Isolation and characterization of *Bacillus thuringiensis* strains native to Assam soil of North East India

Mihir Rabha¹ · Shaswati Sharma¹ · Sumita Acharjee^{1,2} · Bidyut Kumar Sarmah^{1,2}

Received: 2 June 2017 / Accepted: 2 September 2017 / Published online: 9 September 2017
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Abstract We have identified both crystalliferous and acrySTALLIFEROUS Bt isolates from the Assam soil of North East India for the first time. A total of 301 *Bacillus* type colonies were selected based on their appearance and colony morphology. Out of these colonies, 42 isolates had characteristics similar to Bt isolates on MYP (Mannitol Egg Yolk Polymyxin) agar base medium. The ERIC-PCR and 16S rDNA analyses confirmed that 42 isolates are *Bacillus thuringiensis*. Phase contrast microscopy showed that 37 isolates produced crystal endospore during the sporulation phase and 5 acrySTALLIFEROUS isolates were also found. Amplification of *cry* gene was carried out using general Cry primers along with one *cry2* gene specific primer. Out of 42 isolates, 50% of the isolates showed presence of *cry2* gene followed by *cry9* (40.47) and *cry1* (40.47). Moreover, 21.42% of isolates showed the presence of more than one *cry* genes. We also screened these isolates for the possibility of having new Bt genes using universal primer and found two strains having a new type of *CryII* gene with 82 and 85% similarities with the available *CryII* gene sequences. Thus, these new types of Bt gene could be useful for Bt-based bioformulations and generation of transgenic plants.

Keywords *Bacillus thuringiensis* · Cry genes · Crystal protein · ERIC-PCR · North East India

✉ Sumita Acharjee
sumita.aus@gmail.com

✉ Bidyut Kumar Sarmah
bidyutsarmah@yahoo.co.in

¹ Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, Assam 785013, India

² DBT-AAU Centre, Assam Agricultural University, Jorhat, Assam 785013, India

Introduction

Bacillus thuringiensis (Bt) is an agriculturally important bacterial strain having Bt genes which encode for the production of insecticidal proteins in the vegetative stage and during the sporulation phase. There are numbers of crystal-producing Bt strains in nature, though, not all are effective against insect pest (Roh et al. 2007). The crystals are assembled as parasporal bodies expressed by the *cry* genes. So far a large number of Bt strains have been isolated and characterized for their sequence similarities within these *cry* genes. A total of 700 *cry* genes have been isolated till date (Alper et al. 2016) and interestingly, single strains of Bt may contain more than one crystal protein genes (Letowski et al. 2005; Pedro and Ibarra 2010). Although there are variations within the *cry* genes, the three domain of Cry proteins have conserved domains in their tertiary structures of the active toxin.

The proteins expressed from these *cry* genes can be safely used as bio-pesticides to control Lepidoptera, Coleoptera and Diptera pests. The efficacy of these bio-pesticides is low due to proteolytic damage during extraction, un-uniform spray within the crop canopy and degradation by ultraviolet rays of the sun. This lead to the inception of introducing codon-optimized versions of Bt gene in crop plants by genetic transformation procedures so that plants have an inbuilt resistance to target pests. Thus, a large number of *cry* gene expressing Bt transgenic crops was developed and commercially released, including Bt cotton, Bt corn and Bt soybean (Kumar et al. 1996). This Bt crops are widely accepted around the globe and resulted in less insecticidal spray in the field and better economic benefits to the farming community.

In the case of cotton, transgenic line (Bollgard III) expressing Bt Cry1Ab/c, Cry2Ab and Vip3 for durable

resistance to lepidopteran pests in the field was generated. Development of resistance to insecticide, including Bt proteins have been documented (Tabashnik 2015), although a recessive allele governs resistance development within the population. Amongst the Bt genes introduced into crop plants, resistance to Bt Cry2Ab within the Lepidopteran population have been reported in the USA, Australia and India (Gujarat). Three approaches were adopted for delay resistance (a) growing refuge, (b) high dose of Bt expression in crop plants (c) gene pyramiding. Though these strategies are suitable, adopting them is challenging, especially in a country such as India, where the majority of the farmers have small or marginal land holding to spare land for refuge. A suitable alternative is to express novel Bt gene(s) in the crop plant. Therefore, identification of novel insecticidal genes from novel Bt strains and its introduction to crop plant to delay insect resistance development is the need of the hour.

There are several reports on the isolation and characterization of native Bt strains of soil to identify novel toxins that are effective against agricultural pests. The interest to find novel Cry proteins is mainly to increase the range of Bt toxin, to search for toxins with high levels of toxicity and to find a new toxin to delay resistance within the pests due to the use of existing Bt toxins. A large number of Bt strains have been isolated from all over the world in search of novel insecticidal genes which must fit into the current classification and nomenclature based on amino acid sequence identity (Crickmore et al. 1998). Till date, 70 different *cry* gene groups (*cry1* to *cry70*) have been classified, where toxins from each Cry group share less than 40% amino acid sequence similarity with the toxins from other groups (Crickmore et al. 1998). If amino acid sequence similarity is <70% between the groups, a capital letter (*CryIA*, *CryIB* etc.) is given, while a small letter is given (*CryIAa*, *CryIAb* etc.) if the sequence is >70% but <95% amino acid sequence identity (Bravo et al. 2013). Thus, a novel Cry toxin should be less than 90–95% similar to the existing ones.

Based on the reports, novel Bt genes were identified by various methods. First, distinctiveness of the *B. thuringiensis* from its closely related species *B. cereus* because their biochemical and genomic composition are almost similar, except for the fact that *B. thuringiensis* produce a crystal inclusion endospore (Guo et al. 2008). Once the strain is identified, there are various strategies followed to search for novel Bt strains, such as the development of the DNA library (Beard et al. 2001) or use of degenerate primers or partial sequences as probes to hybridize with the library (Porcar and Juarez-Perez 2003) and PCR amplification using general or universal or Cry-specific primers (Noguera and Ibarra 2010; Travers et al. 1987). However, the PCR amplification and sequencing of

amplicon is widely used for identification of novel Bt genes.

To isolate novel Bt gene(s) from Assam soil, we collected soil samples from 14 districts, including soil from rice- and tea-growing regions. This North Eastern state of India, Assam, is rich in biodiversity due to its unique geographical location and climate. The region is completely unexplored or undisturbed due to lack of urbanization and industrialization. Therefore, the chances of finding novel Bt strains was thought to be more than the rest of India. We have used morphological, biochemical and PCR amplification approaches to identify and isolate novel Bt genes. We have identified 42 isolates based on 16S rRNA and amplified *cry* gene using universal primers. We also found two strains having the new type of *CryII* gene.

Materials and methods

Collection and preparations of soil samples

Soil samples were aseptically collected from 14 different locations of rice- and tea-growing belt of Assam, including the Kaziranga National park. Soil samples were collected within the depth of 5 cm from the soil surface. Then 1 g of soil samples from each location were dissolved in 10 ml of sterile distilled water and heated at 80 °C for 10 min to kill all the vegetative cells to eliminate bacteria without spore. Serial dilution was done separately for each sample and aliquots of each dilution were spread on T3 medium (Rebecca et al. 2011). Plates were incubated at 37 °C for overnight and the colonies were observed and subjected to the Gram staining.

Differentiation of isolates with MYP Agar Base

A Mannitol Yolk Polymyxin (MYP) agar (Himedia M636) medium was used to differentiate the *B. thuringiensis* from *B. cereus*. Filter-sterilized Polymyxin B sulfate solution was added to a final concentration of 100 units per ml along with 50 ml of fresh egg yolk emulsion to 450 ml of sterile MYP agar base medium. A single colony from each isolate was picked, streaked on the MYP Agar Base medium and incubated at 30 °C for 24 h.

Genomic DNA and plasmid preparations

The genomic DNA was isolated according to the protocol mentioned by Rebecca et al. (2011) and plasmids were isolated following the method described by Andrup et al. (2008) from a total of 42 isolates. The quantity and purity

of both plasmid and genomic DNA were measured by the Nano drop analyzer of Thermo Scientific.

Amplification of 16S rDNA

Primers for the amplification of 16S rDNA were synthesized accordingly designed by Li et al. (2011). These primers were designed from the conserved regions of available 16S rDNA sequences. The primer sets were forward primer: 5'-TTG TTA GCG GTG AAA TGC GT-3 and reverse primer: 5'-TTG TTA CCC TCT ACG ACG GAC-3'. The reaction was carried out in a thermal cycler with single step pre-denaturation at 94 °C for 2 min, 35 cycles with denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Finally, the amplified product was sequenced and analyzed by NCBI Blastn.

ERIC-PCR on isolates

ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus Sequences PCR) was performed to differentiate between *B. cereus* and *B. thuringiensis* according to the procedure described by the Li et al. (2011). The reaction was carried using primers (forward: 5'-ATGTAAGCTC CTGGGGATTAC-3, reverse: 5'-AAGTAAGTGA CTGGGGTGAGCG-3') to yield a 100 bp product for ERIC-PCR. In a thermal cycler, PCR reaction was performed with single-step pre-denaturation at 94 °C for 2 min, 35 cycles program with denaturation at 94 °C for 50 s, annealing at 52 °C for 1 min, extension at 72 °C for 1 min and the final denaturation at 72 °C for 7 min. The amplified product was analyzed in a 1.5% agarose gel.

Staining of crystal endospore

The Gram-positive isolates were selected for endospore staining to examine the presence of parasporal crystal inclusion bodies by phase-contrast microscopy. A single colony of each isolate was inoculated into 50 ml of T3 Broth medium. The cultures were incubated at 37 °C with 60 rpm for 72 h. After incubation, 1 ml from each liquid culture was transferred to a micro-centrifuge tubes, separately, followed by centrifugation at 4500 rpm for 10 min. The pellet was dissolved in 500 µl of sterile distilled water and 50 µl of aliquot were heat fixed on glass slides and Gram staining was done to observe the under 100× magnification using immersion oil for spore formation.

PCR amplification of *cry* genes

Amplification of *cry* genes was done using available general primers viz, *cry1* (Bravo et al. 1998), *cry2* (Sauka

et al. 2005), *cry3* (Ceron et al. 1995), *cry4* (Apaydin et al. 2005), *Nem-Gral* and *cry9* (Bravo et al. 1998). The reaction profile for *Gral-cry1* were carried out with a pre-denaturation step at 94 °C for 2 min, then a program of 35 cycles with denaturation 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 1 min and final extension with a single step at 72 °C for 5 min. The PCR conditions for other primers were same, except the annealing temperatures (*cry2* at 45 °C, *cry3* at 48 °C, *cry4* at 60 °C, and *spc-cry9* at 51 °C) for each gene. The amplified products of respective primers were analyzed under 1.5% agarose gel.

Western blot analysis of the isolates

Western blot analysis of the crystal spores were extracted from 72 h of incubation at 37 °C. The crystal proteins were purified according to the protocol described by Izabela et al. (2008). The purified proteins were dissolved in 4× Laemmli buffer, boiled for 10 min and 15 µl of proteins were separated on a 12% SDS polyacrylamide gel and stained with coomassie dye. The proteins from isolated having *Cry2* gene were also transferred to nitrocellulose membrane and blocked with 5% skimmed milk for 1 h. Western blotting was performed using rabbit *Cry2Aa* as primary antibody and anti-rabbit IgG (Fc) Alkaline phosphatase conjugate (Promega) as secondary antibody. The hybridized antibody was detected using a 5-bromo-1-chloro-3-indolyl phosphate (BCIP) and Nitroblue tetrazolium (NBT) solution as a substrate.

Identification of novel *cry* genes

For amplification of novel *cry* genes already reported degenerate universal primers were used (Travers et al. 1987). The primer sets were designed from the conserved blocks of *Cry* protein sequences. The primer (forward- 5' TAT GCW CAA GCW GCC AAT YTW CAT YT 3' and reverse- 5' GGR ATA AAT TCA ATT YKR TCW A 3') used for PCR reaction mixtures of a total volume of 25 µl with 1× reaction buffer, 200-µM dNTPs, 2-µM each primer (forward and reverse primer), 60 ng template DNA and 0.5-U Taq polymerase. The reaction was set up with pre-denaturation 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and another single step for the final extension at 72 °C for 7 min. The amplified PCR products were analyzed by 1.5% Agarose gel. The desired amplified products were eluted and purified with the QIAquick gel extraction kit (Qiagen).

Results

Isolation, identification and characterization of isolates

Based on colony morphology of *B. thuringiensis*, 301 isolates were selected for Gram staining. The colonies with rough, irregular, white to off-white in color were selected after overnight incubation at 37 °C. The Gram staining analysis of these 301 isolates showed that only 228 isolates were Gram positive. Since the Gram staining procedure is not suitable to distinguish *B. thuringiensis* from *B. cereus*, therefore, the colonies were streaked on MYP agar base medium and incubated at 30 °C for 24 h. Colonies with pink color yellow hallow were considered as a *B. cereus*, while those without any change were marked as *B. thuringiensis*.

For further confirmation of the isolates, we amplified 16S rDNA and performed ERIC-PCR on the isolates. The 16S rDNA amplification was carried out using specific primers and 42 isolates showed the expected amplicon of 1400 bp (Fig. 1). The amplified products of each isolate were sequenced and analyzed by NCBI BLAST software. The BLAST results revealed complete similarity of 16S rDNA sequences of the existing *B. thuringiensis*. We also performed an ERIC-PCR as described by Li et al. (2011). The isolates (42) revealed desired amplicon of 100 bp (Fig. 2), which confirmed that these isolates are *B. thuringiensis*.

These 42 isolates were incubated at 37 °C for 72 h in the liquid T3 medium to induce crystal endospore. We found

that 37 isolates produced crystal endospore (Fig. 3), while 5 acrystelliferous isolates were also observed under a phase contrast microscopy. The variation in the density of crystal protein(s) production was also recorded, some of the strains produced the more Cry protein(s), while few produced low quantity of crystal protein(s). Most of the crystal structures were cubical and pyramidal in shape, while some were similar to rice grain shape.

Amplification of *cry* genes

The presence of the *cry* genes was detected by performing PCR amplification of genomic DNA using general as well as gene-specific primers. Out of 42 isolates, 15 isolates yielded expected amplicon of 500–1000 bp for *cryI* primers. Generally, *cryI* primers result in PCR products ranging from 250 to 1000 bp size, however, strains producing an amplicon >500 bp are considered as *cryI* positive (Fig. 4).

For the amplification of *cry2* gene, specific primers were designed from the complete coding sequence (AF200816.1) collected from the NCBI. In all, 21 of the 42 isolates showed amplification of a desired amplicon of 700 bp (Fig. 5). Similarly, the *cry3* gene was amplified using general primers and a total of 15 isolates showed the desired amplicon of 700 bp (Fig. 6). Thus, 50% of the isolates showed presence of *cry2* gene followed by *cry9* (40.47) and *cry1* (40.47). The isolates having *cry3*, *cry4* and *Nem-GraI* genes were 35.71, 19.04 and 11.90%, respectively (Table 1). Moreover, 21.42% of isolates showed the presence of more than one *cry* genes (Table 2).

Fig. 1 Amplification of 16S rRNA using genomic DNA from the isolates using specific primers to indicate that these isolates are *Bacillus* spp. Lane B-Blank, N-negative, Lane1-BA01, lane2-BA02, lane3-BA03, lane4-BA04, lane5-BA05, lane6-BA06, lane7-BA07, lane8-BA08, lane9-BA09, lane10-BA10, lane11-BA11, lane12-BA12, lane13-BA13, lane14-BA14, lane15-BA15, lane16-BA16,

lane17-BA17, lane18-BA18, lane19-BA19, lane20-BA20, lane21-BA21, lane22-BA22, lane23-BA24, lane24-BA25, lane25-BA26, lane26-BA29, lane27-BA30, lane28-BA31, lane29-BA33, lane30-BA34, lane31-BA35, lane32-BA36, lane33-BA38, lane34-BA44, lane35-BA46, lane36-BA47, lane37-BA48, lane38-BA50, lane39-BA52, lane40-BA53, lane41-BA54, lane42-BA56

Fig. 2 Molecular typing of the isolates collected from Assam soil by ERIC-PCR analysis. The amplification of 100 bp product indicated that the isolates are *Bacillus thuringiensis*. Lane B-Blank, N-negative, Lane1-BA01, lane2-BA02, lane3-BA03, lane4-BA04, lane5-BA05, lane6-BA06, lane7-BA07, lane8-BA08, lane9-BA09, lane10-BA10, lane11-BA11, lane12-BA12, lane13-BA13, lane14-BA14, lane15-BA15, lane16-BA16, lane17-BA17, lane18-BA18, lane19-BA19,

lane20-BA20, lane21-BA21, lane22-BA22, lane23-BA24, lane24-BA25, lane25-BA26, lane26-BA29, lane27-BA30, lane28-BA31, lane29-BA33, lane30-BA34, lane31-BA35, lane32-BA36, lane33-BA38, lane34-BA44, lane35-BA46, lane36-BA47, lane37-BA48, lane38-BA50, lane39-BA52, lane40-BA53, lane41-BA54, lane42-BA56

Bacterial cells

Crystal proteins

Fig. 3 Microscopic view of crystal proteins produced in the isolates after 72 h of incubation at 37 °C in T3 medium. The malachite green (upper plate) and crystal violet (lower plate) staining showed the presence of Bt crystal proteins indicated by arrow along with the lysed *Bacillus thuringiensis* cells. Bt strains were observed by optical microscopy with a $\times 100$ oil immersion lens

Fig. 4 PCR amplification of the Bt isolates of Assam soil using Gral-cryI primer showed products of more than 500–1000 bp. The amplicons of >500 bp confirmed the presence of gral-cryI gene in the isolates. M-1 kb DNA ladder, B-Blank, N-Negative, lane1-BA12, lane2-BA06, lane3-BA04, lane4-BA05, lane5-BA07, lane6-BA08, lane7-BA03, lane8-BA09, lane9-BA11, lane10-BA01, lane11-BA10, lane12-BA16, lane13-BA13, lane14-BA15

Fig. 5 PCR amplification of the Bt isolates of Assam soil using spc-cry2 primer showed amplification of a 700 bp amplicon specific to spc-cry2 in the isolates. M-DNA ladder, B-Blank, Lane1-BA06, lane2-BA33, lane3-BA12, lane4-BA03, lane4-BA08, lane5-BA31, lane6-BA35, lane7-BA15, lane8-BA02, lane9-BA36, lane10-BA44, lane11-BA47, lane12-BA14, lane13-BA41, lane14-BA18

Fig. 6 PCR amplification of the Bt isolates of Assam soil using *cry3* primer showed amplicon of 700 bp specific for *cry3* gene in the Assam isolates. M-DNA ladder 1 kb, B-blank, N-negative, lane1-BA07, lane2-BA26, lane3-BA12, lane4-BA02, lane5-BA09, lane6-BA10, lane7-BA03, lane8-BA04, lane9-BA11, lane10-BA14, lane11-BA50, Lane12-BA15, lane13-BA16, lane14-BA37

Cry protein expression in the isolates

The total proteins were extracted from various isolates after 72 h of incubation at 37 °C and separated in a 15% SDS PAGE gel. The SDS PAGE gel and the western blot analyses revealed that eight isolates (BA02, BA03, BA06, BA12, BA15, BA20, BA21, BA30) having *cry2* gene based on PCR assays expressed Cry2Aa-like proteins of molecular weight ranged in between 45 and 70 kDa (Fig. 7a, b). We have also extracted Cry1 protein from the PCR-positive isolates (BA02, BA03, BA04, BA05, BA08, BA12, BA14, BA15, BA16, BA20, BA21, BA24, BA26, BA29, BA30, BA31 and BA47) and found that after 72 h of incubation at 37 °C, five isolates (BA04, BA14, BA24, BA29, BA31) expressed a 130 kDa Cry1 protein (Fig. 7c).

PCR amplification to identify novel Bt gene(s)

To amplify the new type of *cry* genes present from the isolates, universal primer was synthesized as described by Noguera and Ibarra (2010). The primers were designed from the conserved blocks of *cry* genes. The PCR result revealed amplification of products in all the isolates, however, isolates showing amplification of a PCR product

Table 2 Combination of *cry* genes of Bt isolates from Assam soil

Combination of <i>cry</i> genes	Isolates
<i>cry1</i> + <i>cry2</i>	BA20, BA21
<i>cry1</i> + <i>cry2</i> + <i>cry3</i>	BA03, BA12, BA08
<i>cry1</i> + <i>cry2</i> + <i>cry9</i>	BA02, BA30, BA31
<i>cry1</i> + <i>cry2</i> + <i>cry9</i> + Nem-Gral	BA15

of 1000 bp were selected (Fig. 8) for elution for gel and sequencing.

Sequencing of Bt genes from the isolates and bioinformatics analyses

The PCR amplicons of 1000 bp were purified from the gel, cloned into a pGEMT-Easy vector and sequenced using the M13 primers. The sequences were analyzed by NCBI BLASTn and two isolates BA04 and BA08 showed sequence similarity of 82 and 85%, respectively, with the *cryII* gene. When these sequences were analyzed by the BLASTx, the similarity percentage in the protein sequence of CryII was 74 and 73% for the isolate BA04 and BA08, respectively. The variation in the amino acid sequences of sample BA04 was found in the N-terminal region with non-specific hits (E value = $6.13e-24$) with delta endotoxin of Bt strain (Pfam00555), while the conserved regions with specific hits (E value = $2.35e-35$) with delta endotoxin of different Bt strain (cd04085). In the case of BA04, we found variation in the C-terminal domain, which is comprised of Domain-III of Bt delta endotoxin. The amino acid sequences of the sample BA08 showed variation in the N-terminal ends with non-specific hit, (E value = $6.83e-08$) with delta endotoxin of Bt strain (pfam03945), while the C-terminal ends showed the specific hit (E value = $1.42e-4$) with delta endotoxin of Bt strain (cd04085).

Table 1 Summary of *Cry* gene isolates of Assam soil

Gene	PCR (+) ve	Percent (%)	Isolates
<i>cry1</i>	17	40.47	BA02, BA03, BA04, BA05, BA08, BA12, BA14, BA15, BA16, BA20, BA21, BA24, BA26, BA29, BA30, BA31, BA47
<i>cry2</i>	21	50.00	BA01, BA02, BA03, BA06, BA08, BA09, BA12, BA15, BA18, BA19, BA20, BA21, BA25, BA30, BA31, BA33, BA34, BA35, BA36, BA48, BA50
<i>cry3</i>	15	35.71	BA02, BA03, BA04, BA05, BA08, BA12, BA13, BA14, BA16, BA18, BA19, BA22, BA24, BA38, BA47
<i>cry4</i>	08	19.04	BA01, BA06, BA21, BA26, BA36, BA38, BA44, BA54
<i>cry9</i>	18	42.85	BA02, BA04, BA11, BA14, BA15, BA17, BA19, BA20, BA24, BA25, BA29, BA30, BA31, BA33, BA46, BA50, BA52, BA56
Nem-gral	05	11.90	BA15, BA26, BA44, BA46, BA53

Fig. 7 Expression of crystal protein in the Bt isolates of Assam soil as observed by SDS-PAGE gel and western blot analyses. **a** Electrophoretic analysis of crude extracts of *B. thuringiensis* isolates on a coomassie blue-stained SDS-polyacrylamide gel. The Cry2 proteins ranging from 45 to 65 kDa were observed on an SDS PAGE gel. M-protein ladder, Lane1-BA02, Lane2-BA04, lane3-BA03, lane4-BA08, lane5-BA12, lane6-BA15, lane7-BA20, lane8-BA21, lane9-BA30. **b** Western blot analyses for expression of Cry2 proteins ranged from 65 to 45 kDa in few Assam isolates and confirmed expression of Cry2 gene. Lane1-BA02, Lane2-BA04, lane3-BA03, lane4-BA08, lane5-BA12, lane6-BA15, lane7-BA20, lane8-BA21, lane9-BA30. **c** SDS PAGE analysis of Cry1 PCR-positive isolates showing 130 kDa protein confirmed the expression of Cry1 gene. Lane1-BA04, Lane2-BA14, lane3-BA24, lane4-BA26, lane5-BA29, lane6-BA31

Discussion

We have combined morphological, biochemical and molecular techniques to characterize local Bt isolates of Assam soil. As the *B. cereus* and *B. thuringiensis* are closely related species and Gram staining was not suitable for distinguishing them, therefore, isolates were cultured on MYP Agar Base medium. The MYP agar (Mossel et al. 1967) medium was used for the identification of clinically important pathogen *B. cereus*, hence the medium

Fig. 8 PCR amplified few selected isolates using *universal* primer to identify novel Bt gene(s) from Assam soil. The presence of a 1000 bp amplicon showed amplification of cry gene suitable for sequencing to identify novel Bt gene present within the Assam isolates. M-DNA ladder, B-Blank, N-Negative, lane1-BA05, lane2-BA02, lane3-BA19, lane4-BA07, lane5-BA06, lane6-BA-03, lane7-BA02, lane8-BA09, lane9-BA10, lane10-BA11, lane11-BA14, lane12-BA15, lane13-BA19, lane14-BA12

was suitable to distinguish them. This medium contains D-mannitol and phenol red (pH indicator), when the fermentation of mannitol leads to the formation of acid by-products it lowers the pH of the medium. The *B. cereus* lack the ability to ferment D-mannitol (Mossel et al. 1967) due to which the pH of the medium increases and phenol red present in the medium turns into pinkish red, while the *B. thuringiensis* can ferment the D-mannitol due to which some organic acids were released and the pH of the medium lowers down. At low pH, the color of phenol red turns to yellow, hence the *Bt* colonies appeared as yellowish colony in the present investigation. The polymyxin B sulfate inhibits the growth of Gram-negative bacteria and the fresh egg yolk was added to check the lecithinase activity and found appropriate to differentiate them in culture (Mossel et al. 1967).

The ERIC-PCR is the Enterobacterial Repetitive Intergenic Consensus sequences polymerase chain reactions. The ERIC sequences are repetitive imperfect palindromes existing in multiple copies in the genome of the bacteria and found to be highly conserved. These sequences were used for differentiating the closely related species. Bacteria are one of the most versatile organisms on earth as they can grow even in extreme climatic conditions and these ERIC sequences may play an important role in the bacterial genome organization and the size of these ERIC sequences ranged from (Lupski and Weinstock 1992; Tobes and Ramos 2005). Previous report support that these ERIC elements were also present in *B. thuringiensis* genome (Shangkuan et al. 2001; Lima et al. 2002) and can be used as a powerful tool to differentiate the bacterial species. We also found this method as simple and fast for the rapid characterization of the *Bt* isolates. In the current study, available primers of ERIC sequence that amplifies a 100 bp amplicon in the case of *B. thuringiensis* isolates were utilized. Out of 45 isolates, 42 showed the expected amplicon

and these isolates were considered as *Bt* isolates of the Assam soil. Li et al. (2011) reported successful characterization of *B. thuringiensis* using ERIC-PCR.

Bacillus thuringiensis has a unique characteristic of production of crystal proteins during the stationary phase of the life cycle. These crystals may comprise more than one Cry toxin that shows toxicity against numbers of insect orders including nematodes, mites, and protozoa (Schnepf et al. 1998; Feitelson 1993; Estruch et al. 1996). We found presence of both crystal endospore forming and acryalliferous *Bt* isolates from Assam soil. There are reports on the presence of acryalliferous *B. thuringiensis*, which lost their crystal protein encoding plasmid during the evolution (Izabela et al. 2008; Tyrell et al. 1981). A non-crystalliferous *B. thuringiensis* sub sp. *israelensis* was also found to form inclusion bodies that are effective against the mosquito larvae (Dai et al. 1994).

We found that the majority (50%) of isolates of Assam has *cry2* gene, followed by *Cry9* (42.85%) and *Cry1* (40.47%) genes. These genes are effective against Lepidopteran pests which are the most common insect of agricultural crops. A similar observation was reported in China (Zhu et al. 2009; Patel et al. 2013) and other parts of India (Letowski et al. 2005). The presence of *cry3*, *cry4* and *Nem-Gral* genes in these isolates was 35.71, 19.04, and 11.90%, respectively. The isolates with coleopteran-specific *cry* gene (*cry3*), and Nematode-genotype (*Nem-Gral*) were also observed. We also found some isolates having more than one gene as reported by pervious workers (Letowski et al. 2005).

The separation of total protein from *Bt* isolates in 12% SDS-PAGE yields protein bands of size ranging from 40 to 130 kDa. In the present study, a similar banding pattern was observed. The size of the *Bt* protoxins ranged from 130 to 140 kDa (Bravo et al. 2007) and becomes active upon the proteolytic cleavage by the insect midgut proteases which releases a 66 kDa of active toxins. This active toxin binds with primary membrane receptor and further oligomerized for disruption of insect midgut. Interestingly, the *Cry2Aa* and *Cry2Ac* were encoded by two operons *Orf1* and *Orf2* (Sasaki et al. 1997; Takashi Yamamoto 2001). The *Orf2* was found to be responsible for crystal formation (Crickmore and Ellar 1992) and *Orf2* produces a 29 kDa protein (15 amino acid residue) of *cry2Aa* toxin (Widner and Whiteley 1989). Therefore, we found the presence of a 66 kDa *Cry2* protein in the isolates. On western blot, we also found lower molecular weight bands which could be cleavage of the protein by proteases or formation of truncated protein during isolation. Sasaki and his co-workers in (1997) also observed 45 kDa protein bands while characterizing the *cry2A* toxins by western blots and concluded that this low molecular weight may be proteolytically cleaved *Cry2* proteins. Espinasse et al.

(2002) reported that when *Cry* proteins were separated on an SDS-PAGE gel, *Cry15* proteins ranged from 40 to 45 kDa, naturally truncated *Cry2* proteins was 66 kDa, *Cry3A* protein ranged from 70 to 73 kDa effective and *Cry1* protoxin was 130–140 kDa in molecular size. They also reported that many isolates contain an unusual crystal protein profile.

In conclusion, for the first time, we have identified and characterized *Bt* isolates from the virgin Assam soil. Moreover, we have been able to identify two novel *Bt* genes from the isolates. A detail study of these novel genes, including isolation of the full length version of these novel genes and efficacy of these genes against insect pests would provide useful information for those working on bio-insecticides using *Bt* proteins and transgenic crop development.

Acknowledgements We are grateful to DBT-AAU Centre for providing the necessary facility to pursue this work. MR acknowledges the Department of Biotechnology, Assam Agricultural University, Jorhat for support to work on this topic. We would also like to thank NFBSFARA, ICAR, National fund for support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

- Alper M, Güneş H, Col B, Tunca H (2016) Bioactivities of cry gene positive *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae) strains on *Ephestia kuehniella* Zeller, 1879 and *Plodia interpunctella* (Hübner 1813) (Lepidoptera: Pyralidae). *Türk Entomol Derg.* 40:365–375
- Andrup L, Barfod KK, Jensen GB, Smidt L (2008) Detection of large plasmids from the *Bacillus cereus* group. *Plasmid* 59(2):139–143
- Apaydin O, Yenidunya AF, Harsa S, Gunes H (2005) Isolation and characterization of *Bacillus thuringiensis* strains from different grain habitats in Turkey. *World J Microbiol Biotechnol* 21:285–292
- Beard CE, Ranasinghe C, Akhurst RJ (2001) Screening for novel *cry* genes by hybridization. *Lett Appl Microbiol* 33:241–245
- Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortíz A, Ortíz M, Lina L, Villalobos FJ, Peña G, Nuñez-Valdez ME, Soberón M, Quintero R (1998) Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Appl Environ Microbiol* 64:4965–4972
- Bravo A, Gill SS, Soberon M (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* 49:423–435
- Bravo A, Gómez I, Porta H, García-Gómez BI, Rodríguez-Almazan C, Pardo L, Soberón M (2013) Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity. *Microb Biotechnol* 6:17–26
- Cerón J, Ortíz A, Quintero R, Güereca L, Bravo A (1995) Specific PCR primers directed to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. *Appl Environ Microbiol* 61:3826–3831
- Crickmore N, Ellar DJ (1992) Involvement of a possible chaperonin in the efficient expression of a cloned *CryIIA* δ -endotoxin gene in *Bacillus thuringiensis*. *Mol Microbiol* 6:1533–1537

- Crickmore N, Zeigler D, Feitelson J, Schnepf E, Van-Rie J, Lereclus D, Baum J, Dean DH (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol* 62:807–813
- Dai JY, Wang B, Luo XX, Yu L, Zhang XM, Yu ZN (1994) Studies on 410 strains of *Bacillus thuringiensis* isolated from soil. *J Huanzhong Agric Univ* 2:144–152
- Espinasse S, Gohar M, Chaufaux J, Buisson C, Perchat S, Sanchis V (2002) Correspondence of high levels of beta-exotoxin I and the presence of Cry1B in *Bacillus thuringiensis*. *Appl Environ Microbiol* 68(9):4182–4186
- Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA, Koziel MG (1996) Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proc Natl Acad Sci* 93:5389–5394
- Feitelson JS (1993) The *Bacillus thuringiensis* family tree. In: Kim L (ed) *Advanced engineered pesticides*. Marcel Dekker, Inc., New York, pp 63–71
- Guo S, Liu M, Peng D, Ji S, Wang P, Yu Z, Sun M (2008) New strategy for isolating novel nematocidal crystal protein genes from *Bacillus thuringiensis* strain YBT-1518. *Appl Environ Microbiol* 74:6997–7001. doi:10.1128/AEM.01346-08
- Izabela S, Dennis KB, Brian AF (2008) Novel isolate of *Bacillus thuringiensis* subsp. *thuringiensis* that produces a quasi-cuboidal crystal of Cry1Ab21 toxic to larvae of *Trichoplusia*. *Appl Environ Microbiol* 74(4):923–930
- Kumar PA, Malik VS, Sharma RP (1996) Insecticidal proteins of *Bacillus thuringiensis*. *Adv Appl Microbiol* 42:1–43
- Letowski J, Bravo A, Brousseau R, Masson L (2005) Assessment of *cryI* gene contents of *B. thuringiensis* strains by use of DNA microassays. *Appl Environ Microbiol* 71(9):5391–5398
- Li H, Liu D, Gao J (2011) Differentiation between *Bacillus thuringiensis* and *Bacillus cereus* by 16S rDNA-PCR and ERIC-PCR. *J Northeast Agric Univ* 18(3):12–15
- Lima ASG, Guidelli AM, Abreu IL, Lemos MVF (2002) Identification of new isolates of *Bacillus thuringiensis* using rep-PCR products and -endotoxin electron microscopy. *Genet Mol Biol* 25:225–229
- Lupski JR, Weinstock GM (1992) Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J Bacteriol* 174:4525–4529
- Mossel DAA, Koopman MJ, Jongerius E (1967) Enumeration of *Bacillus cereus* in foods. *Appl Microbiol* 15:650–653
- Noguera PA, Ibarra JE (2010) Detection of new *cry* genes of *Bacillus thuringiensis* by use of a novel PCR primer system. *Appl Environ Microbiol* 76(18):6150–6155
- Patel KD, Chudasama CJ, Ingle SS (2013) Distribution and diversity analysis of *Bacillus thuringiensis cry* genes in different soil types and geographical regions of India. *J Invertebr Pathol* 112(2):116–121
- Pedro AN, Ibarra JE (2010) Detection of new *cry* genes of *Bacillus thuringiensis* by use of a novel PCR primer system. *Appl Environ Microbiol* 76:6150–6155. doi:10.1128/AEM.00797-10
- Porcar M, Juarez-Perez V (2003) PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. *FEMS Microbiol Rev* 26:419–432
- Rebecca LH, Zothansanga Singh BP, Gurusubramanian G, Senthil NK (2011) DNA finger printing of *Bacillus thuringiensis* based on repetitive DNA sequences using ERIC-PCR. *Sci Vis* 11(3):147–154
- Roh JY, Choi JY, Li MS, Jin BR, Je YH (2007) *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. *J Microbiol Biotechnol* 17(4):547–559
- Sasaki J, Asano S, Hashimoto N, Lay BW, Hastowo S, Bando H, Iizuka T (1997) Characterization of a *cry2A* gene cloned from an isolate of *Bacillus thuringiensis* serovar sotto. *Curr Microbiol* 35:1–8
- Sauka DH, Cozzi JG, Benintende GB (2005) Screening of *cry2* genes in *Bacillus thuringiensis* isolates from Argentina. *Antonie Van Leeuwenhoek* 88:163–165
- Schnepf E, Crickmore N, Rie VJ, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* 62:775–806
- Shangkuan YH, Chang YH, Yang JF, Lin HC, Shaio MF (2001) Molecular characterization of *Bacillus anthracis* using multiplex PCR, ERIC-PCR and RAPD. *Lett Appl Microbiol* 23:139–145
- Tabashnik BE (2015) ABCs of insect resistance to Bt. *PLoS Genet* 11(11):e1005646. doi:10.1371/journal.pgen.1005646
- Tobes R, Ramos JL (2005) REP code: defining bacterial identity in extragenic space. *Environ Microbiol* 7:225–228
- Travers RS, Martin PA, Reinchlderfer CF (1987) Selective process for efficiency isolates of soil *Bacillus* spp. *Appl Environ Microbiol* 53:1263–1266
- Tyrell DJ, Bulla LA, Andrews RE, Kramer KS, Davidson LI, Nardin P (1981) Comparative biochemistry of entomocidal parasporal crystals of selected strains of *Bacillus thuringiensis*. *J Bacteriol* 145:1052–1062
- Widner WR, Whiteley HR (1989) Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *Kurstaki* possess different host range specificities. *J Bacteriol* 171:965–974
- Yamamoto T (2001) One hundred years of *Bacillus thuringiensis* research and development: discovery to transgenic crops. *J Insect Biotechnol Sericol* 70:1–23
- Zhu J, Tan FR, Tang J, Li YY, Zheng AP, Li P (2009) Characterization of insecticidal crystal protein *cry* gene of *Bacillus thuringiensis* from soil of Sichuan Basin, China and cloning of novel haplotypes *cry* gene. *Ann Microbiol* 59(1):1–8