Characterization of \textit{cry1Cb3} and \textit{cry1Fb7} from \textit{Bacillus thuringiensis} subsp. \textit{galleriae}

Abstract: Two \textit{cry}1-type genes encoding insecticidal crystal proteins (ICPs) were detected by PCR-RFLP and cloned from \textit{Bacillus thuringiensis} subsp. \textit{galleriae}. The nucleotide sequences were deposited in GenBank with accession numbers EU679501 and EU679502, and designated as \textit{cry1Fb7} and \textit{cry1Cb3} respectively by \textit{B. thuringiensis} Delta-Endotoxin Nomenclature Committee. \textit{cry1Cb3} shared 99% homology with other \textit{cry1Cb} genes. The existence of two additional stop codons indicated \textit{cry1Cb3} was a silent gene. The \textit{cry1Cb3} was 3531 bp with 38.98% G+C content and its first open reading frame (ORF) encoded a protein of 213 amino acid residues with a calculated molecular weight of 23.8 kDa and a predicted pI value of 4.63. Five amino acid sequence blocks (block 1, block 2, block 3, block 4 and block 5) were found in Cry1Cb3. Translation of \textit{cry1Fb7} revealed an ORF of 3525 bp with 39.12% G+C content and a protein with a calculated molecular weight of 133.2 kDa and a predicted pI value of 5.18. Cry1Fb7 had five amino acid sequence blocks (blocks 1, 2, 3, 4 and 5) and three domains (I, II and III), which consisted of 218 residues (Leu$_{34}$ to Ala$_{252}$), 197 residues (Thr$_{257}$ to Asp$_{454}$), and 138 residues (Ile$_{464}$ to Glu$_{600}$), respectively.

Keywords: \textit{Bacillus thuringiensis} subsp. \textit{galleriae}, PCR-RFLP, cloning, \textit{cry1Cb}, \textit{cry1Fb}

1 Introduction

\textit{Bacillus thuringiensis} is a species of Gram-positive spore-forming bacteria. Research over many years has led to the isolation and description of more than 60,000 strains of these bacteria, which have been subdivided into more than 71 serovars based upon H-serotyping [1-3]. Its characteristic feature is the capacity to produce a complex of insecticidal crystal proteins (ICPs). These proteins are specifically toxic to a number of agricultural pests from the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Phthiraptera (Mallophaga), Acari, Nemathelminthes, Platyhelminthes, and Sarcomastigophora, as well as to various human parasites [4]. To date, genes encoding ICPs have been classified as \textit{cry1} - \textit{cry 74}, \textit{cyt1} - \textit{cyt3}. The current nomenclature, based solely on amino acid identity, allows closely related toxins to be ranked together and removes the necessity for researchers to bioassay each new toxin against a growing series of organisms [5] (also see full toxin list at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Among these, the ICPs toxic to Lepidopteran insects belong to the Cry1, Cry2, and Cry9 groups while the ICPs active against coleopteran insects are the Cry3, Cry7, and Cry8 proteins. The Cry5, Cry12, Cry13, and Cry14 proteins are nematocidal, and the Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins are toxic for dipteran insects. The characterizations for most of the \textit{B. thuringiensis} collections are based on bioassays against various insect larvae without identification of the \textit{cry} genes presented in the \textit{B. thuringiensis} strains [6].

Insecticides derived from \textit{B. thuringiensis} with Cry are acquiring worldwide importance as environmentally desirable alternatives to synthetic chemicals for the control of pests for agricultural, forestry, and public health purposes. Nevertheless, the applications of \textit{B. thuringiensis} products as biopesticides are limited by their narrow host range, low toxicity to the targeted insects, and insects’ resistance [7-9]. Therefore, it is necessary to continuously screen new \textit{cry} genes and perform rational design based on known Cry toxins. In order to prevent the continuous development of insect resistance to \textit{B. thuringiensis}, methods based on
polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) have been developed to detect different cry genes from B. thuringiensis strains [10 – 19]. This rapid means of identifying cry genes by PCR has led to an increase in the number of known cry genes. To date, at least 775 cry gene sequences have been determined (see full toxin list at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

As the largest of these families, cry1 genes encode the 130-138 kDa Lepidopteran-active ICPs that form mainly bipyramidal crystalline inclusions [20]. Most of the commercial B. thuringiensis formulations used for the control of Lepidopteran pests contain toxins of the Cry1A family, and especially Cry1Aa, Cry1Ab and Cry1Ac proteins [18, 21, 22]. In Escherichia coli, the cry1Cb gene produces a protein of approximately 130 kDa toxic to Spodoptera exigua, Trichoplusia ni and Plutella xylostella [18,23]. Cry1Ea is shown to be highly toxic against Spodoptera littoralis and S. exigua [17,24]. Cry1Fa1 is selectively toxic to a tested subset of Lepidopteran insects, including the larvae of Ostrinia nubilalis and S. exigua [20]. Taken together, Cry1 ICPS of B. thuringiensis are generally active against Lepidopteran insects [25-27], but Cry1Ba and Cry1La have additional activity against Coleoptera such as Colorado potato beetle [8,28]. Cry1Ba/Cry1La hybrid toxins have increased activities against this insect species [28].

In the present study, five B. thuringiensis strains (10, 12, 13, 87 and TE-3) were found by PCR-RFLP to harbour five different cry1-type genes: cry1Ac, cry1Cb, cry1Fb, cry1Gb and cry1La. To learn more about the characteristics of cry1C genes and their encoded proteins, two cry1-type genes, cry1Cb3 and cry1Fb7, were cloned and sequenced from B. thuringiensis subsp. galleriae 87.

2 Methods

2.1 Bacterial strains

Five B. thuringiensis strains were used in this study (Table 1). E. coli JM109 was used as the host organism for recombinant DNA cloning and plasmid pMD18-T (TaKaRa) was used as the cloning vector. Throughout the experiments, bacteria were cultivated in Luria-Bertani (LB) liquid medium (1% bactotryptone, 0.5% yeast extract, 1% sodium chloride) at 30 °C (37 °C for E. coli) with constant rotary shaking at 230 rpm.

2.2 Preparation of total DNA

Briefly, cells for total DNA extraction were grown about 10 h in 5 ml LB medium at 30°C with rigorous shaking. A 1.5 ml volume of cells was centrifuged at 11000 g for 2 min and resuspended in 80 μl of Sol I (0.3 M glucose, 25 mM EDTA). The cytoderm was further destroyed by vortex mixer. Then 5 μl of lysozyme (Sigma-Aldrich, 10 mg ml⁻¹) was added and the solution was incubated at 37°C for 30 min with occasional shaking. Next, 420 μl of Sol II (100 mM Tris-HCl, 25 mM EDTA, 0.5 M NaCl, 0.5% SDS, pH 8.0) was added to the solution. After thorough mixing, 500 μl of phenol/chloroform/isopentanol (25 : 24 : 1) was added and extracted one time by centrifuging at 11000 g for 5 min to remove proteins. Precipitation and resuspension of the total DNA sample were then carried out as previously described [29]. The total DNA was analysed by horizontal agarose gel electrophoresis in 0.7% agarose with 1×TAE buffer for 1 to 2 h.

2.3 Preparation of plasmid DNA

Plasmid DNA from B. thuringiensis was extracted by a modified alkaline lysis protocol [30]. Briefly, cells for plasmid DNA preparation were grown about 10 h in 5 ml of LB medium at 30°C under rigorous shaking. A 1.5 ml volume of cells was centrifuged at 12000 rpm for 2 min and resuspended in 200 μl of Sol I (10% glucose, 50 mM Tris-HCl, 20 mM EDTA, pH 8.0). The cytoderm was further destroyed by vortex mixer. Then, 5 μl of lysozyme (Sigma-Aldrich, 10 mg ml⁻¹) was added and the solution was incubated at 37°C for 30 min with occasional shaking. The cells were lysed by 400 μl of Sol II (2% SDS, 0.4 M NaOH). After thorough mixing, 300 μl of ice-cold Sol III (3 M KAc, 2 M HAc) was added to the solution and then centrifuged at 12000 rpm for 5 min. Precipitation and resuspension
of the plasmid DNA sample were then carried out as previously described [29]. The plasmid DNA was analysed by horizontal agarose gel electrophoresis in 0.7% agarose with 1× TAE buffer for 1 to 2 h.

### 2.4 Identification of cry1 genes by PCR-PFLP

Two pairs of universal primers (K5un2/K3un2 and K5un3/K3un3 for cry1-type genes) reported by other authors were used in the analysis [10]. Among them, the sequences of oligonucleotide primers K5un2 and K3un3 are complementary. Total DNA and plasmid DNA extracted from five *B. thuringiensis* strains were used as templates for PCR amplification, which was performed in 50 µl of reaction mixture containing 1 µl of 10 mM for each primer, 1 µl of 10 mM dNTP, 5 µl of 10× PCR reaction buffer, 6 µl of 20 mM MgCl₂, 50 ng of DNA, 5 U of *Taq* DNA polymerase (Fermentas), and sterilized Milli-Q purified water up to 50 µl. PCR was carried out with 30 cycles of 94°C for 1 min, 52 °C for 1 min, and 72°C for 3.5 min. The products were digested with appropriate enzymes, and then separated in 1.5% agarose gels [11].

### 2.5 PCR amplification of cry1-type genes

The oligonucleotide primers 1F, 1Cb-R and 1Fa-R were designed to amplify the open reading frames (ORFs) of cry1C and cry1F genes (Table 2). Plasmid DNA extracted from subsp. *galleriae* 87 was used as templates for PCR under the following conditions: 5 min initial denaturation at 94°C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 53°C for 1 min, and elongation at 72°C for 4 min. Final extension was for 10 min at 72°C. PCR amplification was carried out with *Taq* polymerase (Fermentas) in a DNA thermal cycler. The PCR products of cry1-type genes were analyzed by electrophoresis in a 0.7% agarose gel.

### 2.6 Molecular cloning and sequencing of cry1-type genes

The amplified fragment was purified using a kit from Tiangen Biotech (Beijing) Co., Ltd and ligated with pMD18-T. *E. coli* JM109 cells were used for transformation and selected on LB plates containing ampicillin (100 µg ml⁻¹), X-Gal (20 mg ml⁻¹), and IPTG (200 mg ml⁻¹). White recombinant colonies grown on fresh plates were screened and verified by PCR

<table>
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*Sequences of cry genes were from GenBank. Lowercase in a sequence indicated the base or bases different from those of primers.*
with primers 1F, 1Fa-R, and 1Cb-R, and the PCR-RFLP system of cry1 genes. All DNA manipulations, including ligation, transformation and restriction digestion, were carried out as previously described [29]. Automated DNA sequencing of two clones for each gene was done on the ABI PRISM Model 3730 DNA system.

2.7 DNA sequencing and analysis

The cry1-type genes from B. thuringiensis subsp. galleriae 87 were analyzed and compared with up-to-date GenBank data using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Multiple alignments were carried out utilizing the Clustal-W program in the ANTHEPROT (V5.0) software (http://antheprot-pbil.ibcp.fr/). G+C content of cry genes and molecular weight and pl values of Cry proteins were predicted by DNATools (V6.0.122) software (http://www.crc.dk/dnatoools/). The conserved domain of these cry1-type genes was determined using the SMART software (http://smart.embl-heidelberg.de/).

2.8 Nucleotide sequence accession numbers

The nucleotide sequences of cry1Cb3 and cry1Fb7 obtained in this study were deposited in the GenBank database under the accession numbers EU679502 and EU679501, respectively.

2.9 B. thuringiensis toxin nomenclature

The sequences of the two cry1-type genes and corresponding GenBank accession numbers were submitted to Dr. Neil Crickmore, who then consulted the committee responsible for the B. thuringiensis delta-endotoxin nomenclature database (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/) and assigned respective names to the genes: cry1Cb3 and cry1Fb7.

3 Results

3.1 Identification of cry1 gene classes from B. thuringiensis

Total DNA templates and plasmid DNA templates from five B. thuringiensis strains (10, 12, 13, 87 and TE-3) were used for PCR amplification with universal primers K5un2/K3un2 and K5un3/K3un3. PCR products produced fragments of about 1.6 kb and 1.4 kb (Fig. 1a & Fig. 1b). According to RFLP pattern analysis, five different cry1-type genes were detected in the B. thuringiensis strains (Fig. 1c and Table 1): cry1Ac, cry1La, cry1Gb, cry1Cb and cry1Fb.

3.2 PCR amplification of cry1 genes

To learn more about the characteristics of cry1Cb and cry1Fb genes and their encoded proteins, B. thuringiensis subsp. galleriae 87 was chosen for cloning cry1 genes. Two pairs of primers (1F and 1Cb-R, 1F and 1Fa-R) were designed to match with all known cry1Cb and cry1Fb genes (Table 2). The primers were tested for amplification of PCR product of the expected size using plasmid DNA of B. thuringiensis subsp. galleriae 87 as the template, and PCR products with expected size (3.5 kb) were obtained (Fig. 2a, lane 5 & Fig. 2b, lane 1).

3.3 Cloning and sequence analysis of cry1Cb3

A cry1Cb gene was cloned from B. thuringiensis subsp. galleriae 87 and sequenced (Fig. 2a). It was 3531 bp in size, with 38.98% G+C content. Comparison of the cry1Cb2 sequence with this cry1Cb gene revealed substitutions at the positions 640 of T for A (Leu to stop codon substitution), 1552 of G for A (Arg to Glu substitution), 1563 of A for T (Arg to stop codon substitution), 2074 of A for G (Asp to Gly substitution), and 3029 of A for G (silent mutation). Since it contained two additional stop codons, the cry1Cb was a silent gene. Its first ORF encoded a protein of 213 amino acids with a calculated molecular weight of 23.8 kDa and a predicted pl value of 4.63. This Cry1Cb consisted 28.7% of hydrophilic amino acids, 50.6% of hydrophobic amino acids, 12.2% of acidic amino acids, and 8.5% of basic amino acids (data not shown). BLAST analysis revealed that the deduced amino acid sequence of the first ORF of the cry1Cb gene was 99.8% identity with Cry1Cb1 and Cry1Cb2. Five amino acid sequence blocks (blocks 1–5) were found in the cry1Cb (Fig. 3a). This cry1Cb gene was designated as cry1Cb3 by the B. thuringiensis Delta-Endotoxin Nomenclature Committee.

3.4 Cloning and sequence analysis of cry1Fb7

A cry1F-type gene from B. thuringiensis subsp. galleriae 87 was cloned based on PCR and RFLP pattern (Fig. 2b).
Analysis of two cry1 genes from *Bacillus thuringiensis*  

**Fig. 1** PCR-RFLP analysis of cry1 genes of five *B. thuringiensis* strains (a) Lanes 1–5. PCR amplification using K5un3 and K3un3 as primers and total DNA of 10, 12, 13, 87 and TE-3 as templates; Lanes 6–10. PCR amplification using K5un2 and K3un2 as primers and total DNA of 10, 12, 13, 87 and TE-3 as templates; Lane M. 100 bp plus marker (Fermentas). (b) Lanes 1–5. PCR amplification using K5un3 and K3un3 as primers and plasmid DNA of 10, 12, 13, 87 and TE-3 as templates; Lanes 6–10. PCR amplification using K5un2 and K3un2 as primers and plasmid DNA of 10, 12, 13, 87 and TE-3 as templates; Lane M. 100 bp plus marker (Fermentas). (c) Lanes 1–5. RFLP patterns after *Pst* I and *Xba* I digestion of PCR products of plasmid DNA from 10, 12, 13, 87 and TE-3 with primers K5un2 and K3un2; Lanes 6–9. RFLP patterns after *Pst* I and *Eco* R I digestion of PCR products of plasmid DNA from 10, 12, 87 and TE-3 with primers K5un3 and K3un3; Lanes 10–14. RFLP patterns after *Pst* I and *Xba* I digestion of PCR products of total DNA from 10, 12, 13, 87 and TE-3 with primers K5un2 and K3un2; Lanes 15–18. RFLP patterns after *Pst* I and *Eco* R I digestion of PCR products of total DNA from 12, 13, 87 and TE-3 with primers K5un3 and K3un3; Lane M. 100 bp plus marker (Fermentas).

**Fig. 2** Identification of cry1Cb3 and cry1Fb7 genes from *B. thuringiensis* subsp. *galleriae* 87 (a) Lane 1. RFLP pattern after *Pst* I and *Eco* R I digestion of PCR product of partial-length cry1Cb3 from *B. thuringiensis* subsp. *galleriae* 87 with primers K5un3 and K3un3; Lane 2. RFLP pattern after *Pst* I and *Xba* I digestion of PCR product of partial-length cry1Cb3 from *B. thuringiensis* subsp. *galleriae* 87 with primers K5un2 and K3un2; Lane 3. PCR product of partial-length cry1Cb3 from *B. thuringiensis* subsp. *galleriae* 87 with primers K5un3 and K3un3; Lane 4. PCR product of partial-length cry1Cb3 from *B. thuringiensis* subsp. *galleriae* 87 with primers K5un2 and K3un2; Lane 5. PCR product of full-length cry1Cb3 gene from *B. thuringiensis* subsp. *galleriae* 87 with primers 1F and 1Cb-R; the full-length cry1Cb3 was used as the PCR templates in the samples of Lanes 1-4; Lane M1, 100 bp plus marker (Fermentas); Lane M2, 500–12000 wide range DNA marker (TaKaRa). (b) Lane 1. PCR product of full-length cry1Fb7 from *B. thuringiensis* subsp. *galleriae* 87 with primers 1F and 1Fa-R; Lane 2. PCR product of partial-length cry1Fb7 from *B. thuringiensis* subsp. *galleriae* 87 with primers K5un2 and K3un2; Lane 3. PCR product of partial-length cry1Fb7 from *B. thuringiensis* subsp. *galleriae* 87 with primers K5un3 and K3un3; Lane 4. RFLP pattern after *Pst* I and *Xba* I digestion of PCR product of partial-length cry1Fb7 from *B. thuringiensis* subsp. *galleriae* 87 with primers K5un2 and K3un2; Lane 5. RFLP pattern after *Pst* I and *Eco* R I digestion of PCR product of partial-length cry1Fb7 from *B. thuringiensis* subsp. *galleriae* 87 with primers K5un3 and K3un3; the full-length cry1Cb3 was used as the PCR templates in the samples of Lanes 2-5; Lane M3, 100 bp plus marker (Fermentas); Lane M4, 500–12000 wide range DNA marker (TaKaRa).
In order to delay the continuous development of insect resistance to *B. thuringiensis* insecticides, a search has been undertaken for new *B. thuringiensis* strains harbouring new cry genes [8]. PCR-RFLP identification systems have been successfully used for rapidly detecting and characterizing toxin genes of *B. thuringiensis* [10 – 19].

In the present study, PCR-RFLP was applied to a surveyed five *B. thuringiensis* strains (10, 12, 13, 87 and TE-3) to examine their cry1 gene content and predict their insecticidal activities, which method constitutes the first specifically designed to detect new cry genes [10]. The products of PCR amplification were digested with properly designed restriction enzymes. Based upon their RFLP patterns, the known cry1-type genes were identified and unknown or potential new genes could be found.

In this study, five different cry1 gene profiles (cry1Ac, cry1Cb, cry1Fb, cry1Gb and cry1La) were detected in the *B. thuringiensis* strains. Strains containing cry1Cb produce a protein of approximately 130 kDa toxic to *S. erigua*, *T. ni*, and *P. xylostella* [18, 23], while Cry1F proteins were selectively toxic to a tested subset of Lepidopteran insects, including the larvae of *O. nubilalis* and *S. exigua* [20]. It would be of great interest to obtain cry1C- and cry1F-type genes of native *B. thuringiensis* isolates in order to define

It was designated cry1Fb7 by the *B. thuringiensis* Delta-Endotoxin Nomenclature Committee.

cry1Fb7 had an ORF of 3525 bp with 39.12% G+C content. It encoded a protein of 1174 amino acid residues with a calculated molecular mass of 132.2 kDa and a predicted pI value of 5.18. It consisted of 42.7 and 42.9% hydrophilic amino acids, 36.0 and 36.5% hydrophobic amino acids, 13.0% acidic amino acids, and 11.9 and 12.1% basic amino acids (data not shown).

The existence of five amino acid sequence blocks (blocks 1–5) was confirmed in Cry1Fb7 (Fig. 3b). Bioinformatics analysis by SMART software indicated the deduced amino acid sequence encoded by cry1Fb7 had three domains: domain I consisting of 218 residues (Leu_34 to Ala_252), domain II consisting of 197 residues (Thr_257 to Asp_454), and domain III consisting of 138 residues (Ile_464 to Glu_600).

4 Discussion

Insect resistance has been a key problem in the use of *B. thuringiensis*. The occurrence of insect resistance to Cry proteins has been attributed to a reduction in the affinity of the proteolytically activated Cry toxin for binding to the membrane of midgut epithelial cells in larvae [7].
their distribution, predict their insecticidal activity, and detect new genes or combinations thereof. Hence, two genes (cry1Cb3 and cry1Fb7) were cloned from B. thuringiensis subsp. galleriae 87 and sequenced. Each has the characteristics typical of cry1C and cry1F genes, respectively. We confirmed the existence of five amino acid sequence blocks (blocks 1–5) in Cry1Cb3 and Cry1Fb7 and three domains in Cry1Fb7 [31]. Due to the existence of two additional stop codons in the sequence, the cry1Cb3 should be a silent gene. The sequence of the cry1Fb gene is different at several sites from those of other cry1Fb genes. This might result in different insecticidal activities. However, the potential effects of the substitutions in the three cry1 genes on toxicity or specificity are unclear and require further study.

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Conflict of interest: The authors declare no financial, personal or professional conflicts of interest.

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