Chapter 5

Characterization and Biological Activity of *Bacillus thuringiensis* Isolates that Are Potentially Useful in Insect Pest Control

Analía Alvarez and Flavia del Valle Loto

Additional information is available at the end of the chapter

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1. 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 [1]. The primary action of Cry proteins is to lyse midgut epithelial cells through insertion into the target membrane and form pores [2]. Once ingested, crystals are solubilized in the alkaline environment of midgut lumen and activated by host proteases [3]. On the other hand, the involvement of *Bt* proteases in processing inactive protoxins is also reported [3]. These toxins are also highly specific and completely biodegradable, hence no toxic products are accumulated in the environment. In fact, Calderón et al. [4] suggest the potential use of some crystal proteins as adjuvants for the administration of heterologous antigens.The activity spectrum of *Bt* toxins continually increases as result of the ongoing isolation of new strains around the world.

The fall armyworm, *Spodoptera frugiperda* (*S. frugiperda*) (Lepidoptera: Noctuidae), and the variegated cutworm, *Peridroma saucia* (*P. saucia*) (Lepidoptera: Noctuidae), are two lepidopteran pests that cause severe damage to a variety of crops. While the first one mainly attacks corn, rice, peanuts, cotton, soybeans, alfalfa and forage grasses [5], the second one targets peanuts, sunflowers, soybeans and grapevines, among others [6]. Currently, control of this pest relies on chemical insecticides. Nevertheless, the rapid increases 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 [7,8]. Among these methods the 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 [9]. However, it has been reported that several pests have developed resistance against Cry proteins [9, 10]. The current approach used to delay evolution of resistance to transgenic crops uses a "high dose" and "refuge" strategy [9, 11]. In addition, it is important to use a combination of *cry* genes and/or other genes encoding insecticidal proteins within the same transgenic crop [12, 13]. Due to extensive use of transgenic crops in developing countries based on *cry*-type genes, there is a need for alternative *cry* gene sequences to meet the challenge of novel insect resistance [7]. Crucial to this development is the identification of novel and more active strains with respect to insect pests of economically important crops [14].

The *cry* genes of *Bt* strains are known to be related to their toxicity [15, 16] and identification of these genes by means of PCR has been used to characterize and predict insecticidal activity of the strains [17, 18]. Nevertheless, a more complete characterization should include alternative methods. Phenotypical analysis such as protein profile determination provides useful information for typing and comparative studies [19]. The literature data report the possibility of using the whole-cell protein profile as a discriminating method with potency similar to RAPD with combined DNA patterns [1]. However, there is not always a good correlation between these factors and insecticidal activity of *Bt* strains [20, 21]. In addition, there is a need to develop knowledge about the biological properties and diversity of *Bt* isolates since these data allow a better understanding of the biological factors that determine insecticidal properties. Extracellular factors such as phospholipases, proteases and chitinases have shown to contribute to insecticidal activity of *Bt* [22].

During a screening programme of *Bt* isolates native to Argentina and toxic against Lepidoptera, several strains were characterized according to different biological parameters. In addition, promising isolates regarding their useful in biological control programmes -an environmentally safe technology of pest control- were exhaustively studied [14, 19, 23]. The present work showed most relevant results obtained during a course of those investigations. The discovery of highly pathogenic isolates against devastating insect pests reveals the usefulness of screening studies for novel *Bt* strains.

2. Biochemical characterization of *B. thuringiensis* isolates and assessment of toxicity

Crystalliferous spore-forming bacteria were isolated from both *S. frugiperda* larvae showing disease symptoms and soil samples collected in Argentina [19]. These samples came from maize, sorghum, wheat, grape or sugarcane cultivated fields. Briefly, larvae and soil sample suspensions were made in distilled water, heated at 80 °C for 15 min and then plated onto 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 [24]. From a total of 254 colonies isolated from 490 different environmental samples, 14 were identified as crystal producer strains, giving a mean *Bt* index of 0.05. This result suggested that samples analyzed contained a high background level of other spore-

forming bacteria. One crystalliferous strain came from sorghum cultivated field, while the others came from maize cultivated field. Concerning the source of isolation, 50% of crystal producers came from soil samples and the other 50% came from ill larvae. Interestingly, the last source provided the most pathogenic strains (Table 1).

Bacteria -characterized by conventional microbiological methods- possessed typical cellular and colonial morphologies, as well as physiological, biochemical and nutritional features that resembled *Bacillus* spp. They were motile and produced ellipsoidal endospores, located at sub-terminal position in the sporangia, and formed cream-colored colonies with irregular or circular edges on LB agar.

Phenotypical and molecular characterization	Strains														
	TRC11*	TMAN2*	THM8*	NN1**	TRC10*	RT**	TSA2*	TRC12*	N28**	MAN8**	MAN1**	THM30*	*Bt* 4D1***	LSM**	LQ**
Central spore	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
Sub terminal spore	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-
Growth at pH 9	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+
[b e] Growth in 0.2 % chitin	-	+	+	+	-	+	+	+	-	+	-	-	+	+	+
[b c e] CMC hydrolysis (0.5 %)	+	+	+	+	+	-	-	+	+	+	-	+	+	-	-
[b e] Chitin hydrolysis (0.2 %)	-	-	+	-	-	+	+	-	-	-	-	-	+	+	+
[b] Gelatin hydrolysis (12 %)	+	-	-	-	-	-	+	-	+	-	-	+	-	-	-
[b] Starch hydrolysis (2 %)	-	-	+	+	+	+	+	-	+	-	+	+	+	+	+
Gas production in glucose	-	+	-	+	-	+	-	+	-	-	-	-	+	+	+
[d] clindamycin	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
[d] gentamicin	+	+	-	-	+	-	-	+	+	-	-	-	+	-	-
[d] rifampicin	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+
cry1	-	+	-	+	-	+	-	-	-	-	+	+	+	+	ND
cry2	-	+	+	-	+	+	-	+	+	-	-	+	+	+	ND
cry1Aa						-							+	+	
cry1Ab						+							+	+	
cry1Ac						+							+	+	
cry2Aa						-							+	+	
cry2Ab						+							+	+	

[a] *Asterisks indicate the source of the isolates: *soil, **ill larvae and ***Bacillus Genetic Stock Center*

[b] Expressed in w/v

[c] CMC: carboxy methyl cellulose

[d] Sensitivity to antibiotics was determined by using the routine diffusion plate technique. (+): sensitive and (-): resistant

[e] Growth on chitin and chitin hydrolysis were determined using colloidal chitin according to Kaur et al. (2005). This protocol was also used to analyze CMC hydrolysis

*Strains isolated from soil

**Strains isolated from ill larvae

****B. thuringiensis* var. *kurstaki* 4D1 was provided by the Bacillus Genetic Stock Center (BGSC) (Columbus, Ohio) as well as the others *Bt* reference strains (see below).

ND: no determined (without amplification)

Table 1. Biochemical characteristics that presented variable response among the bacterial isolates. Molecular characterization is also showed (see below).

From a biochemical point of view, the 14 strains were catalase-positive, reduced nitrate and produced acetyl methyl carbinol in Voges-Proskauer broth; growth was observed at pH 7 on LB agar supplemented with 2, 3 and 5% NaCl and on LB agar at 30, 37 and 45 ºC. The strains also hydrolyzed casein and were motile on soft LB agar. Negative results for all strains were obtained in several tests: no growth was observed on LB agar at pH 4 or at 50 ºC and none of the strains hydrolyzed carboxymethyl cellulose (CMC) and urea. Antibiotic sensitivity tests revealed a resistance profile to penicillin, oxacillin, trimethoprim and a sensitive profile to erythromycin, vancomycin, levofloxacin, minocycline, chloramphenicol and teicoplanin. Phenotypic features that presented variability among the strains are showed in Table 1. The positive or negative result of each biochemical assay was entered in a 1-0 matrix. These data were subsequently analyzed through correspondence multivariate analysis, using Multivariate Statistical Package (MSVP) software (version 3.13). A cluster diagram based on these variable biochemical properties (that represented 54% of data variability) revealed that the strains formed two main groups (Figure 1). Group A comprised nine crystalliferous isolates which were clustered together with the reference strain *B. thuringiensis kurstaki* 4D1 (*Bt* 4D1). A second group (B) included three *Bt* strains while the remaining two strains presented more divergent features and hence were not included in any group. Isolates from the same sample and/or the same geographic region differed in their phenotypic features and consequently were not grouped together. This indicates that there was no clear association between *Bt* strains biochemical profile and the environments from which they were obtained [23].

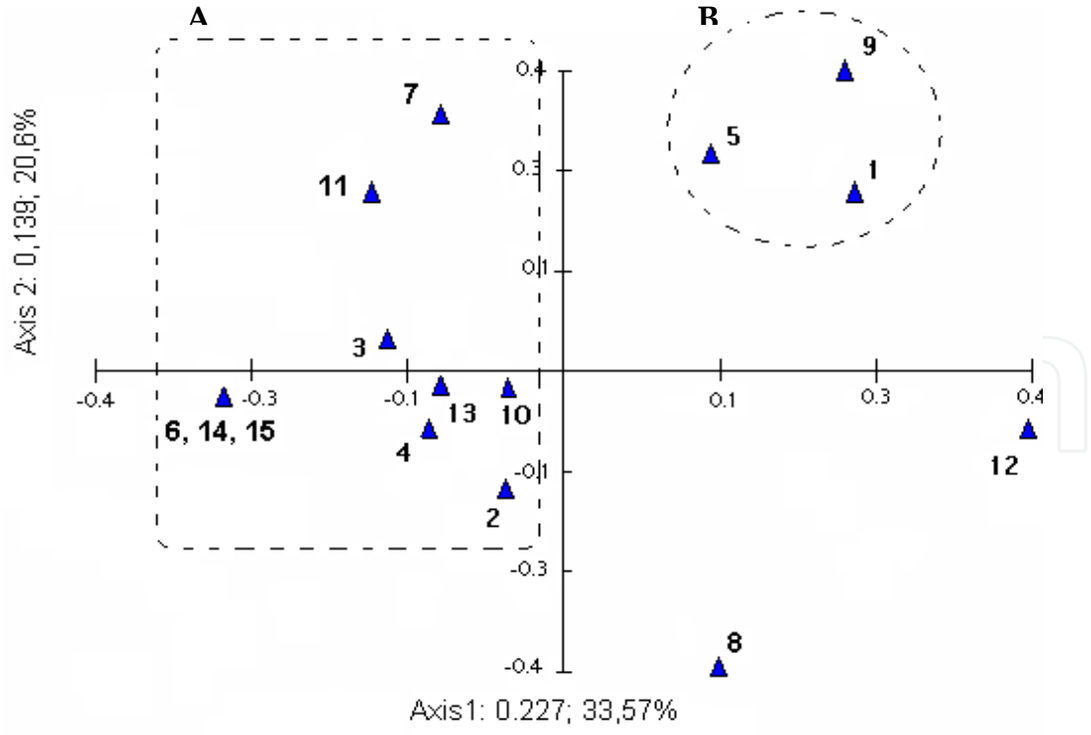

Figure 1. Correspondence multivariate analysis based on biochemical properties of *Bt* strains. 1: TRC11; 2: TMAN2; 3: THM8; 4: NN1; 5: TRC10; 6: *Bt* RT; 7: TSA2; 8: TRC12; 9: N28; 10: MAN8; 11: MAN1; 12: THM30; 13: **Bt* 4D1 (reference strain); 14: LQ and 15: LSM

Although most of the native isolates presented similar biochemical and phenotypical characteristics compared with reference strain *Bt* 4D1 (Group A) (Figure 1), they differed in their toxicity to *S. frugiperda.* Our results showed that the mortality on *S. frugiperda* neonate larvae was variable [19], ranging among values corresponding to Bt strains of bioinsecticides action low to moderate (Figure 2). However, strains named RT, LSM and LQ were found to be highly pathogenic, two of them, even more than reference strain *Bt* 4D1 which was selected for this analysis given it is the most widely used microorganism to control lepidopteran pests [25] (Table 2). This strong biological effect was represented by both a shorter LT_{50} and a higher mortality, which reached 100% in the case of RT strain on *S. frugiperda,* after five days of treatment. This result is extremely relevant considering that *S. frugiperda* is believed a pest with low sensitivity to *Bt* toxins [26]. In addition, when this strain was assayed against first instar larvae of *P. saucia,* reached 93% of mortality (Figure 3) suggesting that RT strain native to Argentina could possibly be employed in biological control of lepidopteran pests [19, 23]. It is important to stress that the high levels of mortality in the present work were 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} [27].

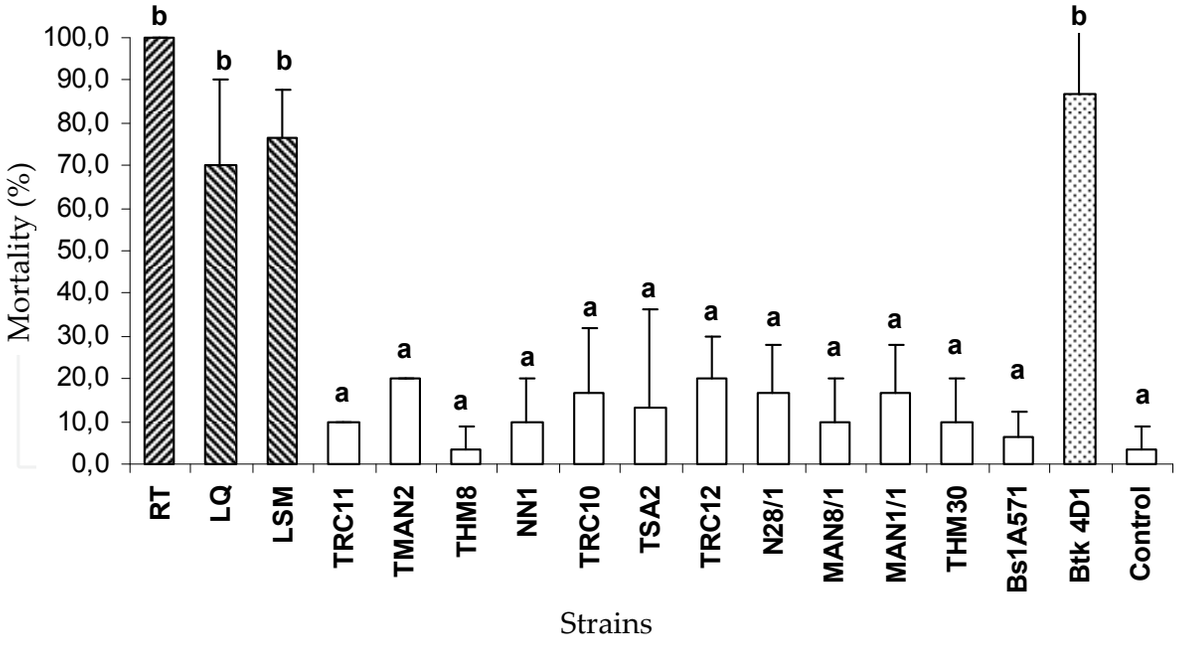

Figure 2. Insecticidal activity of crystalliferous native strains isolated from soil (□) and ill larvae (▨).Bars sharing the same letter were not significantly different ($P > 0.05$, Tukey post-test). Reference strain: *Bt* 4D1 (⁘).Mortality was measured at the 7th day of assay. Ten individuals per treatment were observed and each treatment was repeated 10 times. *B. subtilis* 1A571 *(Bs* 1A571*)* was provided by the Bacillus Genetic Stock Center (BGSC).

Bt strain	*,[a] Mortality (%) ± SD	[b] LT_{50} (h) (95% fiducial limits)	*Specific biomass bound protease activity (± SD) (U g dry wt $^{-1}$)
RT	100 ± 0 a	9.2 (10.4 –16.0)	1.98 ± 98 b
LSM	90.0 ± 7.3 a	37.7 (27.8 – 46.2)	1.80 ± 93 b
LQ	73.0 ± 5.7 c	79.6 (68.2 – 90.7)	1.14 ± 25 a
Bt 4D1	86.0 ± 15.1 b	58.7 (50.4 – 66.0)	946 ± 14 a
control	1.0 ± 3.1 d		

*Values followed by different letters were significantly different (P<0.05, Tukey post-test)
[a] Ten individuals per treatment were observed and each treatment was repeated ten times
[b] 50 % lethal time (LT_{50}) was determined by Probit analysis. Mortality was scored every 24 h during seven days

Table 2. Comparison of mortality and 50% lethal time (LT_{50}) of first instar larvae of *S. frugiperda* among native and reference *Bt* strains. Biomass-bound protease activity of pathogenic isolates are also showed.

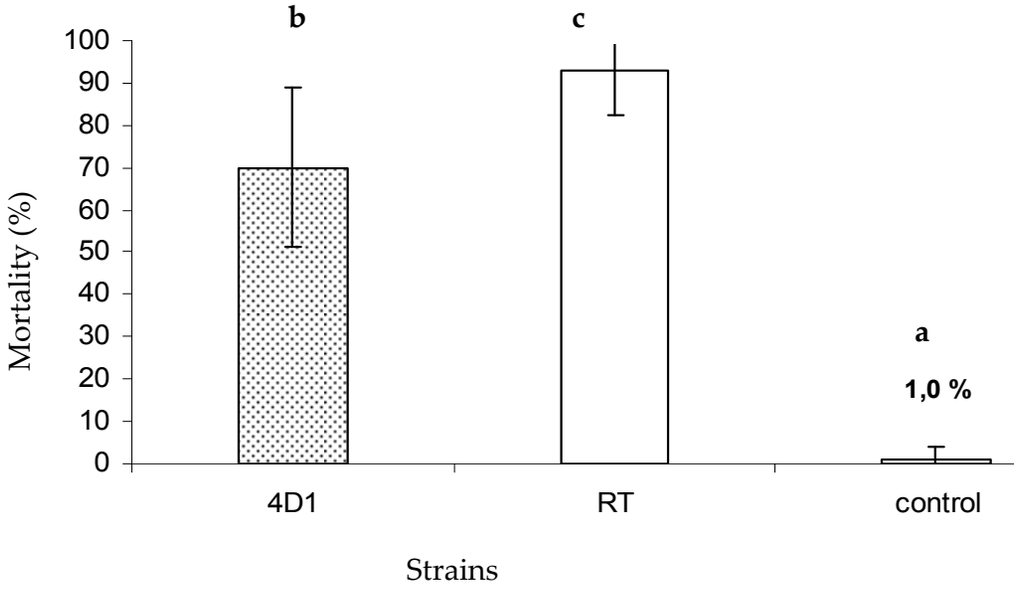

Figure 3. Comparison of insecticidal activity of *Bt* RT and the reference strain *Bt* 4D1 against the first instar larvae of *Peridroma saucia*. Mortality was measured at the 7th day of assay. Ten individuals per treatment were observed and each treatment was repeated 10 times. Mortality differed significantly between all treatments (P < 0.05, Tukey post-test).

3. Numerical analysis of insecticidal crystal proteins of *B. thuringiensis*

In order to differentiate native crystalliferous isolates and to evaluate the relationship between the toxicity assays against *S. frugiperda*, isolated bacteria and parasporal crystals were characterized by the whole-cell protein profiles in SDS-PAGE [19]. Protein bands were individually identified by their specific migration rates in the gels. Once bands were properly and distinctively identified, binary (0/1) matrices were constructed to compare the patterns. Electrophoretic analysis revealed the presence of 53 distinct bands with molecular weights ranging from 266 to 20 kDa (Figure 4). Numerical analysis clearly showed two distinct clusters (Figure 5). Cluster A comprised 11 isolations and the reference strain *B. thuringiensis* var. *thuringiensis* 4A4 (*Bt* 4A4) as well as those crystalliferous isolations that had no or very

low toxicity against *S. frugiperda* first instar larvae. Interestingly, this group of native microorganisms produced proteins from 28 to 31 kDa but not proteins of ~135 and ~65 kDa. These lower molecular mass could correspond to Cyt toxins, entomocidal crystal proteins highly active against Diptera larvae [28]. On the other hand, all isolations with high toxic activity against *S. frugiperda* (RT, LSM and LQ strains) (Table 2) were located in cluster B, as well as the reference strains *Bt* 4D1 and *B. thuringiensis* var. *kurstaki* 4D3 (*Bt* 4D3). The isolation RT had a protein profile similar to *Bt* 4D1 with proteins of ~140 and ~70 kDa. Strains LSM and LQ showed protein bands of ~100 and ~81 kDa. These results demonstrate that the whole cell protein profiling 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.

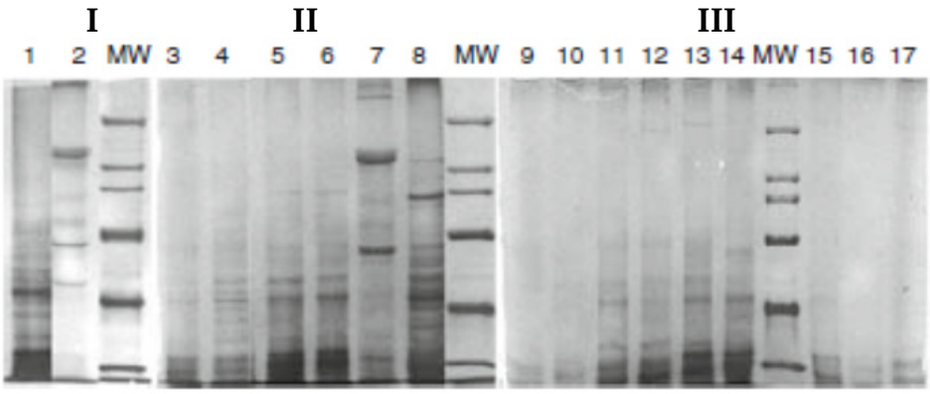

Figure 4. SDS-PAGE of whole-cell protein of crystalliferous strains. Gel I. Lines: 1: *Bt* 4D3, 2: *Bt* 4D1. Gel II. Lines: 3: N28, 4: *Bt* 4A4, 5: LSM, 6: LQ, 7: RT, 8: MAN8. Gel III. Lines: 9: THM8, 10: TMAN2, 11: THM30, 12: NN1, 13: TSA2, 14: MAN1, 15: TRC12, 16: TRC11, 17: TRC10. MW: Molecular weight marker Sigma-Aldrich were rabbit skeletal myosin (200 kDa), *E. coli b*-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. *B. thuringiensis* var. *kurstaki* 4D3 and *B. thuringiensis* var. *thuringiensis* 4A4 were also provided by the Bacillus Genetic Stock Center (BGSC).

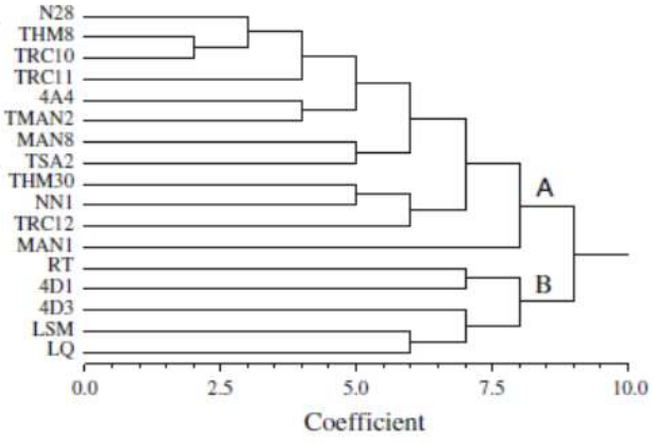

Figure 5. Dendrogram showing the relationship among *Bt* isolates based on electrophoretic whole cell protein patterns. Associations were produced using the simple matching coefficient and the neighbor-joining clustering method.

4. Molecular characterization of *B. thuringiensis* strains and crystal morphology

Although the presence of parasporal crystals is a diagnostic characteristic of *Bt* strains [1], the taxonomic identity of the toxic crystalliferous isolates was confirmed by amplification and partial sequencing of their 16S rDNA genes [19] (Table 3). The partial 16S rDNA sequences were tested by BLAST analysis against the GenBank data base. *Bt* LSM strain showed exact BLAST matches with the sequence from *Bacillus thuringiensis* var *kurstaki* (1 hit, 100% of identity, accession number EF638796). *Bt* LQ strain produced 4 hits (99% of identity, accession number EF638798), all of these corresponding to *Bt* species. Similarly, *Bt* RT (best hits, 13; 99% of identity, accession number EF638795) also shared a close relationship with others *Bt* strains, including *Bt* LSM.

Generally, *B. thuringiensis* insecticidal protein toxin genes (*cry*) reside on large self-transmissible plasmids, and individual *B. thuringiensis* strains can harbor a diverse range of plasmids that can vary in number from 1 to 17 and in size from 2 to 80 MDa [29,30], although it has also been suggested that they are present in the chromosome [31]. In this context, to study the plasmid profiles of *Bt* strains is an important parameter to determine their identity, since the number and size of these is associated with a particular *Bt* strain. Comparison between strains belonging to the same serotype showed a great difference in variability [30]. Some serotypes (e.g., israelensis) showed the same basic pattern among all its strains, while other serotypes (e.g., morrisoni) showed a great diversity of patterns. These results indicate that plasmid patterns are valuable tools to discriminate strains below the serotype level [30]. The profile of extrachromosomal elements in *Bt* is influenced by a number of stressful growth conditions, which determine its stability and heritability (e. i. high temperatures determine the plasmid loss), therefore it is neccesary to take some care . In this study, cultures were routinely grown at 30 °C to avoid this phenomenon. Detection and isolation of plasmid DNA was conducted following the method of Kado and Liu [32]. DNA plasmid samples were electrophoresed on 0.8 % (wt vol^{-1}) agarose gel. Our results showed that selected *Bt* strains present a complex plasmid profile (Figure 6).

In this experiment, the plasmid DNA was not linearized and therefore the same plasmid can produce as many as three different bands in the agarose gel. This made it difficult to determine the precise number of plasmids present in each complex plasmid profile. For this reason, we will refer to the number and size of plasmidic bands rather to plasmids themselves. An intense band above the chromosomal band (C) was observed in *Bt* RT, *Bt* LSM and *Btk* 4D1 suggesting that a large plasmid or plasmids is/are found in this strains which might be responsible for production of parasporal bodies. Compared with the other bacteria, *Bt* LQ presented a very different profile array, suggesting a different *cry*-genotype.

Identification of *cry* genes by means of PCR has been used to predict insecticidal activity of the strains [17,18] and to determine the distribution of *cry* genes within a collection of *B. thuringiensis* strains [20, 33]. In this context, our crystalliferous strains were characterized in terms of presence of *cry1* and *cry2* genes by amplification with general primers. The most toxic *Bt* strains RT, LSM and LQ were characterized through additional PCR with specific

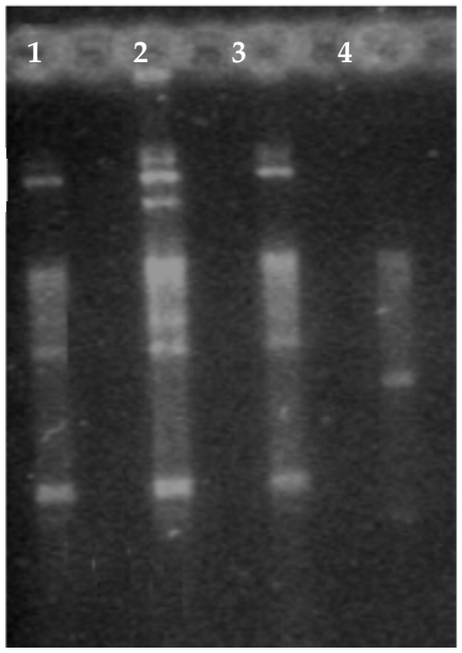

Figure 6. Plasmid profiles of *Bacillus thuringiensis* strains. Lanes: 1: *Bt* RT; 2: *Bt* 4D1; 3: *Bt* LSM; 4: *Bt* LQ. "C" indicate chromosomal DNA.

primers to identify the presence of *cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry2Aa, cry2Ab* and *cry2Ac* genes (Table 3). PCR analysis showed presence of *cry1* and/or *cry2* genes in most of the isolates (Table 1). Specific PCR showed identical *cry* gene profile in both *Bt* LSM and the reference *Bt* 4D1, while *cry* gene content of *Bt* RT was different from them. DNA of *Bt* LQ was not amplified under the current reaction conditions (Table 1).

In addition, amplified fragments corresponding to *cry1* and *cry2* genes from *Bt* RT were sequenced and compared with *cry* genes sequences available from GenBank. This sequences had 99 and 95% identity with *cry1Ab* (EU220269) and *cry2Ab* (EU094885) genes, respectively. As shown in Figure 7, *cry1* 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 (Figure 7A), while *cry2* partial sequence from *Bt* RT lies on a separate diverse branch not only of *cry2* from *Bt* 4D1 but also of the others analyzed *cry2* sequences (Figure 7B). Considering the phylogenetic analysis, it could be expected toxicity mediated by Cry1 rather than Cry2 crystal protein. In fact, *cry2* partial sequence from *Bt* RT shared a 95% homology with *cry2* sequence from a Colombian native *Bt* strain active against *Tecia solanivora* (Lep:Gelechiidae) (EU094885).

As mentioned before, *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 [29] *cry2* encodes for cuboidal proteins, toxic to Lepidoptera and Diptera [39]. Our molecular and electron microscopy analyses of *Bt* RT are in agreement with all this evidence, since this highly pathogenic strain has both genes (Table 1) and both kinds of proteins (Figure 8A). In contrast, and although *Bt* LSM showed amplification products with *cry2* general and specific primers (Table 1) no cuboidal proteins were

Primer pairs	Nucleotide sequence	Reference
16S: 27F 1492R	*5′*-AGAGTTTGATCCTGGCTCAG-3′ 5′-GGTTACCTTGTTACGACTT-3′	[34]
ITS: ISR-1494 ISR-35	*5′*-GTCGTAACAAGGTAGCCGTA -3′ *5′*-CAAGGCATCCACCGT-3′	[35]
Gral-*cry1*	*5′*-CTGGATTTACAGGTGGGGATAT-3′ *5′*-TGAGTCGCTTCGCATATTTGACT-3′	[36]
Gral-*cry2*	*5′*-GAGTTTAATCGACAAGTAGATAATTT-3′ *5′*-GGAAAAGAGAATATAAAAATGGCCAG-3′	[37]
Spe-*cry1*Aa	*5′*-TTATACTTGGTTCAGGCCC-3′ *5′*-TTGGAGCTCTCAAGGTGTAAA-3′	[38]
Spe-*cry1*Ab	*5′*-AACAACTATCTGTTCTTGAC-3′ *5′*-CTCTTATTATACTTACACTAC -3′	
Spe-*cry1*Ac	*5′*- GTTAGATTAAATAGTAGTGG-3′ *5′*- TGTAGCTGGTACTGTATTG-3′	
Spe-*cry*1Ad	*5′*-GTTGATACCCGAGGCACA-3′ *5′*-CCGCTTCCAATAACATCTTTT-3′	
Spe-*cry2*Aa1	*5′*-GTTATTCTTAATGCAGATGAATGGG-3′ *5′*-GAGATTAGTCGCCCCTATGAG-3′	[17]
Spe-*cry2*Ab2	*5′*-GTTATTCTTAATGCAGATGAATGGG-3′ *5′*-TGGCGTTAACAATGGGGGGAGAAAT-3′	
Spe-*cry2*Ac	*5′*-GTTATTCTTAATGCAGATGAATGGG-3′ *5′*-GCGTTGCTAATAGTCCCAACAACA-3′	

Table 3. Primer sequences used in this study.

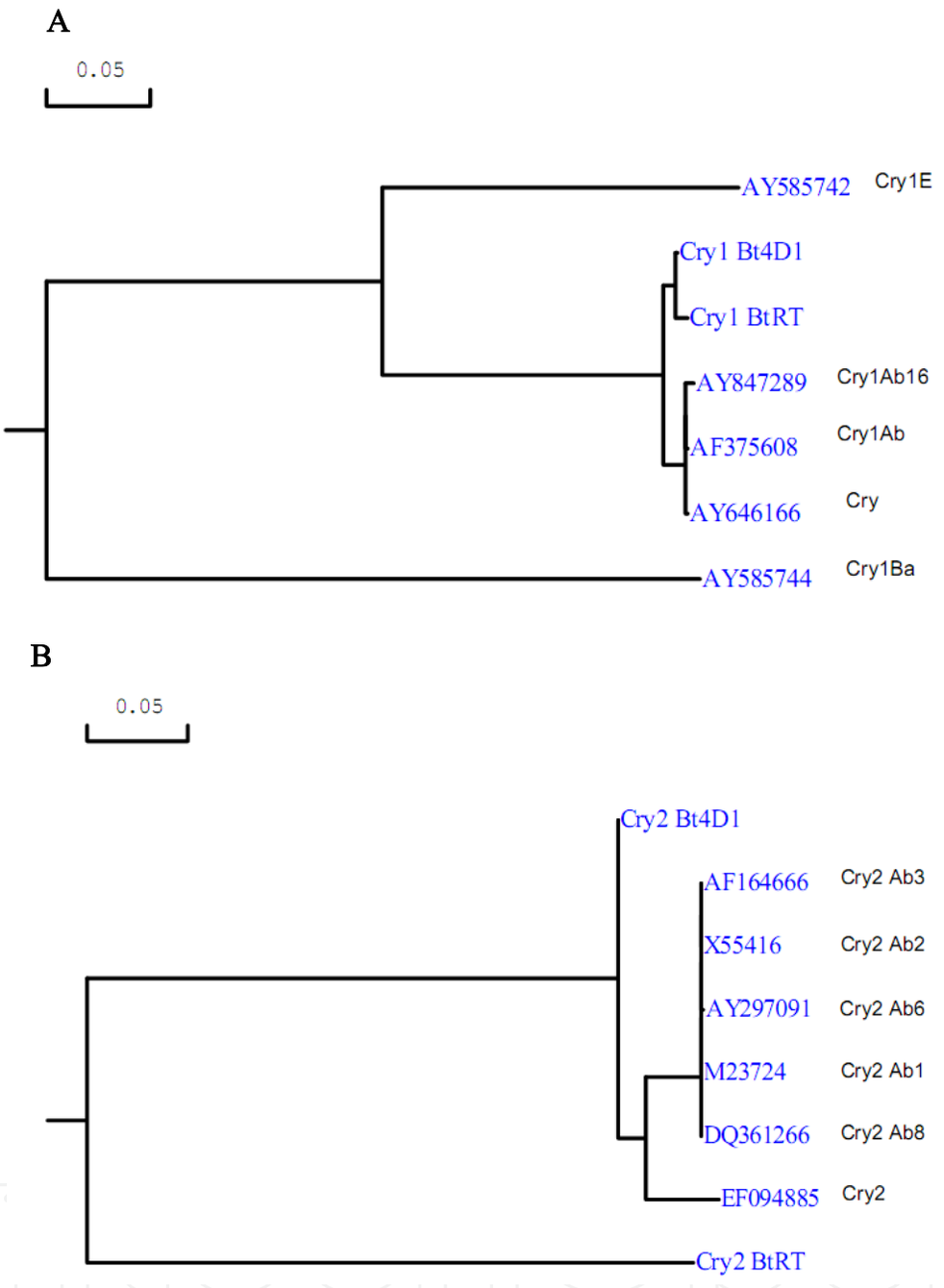

Figure 7. Phylogenetic rooted tree of *cry1* (A) and *cry2* (B) partial sequences from *B. thuringiensis* strains.

identified (Figure 8B). 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 could also explain the lack of cuboidal proteins, the production of these proteins by *Bt* RT under identical experimental conditions argue against this possibility [14]. 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* and *cry2* gene in several attempts (Table 1), despite the

presence of bipyramidal crystals (Figure 8C). Noguera e Ibarra, [40] found that *cry* genes of a *Bt* strain isolated in Argentina that showed elongated bipyramidal crystals [41] presented 98% identity with *cry5Ba* genes. Therefore, *Bt* LQ may have Cry proteins other than Cry1 that form bipyramidal crystals.

From a methodological point of view, washing of crystal suspensions with absolute ethanol/distilled water (Figure 9B) was more appropriate for microscopic observation than washing with distilled water (Figure 9A).

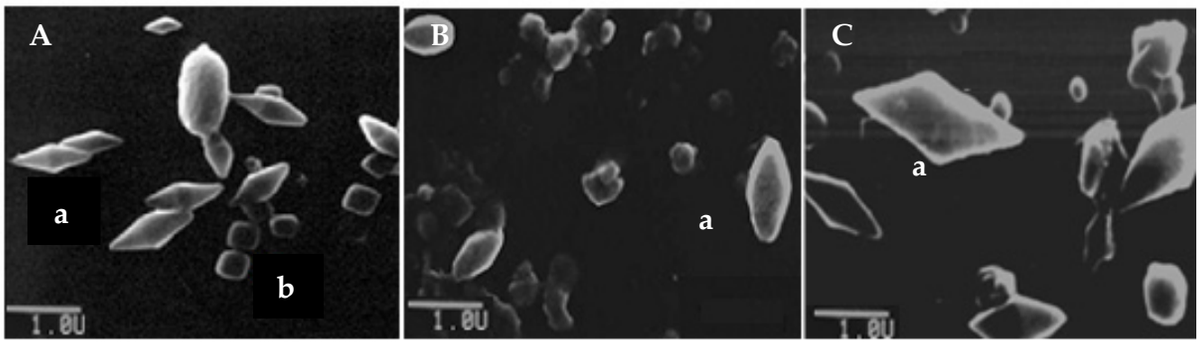

Figure 8. Scanning electron microscopy (SEM) of spore-crystal proteins from *Bt* strains. Concentrated spore-crystal suspensions were placed on a microscope lid and air-dried overnight. Samples were then coated with gold and examined using a scanning electron microscope. A) *Bt* RT; B) *Bt* LSM; C) *Bt* LQ. Both bipyramidal (a) and cuboidal (b) pesticidal crystal proteins are observed. Scale bar: 1 μm.

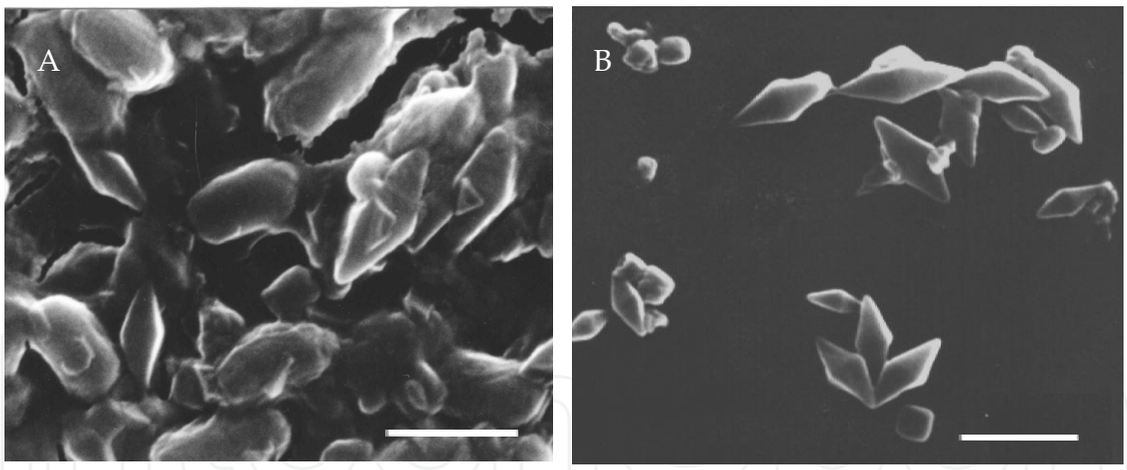

Figure 9. Scanning electron microscopy of spore-crystal proteins from *Bt* RT washed twice either with ethanol/water (1:1, v/v) (A) or with water (B). Scale bar: 1 μm.

As mentioned above, identification of *cry* genes by means of PCR has been used to predict insecticidal activity of the strains [17,18]. However, but since the primers are designed against known genes, the technique presents limitations in the search of novel *cry* genes. Moreover, the reliability of the prediction of insecticidal activity based on PCR results is dependent on the expression of the genes. In this context, 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 [42]. In this context, ITS-PCR was performed as previously described by Daffonchio et al.[31]. Used primer pairs are showed in Table 3. Evaluation of ITS length polymorphism revealed an identical pattern among *Bt* RT, LSM and LQ strains and also with *Bt* 4D1 suggesting that ITS exhibited no polymorphism among the strains (Figure 10). In connection with this, Reyes-Ramirez and Ibarra [43] studied ITS profiles of 31 *Bt* strains and found them to be insufficient to discriminate between isolates.

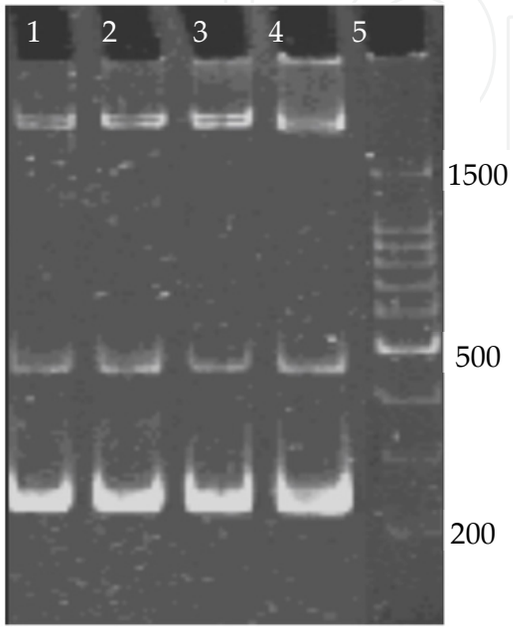

Figure 10. ITS-PCR of *B. thuringiensis* strains. Lanes 1: *Bt* LSM; 2: *Bt* LQ; 3: *Btk* 4D1; 4: *Bt* RT; 5: 100 bp DNA Ladder.

5. Assessment of enzyme activities in *B. thuringiensis*

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. Synthesis of lytic enzymes by *Bacillus* species during the early sporulation phase is one of these properties. Secreted microbial enzymes may function as virulence factor that are essential for survival and spread in the host [44,45]. Our results indicate that the native *Bt* strains responded diversely regarding proteolytic, cellulolytic and/or chitinolytic activity (Table 1). Chitinolytic activity is a contributing factor in *Bt* pathogenicity [21] which our most pathogenic strains possessed. The enzymes involved would act on the peritrophic membrane of the host, which facilitates the entry of pathogens into the haemocoel of susceptible insects [21]. In addition, these strains showed no cellulolytic activity in medium supplemented with carboxymethyl cellulose (CMC), one of the products used as a matrix to protect *Bt* spores against high temperatures and UV exposure prevailing in natural environments [46]. 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 [13].

In *Bt*, high levels of protease activity are associated with both crystal and spore formation [47] and this activity may contribute in processing inactive Cry protoxin to active toxin [3].

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, during our investigations, biomass-bound protease and extracellular protease activities were determined in the toxic strains, which were process according to [48]. Proteolytic activity was assayed by using azocasein as substrate [49]. Table 2 shows that *Bt* RT, *Bt* LSM and *Bt* LQ displayed high biomass-bound protease activity. To our knowledge, the presence of this naturally immobilized enzyme activity has not been reported in *Bt* [18]. On the other hand, extracellular protease activity was observed when crude extracts of *Bt* strains were electrophoresed on SDS-PAGE containing gelatin powder [50]. The gels were then processed according to [51] for proteolysis to occur. Gel was stained with 0.1% (wt vol^{-1}) Coomasie Blue R-250. Proteinase K (10 mg ml^{-1}) was used for comparative analyze. All strains presented a clear zone of proteolytic activity which were larger in both *Bt* RT and *Bt* LSM (Figure 11). Although no correlation between protease activities 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 biomass-bound 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 extracellular and biomass-bound protease activities 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 [18].

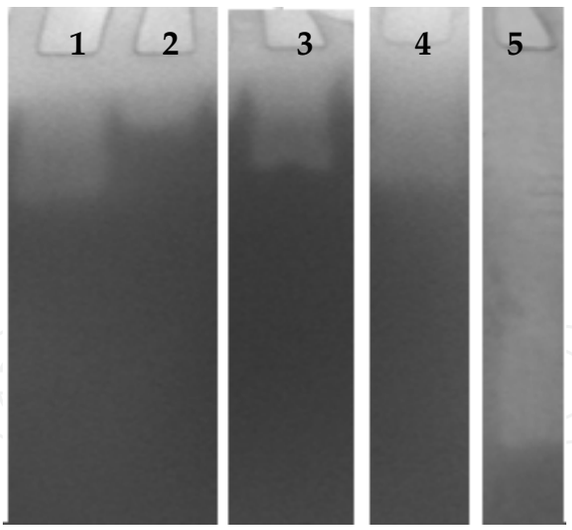

Figure 11. Identification of extracellular protease activity on 10% (wt vol^{-1}) SDS-PAGE containing 0.1% (wt vol^{-1}) gelatin powder. Lanes: 1: *Bt* RT; 2: *Bt* 4D1; 3: *Bt* LQ; 4: *Bt* LSM 5: Proteinase K.

Protein profiles are a useful tool to discriminate among strains, as they provide information about the proximity between species, subspecies and biovars [18, 52]. Considering this, characterization of microorganisms by means of their extracellular isoenzymes showing high polymorphism, as is the case of esterase, is particularly appealing. To determine extracellular esterase profiles, *Bt* strains were processed according to [44]. Briefly, strains

were cultured on LB plates during 48 h at 30 ºC and crude extracts were recovered from solid media. Then, extracts were separated by native-PAGE. Esterase activity was assayed using 1.3 mM of α-naphthyl acetate (C2) derivative as substrate. Known electrophoretic esterase profiles of *Bacillus pumilus* A55 (EF638794.1) (*Bp* A55) were used for comparative analysis. The electrophoretic profiles of esterase activity showed differences among strains (Figure 12). *Bt* LQ showed a unique band/enzyme of 40 kDa as well as *Bt* LSM, but of about 60 kDa while *Bt* RT presented two bands of 95 and 60 kDa. Our results are in accordance with those by Norris [53], since it was possible to differentiate *Bt* strains by comparing the electrophoretic migration profiles of esterase produced during the vegetative growth phase (Figure 12) [13].

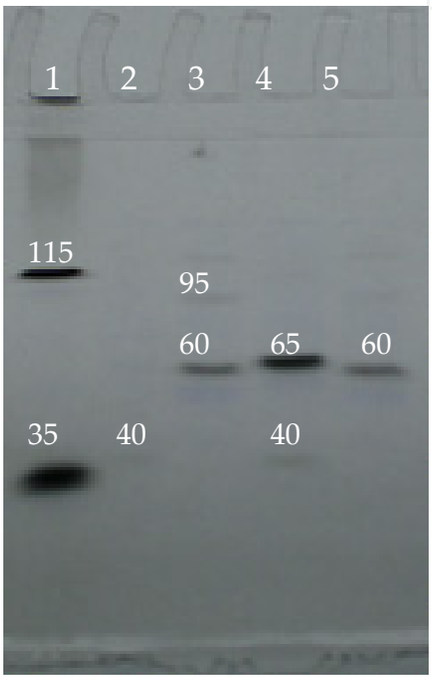

Figure 12. Enzymatic profile of esterase activity in native-PAGE 10%(wt vol^{-1}). Lanes: 1: *Bp* A55; 2: *Bt* LQ; 3: *Bt* RT; 4: *Bt* 4D1; 5: *Bt* LSM. Molecular weight of each band/enzyme is showed in kDa.

6. Conclusion

Lepidoptera causes some of the most devastating insect pests in important crops in America. Since economy of these regions depends largely of agriculture, their control is a priority as well as a necessity. In this context, use of environmentally safe technology to reduce crop damage like *B. thuringiensis* would be extremely valuable. Consequently, we set out to establish and characterize a collection of *Bt* isolates from samples collected in different Argentinean localities in order to find novel strains toxic against insect pests of economically important crops (like soybean and maize).

Fourteen *Bt* strains were isolated and phenotypically, genetically and biologically characterized. Analysis of larvicidal activity indicated that three strains exhibited high toxicity against lepidopteran larvae; this toxicity was in most, higher than that of the reference strain *Bt* 4D1.

The discovery of a highly toxic isolates reveals the usefulness of screening studies for novel *Bt* strains. 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.

Author details

Analía Alvarez and Flavia del Valle Loto
Pilot Plant of Industrial and Microbiological Processes (PROIMI), CONICET, Tucumán, Argentina

Analía Alvarez
Natural Sciences College and Miguel Lillo Institute, National University of Tucumán, Tucumán, Argentina

7. References

[1] Konecka E, Kaznowski A, Ziemnicka J, Ziemnicki K (2007) Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated during epizootics in *Cydia pomonella* L. J. Invertebr. Pathol. 94: 56-63.

[2] Bravo A, Gill S, Soberón M (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 49: 423-435.

[3] Brar S, Verma M, Tyagi R, Surampalli, R Bernabé S, Valéro J (2007). *Bacillus thuringiensis* proteases: Production and role in growth, sporulation and synergism. Process Biochem. 42: 773-790.

[4] Calderón, M, Alcocer González J, Molina M, Tames Guerra R, Rodríguez Padilla (2007). Adjuvant effects of crystal proteins from a Mexican strain of *Bacillus thuringiensis* on the mouse humoral response. Biologicals *35*, 271-276.

[5] Virla E, Alvarez A, Loto F, Pera L, Baigorí M (2008) Fall Armyworm strains (Lepidoptera: Noctuidae) in Argentina, their associate host plants and response to different mortality factors in laboratory. Fla. Entomol. 91: 63-69.

[6] Moreno Fajardo O, Serna Cardona F (2006). Biología de *Peridroma saucia* (Lepidoptera: Noctuidae: Noctuinae) en flores cultivadas del híbrido comercial de *Alstroemeria* spp. Rev. Fac. Nal. Agr. Medellín. 2: 3435-3448.

[7] Berón C, Salerno G (2006) Characterization of *Bacillus thuringiensis* isolates from Argentina that are potentially useful in insect pest control. BioControl 51: 779-794.

[8] Gomes Monnerat R, Cardoso Batista A, Telles De Medeiros P, Soares Martins E, Melatti VM, Praca IB, Dumas VF, Morinaga C, Demo C, Menezes Gomes AC, Falcao R, Siqueira CB, Silva-Werneck JO, Berry C (2007) Screening of Brazilian *Bacillus thuringiensis* isolates active against *Spodoptera frugiperda*, *Plutella xylostella* and *Anticarsia gemmatalis*. Biol. Control 41: 291-295.

[9] Gassmann A, Carrière Y, Tabashnik E (2009) Fitness Costs of Insect Resistance to *Bacillus thuringiensis.* Annu. Rev. Entomol. 54: 147-63.

[10] Tabashnik B, Gassmann A, Crowder D, Carriére Y (2008) Insect resistance to *Bt* crops: evidence versus theory. Nat. Biotechnol. 26: 199-202.

[11] Tabashnik B, Carrière Y (2009) Environmental Impact of Genetically Modified Crops. In: Ferry N, Gatehouse A, editors. Insect Resistance to Genetically Modified Crops. Cambridge: CAB International.74-101pp.

[12] Christou P, Capell T, Kohli A, Gatehouse J, Gatehouse A (2006) Recent developments and future prospects in insect pest control in transgenic crops. Trends Plant. Sci. 11: 302-308.

[13] Sauka D, Benintende G (2008) *Bacillus thuringiensis*: generalidades. Un acercamiento a su empleo en el biocontrol de insectos lepidópteros que son plagas agrícolas. Rev. Argent. Microbiol. 40: 124-140.

[14] Alvarez A, Virla E, Pera L, Baigorí M (2011) Biological characterization of two *Bacillus thuringiensis* strains toxic against *Spodoptera frugiperda.* World J. Microbiol. Biotechnol. 27: 2343-2349.

[15] Carozzi N, Kramer V, Warren G, Evola S, Koziel MG (1991) Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl. Environ. Microbiol. 57: 3057-3061.

[16] Padidam M (1992) The insecticidal crystal protein Cry1A (c) from *Bacillus thuringiensis* is highly toxic for *Heliothis Armigera*. J. Invertebr. Pathol. 59: 109-111.

[17] Ben-Dov E, Zaritski A, Dahan E, Barak Z, Sinai R, Manasherob R, Khamraeb A, Troitskaya E, Dubitsky A, Berezina N, Margalith Y (1997) Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. Appl. Environ. Microbiol. 63: 4883-4890.

[18] Hansen B, Damgaard P, Eilenberg J, Pedersen JC (1998) Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated from leaves and insects. J. Invertebr. Pathol. 71: 106-114.

[19] Alvarez A, Virla E, Pera L, Baigorí M (2009a). Characterization of native *Bacillus thuringiensis* strains and selection of an isolate active against *Spodoptera frugiperda* and *Peridroma saucia*. Biotechnol. Lett. 31: 1899-1903.

[20] Porcar M, Juarez-Perez V (2003) PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol. Rev. 26: 419-432.

[21] Martínez C, Ibarra J, Caballero P (2005) Association analysis between serotype, *cry* gene content, and toxicity to *Helicoverpa armigera* larvae among *Bacillus thuringiensis* isolates native to Spain. J. Invertebr. Pathol. 90: 91-97.

[22] Soberón M, Bravo A (2001) *Bacillus thuringiensis* y sus toxinas insecticidas. In: Microbios en línea. UNAM México. Available vía DIALOG http://www.biblioweb.dgsca.unam.mx/libros/microbios /Cap12 Accessed 1 March 2011.

[23] Alvarez A, Pera L, Loto F, Virla E, Baigori M (2009b) Insecticidal crystal proteins from native *Bacillus thuringiensis*: Numerical analysis and biological activity against *Spodoptera frugiperda*. Biotechnol. Lett. 31: 77-82.

[24] Sharif F, Alaeddinoğlu N (1988) A rapid and simple method for staining of the crystal protein of *Bacillus thuringiensis*. J. Ind. Microbiol. 3: 227-229.

[25] Arango J, Romero M, Orduz S (2002) Diversity of *Bacillus thuringiensis* strains from Colombia with insecticidal activity against *Spodoptera frugiperda* (Lepidoptera: Noctuidae). J. Appl. Microbiol. 92: 466-474.

[26] Del Rincón-Castro M, Méndez-Lozano J, Ibarra J (2006) Caracterización de cepas nativas de *Bacillus thuringiensis* con actividad insecticida hacia el gusano cogollero del maíz *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Folia Entomol. Mex. 45: 157-164.

[27] Seligy V, Rancourt J (1999) Antibiotic MIC/MBC analysis of *Bacillus*-based commercial insecticides: use of bioreduction and DNA-based assays. J. Ind. Microbiol. Biotechnol. 22: 565-574.

[28] Gough J, Kemp D, Akhurst R, Pearson R, Kongsuwan K (2005) Identification and characterization of proteins from *Bacillus thuringiensis* with high toxic activity against the sheep blowfly, *Lucilia cuprina*. J. Invertebr. Pathol. 90: 39-46.

[29] González J, Carlton B. (1980) Patterns of plasmid DNA in crystalliferous and acrystalliferous strains of *Bacillus thuringiensis*. Plasmid 3:92–98.

[30] Reyes-Ramírez IJ (2008) Plasmid patterns of *Bacillus thuringiensis* type strains. Appl. Environ. Microbiol. 74: 125-129.

[31] Kronstad J, Schnepf H, Whiteley H (1983) Diversity of locations for *Bacillus thuringiensis* crystal protein genes. J. Bacteriol. 154:419–428.

[32] Kado C, Liu S (1981) Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. *145:*1365-73.

[33] Chak K, Chao D, Tseng M, Kao S, Tuan, S Feng, T (1994) Determination and distribution of *cry*-type genes of *Bacillus thuringiensis* isolated from Taiwan. Appl. Environ. Microbiol. 60:2415-2420.

[34] Weisburg W, Barns S, Pelletier D, Lane D (1991) 16S Ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697-703.

[35] Daffonchio D, Borin S, Frova G et al (1998) PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. Int. J Syst Bacteriol 48:107–116.

[36] Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A, Ortiz M, Lina L, Villalobos FJ, Peña G, Nuñez-Valdez ME, Soberón M, Quintero R (1998) Characterization of c*ry* genes in Mexican *B. thuringiensis* strain collection. Appl. Environ. Microbiol. 64: 4965-4972.

[37] Ibarra J, del Rincón M, Ordúz S, Noriega D, Benintende G, Monnerat R, Regis L, Oliveira C, Lanz H, Rodriguez M, Sánchez J, Peña G, Bravo A (2003) Diversity of

Bacillus thuringiensis strains from Latin America with insecticidal activity against different mosquito species Appl. Environ. Microbiol. 69:5269–5274.

[38] Cerón J, Covarrubias L, Quintero R, Ortiz M, Aranda E, Lina L, Bravo A (1994) PCR Analysis of the *cryI* Insecticidal Crystal Family Genes from *Bacillus thuringiensis*. Appl. Environm. Microbiol. 60:353-356.

[39] Al-Momani F, Saadoun I, Obeidat M (2002) Molecular characterization of local *Bacillus thuringiensis* strains recovered from Northern Jordan. J. Basic Microbiol. 2: 156-161.

[40] Noguera P, Ibarra J (2010) Detection of New *cry* Genes of *Bacillus thuringiensis* by Use of a Novel PCR Primer System. Appl. Environ. Microbiol. 76: 6150-6155.

[41] Benintende G, López-Meza J, Cozzi J, Ibarra JE (1999) Novel non-toxic isolates of *Bacillus thuringiensis.* Lett .Appl. Microbiol. 29: 151-155.

[42] Daffonchio D, Raddadi N, Merabishvili M (2006) Strategy for identification of *Bacillus* cereus and *Bacillus thuringiensis* strains closely related to *Bacillus anthracis*. Appl. Environ. Microbiol. 72:1295–1301.

[43] Ramírez R, Ibarra J (2005) Fingerprinting of *Bacillus thuringiensis* type strains and isolates by using *Bacillus cereus* group-specific repetitive extragenic palindromic sequence-based PCR analysis. Appl. Environ. Microbiol. 71: 1346-1355.

[44] Stefanova M, Leiva M, Larrinaga L, Coronado M (1999) Metabolic activity of *Trichoderma* spp. isolates for a control of soilborne phytopatogenic fungi. Rev. Fac. Agron. (LUZ) 16: 509-516.

[45] Lengyel MJ, Pekár S, Felföldi G, Patthy A, Gráf L, Fodor A, Venekei I (2004) Comparison of Proteolytic Activities Produced by Entomopathogenic *Photorhabdus* Bacteria: Strain- and Phase-Dependent Heterogeneity in Composition and Activity of Four Enzymes. Appl. Environ. Microbiol. 70: 7311-7320.

[46] Cokmus C, Elcin M (1995) Stability and controlled release properties of carboxymethylcellulose-encapsulated *Bacillus thuringiensis* var. israelensis. Pest. Sci. 45: 351-355.

[47] Ibarra JE, Del Rincón Castro MC, Galindo E, Patiño M, Serrano L, García R, Carrillo JA, Pereyra-Alférez B, Alcázar-Pizaña A, Luna-Olvera H, Galán-Wong L, Pardo L, Muñoz-Garay C, Gómez I, Soberón M, Bravo A (2006) Los microorganismos en el control biológico de insectos y fitopatógenos. Rev. Latinoam. de Microbiol. 48: 113-120.

[48] González C, Martínez A, Vázques F, Baigorí M, Figueroa L (1996) New method of screening and differentiation of exozymes from industrial strains. Biotechnol. Tech. 10: 519-522.

[49] Secades P, Guijarro A (1999) Purification and Characterization of an Extracellular Protease from the Fish Pathogen *Yersinia ruckeri* and Effect of Culture Conditions on Production. Appl. Environ. Microbiol. 65: 3969-3975.

[50] Heussen C, Dowdell E (1980) Electrophoretic analysis of plasminogen activators in polyacrilamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. 102: 196-202.

[51] Ferrero M, Castro G, Abate M, Baigorí M, Siñeriz F (1996) Thermostable alkaline proteases of *Bacillus licheniformis* MIR 29: Isolation, Production and Characterization. Appl. Microbiol. Biotechnol. 45: 327-332.

[52] Berber I (2004) Characterization of *Bacillus* species by numerical analysis of their SDS-PAGE protein profiles. J. Cell Mol. Biol. 3: 33-37.

[53] Norris J (1964) The classification of *Bacillus thuringiensis*. J. Appl. Bacteriol. *27:*39- 447.

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