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# Cloning of *cry2Aa* gene from an indigenous isolate of *Bacillus thuringiensis*

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**Abstract** : By using the *cry2Aa* operon specific primers, DNA fragments of about 4.0 kb were amplified at 55°C annealing temperature without any non-specific amplification from a new Bt strain 47-8 and cloned in pGEM®-T(3.0 kb) vector. Double digestion with *Sal*I and *Sph*I enzymes showed fragments of expected size (3, 4kb) in recombinant clones and one of the positive clones is designated as pTN2A (7.0 kb). Nucleotide sequencing of Bt DNA fragment cloned in pTN2A was carried out with M13F and M13R primers. Totally 983 bases have been sequenced in the insert DNA of pTN2A. Comparison of sequence data obtained from the cloned DNA fragment of Bt strain 47-8 showed 97 to 98 per cent homology to the holotype *cry2Aa operon*. Hence, cloning of *cry2Aa* gene of Bt strain 47-8 is confirmed.
(*Key Words*: *Bacillus thuringiensis*, *Cry2Aa*, Cry2Aa)

*Bacillus thuringiensis* is a spore forming Gram positive bacterium, which produces insecticidal crystal proteins during the sporulation stage of its life cycle. The crystal proteins are highly toxic to insects and nematodes. A given Bt strain may harbor more than one crystal type, and some crystals are comprised of several proteins that have distinct insecticidal activities. Therefore the crystals produced by different Bt strains vary in their levels and spectra of toxicity. The advancement in genetic engineering and biotechnology has led to the cloning of Bt crystal protein (*cry*) gene for the first time in 1981 (Schnepf and Whiteley, (1981). Since then, more than 100 *cry* genes have been successfully cloned from different strains and added to the growing list. Majority of the currently used commercial transgenic plants express one of the two highly homologous *cryI (Ab* or *Ac*) genes to control lepidopteran pests. The reliance on a single kind of Bt protein over the years can lead to the development of resistance in insects (Tabashnik *et al*., 1997).

A highly suitable strategy for delaying Bt resistance development in insects would be expression of multiple toxins (gene pyramiding) or different toxins (gene rotation). In this context *cry2A* genes are potential candidates for resistance management strategies, owing to unique mode of action of the Cry2 toxins compared to other Bt toxins (Lee *et al*., 1997). The *cry2Aa* genes encode approximately 65 KDa proteins, which form cuboidal inclusion (Widner and Whiteley, 1989; Yamamoto and McLauzhlin, 1981). Cry2Aa is toxic to both lepidopterans and dipterans whereas Cry2Ab and Cry2Ac are toxic only to lepidopteran species. The *cry2Aa* and *cry2Ac* genes each occur as the third gene of a three-gene association; the middle gene, or open reading frame (orf)2, in this operon produces a polypeptide which is required for the efficient expression of the *cry2* genes (Aronson, 1993). Neither of the orf1 or orf2 gene products were insecticidal, but the polypeptides may be involved in the assembly of the protein inclusion (Crickmore and Ellar, 1992).

The Cry2Aa is less toxic against lepidopteran larvae when compared to Cry1AC (Chakrabarti *et a* 1998). Sasaki *et al*., (1997) reported significa difference in the level of toxicity among two Cry2A proteins. Variations of a single amino acid ca significantly influence the level of toxicity in Cry protein (Udayasuriyan *et al*., 1994; Rajamohan *et al*., 1996). In this connection, it is an imperative need to search for more potent Cry2Aa proteins from new isolates of Bt and hence the present study was undertaken with the objective to clone the *cry2Aa* gene from a new indigenous Bt strain 47-8.

## Materials and Methods

The chemicals used in this study are of analytical grade and purchased from Hi-media Laboratory Pvt Ltd., Mumbai, India. PCR chemicals, *Sal* enzyme, protein and DNA molecular weight markers were purchased from Bangalore Genei, Bangalore, India. pGEM®-T Vector system was purchased from Promega, USA. Expand ™ High Fidelity PCR system and *Sph* I enzyme were purchased from Boehringer Mannheim, Germany.

### *Bacterial strains*

*B. thuringiensis* strain 47-8 used in this study was obtained from the Bt-Biotechnology laboratory, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, India. The standard Bt strain HD-1 was originally obtained from the *Bacillus* Genetic Stock Centre, Ohio State University, Ohio, Columbus, USA. *E. coli* strain DH5α was purchased from Bangalore Genei, Bangalore, India.

*Analysis of crystal protein profile of Bt strains*

The Bt strains were maintained in T3 agar slants (Martin and Travers, 1989) at 4°C. Bacterial spore suspension was spread on T3 agar plates and grown till complete sporulation. The spore-crystal mixture was scrapped off the plates with ice-cold Tris-EDTA buffer (Tris 10 mM, EDTA 1mM, pH 8.0 with 1 mM PMSF), washed once with ice-cold 0.5 M NaCl at 10,000 rpm for 10 min. followed by two washes with Tris-EDTA buffer at the same speed and time. Finally, the spore-crystal pellet was suspended in sterile distilled water with 1 mM PMSF. The spore-protein mixture was subjected to SDS-PAGE, using a separating gel of 7.5% w/v acrylamide (Laemmli, 1970).

*Isolation and analysis of DNA*

The genomic DNA of Bt and plasmid DNA of *E. coli* were isolated as per the procedures of Kalman *et al.* (1993) & Brinboim and Doly (1979), respectively. The DNA samples were electrophoresed on 0.8 - 1.0% agarose gel, stained with 0.5 $\mu gml^{-1}$ ethidium bromide (Sambrook *et al.*, 1989). $\lambda$/*Hind* III digest was used as a standard marker.

*Amplification and cloning of cry2Aa operon from Bt strain 47-8*

Based on the holotype sequence of *cry2Aa* gene operon, primers were designed to amplify the whole *cry2Aa* gene operon from the Bt strain 47-8. Primer sequences matching to the upstream (250 bases prior to start codon of orf1) and down stream (180 bases away from stop codon) of *cry2Aa* operon were selected. Forward (F) primer: 5'CAAGAAATATGATGTT GATTCTTAGAGC. Reverse (R) primer: 5'AGCTTT AGGTTAACTTGAA ATGATTTC, Polymerase Chain Reaction (PCR) was carried out with Expand™ High Fidelity PCR system (Boehringer Mannheim, German) in 50 µl reaction volume. Each 50 ul reaction mixture contained 100 ng of genomic DNA of *Bt* strain 47-8, primers (F and R) at a final concentration of 1 µM, each dNTP at a final concentration of 200 µM and 2.5 U of High Fidelity enzyme mix (*Taq* DNA polymerase) in 1x Expand HF buffer (with $MgCl_2$). Amplification was accomplished with the DNA thermal cycler (Perkin-Elmer Cetus) by using the step-cycle program (Table-1). Ligation and restriction digestion was carried out as per manufacturer's instructions. Preparation of competent cells of *E. coli* and transformation of *E. coli* were performed as per the standard procedures (Sambrook *et al.*, 1989).

*Nucleotide sequencing of pTN2A*

Nucleotide sequencing of pTN2Aa was obtained by automated sequencing (Bangalaore Genei, Bangalore). Automated DNA sequencing used Sanger method (Sanger and Coulson, 1975) of sequencing with fluorescent labeled DNA fragments. Sequence data were generated with two standard primers (M13F, M13R). The homology analysis was then carried out using Blast N2.0 program.

**Results and Discussion**

*Crystal protein profile of Bt strains HD-1 and 47-8*

To study the cystal protein profile, spore-crystal mixtures prepared from Bt strains 47-8 and HD-1 were subjected to SDS-PAGE. The HD-1 strain showed two bands in Cry1 region (130-140 kDa) and a single band in Cry2 (65-70kDa) region. The native strain 47-8 also showed a crystal protein profile, which is similar to that of the standard strain HD-1 (Figure-1). The Cry proteins get separated into different polypetides when subjected to denaturing agents like SDS. These polypeptides are detected in SDS-PAGE. The standard Bt strain HD1 is known to produce four crystal proteins namely, Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa. The molecular weights of Cry1Aa and Cry1Ac are equal, whereas the molecular weight of Cry1Ab is slightly less, all of them range between 130-140kDa. Molecular weight of Cry2Aa protein is reported to be about 65kDa. Therefore, three bands could be observed for standard strain HD1, as shown in this study. The indigenous strain 47-8 also showed a similar electrophoretic pattern, two bands in Cry1 region and a single band in Cry2 region. In Bt strain 47-8, the single band in Cry2 region matched with the Cry2Aa band of HD1. Therefore, the Cry2 protein of the new Bt strain 47-8 might be similar to that of Cry2Aa.

*Amplification of DNA fragment containing cry2Aa from Bt strain 47-8*

The indigenous isolate 47-8 was grown in 2xYT medium to an optical density of 1.0 at 600 nm. From this genomic DNA was isolated and tested by agarose gel electrophoresis. The electrophoresis showed intact chromosomal DNA. The specific primers designed to amplify Bt DNA fragment containing *cry2Aa* gene operon is expected to yield a product of 3.95 kb. One hundred ng of Bt genomic DNA was used as template DNA in 50µl reaction mixture. The PCR was performed as documented in materials and methods. Agarose gel electrophoresis of PCR products showed predicted size band of approximately 4 kb without any non-specific amplification (Figure-2A). Since the target DNA is the present investigation was about 4.0 kb, a longer extension time (4 min) was used in the PCR cycle, which was divided into two segments. Amplification at 55°C as annealing temperature resulted in the desired amplification of a single band of 4.0 kb.

*Cloning of DNA fragment amplified from Bt strain 47-8*

The amplified DNA fragment was initially resolved on an agarose gel. The selected band was then sliced from the agarose gel and treated with phenol followed by ethanol precipitation. The agarose gel electrophoresis of the eluted PCR product showed the integrity of the DNA. The ligation of amplified DNA (PCR product, 4 kb) with pGEM®-T vector (3 kb) was carried out at 8:1 molar concentrations as per manufacturer's recommendations. Ligation was carried out in 10 µl quantity. The ligated DNA was used to transform the competent cells (100 µl) of *E. coli* strain DH5α. The transformed cells were plated on LB agar plate containing ampicillin, X-Gal, IPTG and incubated at 37°C overnight. The recombinant clones (white colonies) were randomly selected and grown in LB broth containing ampicillin. The plasmid DNA were isolated from the recombinant clones and subjected to restriction analysis. The plasmid DNA isolated from the recombinant clones was subjected to double digestion with *Sal*I and *Sph*I. Agarose gel electrophoresis of the double digested recombinant plasmids showed presence of insert DNA of ~4.0 kb and the vector DNA of ~3 kb (Figure-2B). The recombinant pGEM®-T plasmid is named as pTN2A and used for further studies.

In the present study the *cry2Aa* operon of Bt strain 47-8 was amplified by PCR and the pGEM®T was used to clone the PCR product straightaway. The recognition sequences of *Sal*I and *Sph*I enzymes are present on either side of cloning site of the pGEM®-T vector used for cloning. As per already reported *cry2Aa* operon sequence, the amplified Bt DNA should not have recognition site for these enzymes. Hence, the digestion of the recombinant clones with *Sal*I and *Sph*I enzyme separated the insert (4 kb) and vector (3 kb). The digestion of the positive clones with *Sal*I and *Sph*I yielded fragments of expected size, confirming the intactness of the cloned Bt DNA fragment, in the present study.

*Nucleotide sequence analysis*

To determine the nucleotide sequence of the cloned DNA fragment from Bt strain 47-8, nucleotide sequencing of pTN2A was carried out. Sequence data were generated with M13 forward primer and M13 reverse primer. Sequence obtained with M13F primer showed homology to 71 bases of pGEMZ5f(+) vector followed by 570 bases in the 5'region orf1 of *cry2A* operon. The original sequence data obtained with the M13R primer showed homology to 95 bases in the minus strand of pGEMZ5f(+) followed by 414 bases in the 3' region of minus strand of orf3 of *cry2Aa* operon. Homology of the pTN2A nucleotide sequence (obtained with the M13R primer) to other *cry2A* sequences is given in Table-2.

Till date four *cry2* type genes have been cloned and sequenced (*cry2Aa*, M31738; *cry2Ab*, M23724 *cry2Ac*, *X57252*; *cry2Ad*, AF200816). As per the rules for classifying the Cry proteins, deduced amino acid sequence of the four genes share less than 95 per cent homology and show considerable variation in insect specificities (Crickmore et al., 1998). Nucleotide sequencing results of pTN2A was fed in BlastN2.0 version of multiple sequence alignment programme to find homology with the non-redundant sequences entered in GenBank, EMBL and DDBJ databases. The sequence data (411 bp) of pTN2A generated by M13 primer showed higher (97%) similarity to the *cry2A* sequences than to other *cry2A* sequences, such as *cry2Ab, Ac, Ad* (78-89%). Therefore, the cloning of *cry2Aa* from Bt strain 47-8 in pGEM-T vector is confirmed. Moreover the DNA fragment cloned from Bt strain 47-8 encoded insecticidal proteins of expected size in an acrystalliferous strain of Bt (Lenin *et al.* 2001). Hence the *cry2Aa* cloned from Bt strain 47-8 may be used to transform crop plants and for managing Bt resistance development in insects.

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**Table 1.** PCR step cycle program with two segments

| | Activity | Temperature | Time | No. of cycles |
|---|---|---|---|---|
| | Initial Denaturing | 94°C | 2min | 1 |
| Segment I | | | | |
| | Denaturing | 94°C | 15 sec | 10 |
| | Annealing | 55°C | 30 sec | |
| | Extention | 68°C | 4min | |
| Segment II | | | | |
| | Denaturing | 94°C | 15 sec | 10 |
| | Annealing | 55°C | 30 sec | 20 |
| | Extention | 68°C | 4min | |
| | Final Extention | 72°C | 7min | 1 |

* 10 seconds increments were given for each cycle

**Table 2.** Homology of pTN2A nucleotide sequence to *cry 2A* genes

| S.No | Gene | pTN2A sequence | Identities | Per cent Homology |
|---|---|---|---|---|
| 1 | cry2Aa$_2$ | 96-509 | 402/414 | 97 |
| 2 | cry2Ab | 297-504 | 187/208 | 89 |
| 3 | cry2Ac | 238-504 | 225/267 | 84 |
| | | 151-229 | 62-79 | 78 |
| 4 | cry2Ad | 298-506 | 186/209 | 88 |
| | | 151-223 | 63/75 | 84 |

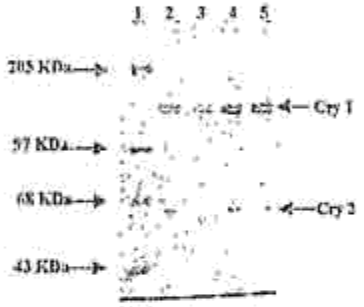



**Fig. I.** SDS-PAGE of crystal proteins of Bt strains HD-I and 47-8
Lane I: Marker, Lane 2 & 4: Strain 47-8: Lane 3 & 5: Strain HD-I

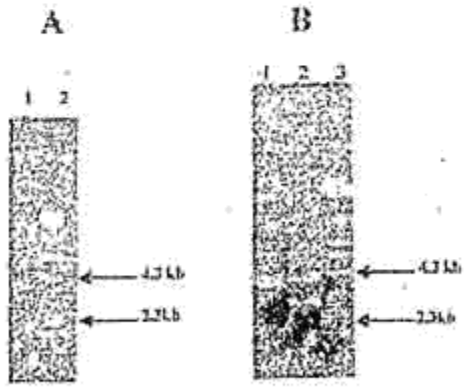
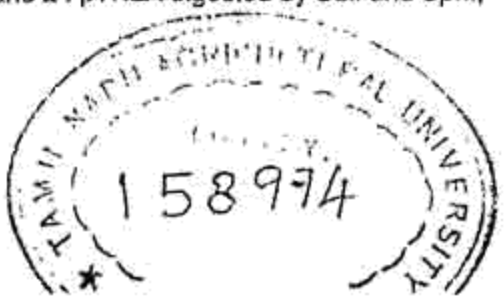



**Fig.2.** Agarose gel electophoresis of DNA

A : Lane I: Eluted DNA: Lane 2: Marker (λDNA/*Hind* III)
B : Lane I: Undigested p TN2A : Lane 2 : pTN2A digested by *Sal* and SphI;
Lane 3 : Marker (λ DNA/*Hind* III)



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