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Cloning and Characterization of a Novel Crystal Protein from a Native *Bacillus thuringiensis* Isolate Highly Active Against *Aedes aegypti*

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Abstract. We characterized a novel *Bacillus thuringiensis* isolate native to Argentina (FCC 41) that exhibits a mosquitocidal activity higher than the reference *B. thuringiensis* subsp. *israelensis*. This isolate shows a rounded crystal harboring two major proteins of about 70–80 kDa. Moreover, we cloned and sequenced the encoding gene of one of the crystal proteins (Cry) consisting of an open reading frame of 2061 pb that encodes a protein of 687 amino acid residues. The deduced amino acid sequence has a predicted relative molecular mass of 78 kDa and is 52% and 45% identical to those of the reported Cry24Aa and Cry24Ba sequences, respectively. The novel Cry protein was designated as Cry24Ca, which also exhibited larvicidal activity against *Aedes aegypti* when its encoding gene was expressed in an *Escherichia coli* host strain.

Blood-sucking insects such as mosquitoes and blackflies are vectors of many human and animal infectious diseases. One of the most important is *Aedes aegypti*, the primary carrier for viruses that cause dengue fever and yellow fever. According to the World Health Organization, despite advances in medical science and new drugs, dengue fever is the most important arboviral disease to man in the world (http://www.who.int/topics/dengue/en). Dengue has become a major international public health concern, spreading geographically in incidence and severity [28]. The control of this disease is based mainly on the elimination of its vector with synthetic products, which has been shown a rapid increase in mosquito resistance to various chemical insecticides. In addition, there has been a growing public concern regarding their potential adverse environmental effects that prompted the development of alternative methods for mosquito control that were compatible with the environment [16]. Although more than 100 bacterial species has been identified as insect pathogens, only certain *Bacillus* species have met success as bioinsecticides [24]. Particularly, formulations based on *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) and *B. sphaericus* (*Bs*) have been shown to be highly effective against mosquito larvae and blackfly larvae. The insecticidal activity of these bacteria is mainly ascribed to proteins produced during sporulation that are included in a crystal complex, which in the case of *Bti* are Cry4A, Cry4B, Cry11A, and Cyt1A [10] and in *Bs* are a single binary protein (Bin) and other reported toxins [9]. The successful use of *Bti* and *Bs* strains in some dipterans control programs stimulated the search for more potent bacterium isolates [10]. Among other examples that demonstrate the value of searching for new mosquitocidal strains, it was reported the isolation of *B. thuringiensis* subsp. *jegathesan* native to Malasya, which produces a very complex parasporal body containing at least seven major polypeptides, including Cry11B. This toxin showed higher toxic activity against mosquito larvae than the previously reported Cry11A of *Bti* [8]. Recently, we have isolated a new mosquitocidal *B. thuringiensis* strain (FCC 41), native to Argentina, which might produce at least one Cry protein unrelated to those of *Bti* [4, 5]. This article describes the characterization of a novel *B. thuringiensis* isolate native to Argentina (FCC 41) that exhibits a mosquitocidal activity higher than the reference *Bti* (HD 567). Moreover, we isolated and sequenced the encoding sequence

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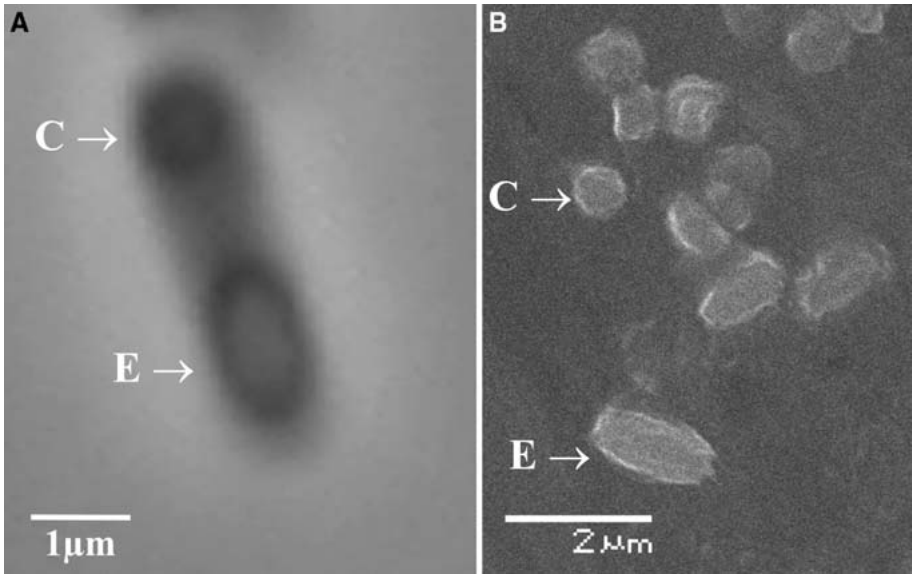

Fig. 1. Micrograph of *B. thuringiensis* FCC 41 strain. (A) Phase-contrast photograph of a sporulating culture; (B) scanning electron micrograph of a crystal. S: spores, C: crystals.

of a novel Cry protein named Cry24Ca. We also showed the insecticidal activity of the recombinant Cry24Ca against *A. aegypti* larvae.

Materials and Methods

Bacterial Strains, Culture Conditions, and Plasmids. The FCC 41 strain was obtained from the FIBA Culture Collection [5]. *B. thuringiensis* subsp. *israelensis* (*Bti*) reference strain (HD 567 strain) was supplied by J. Ibarra (CINVESTAV, Irapuato, Mexico). *B. thuringiensis* cells were cultured 28°C in nutrient broth (Difco). Cells of *Escherichia coli* DH5α and BL21(λDE3):pLysS (Novagen) strains were grown at 37°C in Luria-Bertani (LB) medium, supplemented with the appropriate antibiotic [carbenicillin (Cb), or Cb and chloramphenicol (Cm)] [25]. Plasmids derivative from pGEM-T Easy (Promega) and pRSET A (Invitrogen) were constructed for cloning and expression of the *cry24Ca* gene, respectively [25].

Microscopic Observations. Sporangium cells of *B. thuringiensis* FCC 41 were observed under a phase-contrast microscope. Spore–crystal suspensions of the isolate were air-dried on aluminum mounts. Samples were coated with Au/Pd and examined and photographed in a JEOL JFM 6460LV scanning electron microscope.

Polypeptide Analysis. The FCC 41 strain crystals were purified on discontinuous sucrose gradients [82%, 75%, 70%, 65%, 60%, and 55% (wt/vol)] at 5000 rpm for 15 min in a Beckman Optima™ LE−80K centrifuge, rotor SW 25.1. Polypeptide compositions of the spore–crystal complexes and of purified crystals were determined by sodium dodecyl sulfate–polyacrylamide gel electophoresis (SDS-PAGE) on 10% polyacrylamide gels [17] after visualization with Coomassie blue.

Molecular Methods. DNA modifications and all enzymes were used according to the instructions of the manufacturers and carried out as described [25]. Extraction of total DNA from *B. thuringiensis* cells was done as reported previously [7]. Competent *E. coli* cells and plasmids were prepared by standard procedures [25]. Transformant of *E. coli* strains DH5α and BL21:pLys were selected on LB plates containing Cb, X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and IPTG (isopropyl-beta-D-thiogalactopyranoside), and Cb and Cm, respectively. DNA was analyzed by agarose gel electrophoresis and visualized with ethidium bromide [25].

Table 1. Toxic activity of *B. thuringiensis* FCC 41 native strain against *A. aegypti* four-instar larvae compared with the mosquitocidal reference strain (HD 567)

Strain	LC_{50} (µg/mL)
HD 567 (subsp. *israelensis*)	3.812 (2.921– 4.973)
FCC 41	0.476 (0.139–1.624)

LC_{50}: 50% lethal concentration (in µg) of dry spore–crystal complex per milliliter after 24 h. The data are the average of three assays with the 95% confidence limit, as determined by Probit analysis, in parentheses.

Gene Amplification, Cloning, and Identification. Amplifications were performed with a PTC-100 thermal cycler (MJ Research). From total DNA, we amplified a 900-bp DNA fragment with a two-step polymerase chain reaction (PCR)-based approach [4]. Then a thermal asymmetric interlaced (TAIL) PCR strategy [19] was carried out with six specific primers to amplify the complete coding sequence of the *cry* gene. The nested specific primers used were: SP1 (5′ CGTATAGATTT ACTGGAGAACTGGCATATG 3′), SP2 (5′ TCCTTATACTTTTAT TGGTAGTTTCTTTGA 3′), SP3 (5′ GCCTATAACTTTAGCAT TTCCAGGAGAAGA 3′), SP4 (5′ AGTCCAATTACTGAAATCTGA GAAGGTAGC 3′), SP5 (5′ ATTTGTGTAGGTAATGGATAT TGTACAGAA 3′), and SP6 (5′ TTGTACAGAATCATAAAGTGGA AATAATGT 3′). In addition, two arbitrary degenerate (AD) primers were used: AD3 (5′ WGTGNAGWANCANAG 3′) and AD5 (5′ WCAGNTGWTNGTNCT 3′) [19]. The original 900-bp sequence was assembled in silica with the upstream and downstream flanking sequences originated from the TAIL-PCR methodology. The full-length Cry encoding sequence (2061 bp) was amplified using 0.2 µg of total DNA template in a reaction mixture (total volume: 25 µL) containing 2 µM of the following primers: 24Ca-F (5′ GGATCCG GAGTAGCAAAGATGAATC 3′) and 24Ca-R (5′ GGTACCCCTCTG CTTTAGTAAATACTTTGG 3′); each deoxynucleoside-triphosphate at

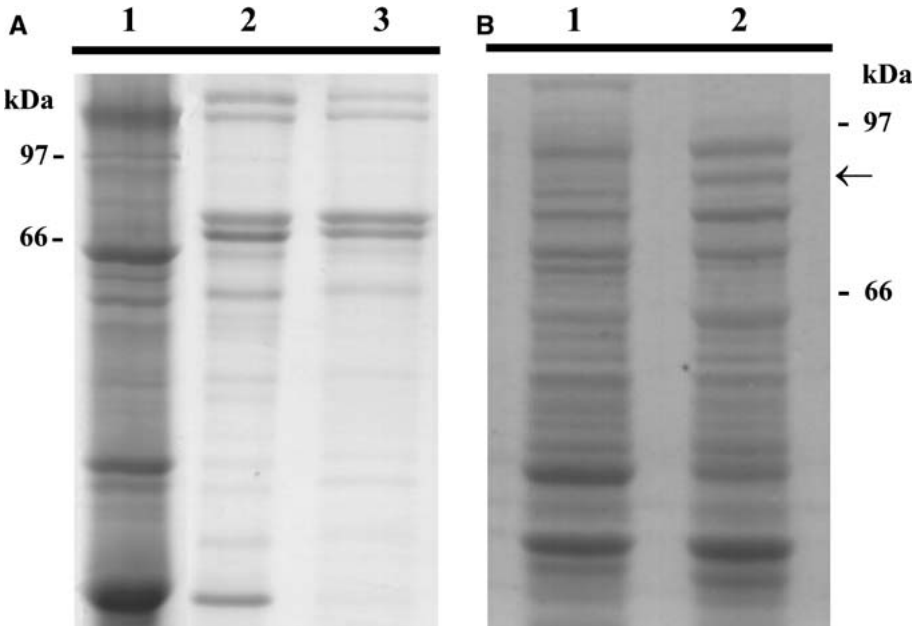

Fig. 2. SDS-PAGE analysis. (A) Lane 1, HD 567 reference strain; lane 2, spore–crystal suspension of *B. thuringiensis* FCC 41 strain; lane 3, FCC 41 purified crystals. (B) Recombinant proteins. Lane 1, crude extract from noninduced *E. coli*; lane 2, crude extract from transformed *E. coli* IPTG-induced cells. Polypeptides were stained with Coomassie blue. The arrow indicates the position of the induced polypeptide. Standard molecular mass (M_r) proteins: phosphorylase B (97.4 kDa), bovine albumin (66 kDa).

a concentration of 400 μM and 0.5 U of high-fidelity *Pfu* DNA polymerase (MCLAB) was dissolved in the corresponding reaction buffer (Promega), under the following conditions: 2 min of denaturation at 94°C, followed by 30 cycles of denaturation for 45 s at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C. An extra extension step consisting of 5 min at 72°C was added after completion of the 30 cycles. DNA sequencing was conducted under BigDyeTM terminator cycling conditions, using an automatic sequencer 3730xl (Macrogen Services, Korea). The amplification products were ligated into the cloning vector pGEM-T Easy vector. The 2061-pb amplicon was ligated between the *Bam*HI and *Kpn*I sites of the expression vector pRSET A. The resulting construct was introduced into *E. coli* strain BL21(λDE3):pLysS.

Bioassays for Mosquito Larvicidal Activity. *Aedes aegypti* larvae were reared at 28°C and a 8:16 dark/light photoperiod. Insecticidal activity of the *B. thuringiensis* FCC 41 strain was determined on mosquito larvae as described previously [20]. Twenty early four-instar larvae were place in 100 mL of dechlorinated water. Six concentrations (1 μg/mL to 100 ng/mL) of the spore–crystal complex were added (three repetitions). The HD 567 strain was included as the positive control. Larvae were incubated at 28°C and examined after 24 h. The mean 50% lethal concentration (LC_{50}) was estimated by Probit analysis in six doses using statistical parameters [14]. All bioassays were performed three times in duplicates for each concentration. Similarly, transformed *E. coli* cells expressing the Cry24Ca protein were tested for mosquitocidal activity, using noninduced BL21pLys cells as a negative control [3]. Recombinant Cry24Ca protein used in each assay was quantified in parallel after separation by SDS-PAGE using an imaging analyzer (Fotodyne System) and its dedicated software.

Sequence Analysis. BLAST X (version 2.2.6) was used for DNA sequence analysis [1]. Known Cry sequences obtained from the nonredundant protein database National Center for Biotechnology (http://www.ncbi.nlm.nih.gov) were used for sequence alignments, generated with the CLUSTAL X software program (version 1.8) [15], and the ProDom program, using the BLOSUM 62 matrix and Pfam database [2]. Dendrograms were compiled from 1000 independent trials of CLUSTAL X. The graphical representation of the tree was generated using the TREVIEW 16 program.

Nucleotide Sequence Accession Number. The nucleotide sequence obtained in this study (*cry24Ca*) has been deposited in the EMBL database under accession no. AM158318. In order to receive a proper designation, the nucleotide sequence obtained was sent to the *Bacillus thuringiensis* Toxin Nomenclature Committee.

Results and Discussion

Native *B. thuringiensis* Isolate Showed High Mosquito Larvicidal Activity. Insecticidal activity of the FCC 41 *B. thuringiensis* isolate, which produces spherical parasporal bodies (Fig. 1), was tested against four-instar *A. aegypti* larvae (Table 1). Spore–crystal complex preparations obtained from autolysed cells of both FCC 41 and HD 567 strains were used in the bioassays. FCC 41 showed significantly higher mosquitocidal activity than that of the reference strain. Similar results were obtained with other Latin American *B. thuringiensis* strains [13].

Polypeptide Analysis. The polypeptide composition of purified parasporal bodies of the FCC 41 isolate was analyzed by SDS-PAGE (Fig. 2A). The polypeptide pattern was different than that of the reference mosquitocidal strain, showing two major polypeptides of M_r between 70 and 80 kDa.

Cloning and Sequence Analysis of a New Crystal Protein Gene. First, a 900-bp DNA fragment was PCR-amplified and cloned from the native isolate FCC 41.

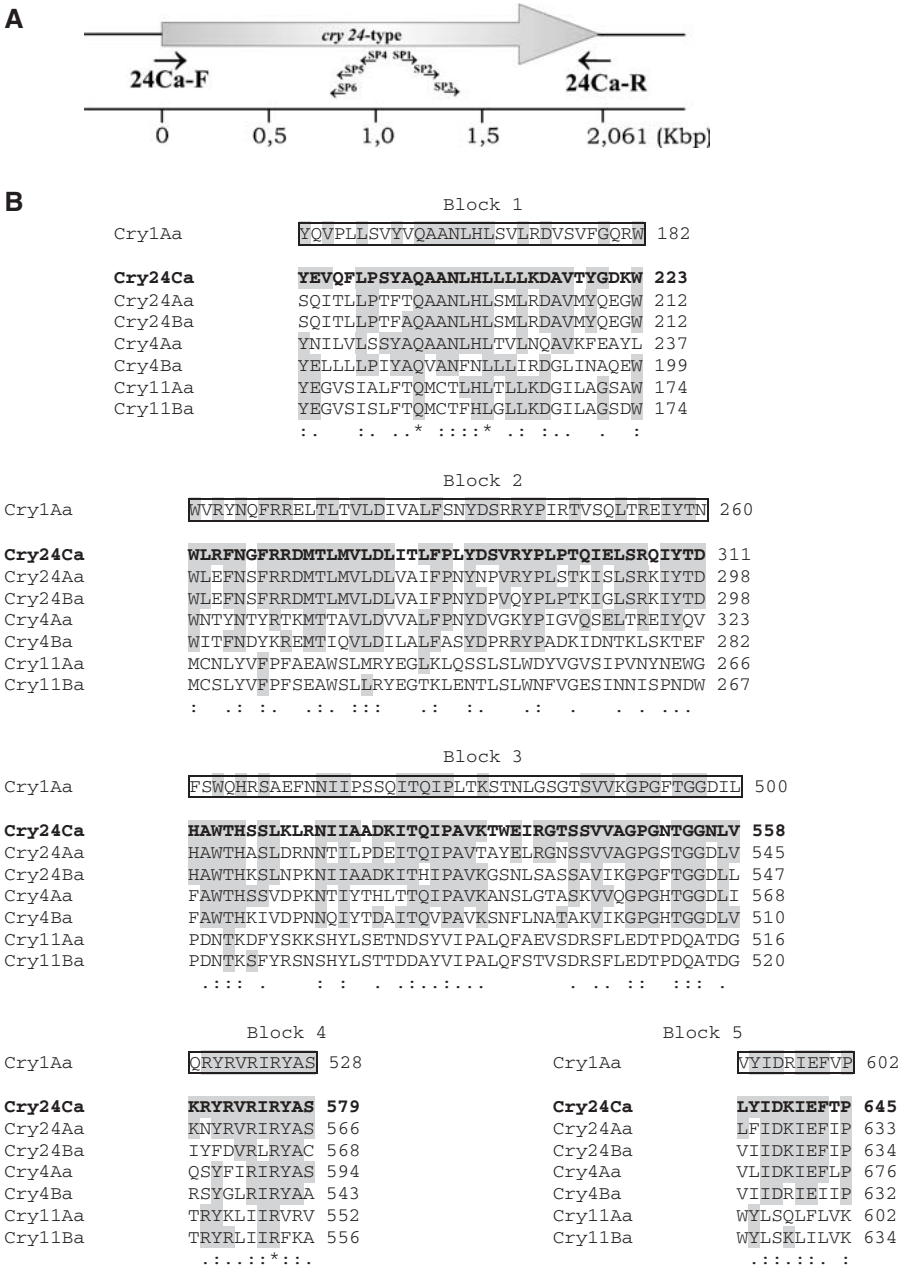

Fig. 3. (A) Schematic diagram of the localization of the nested TAIL-PCR specific primers (SP1–SP6) and PCR reactions (24Ca-F and 24Ca-R) designed for the amplification of the *cry24*-type sequence. (B) Alignment of amino acid sequences of Cry24Ca and other mosquitocidal proteins in the five Cry conserved blocks [26]. Identity in all sequences is marked with an asterisk; in at least six out of nine sequences, it is marked with two dots, and in at least four out of nine sequences, it is marked with a dot. Conserved amino acids with Cry24Ca sequence are indicated in gray. Cry1Aa was included as reference.

Then we obtained a 2061-bp open reading frame (ORF) using a TAIL-PCR upstream and downstream strategy and the primers indicated in Fig. 3A. The full-length sequence encodes a polypeptide of 687 amino acid residues with a M_r of 78 kDa. Sequence alignment analysis revealed that it corresponded to a putative Cry protein, 52% and 45% identical to Cry24Aa from *B. thuringiensis* subsp. *jegathesan* [8] and Cry24Ba from *B. thuringiensis* subsp. *sotto* [22] toxins, respectively. This result suggests that the three proteins might have evolved from a common ancestor (Fig. 4). The novel *cry* gene was designated as *cry24Ca* by the *Bacillus thuringiensis* Pesticide Crystal Protein Nomenclature Committee.

Analysis of the Cry24-like Deduced Aminoacidic Sequence. A CLUSTAL X comparison of the new Cry with other known Cry protein sequences (http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html) helped to identify the characteristic Cry conserved blocks predicted by Schnepf et al. [26]. Although the novel Cry24Ca sequence has weak identity with other

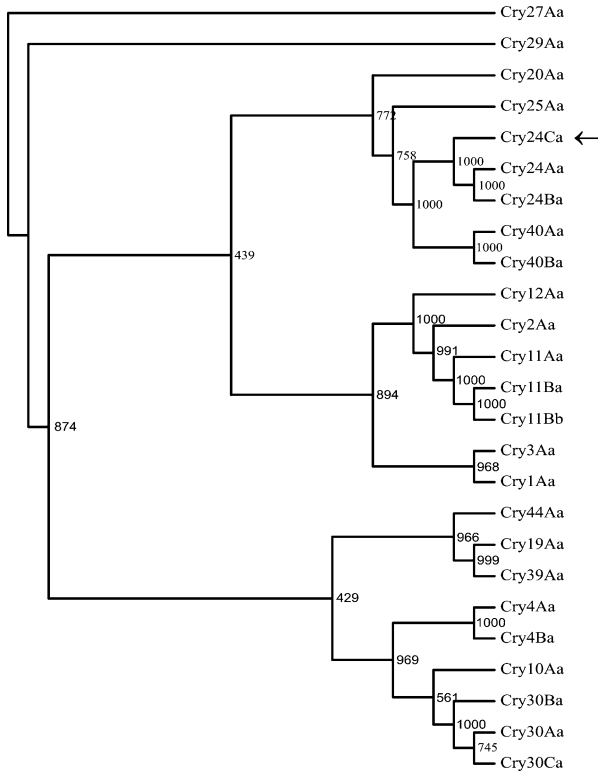

Fig. 4. Phylogenetic analysis of Cry proteins. Neighbor-joining phylograms were constructed after sequence alignment of the deduced amino acid sequences of selected known toxins and Cry24Ca protein by using the CLUSTAL X program and a BLOSSUM matrix. The tree was generated using TREEVIEW. Sequences were obtained from the NCBI database: Cry1Aa (AAA22353), Cry2Aa (M31738), Cry3Aa (M22472), Cry4Aa (Y00423), Cry4Ba (X07423), Cry10Aa (M12662), Cry11Aa (M31737), Cry11Ba (X86902), Cry11Bb (AF017416), Cry12Aa (L07027), Cry19Aa (Y07603), Cry20Aa (U82518), Cry24Aa (U88188), Cry24Ba (BAD32657), Cry25Aa (U88189), Cry27Aa (AB023293), Cry29Aa (AJ251977), Cry30Aa (AJ251978), Cry30Ba (BAD00052), Cry30Ca (BAD67517), Cry39Aa (BAB72016), Cry40Aa (BAB72018), Cry40Ba (BAC77648), and Cry44Aa (BAD08532). The arrow indicates the position of the novel Cry sequence. Numbers at nodes represent the percentages of bootstrap resamplings based on 1000 replicates.

mosquitocidal proteins like Cry2, Cry4, and Cry11 toxins (not shown), a detailed analysis revealed that it has conserved amino acid residues present in the five conserved regions (Fig. 3B). Also, the low relation between Cys and Arg residues proposed as characteristic of mosquitocidal Cry toxins was also found in the Cry24Ca protein sequence (5 Cys and 38 Arg residues) [9].

The analysis of the Cry24Ca amino acid sequence using the ProDom service [27] showed that it is composed of three distinct domains as reported for crystallized Cry proteins (Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, and Cry4Ba) [6, 11, 12, 18, 21]. Particularly, the N-terminal and the C-terminal regions might be composed of 55 and 27 residues, respectively. In addition, the current model for the mechanism of action of the Cry toxins proposes a crucial role for the α4–α5 loop in the toxicity of different Cry toxins. Particularly, in Cry4A and Cry4B, four amino acid residues (Arg-158 in α4, a polar residue at position −166, an aromatic residue at position –170, and a Tyr-202 within the α4–α5 loop) were considered critical for mosquitocidal activity [23]. Interestingly, three of them (an Arg in α4, a polar amino acid, and a Tyr residue within the α4–α5 loop) are found in the novel Cry24Ca.

Toxicity of *cry24Ca* Gene Expressed in *E. coli*. The toxic activity of Cry24Ca protein was assayed in the BL21(λDE3):pLysS *E. coli* strain transformed with a plasmid harboring the *cry24Ca* gene. Cell extract from transformed *E. coli* bacteria induced with IPTG (Fig. 2B), showed insecticidal activity against *A. aegypti* larvae showing a LC_{50} of 2.21 cells × 10^6/mL ± (1.596–3.067), which was equivalent to ∼2–5 μg of recombinant protein. Noninduced cells were used as control and no mortality was observed. This result indicates that Cry24Ca is a mosquitocidal toxin.

In conclusion, this study demonstrates that the novel *B. thuringiensis* isolate native to Argentina (FCC 41) exhibits a different crystal polypeptide pattern and higher mosquitocidal activity than the *Bti* reference. Moreover, we were able to identify and characterized one of the crystal proteins as a novel Cry24Ca that confers toxicity against *A. aegypti*.

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