Novel Bacillus thuringiensis δ-Endotoxin Active against Locusta migratoria manilensis

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A novel δ-endotoxin gene was cloned from a Bacillus thuringiensis strain with activity against Locusta migratoria manilensis by PCR-based genome walking. The sequence of the cry gene was 3,432 bp long, and it encoded a Cry protein of 1,144 amino acid residues with a molecular mass of 129,196.5 kDa, which exhibited 62% homology with Cry7Ba1 in the amino acid sequence. The δ-endotoxin with five conserved sequence blocks in the amino-terminal region was designated Cry7Ca1 (GenBank accession no. EF486523). Protein structure analysis suggested that the activated toxin of Cry7Ca1 has three domains: 227 residues forming 7 α-helices (domain I); 213 residues forming three antiparallel β-sheets (domain II); and 134 residues forming a β-sandwich (domain III). The three domains, respectively, exhibited 47, 44, and 34% sequence identity with corresponding domains of known Cry toxins. SDS-PAGE and Western blot analysis showed that Cry7Ca1, encoded by the full-length open reading frame of the cry gene, the activated toxin 1, which included three domains but without the N-terminal 54 amino acid residues and the C terminus, and the activated toxin 2, which included three domains and N-terminal 54 amino acid residues but without the C terminus, could be expressed in Escherichia coli. The expressed proteins Cry7Ca1 and the activated toxins (toxins 1 and 2) showed significant activity against 2nd instar locusts, and after 7 days of infection, the estimated 50% lethal concentrations (LC50s) were 8.98 μg/ml for the expressed Cry7Ca1, 0.87 μg/ml for the activated toxin 1, and 4.43 μg/ml for the activated toxin 2. The δ-endotoxin also induced histopathological changes in midgut epithelial cells of adult L. migratoria manilensis.

Bacillus thuringiensis is a spore-forming entomopathogenic bacterium that produces parasporal crystals composed of proteins called δ-endotoxins during sporulation (8, 19, 36, 45). It is reported that B. thuringiensis exhibits specific activity against Lepidoptera, Diptera, Coleoptera, Homoptera, Hymenoptera, Mallophaga, nematodes, and other invertebrates (2, 7, 9, 24, 44). B. thuringiensis is a uniquely specific, safe, and effective biological insecticide that has been used for more than 50 years (3, 30). The increased use of B. thuringiensis in biological control and its potential application in medicine have encouraged researchers to find novel strains with different toxic spectra or high specific activity and new functional genes of B. thuringiensis (13, 22, 26, 28).

There are two known classes of δ-endotoxins in B. thuringiensis: the insect-specific insecticidal crystal (Cry) proteins and the Diptera-specific cytolytic (Cyt) proteins. The Cry proteins are encoded by cry genes, include more than 480 different genes belonging to 124 subgroups of 60 groups, and show variable degrees of sequence homology (see the B. thuringiensis [Bt] toxin nomenclature website at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Most of the Cry proteins contain five conserved sequence blocks according to the alignment of the Cry toxins (10), and they are arranged in three distinct domains (domains I to III, from N terminus to C terminus), which play different roles in the entomopathogenic process (14, 15, 17, 27, 31, 37, 39, 46). So far, the three-dimensional structures of some Cry toxins, Cry3Aa (Protein Data Bank [PDB] entry 1DLC) (27), Cry3Bb1 (PDB entry 1J16) (12), Cry1Aa (PDB entry1C1) (17), Cry2Aa (PDB entry 1I5P) (29), Cry4Ba (PDB entry 1W99) (4), Cry4Aa (PDB entry 2C9K) (5), and Cry8Ea1 (PDB entry 3EB7) (18) have been determined by X-ray crystallographic methods. A given Cry protein has a fairly narrow range of target organisms against which it is effective. For example, Cry1-, Cry2-, and Cry9-type toxins are active against Lepidoptera; Cry3, Cry7, Cry8, Cry18, etc., are active against Coleoptera; Cry2, Cry4, Cry10, Cry11, Cry16, etc., are active against Diptera; and Cry5 and Cry6 toxins exhibit activity against plant-parasitic nematodes and plant-pathogenic nematodes (1, 11, 23, 41, 48).

Locusts (Orthoptera) are pests that cause extensive destruction of crops (43, 47). However, the mechanisms by which B. thuringiensis δ-endotoxins act against locusts remain unclear. Quesada-Moraga and Santiago-Alvarez (33) reported the histopathological effect of a strain of B. thuringiensis serovar aizawai on the midgut of the Mediterranean locust Dociostaurus maroccanus. Their research indicated that the midguts of adults fed B. thuringiensis serovar aizawai were in an advanced state of disruption 12 h following exposure and that 54% of newly molted adults died 8 days after treatment. We previously showed the toxic effects of the B. thuringiensis strain BTH-13 against Locusta migratoria manilensis (40). In this paper, we characterized this novel δ-endotoxin from BTH-13 and its activity against L. migratoria manilensis.

MATERIALS AND METHODS

Bacterial strains and plasmids. B. thuringiensis strain BTH-13 active against L. migratoria manilensis was isolated from a Chinese soil sample (40). The plasmid vector pGEM-T Easy was purchased from Promega. Vector pQE30 and Escherichia coli M15 were provided by Ziniu Yu of Huazhong Agricultural University.

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Cloning of crystal protein gene (cry) from BTH-13. The genomic DNA was prepared from strain BTH-13 cells grown as previously described by Gao et al. (13). Extraction of plasmid DNA from E. coli, restriction digestion, ligation reactions, and transformation were performed as recommended by the manufacturers (35). For cloning the cry gene from strain BTH-13, an approximately 1.2-kb cry fragment was amplified from total DNA of BTH-13 by two-step PCR and sequenced (40). On the basis of the sequence of the 1.2-kb fragment, two outer, gene-specific primers (WP-1F and WP-1R) were synthesized, and flanking DNA regions were cloned by genome walking, as recommended by the BD Genome Walker universal kit user’s manual and as previously described by Kongswan et al. (23). BTH-13 genomic DNA was digested by EcoRV, ScaI, and Smal, and the libraries of fragments for PCR amplification were constructed by ligating the digested genomic DNA to the Genome Walker adaptor. The flanking DNA fragments were amplified from the libraries by PCR using the outer adaptor primer (AP1) and the outer, gene-specific primers. The amplification was accomplished in a thermal cycler (PTC-100 programmable thermal controller; MJ Research, Inc.) by using a denaturation step at 94°C for 3 min followed by a 7-cycle program, with each cycle consisting of 94°C for 25 s and 70°C for 3 min, and a 32-cycle program with each cycle containing the steps 94°C for 25 s and 65°C for 3 min. A final extension step of 67°C for 7 min was added. PCR products purified from 1.2% agarose gel with the DNA purification kit (Qiagen) and 65°C for 3 min. A final extension step of 67°C for 7 min was added. PCR products purified from 1.2% agarose gel with the DNA purification kit (Qiagen) and 65°C for 3 min. A final extension step of 67°C for 7 min was added. PCR products purified from 1.2% agarose gel with the DNA purification kit (Qiagen) and 65°C for 3 min. A final extension step of 67°C for 7 min was added. PCR products purified from 1.2% agarose gel with the DNA purification kit (Qiagen) and 65°C for 3 min. A final extension step of 67°C for 7 min was added. PCR products purified from 1.2% agarose gel with the DNA purification kit (Qiagen) and 65°C for 3 min. A final extension step of 67°C for 7 min was added. PCR products purified from 1.2% agarose gel with the DNA purification kit (Qiagen) and 65°C for 3 min. A final extension step of 67°C for 7 min was added. PCR products purified from 1.2% agarose gel with the DNA purification kit (Qiagen) and 65°C for 3 min. A final extension step of 67°C for 7 min was added. PCR products purified from 1.2% agarose gel with the DNA purification kit (Qiagen) and 65°C for 3 min. A final extension step of 67°C for 7 min was added. PCR products purified from 1.2% agarose gel with the DNA purification kit (Qiagen). The antiserum was prepared by using the crystal proteins purified from wild-type strain BTH-13 to immunize a rabbit (40). The purified crystal protein was dissolved in 0.1 N NaOH solution and further separated by SDS-PAGE, and the 129-kDa protein was recovered to immunize a rabbit to obtain rabbit anti-serum. The proteins from the transformants described above were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane on a vertical mini gel (Bio-Rad, Richmond, CA) at room temperature at 20 V for 30 min. The crystal protein and the trypsin-activated toxin from BTH-13 were used as controls. Sequential steps were carried out according to Western blotting procedures previously described (25). The following antibodies were used for immunostaining: rabbit anti-B. thuringiensis serum, goat anti-rabbit, and rabbit anti-goat-alkaline phosphatase. Goat anti-rabbit and rabbit anti-goat-alkaline phosphatase were purchased from Promega.

**RESULTS**

**Cloning and sequence analysis of a novel gene encoding the δ-endotoxin from BTH-13.** In the first genome walking PCR amplification, about 0.9 kb (the upstream of the known frag-
ment) and 3.0 kb (downstream) fragments were obtained from the BTH-13 genomic library, using the outer adaptor primer (AP1) and outer, gene-specific primers WP-1R and WP-1F. The purified PCR products were ligated to the plasmid vector pGEM-T Easy and completely sequenced. The composite sequence of the 0.9-kb, 1.2-kb (known), and 3.0-kb PCR fragments showed an incomplete open reading frame (ORF) of a cry gene. To obtain the full-length cry gene, a 2.1-kb region upstream of the 0.9-kb fragment was cloned from the genomic library by a second genome walking using gene-specific primer Walker-2R and then sequenced. The sequences of the 2.1-, 0.9-, 1.2-, and 3.0-kb fragments were combined to obtain the complete cry gene.

The 3,432 bp of the cry gene ORF encoded 1,144 amino acid residues, and consisted of a molecular mass of 129 kDa. The gene sequence was submitted to GenBank (accession no. ACI44005.1) in amino acid residue sequences (Fig. 1). The homology was low in the amino-terminal regions, but the homology reached as high as 98% between C-terminal portions of the three proteins. Analysis of the three-dimensional structure of Cry7Ca1 based on that of Cry8Ea1 (PDB entry 3EB7) indicated that the activated toxin has three domains comprised of 587 amino acid residues. Domain I consists of 227 residues (Ile55 to Thr281) forming 7 helices. Domain II is formed by three antiparallel sheets with 214 residues (Thr286 to Val498), and domain III consists of 133 residues (Thr510 to Asp642) forming a beta-sandwich. There are 54 amino residues in the N terminus before domain I. The three domains, respectively, exhibited 47, 44, and 34% sequence identity with corresponding domains of known Cry toxins. The predicted secondary structure of the Cry toxin was composed of 27.80% helices, 27.71% sheets, and 44.49% looping structure.

The secondary structure of Cry7Ca1 was predicted with Predict-Protein to compare with the structure of Cry8Ea1 by using default parameters. Top significant hits corresponded with Cry protein structures, with the top hit being Cry8Ea1 (3eb7B.pdb) at 35.3% homology. The PDB file produced by SWISS-MODEL (web-based program) was used to build a model with

FIG. 1. Multiple sequence alignment of the deduced amino acid sequences of Cry7Ca1 (in this study), Cry7Aa1 (GenBank accession no. ABB70817) and Cry7Ba1 (GenBank accession no. ABB70817) using program ClustalW. There are high similarities between the C-terminal portions of the three toxins (not shown), and the presence of five conserved sequence blocks was common to these toxins. The conserved residues in all proteins are indicated by black (identical) and gray (similar) shading.
Swiss-pdb. Diagrammatic representations of the structures were generated by using PyMOL (Fig. 2).

Multiple sequence alignment of Cry toxins indicated that Cry7Ca1 exhibited a primary sequence identity of 35% with the Cry8Ea1 toxin. Using the STRAP program, significant structural divergence was seen by the superposition of the corresponding segments of Cry7Ca1 and Cry8Ea1. Figure 3A shows the superposition of single domains, revealing the greatest diversity in domain II. The same results were also obtained by multiple sequence alignments of Cry7Ca1 and five other structure-known Cry toxins, Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb1, and Cry4Ba, suggesting that the three domains of the six proteins were overlapped, although the branch lengths varied in different domains. In domain I, Cry7Ca1 showed differences between two loops from Cry8Ea1—especially, the α6-α7 loop in Cry7Ca1 is three residues longer than the corresponding loop in Cry8Ea1. In domain II, loop 3 of Cry7Ca1 is four residues longer than the corresponding loop in Cry8Ea1. There are also significant structural differences between loop 1 and loop 2. In domain III, the β19-β20, β20-β23, β18-β19, β13-α9, and β15-β16 loops are different between the two Cry proteins.

**Expression of Cry7Ca1 in E. coli M15.** The expressions of the three recombinant plasmids (pQE-30/7Ca1, pQE-30/toxin1, and pQE-30/toxin2) were detected by SDS-PAGE (Fig. 4A) and Western blot analysis (Fig. 4B). The results showed that Cry7Ca1, the activated toxin 1 and activated toxin 2 (the schematic diagram of three proteins was shown in Fig. 3B) were expressed in E. coli M15, and the molecular masses of the products were 129, 64, and 72 kDa, respectively, which corresponded with the structural analysis. The molecular mass of trypsin-activated toxin from BTH-13 was 64 kDa, which corresponded with that of the expressed activated toxin 1.

**Activity of Cry7Ca1 against L. migratoria manilensis.** The toxicities of Cry7Ca1 and the activated toxins 1 and 2 from the recombinants against the second-instar locusts were analyzed. The results indicated that Cry7Ca1 showed significant activity against L. migratoria manilensis. The bioassay results by Probit analysis are shown in Table 2. The average estimated LC₅₀ values were 8.98 μg/ml for the inclusion protein of Cry7Ca1 from the transformant in E. coli M15, 4.43 μg/ml for the expressed activated toxin 2, 0.87 μg/ml for the expressed activated toxin 1 of Cry7Ca1, and 11.78 μg/ml for the spore-crystal mixtures from B. thuringiensis strain BHT-13 after locusts were exposed for 7 days.

**Histopathological effect of Cry7Ca1 on the midguts of the locusts.** The histopathological effects of the Cry7Ca1 from the

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**FIG. 2.** Ribbon diagrams of the Cry7Ca1 structure and superposition of Cry8Ea1. Shown is a Cry7Ca1 ribbon diagram viewed by using PyMOL. (A) Domain I is red, domain II is yellow, and domain III is blue. (B) Superposition of Cry7Ca1 and Cry8Ea1 by program STRAP shown in stereo pairs. Cry7Ca1 is green, and Cry8Ea1 is blue.

**FIG. 3.** (A) Ribbon diagram showing overlap of individual domain structures of Cry7Ca1 and Cry8Ea1. Domain I is a helix bundle, domain II is a prism of three antiparallel β-sheets, and domain III is a β-sandwich. The structure superpositions of Cry7Ca1 and Cry8Ea1 are colored and labeled in red. (B) Schematic diagram of expressed proteins of Cry7Ca1, activated toxin 1, and activated toxin 2. The thick line and the gray boxes represent the protein sequence, while the arrows and numbers indicate amino acid residue positions in the Cry7Ca1 sequence.
transformant on the midgut of *L. migratoria manilensis* were investigated. Obvious differences were observed between the midguts of locusts fed with Cry7Ca1 toxin (the activated toxin 1) and those of controls under the light microscope. The epithelial cells of the midguts of the activated toxin-treated adults showed a considerable loss or a state of disruption (Fig. 5B), whereas the midguts of untreated adults exhibited uniform morphology and well-defined epithelial cells (Fig. 5A).

**DISCUSSION**

We characterized a novel δ-endotoxin, Cry7Ca1, from *B. thuringiensis* strain BTH-13, that showed toxic activity against *L. migratoria manilensis*. The amino acid sequence of Cry7Ca1 revealed 62% homology with Cry7Ba1, but the similarity was highly embodied between the C-terminal portions of the two toxins and was low in three functional domains (toxin core fragments). The functional domains showed 47, 44, and 34% homology with corresponding domains of known Cry toxins. Moreover, significant structural divergence was seen with the superposition of the corresponding segments of Cry7Ca1 and Cry8Ea1, and the greatest diversity was shown in domain II. The differences in amino acid sequence and secondary structure in the functional domain may imply a specific activity of the δ-endotoxin against pests, and the result was consistent with previous reports (16, 21). Cry7Ca1 and its activated toxin region could be expressed in *E. coli* M15, but the expressed proteins of Cry7Ca1, the activated toxin 1 and activated toxin 2 were not stable according to the results of SDS-PAGE and Western blot analysis. When Cry7Ca1 was expressed in the acrystalliferous *B. thuringiensis* strain BHT-13, Cry7Ca1 could not form parasporal crystals, as expected (data not shown). There are reports that helper proteins possess great potential for improving the production of heterogeneous proteins in *B. thuringiensis* and play an important role in the expression and crystallization of some Cry proteins (38, 42). Therefore, the lack of certain molecule chaperons may affect the expression and crystallization of Cry7Ca1 in the acrystalliferous *B. thuringiensis* strain. Moreover, there might also be some intracellular proteases in the acrystalliferous *B. thuringiensis* strain to degrade Cry7Ca1.

The bioassay results indicated that the expressed three products Cry7Ca1, activated toxin 1, and activated toxin 2 in *E. coli* exhibited activity against *L. migratoria manilensis*. There were reports that the N terminus may prevent the interaction of toxin with target membrane, and the removal of the N-terminal peptide is a necessary step in toxin activation. In addition, the C terminus is believed to be important for crystallization and may by extension apply to the activity of Cry toxin, but it is not required for insecticidal activity (6). Therefore, the cleavages of the N terminus and C terminus may enhance the insecticidal activity of Cry protein. Our previous study showed that the

<table>
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<tr>
<th>Treatment</th>
<th>LC₅₀ in μg/ml (slope ± SE)</th>
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<tbody>
<tr>
<td></td>
<td>6 days</td>
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<tr>
<td>Cry7Ca1</td>
<td>10.57 (0.87 ± 0.44)</td>
</tr>
<tr>
<td>Activated toxin 1</td>
<td>1.34 (0.36 ± 0.37)</td>
</tr>
<tr>
<td>Activated toxin 2</td>
<td>4.60 (0.34 ± 0.27)</td>
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<tr>
<td>Spore-crystal mixture</td>
<td>26.17 (0.69 ± 0.63)</td>
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*With water as a control, there was zero mortality.

*Data were too heterogeneous to allow calculation of 95% confidence intervals.

Protein concentration of the spore-crystal mixtures from cultures of strain BTH-13.
trypsin-activated toxin from BTH-13 had high toxicity to larval and adult locusts (40). As a result, the expressed activated toxin 1, which has the same molecular weight as the trypsin-activated toxin, might have higher insecticidal activity than the other two proteins against the locust. Evidence of the toxic activity of Cry7Ca1 in locusts came from the observed histopathological effects of the 6-endotoxin on the midgut of _L. migratoria manilensis_, which revealed a great loss of epithelial cells in the midguts of the toxin-treated locusts. The histopathological changes were similar to findings described by Quesada-Moraga et al. (33). Song et al.’s study (40) showed that the toxin of _B. thuringiensis_ strain BTH-13 played a role in the death of treated locusts, and the location of the locust-active _Bt_ toxin was found only in the midgut. These results indicated that the mode of action of Cry7Ca1 in locusts may be similar to that reported for other _Bt_ toxins. As a novel toxin from _B. thuringiensis_, Cry7Ca1 toxin could have a potential application for development of _Bt_ insecticide and transgenic plants with activity against locusts. Therefore, it would be interesting to investigate the secondary structure of Cry7Ca1 and its mode of action in locusts, in addition to its receptor proteins in the midguts of locusts.

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