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Characterization of Two Novel *cry8* Genes from *Bacillus thuringiensis* Strain BT185

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Abstract Two novel *cry8*-type genes, *cry8Ea1* and *cry8Fa1*, obtained from a *Holotrichia parallela*–specific *Bacillus thuringiensis* strain, BT185, were characterized. Findings showed that *cry8Ea1* and *cry8Fa1* encoded polypeptides of 1164 and 1174 amino acid residues, respectively. The deduced amino acid sequences of both Cry8Ea1 and Cry8Fa1 polypeptides are the most similar to that of Cry8Ba1. Eight conserved blocks (blocks 1–8) exist in Cry8Ea1 and Cry8Fa1 polypeptides compared with known Cry proteins. Cry8Ea1 and the Cry8Fa1 toxins could form spheric crystals when they were expressed in the acrystalliferous mutant strain HD73$^-$. The spores and crystals from the recombinant strain containing *cry8Ea1* were toxic to *Holotrichia parallela*, with an LC_{50} of 0.0875×10^8 colony-forming units (CFU)/g. However, Cry8Fa1 expressed in the recombinant strain was not toxic to *H. parallela, Anomala corpulenta,* or *H. oblita.*

Introduction

Bacillus thuringiensis, a Gram-positive soil bacterium characterized by the production of parasporal inclusions, has been applied as a biopesticide for control of agricultural, forest, and medical insect pests [2, 6, 9]. The larvacidal activity of *B. thuringiensis* is attributed largely to the crystal protein encoded by the crystal (*cry*) genes.

The crystal proteins currently are classified into two families: Cry and Cyt proteins [6]. Proteins of the Cry3, Cry6, Cry7, Cry8, Cry18 [3–5, 13], and Cry43 classes [11], as well as the binary toxins Cry34A-Cry35A [1], are active against Coleoptera (beetles, including weevils). Among these classes, Cry3-type, Cry8-type, Cry18-type, and Cry43-type are toxic to larvae of the Scarabaeidae family.

Chafers are important insect pests, affecting more than 300 plant species in both Europe and Asia. The larvae destroy the underground parts of the plants, resulting in damage that may kill the plant or cause a significant reduction in productivity and substantial economic loss [12]. Until recently, no Cry chemical has been identified that is toxic to the melolonthine beetle (black Asian chafer), *Holotrichia parallela* (Scarabaeidae).

We previously reported a new *B. thuringiensis* isolate, Bt185, obtained from soil samples, that contains two novel *cry8*-type genes toxic to the larvae of *H. parallela* [12]. In the current study, we characterized the *cry8*-type genes and investigated the toxicity of the two proteins. Characterization included sequence analyses of the *cry* genes, observations using scanning electron microscopy, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and insect bioassays.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Escherichia coli JM110 (*Dam, dcm, sup*E44, *hsdR*17, *thi, leu, rpsL*1, *lacY, galK, galT, ara, tonA, thr, tsx, Δ*[*lac-proAB*]

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[F′, *traD36, proAB, lacI*^{q}ZΔM15]) was used for common transformations, whereas *E. coli* SCS110 (*RpsL*[str^r], *thr, leu, endA, thi*-1, *lacY, galK, galT, ara, tonA, tsx, dam, dcm, supE44, Δ*[*lac-proAB*] [F′ *traD36 proAB lacI*^{q} ZΔM15]) was used to produce nonmethylated plasmid DNA for *B. thuringiensis* transformations.

In the transformation experiments, HD73$^-$, a crystal-negative mutant strain of *B. thuringiensis* subsp. *kurstaki*, was used as a recipient strain. The *B. thuringiensis* isolate Bt185, screened from soil in China, produces spheric crystals and is toxic to *H. parallela* larvae [12]. Plasmid pSTK containing the *cry3Aa* promoter, the STAB-SD sequence, and multiple cloning sites from pET-21b, was derived from plasmid pHT315 by Wang et al. [10] and used to express the novel *cry* genes in the strain HD73$^-$. *Escherichia coli* was incubated at 37°C in Luria-Bertani medium (1% NaCl, 1% tryptone, 0.5% yeast extract). *Bacillus thuringiensis* strains were grown at 30°C in peptone-beef medium (0.5% peptone and 0.3% beef extract). Ampicillin (100 μg/ml) or kanamycin (50 μg/ml) was added to the media, when appropriate, for selection of antibiotic-resistant strains of *E. coli* and *B. thuringiensis*. The initial media pH of all cultures was 7.2.

DNA Manipulations

Plasmid DNA from *B. thuringiensis* was prepared using methods described by Song et al. [8]. Transformation of *B. thuringiensis* HD73$^-$ with the recombinant plasmids was performed using the method described by Wang et al. [10].

Cloning of the Novel *cry8*-Type Gene

In addition to the *cry8Ea* gene in the Bt185 isolate, a 2-kb *Kpn*I fragment (2.340 kb) was cloned and confirmed to be a partial-length novel *cry8*-type gene [12]. In the current research, to clone of this full-length novel gene, total plasmid DNA of Bt185 was digested by endonuclease *Cla*I and ligated into the pBlueScript II SK (+) cloning vector to construct a DNA library. The recombinant plasmids were transformed into *E. coli* JM110 cells, and the transformed cells were grown on Luria-Bertani plates containing ampicillin (100 μg/ml). Specific primers SC8X5 (CTGGAAAGT ATTACGAAGAACT) and SC8X3 (TCCTGGACCTGCA-ATAACA), designed from the *cry8*-type partial gene in the 2-kb *Kpn*I fragment, were used to screen the DNA library. A 2.6-kb *Cla*I fragment subsequently was cloned into the pBlueScript II SK (+) vector to produce a recombinant plasmid pS26, which was sequenced using an automated DNA sequencer (ABI-3730XL, USA). The sequences were analyzed using a Vector NTI Suite 9 (Invitrogen, Carlsbad, CA, USA).

Expression of the Two *cry8*-Type Genes

A *Pyrococcus furiosus* (*pfu*) DNA polymerase (Tiangen, Beijing, China) and the PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) were used for polymerase chain reaction (PCR) consisting of 30 cycles (1 min at 94°C, 1 min at 54°C, 4 min at 72°C) followed by incubation at 72°C for 10 min. The primer pair Pcry8EF5 (CGCGGATCC GATGAGTCCAAATAATCAAAATG)/Pcry8Ea3 (GGCG TCGACCTCTACGTCAACAATCAATTC) was designed to amplify the *cry8Ea1* gene, whereas the primer pair Pcry8EF5 (CGCGGATCCGATGAGTCCAAATAATCAA AATG)/Pcry8Fa3 (GGCCTCGAGCTCTACGTCAACAA TCAATTC) was used to amplify the *cry8Fa1* gene. A *Bam*HI site in the *cry8Fa1* gene was wiped using the primer pair M8FAF (GTGGGCAGAGTTAATGG)/M8FAR (GGT TCCTCTGGTGCAAAGA) and a TaKaRa MutanBEST Kit (TaKaRa, Dalian, China). No change occurred in the amino acid sequence.

The full-length PCR product of *cry8Ea1* was inserted into the *Bam*HI-*Sal*I sites of pSTK, whereas *cry8Fa1* was inserted into the *Bam*HI-*Xho*I sites. The nonmethylated recombinant plasmid, produced in *E. coli* SCS110, was introduced into the *B. thuringiensis* acrystalliferous mutant HD73$^-$ by electroporation. The individual colony of transformants was selected from the Luria-Bertani kanamycin plates and incubated at 30°C until sporulation. The spore–crystal mixtures were washed and resuspended in sterile distilled water for examination by electron microscopy and SDS-PAGE analysis. The experiments were conducted according to Shu et al. [7].

Insect Bioassay

Toxicity activities of the *B. thuringiensis* strains were tested with 5-day-old larvae of *H. parallela*. Larval mortality was scored after a 14-day incubation period, and the 50% median lethal concentration (LC_{50}) was determined by probit analysis. Each assay was repeated three times. The bioassay diet for the *H. parallela* larvae was prepared as described by Yu et al. [12].

Nucleotide Acid Sequence Accession Number

The nucleotide acid sequence data of the *cry8* gene presented in this report have been registered in GenBank. The assigned accession numbers are AY329081 for the *cry8Ea1* gene and AY551093 for *cry8Fa1*.

Results and Discussion

The *cry8Ea* full-length gene and a 2-kb *Kpn*I fragment encoding a C-terminus of a Cry8-like protein with 780

amino acids were cloned in earlier research. In the current investigation, a 2.9-kb *Cla*I fragment (2.903 kb) from recombinant plasmid pS29 was sequenced and found to overlap with the 2-kb *Kpn*I fragment. The 2-kb *Kpn*I and the 2.9-kb *Cla*I fragments were reconstructed to be a 4-kb DNA fragment (4.003 kb).

After sequence analysis of the 4-kb DNA fragment, an open reading frame was identified that corresponded to a polypeptide of 1174 amino acids with a deduced molecular mass of 133.1 kDa. This protein was named Cry8Fa1 by the *B. thuringiensis* Delta-Endotoxin Nomenclature Committee. At comparison of the deduced amino acid sequences with known Cry proteins, Cry8Ea1 and Cry8Fa1 showed high similarity (70% and 63%, respectively) with Cry8Ba. Both Cry8Ea1 and Cry8Fa1 also contained the eight conserved blocks (blocks 1–8) (Fig. 1) present in other typical Cry proteins [6].

Plasmid pSTK-8E, carrying the *cry8Ea1* gene, and plasmid pSTK-8F, carrying the *cry8Fa1* gene, were introduced into strain HD73$^-$ by electroporation. The transformants harboring pSTK-8E and pSTK-8F were named HD8E and HD8F, respectively. Spores of HD73$^-$ and spore–crystal mixtures of HD8E, HD8F, and Bt185 were examined under a scanning electron microscope. The Cry8Ea1 and Cry8Fa1 toxins accumulated in HD8E and HD8F, respectively, and could form spheric crystals (Fig. 2). As indicated by SDS-PAGE analysis, both Cry8Ea1 and Cry8Fa1 toxins had a molecular mass of approximately 133 kDa, similar to the toxins accumulated in wild-type BT185 (Fig. 2e, lanes 2 and 3).

Spores of HD73$^-$ and spore–crystal mixtures of HD8E, HD8F, and Bt185 were tested for insecticidal activity on *H. parallela* larvae to investigate the toxicity of Cry8Ea1 and Cry8Fa1. The insecticidal activity of *B. thuringiensis* is dependent on the quantity and types of δ-endotoxins produced [6].

The results in this study showed that HD73$^-$ and HD8F were not toxic to *H. parallela* larvae. As shown in Table 1, HD8E showed greater activity against *H. parallela* larvae than Bt185. It is possible that the quantity of Cry8Ea1

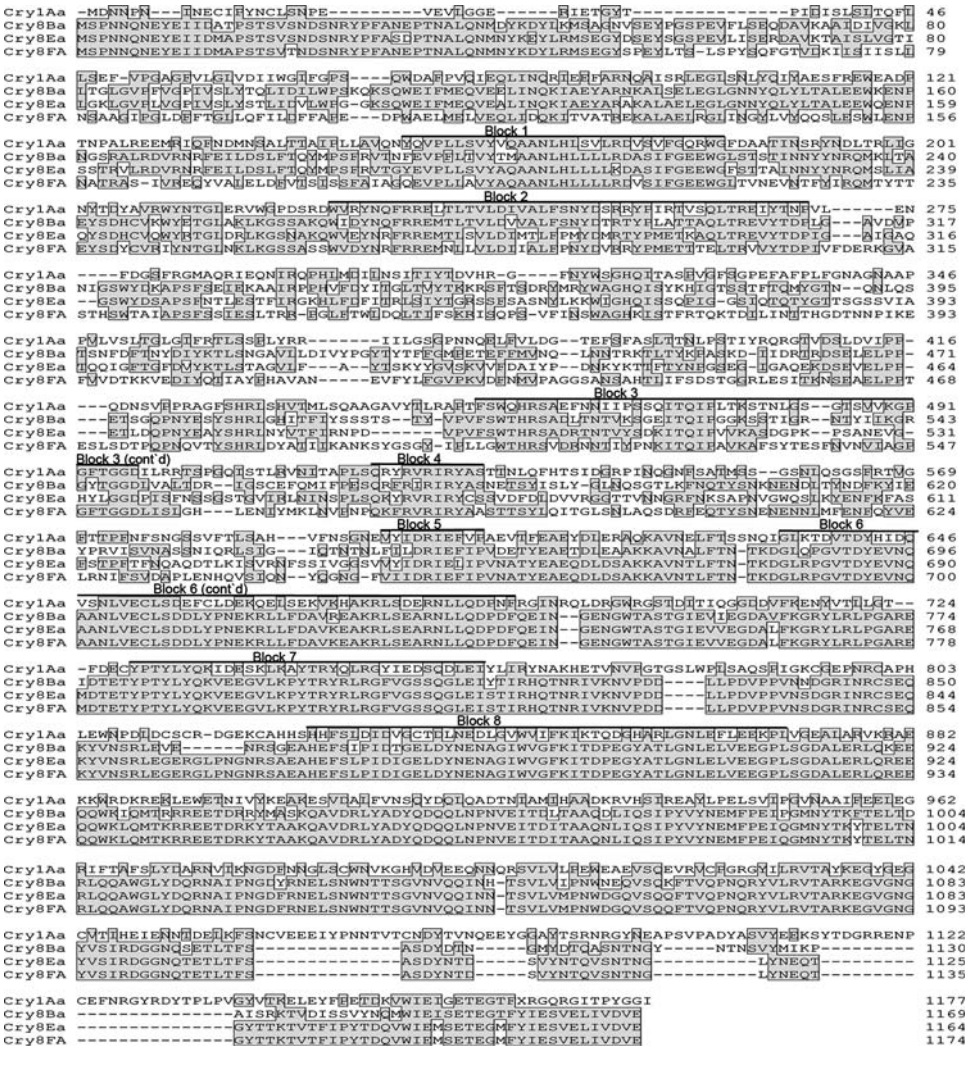

Fig. 1 Comparison of the deduced amino acid sequences Cry1Aa, Cry8Ba, Cry8Ea, and Cry8Fa. The characters in the *gray boxes* are identical amino acids between these toxins. The *bold lines* above the amino acid sequence correspond to the eight conserved blocks found in Cry proteins

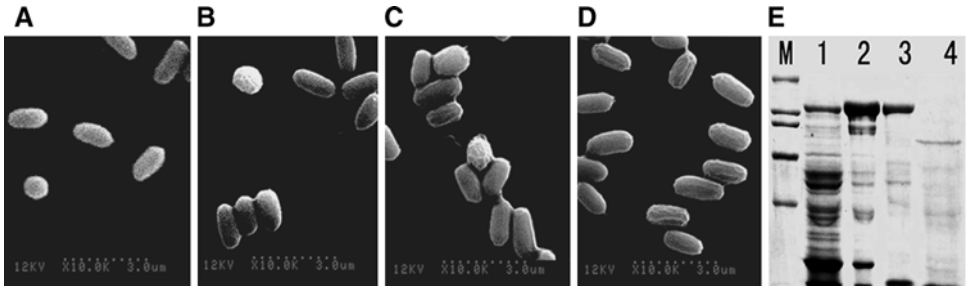

Fig. 2 Scanning electron micrographs and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of spores and crystal mixtures. **a** *Bacillus thuringiensis* strain BT185. **b** Recombinant strain HD8E. **c** Recombinant strain HD8F. **d** *B. thuringiensis* strain HD73^{-}. **e** SDS-PAGE: lane M (marker [212, 119, 97, 66, 40 kDa]), lane 1 (protein components of *B. thuringiensis* strain BT185), lane 2 (protein components of recombinant strain HD8E), lane 3 (protein components of recombinant strain HD8F), lane 4 (protein components of *B. thuringiensis* strain HD73^{-})

Table 1 Larvicidal activity of spores and crystal mixtures of BT185 and HD8E

Strains	$LC_{50}(10^8$ CFU/g)[a]	Slope[b]
BT185	0.1578(0.0617–0.9875)	1.1515 ± 0.1525
HD8E	0.0875(0.0242–0.3875)	1.3724 ± 0.1473

CFU colony-forming unit

[a] The fiducial limits at the 95% level are given in parentheses

[b] Mean ± standard error of the mean

accumulated in Bt185 was lower than in HD8E because Bt185 may express both Cry8Ea1 and Cry8Fa1 polypeptides. Significant differences in the amino acid sequences of the proteins may be responsible for the variability in the toxicity of the Cry molecules. In the current study, the amino acid sequences of the two toxins were found to be identical in the C-terminal region, but the three-domain region, believed to be responsible for the toxicity of the Cry proteins, was significantly different (similarity of only 39%). Our tests did not find HD8F toxic to any Scarabaeidae larvae (*H. parallela, Anomala corpulenta,* or *H. oblita*). The Cry8Fa1 protein may possibly be toxic to insect pests that have not yet been evaluated.

In conclusion, we determined that the novel Cry toxin, Cry8Ea1, demonstrated strong activity against larvae of the Asian chafer, *H. parallela*. To our knowledge, this is the only Cry toxin found to be toxic to this pest. Another *cry* gene, *cry8Fa1*, was cloned from the strain Bt185, but its toxicity to *H. parallela* has not yet been confirmed. Future research will investigate the use of the novel *cry8Ea1* gene in transgenic plants to control *H. parallela* and will include studies to detect of the toxicity of Cry8Fa1.

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