultures were grown for 72 h at 28°C and 200 rpm in a rotating shaker. They were then centrifuged at 1,600 × *g* for 10 min and the supernatant was discarded. The pellet was washed two times and resuspended in sterile distilled water. The bacterial concentration was determined with a Neubauer chamber under an optical microscope.

Secondly, to produce spore–crystal biomass for dose-dependent assays the selected strain and standard control were lyophilized after being cultivated for 72 h in Bt sporulation medium at 28°C and 200 rpm. Cultures (800 mL) were centrifuged at 8,000 × *g* for 20 min, at 4°C. Cell pellets were washed with 1 M/10 mM NaCl/EDTA solution, then with 10 mM EDTA, frozen for 24 h, and lyophilized for at least 24 h (Lyophilizer, model Terroni LS 6000).

Bioassays

Two kinds of bioassays were carried out as described by Arango et al. (2002) and Monnerat et al. (2006)

with some modifications. First, a single-dose test was performed, where the main aim was to identify the Bt strains showing at least 50% toxicity against the fall armyworm. Following this, a dose-dependent assay was done to quantify the toxicity of the strains selected in the single-dose assay. About 50 µL of the culture of each isolate corresponding to 1×10^9 spores mL^{-1} (the approximate amount of crystal were calculated based on the ratio, one spore: one crystal) was added onto the artificial diet surface contained in 24-well polystyrene plates. One-first instar larvae of *S. frugiperda* was added per well, and mortality was recorded after 10 days of incubation under the same laboratory conditions of insects rearing cited above. A total of 72 larvae were tested per strain. Btk strains EG7841, HD-73, 407^+ and Bti IPS-82 were used as standard controls, and sterile distilled water was used as a negative control.

To carry out the dose-dependent assays, the lyophilized samples (280 g) were homogenized in 25 mL of sterile 0.01% Tween 20 solution. These homogenized were then diluted with sterile 0.01% Tween 20 solution to obtain a range of concentrations varying from 0.10 to 10 µg $µL^{-1}$. An aliquot of 40 µL of each dilution was placed on the surface of the solid diet in 24-well plates in three replicates. The standard strains Btk EG7841 and Bti IPS-82 were assayed with the same dilutions, and one bacteria-free control was used. The bioassay was carried out in a similar manner as described above and it was repeated three times to confirm the results.

Biochemical analysis by SDS–PAGE

The protein profile of the strain I4A7 was analyzed by (10% acrylamide) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). For protein extraction, 25 mg of culture powder were placed in a 2 mL tube with 250 µL of sterile distilled water, 0.5 µL of Triton X (final concentration 0.2%), and 250 µL of 0.1 M NaOH. The solution was kept at constant shaking for 1 h at 30°C and then centrifuged at 15,340 × *g*, 4°C, for 30 min. Further, the supernatant containing the proteins was quantified in a spectrophotometer (Amersham Pharmacia Biotech, model Ultrospec 2100 pro). An aliquot of 10 µg of the protein sample was electrophoresed at 25 mA and the gel was stained and fixed in 50% methanol, 10% acetic acid and 0.05% Comassie blue.

PCR *cry* and *cyt* genes analysis

The *cry* and *cyt* genes were firstly amplified from strain I4A7 by polymerase chain reaction (PCR) using the general primers that amplify any gene belonging to the following *cry* families: *cry1*, *cry2*, *cry5*, *cry6*, *cry7*, *cry10*, *cry11*, *cry12*, *cry13*, *cry14*, *cry15*, *cry16*, *cry17*, *cry18*, *cry19*, *cry 20*, *cry21*, *cry22*, *cry24*, *cry25*, *cry26*, *cry27*, *cry28*, *cyt1* and *cyt2* (Ben-Dov et al. 1997; Juaréz-Pérez et al. 1997; Guerchicoff et al. 1997; Porcar and Juaréz-Pérez 2003). In order to amplify particular *cry* genes, it was used specific primers for *cry1*, *cry3*, *cry4*, *cry8* and *cry9* (Ben-Dov et al. 1997; Juaréz-Pérez et al. 1997; Bravo et al. 1998; Masson et al. 1998; Porcar et al. 1999; Wasano et al. 2001; Porcar and Juaréz-Pérez 2003). For that, the strain I4A7 was cultivated in LB medium for 16 h, 30°C and 200 rpm and plasmid extraction was carried out according to Sambrook and Russel (2001). PCR was performed under the following conditions: 10 ng of plasmid DNA; 1.25 U of *Taq* DNA Polymerase (Invitrogen); 200 µM of dNTP; 0.5 µM of each primer, except the primer I (−), which was used at 1 µM concentration; 3 mM $MgCl_2$ and PCR buffer [50 mM KCl, 10 mM Tris–HCl (pH 8.3)] in a final volume of 25 µL. Amplification was performed with a Programmable Thermal Controller (Biometra, model T3000). The temperature conditions used were the same as described by the authors for each primer. An aliquot of 10 µL of the PCR amplicons were electrophoresed in 1% (w/v) agarose gel in Tris–borate buffer and stained with ethidium bromide for visualization.

DNA sequencing reaction and sequence analyses

For DNA sequencing, bands of expected size were extracted from the gel, purified by the GFX™ DNA and gel band purification kit (GE Healthcare) and subjected to sequencing reactions. Amplicons were sequenced in an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were compared to known genes deposited on GeneBank (www.ncbi.nlm.nih.gov/BLAST), using BLASTn (version 2.2.15) (Altschul et al. 1990).

Statistical analysis

The single-dose bioassays were analyzed by ANOVA and Tukey's means comparison analysis, using

ESTAT software (Unesp-Jaboticabal, Brazil). The LC_{50} (lethal concentration required to kill 50% of insects) was calculated by probit analysis (Finney 1971) using Polo-PC (LeOra Software).

Results

Bioassays

Among the 30 strains of Bt subjected to bioassays in this study, only two strains, I4A7 and I19A38, induced mortality above 30% on *S. frugiperda*, and the mortality of I4A7 do not differ from the Btk EG7841 by Tukey's analysis. Mortality means and Tukey's comparison analysis are shown in Table 1. The ANOVA test was significant for the mortality results of these strains ($F_{6,14} = 50.02$, $P < 0.01$).

The LC_{50} obtained in the dose-dependent assay for the I4A7 strain and the two standard strains Btk EG7841 and Bti IPS-82 are shown in Table 2. The standard Btk strain gave a LC_{50} of 0.69 μg cm^{-2}, which is 100 and 111 times lower than the LC_{50} of Bti IPS-82 (69.07 μg cm^{-2}) and I4A7 (76.58 μg cm^{-2}), respectively. The LC_{50} of Bti IPS-82 and I4A7 did not differ by probit analysis ($P < 0.05$).

Biochemical and molecular analyses

Three protein bands of approximately 130, 70 and 29 kDa were detected on SDS–PAGE from Bt strain I4A7 (Fig. 1). The protein profile and size of protein bands are similar to the proteins Cry4A and Cry4B (125–135 kDa), Cry11A (68 kDa) and Cyt1A and Cyt2B (29 kDa) of Bti (Delecluse et al. 2000). PCR products were seen when *cry* primers *cry4Aa*, *4Ba*, *10Aa*, *11Aa*, and *cyt* primers *cyt1Aa* and *cyt2Ba* were used to amplify I4A7 plasmid DNA. The approximate sizes of these amplicons are shown in Fig. 2. Bioinformatics analyses with the PCR amplicon sequence showed high identity of 99–100% with Bti *cry* and *cyt* genes from the database, accession numbers DQ174290 (*cry4Aa*), X07423 (*cry4Ba*),

Table 1 Toxicity of *B. thuringiensis* strains on first-instar of *S. frugiperda*

Strain	*cry* Gene content	Mortality[a] (%)
Btk EG7841[b]	*cry1C*, *cry2A*	100.00 ± 0.00 a
I4A7	*cry4A*, *cry4B*, *cry10A*, *cry11A*, *cyt1A*, *cyt2B*	94.33 ± 2.84 a
Bti IPS-82[b]	*cry4A*, *cry4B*, *cry10A*, *cry11A*, *cyt1A*, *cyt2B*	79.16 ± 2.40 ab
407^{+b}	*cry1Ab*	69.45 ± 5.01 b
I19A38	*cry4A*, *cry4B*, *cry10A*, *cry11A*, *cyt1A*, *cyt2B*	47.22 ± 9.11 c
Btk HD-73[b]	*cry1Ac*	41.67 ± 4.17 c
Negative control	–	11.11 ± 1.39 d

[a] Mean of three replicates ± SE; Mean values followed by the same letter do not differ by Tukey's comparison test ($P < 0.05$)

[b] Standard strains

Table 2 Lethal dose (μg cm^{-2}) of *B. thuringiensis* strains necessary to kill 50% (LC_{50}) of *S. frugiperda* first-instar larvae[a]

Strain	LC_{50} (μg cm^{-2})	CI_{95}[b] of LC_{50}
Btk EG7841	0.69 a	0.42–0.98
Bti IPS82	69.07 b	56.28–81.20
I4A7	76.58 b	57.62–92.00

[a] Values followed by the same letter do not differ among them by Probit analysis

[b] CI_{95}, 95% confidence interval

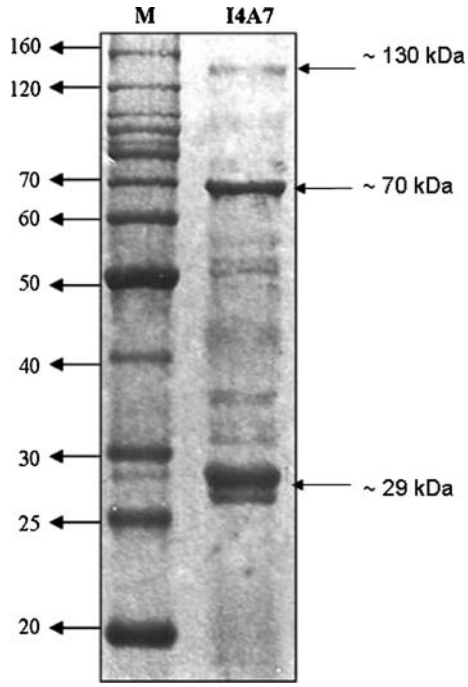

Fig. 1 SDS–PAGE of spore–crystal suspension of *B. thuringiensis* I4A7 strain. M, Molecular weight marker in kDa (Bench Mark™ Protein Ladder, Invitrogen)

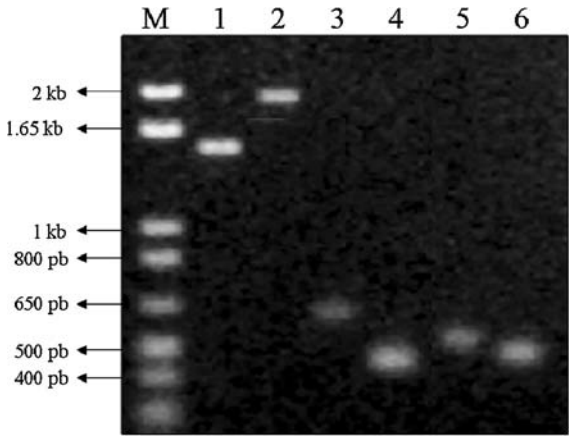

Fig. 2 PCR amplification products of I4A7 strain using cry and cyt primers. M, DNA Ladder plus Marker (Invitrogen); 1, *cry4Aa*; 2, *cry4Ba*; 3, *cry10Aa*; 4, *cry11Aa*; 5, *cyt1Aa*; 6, *cyt2Ba*

AL731825 (*cry10Aa*), AL731825 (*cry11Aa*), DQ302752 (*cyt1Aa*) and AF034926 (*cyt2Ba*).

Discussion

The Bt collection described in this work was systematically screened against the fall armyworm (*S. frugiperda*) larva. From the Bt strains bioassayed, only one Bti-like strain exhibited mortality above 50%. This is the first report about a Bti-like strain showing toxicity towards this lepidopteran species, and interestingly, our results showed that the I4A7 strain was more toxic to *S. frugiperda*, in the single-dose bioassays, than the known lepidopteran toxic strains Btk HD-73 and 407^{+} that harbors *cry1Ac* and *cry1Ab* genes, respectively (Table 1). According to the literature, the Bti serotype is applied in the control of dipteran insects, mainly in vectors of diseases, such as *Aedes aegypti* (Regis et al. 2000). This serotype is also a pathogen for Coleoptera and Lepidoptera species, besides its widely reported toxicity against mosquitoes and black flies (Ignoffo et al. 1981; Mendéz-López et al. 2003; Martins et al. 2007).

Although, the question that needs an answer is how a Bti-like strain could be toxic to a lepidopteran pest. Ignoffo et al. (1981) reported that Bti (spore + crystal suspension) was toxic against *Trichoplusia ni* (Lepidoptera: Noctuidae), *Heliothis zea* (Lepidoptera: Noctuidae) and *Heliothis virescens* (Lepidoptera: Noctuidae), and these species belong to same family of *S. frugiperda*. However, according to Puntambekar et al. (1997), Bti was not toxic to first-instar larvae of *S. litura*, a species belonging to the same genus of the fall armyworm. These differences in toxicity could be explained by the variation in toxin membrane receptors that are present in the midgut of these insects. Pigott and Ellar (2007) reported that toxin membrane receptors could vary between Lepidoptera species, even if these species are closely related. This evidence would explain the narrow specificity presented by Bt toxins, and by this way one or more Bti toxin could be specific for some midgut membrane receptor of *S. frugiperda* and not for other lepidopteran species.

About the Bti toxins, the first possibility that should be considered is the possible action of Cyt toxin against *S. frugiperda*. As reported by Butko et al. (1997) the Cyt toxins are very broad and not depend on specific receptors on target cells. In accordance with this fact, Sayyed et al. (2001) showed that Cyt1A toxin show high toxicity against *Plutella xylostella* (Lepidoptera: Plutellidae). Other report confirms the wide spectrum of action of Cyt toxin, as reported by Federici and Bauer (1998), the Cyt1A protein was toxic to the coleopteran pest *Chrysomela scripta* (Coleoptera: Chrysomelidae). Based on these reports it is not difficult to accept that Cyt toxins could affect *S. frugiperda*. Another point should be considered is the possibility of Cyt toxin could act as membrane receptor for other Bti Cry toxins, at least for Cry11A in *Aedes aegypti* (Diptera: Culicidae) brush border membrane vesicles, as reported by Perez et al. (2005).

Other hypothesis is the possibility of synergical activity of all Bti toxins on *S. frugiperda*. Crickmore et al. (1995) and Poncet et al. (1995) reported that the Bti toxins acting alone are less toxic than when they are assayed in any combination, however, the Bti wild-type crystal showed the high mortality in Diptera insects. Moreover these possibilities, other molecules apart from Cry and Cyt toxins, for instance chitinases could be involved with the biological process of *S. frugiperda* mortality (Barboza-Corona et al. 2003).

These hypotheses should be tested in a future work firstly verifying if Cry and/or Cyt toxins reported in this paper are really responsible for the mortality when tested against fall armyworm. If not, testing for other proteins, like chitinases, is a possible way to find out the protein responsible for this insect mortality. Despite that, the finding that *S. frugiperda* is susceptible to this Bti-like strain opens up new perspectives,

since this strain could possibly be used to suppress future cases of resistance to other Bt strains in this pest.

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