**Short Communications**

PCR based detection of *cry* genes in indigenous strains of *Bacillus thuringiensis* isolated from the soils of Rajasthan

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Novel *cry* genes are potential candidates for resistance management strategies due to their different structure and mode of action. Therefore, it is desirable to clone and express novel *cry* genes from several novel isolates of *Bacillus thuringiensis* (Bt). Eight different Bt strains (namely IS1-IS8) were isolated from the desert part of Rajasthan and a simplified PCR method was developed for extensive analysis of *cry* and *cyt* genes in the native isolates. PCR results demonstrated that *cry*1 type genes (100%) were the most abundant genes in the indigenous Bt strains, followed by *vip3A* (87.5%), *cry*2 (75%), *cry*9 (62%), *cry*3 (50%), *cry*11 (37.5%), *cry*7-8 (37.5%), *cry*5,12,14, 21 (25%), *cyt*1 (25%), *cyt*4 (12.5%) and *cry*2 (12.5%). Further, PCR with degenerate primers also showed the presence of *cry* type genes. The toxicological characterization of these novel *cry* genes will have huge importance in transgenic technology and will be useful in transgenesis of crop plants for better resistance management.

**Keywords:** *Bacillus thuringiensis*, cloning and sequencing, δ-endotoxin, PCR

*Bacillus thuringiensis* (Bt), is a Gram-positive, spore-forming soil bacterium that produces parasporal insecticidal crystal proteins known as δ-endotoxins during the stationary and sporulation phase of growth cycle. These are intercellular proteins and have been used for many years as potent biological insecticides. Molecular effectiveness of Bt toxins is 300 times higher than synthetic pyrethroids and 80,000 times stronger than organophosphates. Search for novel Bt strains may lead to the discovery of novel insecticidal proteins with higher toxicity which can provide alternatives to cope up with the emergence of resistant insect populations. Therefore, it is essential to isolate a large number of Bt strains from diverse geographical conditions and cloning of many types of insecticidal crystal protein genes.

The identification and characterization of Bt *cry* and *cyt* genes by PCR offers several advantages in terms of rapidity and reproducibility. Different Bt strains available in India are valuable for identification of indigenous and novel Bt genes, which could encode more effective toxins due to sequence variations. In the present study, eight novel strains of Bt (IS1-IS8) were isolated and characterized form the desert soils of Rajasthan.

Bt isolates were isolated from the soil according to the methods of Ohba et al. and Santana et al., and cultures were maintained in the laboratory. The soil samples used for the isolation of Bt strains were collected from different parts of Rajasthan including the desert parts of Barmer and Jaisalmer, where Bt has never been applied as an insecticidal spray. A total of 60 bacterial colonies were subcultured from various soil samples and subjected to microscopic observation for the presence of proteinaceous crystals. From 60 bacterial colonies observed through bright field microscopy, 8 isolates were identified as Bt based on the presence of crystalline inclusions and named as IS1-IS8. The total genomic DNAs of Bt strains were isolated according to standard protocols. Agarose gel carried out as per the standard procedure. The comparison of the partial 16S rDNA sequence of Bt isolates (IS1-IS8) by the “Ribosomal Database Project II Sequence match” revealed that the isolates showed significant sequence identity with Bt type strains as well as very high homology to Bt strains in BLAST analysis, which confirmed that all the bacterial isolates (IS1-IS8) are *B. thuringiensis* and belong to group Firmicutes (data not shown).

Genomic DNA isolated from Bt strains were used as template for the PCR amplification to screen different *cry*-type genes. Amplification of respective *cry* genes was done using specific primers from Table 1 as per the earlier prescribed protocols. Amplification of expected size of PCR products in different primer pairs (Fig. 1) indicated the presence of the above mentioned *cry* type genes in Bt strains (IS1-IS8) (Table 2). Strains containing *cry*1 type genes were the most abundant in the indigenous isolates since all the strains were harbouring these genes, followed by *vip3A* (87.5%), *cry*2 (75%), *cry*9 (62%), *cry*3 (50%), *cry*11 (37.5%), *cry*7-8 (37.5%), *cry*5,12,14, 21 (25%), *cyt*1 (25%), *cyt*4 (12.5%) and *cry*2 (12.5%). Further, PCR with degenerate primers also showed the presence of *cry* type genes. The toxicological characterization of these novel *cry* genes will have huge importance in transgenic technology and will be useful in transgenesis of crop plants for better resistance management.

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cry1 (37.5%), cry7-8 (37.5%), cry5,12,14,21 (25%), cyt1 (25%), cry4 (12.5%) and cyt2 (12.5%) as detected by PCR. The characterization of these isolates by PCR showed that cry1, vip3 and cry2 type genes were found in 8, 7 and 6 strains, respectively. Ben-Dov et al.5 also reported that strains containing cry1 genes were the most abundant in Bt collections. Further, the sequence analysis of partial cry1 and cry2 showed the maximum similarity with known cry1 and cry2A genes from the nucleotide repository that confirmed the presence of cry genes in native Bt isolates (IS1-IS8) (data not shown). These results suggested that the PCR based cry gene screening with other screening primers are likely to be accurate.

![Agarose gel electrophoresis of DNA fragments amplified from Bt strains IS1-IS8 by PCR](image)

**Table 1—Oligonucleotide primers used for screening of partial cry type genes**

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Un1F</td>
<td>CATGATTGACACTGTCCTTCCCATTT</td>
<td>Partial cry1 gene</td>
</tr>
<tr>
<td>2</td>
<td>Un1R</td>
<td>TTTGTGACACTTCTGCTTCCCATT</td>
<td>(277 bp)</td>
</tr>
<tr>
<td>3</td>
<td>Un2F</td>
<td>GGTATTCTTAATGCAAGATGATGGAAGGG</td>
<td>Partial cry2 gene</td>
</tr>
<tr>
<td>4</td>
<td>Un2R</td>
<td>CCGATAAAAATATCTGTGGGAAGGATTG</td>
<td>(689–701 bp)</td>
</tr>
<tr>
<td>5</td>
<td>Un3F</td>
<td>CGCTTACGTCAGAGATGACTCAAACC</td>
<td>Partial cry3 gene</td>
</tr>
<tr>
<td>6</td>
<td>Un3R</td>
<td>CATCCTGTTCTTGAGGGAACATTG</td>
<td>(589-604bp)</td>
</tr>
<tr>
<td>7</td>
<td>Un4F</td>
<td>GCATATGATGTAGCGAAACAAAGCC</td>
<td>Partial cry4 gene</td>
</tr>
<tr>
<td>8</td>
<td>Un4R</td>
<td>GCGTGACATACCCATTTCCAGGTCC</td>
<td>(439 bp)</td>
</tr>
<tr>
<td>9</td>
<td>Un5F</td>
<td>TTAGTCAATATTGCGAATTCAAGCAAA</td>
<td>Partial cry 5, 12, 14, 21 genes</td>
</tr>
<tr>
<td>10</td>
<td>Un5R</td>
<td>AAGACCCAAATTCAATACCGGTT</td>
<td>(474-489 bp)</td>
</tr>
<tr>
<td>11</td>
<td>Un7-8F</td>
<td>AAGACGATGCTACCTGTTTAC</td>
<td>Partial cry 7-8 gene</td>
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<td>Un7-8R</td>
<td>CTTCTAACCCTTAAGTTTAC</td>
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<td>13</td>
<td>Un9F</td>
<td>CGGTGTTGACTATTAGCCAGGGCAG</td>
<td>Partial cry9 genes</td>
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<td>14</td>
<td>Un9R</td>
<td>GTGTGAGCCGCTTCACAGCAATCC</td>
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<td>15</td>
<td>Un11F</td>
<td>TTTACCTGCATACCTTTAAGC</td>
<td>Partial cry11 genes</td>
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<tr>
<td>16</td>
<td>Un11R</td>
<td>AGCTATGGCCTAAGGGGA</td>
<td>(305 bp)</td>
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<tr>
<td>17</td>
<td>VipF</td>
<td>CCTCGATGTTGATGATGATA</td>
<td>Partial vip3 genes</td>
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<td>18</td>
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<td>19</td>
<td>Cyt1F</td>
<td>AACCCCTCAATCAACAGCAAGG</td>
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<td>20</td>
<td>Cyt1R</td>
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<td>21</td>
<td>Cyt2F</td>
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<td>22</td>
<td>Cyt2R</td>
<td>TTTTACACATTTTACACATAC</td>
<td>(469 bp)</td>
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<table>
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<tr>
<th>Genes</th>
<th>IS1</th>
<th>IS2</th>
<th>IS3</th>
<th>IS4</th>
<th>IS5</th>
<th>IS6</th>
<th>IS7</th>
<th>IS8</th>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cry5,12,14,21</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cry 7-8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>cry9</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>cry11</td>
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<td>+</td>
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<tr>
<td>vip3A</td>
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<tr>
<td>cyt2</td>
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<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

+ indicate the presence of cry gene by PCR.

**Table 2—Distribution of cry, cyt and vip type genes in Bt isolates (IS1-IS8)**

**Fig. 1 (a-d)—Agarose gel electrophoresis of DNA fragments amplified from Bt strains IS1-IS8 by PCR: a. Amplified with cry3 specific primers; b. Amplified with vip specific primers; c. Amplified with degenerate PCR primers OL1F and OL5R; & d. Amplified with degenerate PCR primers OL1F and OL4R. (M, Mol wt marker; lanes 1-8, Bt strains IS1-IS8)**
Further confirmation of the presence of cry genes was done by amplification of partial cry genes by using degenerate primers. These primers were designed based on the consensus amino acid sequence from 15 different cry protein families by reducing the degeneracy in primer sequence\textsuperscript{7}. For amplification of cry genes with degenerate primers, a touchdown PCR was developed with the following amplification steps: initial denaturation at 94°C for 5 min, followed by first 10 cycles consisting of denaturation at 94°C for 30 sec, annealing at temperature of 55°C to 45°C (decreasing 1°C/cycle) for 30 sec and primer extension at 72°C for 1 min; then additional 20 cycle consisting of denaturation at 94°C for 30 sec, annealing at temperature 45°C for 30 sec and primer extension at 72°C for 1 min, and final extension for 7 min. The PCR products obtained by different degenerate primers were visualized by agarose gel electrophoresis for the presence of different cry genes (Fig. 1). The results obtained from each primer set were summarized in Table 3. The cry gene screening results obtained from the degenerate primers also confirmed the presence of cry genes in indigenous isolates along with the gene specific screening primers. Moreover, these strains showed some distinct amplicon with degenerate primers, which indicate the presence of some new cry type genes (data not shown).

The characterization of Bt strain collections for the presence of cry genes by PCR has been reported earlier also. Seifinejad et al\textsuperscript{12} reported 82.6% frequency of Vip3A, followed by Cry2A (56.5%), Cry1 (49%) and Cry9 (30%) in Iranian Bt strain collection. Arrieta et al\textsuperscript{13} reported that out of 105 Bt strain collection, PCR analyses confirmed the presence the vip3 (78 strains), cry2 (82 strains), cry1 (45 strains) and cry3-cry7 genes (29 strains) where as a total of 13 strains did not amplified with any of the cry primers used: cry1, cry2, cry3-7, cry5, cry11, cry12 and cry14. Beard et al\textsuperscript{14} reported the 87% frequency of Vip proteins in Australian Bt collection of 188 strains. In the present study also, seven of the eight isolates showed amplification with primers specific to vip3 and thus supports the previous studies.

Several PCR-based methodologies with universal primers and sets of primers directed against specific regions of type specific cry genes, and these approaches, allow the detection of cry genes and prediction of their insecticidal activities. However, these approaches (mostly multiplex PCR) are mainly focused on screening most currently known cry genes or gene families and do not ensure isolation of novel genes belonging to un-described groups\textsuperscript{15}. Beron et al\textsuperscript{16} designed five degenerate primers for detection of novel cry genes from B. thuringiensis strains by employing a two-step PCR-based approach, which allows amplification of unknown cry-related sequences along with known cry sequences that can be characterized after DNA sequencing. In the present study, unknown PCR amplicons were generated by degenerate primers along with known PCR amplicons, these might be novel cry type genes.

The use of PCR has greatly improved cry gene detection; however, this method is mostly limited to members of previously described gene families and requires a large number of primers. Also, universal degenerate primers were designed to amplify all the members of different subfamilies of cry genes. Although the use of these degenerate oligonucleotides increases the probability of amplifying novel genes, the efficiency is restricted to detection of closely related genes in the same group\textsuperscript{17}. The possibility of detecting and characterizing unknown cry sequences, which was proved by characterization of novel cry-related sequences from native Bt strains, was a clear demonstration of the potential of these set of primers\textsuperscript{17}.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amplified cry type gene with degenerate primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS1</td>
<td>cry1 Aa * cry3 Aa * cry4 Aa * cry7 Aa * cry8 Aa * cry9 Aa * cry11 Aa * cry12 Aa</td>
</tr>
<tr>
<td>IS2</td>
<td>cry1 Aa * cry3 Aa * cry4 Aa * cry7 Aa * cry8 Aa * cry9 Aa * cry11 Aa * cry12 Aa</td>
</tr>
<tr>
<td>IS3</td>
<td>cry1 Aa * cry3 Aa * cry4 Aa * cry7 Aa * cry8 Aa * cry9 Aa * cry11 Aa * cry12 Aa</td>
</tr>
<tr>
<td>IS4</td>
<td>cry1 Aa * cry3 Aa * cry4 Aa * cry7 Aa * cry8 Aa * cry9 Aa * cry11 Aa * cry12 Aa</td>
</tr>
<tr>
<td>IS5</td>
<td>cry1 Aa * cry3 Aa * cry4 Aa * cry7 Aa * cry8 Aa * cry9 Aa * cry11 Aa * cry12 Aa</td>
</tr>
<tr>
<td>IS6</td>
<td>cry1 Aa * cry3 Aa * cry4 Aa * cry7 Aa * cry8 Aa * cry9 Aa * cry11 Aa * cry12 Aa</td>
</tr>
<tr>
<td>IS7</td>
<td>cry1 Aa * cry3 Aa * cry4 Aa * cry7 Aa * cry8 Aa * cry9 Aa * cry11 Aa * cry12 Aa</td>
</tr>
<tr>
<td>IS8</td>
<td>cry1 Aa * cry3 Aa * cry4 Aa * cry7 Aa * cry8 Aa * cry9 Aa * cry11 Aa * cry12 Aa</td>
</tr>
</tbody>
</table>

* shows the presence of amplicon in the selected Bt isolate
The results of the present investigation suggest the presence of diversity in native Bt isolates of Rajasthan. Results of PCR based screening of cry genes and their partial sequencing confirmed the presence of novel genes in the Bt isolates (IS1-IS8). Further studies on cloning and characterization of those novel cry genes from these new isolates of Bt will be useful and open new opportunities in the area of integrated pest management for sustainable agriculture.

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References
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