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Identification, Cloning and Expression of an Insecticide *cry8* Gene from *Bacillus thuringiensis* INTA Fr7-4

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Key Words
Bacillus thuringiensis · *cry8* · Expression

Abstract
Insecticidal activity of *Bacillus thuringiensis* is attributed largely to the crystal proteins. These were found with specific toxic activity against insects in different orders. A novel *cry8* gene from *B. thuringiensis* strain INTA Fr7-4 from Argentina was characterized. The encoded protein, Cry8Qa2, is 1184 amino acids long and its sequence is more similar to those of Cry8F class. We cloned and expressed the protein in an acrystalliferous strain of *B. thuringiensis* using two differentially regulated promoters. The recombinant strains produced ovoid crystals with low toxicity against larvae of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). The morphology and insecticidal properties of these crystals resembled those produced by the native strain INTA Fr7-4.

Introduction

Bacillus thuringiensis is a spore-forming, Gram-positive, ubiquitous bacterium, characterized by the production of proteinaceus parasporal inclusions known as 'crystals' [Sauka and Benintende, 2008]. *B. thuringiensis* crystals present a variety of shapes including bipyramidal, cubic, rhomboid, ovoid, rectangular, triangular and irregular forms [Iriarte and Caballero, 2001]. Some crystals are toxic by ingestion to many insect species including pests of agriculture and forestry, and vectors of human and animal diseases [Sauka and Benintende, 2008].

Conventionally, the control of insects has relied mostly on the application of chemical insecticides. However, such insecticides have numerous disadvantages, mainly their toxicity against beneficial insects and the hazards associated with public health and the environment. On the other hand, *B. thuringiensis*-based pesticides have been commercialized for decades as an environmentally friendly alternative [Roh et al., 2007]. Insecticidal activity of *B. thuringiensis* is attributed largely to the crystal (Cry) proteins. These proteins have specific toxic activity against insects in the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera, and also against mites, nematodes, flatworms and protozoa [Sauka and Benintende, 2008]. With the advent of agrobiotechnology, alternative systems to the direct application of the bacterium in the field have been developed. One of the most important was

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the generation of transgenic plants expressing Cry proteins in their tissues, including corn, cotton, soybean and canola as leading exponents [Zaritsky et al., 2010].

Cry proteins are classified according to their amino acid sequence similarity in 72 major groups subdivided into classes and subclasses [Crickmore et al., 2012]. The *cry* genes range in length from approximately 1,900 bp (*cry2Ab*) to 3,600 bp (e.g. *cry1Aa*) and the molecular weight of their protein products varies accordingly, between 50 and 140 kDa [Sauka and Benintende, 2008].

B. thuringiensis strains may harbor a single *cry* gene, such as *B. thuringiensis* serovar *kurstaki* HD-73, with only one *cry1Ac* gene [González et al., 1981], or present complex gene profiles, with up to eight different *cry* genes [Bravo et al., 1998; Jouzani et al., 2008; Juárez-Pérez et al., 1997; Uribe et al., 2003]. In general, a correlation can be established between the classes of *cry* genes and the orders of insects to which the susceptible species belong. Therefore, the *cry* gene repertoire of a *B. thuringiensis* strain can be used to predict the insecticidal activity of the strain [Porcar and Juárez-Pérez, 2003].

The search for *B. thuringiensis* genes encoding new insecticidal proteins is a very active research area which is reflected in the continuous increasing number of *cry* genes deposited in databases. To date, more than 600 different *cry* genes have been reported [Crickmore et al., 2012].

A large collection of *B. thuringiensis* isolates from Argentina is maintained at IMYZA-INTA. In the long term, our goal is to find new insecticidal proteins from the local *B. thuringiensis* insecticidal gene pool to serve as a supply for the development of new tools for crop protection. The entries of the collection have been characterized to different extents [e.g., Benintende et al., 2000; Franco-Rivera et al., 2004; Sauka et al., 2010a].

A screening of biological activity is routinely undertaken for each strain entering to the collection. At this stage, a strain called INTA Fr7-4 showed an ovoid crystal and a low toxicity level against larvae of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). Recently, we have reported the complete sequence of three plasmids of this strain [Amadio et al., 2009]. INTA Fr7-4 was selected to study because its plasmid profile differs from those of some of the reference strains of the species.

The sequence of the INTA Fr7-4 plasmids was obtained by a shotgun sequencing method using a shotgun library of total plasmid DNA. Although the closure of the sequenced plasmids did not include any *cry* gene, some reads that could not be assembled in any plasmid presented similarity to known *B. thuringiensis cry8* genes, suggesting that INTA Fr7-4 could harbor a representative of this class of insecticidal genes.

In this paper we report on the cloning of a novel *cry8* gene from the Argentinean INTA Fr7-4 isolate and the expression of the gene product in an acrystalliferous *B. thuringiensis* strain. In addition, phenotypic characterization and the results of surveying other *cry* genes are also presented.

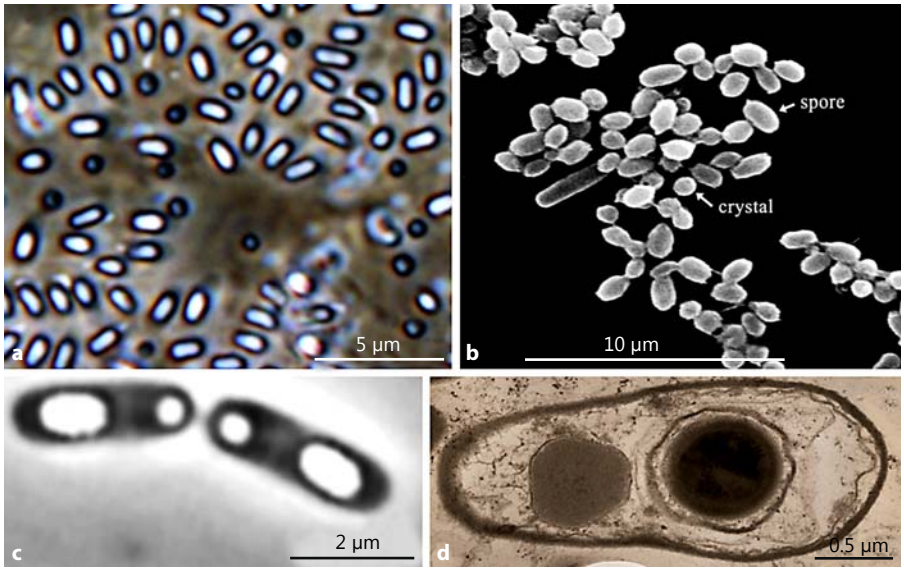
Results and Discussion

Ultrastructural Analysis of Crystals Produced by B. thuringiensis *INTA Fr7-4 and Screening of Insecticidal Genes*

Crystalline inclusions of INTA Fr7-4 were observed under phase-contrast microscopy as ovoid crystals (fig. 1). When ultrathin sections of sporulating cells were analyzed under transmission electron microscopy, uniform ovoid crystals were visible, completely separated from the spores, and ranging in size from 0.7 to 0.8 μm (fig. 1). These dimensions were verified under scanning electron microscopy where, again, only ovoid crystals were observed (fig. 1). On the basis of their morphology, these crystals resembled the parasporal crystals of the mosquitocidal *B. thuringiensis* serovar *israelensis* [Ibarra et al., 2003], and those produced by some Lepidoptera-toxic strains [Wasano and Ohba, 1998]. This observation, together with results of preliminary studies in which INTA Fr7-4 showed low activity against Lepidoptera larvae, led us to investigate the presence of some representative genes encoding insecticidal proteins toxic to Diptera and Lepidoptera that could be forming the ovoid crystals. No positive results were obtained in PCR reactions using general primers for either Diptera-specific *cry4*, *cry11*, *cyt1* and *cyt2* or the Lepidoptera-specific *cry1*, *cry2* and *cry9* genes. That is, INTA Fr7-4 does not appear to contain any of the most frequently insecticidal genes associated to this type of crystals. Therefore, these results suggested that the crystal could be composed of other Cry protein(s) that also could account for low toxicity against *A. gemmatalis*.

Sequence Analysis of Cry8 INTA Fr7-4 Protein

In a previous work, we carried out a shotgun sequencing of the plasmid content of INTA Fr7-4. As a result, three plasmids were completely sequenced [Amadio et al., 2009]. Some of the reads that could not be assembled into a plasmid showed significant similarity to *cry8* genes. The presence of a *cry8* gene in this strain could be respon-



Color version available online

Fig. 1. Micrographs of *B. thuringiensis* INTA Fr7-4. **a** Phase-contrast micrograph of spores and crystals. **b** Scanning electron micrograph of spores and ovoid crystals. **c** Phase-contrast micrograph of sporulating cells, showing crystals and spores. **d** Transmission electron micrograph of a sporulating cell, showing a spore and an ovoid crystalline inclusion.

Table 1. Primers used for INTA Fr7-4 *cry8* gene, *cry1Ac* and *cry3Aa* promoters

cry8 Fr7-4	Oligonucleotide sequence	Position	Tm, °C[a]
Primers			
C8-F01	ATGAGTCCAAATAATCAAAATG	1–22	47.3
C8-R01	CTCTACGTCTACAATCAATTCTAG	3529–3552	50.4
P8-07-F01	TTTCCGTATCAATTTGTCG	2344–2362	48.4
P8-07-F02	AACGAGTTCATCTGATTGG	762–780	50.2
P8-07-R02	ATGTTAAAGGCTCACCTG	1844–1862	50.0
P8-07XR01	GGAGTATGGGTTGGCTTT	2698–2715	52.6
Cry8-04inv192	GATCAACTGTGCCAAACTGG	192–212	54.9
Promoters			
Cry1Ac_promF1	TGGGAAGAACGTGAGGGCTGG	424–444[b]	61.7
Cry1Ac_promR1	AAGTTACCTCCATCTCTTTTATTAAG	975–1000[b]	50.6
Cry3A_promF1	TCTCTACGGTGTACTGGGGC	113–132[c]	58.2
Cry3A_promR1	TTTTCTTCCTCCCTTTCTTATC	1520–1541[c]	50.2

[a] Tm, melting temperature of the primer. [b] Relative to GenBank AF039908. [c] Relative to GenBank L03393.

sible of the ovoid crystals. To obtain the complete *cry8* gene, we designed primers based on a consensus sequence derived from the multiple alignment of the following *cry8* genes (Genbank accession No.) (table 1): *cry8Aa1* (U04364), *cry8Ba1* (U04365), *cry8Bb1* (AX543924), *cry8Bc1* (AX543926), *cry8Ca1* (U04366), *cry8Ca2* (AY518201) and *cry8Da1* (AB089299). A PCR product of about 3.5 kb was obtained using a plasmid preparation as template and cloned into pGEM®-T-Easy vector. We confirmed the identity of the clone and obtained its complete sequence using a primer walking strategy.

The INTA Fr7-4 *cry8* gene is 3,555 bp long and encodes a protein of 1184 amino acid residues with a predicted molecular weight of 132.7 kDa. The protein matches the molecular mass calculated for other Cry8 proteins reported previously [Asano et al., 2003; Shu et al., 2009]. The DNA nucleotide sequence of the *cry8* gene from *B. thuringiensis* INTA Fr7-4 has been deposited in the GenBank databases under accession No. KC152468. The protein was named Cry8Qa2 by the *B. thuringiensis* Delta-Endotoxin Nomenclature Committee. The identity values of Cry8 INTA Fr7-4 sequence with members of

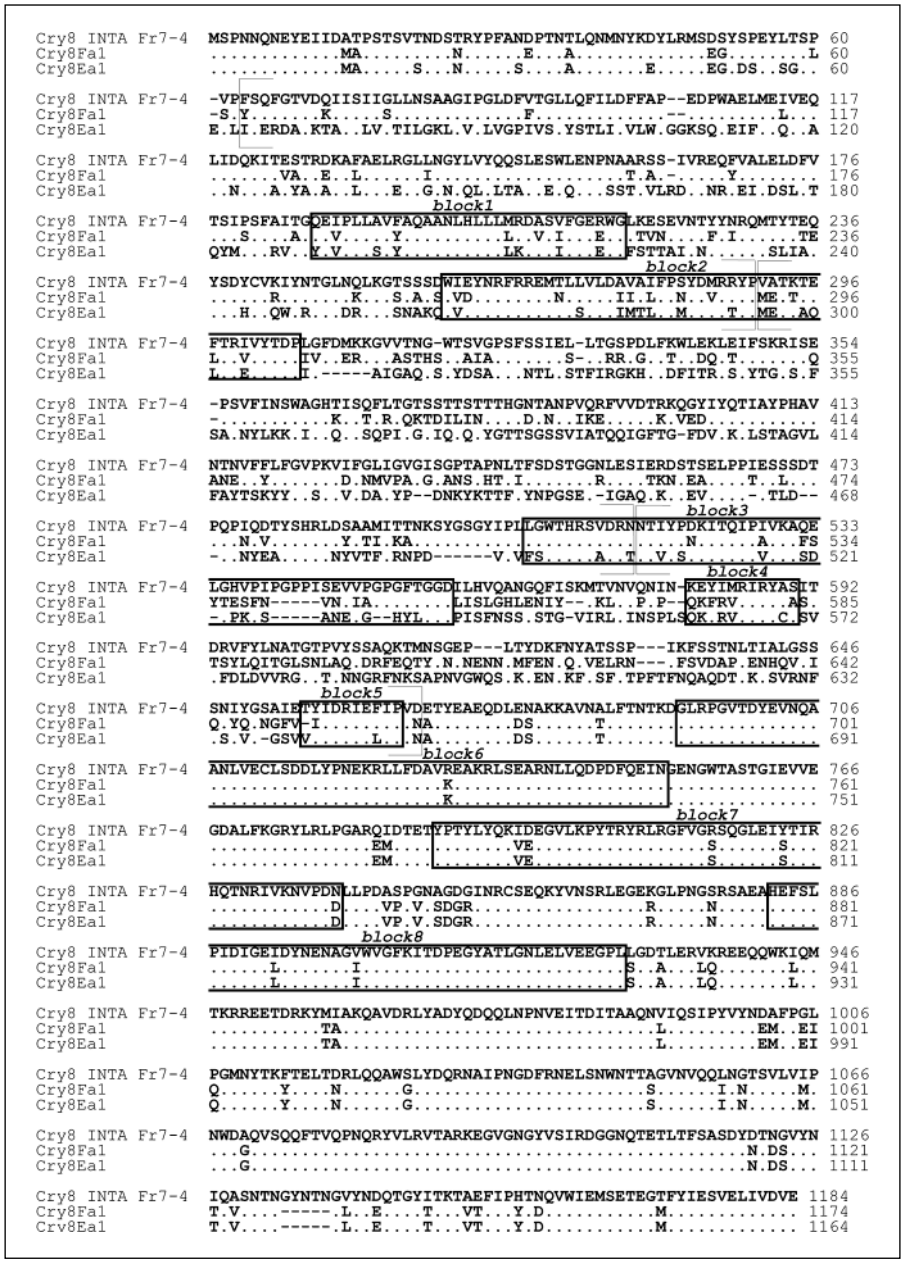


Fig. 2. Protein sequence alignment of Cry8 INTA Fr7-4, Cry8Fa1 and Cry8Ea1. Cry8Fa1 is the reported protein with highest similarity to Cry8 INTA Fr7-4 and Cry8Ea1 is the only Cry8 protein with solved structure. The black boxes indicate regions that correspond to the eight conserved blocks found in Cry8 proteins. The regions between black brackets correspond to each of the three domains of the proteins in sequential order.

the Cry8 class ranges from 46.3% (Cry8Ma2) to 75.4% (Cry8Fa1).

An alignment between Cry8 INTA Fr7-4 and other Cry8 proteins is shown in figure 2. Cry8 INTA Fr7-4 contained the five blocks (1–5) characteristic of other Cry proteins and the three blocks (6–8) present in toxins that are cleaved in the C-terminal region [Schnepf et al., 1998]. Recently, Guo et al. [2009] determined the crystal structure of the active Cry8Ea1 being the first crystallographic study made for Cry8 toxins. The overall folding pattern of the protein is a three-domain structure, similar to Cry toxins crystallized previously [Schnepf et al., 1998]. As the identity percentage between Cry8Ea1 and Cry8 INTA Fr7-4 is about 62%, a reasonable prediction structure of Cry8 INTA Fr7-4 is possible and a comparison of their putative structure could be done. According to this, conserved block 1 of Cry8 INTA Fr7-4 would be presumably included in domain I (Phe^{63}–Pro^{290}), conserved block 2 in domain I and II (Val^{291}–Asn^{515}), conserved block 3 in domain II and III (Asn^{516}–D^{667}), and conserved blocks 4

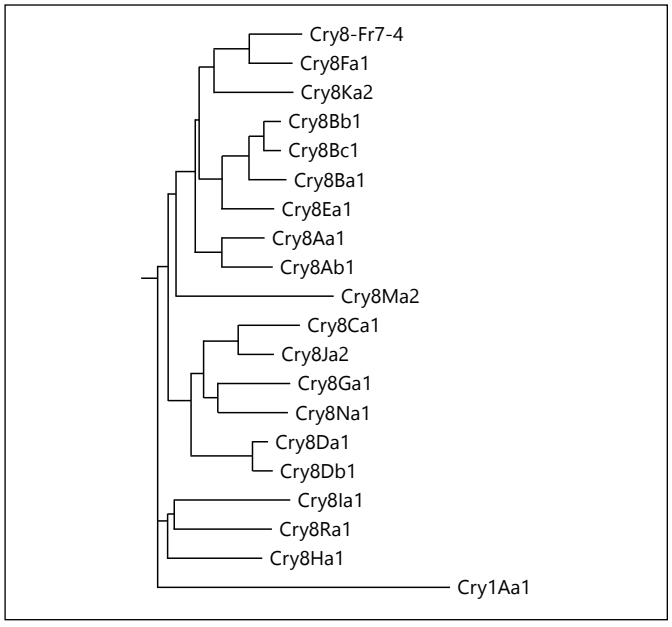


Fig. 3. Neighbor-joining tree showing phylogenetic relationship among different available Cry8 proteins. Cry1Aa1 was included as out group.

and 5 also in domain III (fig. 2). The amino acid residues located into domains II and III were less conserved in the alignment when compared to other Cry8 proteins. Both domains have been related to target specificity of a Cry toxin [de Maagd et al., 1999; Herrero et al., 2004; Schnepf et al., 1998], and more specifically, domain II is thought to be important in receptor recognition and binding [Schnepf et al., 1998].

A member of each subclass of Cry8 proteins with public accession in GenBank and the novel Cry8 Fr7-4 were aligned and a phylogenetic tree was created by the neighbor-joining method. Three main clusters could be distinguished (fig. 3). Cry8 INTA Fr7-4 formed a subcluster with Cry8Fa1 and Cry8Ka2. These proteins are produced by the Chinese *B. thuringiensis* strain Bt185 and the Argentinean *B. thuringiensis* serovar *kenyae* strain LBIT-597, respectively [Noguera and Ibarra, 2010; Shu et al., 2009].

Expression of INTA Fr7-4 cry8 Gene in B. thuringiensis

The INTA Fr7-4 *cry8* gene was cloned in the shuttle vector pHT3101 [Lereclus et al., 1989]. This plasmid does not carry a promoter to drive the expression of the cloned gene. Since there was no *cry8* promoter reported at the moment of the expression design, we tested known promoters of two other *cry* genes to drive INTA Fr7-4 *cry8*: the sporulation-dependent promoter of *cry1Ac* and the sporulation-independent promoter of *cry3Aa*. The corresponding plasmid constructs were named pHT1Ac-cry8 and pHTIIIA-cry8. These plasmids were used to transform the acrystalliferous strain *B. thuringiensis* 4Q7.

Cry8 INTA Fr7-4 expression was analyzed by SDS-PAGE, and the presence of crystals in recombinant strains was monitored by phase-contrast microscopy (fig. 4). The strain 4Q7 transformed with plasmid vector pHT3101 was used as negative control. A protein of ca. 130 kDa was observed in recombinants carrying INTA Fr7-4 *cry8* gene (fig. 4). This protein has the same molecular weight to that observed in strain INTA Fr7-4 (fig. 4). On the other hand, no ca. 130-kDa protein was produced by the control strain 4Q7 transformed with the plasmid without *cry8* gene (fig. 4). These results indicate that the promoters tested were active in strain 4Q7 and that the levels of Cry8 attained at the sporulation phase of the culture seemed to be similar with either promoter. However, a higher level of vegetative cells were observed in the recombinant carrying pHT1Ac-cry8 (data not shown) which can also be evidenced by the more complex mixture of proteins in the gel (fig. 4). Proteins in the range of 70–130 kDa are produced in both recombinants, and these bands could correspond to degradation products of the Cry8 protein. The analysis of the recombinants by SDS-PAGE correlated with the presence of inclusions in sporangia observed by phase-contrast microscopy (fig. 4). Inclusions present in recombinants carrying *cry8* under control of each promoter were similar in shape to crystals produced by strain INTA Fr7-4. However, they were slightly smaller and refract light differently.

In the present study we used an acrystalliferous strain derived from *B. thuringiensis* serovar *israelensis*, which has activity against Diptera, carrying four *cry* genes (*cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa*), two *cyt* genes (*cyt1Aa* y *cyt2Ba*) and a *cyt*-like gene [Berry et al., 2002]. The insecticidal genes of *B. thuringiensis israelensis* produce crystals very different to that of INTA Fr7-4 and it is possible that 4Q7 lacks factors needed for optimal production of Cry8-containing crystals or those factors are considerably different. Recently, a 54-kDa protein was described as essential for crystal aggregation in *B. thuringiensis israelensis* [Diaz-Mendoza et al., 2012]. Furthermore, the protein P20 needed for crystallization of proteins Cyt1A and Cry4B [Adams et al., 1989; Dervyn et al., 1995; Visick and Whiteley, 1991]. Therefore, it is likely that Cry8 proteins require specific or common factors involved in crystal formation for the stable aggregation of crystals that may be absent in 4Q7. Nev-

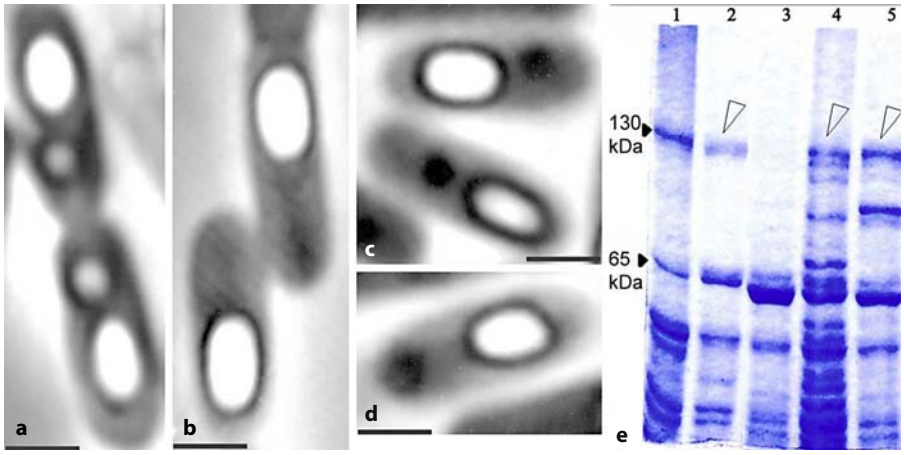


Fig. 4. Phase-contrast micrographs and SDS-PAGE analysis of spore and crystal mixtures. **a** *B. thuringiensis* INTA Fr7-4 in LB agar at 24 h of growth. **b** Recombinant 4Q7/pHT3101 in LB agar at 48 h of growth. **c** Recombinant 4Q7/pHT1Ac-cry8 in LB agar at 48 h of growth. **d** Recombinant 4Q7/pHTIIIA-cry8 in LB agar at 48 h of growth. **e** Expression analysis of INTA Fr7-4 *cry8* gene in LB broth at 72 h of growth by SDS-PAGE: protein components of *B. thuringiensis* serovar *kurstaki* HD-1 (lane 1), *B. thuringiensis* INTA Fr7-4 (lane 2), recombinant 4Q7/pHT3101 (lane 3), recombinant 4Q7/pHT1Ac-cry8 (lane 4), recombinant 4Q7/pHTIIIA-cry8 (lane 5). Cry1 (130 kDa) and Cry2 (65 kDa) typical proteins of HD-1 strain were indicated by the closed arrows and the Cry8 INTA Fr7-4 proteins indicated by the open arrows.

Table 2. Toxic activity of *B. thuringiensis* spore-crystal complexes against neonate *A. gemmatalis* larvae (mean ± SEM values)

Strain	Mortality, %
INTA Fr7-4	13.8±3.0
4Q7/pHT1Ac-cry8	14.6±2.5
4Q7/pHTIIIA-cry8	8.6±1.6
4Q7	6.0±2.1

ertheless, the expression of *cry8* INTA Fr7-4 under control of two differently regulated promoters produced inclusions that could be used in bioassays.

Insect Bioassays with Cry8 INTA Fr7-4

To evaluate the toxicity of the new protein, spores of 4Q7 and spore-crystal complexes of 4Q7/pHT1Ac-cry8, 4Q7/pHTIIIA-cry8 and INTA Fr7-4 were tested for insecticidal activity on neonate larvae of *A. gemmatalis*. The larvicidal activities rates corrected by natural mortality using Abbott's formula are shown in table 2. Cry8 INTA Fr7-4 expressed in 4Q7/pHT1Ac-cry8 showed the highest toxic activity against *A. gemmatalis* larvae, at a level comparable to that of the wild-type strain. In contrast, these values were significantly different when compared to that obtained from the 4Q7/pHTIIIA-cry8 construct. It is possible that the amount of Cry8 produced per cell in 4Q7/pHTIIIA-cry8 was lower than in 4Q7/pHT1Ac-cry8 because of different strength or moment of activation of the promoters. At the concentration evaluated, strain 4Q7 had a basal level of insecticidal activity. This may be due to unspecific virulence factors; different to specific Cry present in *B. thuringiensis* [Sauka and Benintende, 2008]. Insecticidal activity of *B. thuringiensis* is dependent on the quantity and types of Cry proteins produced [Sauka and Benintende, 2008].

Our tests demonstrated that the Cry8 protein from INTA Fr7-4 is responsible for a low insecticidal activity against *A. gemmatalis* larvae. To our knowledge, there was only one report where a Cry8 protein showed toxicity against Lepidoptera larvae. In that study, Cry8Da1 showed a weak but substantial mortality in larva of *Plutella xylostella* (Lepidoptera: Yponomeutidae) [van Frankenhuyzen and Nystrom, 2002]. The Cry8 INTA Fr7-4 protein may also be toxic to insect pests that have not yet been evaluated. For example, Cry8-type toxins have been described as toxic to coleopteran pests (mainly members from Scarabaeidae family) [Shu et al., 2009]. Further studies are needed to determine toxicity of Cry8 INTA Fr7-4 against scarab species. However, we tested the insecticidal activity of a spore-crystal complex of INTA Fr7-4 against *Anthonomus grandis* (Coleoptera: Curculionidae) without finding any mortality level (data not shown).

In conclusion, INTA Fr7-4 encodes a new *cry8* gene whose protein product of about 130 kDa is capable of producing ovoid crystals with a low level of toxicity to Lepidoptera.

Experimental Procedures

B. thuringiensis *Strains and Culture Conditions*

B. thuringiensis INTA Fr7-4 was obtained from a soil sample of an orchard of the province of Misiones, Argentina. This isolate was primarily identified by the presence of parasporal inclusions observed under phase-contrast microscopy. *B. thuringiensis* serovar *kurstaki* HD-1 was kindly provided by the United States Department of Agriculture (USDA), Agricultural Research Service (Peoria, Ill., USA) and *B. thuringiensis* serovar *tenebrionis* DSM2803 and the acrystalliferous 4Q7 strain from serovar *israelensis*, by the Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional's (CINVESTAV's) stock collection (Irapuato, Mexico). The strains were grown in BM medium [Benintende and Cozzi, 1996], at 340 rpm and 30°C, during 72 h or until complete autolysis was observed. Spore-crystal complexes were obtained by centrifugation at 12,000 *g* and 4°C for 15 min, and pellets were freeze-dried. Powders of spore-crystal complexes were kept at –20°C until use. For some experiments, Luria-Bertani's (LB) medium was used as indicated.

Electron Microscopy

Scanning and transmission electron microscopy images of INTA Fr7-4 were obtained by following the procedure described by Benintende et al. [2000].

Screening of Lepidopteran and Dipteran-Specific Insecticidal Genes

INTA Fr7-4 was grown on nutrient agar plates for 16 h. A loopful of vegetative cells was transferred to 100 µl of water and boiled for 10 min to make bacterial DNA accessible for PCR amplification. The lysate was centrifuged (5 s at 15,700 *g*; Eppendorf model 5415R centrifuge) and 5 µl of supernatant was used as a DNA template for PCR. Screening of *cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1* and *cyt2* genes was carried out as previously described [Ibarra et al., 2003; Juárez-Pérez et al., 1997; Sauka et al., 2005, 2010a, 2010b].

Amplification and Sequencing of cry8 Gene

The *cry8* gene of INTA Fr7-4 was amplified by PCR from start to stop codons with primers C8-F01 and C8-R01 (table 1). Total plasmid DNA obtained by an alkaline lysis procedure was used as template for PCR [Amadio et al., 2009]. For PCR amplification, AccuPrime™ High Fidelity *Taq* DNA Polymerase (Invitrogen) was used following the manufacturer's instructions. PCR consisted of 35 cycles (10 s at 94°C, 10 s at 45°C, 3 min 30 s at 68°C) followed by incubation at 68°C for 10 min. The annealing temperature was calculated with FastPCR software according to thermodynamical properties of the primers [Kalendar et al., 2009]. The amplified fragment of about 3.5 kb was cloned in pGEM®-T-Easy vector (Promega) following the manufacturer's instructions. Initial sequences were obtained with universal primers T7 and SP6. To determine the complete sequence, a primer walking strategy was followed. Subsequent primers (table 1) were designed upon newly obtained sequences, using the function included in Gap4 program from Staden Package software [Staden et al., 1999]. The complete *cry8* gene sequence was assembled using Gap4. Three independent clones were sequenced to confirm amplification from a single locus.

Comparison of B. thuringiensis *Cry8 Proteins*

The predicted amino acid sequence of Cry8 INTA Fr7-4 was aligned with one member of each subclass of Cry8 proteins available at the Bt toxin nomenclature website [Crickmore et al., 2012]. The multiple sequence alignment was generated using ClustalX2 [Larkin et al., 2007] software and the construction of the phylogenetic tree was carried out using the Jones et al. [1992] model of amino acid change to compute the distance matrix and the neighbor-joining method of Saitou and Nei [1987] of clustering. Cry1Aa1 (GenBank Accession AAA22353) was used as out-group. TreeView was used to plot the rooted tree diagram [Page, 1996].

Cloning and Expression of cry8 INTA Fr7-4 Gene in a B. thuringiensis *Expression System*

To express *cry8* INTA Fr7-4 in *B. thuringiensis*, the gene was cloned in the shuttle vector pHT3101 [Lereclus et al., 1989]. It contains the polylinker from vector pUC18, and can replicate in both *Escherichia coli* and *B. thuringiensis*, expressing resistance to ampicillin and erythromycin, respectively. Two constructs were made that differ in the promoter sequence inserted upstream of *cry8* INTA Fr7-4. Plasmid pHT1Ac-cry8 carries the sporulation-dependent promoter of *cry1Ac* gene from *B. thuringiensis* serovar *kurstaki* HD-1 (GenBank Accession AF039908), while plasmid pHTIIIA-cry8 contains the sporulation-independent promoter of *cry3Aa* gene from *B. thuringiensis* serovar *tenebrionis* DSM2803 (GenBank Accession L03393). The promoters were amplified as PCR products containing sequences immediately upstream of the start codon of the respective genes, 577 bp long for *cry1Ac* (primers Cry1Ac_promF1 and Cry1Ac_promR1; table 1), and 1,429 bp long for *cry3Aa* (primers Cry3A_promF1 and Cry3A_promR1; table 1). Plasmid preparation from the corresponding *B. thuringiensis* strains were used as template for PCR amplification with AccuPrime™ High Fidelity *Taq* DNA Polymerase. The PCR products were cloned into pGEM®-T-Easy vector and sequenced. To bring together the promoter and the *cry8* INTA Fr7-4 ORF into vector pHT3101, we used the following strategy: the promoter and *cry8* sequences were re-amplified from the corresponding recombinant pGEM clones using *Pfu* DNA polymerase and a mix of the PCR products were ligated using T4 DNA ligase (Promega). The ligation reaction was used directly as template to selectively amplify the correct ligation product 5′-promoter-*cry8*-3′, by using promoter forward and *cry8* reverse primers (table 1). PCR products of the expected size were gel-purified using Sephaglass BandPrep kit (Amersham) and cloned into pGEM®-T-Easy vector. The promoter-*cry8* constructs were excised from the vector backbone with restriction enzymes *Sph*I and *Sac*I (Promega), gel-purified and ligated to plasmid pHT3101 digested with the same enzymes.

Plasmids pHT1Ac-cry8 and pHTIIIA-cry8 were transformed into *E. coli da*$^-$ *m*$^-$ *dcm*$^-$ to obtain unmethylated plasmid DNA required to transform *B. thuringiensis* [Macaluso and Mettus, 1991]. The unmethylated recombinant plasmids and parental plasmid pHT3101 were introduced into the acrystalliferous *B. thuringiensis* 4Q7 strain by electroporation as described [Mahillon and Lereclus, 2000]. Transformants for each plasmid were selected by erythro-

mycin resistance in LB plates incubated at 30°C. The presence of parasporal inclusions was confirmed under phase-contrast microscopy. The Cry8 INTA Fr7-4 expression was also visualized by SDS-PAGE following standard methods [Sambrook et al., 1989].

Insect Bioassays

Toxicity of *B. thuringiensis* spore-crystal suspensions was qualitatively analyzed by bioassays against neonate larvae of *A. gemmatalis* Hüb. (Lepidoptera: Noctuidae). Four milliliters of cultures containing spore or spore-crystal suspensions (4.5×10^6 CFU/ml) were incorporated into polypropylene conical tubes containing a thermostatized (40°C) artificial diet for *A. gemmatalis* [Greene et al., 1976] and poured into each well of a 24-well plate (Nunc 143982). Sterile BM medium were added to the control. 24 *A. gemmatalis* larvae were used per assay (5 replicates). Mortality was registered after 5 days at 29°C. *A. gemmatalis* larvae were considered dead if they failed to respond to gentle probing. Data were corrected for control mortality using Abbott [1925] correction factor.

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