Pakistan J. Zool., vol. 45(3), pp. 779-791, 2013

Molecular Characterization of *cry4B* Gene from Local Isolates of *Bacillus thuringiensis* Encoding 70kDa Crystal Protein

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Abstract.- The toxic region (2.0 kb) of *cry4B* gene amplified from six different local isolates of *Bacillus thuringiensis* (DAB *Bt* 1-6) was cloned in pTZ57R/T. *E. coli* DH5α were transformed with this recombinant plasmid. The toxic region was restricted with *Eco*R1 and *Hin*dIII, and ligated in the expression vector pT7-7. *E. coli* BL21C were transformed with the recombinant DNA. The expression profile of recombinant organism containing *cry4B* gene was studied. The expression conditions were optimised with respect to IPTG concentration, time of induction and incubation temperature. It was found that the high level of expression occurred at 0.5mM of IPTG, at 37°C for 3 hours. The toxicity of genetically modified organisms and crude recombinant Cry4B proteins was determined against third instar larvae of mosquito *Anopheles stephensi*. The LC_{50} of *E. coli* transformed with *cry4B* gene isolated form six *Bt* isolates against 3rd instar larvae of *Anopheles stephensi* ranged between 175±3.34-288±3.02 µg/ml, whereas that of crude preparation of Cry4B ranged between 363±2.67-566±2.96 ng/ml. Of all the isolates DAB *Bt* 5 was found to be the most toxic isolate.

Keywords: *Bacillus thuringiensis, cry4B* gene, transformation, expression optimization, IPTG.

INTRODUCTION

The resistance developed by various insects against conventional insecticides has necessitated the search for bio-insecticides. The discovery of insecticidal toxins from *Bacillus thuringiensis* has been warmly welcomed by WHO as the most effective biological control agent after its successful *in vivo* trials on phytophagous and vector insects of Diptera, Lepidoptera and Coleoptera etc. (Ferré *et al*., 1991; Promdonkoy *et al.*, 2005).

Bacillus thuringiensis (*Bt*) is a Gram positive, rod shaped bacterium which has an important feature to encode insecticidal parasporal crystal proteins (ICPs), δ -endotoxins in the form of inclusion bodies during sporulation. These endotoxins are classified as crystal (Cry) proteins and cytolytic (Cyt) proteins on the basis of their mode of action and amino acid sequence specificity for epithelial receptor binding sites present inside the insect gut (Schnepf and Whiteley, 1981; Promdonkoy *et al.*, 2005). There are 30 known Cry and Cyt proteins according to the revised edition of nomenclature system by White *et al.* (1998) having various sub groups on the basis of the amino acid sequence homology percentage of their encoded protein products. *Bt* subsp. *israelensis* (*Bti*) known to be toxic to *Aedes* and *Anopheles* mosquito larvae (Abdullah *et al*., 2003) causing agents for malaria and filariasis, encodes different plasmid borne insecticidal toxins like Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa etc. (Abdullah *et al*., 2003). Cry4B is actually a 130-kDa protein secreted into the cytoplasm as protoxin, while the active toxins comprises just 65-70 kDa protein after cleavage of C-terminal and some portion of N- terminal protoxin inside insect gut by protease enzyme under alkaline pH conditions (Barusrux *et al*., 2003). This active toxin then binds to its specific receptors (lacking aminopeptidase activity) inside insect gut (Abdullah *et al*., 2006) and causes pore formation in epithelial cell surface, which ultimately leads to disturbance in electric potential across the membrane due to leakage of ions and finally death of insect larvae due to swelling of epithelial cells of midgut. This interaction of Cry toxins with cell surface receptors is mediated by various cellular lipids having unsaturated acyl lipids like phospholipids. This lipid-protein complex works like detergent to disrupt the integrity of membrane and finally leads to cell lysis (Thomas and Ellar, 1983).

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0030-9923/2013/0003-0779 $ 8.00/0

In the present study the toxic region of *cry4B* gene from the local *Bt* isolates was amplified by using specific primers. The amplified gene was cloned and sequence analysis was done. The toxic region was cloned in pT7-7 and expressed in *Escherichia coli* BL21C. The expression conditions were studied with respect to IPTG concentrations, time of induction and incubation temperature for high level expression of toxic region of *cry4B* gene. Positive controls of *cry4B* gene *viz.,* IPS78 and HD500 were analyzed in parallel.

MATERIALS AND METHODS

Bacterial strains and plasmids

Six most toxic local *Bt* isolates *(*DAB-*Bt* 1-6) which were previously characterized for the toxicity against larvae of mosquito *Anopheles stephensi* and the presence of *cry4* gene (Bukhari and Shakoori, 2010) were used to amplify the 2.0 kb toxic region of *cry4B* gene. The reference strains HD500 and IPS78 of *cry4* gene kindly provided by Prof. Dr. Daniel R. Zeigler, Director, Bacillus Genetic Stock Centre, Columbus, Ohio, USA, were used as positive controls. All the *Bt* strains were maintained on LB agar medium. *E. coli* DH5α competent cells and pTZ57R/T (Fermentas) were used for the initial cloning and sequencing of the *cry4B* toxic region. The pT7-7 and *E. coli* BL21C were used for the expression of toxic region of *cryB* gene. *E. coli* transformants positive for the toxic region of *cry4B* gene were selected on the medium containing X-gal (270 µg/ml), IPTG (120 µg/ml) and ampicillin (100 µg/ml).

Amplification of toxic region of cry4B gene

The toxic region of *cry4B* gene (2.0 Kb) was amplified from six local *B.t* isolates as well as from HD500 and IPS78 as positive control. Following steps were followed for amplification of *cry4B* gene.

DNA isolation

Two loops full of 24 hours *Bt* culture from LB agar plate were suspended in 100 µl of autoclaved distilled water by vortex mixing and centrifuged at 10,000rpm (3500 x g) for 5 min to get a pellet to which 200 µl of detergent solution (10 mM Tris- HCl pH 8.0, 3 mM $MgCl_2$, 200 µl Triton X100, 6.6 ml autoclaved distilled water) and 3 µl of proteinase K (20 mg/ml) was added and incubated at 45°C for 30 min in water bath. To the reaction mixture 400 µl of phenol: chloroform (1:1) was added and gently mixed by inverting the tube, centrifuged at 6500 rpm (5000 x g) for 10 min at room temperature. The supernatant was taken in another set of tubes to which 1 ml of chilled ethanol was added and kept on ice for 10 min. DNA was precipitated, which was pelleted at 6000rpm (5000 x g)**.** The pellet was later washed with 70% ethanol and dissolved in 100 µl of distilled water.

Primers

In order to clone the toxic region (2.0 kb) of *cry4B* gene, a forward and reverse primer was designed based on the nucleotide sequence of full length *cry4B* gene. Mutation was introduced at the 3' end of the gene by changing the nucleotide at the 5' end of the reverse primer to introduce the stop codon within full length *cry4B* gene. Following primers were used for the amplification of *cry4B* (2.0 kb) gene.

F 5` ATGAATTCAGGCTATCCATGGTTAGCG 3`
R 5` CTAAATACATTCCACAAGATTTGCGGCTTGA 3 `

Optimization for amplification

For optimization of PCR conditions different DNA concentrations (*viz.*, 50, 100, 150, 200, 250 and 300 ng), dNTPs (ranging from 100 µM to 300 µM, with the difference of 50 µM at each step), $MgCl_2$ (*viz.*, ranged between 1.5 mM and 3.5 mM), primers (*viz.*, 50pmoles, 100pmoles, 150pmoles and 200pmoles) and taq DNA polymerase (*viz.*, 0.5 unit, 1 unit, 1.5 units, 2.0 units and 2.5 units) were used to amplify the 2.0 kb *cry4B* gene in 50 µl PCR reaction mixture, using Fermentas PCR reagents #EP0402 at different annealing temperatures *viz.*, 48°C, 50°C, 52°C, 54°C, 55°C, 56°C and 58°C.

PCR reaction cycle

PCR was performed in thermal cycler (Applied Biosystem 2720) by initial denaturation at 94°C for 5min followed by 30 cycles each of denaturation at 94°C for 2 min, annealing at 50°C for 2 min, and extension at 72°C for 2 min, with final extension at 72°C for 5 min.

Cloning and sequencing of cry4B *gene*

PCR products of *cry4B* genes (2.0 kb) were visualized in 1% agarose gel and then isolated from the low melting point agarose gels following method described by Sambrook *et al.* (1989). Cloning of *cry4B* gene was done in pTZ57R (T/A cloning vector). The T/A cloning vector pTZ57R/T, already digested with *Eco*R1 and *Hin*dIII, was used to ligate PCR product using Fermentas DNA ligation kit (#K1214). For ligation pTZ57R/T and DNA were used in 3:1 ratio. Ligation reaction was done at 22°C for 18 h. Competent cells of *E. coli* DH5α (200 µl), prepared by the method described in Sambrook *et al.* (1989), were transformed with the recombinant plasmid.

The transformed cells (200 µl) were spread on dried LB agar plates containing X-gal (270 µg/ml), IPTG (120 µg/ml) and ampicillin (100 µg/ml). The plates were incubated at 37°C for 24 h to screen blue and white colonies. Cloning was confirmed by colony PCR and restriction analysis of recombinant plasmid DNA containing *cry4B* gene.

Cloned *cry4B* genes were sequenced to check the homology with already sequenced genes in the database, and to find out the mutations present in genes cloned from local *Bt* isolates. Automated DNA sequencing system (CEQ System, Ver.9.0.25) was used. This method is based on dye terminator chemistry, in which each of four dideoxynucleotides is labeled with a different fluourochrome (Prober *et al*., 1987; Lee *et al*., 1992). The raw sequencing data collected by the system was processed by associated software to get an electropherogram and finally the DNA sequence in text format.

Expression of toxic region cry4B *gene*

For the expression of toxic region *cry4B* gene the *E. coli* BL21C was used as host for plasmid propagation, whereas pT7-7 was used as an expression vector. For cloning in expression vector the amplified and purified *cry4B* (2.0 kb) gene was ligated using T4 ligase in the expression vector, pT7-7 cut with *Eco*R1 and *Hin*dIII. *E. coli* BL21C cells were transformed with pT7-7 containing *cry4B* gene (2.0kb) along with pT7-7 plasmid with out *cry4B* used as control. The transformed cells (200 µl) were spread on dried LB agar ampicillin (100 µg/ml) plate. The plates were then incubated at 37°C for 24 h to screen colonies for further processing. Screening of positive clones was done by colony PCR and digestion of recombinant plasmids with *Eco*R1 and *Hin*dIII.

The expressed protein was isolated by taking 1.5ml culture and spun at 13,000rpm (6500 x g) for 5 min. Pellets were washed once with autoclaved distilled water, and then sonicated in 140 µl of lysis buffer (1% SDS, 0.01% mercaptoethanol) before being boiled for 10 min. During boiling the tubes were again sonicated briefly for solubilization of crystals. All tubes were again spun for 10 min at 13,000rpm (6700 x g) and supernatants were removed very carefully in new tubes with out disturbing the pellet. The expressed protein was analyzed by SDS-PAGE, for which 15 µl sample was run on 12% SDS-PAGE (5% stacking and 12% resolving) along with the control sample.

Optimization of the expression of cry4B *gene*

For good expression of recombinant Cry4B protein, different conditions such as concentration of IPTG, incubation time, and incubation temperature were optimized. Different concentrations of IPTG ranging 0.25, 0.5, 1.0, and 1.5, 2.5, 3.0 mM were used for determination of optimum IPTG concentration during incubation at 37°C for 3 h.

Likewise seven different incubation temperatures (*viz*., 28°C, 30°C, 32°C, 35°C, 37°C, 40°C, and 45°C) were tried at IPTG concentration of 0.5 mM for 3 h for determination of temperature for optimum growth. The period of incubation was also varied (3, 5, 7 and 10 h) at IPTG concentration of 0.5 mM and incubation temperature at 37°C to determine the optimum incubation time for maximum expression. BL21C transformed with pT7-7with out insert was used as controls.

Extraction of total cellular proteins from Bt

Total cellular protein was isolated from the wild type strains of *Bt* after 48 h growth period at 37^{o}C to determine the concentration of various sporulation specific proteins along with other normal cellular proteins. After 48 h *Bt* sample from plate was firstly washed with 0.5N NaCl and then treated with lysis solution having SDS and

mercaptoethanol. Then heat shock was provided to these samples twice in boiling water bath (100°C for 5 min). After centrifugation, the supernatant having soluble cellular proteins was separated and the amount of protein was determined by Lowry *et al.* (1951) method. Finally the 50 µl of soluble protein aliquote was heated at 100°C for 5 min with 10 µl of 5X sample buffer and loaded to 12% Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) with an acrylamide and N,N-methylene bisacrylamide ratio 29:1 as described by Laemmli (1970).

Bioassays

Mosquitocidal activity of expressed Cry4B protein was determined by using *E. coli* transformed with *cry4B* gene and with total proteins expressed by *E. coli* transformed with toxic region of *cry4B* gene.

E. coli *transformed with* cry4B *gene*

Bioassays with transformed organisms were done to check the toxicity of cloned *cry4B* gene and their expressed proteins against third instar larvae of *Anopheles stephensi*. The cell pellet was washed thoroughly with buffer (10 mM Tris-HCl pH 7.2, 10 mM NaCl and 1% Tween-20), disrupted by sonication at 4°C, and cell-free extract prepared by centrifugation. Different concentrations of transformed organisms *viz.* 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml … and upto 500 µg/ml were used in triplicate. The transformed organisms was first grown in 250 ml LB broth supplemented with ampicillin (100 µg/ml) and IPTG (0.5 mM) and incubated at 37°C for 3 hours in shaking incubator. Cells were harvested by centrifugation at 10,000rpm (3500 x g) and washed twice with autoclaved distilled water. Pellet was then weighed and desired concentrations *viz.*, 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml ….and upto 5 00µg/ml were added in sterile cups containing 20 ml autoclaved distilled water. Twenty mosquito larvae (*Anopheles stephensi*) were kept in this water at 25°C for 24 hours to check the toxicity of the organism, expressing Cry4B protein. The number of dead larvae was counted and % mortality was calculated.

Total expressed protein of E. coli *transformed with toxic region of* cry4B *gene*

Bioassay with total protein expressed by transformed organisms was done against *Anopheles stephensi* larvae. The transformed organism was first grown in 250 ml LB broth supplemented with Ampicillin (100 µg/ml) and IPTG (0.5 mM) and incubated for 3 h at 37°C in shaking incubator. Different concentrations (*viz.*, 100 ng, 200 ng/ml, 300 ng/ml, ……1000 ng/ml) of total protein of recombinant organism were added in sterile cups containing 20 ml autoclaved distilled water and 20 third instar larvae of mosquito, *Anopheles stephensi* at 25°C for 24 h. The number of dead larvae was counted and % mortality was calculated.

RESULTS

The isolated DNA (Fig. 1) was optimally amplified for amplification of *cry4B* gene (Fig.2) using 250ng of DNA, 200 µM of dNTPs 2.5mM of $MgCl_2$, 150pmoles of primer concentration and IU of Taq DNA polymerase.

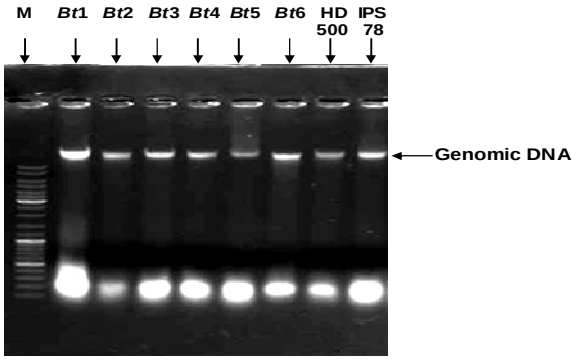

Fig. 1. DNA extraction from DAB *Bt*.1-6 (Lanes 1-6); HD 500 and IPS78 are control strains. M, DNA marker (1 Kb).

E. coli DH5ά cells were transformed with recombinant DNA comprising *cry4B* gene (2 kb) ligated in pTZ57R vector. The clones were later confirmed by colony PCR and restriction endonucleases digestion with *Eco*RI and *Hin*dIII (Fig. 3).

The toxic region of *cry4B* gene from DAB *Bt*1-6 was aligned with already known sequences using BLAST (Basic Local Alignment Search Tool)

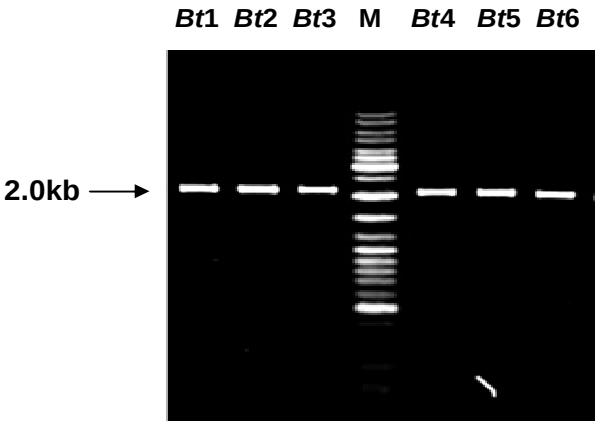

Fig. 2. PCR product of *cry4B* gene (2Kb) from *Bt* isolates. M, DNA Marker (1Kb).

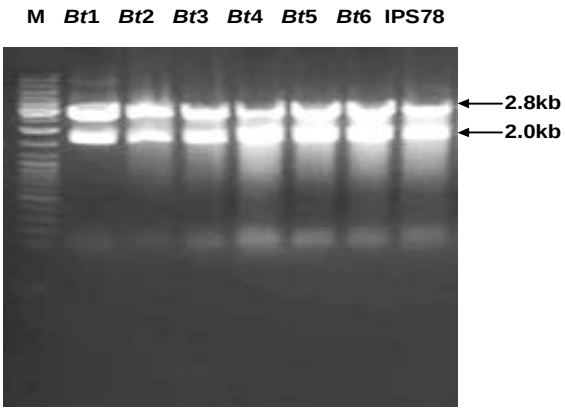

Fig. 3. Restriction digestion of recombinant plasmid containing 2.0 Kb toxic region *cry4B* gene of *Bt* isolates, *Bt* 1-6 and IPS78, with *Eco*RI and *Hin*dIII.

which showed 99% homology with toxic region of full length 130 kDa crystal protein emb|X07423.1|BTTOXD2 *Bacillus thuringiensis israelensis* bt8 gene for (mosquito-specific toxin).

Protein profile of wild type Bt isolates

Total cellular proteins of locally isolated strains of *Bt* were obtained after 48 hours of incubation time for sporulation. Figure 4 shows the different protein bands of various molecular weights obtained from total cell of *Bt* after running 12% SDS-PAGE. According to the gel, a large number of bands were present in total protein profiles of all six *Bt* isolates. Among these the most prominent protein bands with molecular weights were 130 kDa, 70 kDa, and 40 kDa. The same case was observed with positive control samples of HD500 and IPS78.

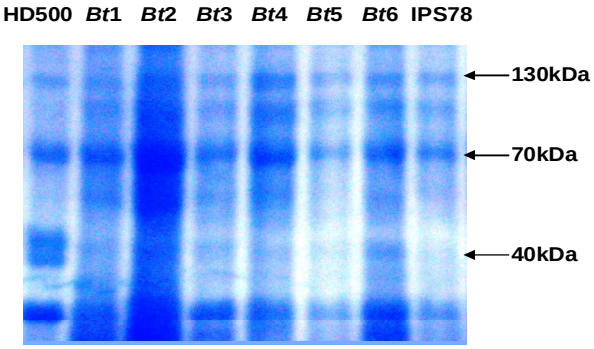

Fig. 4. Polyacrylamide gel electrophoretic pattern of total proteins of sporulated *Bt* isolates. The lanes from left to right show protein pattern of HD 500, DAB *Bt* 1-6 and IPS78.

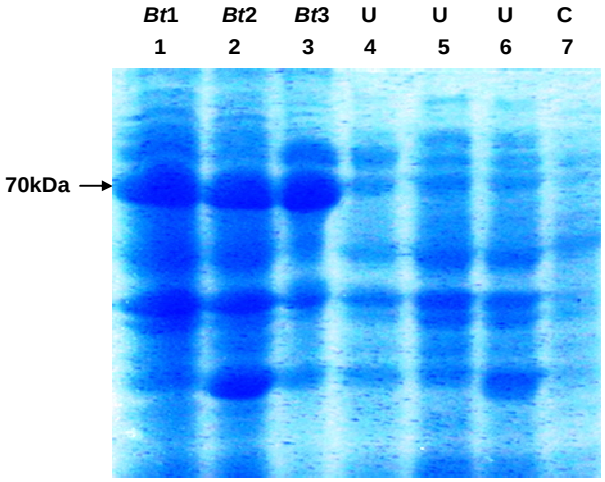

Fig. 5. Optimized protein expression profile of induced *E. coli* samples with 0.5 mM of IPTG in comparison to uninduced *E. coli* samples (lane 4, 5, 6) and control sample (lane 7) did not have any insert of *cry4B* gene but have vector grown at 37°C for 3 hours. The induced 70 kDa Cry4B band is very prominent in induced samples (lane 1, 2, 3) in comparison to all other samples

Expression of cry4B *gene in* E. coli *BL21C*

It was found that incubation in the presence of 0.5 mM of IPTG at 37°C for 3 hours resulted in over-expression of *cry4B* gene. *E. coli* transformed

with pT7-7 without *cry4B* gene was used as control. The total cell protein was isolated and run on 12% SDS–PAGE. The expressed 70 kDa Cry4B protein bands were present in the total protein profile of all the organisms (BL21C transformed with pT7-7 containing *cry4B* gene) and absent in the protein profile of control containing only transformed expression vector (pT7-7) without *cry4B* gene (Fig. 5).

Toxic region (N terminal domain-I) of Cry4B protein

Amino acids sequence of toxic region (N-terminal) of Cry4B protein of DAB *Bt* 1-6 was aligned with Cry4B protein of positive control HD500 (Table I, Fig. 6). Cry4B of DAB *Bt* 1 showed six amino acid differences at the position 32 (Gln → Thr) , 50 (Lys → Phe), 100 (Ile → Thr), 138 (Asn → Ser), 225 (Ile → Phe) and 263 (Leu → Arg). Cry4B of DAB *Bt*1, 2 two common changes were found at position 32 (Gln → Thr) and 263 (Leu → Arg), the other five changes in Cry4B of DAB *Bt* 2 at position 66 (Val → Phe), 108 (Val → Ser) , 145 (Ala → Phe),197 (Gln → Pro) and 216 (Tyr → Asp). Cry4B of DAB *Bt* 3 three amino acid changes were found at position 7 (Lys → Phe), 198 (Glu → Gly), and 199 (Trp → Gly). Three amino acid changes were detected in Cry4B from DAB *Bt* 4 at position 159 (Glu → Gly), 179 (Arg → Gly), and 195 (Asn → Lys). Cry4B of DAB *Bt* 5 a total of sixteen mutations were found at positions 63 (Ala→Pro), 64 (Gly → Pro), 72 (Ala → Gly), 86 (Glu → Pro),89 (Trp → Gly), 99 (Leu → Phe), 125 (Gln → Pro), 145 (Ala → Pro), , 177 (Ile → Pro), 181 (Val → Thr),216 (Tyr → Asp), 234 (Val → Pro),236 (Arg → Gly), 238 (Lys → Pro) 242 (Gln → Leu), and 250 (Lys → Phe). Cry4B of DAB *Bt* 6 eight mutations were found at positions 39 (Ala → Gly), 55 (Ile → Pro), 77 (Leu → Thr), 142 (Tyr → Phe), 159 (Glu → Gly), 199 (Trp → Gly), 263 (Leu → Phe) and 266 (Ser → Gly) (Fig. 6, Table I). Figure 7 shows a dendrogram describing the relatedness of all the Cry4B toxins believed to share the common three-domain structure.

Phylogenetic relationship of Cry4B protein of DAB Bt 1-6.

The homology of amino acid sequence of Cry4B protein from DAB *Bt* 1-6 and HD500 (positive control) was determined. A dendrogram was made to describe the relatedness of Cry4B toxins of six *Bt* isolates (DAB *Bt* 1-6) and HD500 (positive control). The rectangular phylogenetic tree based on topological Algorithm. The distance matrices are based on amino acid sequences of different Cry4B proteins. Cry4B of DAB *Bt* 1 showed close resemblance with Cry4B of HD500. Cry4B of DAB *Bt* 5 showed sequence homology with Cry4B of DAB *Bt* 6. Cry4B of DAB *Bt* 4 showed resemblance to Cry4B of DAB *Bt* 3 and further with Cry4B of DAB *Bt* 2 (Fig. 7).

*Toxicity of recombinant organism (*E. coli *BL21C transformed with plasmid containing toxic region of* cry4B *gene)*

Different concentrations of transformed organism were added in 20 ml water containing 20 mosquito larvae of 3rd instar *Anopheles stephensi*. Toxicity was calculated as percentage mortality of triplicate readings. It was found that *cry4B* of DAB *Bt* 5 was the most toxic, whereas *Bt* 2 and 3 were least toxic (Table II).The toxicity of *Bt* isolates with *cry4B* can be graded as DAB *Bt* 5 > 6> 1 = 4 > 2=3.

Toxicity of crude recombinant Cry4B protein

The toxicity of crude recombinant protein increased several folds compared with that of organism transformed with specific gene. Biotoxicity assays with expressed toxic region of Cry4B protein cloned from six different local *Bt* isolates showed that Cry4B protein of DAB *Bt* 5 (SBS *Bt* 45) was the most toxic, while Cry4B of DAB *Bt* 1 (SBS *Bt* 23) showed almost the same level of toxicity (Table II, Fig. 8.)

DISCUSSION

Cry4B is a dipteran-specific δ-endotoxin produced by plasmid borne gene present on 127,923bp mega plasmid known as pBtoxis. Full length 3.4 kb *cry4B* gene having coding region from 157 to 3564 encodes 128 kDa Cry4B protein, while 2.0 Kb encodes just 70 kDa toxic region of Cry4B protein (Knowles, 1994) and is supposed to be toxic to dipteran insects. Cry4B protein has 30% sequence

Table I.- Mutational analysis of toxic region of Cry4B protein of *Bt* isolates DAB *Bt*1-6 (SBS *Bt*- 23, 29, 34, 37, 45 and 47, respectively).

Amino acids no.	Change	DAB *Bt* 1	DAB *Bt* 2	DAB *Bt* 3	DAB *Bt* 4	DAB *Bt* 5	DAB *Bt* 6
7	Lys → Phe			+			
32	Gln → Thr	+	+				
39	Ala → Gly						+
50	Lys → Phe	+					
55	Ile → Pro						+
63	Ala → Pro					+	
64	Gly → Pro					+	
66	Val → Phe		+				
72	Ala → Gly					+	
77	Leu → Thr						+
86	Glu → Pro					+	
89	Trp → Gly					+	
99	Leu → Phe					+	
100	Ile → Thr	+					
108	Val → Ser		+				
125	Gln → Pro					+	
138	Asn → Ser	+					
142	Tyr → Phe						+
145	Ala → Phe		+			-	
	Ala → Pro		-			+	
157	Leu → Phe						
159	Glu → Gly				+		+
177	Ile → Pro					+	
179	Arg → Gly				+		
181	Val → Thr					+	
195	Asn → Lys				+		
197	Gln → Pro		+				
198	Glu → Gly			+			
199	Trp → Gly			+			+
216	Tyr → Asp		+			+	
225	Ile → Phe	+					
234	Val → Pro					+	
236	Arg → Gly					+	
238	Lys → Pro					+	
242	Gln → Leu					+	
250	Lys → Phe					+	
263	Leu → Arg	+	+				-
	Leu → Phe						+
266	Ser → Gly						+

identity to Cry4A protein (Chungjatupornchai *et al*., 1988; Ward and Ellar, 1988) and Cry4A and Cry4B proteins are structurally similar to Cry1 proteins and are proteolytically cleaved to smaller toxic fragments. The C-terminal halves of these proteins are similar to C-terminal halves of Cry1 proteins suggesting the localization of toxicity in the N-terminal halves (Chunjatpornchai *et al*., 1988; Pao-intara *et al*., 1988). Cry4B protein gene is a plasmid borne gene in wild type *Bt* whose transcription is controlled by sigma 35 dependent promoter during sporulation phase of cell cycle **(**Gonzalez and Carlton, 1992; Yoshisue *et al*., 1993**)**. The Cry4B protein is toxic against mosquito at larval stage and ineffective at adult stage (Pazos, 2007).

For best PCR amplification, freshly isolated DNA was used in each case. Stored DNA samples for more than two months were found to be not

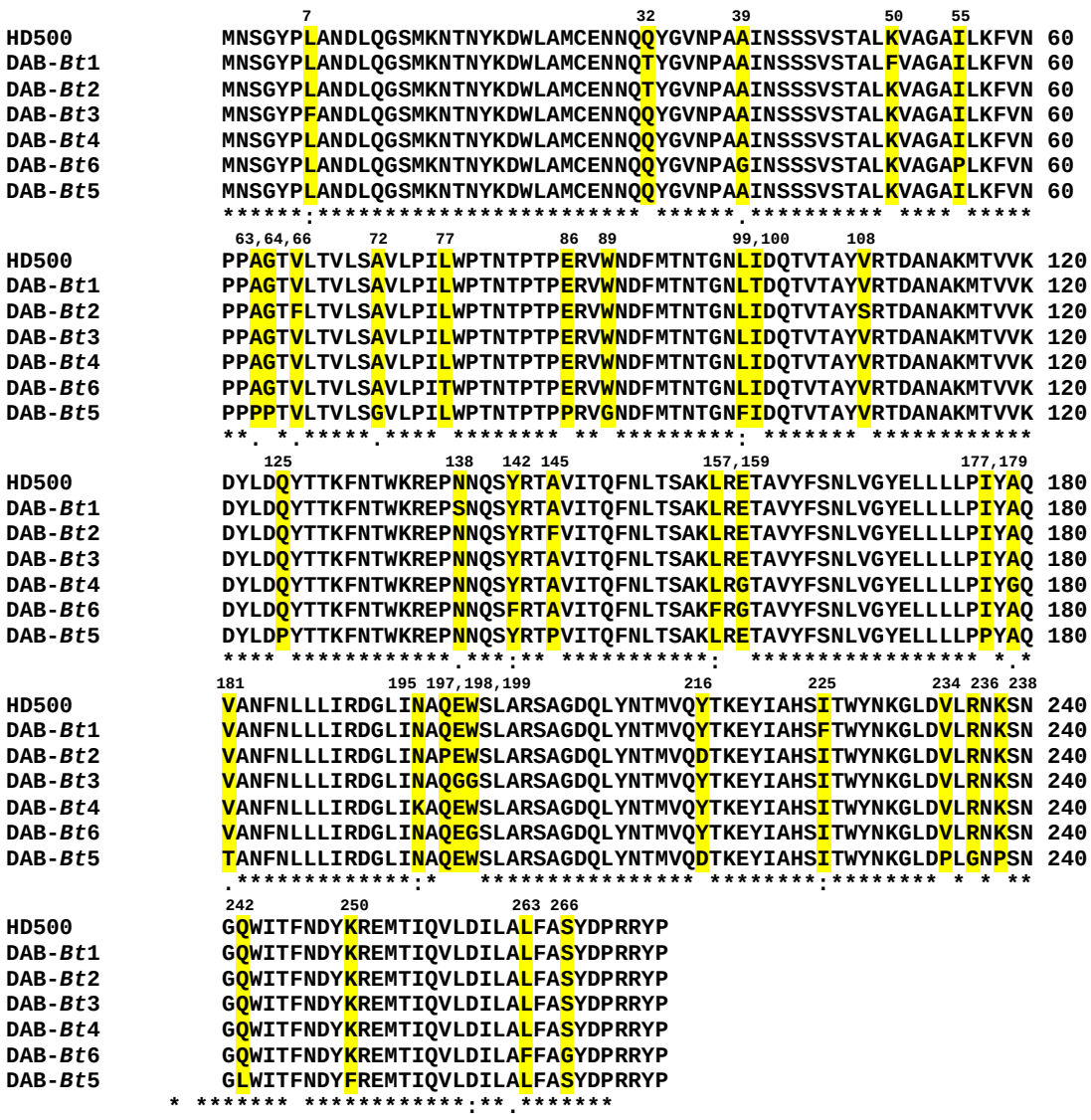

Fig. 6. Alignment of toxic region (N terminal domain I) of Cry4B proteins of DAB *Bt*1-6. The sequences have been compared with corresponding region of HD500 Cry4B protein.

good for PCR. Historically PCR has been used successfully to detect *cry* genes and to determine their distribution among natural *Bt* population (Ben-Dov *et al*., 1997; Juárez-Pérez *et al*., 1997; Bravo *et al*., 1998).

Delta-endotoxins are environmentally labile, which is why the most successful systems to produce them as bioinsecticides are those in which they remain encapsulated and protected from degradation. The ICPs are highly susceptible to various factors, such as ultraviolet light, heat, and humidity cycles. Hence, expression of *cry* genes in organisms that make the toxins more stable in the environment was proposed as a way to overcome this problem.

Various types of proteins ranging from 130 kDa to 40 kDa were isolated after 48 hours incubation. The protein profile pattern was similar to the control type *Bt* HD500 and IPS78. Mostly the molecular weight of the Cry proteins were 135 kDa

Table II.- LC_{50} values of *E. coli* BL21C transformed with plasmid containing *cry4B* and Cry4B recombinant crude protein gene against 3rd instar larvae of *Anopheles stephensi*. The preparation were added in water (n=3).

Bt isolate	LC_{50} (µg/ml)	LC_{50} (ng/ml)
DAB *Bt*1	250±2.43	444±2.57
DAB *Bt*2	263±1.99	465±3.86
DAB *Bt*3	288±3.02	423±1.99
DAB *Bt*4	227±2.08	377±3.02
DAB *Bt*5	175±3.34	363±2.67
DAB *Bt*6	219±2.99	476±2.97
IPS 78	245±1.84	490±2.59
HD 500	272±2.76	566±2.96
Zero dose	-	-

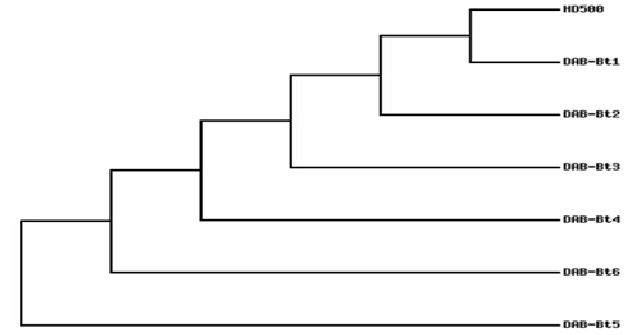

Fig. 7. Dendrogram showing the relatedness of Cry4B toxins of six *Bt* isolates (DAB *Bt* 1-6) and HD500 (positive control).

(Cry4A), 130 kDa (Cry4B), and 68 kDa (Cry4D) (Cappello *et al*., 2006). The level of expression of Cry proteins was relatively high and other cellular and non-Cry proteins of low molecular weights were present in low concentration. This indicated that the 48 hours are sufficient for the *Bt* cells to sporulate and produce high Cry proteins. At this level normally the proteins required for cellular and metabolic functions are present in excess. The protein was isolated by using SDS and β-mercaptoethanol as detergent and denaturing agent because Cry proteins are mostly water insoluble due to high level of cysteine residues present in C terminal portion. These cysteine molecules are actively involved in sulphur bridge formation and providing more complex structure to the proteins. Thus mercaptoethanol disrupted these sulphur bridges and SDS provided negative charge to the protein and made it linear to be resolved on SDS PAGE effectively (Höfte and Whiteley, 1989; Laemmli, 1970).

E. coli is an effective expression system for *cry* genes, because the parameters of the biosynthetic processes are better understood in this bacterium. There are several reports on characterization, cloning and expression of novel *Bt* toxin genes (Ben-Dov *et al.,* 1995; Brizzard *et al*., 1991; Ge *et al*., 1990; McPherson *et al*., 1988; Sanchis *et al*., 1989; Shimizu *et al*., 1988). The use of *E. coli* as an expression host allows the selective production of Cry proteins with particular biocidal specificity for their individual study. In addition, it can also be used as an effective vehicle for industrial production of crystal proteins, functionally improved by genetic and protein engineering (Aronson, 1995; Bosch *et al*., 1994; Caramori *et al*., 1991; De Maagd *et al*., 1996). Ge *et al.* (1991) have optimized the culture conditions for suitable hyper-expression in *E. coli* of the gene *cry1Ac73* encoding the Cry1Ac insecticidal crystal protein. P*tac* promoter was found to have the best performance for the production of protoxins to high levels on pBR322 derivatives (pKK223–3) in *E. coli* host JM103. The expression in *E. coli* of the truncated *cry1A* gene versions encoding the ICP toxic fragments has also been achieved (Kalman *et al.*, 1995; Vazquez *et al*., 1995).

In the present work, we cloned the toxic region of the *cry4B* gene in *E. coli* strain BL21C to study the expression pattern. The pTZ57R/T vector was used to clone the 2.0 Kb *cry4B* gene and pT7-7 vector was used as an expression vector to transfer the cloned *cry4B* gene into the *E. coli* host organism after restriction digestion by using *Eco*R1 and *Hin*dIII restriction enzymes. Various promoters are used to increase the expression level of the inserted gene like heterologous promoter was used for amylase gene of *B. subtilis*, *cry3Aa* and *cry3Bb* **(**Chak *et al*., 1994; Agaisse and Lereclus, 1995). In our case, we used T7 promoter in pT7-7 for controlling the expression of *cry4B* gene inserted next to it. The T7 promoter is recognized by the viral T7 RNA polymerase encoded by genomic DNA of the *E. coli* and is under the strict control of strong *lacuv* promoter. Now, in *E. coli* the

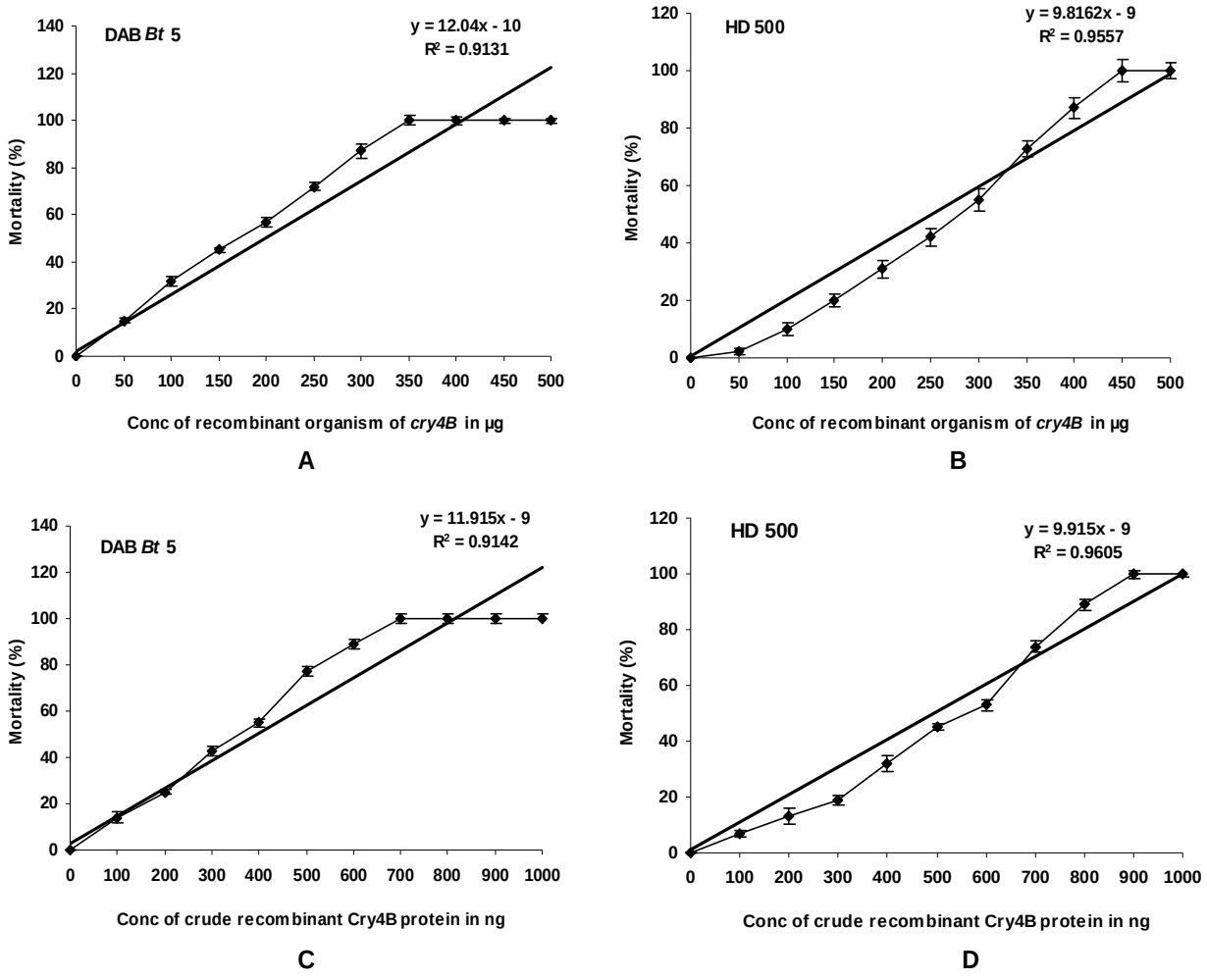

Fig. 8. Toxicity (% mortality) of recombinant organisms containing *cry4B* gene (- DAB Bt 5 (A), HD500 (B)), and crude recombinant Cry4B protein (C, D) of *Bt* isolate (DAB Bt5) and control Bt strain (HD500), against 3rd instar larvae of *Anopheles stephensis*.

sporulation dependent *cry4B* gene becomes independent of sporulation phase and expressed as normal cellular gene in *E. coli.*

Finally, we optimized the expression of the Cry4B protein into the *E. coli* host with respect to the incubation time, incubation temperature and IPTG concentration. Maximum expression was obtained in induced as compared to uninduced samples after 3 hours of incubation time, at 37°C and by using 0.5 mM IPTG concentration. The presence of proteins in uninduced samples could be because of some leakage of inducible T7 RNA polymerase gene promoter. This kind of constitutive expression has also been reported by Ge *et al*. (1990). As it was reported that T7 promoter is recognised by viral RNA polymerase encoded by host genomic DNA. In host cell, chromosomal DNA encodes viral RNA polymerase is itself under strict regulation of *lacuv* promoter. IPTG is an artificial inducer of this promoter being having structural homology to the natural inducer lactose. This inducer activates *lacuv* promoter which in turn transcribes more RNA polymerase gene and later on more viral RNA polymerases were translated from these transcripts by using host cell machinery. Thus large number of RNA polymerases now bind to T7

promoters to transcribe the downstream located gene accordingly and by this switching and indirect activation by IPTG, expression of the cloned *cry4B* gene was enhanced exponentially within very short time period of 3 hours. Thus by using *E. coli* as host cell, we can get the expression of sporulation dependent genes even earlier than normal cellular gene expression by induction mechanism.

The activated region of Cry4B of the δ-endotoxin is composed of three structural domains. The N-terminal helical domain is involved in membrane insertion and pore formation. The second and third domains are involved in receptor binding. The N-terminal domain I, a seven helix bundle involved in membrane insertion and pore formation (Li *et al*., 1991; Grochulski *et al*., 1995; Morse *et al*., 2001). Cry4B δ-endotoxin produced protease-resistant products of ca. 47 kDa and ca. 21 kDa after tryptic activation. The 21-kDa fragment contained the N-terminal five-helix bundle (ά1-ά 5), is the only candidate for membrane insertion and pore formation (Puntheeranurak *et al.*, 2001). Angsuthanasombat *et al.* (1991) reported the removal of the C-terminal half of the 130-kDa Cry4B protoxin, the activated molecule had undergone proteolytic activation producing two main sets of cleavage products at ca. 47 kDa and ca. 21 kDa. Amino acid sequence alignment of toxic region of Cry4B of DAB *Bt* 1-6 with already reported sequences in GenBank database showed the maximum homology with the reported sequence. P05519 CR4BA_BACTI Pesticidal crystal protein Cry4Ba (Insecticidal delta-endotoxin CryIVB(a) (crystaline entomocidal protoxin). Mutations were found at different positions in toxic region of Cry4B and most of them were found in N-terminal helical domain which is involved in membrane insertion and pore formation. The mutation in toxic region especially in pore forming unit (α helices) are most important as for as the toxicity is concerned. The mutation of one charged residue (Arg-158) in α4 of Cry4B was found to considerably affect toxicity (Sramala *et al.*, 2001).

Biotoxicity assay with *E. coli* BL21C transformed with toxic region of Cry4B against third instar larvae of mosquito, *Anopheles stephensi* showed that Cry4B of DAB *Bt*5 was more toxic (LC_{50}=175µg/ml) than control strains IPS78 and HD500 (LC_{50}=245µg/ml and 272=µg/ml, respectively). Cry4B of DAB *Bt*1-4 showed almost the same toxicity as positive controls. Biotoxicity assay with crude recombinant Cry4B protein of *Bt* isolates (DAB *Bt*1 6) showed that Cry4B of DAB *Bt*5 was the most toxic (LC_{50}=363ng/ml) followed by that of DAB *Bt*4 and 6. All the six recombinant Cry4B proteins were more toxic than the positive controls. Chayaratanasin *et al*. (2007) reported high level of soluble expression Cry4Ba in *E. coli* and characterized the high mosquito-larvicidal activity of Cry4Ba domain III fragment of Cry4Ba.

To conclude, the present study was molecular characterization of toxic region of *cry4B* gene from local *Bt* isolates and the determination of toxicity of N-terminal domain of Cry4B protein as bioinsecticide against mosquito larvae.

ACKNOWLEDGMENTS

We are grateful to Prof Dr. Daniel R. Zeigler, Director, Bacillus Genetic Stock Centre, Columbus, Ohio, USA for providing us positive strains of *Bacillus thuringiensis* and to Ms. Ghazala, Director, Malaria Centre Birdwood Road, Lahore, for providing mosquito larvae for bioassays.

REFERENCES

ABDULLAH, M. A. F., VALAITIS, A. P. AND DEAN, D. H. 2006. Identification of a *Bacillus thuringiensis* Cry11Ba toxin – binding aminopeptidase from the mosquito, *Anopheles quadrimaculatus*. *BMC Biochem*., **7:** 16 doi:10.1186/1471-2091-7-16.

ABDULLAH, M.A., ALZATE, O. MOHAMMAD**,** M. MCNALL, R.J., ADANG, M.J. AND DEAN, D.H., 2003. Introduction of C*ulex* toxicity into *Bacillus thuringiensis* Cry4Ba by protein engineering. *Appl. environ. Microbiol*., **69:** 5343-5353.

AGAISSE, H. AND LERECLUS. D., 1995. How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? *J. Bacteriol*., **177:** 6027-6032.

ANGSUTHANASOMBAT, C., CRICKMORE, N. AND ELLAR, D.J., 1991. Cytotoxicity of a cloned *Bacillus thuringiensis* subsp. *israelensis* CryIVB toxin to an *Aedes aegypti* cell line. *FEMS Microbiol. Lett.,* **67:** 273-276.

ARONSON, A.I., 1995. The protoxin composition of *Bacillus thuringiensis* insecticidal inclusion affects solubility and toxicity. *Appl. environ. Microbiol*., **61:** 4057-4060.

BARUSRUX, S., SRAMALA, I., KATZENMEIER, G.,

BUNYARATVEJ, A., PANYIM, S. AND ANGSUTHANASOMBAT, 2003. *Ex vivo* cytotoxicity of the *Bacillus thuringiensis* Cry4B δ-Endotoxin to isolated midguts of *Aedes aegypti* larvae. *J. Biochem. Mol. Biol*., **36:** 294 – 298.

BEN-DOV, E., BOUSSIBA, S. AND ZARITSKY, A., 1995. Mosquito larvicidal activity of *Escherichia coli* with combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. *J. Baceriolt*., **177:** 2851-2857.

BEN-DOV, E., ZARITSKY, A., DAHAN, E., BARAK, Z., SINAI, R., MANASHEROB, R., KHAMRAEV, A., TROITSKAYA, E., DUBITSKY, A., BEREZINA, N. AND MARGALITH, Y., 1997. Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. *Appl. environ. Microbiol*., **63:**4883–4890.

BOSCH, D., SCHIPPER, B., VAN DER KLEIJ, H., DE MAAGD, R.A. AND STIEK-EMA, W.J., 1994. Recombinant *Bacillus thuringiensis* crystal proteins with new properties: possibilities for resistance management. *Biotechnology*, **12:** 915–918.

BRAVO, A., SARABIA, S., LOPEZ, L., ONTIVEROS, H., ABARCA, C., ORTIZ, A., ORTIZ, M., LINA, L., VILLALOBOS, F.J., PENA, G., NUNEZ-VALDEZ, M.E., SOBERON, M. AND QUINTERO, R., 1998. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Appl. environ. Microbiol*., **64:**4965–4972.

BRIZZARD, B.L., SCHNEPF, H.E. AND KRONSTAD, J.W., 1991. Expression of cryIB crystal protein gene of *Bacillus thuringiensis*. *Mol. Gen. Genet*., **231:** 59-64.

BUKHARI, D.A. AND SHAKOORI, A.R., 2010. Isolation and Molecular Characterization of *cry*4 harbouring *Bacillus thuringiensis* isolates from Pakistan and mosquitocidal activity of their spores and total proteins. *Pakistan J. Zool.*, **42(1):** 1-15.

CAPPELLO, M., BUNGIRO, R. D. HARRISON, L. M. BISCHOF, L. J. GRIFFITTS, J. S. BARROWS, B.D. AND AROIAN. R.V**.** 2006. A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicum. PNAS*., **103**: 15154 – 15159.

CARAMORI, T., ALBERTINI, A.M. AND GALZZI, A., 1991. *In vivo* generation of hybrids between two *Bacillus thuringiensis* insect-toxin encoding genes. *Gene*, **98:** 37-44.

CHAK, K.F., TSENG, M.Y. AND YAMMOTO, T., 1994. Expression of the crystal protein gene under the control of the a-amylase promoter in the *Bacillus thuringiensis* strains. *Appl. environ. Microbiol*. **60:** 2304-2310.

CHAYARATANASIN, P., MOONSOM, S., SAKDEE, S., CHAISRI, U., KATZENMEIER, G. AND ANGSUTHANASOMBAT, C., 2007. High level of soluble expression in *Escherichia coli* and characterisation of the cloned *Bacillus thuringiensis* Cry4Ba domain III fragment. *J. Biochem. mol. Biol*., **40:** 58-64.

CHUNGJATUPORNCHAI, W.H., HOFTE, H., SEURINK, J., ANGSUTHANASOMBAT, C. AND VAECK, M., 1988. Common features of *Bacillus thuringiensis* toxins specific for Diptera and Lepidoptera. *Europ. J. Biochem*., **173:** 9-16.

DE-MGAAD, P.H., LARSEN, H.D., HANSEN, B.H., BRESCIANI, J. AND JØRGENSEN, K., 1996. Enterotoxin-producing strains of *Bacillus thuringiensis* isolated from food. *Lett. appl. Microbiol*., **23:**146–150.

FERRÉ, J., REAL, M.D., VAN RIE, J., JANSENS, S. AND PEFEROEN, M., 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. *Proc. natl. Acad. Sci. USA*, **88:** 5119-5123.

GE, A. Z., PFISTER, R. M. AND DEAN**,** D. H., 1990. Hyperexpression of a *Bacillus thuringiensis* delta-endotoxin-encoding gene in *Escherichia coli*: properties of the product. *Gene*, **93:**49-54.

GE, A.Z., RIVERS, D., MILINE, R. AND DEAN, D.H., 1991. Functional domains of *Bacillus thuringiensis* insecticidal crystal proteins. *J. biol. Chem.*, **266:** 17954-17958.

GONZALEZ, J. M., JR., AND CARLTON, B.C., 1992. Plasmid transfer in *Bacillus thuringiensis.* In: *Genetic Engineering. A celebration and a New Generation* (eds. U.N. Streips, S.H. Goodgot, W.R. Guild and G.A. Wilson), Marcel-Dekker. New York, pp. 85–95.

GROCHULSKI, P., MASSON, L., BORISOVA, S., PUSZTAI-CAREY, M., SCHWARTZ, J.L., BROUSSEU, R. AND CYGLER, M., 1995. *Bacillus thuringiensis* CryIA (a) insecticidal toxin: Crystal structure and channel formation. *J. mol. Biol*., **254:** 447-464.

HÖFTE, H. AND WHITELEY, H.R., 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev*., **53:** 242-255.

JUA´REZ-PE´REZ, V. M., FERRANDIS, M.D. AND FRUTOS, R., 1997. PCR-based approach for detection of novel *Bacillus thuringiensis cry* genes. *Appl. environ. Microbiol*., **63:** 2997–3002.

KALMAN, S., KIEHNE, K.L., COOPER, N., REYNOSO, M., S. AND YAMAMOTO, T., 1995. Enhanced production of insecticidal proteins in *Bacillus thuringiensis* strains carrying an additional crystal protein gene in the chromosomes. *Appl. environ. Microbiol*., **61:** 3063-3068.

KNOWLES, B.H., 1994. Mechanism of action of *Bacillus thuringiensis* Insecticidal δ endotoxins. *Adv. Insect Physiol*., **4:** 275-308.

LAEMMLI, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227:** 680-685.

LEE, M.K., MILNE, R.E., GE, A.Z. AND DEAN, D.H., 1992. Localization of a *Bombyx mori* receptor binding region

on a *Bacillus thuringiensis* 8-endotoxin. *J. biol. Chem*., **267:** 3115-3121.

LI, J., CARROLL, J. AND ELLAR, D.J., 1991. Crystal Structure of insecticidal d-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolutions. *Nature*, **353:** 815-821.

MCPHERSON, S.A., PERLACK, F.J., FUCHS, R.L., MARRONE, P.G., LAVRIK, P.B. AND FISCHHOFF, D.A., 1988. Characterization of the coleopteran-specific protein gene of *Bacillus thuringiensis* var *tenebrionsis*. *Biotechnology*, **6:** 61-66.

MORSE, R.J., YAMAMOTO, T. AND STROUD, R. M., 2001. Structure of Cry2Aa suggests an unexpected receptor binding epitope. *Structure* (*Camb.*), **9:** 409-417.

PAO-INTARA M., ANGSUTHANASOMBAT, C. AND PANYIM, S., 1988. The mosquito larvicidal activity of 130kDa delta-endotoxin of *Bacillus thuringiensis* var *israelensis* resides in the 72kDa amino-terminal fragment *Biochem. biophys. Res. Commun.*, **153:** 294-300.

PAZOS, L.S.A., 2007. Three dimensional structure of *Bacillus thuringiensis* toxin. *Acta Biol. Colomb. Rev.*, **12:** 19-32.

PROBER, J.M., TRAINER, G.L., DAM, R.J., BOBBS, F.W., ROBERTSON, C.W., ZAGURSKY, R.J., COCUZZ, A.J., JENSEN, M.A. AND BAUMEISTER, K., 1987. A system for rapid DNA sequencing with flurorescent chain terminating dideoxynucleotides. *Science*, **238**, 336-341.

PROMDONKOY, B., PROMDONKOY, P. AND PANYIM, S., 2005. Co-expression of *Bacillus thuringiensis* Cry4Ba and Cyt2Aa2 in *Escherichia coli* revealed high synergism against *Aedes aegypti* and *Culex quinquefasciatus* larvae. *FEMS Microbiol. Lett*., **252:** 121–126

PUNTHEERANURAK, T., LEETACHEEWA, S., KATZENMEIER, G., KRITTANAI, C., PANYIM, S. AND ANGSUTHANASOMBAT, C., 2001. Expression and biochemical characterization of the *Bacillus thuringiensis* Cry4B α1- α5 pore-forming fragment. *J. Biochem. mol. Biol*., **34:** 293-298.

SAMBROOK, J., FRITSCH, E.F. AND MANIATIS, T., 1989 In: *Molecular cloning, a laboratory manual*. Cold Spring Harbor Laboratroy, Cold Spring Harbor, N.Y.

SANCHIS, V., LERCLUS, D., MENOU, G., CHAUFAUX, S. AND LECADET, M.M., 1989 Nucleotide sequence and analysis of N-terminal coding region of Spodoptera-active S-endotoxin gene of *Bacillus thuringiensis aizawai* 7.29. *Mol. Microbiol*., **3:** 229-238.

SCHNEPF, H. E. AND WHITELEY, H.R., 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. *Proc. natl. Acad. Sci. USA*, **78:** 2893-2897.

SHIMIZU, M., OSHIE, K., NAKAMURA, K.,Y TAKADA, Y., OEDA, K. AND OHKAWA, H., 1988. Cloning and expression in *E. coli* of the 135-kDa insecticidal protein gene from *Bacillus thuringiensis* subsp *aizawai* IPL7. *Agric. biol. Chem*., **52:** 1565–1573.

SRAMALA, I., LEETACHEWA, S., KRITTANAI, C., KATZENMEIER, G., PANYIM, S. AND ANGSUTHANASOMBAT, C., 2001. Screening charged residues in helix 4 of the *Bacillus thuringiensis* Cry4B toxin reveals one critical residue for larvicidal activity. *J. Biochem. mol. Biol. Biophys*.**, 5:**219-225.

THOMAS, W.E. AND ELLAR, D.J., 1983. Mechanism of action of *Bacillus thuringiensis var israelensis* insecticidal δ – endotoxin. *FEBS Lett*., **154:** 362- 368.

VA´ZQUEZ, R., PRIETO, D., OLO´RIZ, M.I., DE LA RIVA, G.A. AND SELMAN-HOUSEIN, G., 1995. Heterologous expression of recombinant d-endotoxins from *Bacillus thuringiensis* in *E. coli*: comparative study in three transcriptional systems. *Rev. Lat-Am. Microbiol.*, **37**: 237–244.

WARD, E.S. AND ELLAR, D.J., 1988. Cloning and expression of two homologous genes of *Bacillus thuringiensis* subsp. *israelensis* which encode 130-kilodalton mosquitocidal proteins. *J. Bact*., **170:** 727-735.

WHITE, J.A., MALTAIS, L,J. AND NEBERT, D.W., 1998. *An increasingly urgent need for standardized nomenclature.* http//gentics.Nature.com/web_special/nomen/noman_article.html.

YOSHISUE, H., NISHIMOTO, T. SAKAI, H. AND KOMANO, T., 1993. Identification of a promoter for the crystal protein-encoding gene *cryIVB* from *Bacillus thuringiensis* subsp. *israelensis*. *Gene*, **137:** 247-251.

(Received 9 March 2012, revised 12 April 2013)