Characterization and evaluation of two indigenous *Bacillus thuringiensis* isolates against *Helicoverpa armigera* Hubner

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ABSTRACT: Two isolates of *Bacillus thuringiensis* isolated from dead lepidopteran larvae from a tea garden in Jorhat, Assam and one isolate from soil sample from Rajasthan, obtained in a nationwide screening program showed bipyramidal crystal morphology. These two isolates named as NBAII-BTAS and NBAII-BTG4 were characterized by their high level of toxicity against diamond back moth (*Plutella xylostella*). The PCR amplification of these two isolates revealed the expected size of the PCR product for *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1E*, *cry1G*, *cry1I*, and *cry2* of 390 bps, 1111 bps, 238 bps, 540 bps, 300 bps, 468 bps, and 1170 bps respectively. Purified *cry* proteins from each of these two cultures were subjected to SDS-PAGE analysis, where, two distinct bands of 130-140 Kda and 65 Kda corresponding to *cry1* and *cry2* proteins were observed. Toxicity studies was carried out using trypsin activated purified proteins against *Helicoverpa armigera*, where NBAII-BTG4 derived crystal proteins displayed more toxicity (0.93µg/ml) than NBAII-BTAS.

KEY WORDS: *cry* genes, PCR, specific *cry* primers, bipyramidal, toxicity

INTRODUCTION

One of the most promising alternatives to the use of chemical pesticides is the applications of *Bacillus thuringiensis* (Beron and Salerno, 2006). Recent discoveries of new varieties of *B. thuringiensis* suggest that naturally occurring microorganism found in the soil may provide a treasure chest of microbes with untapped, unknown potential for agriculture. In 1987, two scientists at the US Department of Agriculture (USDA) announced the discovery of 72 new varieties of *B. thuringiensis*. Since, only about 24 varieties of *B. thuringiensis* were previously known, the identification of new *B. thuringiensis* germplasm could radically change (Schneper *et al*., 1998). More than 300 ICPs have been cloned, sequenced and classified into 53 groups based on amino acid homology. Establishment of a *B. thuringiensis* collection is to have a method which allows for rapid and accurate characterization of its crystal inclusions so that its specificity and toxicity is determined. Analysis of these genes by bioassay has proved to be an exhaustive, time-consuming process, since, it is necessary to screen all target insect isolates. Different methods have been developed in an effort to reduce the number of bioassays, such as (i) Southern blot analysis in search of known homologous genes (Kronstad and Whiteley, 1986), (ii) analysis of reactivity to different monoclonal antibodies (Hofte *et al*., 1988) or (iii) electrophoretic analysis of PCR products using specific primers (Carozzi *et al*., 1991). From the above-mentioned methods, PCR analysis is considered to be the best choice, because, it allows rapid determination of the presence or absence of a sequence and it is highly sensitive, relatively fast, and can easily be used on a routine basis (Ceron *et al*., 1994; 1995).

PCR-based methodologies with universal primers and sets of primers directed against specific regions of type-specific *cry* genes have been proposed, and these approaches allow the detection of *cry* genes and prediction of their insecticidal activities (Ceron *et al*., 1995; Ben Dov *et al*., 1999; Beron *et al*., 2005). Recently, Aly (2007) and Thammasittirong and Attathom (2008) designed primers to detect specific *cry* genes based on the nucleotide sequences available at NCBI and proposed that *cry* specific genes could be identified using PCR techniques. *B. thuringiensis* strains isolated from avocado orchards exhibit a low toxic activity towards *Argyrotaenia* sp. larvae, in spite of their specific *cry* gene content (Rosas-Garcíá *et al*., 2007).
During screening for native \textit{B. thuringiensis} isolates, two bipyramidal forming isolates were encountered from soil and insect cadaver respectively. We undertook a study to characterize these two isolates for their \textit{cry} gene profile using PCR based methodologies and also evaluate their toxicity against \textit{Helicoverpa armigera}. We used the standard HD-1 strain for comparison.

**MATERIALS AND METHODS**

**Bacterial strains**

Two indigenous \textit{B. thuringiensis} isolates NBAII-BT-G4 (from rhizosphere soil of groundnut, Sri Ganganagar, Rajasthan) and NBAII-BT-AS (purified from dead lepidopteran larvae, Jorhat, Asssam) were used in the study. The isolation was carried out according to the protocol of Obeidat \textit{et al.} (2004). Microscopic analysis revealed that they produced bipyramidal crystals, spore forming and Gram positive as revealed in Gram staining and Amido black staining. They were previously found to be toxic to diamondback moth (\textit{Plutella xylostella}). They were identified and characterized by PCR reactions with universal and specific primers.

**DNA extraction**

The cultures of two \textit{B. thuringiensis} isolates, namely NBAII-BTAS and NBAII-BTG4, were grown overnight in LB broth in rotary shaker at 250 rpm. 3ml of culture was pelleted and DNA was extracted by HiPura reagents (HiMedia) with mini-prep column formation as per Sambrook (2001). Integrity of DNA was tested by running in 1.25% agarose gel for 1hr at 100V.

**PCR amplification and primers designed of \textit{cry} genes**

Polymerase chain reaction was carried out in a Thermo cycler (Quantrarus) for 30 reaction cycles each. PCR reactions were carried out in 25 il containing 50 ng of DNA mixed with 1X Taq reaction buffer, dNTP mix- 50 mM, Primer- 0.4 µM (Both forward & reverse), Taq DNA Polymerase (Genei)- 1.5 U, Mgcl₂- 14 i M. Template DNA was denatured for one minute at 94°C, one minute of annealing at 54-59°C and elongation at 72°C. An extra denaturation and elongation step was provided at 94°C for 2.5 minutes and at 72°C for 5 minutes respectively (Mahadi \textit{et al.}, 1998). PCR products were separated by running on 1.25% agarose gel with 4il/100ml of ethidium bromide for 1hr at 100V. The fragments were visualized under gel documentation unit (DNR MiniLumi). Sizes of PCR product was determined by the ladder of 100 bps and of 1 kb as required in each according to the sizes. The specific primers are designed by Aly (2007), and the universal primers by Bravo \textit{et al.} (1998) and Thammasittrong and Attathom (2008) (Table 1).

**Purification of \textit{cry} proteins and SDS-PAGE analysis**

Whole cell proteins were isolated from sporulating bacterial culture by centrifugation and dissolution in specific buffers. Respective cultures were allowed to grow on T3 agar medium containing (per litre) tryptose 3g, peptone 2g, yeast extract 15g, magnesium chloride 0.005g, agar powder 15g, sodium phosphate 0.05M and pH 6.8 at 30°C for 5 days until sporulation. The lawn culture was scraped using sterile cotton swabs and dissolved in equal amount of sterile water, washed twice with distilled water (8000 rpm for 8 minutes), dissolves in 500µl of lysis buffer containing 100mM Tris HCl (pH 7.0), 20mM EDTA, 5mg/ml lysozyme, 2% SDS and it was centrifuged at 8000 rpm for 7 minutes. Pellet was resuspended in 200µl resuspension buffer (0.1% SDS + 10mM EDTA), further diluted with treatment buffer (2.0%SDS +5% ß mercaptoetranol+130mM Tris HCl, pH 10.0) and incubated at 90°C for 7 minutes (Morris \textit{et al.}, 1998). Protein concentration was measured as per Lowry \textit{et al.} (1951) and used for SDS PAGE. Approximately, 150g of proteins were loaded in each well of 12% stacking gel and run at 50V for 4 hours in denaturing conditions. The gel was stained for 1 hour in staining solution and destained overnight. Gel was visualized in a gel documentation system (DNR Mini-Lumi, Israel).

**Activation of \textit{cry} proteins (protoxins)**

Protoxins were converted to active toxins by trypsin digestion. Since, trypsin works at neutral pH, the pH 10.0 of the solubilized protoxin was adjusted to pH 7.0 using 1N HCl. 1µg of trypsin (stock: 1mg/ml in distilled deionized water) for each 20 µg of protoxin was mixed well and incubated at 37°C for 3 hours.

**Biotoxicity analysis of \textit{cry} protein**

NBAII-BTAS and NBAII-BTG4 were screened against \textit{H. armigera} using the semi-synthetic diet bioassay method. The concentration of the activated \textit{cry} proteins was adjusted to 2mg/ml and then dilutions were made up to 10⁻⁶. Around 100µl were applied to each vial containing about 5ml diet and air dried. Two second instar larvae of \textit{H. armigera} were introduced into each vial and 10 replications were maintained for each dilution. Larval death was recorded up to 92 hours and \textit{LC₅₀} values were calculated by subjecting the data to probit analysis using SPSS software version 10.
RESULTS AND DISCUSSION

Isolation

The two *B. thuringiensis* isolates were purified from soil and insect cadaver during routine screening. The isolate NBAII-BTG4 was isolated from rhizosphere soil (Rajasthan) during screening of 158 soil samples whereas the NBAII-BTAS was isolated from an infected lepidopteran larvae from Assam. Microscopic analysis revealed that the indigenous *B. thuringiensis* isolates NBAIIBT-G4 and NBAIIBT-AS were spore forming, Gram positive and produced bipyramidal crystals.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Genes recognized</th>
<th>Product Size (bp)</th>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal <em>cry</em> 1 primers</td>
<td><em>cry</em> 1A, <em>cry</em> 1B, <em>cry</em> 1C etc.</td>
<td>558</td>
<td>5’CTGGATTAGGTTGAGGATGAT’3 5’TGGATGTCCCTCCGATATTGTGACT’3</td>
<td>52</td>
<td>Bravos et al, 1998</td>
</tr>
</tbody>
</table>

**Table 1. Primers used in the study for identification of *cry* genes**

*B. thuringiensis* was isolated from many environmental sites, e.g. soil, insect habitats, insect larvae, stored product and leaf surface (Martin and Travers, 1989; Ohba and Aizawa, 1986 and Smith and Couche, 1991). Bipyramidal shaped crystals show a greater degree of toxicity than all other types and the majority of lepidopteran active *B. thuringiensis* produce such bipyramidal crystal inclusions (Chilcott and Wigley, 1994).

**PCR studies**

To date, specific DNA sequences of a known *cry* gene sequence used either as probes or PCR primers to

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identify the potential cry gene sequences have been used as a common strategy for cloning cry genes (Shang Kuo et al., 2000). Carozzi et al., (1991) initially performed PCR analysis to identify different delta-endotoxins and reported the sequences of 12 PCR primers that distinguished three major classes of ICP genes (cryI, cryIII, and cryIV). In the present study, analysis of cry genes present in the two indigenous Bt isolates namely NBAIIBTAS and NBAII-BTG4 was characterized based on primers indicated in Table 1. BT-HD-1 was used as positive standard for the cry genes-cry1, cry2, cry1Aa, cry1Ab and cry1Ac. For the other cry genes namely cry1I, cry1G and cry1E, we only did a preliminary study using universal primers and standard validation was done only based on the size of the PCR product as mentioned in the quoted papers. The results of the PCR studies revealed that both the isolates expressed the cry1 gene (558bp) but NBAII-BTG4 also expressed the cry2 gene (1.17kb) which is supposed to have dual toxicity against Lepidopterans and Dipterans (Fig. 1). The cry gene profile of the two Bt isolates were also analyzed using cry specific primers and the PCR analysis revealed that the two isolates harbored cry1A, cry1Ab (1111bp) and cry1Ac (238bp) genes, but, the NBAII-BTG4 also expressed the cry1Aa (398bp) gene (Fig. 2 to 4).

Analysis was also carried out using the universal cry primers for detection of other cry genes. Results established that both were positive for cry1I which is having supposed to have dual toxicity against Lepidopterans and Coleopterans (Fig. 5). The NBAIIBTAS isolate, however, also showed positive for the genes cry1E (Fig. 6) and cry1G (Fig. 7). The comprehensive cry gene profile of the two isolates is indicated in Table 2.

PCR-based techniques have been proposed to identify different cry genes in B. thuringiensis strains (Porcar and Juarez-Perez, 2003). The insecticidal pathogenicity of B. thuringiensis can be predicted from the presence of cry genes in the bacterial genome (Carozzi et al., 1991 and Ben Dov et al., 1999). The profile of B. thuringiensis cry genes can be estimated by the polymerase chain reaction (PCR) method with specific primers (Konecka et al., 2007).

We also undertook partial sequencing of the specific primer products for cry1Aa, cry1Ab and cry1Ac. Blast analysis revealed that the cry1Aa gene of NBAII-BTG4 shares 99 per cent homology with the reference sequence of B. thuringiensis strain BLB1 cry1Aa gene (GenBank Acc. No. GU322940.1). Similarly, for cry1Ab and cry1Ac specific genes partial PCR products share a homology of
at least 99% for the reference strain GU322940.1 and EU250285.1 respectively.

The GenBank accession numbers assigned for cry1Aa from NBAII-BTG4 is JN120765 and cry1Ac from NBAII-BTAS is JN120764. Similarly, cry1Ab partial cds obtained from NBAII-BTG4 and NBAII-BTAS were submitted and the respective accession numbers are JN120763 and JF501457.

**SDS-PAGE analysis**

The crystalline delta-endotoxins are predominantly synthesized as long inactive protoxins that are activated by proteolysis in the insect gut. These toxins include cry1, cry4A, cry4B having molecular weights ranging from 130-140 kDa and are processed to active 65-70 kDa toxins while, cry2A, cry3A, cry10A and cry11A are naturally truncated toxins with molecular weights ranging from 65-80 kDa. However, as observed in cry2A and cry3A, proteolytic cleavage at the N and C termini can also process these naturally truncated toxins to active 60-65 kDa toxins (Gill *et al.*, 1992; Hofte and Whiteley, 1989). In our studies SDS-PAGE analysis of whole cell protein was carried out and the results revealed that the strains synthesize a protein or a group of proteins with molecular weights between 130 and 140 kDa (consistent with the presence of a cry1 gene), and a further protein of 65 kDa (consistent with the presence of a cry2 gene) (Fig. 9).

**Toxicity studies**

The trypsin activated toxins of the indigenous *Bt* namely NBAII-BTG4, NBAII-BTAS was evaluated against second instar larvae of *H. armigera* and it was observed that the standard *Bacillus thuringiensis kurstaki* HD-1 and NBAII-BTG4 were on par showing a LC$_{50}$ value of 0.92 and 0.93 µg/ml respectively (Fig. 9). The least toxic was NBAII-BTAS which showed a LC$_{50}$ value of 4.6 µg/ml. The lowest LC$_{50}$ values (highly toxic) were recorded in the *Btk* isolates (BtNg13, BtAm2 and BtPl4) from Tamil Nadu (0.03–1.82 mg ml$^{-1}$) when tested against *H. armigera* (Anitha *et al.*, 2011). Toxicity and larval growth inhibition of 11 insecticidal proteins of *B. thuringiensis* against neonate larvae of *H. armigera*, revealed that the most active toxins were cry 1Ac and cry2Aa1 with LC$_{50}$ values of 3.5 and 6.3 ig/ml, respectively (Avilla *et al.*, 2005). The cry I proteins belonging to different subgroups could exert different insecticidal properties against different lepidopteran pests (Gill *et al.*, 1992). In our studies, the isolate NBAII-BTG4 carried diverse types of cry genes and was more toxic. Carrozi *et al.* (1991) has stated that one can predict insecticidal activity of *B. thuringiensis* strains by polymerase chain reaction product profiles.

The present study established that cry gene profiling of new *B. thuringiensis* isolates can be readily done using PCR based techniques. Based on the cry gene profile one can determine their toxicity status.

**ACKNOWLEDGEMENT**

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**REFERENCES**


Table 2. cry gene profile of the indigenous Bacillus thuringiensis isolates NBAII-BT-AS and NBAII-BT-G4

<table>
<thead>
<tr>
<th>Genes</th>
<th>NBAII-BTAS</th>
<th>NBAII-BTG4</th>
<th>Active against</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal cry1 primers</td>
<td>✓</td>
<td>✓</td>
<td>Lepidopteran</td>
</tr>
<tr>
<td>Universal cry2 primers</td>
<td>✓</td>
<td></td>
<td>Lepidopteran &amp; Dipteran</td>
</tr>
<tr>
<td>Universal cry1A primers</td>
<td>✓</td>
<td>✓</td>
<td>Lepidopteran</td>
</tr>
<tr>
<td>cry1Aa specific primers</td>
<td>✓</td>
<td></td>
<td>Lepidopteran</td>
</tr>
<tr>
<td>cry1Ab specific primers</td>
<td>✓</td>
<td>✓</td>
<td>Lepidopteran</td>
</tr>
<tr>
<td>cry1Ac specific primers</td>
<td>✓</td>
<td>✓</td>
<td>Lepidopteran</td>
</tr>
<tr>
<td>Universal cry1B primers</td>
<td></td>
<td></td>
<td>Lepidopteran &amp; Coleopteran</td>
</tr>
<tr>
<td>Universal cry1C primers</td>
<td>✓</td>
<td></td>
<td>Lepidopteran</td>
</tr>
<tr>
<td>Universal cry1E primers</td>
<td></td>
<td></td>
<td>Lepidopteran</td>
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<tr>
<td>Universal cry1F primers</td>
<td></td>
<td></td>
<td>Lepidopteran</td>
</tr>
<tr>
<td>Universal cry1G primers</td>
<td>✓</td>
<td></td>
<td>Lepidopteran</td>
</tr>
<tr>
<td>Universal cry1H primers</td>
<td>✓</td>
<td>✓</td>
<td>Lepidopteran &amp; Coleopteran</td>
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<tr>
<td>Universal cry1I primers</td>
<td></td>
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<td>Lepidopteran</td>
</tr>
<tr>
<td>Universal cry3 primers</td>
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<td></td>
<td>Coleopteran</td>
</tr>
<tr>
<td>Universal cry9 primers</td>
<td></td>
<td></td>
<td>Lepidopteran</td>
</tr>
</tbody>
</table>


Porcar, M. and Juarez/Perez, V. 2003. PCR-based identification of Bacillus thuringiensis pesticidal crystal genes. FEMS Microbiology Reviews, 26: 419–432.


