World J Microbiol Biotechnol (2011) 27:2343–2349
DOI 10.1007/s11274-011-0701-y

ORIGINAL PAPER

# Biological characterization of two *Bacillus thuringiensis* strains toxic against *Spodoptera frugiperda*

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Received: 3 December 2010 / Accepted: 18 February 2011 / Published online: 2 March 2011


**Abstract** Two *Bacillus thuringiensis* strains isolated from diseased *Spodoptera frugiperda* larvae collected in the northwest of Argentina were molecularly and phenotypically characterized. Insecticidal activity against *Spodoptera frugiperda* larvae was also determined. Both strains were highly toxic against first instar larvae. One strain (*Bacillus thuringiensis* LSM) was found to be even more toxic than the reference strain *Bacillus thuringiensis* var. *kurstaki* 4D1. This strong biological effect was represented by both a higher mortality which reached 90%, and a shorter $LT_{50}$. Molecular characterization showed that *Bacillus thuringiensis* LSM carried a *cry* gene profile identical to that of *Bacillus thuringiensis* var. *kurstaki* 4D1. Evaluation of length polymorphism of the intergenic transcribed spacers between the 16S and 23S rDNA genes revealed an identical pattern between native strains and *Bacillus thuringiensis* var. *kurstaki* 4D1. In contrast, phenotypic characterization allowed differentiation among the isolates by means of their extracellular esterase profiles. Lytic activity that would contribute to *Bacillus thuringiensis* effectiveness was also studied in both strains. Analyses like those presented in the current study are essential to identify the most toxic strains and to allow the exploitation of local biodiversity for its application in biological control programmes.

**Keywords** *Bacillus thuringiensis* · Phenotypic properties · *Spodoptera frugiperda* · Biological control

## Introduction

*Bacillus thuringiensis* (*Bt*) is a spore-forming bacterium well-known for its insecticidal properties associated with its ability to produce crystal inclusions during sporulation. These inclusions are proteins encoded by *cry* genes and have shown to be toxic to a variety of insects and other organisms like nematodes and protozoa (Konecka et al. 2007). The primary action of Cry proteins is to lyse midgut epithelial cells through insertion into the target membrane and form pores (Bravo et al. 2007). Once ingested, crystals are solubilized in the alkaline environment of midgut lumen and activated by host proteases (Brar et al. 2007). The activity spectrum of *Bt* toxins continually increases as the result of the ongoing isolation of new strains around the world.

The fall armyworm, *Spodoptera frugiperda* (*S. frugiperda*) (Lepidoptera: Noctuidae) is an agricultural pest that causes severe damage to a variety of crops like corn, rice, peanuts, cotton, soybeans, alfalfa and forage grasses, among others (Virla et al. 2008). Currently, control of this pest relies on chemical insecticides. Nevertheless, the rapid increase in resistance to insecticides together with the potential adverse environmental effects produced by these chemicals have encouraged the development of alternative methods for Lepidoptera control (Berón and Salerno 2006; Gomes Monnerat et al. 2007). Among these methods the

**Electronic supplementary material** The online version of this article (doi:10.1007/s11274-011-0701-y) contains supplementary material, which is available to authorized users.

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use of *Bt* as a biocontrol agent has shown to be extremely valuable. The diversity of Cry toxins produced by *Bt* allows the formulation of a variety of bioinsecticides by using the bacteria themselves or by expressing their toxin genes in transgenic plants. To date, many plant species have been genetically modified with *cry* genes, resulting in transgenic plants with a high level of resistance to insect pests (Christou et al. 2006; Gassmann et al. 2009). However, it has been reported that several pests have developed resistance against Cry proteins (Sauka and Benintende 2008; Tabashnik et al. 2008; Gassmann et al. 2009). The current approach used to delay evolution of resistance to transgenic crops uses a "high dose" (ensuring that the toxins are expressed at a level that is likely to kill resistant heterozygotes) and "refuge" strategy (non-transgenic crops maintained to preserve susceptible genes within treated insect populations) (Ffrench-Constant et al. 2004; Gassmann et al. 2009; Tabashnik and Carrière 2009). In addition, it is important to use a combination of *cry* genes and/or other genes encoding insecticidal proteins within the same transgenic crop (Christou et al. 2006; Sauka and Benintende 2008). Due to extensive use of transgenic crops in developing countries (ca. 42 million ha, James 2007) based on *cry*-type genes, there is a need for alternative *cry* gene sequences to meet the challenge of insect resistance (Berón and Salerno 2006). Crucial to this development is the identification of novel and more active strains with respect to insect pests of economically important crops.

The *cry* genes of *Bt* strains are known to be related to their toxicity (Carozzi et al. 1991; Padidam 1992) and identification of these genes by means of PCR has been used to predict insecticidal activity of the strains (Ben-Dov et al. 1997; Hansen et al. 1998). Nevertheless, a more complete characterization of *Bt* strains should include alternative PCR fingerprinting methods. Among them, assessment of length polymorphism of intergenic transcribed spacers (ITS) between the 16S and 23S rDNA genes has been shown to be an important tool for differentiating bacterial species and even prokaryotic strains (Daffonchio et al. 2006). Alternative sources of evidence for typing and comparative studies are the phenotypic characteristics (Konecka et al. 2007). In addition, these studies allow a better understanding of effectiveness of *Bt*, such as detection of lytic activity.

During a screening programme of *Bt* strains native to Argentina and toxic against Lepidoptera, several isolates were characterized according to different biological parameters (Alvarez et al. 2009a, b). Two of these strains showed promising results regarding toxicity against *S. frugiperda* in preliminary analyses. The present paper molecularly and phenotypically characterizes these strains. In addition, their toxicity against *S. frugiperda* is assessed with a larger scale assay.

## Materials and methods

### Microorganisms

*Bacillus thuringiensis* var. *kurstaki* 4D1 (*Bt* 4D1) was used as reference strain and provided by the Bacillus Genetic Stock Center (BGSC), Columbus, Ohio, USA. This strain was chosen because it is currently the most widely used microorganism to control lepidopteran pests (Arango et al. 2002).

*Bt* LSM and *Bt* LQ strains were isolated from *S. frugiperda* larvae showing disease symptoms (Alvarez et al. 2009a). Briefly, larval sample suspensions were made in distilled water, heated at 80°C for 15 min and then plated onto Luria–Bertani (LB) agar. Several colonies were then analysed for the presence of parasporal crystals by microscopic examination (Sharif and Alaeddinoğlu 1988). Among others, *Bt* LSM and *Bt* LQ presented parasporal crystals and they were selected for further studies. Previously, taxonomic identity of both strains had been confirmed by amplification and partial sequencing of their 16S rDNA genes (Alvarez et al. 2009a).

### Phenotypic analysis

*Bt* LSM and *Bt* LQ were characterized by conventional microbiological methods, like Gram staining, shape and position of spores, colony morphology and cell motility. The following characteristics were also studied: catalase production, nitrate reduction, gas production in glucose, degradation of 2% starch, 2% casein and 12% gelatine (wt $vol^{-1}$), acetyl methyl carbinol production, growth on 2% (wt $vol^{-1}$) LB agar at 30, 37, 45 and 50°C, NaCl requirement (2, 3, 5, 7 and 10% wt $vol^{-1}$) and growth on 2% (wt $vol^{-1}$) LB agar at different pH (4, 7 and 9).

Growth on 0.2% chitin (wt $vol^{-1}$) and chitin hydrolysis were determined using colloidal chitin as the sole carbon source according to Kaur et al. (2005). This protocol was also used to assay hydrolysis of 0.5% (wt $vol^{-1}$) carboxymethylcellulose (CMC). Sensitivity to antibiotics was determined by using the routine diffusion plate technique testing the following antibiotics: penicillin, oxacillin, erythromycin, trimethoprim, vancomycin, levofloxacin, minocycline, chloramphenicol, teicoplanin, clindamycin, gentamicin and rifampicin.

### Esterase profile

*Bt* strains were processed according to Gonzalez et al. (1996). Briefly, strains were cultured on LB plates during 48 h at 30°C and crude extracts were recovered from solid media. Then, extracts were separated by native-PAGE using a 10% (wt $vol^{-1}$) polyacrylamide gel. Esterase

activity was assayed using 1.3 mM of α-naphthyl acetate (C2) derivative as substrate. Released naphthol was bound with 1 mM Fast Blue to give a coloured product. Reactions were carried out at 37°C in shaken plates containing 100 mM phosphate buffer (pH 7). Known electrophoretic esterase profiles of *Bacillus pumilus* A55 (*Bp* A55) (Loto 2006 unpublished work) were used for comparative analysis.

### DNA preparation and PCR amplification

Strains were characterized in terms of presence of *cry1* and *cry2* genes by amplification with general primers (Bravo et al. 1998; Ibarra et al. 2003). Additional PCR was carried out with specific primers in order to identify the presence of *cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry2Aa, cry2Ab* and *cry2Ac* genes (Ben-Dov et al. 1997; Bravo et al. 1998). ITS-PCR was performed as previously described by Daffonchio et al. (1998).

Total DNA was extracted from cells harvested in the mid-exponential growth phase as described by Miller (1972). PCR amplification was performed in a 25-µl reaction mixture containing 2.5 µl 10 × STR reaction buffer (Promega), 20 ng of total DNA, 0.5 µM of each primer and 1 U of Taq DNA polymerase (Promega). *cry*-PCR products were analysed by electrophoresis in 2% (wt $vol^{-1}$) agarose gel and ITS-PCR products by native PAGE (6% wt $vol^{-1}$). Gels were stained with ethidium bromide. PCR fragments were run and compared with reference strain *Bt* 4D1.

### Bioassays

All experiments were conducted in a climate-controlled room at 25°C ± 0.5 with a photoperiod of 14/10 (light/dark) and 70% ± 15 relative humidity. Data were recorded hourly by a data logger. Insecticidal activity was tested against *S. frugiperda* grown on an artificial diet previously immersed in a spore-crystal suspension. The artificial diet contained the following constituents (g $l^{-1}$): bean flour, 150; wheat germ, 35; beer-brewing yeast, 30; ascorbic acid, 6; sorbic acid, 1.6; nipagin, 2; agar, 22 and 2 ml formaldehyde. Spore-crystal suspensions were prepared by suspending a five-day culture ($OD_{610} = 2$, ca. $6 \times 10^7$ c.f.u. $ml^{-1}$) and grown on LB agar at 30°C in sterilized water. Ten replicates of 10 neonate larvae were held in separate 2-ml Eppendorf vials. Each larva was supplied with a piece of artificial diet (approximately 0.25 $cm^2$) soaked with 100 µl of a *Bt* spore-crystal suspension. Then, the diet was left to dry for 30 min under sterile conditions before the bioassay was started. All *Bt* strains contained a similar number of crystals per field (100×). Control groups were fed with a similar piece of artificial diet but soaked with 100 µl of sterile distilled water. Mortality was scored every 24 h over 7 days.

Survival was analysed using ANOVA [Tukey post-test ($P < 0.05$)]. 50% lethal times ($LT_{50}$) were determined with Probit analysis. Data were analysed using deviance and Pearson's chi-square goodness-of-fit tests.

### Electron microscopy

Concentrated spore-crystal suspensions of native strains were washed with absolute ethanol/distilled water (1:1, vol $vol^{-1}$). Suspensions were placed on a microscope lid and air-dried overnight for scanning electron microscopy (SEM). Then, samples were coated with gold and examined using a Jeol scanning microscope.

## Results

### Phenotypic characterization

Two *Bt* strains had previously been isolated from diseased *S. frugiperda* larvae collected from maize (*Bt* LSM) and sorghum (*Bt* LQ) fields in the northwest of Argentina (Alvarez et al. 2009a). The microorganisms showed bipyramidal crystals and possessed typical colonial morphologies, as well as physiological, biochemical and nutritional features that resembled *Bacillus* spp. Bacteria were motile and produced ellipsoidal endospores, located at the subterminal position in the sporangia, and formed cream-coloured colonies with irregular edges on LB agar. From a biochemical point of view, *Bt* strains were catalase-positive, reduced nitrate, produced gas in glucose and acetyl methyl carbinol in Voges-Proskauer broth. Growth was observed at pH 7 and 9 on LB agar supplemented with 2, 3 and 5% NaCl, on LB agar at 30, 37 and 45°C and on minimum culture medium supplemented with chitin. Bacteria also hydrolysed casein, chitin and starch and were motile on soft LB agar. Negative results were obtained in the following tests: no growth was observed on LB agar at pH 4 or at 50°C and neither of the strains hydrolysed CMC and urea.

Antibiotic sensitivity tests revealed a resistance profile against gentamicin, penicillin, oxacillin, trimethoprim and a sensitive profile against clindamycin, rifampicin, erythromycin, vancomycin, levofloxacin, minocycline, chloramphenicol and teicoplanin.

Most of the morphological, biochemical and physiological features agreed with reference strain *Bt* 4D1. Nevertheless, *Bt* LSM and *Bt* LQ differed from *Bt* 4D1 regarding resistance against gentamicin and the lack of hydrolytic activity towards CMC.

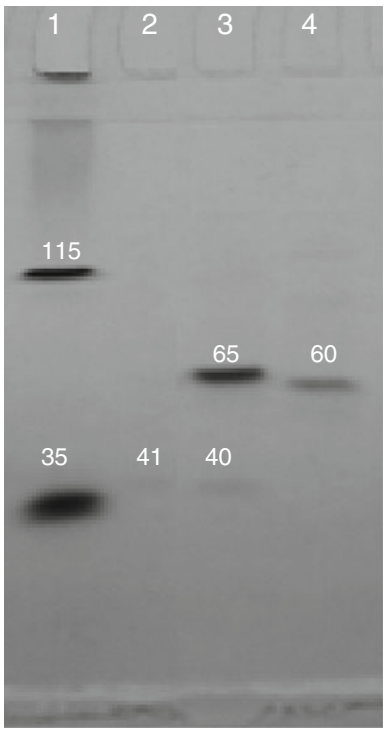


**Fig. 1** Enzymatic profile of esterase activity in native PAGE. Lanes 1 *B. pumilus* A55; 2 *Bt* LQ; 3 *Bt* 4D1; 4 *Bt* LSM. Molecular weight of each band/enzyme is showed in kDa

Electrophoretic profiles of esterase activity showed differences among strains (Fig. 1). *Bt* LSM and *Bt* LQ presented a unique band/enzyme of about 60 and 41 kDa, respectively.

### PCR *cry* gene and ITS characterization

PCR analysis showed the presence of *cry1* and *cry2* genes in *Bt* LSM but not in *Bt* LQ under the current reaction conditions (Online Resource 1). *Bt* LSM was characterized by additional specific PCR and compared with *Bt* 4D1. Both *Bt* LSM and the reference strain showed *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa* and *cry2Ab* genes (Online Resources 2,3,4,5 and 6).

Evaluation of ITS length polymorphism revealed an identical pattern among the native strains and also with *Bt* 4D1 (Fig. 2).

### Insecticidal activity

The *Bt* strains analysed produced 90% (*Bt* LSM) and 73% (*Bt* LQ) mortality against *S. frugiperda* neonate larvae (Table 1), compared with 86% mortality produced by *Bt* 4D1. According to the 50% lethal time and the fiducial limit, the spore-crystal suspension of *Bt* LSM killed the larvae faster than the reference strain (Table 1).

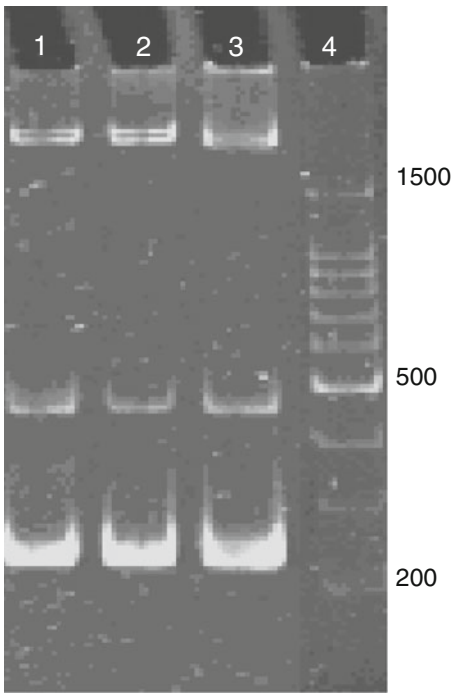


**Fig. 2** ITS-PCR of *B. thuringiensis* strains. Lanes 1 LSM; 2 LQ; 3 4D1; 4 100 bp DNA Ladder

**Table 1** Comparison of mortality and 50% lethal time ($LT_{50}$) of first instar larvae of *S. frugiperda* among native and reference *B. thuringiensis* (*Bt*) strains

| *Bt* strain | Mortality (%) ± SD* | $LT_{50}$ (h) (95% fiducial limits) |
|---|---|---|
| *Bt* LSM | 90.0 ± 7.3b | 37.7 (27.8–46.2) |
| *Bt* 4D1 | 86.0 ± 15.1b | 58.7 (50.4–66.0) |
| *Bt* LQ | 73.0 ± 5.7c | 79.6 (68.2–90.7) |
| Control | 1.0 ± 3.1a | |

* Values followed by different letters (a, b, c) were significantly different ($P < 0.05$, Tukey post-test)

### Microscopic examination of crystal proteins

Scanning electron microscopy allowed observation of bipyramidal crystal proteins in *Bt* LSM and *Bt* LQ strains (Fig. 3a, b).

## Discussion

Lepidoptera cause some of the most devastating insect damage to economically important crops. In this context it is important to look for novel or more potent insecticidal *Bt* strains useful in biological control. Phenotypic characterization of selected strains allows identification of properties that are relevant at the moment of selecting bacteria for their use in environmental and agricultural microbiology.

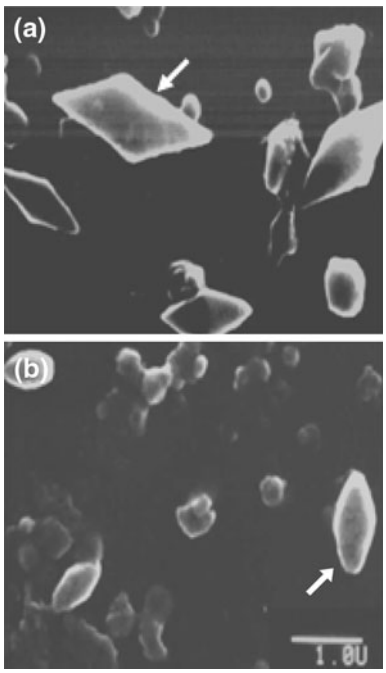


**Fig. 3** Scanning electron micrographs of spore-crystal complexes of native strains: *B. thuringiensis* LSM (**a**) and *B. thuringiensis* LQ (**b**). *Arrows* indicate bipyramidal crystal proteins. *Scale bar* 1 μm

Synthesis of lytic enzymes by *Bacillus* species during the early sporulation phase is one of these properties, since materials applied in formulations with *Bt* could be adversely affected by this activity. Our results indicate that the native isolates assayed showed no cellulolytic activity in medium supplemented with carboxymethylcellulose (CMC), one of the products used as a matrix to protect *Bt* spores against high temperatures and U.V. exposure prevailing in natural environments (Cokmus and Elcin 1995). This lack of cellulolytic activity is a desirable property given that gelled CMC will not be degraded at the time of *Bt* formulation, and therefore it can be employed for this purpose.

Protein profiles are a useful tool to discriminate among strains, as they provide information about the proximity between species, subspecies and biovars (Alvarez et al. 2009a; Berber 2004). Considering this, characterization of microorganisms by means of their extracellular isoenzymes showing high polymorphism, as is the case of esterases, is particularly appealing. Our results are in accordance with those by Norris (1964), since it was possible to differentiate *Bt* strains by comparing the electrophoretic migration profiles of esterases produced during the vegetative growth phase (Fig. 1). Another tool to detect differences between species and even between strains is evaluation of the ITS genomic region (Gürtler and Stanisich 1996). However, the length of the amplified ITS exhibited no polymorphism among the strains (Fig. 2). In connection with this, Reyes-Ramirez and Ibarra (2005) studied ITS profiles of 31 *Bt* strains and found them to be insufficient to discriminate between isolates.

The similarity between native isolates and *Bt* 4D1 regarding biochemical and phenotypical characteristics was also found for toxicity against *S. frugiperda.* Moreover, *Bt* LSM was found to be even more toxic than reference strain *Bt* 4D1 (Table 1), which was selected for this analysis given it is the most widely used microorganism to control lepidopteran pests (Arango et al. 2002). The higher toxicity of *Bt* LSM was represented by both a shorter $LT_{50}$ and a higher mortality which reached 90% (Table 1). Although *S. frugiperda* was believed to be a pest with low sensitivity to *Bt* toxins (Del Rincón-Castro et al. 2006), we recently found a strain that killed 100% of *S. frugiperda* neonate larvae (Alvarez et al. 2009b). The present study is in agreement with these previous results showing that *Bt* native to Argentina could possibly be employed in biological control of lepidopteran pests (Alvarez et al. 2009a, b). It is important to stress that the high level of mortality in the present work was obtained with a concentration of a spore-crystal suspension that was lower than some commercial *Bt* formulations; while our crystal spore suspensions presented a dose of $10^7$ c.f.u. $ml^{-1}$, *Bt* kurstaki preparations generally present a dose of $10^9$ c.f.u. $ml^{-1}$ (Seligy and Rancourt 1999).

*Cry* genes are a family of genes associated with the toxicity of *Bt* against insects. While *cry1* encodes for proteins forming bipyramidal crystals and are related to toxicity to Lepidoptera (Bravo et al. 1998), *cry2* encodes for cuboidal proteins, toxic to Lepidoptera and Diptera (Al-Momani et al. 2002). Electron microscopy analysis allowed identification of the bipyramidal proteins in both strains (Fig. 3a, b). Although *cry2* was amplified with general and specific primers in *Bt* LSM (Online Resource 1) no cuboidal proteins were identified (Fig. 3a). This suggests that a modification in the regulation of the gene would be responsible for the lack of protein product of this gene. Although the experimental growth conditions employed in this study could also explain the lack of cuboidal proteins, the production of these proteins by another *Bt* strain under identical experimental conditions argue against this possibility (Alvarez et al. 2009b). Cloning and sequencing the putative toxins with surrogate production made help clarify this issue as well as to confirm toxicity. In addition, *Bt* LQ showed no amplification products of *cry1* in several attempts (Online Resource 1), despite the presence of bipyramidal crystals (Fig. 3b). Noguera and Ibarra (2010) found that *cry* genes of a *Bt* strain isolated in Argentina that showed elongated bipyramidal crystals (Benintende et al. 1999) presented 98% identity with *cry5Ba* genes. Therefore, *Bt* LQ may have Cry proteins other than Cry 1 that form bipyramidal crystals.

We characterized two *Bt* strains which were highly pathogenic against *S. frugiperda*, an important agricultural pest found world-wide. The future application of these strains in biological control programmes requires optimization of the production conditions of the microorganisms using low-cost substrates. In this context characterization of phenotypic and biochemical properties as evaluated in this study is highly relevant.

**Acknowledgments** The authors would like to thank the following people: Dr Dan Zeigler for providing *Bacillus thuringiensis*, Dr Santiago A Catalano and Flavia Loto for helpful comments on the manuscript. The present work was supported by PIP 6062-Consejo Nacional de Investigaciones Científicas y Técnicas and PICTO-Universidad Nacional de Tucumán 761 grants.

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