Molecular characterization and PCR-based screening of cry genes from Bacillus thuringiensis strains

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Abstract Novel cry genes are potential candidates for resistance management strategies, due to their different structures and modes of action. Therefore, it is desirable to clone and express novel cry genes from several new isolates of Bacillus thuringiensis (Bt). In the present study, 28 Bt strains were characterized at morphological and molecular level. All these strains are Gram positive, endospore forming and had shown different crystal morphologies when viewed under the microscope. The ARDRA (16S rDNA PCR-RFLP technique) with AluI, HaeIII, HinfI and TaqI produced unique and distinguishable restriction patterns used for the molecular characterization of these isolates. Based on UPGMA clustering analysis, Bt strains showed significant molecular diversity and the dendrogram obtained differentiated 28 Bt strains into 1 major cluster at a similarity coefficient 0.56. PCR analysis demonstrated that the Bt strains showed diverse cry gene profiles with several genes per strain. The Bt strain G3C1 showed the presence of maximum cry-type genes by PCR. The toxicological characterization of these 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 • Delta-endotoxin • cry genes • PCR • 16S rDNA • ARDRA • Screening

Abbreviations

Bt Bacillus thuringiensis
Cry protein Crystal protein
PCR Polymerase chain reaction
ARDRA Amplified ribosomal DNA restriction analysis
UPGMA Unweighted pair-group method for arithmetic average

Introduction

Bacillus thuringiensis (Bt), is a Gram-positive, spore-forming soil bacterium that forms parasporal insecticidal crystal proteins during the stationary and sporulation phase of its growth cycle. These proteins are termed delta-endotoxins because of their intracellular location and have been used for many years as successful biological insecticides (Schneef et al. 1998). Commercial Bt-based bio-insecticides used worldwide are applied at 10–50 g/acre or about $10^{20}$ molecules/acre, while chemical pesticides such as organophosphates and synthetic pyrethroids are applied about $8 \times 10^{24}$ molecules/acre and $3 \times 10^{22}$ molecules/acre, respectively. Thus, molecular potency of these toxins is 80,000 times better than organophosphates and 300 times greater than synthetic pyrethroids (Feitelson et al. 1992). Search for novel Bt strains may lead to the discovery of novel insecticidal proteins with higher toxicity which will be important for providing alternatives to cope up with the emergence of resistant insect populations. Therefore, there is a need of isolation of large number of Bt strains from diverse geographical conditions and cloning of many new types of insecticidal crystal proteins genes (Ramalakshmi and Udayasuriyan 2010).

The polymerase chain reaction (PCR) has been widely used to characterize the Bt strain collections (Ceron et al.
This technique is a highly sensitive method for rapid detection and identification of target cry gene sequences requires very small amounts of DNA and allows simultaneous screening of many Bt strains to classify them and predict their insecticidal activities. The efficacy of PCR in identifying the large family of cry genes is based on the presence of conserved regions. Another strategy for the screening is based on the multiplex PCR which uses more than two primers in a mixture of the same reaction (Juarez-Perez et al. 1997).

Continuous exposure to a single kind of Bt toxin can lead to resistance development in insects. Routine replacement of cry genes or pyramiding of cry genes could be useful for effective control of insect pests by Bt transgenic plants. Variation of a single amino acid can significantly influence the level of toxicity in Cry proteins (Udayasuriyan et al. 1994). The applications of Bt products as biopesticides are limited by their narrow host range, low toxicity to the targeted insects and the resistance from insects. Therefore, it is necessary to continuously screen novel cry genes and perform rational design based on a known Cry toxin (Lin et al. 2008). In general, the type of cry and cyt genes present in a strain correlates to some extent with its insecticidal activity (Porcar and Juarez-Perez 2003). Since India is very rich in biodiversity and genetic resources, different Bt strains available in the country are valuable source for identification of indigenous novel Bt genes, which could encode more potent toxins due to sequence variations (Jain et al. 2006). In the present study, 28 strains of Bt were characterized and screened for novel cry genes for better resistance management.

### Materials and methods

#### Bacterial strains, chemicals and oligonucleotide primers

The Bt were isolated from the northeast region of India (Table 1). PCR chemicals, oligonucleotide primers and restriction enzymes were procured from Bangalore Genei, Pvt. Ltd., India, and AB genes Pvt. Ltd., UK. Microscopic examinations of the isolated strains were used for the characterization of Bt by physiological methods of staining such as Gram, endospore and crystal staining.

#### Molecular characterization using ARDRA of the 16S rDNA region

The total genomic DNA of Bt strains were isolated according to standard protocols (Kalman et al. 1993). The total DNA pattern of all Bt strains were analyzed on agarose gel. Genomic DNA isolated from Bt strains were used as template for the PCR amplification. Amplification of 16S rDNA region was performed with universal primers, 27F (5′AGAGTTTGATCCTGCGTCA3′) and 1492R (5′ACGGCTACCTTGTACGACTT3′). Each 40 μl reaction mixture contained 50 ng of genomic DNA of Bt strain, 50 ng of forward and reverse primers each, each dNTP at a final concentration of 200 μM and 1 U of Taq polymerase in 1× Taq buffer (with 15 μM MgCl₂). Amplification was accomplished with the thermal cycler (Eppendorf, Germany). The PCR was performed with Taq polymerase for 30 cycles as follows: 94°C for 1 min, 60°C for 45 s and 72°C for 1 min., the final extension was performed for 10 min at 72°C. The 16S rDNA amplicons were digested using four different restriction endonucleases viz HinFI, HaeIII, AluI and TaqI (Cihan et al. 2012). The restriction-digested products were analyzed on 2% agarose gel prepared in 1× TAE buffer containing 0.5 μg/ml of ethidium bromide. Electrophoresis was carried out at 100 V for 3 h in 1× TAE electrophoresis buffer. Data analysis was done using NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) software and SIMUQUAL Jaccard’s similarity coefficient (Rohlf 1997).

#### PCR-based cry gene screening in indigenous Bt strains

Genomic DNA of the Bt was used in PCR with cry gene-specific screening primers (Table 2). The PCR was accomplished using an Eppendorf thermal cycler as prescribed by Jain et al. (2012).

#### Results and discussion

#### Characterization of indigenous Bt strains

All the Bt isolates were found to be Gram positive and endospore forming. Similarly different crystal morphologies were seen blue under the white background and showed bi-pyramidal, spherical, cuboidal, rectangular, irregular crystals, etc. The shape of the crystalline inclusions varied among the 28 Bt strains. Shishir et al. (2012) identified Bt isolates based on their hemolytic activity, presence of parasporal crystal proteins and crystal protein profile and observed five different types of parasporal crystal proteins such as spherical, bi-pyramidal, irregular pointed, cuboidal and irregular shaped which formed irregular white colonies with a pink background among the isolates which indicates the diversity of the local Bt isolates. Similarly, Unalmis et al. (2015) isolated Bt-like colonies and characterized them on the basis of Gram
staining, spore staining and crystal staining. From 60 bacterial colonies observed through bright field microscopy, 28 isolates were identified as Bt based on the presence of crystalline inclusions.

**Molecular characterization of indigenous Bt strains using ARDRA**

Amplified rDNA (Ribosomal DNA) restriction analysis (ARDRA) is the extension of the technique of RFLP (restriction fragment length polymorphism) to the gene encoding the small ribosomal subunit (16S) of bacteria. The technique involves an enzymatic amplification using primers directed at the conserved regions at the ends of the 16S rDNA, followed by digestion using restriction enzymes. The pattern obtained is said to be representative of the species analyzed and important for their molecular characterization. In the present study ARDRA produced a fingerprint based on length polymorphism for molecular characterization of native Bt strains. Four restriction endonucleases viz., *Hinf*I, *Hae*III, *Alu*I and *Taq*I were used for restriction fragment analysis of amplified 16S rDNA. The banding patterns of the representative Bt are shown in Fig. 1, with standard molecular weight marker. Totally 20 bands of varying sizes were observed in all the 28 strains when digested with four restriction enzymes. When digested with *Hae*III, 7 different DNA fragments were obtained, whereas 3 different DNA fragments were obtained with *Hinf*I endonuclease, *Taq*I digestion resulted 6 different DNA fragments while *Alu*I resulted in 4 different DNA fragments.

Genetic similarity estimates the variations based on ARDRA banding patterns, which were calculated using method of Jaccard’s coefficient analysis. The similarity coefficient matrix generated was subjected to algorithm “Unweighted Pair Group Method for Arithmetic Average (UPGMA)” to generate clusters using NTSYS 2.02 pc program. The pairwise comparison of ARDRA patterns based on both shared and unique amplification products was made to generate a similarity matrix. Similarity indices

| Sl. no. | Location                  | Isolate code | Lat and long |
|--------|---------------------------|--------------|--------------|
| 1      | Borbam tea estate         | S20C4        | N = 26°47.704; E = 94°31.868 |
| 2      | Borbam tea estate         | S22C1        | N = 26°48.056; E = 94°31.801 |
| 3      | Jogduwar, small tea garden| S29C2        | N = 26°50.209; E = 94°26.551 |
| 4      | Kharuapather, rice field  | D9C1         | N = 27°51.35; E = 95°22.812 |
| 5      | Borbam tea estate         | S23C1        | N = 26°48.056; E = 94°31.801 |
| 6      | Borbam tea estate         | S21C1        | N = 26°47.704; E = 94°31.868 |
| 7      | Borbam tea estate         | S20C3        | N = 26°47.704; E = 94°31.868 |
| 8      | Gorjugonia forest         | J11C1        | N = 26°41.455; E = 94°08.605 |
| 9      | Baruah bagan, tea garden  | S11C1        | N = 26°48.051; E = 94°33.483 |
| 10     | Nabaruwara, rice field    | T2C2         | N = 27°74.633; E = 95°55.391 |
| 11     | Nabaruwara, rice field    | T5C1         | N = 27°74.908; E = 95°56.913 |
| 12     | Amguri, tea garden        | S16C2        | N = 26°48.494; E = 94°32.421 |
| 13     | Charing, vegetable land   | S6C3         | N = 26°48.494; E = 94°32.421 |
| 14     | Nabaruwara, rice field    | T3C2         | N = 27°74.369; E = 95°55.364 |
| 15     | Hatigarih, rice field     | T7C1         | N = 27°62.579; E = 95°69.452 |
| 16     | Hatigarih, rice field     | T8C2         | N = 27°62.606; E = 95°69.345 |
| 17     | Khowang, rice field       | D1C1         | N = 26°27.278; E = 94°81.613 |
| 18     | Tengakhat, rice field     | D12C1        | N = 27°41.047; E = 95°18.225 |
| 19     | Charaihah tea garden      | J10C1        | N = 26°40.893; E = 94°08.557 |
| 20     | Sessa tiniali, rice field | D5C1         | N = 27°25.843; E = 94°78.486 |
| 21     | Borbam tea estate         | S19C1        | N = 26°47.704; E = 94°31.868 |
| 22     | Sessa tiniali, rice field | D4C1         | N = 27°55.792; E = 94°32.764 |
| 23     | Nakhona gaon, rice field  | S4C2         | N = 26°55.792; E = 94°32.764 |
| 24     | Golaghat, tea estate      | G1C1         | N = 26°63.334; E = 93°91.192 |
| 25     | Nakhona gaon, rice field  | S4C1         | N = 26°55.792; E = 94°32.764 |
| 26     | Gorjugonia forest         | J11C3        | N = 26°41.455; E = 94°08.605 |
| 27     | Golaghat, rice fields     | G2C1         | N = 26.44788; E = 94.01534 |
| 28     | Golaghat, vegetables      | G3C1         | N = 26.29748; E = 94.08121 |
Table 2: Oligonucleotide primers used for screening of partial cry-type genes

| Sl. no. | Name   | Sequence (5’→3’)                        | Amplification                  |
|---------|--------|-----------------------------------------|--------------------------------|
| 1       | Un1F   | CATGATTCTATGCAGGAGATAA AC               | Partial cry1 gene (277 bp)     |
| 2       | Un1R   | GTTGACACTTCTGCGCTCCATT                   |                                |
| 3       | Un2F   | GGTATCTTAATGCGAGATGGAATGGG              | Partial cry2 gene (689–701 bp) |
| 4       | Un2R   | CGGATTAAATAATCTGGGAAATAGF               |                                |
| 5       | Un3F   | CGTTATCGCAGAGAGATGACATTAAC              | Partial cry3 gene (589–604 bp) |
| 6       | Un3R   | CATCTGTTGTTTCTGGGAGGAAT                |                                |
| 7       | Un4F   | GCAATGATGTAGCGAAACACGCC                | Partial cry4 gene (439 bp)     |
| 8       | Un4R   | GCGTGACATACCCATTTCCAGGTC                |                                |
| 9       | Un5F   | TTAGGTTAAATTGGCTCAATCGAAGCAA            | Partial cry 5, 12, 14, 21 genes (474–489 bp) |
| 10      | Un5R   | AAGACCAAAATTCAATACCAGGGTT              |                                |
| 11      | Un7-8F | AAGCAGTGAAATGCTTGTTTAC                 | Partial cry 7–8 gene (420 bp)  |
| 12      | Un7-8R | CTCTATAACCTTTGACTAC                  |                                |
| 13      | Un9F   | CGGTTATCGACTTTAGCGAGGGCG               | Partial cry9 genes (351–359 bp) |
| 14      | Un9R   | GTGAGGCGCTTCAGGCAATCC                 |                                |
| 15      | Un11F  | TCTCAACCAAATTCCACAGGC                | Partial cry11 genes (305 bp)   |
| 16      | Un11R  | AGCTATGGAATGCTTGTTTAC                 |                                |
| 17      | VipF   | CTAATGGGCTAAAGGGAAA                   | Partial vip3 genes (1000 bp)   |
| 18      | VipR   | CATCTGTTGTTTCTGGGAGGAAT              |                                |
| 19      | Cyt1F  | AATATGTTTCAATAGCGAGCCAGGG             | Partial cyt1 genes (522–525 bp) |
| 20      | Cyt1R  | GGTAGCAAAATACAGCAGCACC                |                                |
| 21      | Cyt2F  | AATACATTCTCAAGAGCTA                   | Partial cyt2 genes (469 bp)     |
| 22      | Cyt2R  | TTTGTTGTTAATCTGTC                    |                                |

Fig. 1: Agarose gel electrophoresis of a PCR amplification of 16S rDNA region from native Bt strains. b ARDRA patterns of 16S rDNA sequence of Bt strains by restriction enzyme AluI. M1 500 bp DNA ladder, Bt strains 1 S4C1, 2 S16C2, 3 S4C2, 4 D4C1, 5 T5C1, 6 S20C4, 7 G1C1, 8 S19C1, 9 S22C1, 10 S20C3, 11 D9C1, 12 T8C2, 13 T2C2, 14 S21C1, 15 D1C1, 16 G3C1, 17 J11C1, 18 D12C1, 19 T3C2, 20 G2C1, 21 D5C1, 22 S29C2, 23 J11C3, 24 T7C1, 25 S23C1, 26 S6C3, 27 S11C1, 28 J10C1
established on the basis 20 bands of 4 restriction enzymes ranged from 0.11 to 0.93.

The dendrogram (Fig. 2) is a close representation of the values obtained in the similarity matrix. The dendrogram depicted the relationship among the Bt strains and clearly divided into one major cluster at a similarity coefficient 0.56. The dendrogram clearly indicated that S6C3 and S23C1 were different from remaining strains, hence these are more diverse. The first (A) cluster was divided into two sub-clusters A1 and A2. Sub-cluster A1 included 24 strains and further divided into two sub-clusters A1a and A1b. Sub-cluster A1a included strains J11C3, G2C1, S20C4, T7C1, S21C1, T8C2, D12C1, S20C3, D9C1, D5C1, S22C1, T5C1, J11C1, D4C1, and S4C1. S4C1, D4C1 and J11C1 and sub-cluster A1b comprises only one strain S11C1. Sub-cluster A2 comprises 2 strains S16C2 and S4C2.

Molecular techniques have helped to develop easy and rapid methods to perform microbial characterization at genus, species and even at strain level. Molecular markers have been found to be strain specific and have proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. Identification of Bacillus species using conventional sequencing methods can divulge their taxonomic affiliation, but there are certain groups of Bacillus where alternate methods like ARDRA and PCR fingerprinting can expose the exact lineage of the species rapidly.

Saadaoui et al. (2009) reported a new Bacillus thuringiensis kurstaki strain BLB1, isolated from a Tunisian soil sample showed the same 16S rDNA ARDRA profile than HD1 using TaqI, AluI, MboII, and MspI restriction enzymes. Gowdaman et al. (2014) reported that a collection of 171 soil bacterial isolates was analyzed for the occurrence of genus Bacillus using group-specific primers and ARDRA was performed for the Bacillus-positive isolates with standard Bacillus strains. Sangeetha et al. (2016) analyzed 15 bacterial isolates by ARDRA and used eight restriction endonucleases (CfoI, HinfI, RsaI, Ddel, Sau3AI, AluI, HaeIII, and MspI) forming two heterogenous main clusters after analysis by unweighted pair-group method using arithmetic averages.

**PCR-based screening of cry genes in native Bt strains**

Amplification of expected size of PCR products in different primer pairs (Fig. 3) indicated the presence of the above-mentioned cry-type genes in Bt strains (Table 3). The primers cry2, cry3, cry5 and cry7–8 did not show any amplification in PCR-based cry gene screening. The strains containing cry1-type genes were the most abundant (100%) in the indigenous Bt strains since all the strains were harboring these genes followed by cry4 (84.14%), cry9 (64.28%), cry11 (39.28%), cry2 (39.28%), and vip3A (25%) (Table 4). Agarose gel electrophoresis showed non-
specific amplification along with specific partial cry gene amplicon which was also observed in many published research findings using the same screening primers.

Jain et al. (2012) observed the frequency of cry-type genes in eight Bt strains (IS1–IS8) with the result that the cry1 type genes were most abundant in the indigenous isolates since all the strains were harboring these genes, followed by vip3A (87.5%), cry2 (75%), cry9 (62%), cry3 (50%), cry11 (37.5%), cry7–8 (37.5%), cry5, 12, 14, 21 (25%), cry1 (25%), cry4 (12.5%) and cry2 (12.5%) as detected by PCR. Patel et al. (2012) reported the diversity of cry genes from different soil types and climatic environments and reported the presence of cry1, cry2, cry3, 7, 8, cry4, cry5, 12, 14, 21, cry11, cry13 and cyt1 genes from Bt, whereas absence of cry3 and cry13 genes were reported in the isolates of non-agricultural samples. Similarly, Salekjalali et al. (2012) identified isolates harboring different cry-type genes through PCR and found 47% of the strains amplified with the cry1 primer, 29% with cry3 and 13% with cry4. Salama et al. (2015) reported that cry1 gene is the most abundant in these isolates (83.33%) among tested cry-type genes, followed by cry1 gene subfamilies (cry1B and cry1C) with percentage of 38.88 and 77.77%, respectively. The tested isolates showed the presence of cry2A gene, but not all of these isolates were positive for cry2 gene (55.55%). Only 27.77 and 16.66% of the tested isolates harbor cry4 and cry3 genes, respectively.

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. The results of the present study suggest the presence of diversity in the native Bt isolates. 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.

**Fig. 3** Agarose gel electrophoresis of **a** DNA isolation from native Bt strains. **b** PCR amplification of partial cry1 gene from Bt strains. **c** PCR amplification of partial cry4 gene from Bt strains. MI 500 bp DNA ladder, Bt strains 1 S4C1, 2 S16C2, 3 S4C2, 4 D4C1, 5 T5C1, 6 S20C4, 7 G1C1, 8 S19C1, 9 S22C1, 10 S20C3, 11 D9C1, 12 T8C2, 13 T2C2, 14 S21C1, 15 D1C1, 16 G3C1, 17 J11C1, 18 D12C1, 19 T3C2, 20 G2C1, 21 DSC1, 22 S29C2, 23 J11C3, 24 T7C1, 25 S23C1, 26 S6C3, 27 S11C1, 28 J10C1.
Table 3 Distribution of cry and cyt-type genes in native Bt isolates

| Genes | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 |
|-------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| cry1  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry2  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry3  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry4  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry5  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry6  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry7  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry8  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry9  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry10 | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cry11 | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cyt1  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| cyt2  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Vip1  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |

Bt strains 1-28 represent 1. D1C1, 2. T7C1, 3. J11C1, 4. S6C3, 5. T8C2, 6. S19C1, 7. S23C1, 8. T5C1, 9. S4C1, 10. S20C3, 11. G1C1, 12. J11C1, 13. J12C1, 14. S6C4, 15. T5C2, 16. S11C1, 17. T1C1, 18. S11C1, 19. T1C1, 20. S11C2, 21. D1C1, 22. D2C1, 23. S2C4, 24. S2C5, 25. S3C2, 26. T2C1, 27. T2C2, 28. S4C2

* indicates the presence of cry gene by PCR.
Table 4 Frequency of cry-, cyt- and vip-type genes in Bt strains

| Genes | Frequency (%) |
|-------|---------------|
| Cry 1 | 100           |
| Cry 4 | 82.14         |
| Cry 9 | 64.28         |
| Cry 11| 39.28         |
| Cyt 1 | 32.14         |
| Cyt 2 | 39.28         |
| Vip   | 25            |

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Compliance with ethical standards

Conflict of interest All the authors declare that there is no conflict of interest.

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