RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY

International Microbiology (2012) 15:209-216
DOI: 10.2436/20.1501.01.174 ISSN 1139-6709 **www.im.microbios.org**

New combinations of *cry* genes from *Bacillus thuringiensis* strains isolated from northwestern Mexico

Gretel Mendoza,[1] Amelia Portillo,[2] Efraín Arias,[1] Rosa M. Ribas,[3] Jorge Olmos[1]*

[1]Department of Marine Biotechnology, Center for Scientific Research and Education (CICESE), Ensenada, B.C., Mexico. [2]Faculty of Sciences, Autonomous University of Baja California, Ensenada, B.C., Mexico. [3]Department of Microbiology, School of Biological Sciences, National Technical Institute, Mexico, D.F., Mexico

Received 8 October 2012 · Accepted 7 November 2012

Summary. Twenty eight *Bacillus thuringiensis* strains isolated from the Tijuana-Ensenada region of northwestern Mexico were analyzed to determine the distribution of *cry* and *cyt* genes. Crystal production by the strains was examined by scanning electron microscopy, which showed the predominance of cubic crystals. Alkaline-dissolved and trypsin activated crystals were also analyzed by SDS-PAGE, yielding bands of 40–200 kDa. The *cry1* and *cry2* genes were molecularly characterized using general and newly designed specific primers in addition to other oligonucleotides (*cry3*, *cry4*, *cry8*, *cry9*, *cry11*, *Nem*, *cry25*, *cry29* and *cyt*), resulting in the identification of novel gene combinations. The use of specific primers for *cry1A*, *cry1B*, *cry1C*, *cry1D*, *cry1E*, *cry1F* and *cry2Aa*, *cry2Ab*, *cry2Ac*, *cry2Ad* showed differences in the distribution of *cry1* (36 %), *cry2* (71 %), and *cyt* (40 %) in strains from Tijuana-Ensenada compared to other previously studied regions. Bioassays were conducted on *Manduca sexta* larvae to analyze the Cry insecticidal capacity of the isolated strains. The hemolytic activity of the Cyt toxin from the same strains was assessed in human erythrocytes. [**Int Microbiol** 2012; 15(4):209-216]

Keywords: *Bacillus thuringiensis* · Cry proteins · Cyt proteins · insecticidal activity

Introduction

According to the Mexican Commission for the Knowledge and Use of Biodiversity [http://www.conabio.gob.mx/conocimiento/manglares/doctos/manglaresMexico.pdf], Mexico is a megadiverse country, the fourth richest nation in biodiversity after Colombia, Brazil, and Indonesia. The diversity of insects in Mexico is estimated at about 110,000 species, of which 10,000 belong to Hemiptera, 20,000 to Diptera, 21,000 to Hymenoptera, and more than 35,000 to Coleoptera [Morón and Valenzuela, 1993, *Revista de la Sociedad Mexicana de Historia Natural*].

***Corresponding author:** J. Olmos
Department of Marine Biotechnology
PO Box 430222
San Diego, CA 92143-0222, USA
Tel + 52-6461750500. Fax 52-6461750534
E-mail: jolmos@cicese.mx

Bacillus thuringiensis (Bt) is a ubiquitous gram-positive, spore-forming bacterium that has been isolated all over the world from many different habitats, including soil, water, plant leaves, dead insects, and cobwebs [1,4,10]. During spore synthesis, Bt also produces a mixture of δ-endotoxins, known as Cry and Cyt toxins [5,12]. These proteins form crystalline parasporal inclusions bodies in the mother-cell compartment [2]. Genes encoding the Bt Cry and Cyt toxins are frequently located on plasmids [6,14], implying a high degree of genetic plasticity that results in a wide variety of Bt strains and crystal protein diversity [3,14]. Cry proteins show toxic activity against Lepidoptera, Coleoptera, Diptera, Hymenoptera, mites, and other invertebrates as well as against nematodes, flatworms, and protozoa [1,2,11]. In contrast, Cyt proteins specifically target dipteran insects. Importantly, Cry and Cyt proteins, including their solubilized and trypsin-activated forms, are not in any way toxic for mammals, birds, amphibians, and reptiles [7,13].

The variation in the frequency distribution of the *cry* and *cyt* genes in Bt isolates, even those from the same country, is well recognized and likely reflects differences in biological, geographical, and ecological conditions, but it may also be directly related to the diversity of insects from region to region [15]. Based on these observations, the co-evolution of *cry* genes and insects has been proposed [1,14,15]. In this study, 28 Bt isolates were analyzed with newly developed primers to determine the frequency of *cry1*, *cry2*, and *cyt* genes in strains from northwestern Mexico. Our results differ from those of a previously reported study but contribute to the pool of knowledge on the distribution of *cry* and *cyt* genes.

Materials and methods

Isolation of *Bacillus thuringiensis* strains. Bt strains used to produce the commercial agricultural insecticides DiPe1, containing spores and crystals of Bt var. *kurstaki*, and Xentari DF, containing spores and crystals of Bt var. *aizawai*, were used as controls. Samples were obtained from different geographical locations of Tijuana-Ensenada, in western Mexico. Each of the 50 isolates was grown in 50 ml of SP liquid medium [9] for 30 °C at 96 h with constant agitation at 275 rpm. The production of Bt spores and crystals was screened every 24 h by phase-contrast microscopy and malachite green staining (malachite green oxalate salt, Sigma). Spores and crystal inclusions were harvested after sporulation from 28 positive Bt isolates by centrifugation for 10 min at 10,000 rpm, in 50-ml conical tubes. The pellets were washed with a buffer consisting of 0.5 M NaCl, 0.01 M EDTA, pH 8.0, and centrifuged for 10 min at 10,000 rpm. The procedure was repeated twice. The pellets were then washed with 20 ml of 0.1 M phenylmethylsulfonil fluoride (PMSF) per 50-ml culture, twice repeating the procedure, and maintained at –20°C until the activation of Cry proteins.

Identification of crystal morphology by scanning electron microscopy. Crystal-spore pellets were thawed, washed twice with 5 ml of deionized water, and centrifuged at 10,000 rpm for 10 min. The crystals were suspended in 0.5 ml of deionized water and 10-µl aliquots were placed on glass slides, which were allowed to air dry. The samples were processed in the Nanoscience and Nanotechnology Center-UNAM, by coating with a gold layer using a vacuum evaporator (JEOL: JEE-400). They were then observed in a JSM-5300 scanning electron microscope.

DNA purification. Bt strains were grown in LB medium for 12 h [1]. Total DNA was then extracted according to a previously reported protocol [15] in cells harvested by centrifugation at 9000 ×*g* for 10 min.

Molecular characterization by PCR of cry and cyt genes. The *cry* and *cyt* genes from Bt strains were identified using the following specific and conserved previously reported primers [1,5,15]: *cry1A, cry1D, cry3,*

Table 1. Characteristics of the general and specific primers used in the identification of *cry* genes

Primer pair	Sequence	Gene recognized	Annealing temp. (°C)	Product size (bp)	Accession no.
Gral-cry1	5´-GCGGTGAATGCTCTGTTT (f) 5´-TTTATCTGCCGCATGAATC (r)	*cry1A, cry1B, cry1C, cry1D cry1E, cry1F*	50	990	EF102874.1
Gral-cry2	5´-ACCTTTATTTGCACAGGCA (f) 5´-AATATCTGAAAAACGAGCTC (r)	*cry2Aa,cry2Ab cry2Ac, cry2Ad*	50	1249	AF273218.1
spe-cry1B	5′-CTTCATCACGATGGAGTAA (f) 5′ -CATAATTTGGTCGTTCTGTT (r)	*cry1B*	50	369	EF102874.1
spe-cry1C	5′-CAAAGATCTGGAACACCTT (f) 5′-CAAACTCTAAATCCTTTCAC (r)	*cry1C*	50	131	AY955268.1
spe-cry1E	5′-GAACCAAGACGAACTATTG (f) 5′- TGAATGAACCCTACTCCC (r)	*cry1E*	50	144	173252.1
spe-cry1F	5′-GCAGGAAGTGATTCATGG (f) 5′-CAATGTGAATGTACTTTGCG (r)	*cry1F*	50	432	EU679501.1
spe-cry2Aa	5′-CAAGCGAATATAAGGGAGT (f) 5′ TAGCGCCAGAAGATACCA (r)	*cry2Aa*	50	460	AF273218.1
spe-cry2Ab	5′-CACCTGGTGGAGCACGAG (f) 5′-GTCTACGATGAATGTCCC (r)	*cry2Ab*	50	771	AF336115.1
spe-cry2Ac	5′-GCAGACACCCTTGGTCGT(f) 5′-TGGCAACGCCCTCCCGAT(r)	*cry2Ac*	50	841	EU360896.1
spe-cry2Ad	5′-TCAAAATCACCTGAGAAA(f) 5′-ATTAGGACCCCCTATAC (r)	*cry2Ad*	50	442	DQ358053.1

cry4, nem (cry5, cry12, cry14, cry21), *cry8, cry9, cry10, cry11, cry25, cry29* and *cyt*. In addition, new *cry1* and *cry2* primers were designed from specific and conserved sequences. The *cry* and *cyt* genes were amplified according to previously reported conditions [1,5,15] and annealing temperatures (Table 1).

Protein electrophoresis. Denaturing SDS-PAGE [8] was performed using a 10 % separating gel (wt/vol). Samples were run at 25 mA for approximately 40 min and at 30 mA for approximately 1.5 h. The gel was stained with 0.4 % Coomassie brilliant blue R250 (Sigma, St. Louis, MO, USA). The molecular masses of the proteins of interest were determined using a commercial molecular mass marker as reference (Precision Plus Protein All Blue Standard from BioRad).

Solubilization and trypsin activation of crystal inclusions. The crystal-spore pellets were thawed, washed twice with 5 ml of deionized water, and centrifuged at 10,000 rpm for 10 min. The pellets were suspended in TNT buffer (20 mM Tris, 300 mM NaCl, Triton X-100 at 0.1 %, pH 7.2), incubated at 37 °C for 30 min, and sonicated for 6 min at 20 Watts. The proteins were solubilized at an alkaline pH of 10.5 either with 0.5 M sodium carbonate-sodium bicarbonate or with 0.1 M sodium hydroxide and reducing conditions obtained with 0.2 % β-mercaptoethanol. Solubilized proteins were recovered from the supernatant after centrifugation of the samples at 10,000 rpm for 10 min and then analyzed by SDS-PAGE. They were then activated by incubation with different trypsin concentrations (5–50 µg/ml, final concentrations) for 30 min to 2.5 h at 37 °C. The reaction was stopped by the addition of 1 mM PMSF (final concentration). Activated toxins were recovered from the supernatant after centrifugation at 10,000 rpm for 10 min and stored at –70ºC until used in the toxin assays. The presence of activated Cry proteins was confirmed by SDS-PAGE (data not shown). The amount of protein was quantified according to the Lowry method, using bovine serum albumin as reference.

Insecticidal activity. The toxicity of the activated Cry proteins was tested in *Manduca sexta* larvae. Toxin concentrations of 2000, 1000, and 100 ng/cm^2 were placed in each well of a 24-well plate containing artificial food for *Manduca sexta* larvae and maintained under sterile conditions. The plates were allowed to dry, sealed with plastic wrap, and incubated for 7 days under a photoperiod of 12 h light/12 h darkness at 26 ºC and a relative humidity of 60 %. The number of dead larvae per plate was counted. Three repetitions, involving 24 larvae per concentration, were prepared and analyzed.

Hemolytic activity. Hemolytic activity was assayed as previously described [16]. One hundred µl of a 0.1 % erythrocyte suspension was added to each well of a U-bottomed 96-well plates, followed by the addition of 100 µl of activated toxin at concentrations of 5, 2.5, and 1.25 µg/ml. The negative control was not inoculated with toxins. The positive control for hemolysis was prepared by mixing 100 µl of the erythrocyte suspension with a final concentration of 1 % Triton X-100. Hemolytic activity in each well was quantified using an automatic blood cell counter after incubation of the samples for 24 h at 37 °C. The test was performed in triplicate.

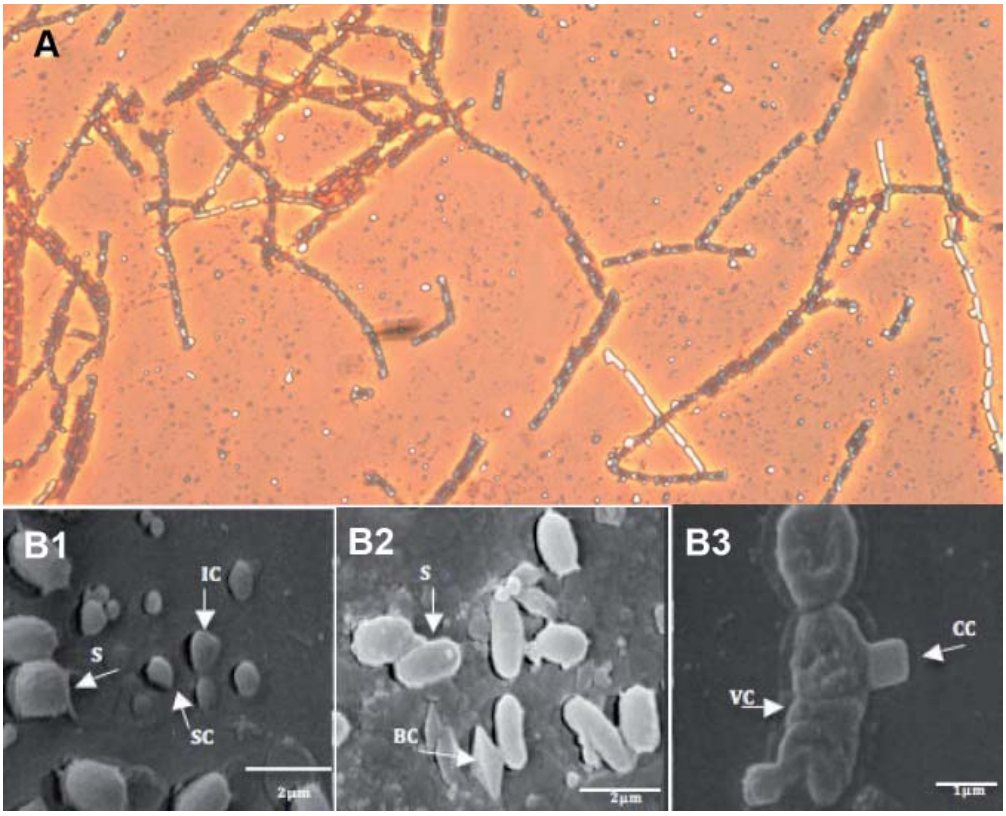

Fig. 1. (**A**) Phase-contrast microscopy. Vegetative cells, spores, and crystals (dark and white dots) of different sizes and shapes are shown outside strain 18-5 cells. (**B**) Electron microscopy of the isolated crystals: (B1) Scanning electron microscopy (SEM) images from strain 10-2; SC-IC, spherical, irregular crystals, and S, spores. (B2) SEM images from strain M1#8; BC, bipyramidal crystals, and S, spores. (B3) SEM images from strain 8-3; CC, cubic crystals, S, spores, and VC, vegetative cells.

Results

Isolation of *Bacillus thuringiensis* strains and crystal characterization. Forty nine positive *Bacillus* strains were isolated by applying the temperature spore resistance methodology to 73 samples. Crystals produced by the cultured strains were analyzed by phase-contrast (Fig. 1A) and scanning electron (Fig. 1B) microscopy. The former showed vegetative cells as well as spores and crystals (dark and white dots) of different sizes and shapes. Figure 2 shows the frequency of the diverse crystal morphology encountered in some of the 28 isolated strains.

Identification of *cry* and *cyt* in the isolated strains. Figure 3 summarizes the *cry* and *cyt* genes determined in the 28 Bt isolates using both sets of primers in Table 1. As seen in the figure, the analyzed Bt strains showed little diversity in their *cry* genes. The most frequently occurring gene was *cry2*, detected in 20 of the 28 isolates and accounting for almost 71 % of all *cry* genes, followed by *cry1,* contained in 10 of the 28 Bt isolates and representing 36 % of the total *cry* genes. Three other *cry* genes, *cry11*, *cry8*, and *cry3*, were also identified in isolates from this region of Mexico, in 20 %, 11 % and 11 % of the strains, respectively. Four of the 28 Bt isolated strains did not amplify with any of the tested *cry* oligonucleotides, indicating the presence of new genes (Table 2). Amplification of *cyt* genes yielded a frequency of 40 % in the 28 Bt isolated strains. Interestingly, the diversity and frequency distribution of *cry* and *cyt* genes differed from others regions of Mexico and from other countries, most likely reflecting differences in the geographical and climate conditions. The predominance of cubic crystals in the 28 Bt isolates matched well with that of *cry2* (Figs. 2 and 3).

Analysis of *cry* gene profiles in the isolated strains. The study of *cry* gene profiles from Bt isolates showed the presence of two or more *cry* gene combinations (Table 3). *cry2Aa*, the most abundant gene identified, was detected in 20 of the 28 strains, while *cry2Ab* occurred in 18 of the 28 strains (Table 2). *cry2Ac* was detected in 4 of the Bt strains. Ten of the 28 isolates were positive for *cry1* genes, with c*ry1A* determined in 10 of the 28 strains, followed by *cry1B*, *cry1C*, and *cry1D* genes, each of which were detected in 3, 1, and 3 of the 28 isolates (Table 2). Sixteen different *cry* and *cyt* gene profiles, including *cry1, cry2, cry3, cry8, cry11*, and *cyt*, were present in our collection. The most abundant profile were the combination *cry2Aa*/*cry2Ab* and combination *cry2Aa*/*cry2Ab*/*cry2Ac* (Table 3).

Characterization of Cry protein profiles. To characterize the Cry protein profiles from each of the 28 Bt isolated strains, SDS-PAGE was performed after alkaline pH solubilization and trypsin digestion of the protoxins (see Materials and methods). Bands between 40 and 200 kDa were obtained and are described together with the protein profiles in Table 2.

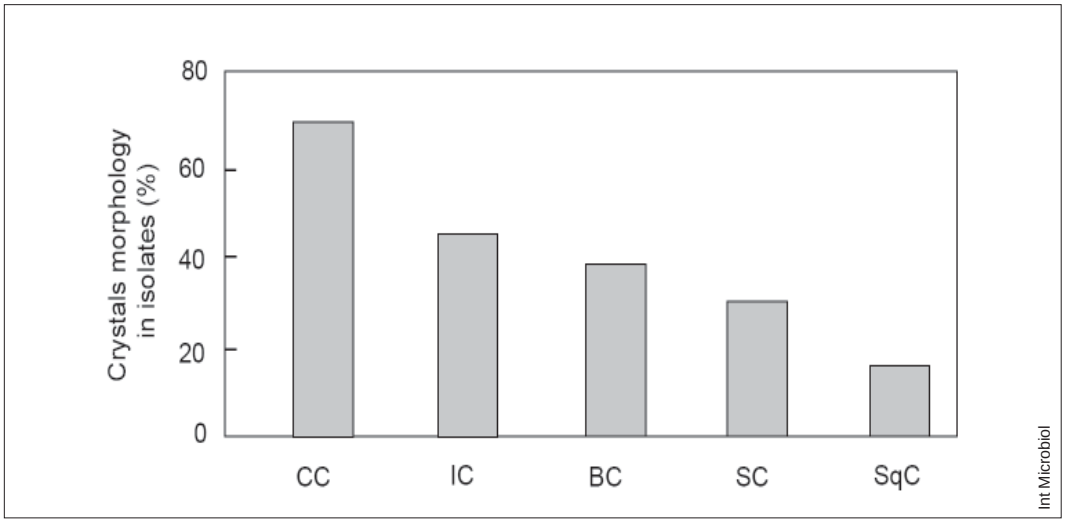

Fig. 2. The crystals composition (% abundance) of Bt strains isolated from Tijuana-Ensenada, in western Mexico. CC: cubic crystals (68 %). IC: irregular crystals (43 %). BC: bipyramidal crystals (36 %). SC: spherical crystals (28 %). SqC: square crystals (14 %).

Table 2. Protein size, gene profiles, and insecticidal and hemolytic activity of the isolated strains

Strain	Protein profile	*cry* and *cyt* genes identification	Crystal shape*	Insecticidal activity (ng/cm^2) 2000, 1000, 100	Hemolytic activity (µg/ml) 5, 2.5, 1.25
M1#4	200, 130, 88, 75, 65, 44	*cry2Aa, cry2Ab, cry2Ac, cyt*	CC, IC	nt	+ – –
M1#7	200, 160, 140, 88, 75	*cry1A, cry1B, cry2Aa, cry2Ab*	BC, CC	+ + –	– – –
M1#8	160, 140, 88	*cry1A, cry2Aa, cry2Ab*	BC	+ + –	– – –
M2#2	180, 150, 120, 100, 88, 65	*cry2Aa, cry2Ab, cry8*	CC, SC	nt	– – –
M2#7	88, 70, 65, 50	*cry2Aa, cry2Ab, cyt*	CC, IC	nt	– – –
1-2	200, 130, 100, 88, 75, 65	*cry1A, cry1D, cry2Aa, cry2Ab, cry8*	BC, CC	+ + +	– – –
2-2	200, 88, 75	*cry2Aa, cry2Ab*	CC, IC	nt	– – –
4-2	200, 88, 75, 68, 65, 62, 50	*cry2Aa, cry2Ab, cry2Ac, cyt*	CC, SC	nt	– – –
4-5	200, 88, 75, 68, 65, 62	*cry2Aa, cry2Ab*	CC	nt	– – –
5-4	150, 120, 75, 65, 55, 48	*cry2Aa, cry2Ab, cry2Ac, cyt*	CC, IC	nt	+ – –
6-1	250, 130, 88, 75, 65	*cry1A, cry2Aa, cry2Ab*	BC, CC, SC	+ – –	– – –
6-3	88, 75, 65, 55, 48		SC, IC	nt	– – –
6-4	200, 88, 75, 70, 44		CC	nt	– – –
7-2	200, 88, 70	*cry1A, cry1B, cry2Aa, cry2Ab*	BC, CC	+ + –	– – –
7-3	210, 40	*cry11, cyt*	SC, IC	nt	+ + +
8-3	130, 88, 75, 65, 50	*cry1A, cry2Aa, cry2Ab, cyt*	BC, CC	+ + –	+ + –
8-4	200, 130, 120, 100, 88, 70, 65, 50	*cry1A, cry1D, cry8, cry11, cyt*	BC, IC, SC	+ + +	+ – –
9-2	140, 110, 75	*cry2Aa, cry2Ab, cry11*	CC, SqC, SC	nt	– – –
10-2	150, 88, 70, 55		IC, SC	nt	– – –
11-4	200, 88, 70, 67	*cry2Aa, cry2Ab*	CC	nt	– – –
12-2	200, 88, 70, 67, 66, 44	*cry1Aa, cry1C, cry2Aa, cry3, cyt*	BC, IC	+ + –	– – –
12-6	200, 88, 75	*cry3, cry11*	SqC	nt	– – –
13-4	80		CC	nt	– – –
14-1	200, 55, 47, 40	*cry3, cry11, cyt*	IC, SqC	nt	+ + –
17-3	160, 88, 72, 44	*cry1A, cry1B, cry2Aa, cyt*	BC, IC, CC	+ + –	+ + –
18-2	130, 88, 70	*cry2Aa, cry2Ab, cry2Ac*	CC	nt	– – –
18-5	100, 75, 50	*cry1A, cry1D, cry2Aa, cry2Ab*	BC, SqC	+ + +	– – –
19-1	88, 75, 50	*cry2Aa, cry2Ab, cry2Ac, cyt*	CC, IC	nt	+ + +

–: No amplification and no activity; +: positive activity; nt: not tested.
*BC: bipyramidal crystal. CC: cubic crystal. IC: irregular crystal. SC: spherical crystal. SqC: square crystal.

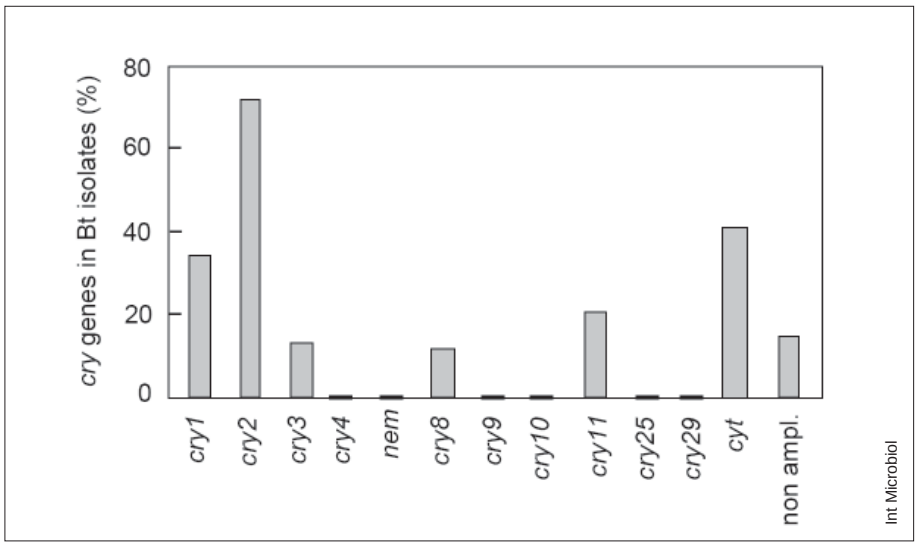

Fig. 3. The *cry* and *cyt* genes detected in the 28 Bt isolates.

Insecticidal activity on *Manduca sexta* larvae. Bioassays were carried out with Cry proteins produced from the isolated strains (Table 2). The toxic effects on *Manduca sexta* larvae were measured by including pre-defined concentrations of the proteins in the larval diet, with feeding carried out for a period of 7 days. The results were considered positive when 50 % of the larvae in the test well had died at the end of the experiment. Based on this criterion, strains 1-2, 8-4, and 18-5, containing the *cry* gene profile *cry1A*/*cry1D*, were the most toxic against *Manduca sexta*, as their toxins were effective even at the lowest concentration (Table 2).

Hemolytic activity on human erythrocytes. Hemolytic activity on human erythrocytes was determined in 11 of the 28 Bt isolates, in agreement with the number of Bt strains harboring *cyt* genes (Table 2). Strains 7-3 and 19-1 had hemolytic activity at toxin concentrations of 5, 2.5, and 1.25 µg/ml, and strains 8-3, 14-1, and 17-3 at 5 and 2.25 µg/ml. Strains M1#4, M2#7, 4-2, 5-4, 8-4 and 12-2, while also positive, showed hemolytic activity only at the highest toxin concentration (Table 2).

Discussion

The co-evolution of toxins and insects has been postulated to account for the high degree of variability of Bt strains, which produce a broad range of Cry and Cyt proteins active against most insect pests. In 1998, a collection of Bt strains isolated from soil samples taken throughout Mexico, with the exception of the Baja California peninsula, was described [1]. In that study, the *cry1* gene was determined to be the most abundant (49.5 %) in the isolated strains, followed by *cry3* (20 %) and *cry4* (10 %). However, the presence of *cry2* was not analyzed and Bt strain selection was largely based on phase-contrast microscopy. This approach favors isolates producing bipyramidal crystals (Cry1 toxin), which are more readily distinguished than cubic (Cry2 toxin), rhomboid, oval, or irregular crystals. In the present work, strain selection based on Bt crystal production was carried out by phase-contrast and scanning electron microscopy. Surprisingly, among the 28 Bt isolates cubic crystals, indicative of Cry2 toxins, were the most abundant (68 %) whereas bipyramidal crystals, formed by Cry1 toxins, were produced by 38 % of the strains and were thus the third most abundant type. Accordingly, the strains isolated in this study differed from those obtained from other areas in Mexico, where bipyramidal was the most abundant crystal type [1]. To corroborate our scanning electron microscopy results of crystal characterization, the *cry* genes of the isolates were identified using general primers as well as primers specifically designed for this study (Table 1). The latter principally targeted the *cry1*, *cry2*, and *cyt* gene combinations typical of this geographical area. As shown in Fig. 3, the results confirmed *cry2* as the most abundant gene in the 28 Bt isolates. Additionally, the predicted size of the Cry2 protein (50–75 kDa) was almost always confirmed by the protein profiles determined by SDS-PAGE, thus supporting *cry2* gene identification.

On the other hand, the detection of *cry1* in 36 % of the isolates is in agreement with previously reported results [1], suggesting a similar distribution of this gene throughout Mexico. Other *cry* genes highly distributed in this region were

Table 3. The *cry* and *cyt* gene combination profiles in the isolated strains

cry and *cyt* gene profiles	No. of strains
cry1A, cry1B, cry2Aa, cry2Ab	2
cry1A, cry1B, cry2Aa, cyt	1
cry1A, cry1C, cry2Aa, cry3, cyt	1
cry1A, cry1D, cry2Aa, cry2Ab	1
cry1A, cry1D, cry2Aa, cry2Ab, cry8	1
cry1A, cry1D, cry8, cry11, cyt	1
cry1A, cry2Aa, cry2Ab	2
cry2Aa, cry2Ab	3
cry2Aa, cry2Ab, cry8	1
cry2Aa, cry2Ab, cry11	1
cry2Aa, cry2Ab, cyt	1
cry2Aa, cry2Ab, cry2Ac	1
cry2Aa, cry2Ab, cry2Ac, cyt	3
cry3, cry11	1
cry3, cry11, cyt	1
cry11, cyt	1

cry11 (20 %), *cry8* (11 %), and *cry3* (11 %) (Fig. 3). While the predominance of *cry1* has been reported in other countries, a large proportion of those samples were also positive for *cry2*. For example, in a 2003 report from China [17], *cry1*, *cry2*, and *cry9* were detected, respectively, in 76.5 %, 70 %, and 15.5 % of the Bt strains. In Thailand, a 2008 report [15] found that these same genes were present in 81.3 %, 80.6 % and 37.3 % of the isolates. Our findings in 28 Bt strains isolated from the Tijuana-Ensenada region of northwestern Mexico complement current knowledge on *cry* gene distribution.

Also of interest is the relatively high abundance (40 %) of the *cyt* gene in the 28 Bt isolates, as this was not the case in other studies [1,18]. As shown in Table 2 and Fig. 3, *cyt* was the second most frequently amplified gene in this study. This predominance of Cyt is surprising since these proteins are toxic to mosquitoes, which inhabit regions characterized by high levels of rain and/or humidity. However, in Tijuana-Ensenada rain is scarce, there are no tropical forests or large lagoons, and mosquitoes are uncommon. Nevertheless, this region is the only zone in Mexico that is influenced by cold sea water, which results in humidity levels of 50–75 % at least 15 h a day throughout the year, with temperatures fluctuating from 15 to 22 °C. Therefore, the high percentage of Cyt proteins in Bt isolates from this area seems to be related to the high humidity rather than to an abundance of mosquitoes. In addition, the percentage of Bt isolates expressing the *cyt* gene is one of the highest reported thus far.

The distribution of *cry2* and *cyt* in the 28 Bt isolates obtained in this study versus isolates from other areas [1,5] is a topic that deserves careful study in relation to insect distribution and the respective environmental conditions. Analyses of the insecticidal capacity of isolated strains will no doubt yield new and more potent Cry and Cyt toxin combinations. To the best of our knowledge, protein combinations such as Cry2Aa/Cyt, which kill Lepidoptera and Diptera, have not been reported (Table 2). Thus, Bt isolates of this type are good candidates as a multifunctional insecticide, with the advantages of diminishing the need for strain combination and avoiding growth competence issues as well as the need for DNA recombinant technology. Additionally, our results highlight the benefits to be gained by searching for Bt strains in new and extreme geographical areas. Finally, although some of the 28 Bt isolates did not amplify with any of the oligonucleotides tested, they were nonetheless classified as Bt based on their protein profiles and crystal production (Table 2). These strains are likely to be sources of new Cry proteins.

Acknowledgements. This work was supported by the Consejo Nacional de Ciencia y Tecnología, CONACYT-202918. We thank Israel Gradilla for technical assistance and Christian Hernández for editing of the figures.

Competing interest. None declared.

References

1. Bravo A, Sarabia S, López L, Ontiveros H, Abarca C, Ortiz A, Ortiz M, Lina L, Villalobos FJ, Peña G, Núñez-Valdez ME, Soberón M, Quintero R (1998) Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. Appl Environ Microbiol 64:4965-4972
2. Bravo A, Gill SS, Soberón M (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 49:423-435
3. Broderick N, Raffa KF, Handelsman J (2006) Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. Proc Natl Acad Sci USA 103:15196-15199
4. Gao M, Li R, Dai S, Wu Y, Yi D (2008) Diversity of *Bacillus thuringiensis* strains from soil in China and their pesticidal activities. Biol Control 44:380-388

5. Ibarra JE, del Rincón MC, Ordúz S, Noriega D, Benintende G, Monnerat R, Regis L, de Oliveira CMF, Lanz H, Rodriguez MH, 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
6. Ito A, Sasaguri Y, Kitada S, Kusaka Y, Kuwano K, Masutomi K, Mizuki E, Akao T, Ohba M (2004) A *Bacillus thuringiensis* crystal protein with selective cytocidal action to human cells. J Biol Chem 279:21282-21286
7. Jiménez-Juárez N, Muñoz-Garay C, Gómez I, Gill SS, Soberón M, Bravo A (2008) The pre-pore from *Bacillus thuringiensis* Cry1Ab toxin is necessary to induce insect death in *Manduca sexta*. Peptides 29:318-323
8. Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685
9. Lereclus D, Agaisse H, Gominet M, Chaufaux J (1995) Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis spo0A* mutant. Nature Biotechnol 13:67-71
10. Martínez-Alonso M, Escolano J, Montesinos E, Gaju N (2010) Diversity of the bacterial community in the surface soil of a pear orchard based on 16S rRNA gene analysis. Int Microbiol 13:123-134
11. Mora I, Cabrefiga J, Montesinos E (2011) Antimicrobial peptide genes in *Bacillus* strains from plant environments. Int Microbiol 14:213-223
12. Palma L, Hernández-Rodríguez CS, Maeztu M, Hernández-Martínez P, de Escudero IR, Escriche B, Muñoz D, Van Rie J, Ferré J, Caballero P (2012) Vip3C, a novel class of vegetative insecticidal proteins from *Bacillus thuringiensis*. Appl Environ Microbiol 78:7163-7165
13. Pérez C, Fernández LE, Sun J, Folch JL, Gill SS, Soberón M, Bravo A (2005) *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. Proc Natl Acad Sci USA 102:18303-18308
14. Sarrafzadeh MH, Bigey F, Capariccio B, Mehrnia MR, Guiraud JP, Navarro JM (2007) Simple indicators of plasmid loss during fermentation of *Bacillus thuringiensis*. Enzyme Microb Tech 40:1052-1058
15. Thammasittirong A, Attathom T (2008) PCR-based method for the detection of *cry* genes in local isolates of *Bacillus thuringiensis* from Thailand. J Invertebr Pathol 98:121-126
16. Thomas WE, Ellar DJ (1983) *Bacillus thuringiensis* var *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. J Cell Sci 60:181-197
17. Wang J, Boets A, Van Rie J, Ren G (2003) Characterization of *cry1, cry2,* and *cry9* genes in *Bacillus thuringiensis* isolates from China. J Invertebr Pathol 82:63-71
18. Wu Y, Gao M, Dai S, Yi D, Fan H (2008) Investigation of the *cyt* gene in *Bacillus thuringiensis* and the biological activities of Bt isolates from the soil of China. Biol Control 47:335-339