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ORIGINAL RESEARCH PAPER

# Insecticidal crystal proteins from native *Bacillus thuringiensis*: numerical analysis and biological activity against *Spodoptera frugiperda*

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**Abstract** Fourteen strains of *Bacillus thuringiensis* collected from both larvae showing disease symptoms and soil samples in northwest Argentina were characterized by insecticidal activity against *Spodoptera frugiperda.* First instar larvae and protein profile SDS-PAGE analysis of whole cell proteins not only allowed the differentiation of native *Bacillus thuringiensis* but also revealed the possibility of applying protein profile analysis in classification of toxicity patterns. Cluster analysis showed that there were two main groups. Interestingly, one of them only contained the most pathogenic native strains. The biomass-bound protease activity of native pathogenic isolates and the reference strain *Bt* 4D1 is also reported.

**Keywords** *Bacillus thuringiensis* · Biological activity · Biopesticide · Crystal toxin · Numerical analysis · *Spodoptera frugiperda*

## Introduction

Insecticidal crystal proteins from *Bacillus thuringiensis* (*Bt*) are a group of proteins with activity against insects of different orders such as Lepidoptera, Coleoptera, Diptera and nematodes. These pesticidal delta-endotoxins are produced during sporulation and are accumulated both as an inclusion and as part of the spore coat. Their primary action is to lyse midgut epithelial cells by inserting into the target membrane and forming pores (Bravo et al. 2007). Once ingested, crystals are solubilized in the alkaline and reducing environment of the midgut lumen and are activated by host proteases. On the other hand, the involvement of *Bt* proteases in processing inactive protoxins is also reported (Brar et al. 2007). These toxins are also highly specific and completely biodegradable, so no toxic products are accumulated in the environment. In fact, Calderón et al. (2007) suggest the potential use of some crystal proteins as adjuvants for the administration of heterologous antigens.

The fall armyworm, *Spodoptera frugiperda* (*Sf*) (Lepidoptera: Noctuidae), is an agricultural pest that causes damage to corn, rice, peanuts, cotton, soybeans, alfalfa and forage grasses. Currently, control relies on both transgenic plants and chemical insecticides. However, with the development of resistance and increasing concerns about human health and environmental residues, the utilization of entomopathogenic agents such as *Bt* is an attractive alternative to control this economically important pest. Thus, it is crucial to identify and classify the most active strains with respect to a given target insect. There are a number of methods to characterize isolates such as PCR amplification based on sequences of known crystal protein genes, the use

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of monoclonal antibodies and plasmid profiles. However, there is not always a good correlation between any of these factors and reported insecticidal activity (Hongyu et al. 2000; Porcar and Juarez-Perez 2003; Martínez et al. 2005).

The main objectives of this study are to differentiate native crystalliferous strains and to evaluate the relationship between the toxicity assays against *Sf* first instar larvae and SDS-PAGE patterns of whole cell proteins. The biomass-bound protease activity of native pathogenic isolates and the reference strain 4D1 was also determined.

## Materials and methods

### Micro-organisms

*Bacillus thuringiensis* var. *kurstaki* 4D1, *Bacillus thuringiensis* var. *kurstaki* 4D3, *Bacillus thuringiensis* var. *thuringiensis* 4A4 and *Bacillus subtilis* 1A571 were provided by Bacillus Genetic Stock Center, Columbus, Ohio.

Bacteria were isolated from both larvae showing disease symptoms and soil samples collected in northwest Argentina. These samples came from maize, sorghum, wheat, grape or sugarcane cultivated fields. Suspensions were heated at 80°C for 15 min and plated on Luria-Bertani (LB) agar. Plates were incubated at either 30 or 55°C for 24 h. Colonies that did not grow at 55°C were then analyzed for the presence of parasporal crystals by microscopic examination (Sharif and Alaeddinoğlu 1988).

### Bioassays

All experiments were conducted in a climate-controlled room at 25°C, with a photoperiod of 12:12 (light/dark), and 65% relative humidity. The insecticidal activity was tested against *Sf* first instar larvae on an artificial diet previously soaked in a spore-crystal suspension. Artificial diet comprised: 150 g bean flour, 35 g wheat germ, 30 g beer-brewing yeast, 6 g ascorbic acid, 1.6 g sorbic acid, 2 g nipagin, 2 ml formaldehyde, 22 g agar and 1,000 ml water. The bacterial inoculum was prepared by suspending in sterilized water a 5 day culture ($DO_{610} = 2$, ca. $6 \times 10^7$ c.f.u. $ml^{-1}$) grown on a LB agar plate at 30°C. Ten individuals were observed per treatment, each treatment was replicated three times. The control groups were fed with an artificial diet soaked in either sterilized water or *B. subtilis* (1A571) suspension. Mortality was scored after 7 days. The 50% lethal doses ($LD_{50}$) were determined by Probit analysis. Data were analyzed by Pearson and deviance chi-square goodness-of-fit tests.

### Protein electrophoresis

Biomass containing sporulated cells and crystalline inclusions was harvested from LB agar cultures as described above. SDS-PAGE was performed by the method of Laemmli, using 10% (v/v) polyacrylamide gel. Spore-crystal mixtures were washed once in 1 ml 1.5 M NaCl. Concentrated suspensions in sample loading buffer 2× (25 mM Tris/HCl pH 6.8, 1.28 M 2-mercaptoethanol, 2.89 mM Bromophenol Blue, 0.138 M SDS, 2.17 M glycerol) and were boiled for 10 min. Then aliquots containing ca. 30 µg protein $ml^{-1}$ were loaded in each well. Protein standards from Sigma-Aldrich were rabbit skeletal myosin (200 kDa), *E. coli* β-galactosidase (116.25 kDa), rabbit muscle phosphorylase b (97.4 kDa), Bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa) and bovine carbonic anhydrase (31 kDa). Gels were stained with silver reagent.

Protein bands were individually identified by their specific migration rates in the electrophoretic analyses. Once bands were properly and distinctively identified, binary (0/1) matrices were constructed to compare the patterns. Simple matching similarity coefficients were generated by the SIMQUAL subroutine from the NTSYS-pc 2.02f (Applied Biostatistics, Inc). For cluster analysis the neighbor-joining method was used.

### Protein determination

To 100 µl sample, 1,000 µl Coomassie Blue G-250 reagent was added, held for 10 min at room temperature and the protein concentration was then estimated at 590 nm using BSA (fraction V) as standard.

### Biomass-bound protease activity determination

After 48 h incubation, colonies were scraped off and washed twice in 1 ml of 500 mM phosphate buffer

(pH 7). This suspension was used as source of enzyme. Proteolytic activity was assayed according to Secades and Guijarro (1999) by using azocasein (Sigma) as substrate. Briefly, 120 µl of a suitable dilution of a biomass suspension was added to 480 µl azocasein (1%, w/v) in reaction buffer (Tris buffer containing $MgCl_2$ at 5 mM]) and the mixture was shaken (150 rpm) at 30°C for 30 min. The reaction was terminated by adding 600 µl 10% (w/v) trichloroacetic acid and left for 30 min on ice, followed by centrifugation at 15,000*g*, at 4°C for 10 min. The supernatant, 0.8 ml, was neutralized by adding 200 µl 1.8 M NaOH and the absorbance at 420 nm ($A_{420}$) was then measured. One unit of enzyme activity was defined as the amount which yielded an increase in $A_{420}$ of 0.01 in 30 min at 30°C. Specific activity was expressed as units per g dry wt. For each strain tested, a calibration curve was generated with its optical density measured at 610 nm and the corresponding washed dry biomass developed in LB medium.

### DNA preparation and PCR amplification

Total DNA was extracted from cells harvested in the mid-exponential growth phase as described by Miller. PCR amplification was performed in a 25 µl reaction mix containing 2.5 µl 10× STR reaction buffer (Promega), 20 ng total DNA, 0.5 µM of each primer and 1 U of Taq DNA polymerase (Promega). Primers 27 F and 1492 R (Table 1) were used to generate partial sequences of 16S rDNA. Samples were amplified as follows: 5 min at 94°C; 35 cycles of 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C; and, at the end, 7 min at 72°C for final extension. General primers designed for detection of *cry1* and *cry2* genes, and specific primers for identification of spe-*cry9*C gene, were also used (Table 1). Amplifications were performed as follows: 2 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C; and, at the end, 5 min at 72°C for final extension. PCR products were analyzed by electrophoresis in 2% (wt $vol^{-1}$) agarose gels.

### DNA sequencing and data analysis

DNA sequencing was carried out by Macrogen Services. Sequences were compared and aligned with sequences from the GenBank database by using the BLAST program of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) network server. Phylogenetic rooted trees were generated by the fast alignment option from the DNAMAN 4.03 (Lynnon Biosoft).

### Nucleotide sequence accession numbers

The partial nucleotide sequence of the 16S rDNA gene from *Bt* RT, *Bt* LSM and *Bt* LQ as well as the partial nucleotide sequence of *cry 1* and *cry 2* from *Bt* RT were deposited in GenBank database under accession numbers EF638795, EF638796, EF638798, EU327754, and EU327755, respectively.

## Results

### Insecticidal activity of crystalliferous native strains

The presence of crystalliferous spore-forming microorganisms was examined in samples collected from maize, sorghum, wheat, grape or sugarcane cultivated fields in northwest Argentina. From a total of 254

**Table 1** Primer sequences used in this study

| Primer pairs | Nucleotide sequence | Reference |
|---|---|---|
| 7F | 5′-AGAGTTTGATCCTGGCTCAG-3′ | Weisburg et al. (1991) |
| 1492R | 5′-GGTTACCTTGTTACGACTT-3′ | Weisburg et al. (1991) |
| Gral cry 1 | 5-CTGGATTTACAGGTGGGGATAT-3 | Bravo et al. (1998) |
| | 5′-TGAGTCGCTTCGCATATTTGACT-3′ | Bravo et al. (1998) |
| Gral cry 2 | 5′-GAGTTTAATCGACAAGTAGATAATTT-3′ | Ibarra et al. (2003) |
| | 5′-GGAAAAGAGAATATAAAAATGGCCAG-3′ | Ibarra et al. (2003) |
| spe-cry9C | 5′-CTGGTCCGTTCAATCC-3′ | Bravo et al. (1998) |
| | 5′-CCGCTTCCAATAACATCTTTT-3′ | Bravo et al. (1998) |

colonies, 14 were identified as crystal producer strains, giving a mean *Bt* index of 0.055. This result suggested that samples analyzed contained a high background level of other spore-forming bacteria. The crystalliferous strain LQ came from sorghum cultivated field, while the others came from maize cultivated field. Concerning the source of isolation, 50% of crystal producer strains came from soil samples and the other 50% came from ill larvae. Interestingly, the last source provided the most pathogenic strains (Fig. 1).

As expected, the great majority of the selected strains showed no or very low toxicity against *Sf* first instar larvae. Only three isolations produced mortalities between 53 and 100% (Fig. 1). These strains were further assayed with serial dilutions of spore-crystal suspensions to estimate their $LD_{50}$ (Table 2). According to this parameter and their fiducial limits, the isolation *Bt* RT was the most toxic showing potency similar to the reference strain *Bt* 4D1. In addition, the crystalliferous strains LSM and LQ displayed $LD_{50}$ 15 and 7 times higher than that of the isolation *Bt* RT, respectively.

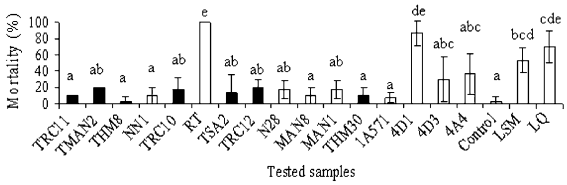


**Fig. 1** Insecticidal activity of crystalliferous native strains isolated from soil (■) and ill larvae (□) against *Sf* first instar larvae. Bars sharing the same letter were not significantly different ($P > 0.05$, Tukey test)

**Table 2** Estimated 50% lethal dose and biomass-bound protease activity of pathogenic native *Bt* strains

| *Bt* strains | $LD_{50}$ (95% fiducial limits) ($\times 10^6$ c.f.u. $ml^{-1}$) | Specific biomass-bound protease activity (±SD) (U g dry wt $^{-1}$) |
|---|---|---|
| RT | 3.6 (0.9–8.5) | 1,988 ± 98 b |
| LQ | 27.8 (13.9–46.0) | 1,140 ± 25 a |
| LSM | 55.9 (36.5–129.2) | 1,809 ± 93 b |
| 4D1 | 4.4 (1.2–8.8) | 946 ± 14 a |

[a] Values of specific biomass-bound protease activity that sharing the same letter were not significantly different ($P > 0.05$, Tukey test)

## SDS-PAGE analysis

Newly isolated bacteria and parasporal crystals were characterized by the protein bands in SDS-PAGE. Electrophoretic analysis revealed the presence of 53 distinct protein bands with molecular weights ranging from 266 to 20 kDa (Fig. 2a). The whole cell protein profiling allowed the differentiation of native *Bt* at strain level. Numerical analysis clearly showed two distinct clusters (Fig. 2b). Cluster A comprised 11 isolations and the reference strain 4A4. Interestingly, this group of native microorganisms produced proteins from 28 to 31 kDa but not proteins of ~135 and 65 kDa. Cluster B included the reference strains *Bt* 4D1 and 4D3 as well as 3 native strains. One of them, the isolation *Bt* RT, had a protein profile similar to *Bt* 4D1 with proteins of ~140 and ~70 kDa. Strains *Bt* LSM and *Bt* LQ showed protein bands of ~100 and ~81 kDa.

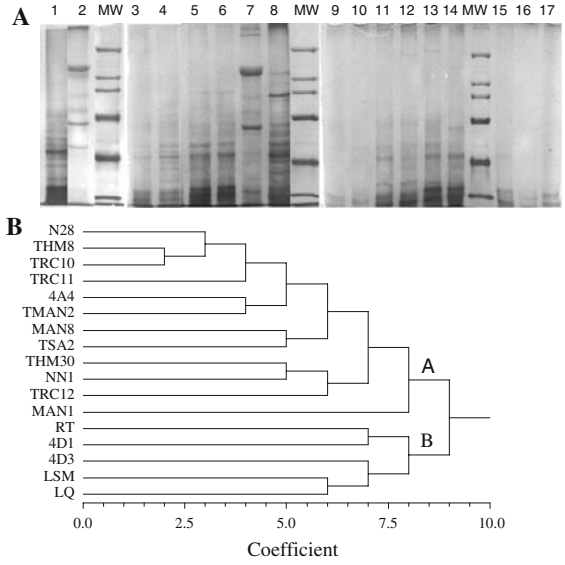


**Fig. 2** (**a**) SDS-PAGE of whole-cell protein of *Bt* strains. Gel I. Lines: **1**: 4D3, **2:** 4D1. Gel II. Lines: **3**: N28, **4:** 4A4, **5**: LSM, **6:** LQ, **7:** RT, **8**: MAN8. Gel III. Lines: **9:** THM8, **10**: TMAN2, **11**: THM30, **12**: NN1, **13**: TSA2, **14**: MAN1, **15:** TRC12, **16**: TRC11, **17**: TRC10. **MW:** Molecular weight marker (see the Materials and Methods section). (**b**) Dendrogram showing the relationship among *Bt* isolates based on electrophoretic whole-cell protein patterns. Associations were produced using the simple matching coefficient and the neighbor-joining clustering method

Biomass-bound protease activity of pathogenic strains

In addition to Cry protein, *Bt* is also an excellent source of protease activities. Table 2 shows that native isolations *Bt* RT, *Bt* LSM and *Bt* LQ as well as the reference strain *Bt* 4D1 displayed a biomass-bound protease activity. *Bt* RT has the highest proteolytic activity, significantly different to that achieved for *Bt* 4D1.

Characterization of *Bt* RT crystal proteins

Both bipyramidal and cuboidal crystal proteins produced by *Bt* RT were observed by scanning electron microscopy (data not shown). PCR analysis showed the presence of *cry1* and *cry2* genes but not spe-*cry9*C. The corresponding amplified fragments, sequenced and compared with *cry* genes sequences available from GenBank, had 99 and 95% identity with *cry1Ab* (EU220269) and *cry2Ab* (EU094885) genes, respectively. As shown in Fig. 3, *cry 1* and *cry2* partial sequences from *Bt* RT and *Bt* 4D1 were also aligned with five and six GenBank published cry sequences, respectively. The phylogenetic analysis revealed that *cry* 1 partial sequences from *Bt* RT and *Bt* 4D1 possess almost the same level of evolutionary distance (Fig. 3a), while *cry* 2 partial sequence from *Bt* RT lies on a separate diverse branch not only of *cry* 2 from *Bt* 4D1 but also of the others analyzed *cry* 2 sequences (Fig. 3 b).

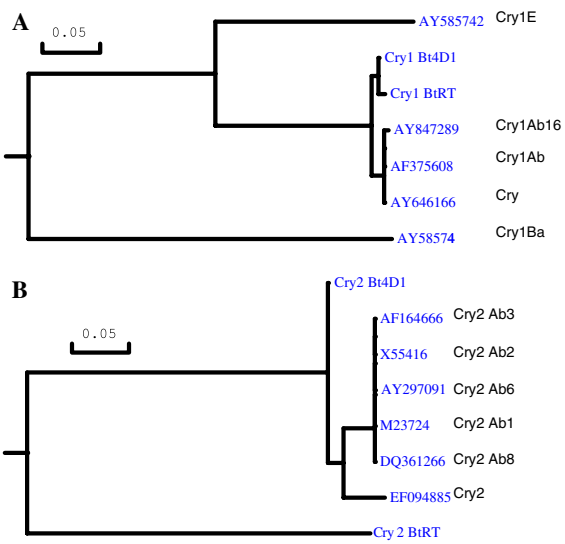


**Fig. 3** Phylogenetic rooted tree of *cry*1 (**a**) and *cry* 2 (**b**) partial sequences from *B. thuringiensis* strains

## Discussion

The identification and classification of bacteria are of crucial importance in environmental, industrial and agricultural microbiology. In this context, biochemical and molecular markers are versatile and highly informative tools. Our results showed that SDS-PAGE analysis of whole cell proteins not only allowed the differentiation of *Bt* at strain level but also revealed a possibility to apply protein profile analysis in classification of toxicity patterns. Cluster analysis displayed two main groups. Cluster A included the reference strain 4A4 as well as those crystalliferous isolations that had no or very low toxicity against *Sf* first instar larvae. PAGE analysis showed no bands of about 135 and 65 kDa. However, sporulated cultures of these native microorganisms revealed protein bands of around 28 kDa. This molecular mass could correspond to Cyt toxins, entomocidal crystal proteins highly active against Diptera larvae (Gough et al. 2005). On the other hand, all isolations with high toxic activity against *Sf* were located in cluster B. Strain *Bt* RT displayed a level of similarity of 88% with both *Bt* LSM and *Bt* LQ, which showed a higher level of similarity between them (96%) (data not shown). In dose-response experiments, the isolation *Bt* LSM and *Bt* LQ exhibited higher $LD_{50}$ value than that of *Bt* RT, probably because a different crystal proteins composition. According to electrophoretic data, *Bt* LSM as well as *Bt* LQ revealed proteins bands of around 100 and 81 kDa. While *Bt* RT, like the reference strain *Bt* 4D1, produced $\sim$140 and $\sim$70 kDa protein bands, which is consistent with the presence of at least one *cry1* and one *cry2* family gene. In fact, the sequence of PCR fragments generated with general primers designed for detection of those genes showed 99 and 95% homology with *cry1 Ab* and *cry2 Ab*, respectively. In addition, these crystalline entomocidal protoxins are also consistent with the production of bipyramidal and cuboidal crystals, associate with Cry1 and Cry2 toxins, respectively (Schnepf et al. 1998). Besides, considering the phylogenetic analysis, it could be expected toxicity mediated by Cry 1 rather than Cry2 crystal protein. In fact, *cry* 2 partial sequence from *Bt* RT shared a 95 % homology with

*cry* 2 sequence from a Colombian native *Bt* strain active against *Tecia solanivora* (Lep: Gelechiidae) (EU094885).

With respect to taxonomic identities, partial 16S rDNA sequences from native pathogenic strains were tested by BLAST analysis against the GenBank data base. Strain *Bt* LSM showed exact BLAST matches with the sequence from *Bacillus thuringiensis* serovar *kurtaki* (1 hit, 100% of identity, accession number EU 153549). Strain *Bt* LQ produced 4 hits (99% of identity), all of these corresponding to *Bt* species. Similarly, *Bt* RT (best hits, 13; 99% of identity) also shared a close relationship with others *Bt* strains, including *Bt* LSM.

Lepidopteran-active toxins are obtained via proteolytic processing which has been reported to proceed by removal of both *N*- and *C*-terminal peptides (Bravo et al. 2007). The composition and/or the activities of midgut proteases influence the insect susceptibility to Cry toxins and may provide the basis for *Bt* specificity and resistance development (Bah et al. 2004; Rausell et al. 2007). While there is a reasonable understanding of soluble midgut proteases in toxin activation, little is known about the role of *Bt* protease in entomotoxicity. In this connection, Brar et al. (2007) clearly explain the scenario in which *Bt* proteolytic enzymes can process or degrade the protoxin. They also discussed the use of *Bt* protease activity as a potential indicator of entomotoxicity. During our investigations, high biomass-bound protease activity was detected in the presence of previously washed cells of *Bt* RT, *Bt* LSM, *Bt* LQ and *Bt* 4D1. To our knowledge, the presence of this naturally immobilized enzyme activity has not been reported in *Bt*. Although no correlation between biomass-bound protease activity and mortality values was initially detected, this result could be complementary information to consider in commercial *Bt* formulations, since the cell structure may act as a natural matrix able to protect the enzymes from the possible negative action of external agents; and therefore it could be that an increased percentage of *Bt* protease may actually reach the larvae midgut. Finally, it would be useful to explore the role of the biomass-bound protease activity in crystal protein modification during *Bt* fermentation, the synergy of this protease source with insect entomotoxicity and the possible addition of vegetative cells in the final *Bt* formulation.

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