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Characterization of *cry* Genes from Native *Bacillus thuringiensis* (Bt) isolates

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ABSTRACT

In this study, cry genes were amplified by *cry4* gene primers from the isolates of Bt-23, Bt-133, Bt-190, and Bt-242 and sequences was carried out. Different computer algorithms were used to analyze the nucleotide sequences obtained from the isolates Bt-23, Bt-133, Bt-190 and Bt-242. Homology search of nucleotide sequences obtained from isolates Bt-23, Bt-133, Bt-190, and Bt-242 with showed homology with *cry4A*, *cry4D*, *cry4*BLB and other dipteran specific insecticidal genes, There were five open reading frames in Bt-23*cry4*, with the longest one located on +3 strand starting from base 279 to 683, having a length of 405 bases. The A+T and G+C base pair composition was 60.6 per cent and 39.4 per cent in Bt-23*cry4*, while Bt-133*cry4* A+T was 61 per cent and G+C was 38.8 per cent. Bt-190*cry4* was comparatively rich in A+T (64.3%) among all sequences with very low G+C (35.7%). In contrast, Bt-242cry4 was comparatively low in A+T content (57.2%) than the other gene sequences.

Key words: *Bacillus thuringiensis*, *cry*4 gene, nucleotide sequences, Open reading frame

Bacillus thuringiensis (Bt) is a gram positive, aerobic, endospore-forming bacterium. It is recognized by its parasporal body (known as crystal protein) encoded by cry genes possesses insecticidal properties. Insecticidal crystal proteins, endospores or beta-exotoxins dominate bioactivity of *Bacillus thuringiensis.* Bt strains have also been classified on the basis of toxicity towards target insects. Bt strains active against a range of invertebrates including Hymenoptera, Homoptera, Mallophaga, Orthoptera, nematodes and mites have been reported (Marroquin *et al*., 2000). Insect bioassays, however, being time-consuming and exhaustive, pose a limitation for rapid preliminary screening of large Bt collections. Similarly, biochemical testing and flagellar antigen typing, though useful for determination of subspecies do not directly reflect the specific *cry* genes present in a particular Bt strain. *cry* gene profiling could be used for classification of Bt strains (Wang *et al*., 2003).

Many *cry* genes have shown the presence of five conserved blocks of homology, which are of use in their identification. Earlier, *cry* genes were classified into four major classes: *cry*I (Lepidoptera specific), *cry*II (Lepidoptera and Diptera specific), *cry*III (Coleoptera specific) and *cry*IV (Diptera specific), on the basis of sequence homology and spectrum of insecticidal activity (Hofte and Whiteley, 1989; Feitelson *et al.*, 1992). New genes encoding a diverse set of proteins without a common insecticidal activity each received the name *cry*V, based on the next available Roman numeral (Shin *et al.*, 1995).

However, the nomenclature of Hofte and Whiteley (1989) failed to accommodate toxins that belonged to same class but with a different insecticidal spectrum. Hence, Crickmore *et al.* (1998) introduced a system of classification based on amino acid homology, where each protoxin acquired a name consisting of the *Cry* (or Cyt) and four hierarchical ranks consisting of Arabic numbers, capital letters, lower case letters and Arabic numbers (e.g. *Cry22Aa1*). Thus, proteins of less than 45 per cent homology differ in primary rank (*Cry1*, *Cry2* etc.), and 78 per cent and 95 per cent identity constitute the border for secondary (*Cry1A*, *Cry1B*) and tertiary ranks (*Cry1Aa*, Cry1Ba), respectively. Quaternary ranks are given to those proteins, which are more than 95 per cent similar in amino acid sequence (*Cry1Aa1* and

Cry1Aa2).So Attempt have been made to characterize dipteran specific *cry* genes from *B. thuringiensis* isolated from the Western Ghats of Kerala.

MATERIALS AND METHODS

Isolation of total DNA from *Bacillus thuringiensis*

The total DNA was isolated from *Bacillus thuringiensis* isolates following the protocol of Sambrook and Russel (2001). Agarose gel electrophoresis was performed based on the method described by Sambrook *et al*. (1989) to check the quality of DNA and also to separate the amplified products. The purity of DNA was further analysed by using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The absorbance of nucleic acid sample was measured at a wavelength of 260 nm and 280 nm. 260/280 ratio was used to assess the purity of nucleic acids.

PCR reaction

Amplification of *cry* gene from native *Bacillus thuringiensis*

The total DNA extracted from *B. thuringiensis* isolates was amplified by PCR using *cry*4 specific primers *viz.*, F.P. 5'GCATATGATGTAGCGAAACAAGCC3', R.P. 5'GCGTGACATACCCATTTCCAGGTCC3' (Bendov *et al.*, 1997). Polymerase chain reaction was carried out using *cry* gene specific primers in Eppendorf Master Cycler, Gradient (Eppendorf, Germany).

Thermal cycler programme

Steps followed in PCR, temperature profile and time duration of each step for amplification of *cry*4 are given in table 1.

Table 1. Temperature profile for amplification of *cry*4 gene by PCR

Sl. No	Step	Temperature	Time (min)
1	Initial denaturation	94°C	2.00
2	Denaturation	94°C	0.45
3	Annealing	53°C	1.00
4	Primer extension	72°C	1.00
5	Step 2 to 4	29 cycles	
6	Final extension	72°C	10.00

Sequencing of *cry*4 amplicons

*cry*4 amplicons obtained from the isolates Bt-23, Bt-133, Bt-190 and Bt-242 were sequenced. The eluted products of 800bp from the isolates Bt-23, 400bp in the isolates Bt-133, Bt-190 and Bt-242 were directly sequenced using the corresponding forward and reverse primers at Chromous Biotech PVT. LTD.

Theoretical analysis of sequence

Nucleic acid sequence analysis

The Blastn programme (http://www.ncbi.nm.nih.gov/blast/) was used to find out the homology of nucleotide sequences (Altschul *et al*., 1997). To find the open reading frame of the nucleotide sequence, the programme ORF finder of NCBI was used (www.ncbi.nlm.gov/gorf/gorf). Nucleotide composition of the given sequence was determined by nucleotide statistics (NASTATS) tool offered by Biology Workbench (http://seqtool.sdsc.edu/).

RESULTS AND DISCUSSION

Amplification of *cry*4 gene from native isolates of selected *B. thuringiensis* isolates

Amplification of *cry*4 was carried out by PCR in native isolates viz., Bt-23, produced two amplicons of 400 and 800bp. Three isolates namely Bt-133, Bt-242 and Bt-190 yielded a single amplicon of size 400bp.

Gel elution and Sequencing of *cry*4 amplicons

The *cry*4 amplicons of 400bp from isolates Bt-133, Bt-190, Bt-242 and 800bp amplicon from isolate Bt-23 were eluted from the gel the products showed good concentration on agarose gel suggesting that recovery of the PCR product was good (Fig 1.). Eluted product of 400bp from native isolates Bt-23, Bt-133, Bt-190 and Bt-242 were sequenced. Number of nucleotides and amino acids of each sequence are presented in Table 2.

Table 2. Details of amplicons from native *B. thuringiensis* isolates used for sequencing

Sl. No.	Sequence name	Nucleotide
1	Bt-23*cry*4	780
2	Bt-133*cry*4	356
3	Bt-190*cry*4	364
4	Bt- 242*cry*4	362

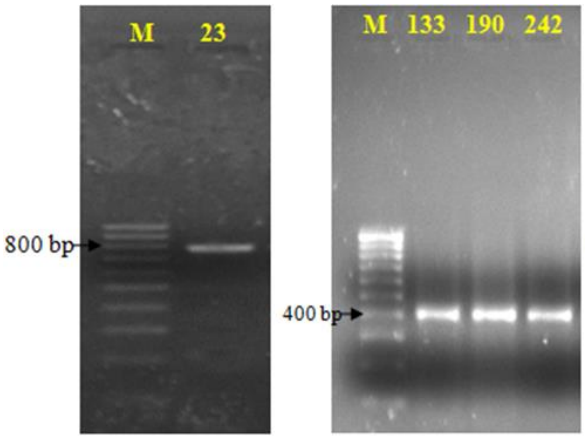

Fig 1. Eluted bands of *cry*4 gene amplicons

Theoretical sequence analysis

Different computer algorithms were used to analyze the nucleotide sequences obtained from the isolates Bt-23, Bt-133, Bt-190 and Bt-242.

Nucleotide Blast

Homology search of nucleotide sequences obtained from isolates Bt-23, Bt-133, Bt-190, and Bt-242 with other reported *cry* gene sequences was carried out. All the four sequences (Bt-23*cry*4, Bt-133*cry*4, Bt-190*cry*4 and Bt-242*cry*4 showed homology with *cry*4A, *cry*4D, *cry*4BLB and other dipteran specific insecticidal genes, truncated pesticidal crystal protein genes and *Bacillus thuringiensis* subsp. *israelensis* 130kDa pesticidal *cry* gene sequences in NCBI databank. However, Bt-23*cry*4 also showed homology with *B. cereus* genome.

Open reading frame

The sequences were translated in all six opening reading frames (http://www.ncbi.nlm.nih.gov.ORF finder). There were five open reading frames in Bt-23*cry*4, with the longest one located on +3 strand starting from base 279 to 683, having a length of 405 bases, second one on +2 strand starting from the base 581 to 781, with a length of 201 bases, the remaining ORFs were starting from 227 to 346, 2 to 118 and 110 to 211 with 120 bases, 117 bases, 102 bases length on -2, -2 and +2 strands respectively. Bt-133*cry*4 had two open reading frames, with the longer one on -3 strand with a length of 237 bases, starting from base 40 to 276 and the other on +3 strand with a length of 102 bases starting from base 93 to 194. Bt-190*cry*4 possessed two open reading frames with the longest one located on -1 strand with a length of 285bp, starting from base 2 to 286. The second one was encoded on the +1 strand, starting from base 103 to 216, with a length of 114bp. There were four ORFs in Bt-242*cry*4, with the largest one located on -3 strand, having a length of 288bp starting from base 1 to 288. The others were located on the +1, +3 and -1 strands, with a length of 186bp, 183bp and 180bp respectively (Table 3). Nucleotide composition of the above sequences was determined using Biology Work Bench (http://seqtool.sds.edu/). The A+T and G+C base pair composition was 60.6 per cent and 39.4 per cent in Bt-23*cry*4, while Bt-133*cry*4 A+T was 61 per cent and G+C was 38.8 per cent.

Table 3. Open reading frames of *cry* gene fragments from native isolates

Sl. No.	Gene	No. of ORFs	Location	Length (bp)	Reading frame
1	Bt-23*cry*4	5	279-683	405	+3
			581-781	201	+2
			227-346	120	-2
			2-118	117	-2
			110-211	102	+2
2	Bt-133*cry*4	2	40-276	237	-3
			93-194	102	+3
3	Bt-190*cry*4	2	2-286	285	-1
			103-216	114	+1
4	Bt-242*cry*4	4	1-288	288	-3
			1-186	186	+1
			180-361	183	+3
			51-230	180	-1

Table 4. Nucleotide statistics of *cry* gene fragments from native *B. thuringiensis* isolates

Sl. No.	Gene sequence	Nitrogen base percentage (%)					
		A	T	G	C	A and T	G and C
1	Bt-23*cry*4	27.0	33.6	20.1	19.3	60.6	39.4
2	Bt-133*cry*4	30.3	30.9	14.9	23.9	61.2	38.8
3	Bt-190*cry*4	32.1	32.1	13.2	22.5	64.3	35.7
4	Bt-242*cry*4	24.9	32.3	18.0	24.9	57.2	42.8

Bt-190*cry*4 was comparatively rich in A+T (64.3%) among all sequences with very low G+C (35.7%). In contrast, Bt-242*cry*4 was comparatively low in A+T content (57.2%) than the other gene sequences (Table 4). The Analysis of the sequence data of the four amplified products by Blastn revealed that they were partial *cry*4 genes. Blastn is a algorithm that finds region of local similarity between nucleotide sequences. The homology search revealed that all the four amplified sequences shared significant homology with *cry*4A, *cry*4D, *cry*4BLB, and *cry* gene sequence of *Bacillus thuringiensis* subsp. *israelensis*. Hundred per cent identity was obtained with that of *cry*4A gene sequence from *B. thuringiensis*, *cry*4BLB gene sequences from *B. thuringiensis* serovar *israelensis* delta endotoxin gene of *B. thuringiensis* and other insecticidal toxin *cry* genes reported in NCBI biological databases.

However the query coverage was low, ranging from 4 to 6 per cent for different sequences. This could be due to high amount of variability in the *cry*4 gene among different isolates. In the case of Bt-23 *cry*4, the sequence showed homology with *Bacillus cereus* genome also. This could probably be due to the genetic similarity between the two species, which has already been reported (Ash *et al*., 1991).

ORF analysis revealed that out of the five ORF in Bt-23*cry*4 sequence, three of them were located on the plus strands (+3, +2 and +2) and two were located on minus (-2, -2). The longest ORF was on +3 with a length of 405bp. In Bt-133*cry*4 sequence had only two reading frames. The longest one was reading frame -3 with length of 237bp. The longest ORF of Bt-190*cry*4 sequence was located on -1 with 285bp length and Bt-242*cry*4 sequence encoded the longest ORF on -3 reading frame with a length of 288bp. The analysis for discovering nitrogen base composition revealed that all the *cry*4 sequences were rich in A+T. Johnson *et al.* (1996) made similar observations in which the cloned sequences of *cry* genes were rich in A+T compared to C+G bases. Phylogenetic analysis of the sequences showed all four isolates shared a common ancestor. Bt-23*cry*4 and Bt-242*cry*4 were clustered together, indicating strong phylogenic similarity.

CONCLUSION

Toxic effect of crystal protein is highly dependent upon the solubility of the crystal protein and membrane receptor-binding sites. The solubility is again based upon the amino acid residues present in the protein. In this study, amino acid analysis revealed that the hydrophobic amino acid leucine was highest (18.3%), followed by hydrophilic amino acid serine with 9.05 per cent in Bt-23*cry*4. Bt-133*cry*4 possessed a high molar percentage of the polar amino acid threonine (12.61). Analysis of Bt-190*cry*4 and Bt-242*cry*4 revealed, those were rich in non-polar amino acids leucine (26.72%) and isoleucine (12.07%).

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