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Sequence and Expression of Two *cry8* Genes from *Bacillus thuringiensis* INTA Fr7-4, a Native Strain from Argentina

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Key Words
Bacillus thuringiensis · *cry8* · Expression

Abstract
We found and characterized two *cry8* genes from the *Bacillus thuringiensis* strain INTA Fr7-4 isolated in Argentina. These genes, *cry8Kb3* and *cry8Pa3*, are located in a tandem array within a 13,200-bp DNA segment sequenced from a preparation of total DNA. They encode 1,169- and 1,176-amino-acid proteins, respectively. Both genes were cloned with their promoter sequences and the proteins were expressed separately in an acrystalliferous strain of *B. thuringiensis* leading to the formation of ovoid crystals in the recombinant strains. The toxicity against larvae of *Anthonomus grandis* Bh. (Coleoptera: Curculionidae) of a spore-crystal suspension from the recombinant strain containing *cry8Pa3* was similar to that of the parent strain INTA Fr7-4.

Introduction

Bacillus thuringiensis is a ubiquitous Gram-positive, spore-forming bacterium, which produces parasporal inclusions or crystals at the sporulation phase of its life cycle. *B. thuringiensis* crystals are formed by δ-endotoxin proteins known as Cry proteins, which show toxic activity against several insect orders, mainly Lepidoptera, Diptera, and Coleoptera [Sauka and Benintende, 2008]. Toxicity to insects is mediated by activation of the Cry protoxin via proteolysis in the midgut of a susceptible insect. Subsequently, the active toxin forms pores in the membrane of epithelial cells lining the midgut, leading to their lysis [Bravo et al., 2007].

B. thuringiensis-based pesticides have been commercialized for many decades [Roh et al., 2007]. These pesticides represent an effective alternative to chemical insecticides to avoid toxicity against non-target insects and risks associated with the environment and human health.

More than 700 *cry* genes have been reported to date [Crickmore et al., 2014]. The current classification of Cry proteins in 73 major groups is based on their amino acid sequence similarity. The established groups are subdivided into classes and subclasses. To some extent, there is a correlation between the classes of Cry proteins and the orders of insects to which the susceptible species belong [Sauka and Benintende, 2008].

Cry8 proteins in *B. thuringiensis* strains have molecular weights of about 130 kDa. After proteolysis-mediated activation, the active toxin has a molecular weight of 60–70 kDa. Cry8 toxins are known to be toxic to coleopteran

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Table 1. Primers used for cloning and sequencing of INTA Fr7-4 *cry8Kb3, cry8Pa3* and their promoters

INTA Fr7-4	Oligonucleotide sequence	Position[a]	Tm, °C[b]
C801-F1	CCTCCTGTAAACAATGATGG	2,887–2,906	51.3
C801-F2	TACAAGCGTCGAATACAAAC	3,716–3,735	50.9
C801-R1	AGTAATTCCAGTTGTAATCCC	583–603	50
C801-R2	TCAGTTGTAGCAGCGTAAC	2,117–2,135	52.6
C802-F1	CGATAGTGAATATGCTGGTTC	9,705–9,725	51.2
C802-F2	GGAATATGGGTTGGATTT	12,223–12,240	47.4
C802-R1	TTTCCGTATCAATTTGTCG	11,869–11,887	48.4
C802-R2	TGATACAGATACGTTGGATACG	11,888–11,909	51.6
P01-F	TCTACAATCATTCGCACAAG	163–182	50.7
P01-R	ATAGAGAAGAAACCGCAAGG	4,002–4,021	52.5
P02-F	GGTCGTCATATTCAGGTACG	9,150–9,169	52.7
P02-R	TAACCATAGCCCAATCCAC	13,186–13,204	52.2

[a] Position relative to GenBank KJ123823 (sequence of 13,200 bp reported in this work). [b] Tm, melting temperature of the primer.

species, e.g. *Anomala cuprea* [Asano et al., 2003], *Holotrichia parallela* [Shu et al., 2009], *Anthonomus grandis* [Oliveira et al., 2011], and also to some lepidopteran pests, e.g. *Anticarsia gemmatalis* [Amadio et al., 2013].

A large collection of *B. thuringiensis* isolates from Argentina is maintained at the Instituto de Microbiología y Zoología Agrícola (IMyZA) of the Instituto Nacional de Tecnología Agropecuaria (INTA). We have previously reported the sequence and characterization of three plasmids carried by the *B. thuringiesis* strain INTA Fr7-4 [Amadio et al., 2009]. We have further studied the same *B. thuringiesis* strain at the phenotypic and genotypic levels and found the *cry8Qa2* gene, which codes for a protein with low toxicity against *A. gemmatalis* (Lepidoptera) [Amadio et al., 2013].

In this paper, we present results of the cloning and heterologous expression of two additional *cry8* genes, localized in a tandem array in strain INTA Fr7-4. In addition, the toxicity of the two protein products was evaluated against two coleopteran species.

Results and Discussion

Sequence Analysis of cry8Kb3 and cry8Pa3 from INTA Fr7-4

We have previously reported that *B. thuringiensis* strain INTA Fr7-4 encodes the *cry8Qa2* gene [Amadio et al., 2013]. This gene was sequenced from a cloned PCR product of 3,555 bp amplified with *cry8*-specific primers C8F01 and C8R01 (table 1). Sequences obtained from screening additional clones allowed us to identify another two different *cry8* genes coded in strain INTA Fr7-4 on the basis of their similarity with members of this class. These genes were named *cry8Kb3 and cry8Pa3* by the *B. thuringiensis* Delta-Endotoxin Nomenclature Committee [http://www.btnomenclature.info]. The *cry8Kb3* gene is 3,510 bp long and encodes a 1,169-amino-acid protein with a predicted molecular weight of 131,470 Da. The length of the *cry8Pa3* gene is 3,531 bp, encoding a protein of 1,176 amino acid residues with 132,769 Da of predicted molecular weight. The sizes of both gene products are within the range calculated for other Cry8 proteins [e.g. Amadio et al., 2013; Ogiwara et al., 1995; Shu et al., 2009]. Alignment of the amino acid sequences of Cry8Kb3 and Cry8Pa3 shows that their sequences share an overall 63% sequence identity (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000365929). While their C-terminal regions are almost identical, the less conserved region is a 300-amino-acid long portion coinciding with domains II and III, previously related to target specificity of *cry* proteins [De Maagd et al., 1999; Herrero et al., 2004; Schnepf et al., 1998]. The amino acid sequence differences between Cry8Kb3 and Cry8Pa3 and their different levels of toxicity against *A. grandis* (see below) suggest that each protein could be targeting different insect species. The amino acid sequence of Cry8Kb3 resulted identical to reported proteins with GenBank accession Nos. HM123758 (Cry8Kb1) and KC156675 (Cry8Kb2), although the nucleotide sequence of the gene is identical only to that of Cry8Kb2. The amino acid sequence of Cry8Pa3 resulted identical to a reported pro-

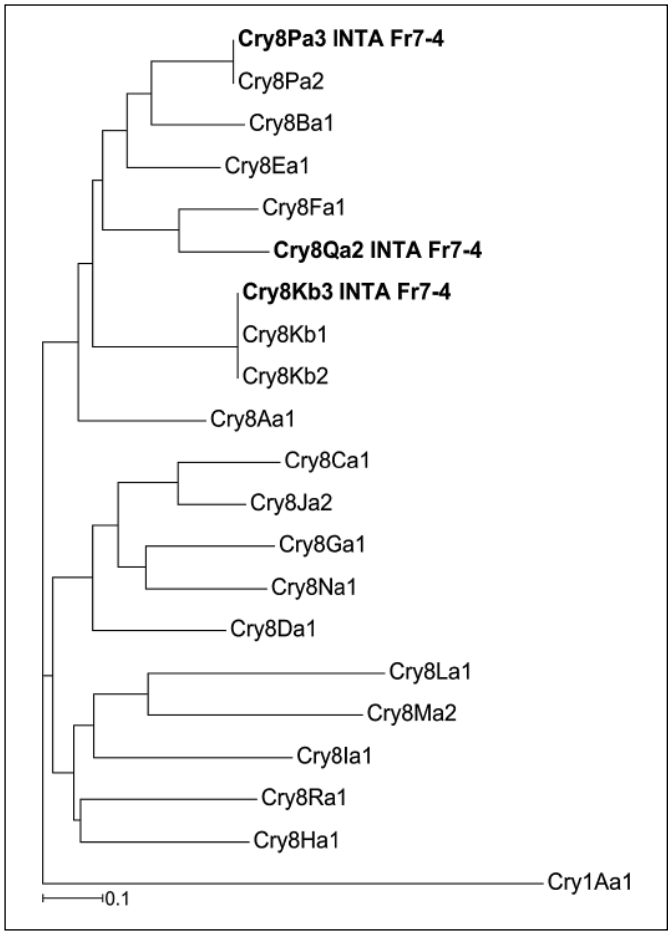

Fig. 1. Neighbor-joining tree showing phylogenetic relationship among different available Cry8 proteins and the three Cry8 proteins from INTA Fr7-4. Cry8Kb3 and Cry8Pa3 are reported in this work. Cry8Qa2 was previously reported [Amadio et al., 2013]. Cry1Aa1 sequence was included as out group.

tein with accession No. HQ413324 (Cry8Pa2), whereas the alignment of their nucleotide sequences presents 4 mismatches. To our knowledge, there are no published studies on the sequences deposited in GenBank.

Since the *cry8* genes found in *B. thuringiensis* INTA Fr7-4 are not encoded in any of its three known plasmids [Amadio et al., 2009], it is possible that other non-characterized plasmid(s) could exist in this strain.

Phylogenetic Analysis

A phylogenetic tree was created by the Neighbor Joining method using the amino acid sequences of one representative member for every Cry8 subclass found in GenBank and the amino acid sequences of the two Cry8 proteins reported here. As shown in figure 1, three main groups can be distinguished. INTA Fr7-4 Cry8Kb3 and Cry8Pa3 were both included in one of these groups, along with the previously reported Cry8Qa2. Remarkably, *B. thuringiensis* Chinese strain ST8 carries three *cry8* genes belonging to the same subclasses as those found in INTA Fr7-4, namely *cry8Kb*, *cry8Pa* and *cry8Qa* [Crickmore et al., 2014]. This suggests that the *cry8* gene content of these strains might be evolving from a common ancestral arrangement. In another *cry8*-containing strain, BT185 [Shu et al., 2009; Yu et al., 2006], the genes (*cry8Fa1*, *cry8Ea1* and *cry8Ha1*) are representative members of more than one phylogenetic group within the tree shown in figure 1.

cry8Kb3 and cry8Pa3 Are Coded in the Same DNA Strand

Since different genes with very similar sequences may arise by gene duplication [Zhang, 2003], we followed a strategy based on PCR to explore the possibility that the two *cry8* genes were located closely. Based on the nucleotide sequence differences of *cry8Kb3* and *cry8Pa3*, specific internal sense and antisense primers were designed for each gene and used in all possible primer pair combinations in long PCR reactions. The amplification of large DNA fragments with several primer pair combinations suggested the proximity of the two *cry8* genes. Specifically, testing forward primers of *cry8Kb3* with reverse primers of *cry8Pa3* generated an amplified DNA fragment of around 8.5 kbp (online suppl. fig. 2).

One of the PCR products was sequenced following a primer walking strategy and proved to correspond to the targeted loci. The intergenic region between *cry8Kb3* and *cry8Pa3* has a size of 5,659 bp. The assembly of this region to the coding sequence of the genes resulted in a 13,204-bp fragment (deposited in GenBank under accession No. KJ123823). We thus concluded that both genes are encoded in the same DNA strand, with a relative position of *cry8Kb3* upstream of *cry8Pa3*.

Examining the DNA sequences near the *cry8* genes for the presence of additional genes resulted in the identification of four ORFs longer than 100 codons. One ORF immediately upstream of *cry8Pa3* (fig. 2) is highly similar to a gene named *orf1*, which forms an operon with *cry8Ea1* [Du et al., 2012]. Likewise, other *cry* genes, e.g. *cry2A*, *cry2C, cry11A* [Dervyn et al., 1995; Widner and Whiteley, 1989; Wu et al., 1991], form operons with genes homologous to *orf1*. A BLAST search with the other three ORFs showed that they match fragments of some *B. thuringiensis* gene entries in GenBank (data not shown). However, we could not clearly identify or correlate those ORFs to

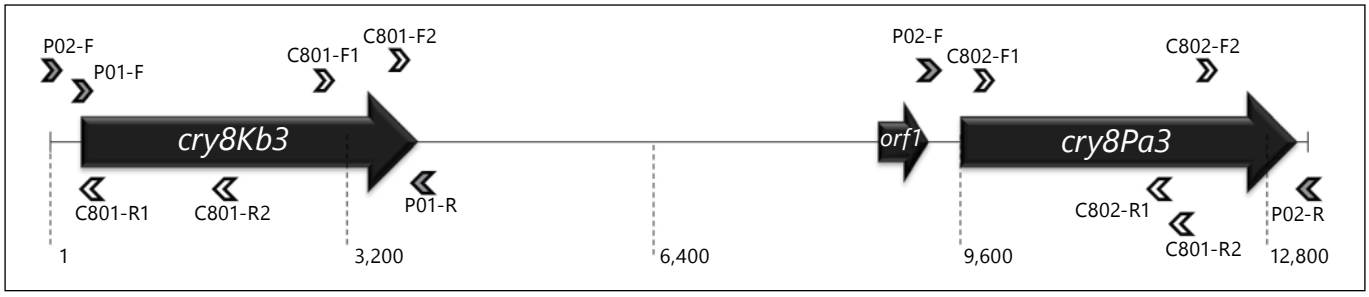

Fig. 2. Scheme of INTA Fr7-4 *cry8Kb3* and *cry8Pa3* genes with their corresponding intergenic sequences and primer binding sites. The *cry8* genes and *orf1* are represented with black arrows. The gray arrowheads indicate the primers used for cloning the genes. The white arrowheads indicate the primers used to determine proximity between genes.

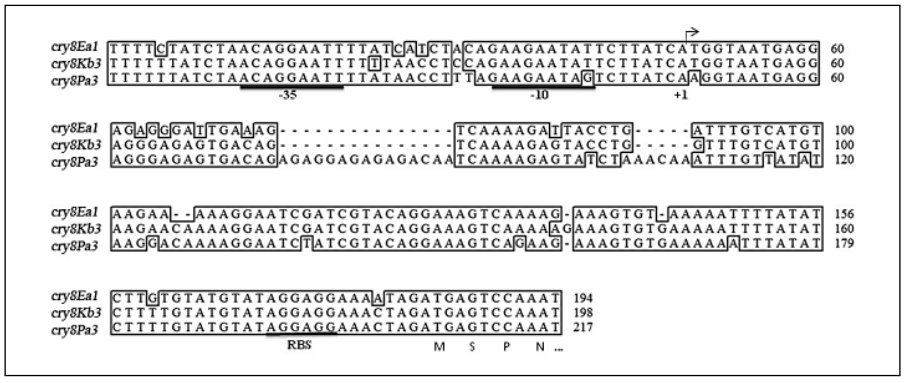

Fig. 3. Nucleotide sequence alignment of the promoter region of *cry8Kb3*, *cry8Pa3* and *cry8Ea1*. Transcriptional initiation (+1) is marked. The putative promoter signals –35 and –10 and the putative RBS of the *cry8* are underlined. The boxed-in residues comprise positions of identity.

true genes. Interestingly, one of the ORFs was highly similar to a fragment of a transposase gene, suggesting the occurrence of transposition events associated with this region [Mahillon et al., 1994].

We then compared the regions of ca. 200 bp upstream of the start codon of *cry8Kb3* and *cry8Pa3* and aligned them with the corresponding sequence of *cry8Ea1*, which is known to include a functional promoter [Du et al., 2012]. The promoter region (–35 and –10), start of transcription (+1) and ribosome binding site (RBS) are strictly conserved between the genes except for two nucleotide changes in *cry8Pa3* (fig. 3). However, there are some mismatches in the overall sequence. The most remarkable differences are found in the *cry8Pa3* sequence as two small insertions, of 15 and 5 bp (fig. 3). Those insertions are going to be present in the 5′-UTR of the predicted *cry8Pa3* mRNA.

Expression of cry8Kb3 and cry8Pa3 in B. thuringiensis 4Q7

To further study the *cry8Kb3* and *cry8Pa3* genes, their coding sequences were individually cloned along with the corresponding promoter region in the shuttle vector pHT3101 [Lereclus et al., 1989]. The corresponding plasmid constructs were confirmed by restriction enzyme analysis and DNA sequencing and named pHT*cry8Kb3* and pHT*cry8Pa3* (see Experimental Procedures). Purified plasmids from *Escherichia coli* were used to transform the acrystalliferous strain *B. thuringiensis* 4Q7 to determine their ability to direct synthesis of the Cry8 proteins and generate crystals.

The expression of Cry8Kb3 and Cry8Pa3 was analyzed by SDS-PAGE, and the presence of crystals in recombinant bacterial strains was monitored by phase-contrast microscopy (fig. 4). The strain 4Q7 transformed with the plasmid vector pHT3101 without insert was used as negative control. After 72 h of growth in BM broth, autolysed cultures of recombinants carrying *cry8Kb3* or *cry8Pa3* showed electrophoretic bands corresponding to proteins of ca. 130 kDa similar to that observed from strain INTA Fr7-4 (fig. 4e, compare lane 1 to lanes 3 and 4). On the other hand, no ca. 130 kDa protein was produced by the control strain 4Q7 transformed with the plasmid with no *cry8* (fig. 4e, lane 2). These results indicate that promoters of both genes were active in strain 4Q7. The results of

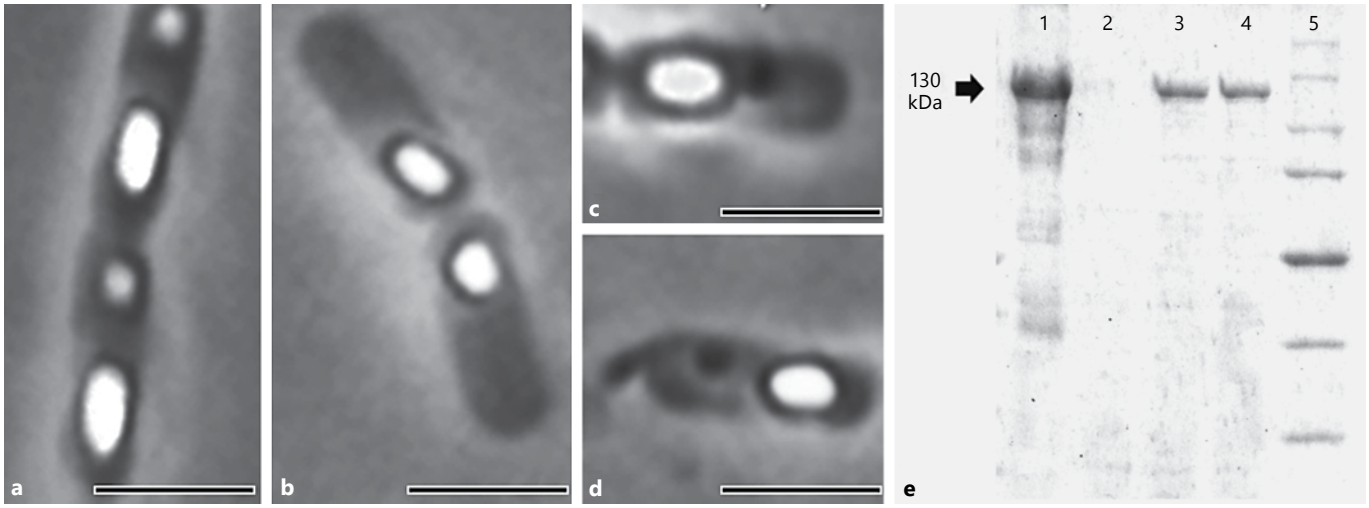

Fig. 4. a–d Phase-contrast micrographs and SDS-PAGE analysis of spore and crystal mixtures. Cultures in BM agar at 24 h of growth of (**a**) *B. thuringiensis* INTA Fr7-4, (**b**) recombinant 4Q7/pHT3101, (**c**) recombinant 4Q7/pHT*cry8Kb3*, and (**d**) recombinant 4Q7/pHT*cry8Pa3*. Scale bar 2 μm. **e** Expression analysis of INTA Fr7-4 *cry8* genes in BM broth at 72 h of growth by SDS-PAGE: *B. thuringiensis* INTA Fr7-4 (lane 1), recombinant 4Q7/pHT3101 (lane 2), recombinant 4Q7/pHT1*cry8Kb3* (lane 3), recombinant 4Q7/pHT*cry8Pa3* (lane 4), and molecular marker (lane 5).

SDS-PAGE correlated with the presence of inclusions in sporangia observed by phase-contrast microscopy at 24 h of growth in BM plates (fig. 4a–d). The parasporal inclusions present in 4Q7 recombinants carrying *cry8Kb3* and *cry8Pa3* under control of their own promoters were similar in shape to the crystals produced by strain INTA Fr7-4. However, the crystals were smaller and refracted light differently. These differential properties were also observed in crystals formed upon expression of *cry8Qa2*, the other *cry8* gene cloned from INTA Fr7-4, in strain 4Q7 [Amadio et al., 2013]. These differences in crystal appearances may be attributed to the lower level of expression of *cry8* genes in 4Q7. Alternatively, it is possible that *B. thuringiensis* INTA Fr7-4 crystals are formed by more than one *cry*8 gene product and that the interaction between them may stabilize the crystal structure.

On the other hand, some *cry* genes are transcribed as integral members of operons and their expression depends on the expression of other gene or genes within the operon [Crickmore and Ellar, 1992; Dervyn et al., 1995; Widner and Whiteley, 1989; Wu et al., 1991]. *Orf1* and similar genes seem to encode proteins which play a role as chaperones that assist the proper folding of Cry proteins. Dervyn et al. [1995] proposed that Cry11A of *B. thuringiensis* serovar *israelensis* is possibly targeted by P19, a putative chaperone encoded by a gene immediately upstream of *cry11A*, within the same transcriptional unit. *p19*, also known as *orf1*, has homologs in different *B. thuringiensis* isolates, in the same position relative to other *cry* genes, including *cry8* [Du et al., 2012, and this report]. Du et al. [2012] showed that *orf1* and *cry8Ea1* of *B. thuringiensis* strain BT185 form an operon and that *cry8* is transcribed alternatively as a bicistron or as a monocistron from an independent promoter within the intergenic region. Figure 2 shows *orf1* localized upstream of *cry8Pa3*. We have evidence that a gene homologous to *orf1* occurs at equivalent position upstream of *cry8Kb3* (data not shown). In the present work, the recombinant *cry8* genes from INTA Fr7-4 were expressed without any *orf1* gene product. This may have been another reason for obtaining crystal structures different in appearance from the crystals of the wild-type strain. Work is underway in our laboratory to test this hypothesis.

Insect Bioassays of Wild-Type and Recombinant Cry8 Proteins

The amino acid identity range between Cry8Kb proteins and their closest relatives of the class Cry8Ka is 65.7–89.2%. Cry8Ka toxins have been reported to be toxic to the cotton boll weevil, *A. grandis* (Coleoptera: Curculionidae) [Nakasu et al., 2010]. Therefore, we included this insect species in the bioassays to determine the toxicity of the three Cry8 proteins found in INTA Fr7-4. Spores of 4Q7/pHT3101 and spore-crystal complexes obtained from autolysed cultures of 4Q7/pHT*cry8Kb3*, 4Q7/pHT*cry8Pa3* and INTA Fr7-4 were fed to second-instar

Table 2. Toxic activity of *B. thuringiensis* spore-crystal complexes against neonate *A. grandis* larvae

Strain	Mortality, %
INTA Fr7-4	38.7±1.8[c]
4Q7/pHT*cry8Kb3*	5.3±3.0[a]
4Q7/pHT*cry8Pa3*	36.0±2.6[b, c]
4Q7/pHT*cry8Qa2*	21.4±3.6[a, b]
4Q7/pHT3101	14.9±3.8[a]

Values represent mean ± SEM. [a–c] A common letter indicates no significant difference at p = 0.05 or lower.

and neonate larvae of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) and *A. grandis* respectively. The spore-crystal complex of a recombinant *B. thuringiensis* strain that produces Cry8Qa2 (4Q7/pHT1Ac-*cry8* [Amadio et al., 2013]) was also included. Thereby, the three *cry8* genes reported from strain INTA Fr7-4 were separately evaluated and compared with the activity of the crystals from INTA Fr7-4.

Bioassay results showed that the Cry8Pa3, Cry8Qa2 and INTA Fr7-4 preparations had larvicidal toxicity against *A. grandis* (table 2), but that none of the preparations had toxicity against *A. diaperinus* (data not shown). The mortality rates of the natural mortality control were always less than 10%. The recombinant Cry8Pa3 expressed in 4Q7 showed the highest toxic activity against *A. grandis* larvae, in a level comparable to that of the preparations of the wild-type strain and followed closely by pHT1Ac-*cry8* (Cry8Qa2). The mortality rate of the recombinant Cry8Pa3 was significantly higher than that of the recombinant Cry8Kb3 but not from Cry8Qa2. The toxic activity against *A. grandis* found here was obtained after using much higher concentrations of the spore-crystal preparations than in Amadio et al. [2013]. The toxicity of *B. thuringiensis* against insects relies on the quantity and types of Cry proteins produced [Sauka and Benintende, 2008]. At the concentrations tested, 4Q7/pHT3101 (control without insert) and 4Q7/pHT*cry8Kb3* showed mortality rates that did not differ significantly. These results suggest that Cry8Kb3 has no insecticidal activity against *A. grandis*. Moreover, 4Q7/pHT3101 may harbor other unspecific virulence factors responsible of these low mortality rates, as also found in *B. thuringiensis* species [Sauka and Benintende, 2008].

The bioassay results suggest that Cry8Pa3 might be the main responsible for the insecticidal activity of INTA Fr7-4 against *A. grandis* larvae since the recombinant protein showed a level of toxicity similar to that of the parent strain. Cry8-type toxins have been described as toxic to coleopteran pests (mainly members of the Scarabaeidae family) [Asano et al., 2003; Shu et al., 2009], and to some lepidopteran pests [Amadio et al., 2013]. To our knowledge, there was only one work where Cry8 proteins, Cry8Ka in particular, showed toxicity against *A. grandis* larvae [Oliveira et al., 2011]. Cry8 proteins from INTA Fr7-4 could also be toxic to insect pests that have not yet been evaluated (e.g. scarab species).

In conclusion, we found three *cry* genes in *B. thuringiensis* INTA Fr7-4: two *cry8* genes arranged in tandem and the *cry8Qa2* previously reported. Each of the three *cry8* genes code for proteins with different amino acid sequences and could be expressed in an acrystalliferous *B. thuringiensis* strain. We demonstrated that each of these Cry8 proteins can individually generate ovoid crystals similar to the wild-type strain, although smaller. All three proteins expressed separately with their promoter sequences have similar molecular weight of 130 kDa. Only one of the three recombinant Cry8 proteins, Cry8Pa3, showed toxicity levels against *A. grandis* larvae equivalent to preparations of crystals and spores of INTA Fr7-4.

Experimental Procedures

B. thuringiensis Strains and Culture Conditions

B. thuringiensis INTA Fr7-4 is a bacterial isolate obtained from a soil sample of an orchard of the province of Misiones, Argentina. The acrystalliferous 4Q7 strain from serovar *israelensis* was kindly provided by the Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV, Irapuato, Mexico). The strains were grown in BM medium [Benintende and Cozzi, 1996] at 250 rpm and 30°C for 48 and 72 h (till autolysis), or in LB plates for 24 h, depending on the assay.

Amplification and Sequencing of the cry8 Genes and Intergenic Region

The *cry8Kb3* and *cry8Pa3* genes were identified as independent clones from a 3.5-kbp PCR product, cloned in plasmid vector pGEM®-T Easy (Promega), which was amplified with *cry8*-specific primers C8F01 and C8R01, and using a plasmid DNA preparation of INTA Fr7-4 as template, as described in Amadio et al. [2013].

To determine the complete sequence of the *cry8* genes, a primer walking strategy was followed. Sequences were assembled using the Gap4 program from the Staden Package software [Staden et al., 1999] and subsequent primers were designed upon newly obtained sequences, using the function included in the program.

To explore the possibility that the *cry8* genes were located in tandem, within a PCR-amplifiable distance, internal sense and antisense primers specific for one gene (table 1: C801-F1, C801-F2, C801-R1, C801-R2) were assessed in combination with primers

specific for the other gene (table 1: C802-F1, C802-F2, C802-R1, C802-R2). Total plasmid DNA obtained by an alkaline lysis procedure was used as template [Amadio et al., 2009] and AccuPrime™ High Fidelity Taq DNA Polymerase (Invitrogen) was used following Jones et al. [2006] for long PCR. The annealing temperature was calculated using the FastPCR software according to the thermodynamic properties of the primers [Kalendar et al., 2009]. A PCR product of about 9 kbp, amplified with primers C801-F1 and C802-R1, was sequenced following a primer walking strategy to find the nucleotide sequences between *cry8Kb3* and *cry8Pa3*. Sequences were assembled using Gap4. The ORFs were then analyzed using Artemis [Rutherford et al., 2000], and the BLAST online tool at NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Cloning and Expression of INTA Fr7-4 cry8 Genes in B. thuringiensis 4Q7

Cloning cry8Kb3. The *cry8Kb3* promoter sequence was obtained as part of a PCR product amplified with primers P02F and C801-R1. Primer P01F was designed starting 219 bp upstream of the start codon of *cry8Kb3* (fig. 2) and used with P01R to amplify the gene up to 120 bp downstream of the stop codon.

Cloning cry8Pa3. Sequences downstream of *cry8Pa3* were obtained as part of a long PCR product successfully amplified after surveying a panel of primers specific for *B. thuringiensis* sequences in combination with C802-F2. Primer P02R was designed 120 bp downstream of the stop codon of *cry8Pa3* and used in combination with P02F to amplify the *cry8Pa3* gene from 400 bp upstream of the start codon.

The PCR amplified products were cloned into pGEM®-T Easy vector (Promega) and excised with restriction enzymes *Sph*I and *Sac*I (Promega) and gel-purified. The corresponding *Sph*I-*Sac*I fragments were ligated to the shuttle vector pHT3101 [Lereclus et al., 1989] previously digested with the same enzymes. The shuttle vector used can replicate in both *E. coli* and *B. thuringiensis*, expressing resistance to ampicillin and erythromycin, respectively. The vector and the digested DNA were ligated with T4 DNA ligase (Promega) and the ligation reaction was used to transform *E. coli* XL1. By means of DNA sequencing, we confirmed that the constructs, pHT*cry8Kb3* and pHT*cry8Pa3*, carried the *cry8Kb3* and *cry8Pa3* coding sequences and their corresponding promoters. The purified recombinant plasmids and parental plasmid pHT3101 from *E. coli* were used to transform the acrystalliferous *B. thuringiensis* 4Q7 strain by electroporation, as previously described [Mahillon and Lereclus, 2000]. Transformed bacteria were selected by erythromycin resistance in LB plates incubated at 30°C. Crystals of Cry8Kb3 and Cry8Pa3 were detected by phase-contrast microscopy and photographed with a digital camera CoolSnap-Pro cf. The expressions of proteins by the different *B. thuringiensis* strains were determined by SDS-PAGE using a 10% acrylamide/bisacrylamide gel, following standard methods [Sambrook et al., 1989]. Briefly, 1-ml samples of autolysed cultures were centrifuged at 13,000 rpm for 1 min, followed by a step of washing with distilled water and resuspension in 40 µl of 2× sample buffer. After heat-denaturalization for 10 min, 10 µl of each sample was loaded into the gel.

Comparison of B. thuringiensis Cry8 Proteins

The predicted amino acid sequence of Cry8Kb3 and Cry8Pa3 INTA Fr7-4 were aligned with one member of each subclass of Cry8 proteins available at the *B. thuringiensis* toxin nomenclature website [Crickmore et al., 2013]. The multiple sequence alignment was generated using the ClustalX2 [Larkin et al., 2007] software. The model of amino acid change used to compute the distance matrix was that of Jones et al. [1992] and the Neighbor Joining method of Saitou and Nei [1987] of clustering to construct the phylogenetic tree. TreeView was used to plot the rooted tree diagram [Page, 1996]. Cry1Aa1 sequence (GenBank accession No. AAA22353) was used as out group.

Insect Toxicity Assays

The toxicity of *B. thuringiensis* spore-crystal suspensions obtained from autolysed cultures was analyzed by bioassays against second-instar larvae of *A. diaperinus* Pz. (Coleoptera: Tenebrionidae) and neonate larvae of *A. grandis* Bh. (Coleoptera: Curculionidae). To this end, 6 and 4 ml of cultures containing spore or spore-crystal suspensions (1.1×10^8 cfu/ml) were incorporated into polypropylene conical tubes containing 34 and 36 ml of a thermostatized (60°C) artificial diet for *A. diaperinus* and *A. grandis*, respectively. 400 µl of each mix were poured into each well of a 24-well plate (Nunc 143982). Sterile BM medium was added to the control. 48 larvae were used per assay (three repetitions in different days). Mortality was recorded after 7 days at 29°C. *A. diaperinus* and *A. grandis* larvae were considered dead if they failed to respond to gentle probing. Schneider-Orelli's formula [Schneider-Orelli, 1947] was used to calculate corrected mortality in comparison to the untreated control. Finally, data went through ANOVA and Tukey's test ($p < 0.05$) for means comparison.

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References

Amadio AF, Benintende GB, Zandomeni RO: Complete sequence of three plasmids from *Bacillus thuringiensis* INTA-FR7-4 environmental isolate and comparison with related plasmids from the *Bacillus cereus* group. Plasmid 2009;62:172–182.

Amadio AF, Navas LE, Sauka DH, Berretta MF, Benintende GB, Zandomeni RO: Identification, cloning and expression of an insecticide *cry8* gene from *Bacillus thuringiensis* INTA Fr7-4. J Mol Microbiol Biotechnol 2013;23:401–409.

Asano S, Yamashita C, Iizuka T, Takeuchi K, Yamanaka S, Cerf D, Yamamoto T: A strain of *Bacillus thuringiensis* subsp. *galleriae* containing a novel *cry8* gene highly toxic to *Anomala cuprea* (Coleoptera: Scarabaeidae). Biol Control 2003;28:191–196.

Benintende G, Cozzi J: Actividad insecticida de distintas fracciones de cultivos líquidos de *Bacillus moritai* (Aizawa y Fujiyoshi) en *Musca domestica* L. (Diptera: Muscidae). RIA 1996; 27:27–32.

Bravo A, Gill SS, Soberón M: Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 2007;49:423–435.

Crickmore N, Baum J, Bravo A, Lereclus D, Narva K, Sampson K, Schnep E, Sun M, Zeigler DR: *Bacillus thuringiensis* toxin nomenclature. 2014. http://www.btnomenclature.info.

Crickmore N, Ellar DJ: Involvement of a possible chaperonin in the efficient expression of a cloned CryIIA δ-endotoxin gene in *Bacillus thuringiensis*. Mol Microbiol 1992;6:1533–1537.

De Maagd RA, Bakker P, Staykov N, Dukiandjiev S, Stiekema W, Bosch D: Identification of *Bacillus thuringiensis* δ-endotoxin Cry1C domain III amino acid residues involved in insect specificity. Appl Environ Microbiol 1999; 65:4369–4374.

Dervyn E, Poncet S, Klier A, Rapoport G: Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. J Bacteriol 1995;177:2283–2291.

Du L, Qiu L, Peng Q, Lereclus D, Zhang J, Song F, Huang D: Identification of the promoter in the intergenic region between orf1 and *cry8Ea1* controlled by sigma H factor. Appl Environ Microbiol 2012;78:4164–4168.

Herrero S, Gonzalez-Cabrera J, Ferre J, Bakker PL, de Maagd RA: Mutations in the *Bacillus thuringiensis* Cry1Ca toxin demonstrate the role of domains II and III in specificity towards *Spodoptera exigua* larvae. Biochem J 2004;384:507–513.

Jones DT, Taylor WR, Thornton JM: The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 1992; 8:275–282.

Jones LR, Zandomeni RO, Weber EL: A long distance RT-PCR able to amplify the Pestivirus genome. J Virol Methods 2006;134:197–204.

Kalendar R, Lee D, Schulman AH: FastPCR software for PCR primer and probe design and repeat search. Genes Genomes Genomics 2009;3:1–14.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG: Clustal W and Clustal X version 2.0. Bioinformatics 2007;23: 2947–2948.

Lereclus D, Arantes O, Chaufaux J, Lecadet M: Transformation and expression of a cloned δ-endotoxin gene in *Bacillus thuringiensis*. FEMS Microbiol Lett 1989;51:211–217.

Mahillon J, Lereclus D: Electroporation of *Bacillus thuringiensis* and *Bacillus cereus*; in Eynard N, Teissie J (eds): Electro-Transformation of Bacteria. Springer Lab Manual. Berlin, Springer, 2000, pp 242–252.

Mahillon J, Rezsöhazy R, Hallet B, Delcour J: IS231 and other *Bacillus thuringiensis* transposable elements: a review. Genetica 1994;93: 13–26.

Nakasu EY, Firmino AA, Dias SC, Rocha TL, Ramos HB, Oliveira GR, Lucena W, Carlini CR, Grossi-de-Sá MF: Analysis of Cry8Ka5-binding proteins from *Anthonomus grandis* (Coleoptera: Curculionidae) midgut. J Invertebr Pathol 2010;104:227–230.

Ogiwara K, Hori H, Minami M, Takeuchi K, Sato R, Ohba M, Iwahana H: Nucleotide sequence of the gene encoding novel δ-endotoxin from *Bacillus thuringiensis* serovar *japonensis* strain Buibui specific to scarabaeid beetles. Curr Microbiol 1995;30:227–235.

Oliveira GR, Silva MC, Lucena WA, Nakasu EY, Firmino AA, Beneventi MA, Souza DS, Gomes JE Jr, de Souza JD Jr, Rigden DJ, Ramos HB, Soccol CR, Grossi-de-Sa MF: Improving Cry8Ka toxin activity towards the cotton boll weevil *(Anthonomus grandis)*. BMC Biotechnol 2011;11:85.

Page RDM: TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 1996;12:357–358.

Roh JY, Jae YC, Ming SL, Byung RJ, Yeon HJ: *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. J Microbiol Biotechnol 2007;17:547–559.

Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B: Artemis: sequence visualization and annotation. Bioinformatics 2000;16:944–945.

Saitou N, Nei M: The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–425.

Sambrook J, Fritsch E, Maniatis T: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1989.

Sauka D, Benintende G: *Bacillus thuringiensis*: general aspects. An approach to its use in the biological control of lepidopteran insects behaving as agricultural pests. Rev Argent Microbiol 2008;40:124–140.

Schneider-Orelli O: Entomologisches Praktikum, ed 2. Aarau, Sauerlander, 1947.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH: *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 1998;62: 775–806.

Shu C, Yu H, Wang R, Fen S, Su X, Huang D, Zhang J, Song F: Characterization of two novel *cry8* genes from *Bacillus thuringiensis* strain BT185. Curr Microbiol 2009;58:389–392.

Staden R, Beal KF, Bonfield JK: The Staden package, 1998. Methods Mol Biol 1999;132:115–130.

Widner WR, Whiteley HR: Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. J Bacteriol 1989;171:965–974.

Wu D, Cao XL, Bay YY, Aronson AI: Sequence of an operon containing a novel δ-endotoxin gene from *Bacillus thuringiensis*. FEMS Microbiol Lett 1991;81:31–36.

Yu H, Zhang J, Huang D, Gao J, Song F: Characterization of *Bacillus thuringiensis* strain Bt185 toxic to the Asian cockchafer, *Holotrichia parallela*. Curr Microbiol 2006;53:13–17.

Zhang J: Evolution by gene duplication: an update. Trends Ecol Evol 2003;18:292–298.