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Cloning and Sequencing of a Coleopteran specific Novel *cry* gene of a local isolate of *Bacillus thuringiensis*

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Abstract: *A cry gene, obtained from a locally isolated Bt. strain, CAMB # 30382 (isolated from grain dust of Shakargarh, Punjab, Pakistan), highly effective against Tribolium castaneum (Commonly known as red flour beetle), was amplified through Polymerase chain reaction (PCR) by using specific primers. The gene was ligated to a cloning vector pGEM-T and was cloned into an E.coli strain DH5α. The positive clones were screened for the cry gene content.*

The partial sequencing of the cloned gene from this strain was accomplished and sequence homology was analyzed. The alignment of the sequenced gene with gene sequences present in gene data bank showed two silent mutations, at nucleotide 676 and 701. However, two other nucleotide changes at position 2887 and 2902 of holotype cryIIIf (cry8Ca) gene were resulting from amino acid change near 3′ end were observed. These changes were causing change in the stretch of amino acids from valine, tyrosine, serine & glutamine in the holotype cryIIIf to glycine, phenylalanine, alanine & asparginine respectively in the clone from CAMB isolate 30382. This change may have drastic effect on the toxicity spectrum of the Cry protein isolated from this local isolate in comparison with the Cry protein from the Buibui. serovar japonesis. Partial sequencing has shown it to be a varient of cryIII gene, which may be a novel cry gene.

Key Words; *Cloning, Bacillus thuringiensis, cry gene, Sequencing, Coleopteran.*

I. Introduction

Bacillus thuringiensis is a gram-positive bacterium, widely used in agriculture as a biological pesticide. The protein inclusion is composed of one or more types of delta-endotoxins Cry and Cyt proteins. Many *Bacillus thuringiensis* with different host spectra have been identified (Burges 1981). The delta-endotoxins are mostly used in agriculture by organic and other growers to control agronomically important pests (Dulmage 1981; Guillet et al., 1990; and Mulla 1990).

Bt. produces several insecticidal crystalline proteins (ICP/Cry proteins) at the time of sporulation. The β-exotoxins and δ-endotoxins are used for the control of pests and vectors of diseases. The crystalline delta endotoxins are predominantly synthesized as long, inactive protoxins that are activated by proteolysis in the insect gut. The examples include Cry 1, Cry4A, Cry4B, having molecular weights of 130 to 140 kDa are processed *Bacillus thuringienesis* to active 65 to 70 kDa toxins (Gill et al., 1992,; Hofte and whitely; 1989) while Cry2A, Cry3A, Cry10A and Cry11A are naturally truncated toxins with molecular weights ranging from 65-80 kDa. Sequence analyses of many genes have suggested that significant changes in the activity spectrum can be attributed to comparatively small changes in amino acid sequences (Rahat 1998).

The red flour beetle, *Tribolium castaneum* is an important pest of stored grains. These beetles live mainly in grain store mills and bird nests (Roth and Kurtz 2008).Both Larvae and adults of the red flour beetle feed on broken kernels and grain dust. These stored pests are mostly get rid of in the house in infected cereal or flour while some red flour beetles survive on food material in cervices, furniture and cabinet cracks and can increase their descendents (Haque et al., 2000).

Bacillus thuringienesis contain valuable environment-proteins of cry genes. So it is considered as friendly bio pesticide, which constitutes 90% of the world significance by production of resistant crops, such bio pesticide commercially. Its insecticidal properties were analyzed against maize, cotton, potato,rice etc (Kumar 2002).

Incorporation of Bt technology into an integrated pest management is the preferred strategy to achieve effective insect control while minimizing target resistance (Andow *et al*., 2001).

To increase the effacacy and persistance of Bt. toxins for field use, crystal proteins genes (cry genes) have been cloned and expressed in *E. coli*, *Bacillus subtitles*, *Pseudomonas fluorescens*, *Clavibacter*, *Baculovirus* and nuclear polyhedrons virus (Haider and Ellar, 1988; Hofte *et al*.,1987; Honee *et al*.,1988; Sen *et al*.,1988; Ge *et al*.,1990; Merryweather *et al*.,1990; Martins *et al*.,1990)

The first cloning of Cry IAa gene from *Bacillus thuringiensis* subsps. *kurstaki* was reported by Schenpf and Whiteley 1981.It has been established that the delta-endotoxin genes whose products are specifically active on different orders of insects show only limited similarity and are distantly related (Hernest et al., 1986). .

The *cryIII*A gene was cloned and sequenced by Sekar et al., 1987 and Mc Pherson et al., 1988 from Bt. *var tenebrionis*, which showed 69 percent homology with *cry III*B gene from Bt. var tolworthi (Sick et al., 1990).

II. Material And Methods

bacterial strains and media

Based on molecular characterization, a *Bacillus thuringiensis* strain C.E.M.B 30382, containing *cryIII* gene, highly effective against *Tribolium casatenium* was isolated and selected for gene cloning and sequencing. The Bt. strain isolated from the local environment was cultivated in S.P.Y medium having the composition in g/l: ammonium sulphate 20, potassium dihydrogen phosphste 6, dipotassium hydrogen phosphate 14, sodium citrate 1, magnesium sulphate 0.29, yeast extrat 1 and glucose (sigma) 6 was used as a carbon source.
E.coli strain DH5-alpha was chosen as recipient strain and grown in LB medium containing g/l: tryptone (Difco) 10.0, Yeast extract (Difco) 5.0, NaCl 10.0, and ampicillin 50 ug/ml at 30 ^{o}C for 24 hours.

DNA isolation and gene identification

To isolate and identify the gene of interest, the total genomic DNA was isolated by the method of Kronstad et al., (1983), (fig 1). An overnight culture from a single colony of bacterial cells was diluted in SPY medium in 1:100 ratio. Cells were grown at 35 ^{o}C with shaking to optical density 0.8 at 600 nm in 2.8 Litre flask with shaking 200 rpm. Cells were harvested by centrifugation at 4^{o}C at 7K rpm for 10 min. The cell pellet was washed with solution containing 100mM Tris-HCl (pH 7.9) and 10 mM EDTA. Lysozyme was added to give a concentration of 0.5 mg/ml. Mixture was incubated at 37^{o}C for 20 minutes. 6.25 ml of solution containing 100 mM tris-HCl (pH 7.5), 100 mM NaCl and 2% SDS was were added to lyse the cells. Preparation was mixed by gently inverting the tubes several times. Incubation was given at 60 ^{0}C, till the preparation was clear. Extraction was done with Phenol-Chloroform mixture. Aqueous layer was removed with the wide bore pippette. 2.5 volume of chilled Ehanol was added and DNA was spooled out with a glass rod. DNA was rinsed with 70% Ethanol, air dried and resuspended in 500 ul to 1ml of T.E (10mM Tris-HCL pH 7.9,1mM EDTA). DNA was dialyzed extensively against cold TE buffer at 4^{0}C and concentration measured by taking O.D.260.

Gene amplification through Polymerase Chain Reaction

To amplify the gene of interest, Polymerase Chain reaction was done by a modification by saiki et.al., (1988). A total volume of 50 ul contained 2.5 mM MgCl2, 10mM tris-HCl pH 8.0, 100 uM of each deoxyribonucleotide triphosphate (dNTPs), 50 pmol of each forward and reverse primer, one unit Taq DNA polymerase and 5-100 ng of DNA template. Overlaid 50-ul light mineral oil on the reaction mixture. The mixtures were amplified in 35 cycles by programming the PCR machine (M J research) according to the expected length of the PCR product.
To amplify 3.4 kbps, DNA fragment from the genomic DNA of isolate 30385, the PCR programme was as follows:

1 Cycle
95^{0}C 5 minutes
35 Cycles
95^{0}C 1 minute
59^{0}C 1 minute
72^{0}C 4 minutes
1 Cycle
95^{0}C 1 minute
59^{0}C 1 minute
72^{0}C 4 minutes

Once the PCR reaction was complete, it was kept at 4^{0}C till further processing.

Purification of DNA fragments

DNA fragments were isolated from the agarose gel using Qiaquick gel extraction kit (cat #28704). The specific DNA samples were run through 1% agarose gel. The required fragment of 3.48 kb was cut out of the gel under UV light (by using UV transilluminator), and transferred to an eppendorf tube. The gel slice was weighed and three times volume of buffer QG was added from the kit. In addition it was incubated at 50 C in a water bath for approximately 10 minutes. Now added equal volume of isopropanol and mixed gently. Applied this mixture to Qiaquick column and centrifuged for 1 minute. Discarded the flow through and added 0.75 ml buffer PE (wash buffer) to the column and centrifuged for 1 minute. Discarded the flow through and eluted the DNA bound with column, with 33-50-ul water.

Plasmid DNA Isolation.

Plamid DNA was isolated by alkaline lysis method (Brinboin and Dolly, 1979). Single bacterial colony was used to inoculate 5 ml of LB medium containing the antibiotic ampicillin and grown at 35 ^{0}C with vigorous shaking for 12-16 hours (overnight). 1.5 ml of overnight culture was shifted to a micro centrifuge tube and centrifuged at 12000 rpm for 3 minutes. The supernatant was decanted and the pellet was re-suspended in 100 ul of ice cold cell suspension buffer (25M Tris Hcl pH 8.0,10 mM EDTA, 50mM glucose,2 mg/ml lysozyme) and incubated for 5 minutes. Then 200 ul of a freshly prepared cell lysis solution (1% SDS, 0.2 % NaOH) was added. After immediately mixing by inverting the tube several times and 5 min. incubation of lysate at room temperature, 150 ml of ice-cold 3M potassium acetate solution (pH 4.8) was added and again mixed by inversion followed by 15 minutes incubation on ice. Centrifugation was done at 12000 rpm for 15 minutes and clear supernatant was extracted with an equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture. Extracted aqueous phase was carried out at -20 ^{0}C for 15 minutes followed by centrifugation at 12000xg for 15 minutes. The pellet was rinsed with ice-cold 70% Ethanol, air dried and re-suspended in 25 ul of nuclease free water.

Automated DNA sequencing

Automated DNA sequencing (ABI) from Applied Biosystems was used along with ABI PRISM Ready reaction DyeDeoxy terminator sequencing Kit according to manufacturer's instructions. This method is based on dye terminator chemistry, in which each of the four dideoxynucleotides is labeled with a different flourochrome (Prober et al., 1987; Lee et al., 1992). The ABI 377 can simultaneously detect fluorescence at four different wave lengths, set to coincide with the emission of four different fluorescent dyes. The reaction mixture is run in a single capillary so that color of each band is passing the detector represents the DNA sequences.

Performing the sequences reaction simply required the mixing of DNA (0.8 ug), primer (~ 3.2 pmol) and water with an aliquot of premixed reagents from the kit followed by 25 cycles in a thermal cycler (Perkin-Elmer). The unincorporated, labeled nucleotides were removed by ethanol precipitation and the samples air dried. Just prior to gel electrophoresis, the sample was re-suspended in a gel loaded buffer (TSR Template Solubilizing Reagent) and heated to denature the DNA.

The raw sequence data collected by the system were processed by associated software to get an electropherogram and finally the DNA sequence in the text form.

Homology Studies of Sequenced Nucleotides

Homology studies of the nucleotide sequences of the clones with known nucleotide sequences present in gene data bank was done through standard nucleotide-nucleotide BLAST (Basic Local Alignment Search Tool) software available at NCBI website.

www.ncbi.nlm.gov/home/Blast

Nucleotide sequences were put in FASTA format and a non redundant search was conducted. The results were obtained at HTML document showing the statistical score values for homology along with nucleotide to nucleotide homology.

Electro competent Cell Preparation

A single colony from freshly growing plate was inoculated into 5ml of LB medium and grown with vigorous shaking at 37C until O.D was 0.8 at 550nm. Cells were harvested by centrifugation and pellet washed twice by re-suspension in ice-cold 10% sterile glycerol in sterilized de-ionized water. The cell pellet was then re-suspended in 1ml of 10% glycerol to bring the final cell density to 200-250 O.D 550 units. Cells were aliquoted in 100ul aliquots and stored at -70 C.

Cloning procedure

Total genomic DNA, isolated from the Bt. strain 30382, was used to amplify a 3.48 kbp full length cry3 type gene by using specific primers. Ligation of the amplified fragments was done in pGEM-T vector according to the instructions supplied with kit (Promega). The ligation mixture contained 11 ul sample containing 60 ng of vector, 180 ng of insert and 5 ul of Buffer (Progema). Ligation reaction was achieved with 3 units of T4 DNA ligase (Promega) at 16 C for 24 hours. The *E.coli* DH5α (alpha) was used for high ligation mixture transformations. For the transformation of ligated DNA, the ligation mixture was ethanol precipitated, washed with 70 % ethanol, dried and resuspended in 5 ul of ionized water. Half of the resuspended ligation mixture was used in electroporation of 100 ul of DH5α cells. Pulse for electroporation was given at 2.5 K.V voltage, 200 ohms resistance & 25 μF capacitance. After electroporation, cells were immediately shifted to SOC Medium (MgCl 2. 6H2O, MgSO4.7H2O) & grown for 1 hour. The transformants were selected on LB agar plates containing X-gal, IPTG, at 40 ug/ml concentration each and Ampicillin at the concentration of 100 ug/ml for Blue/white colony selection. White colonies analyzed contained the recombinant plasmid. The positive

clones were also confirmed through PCR amplification using forward and reverse primers. The positive clones were also confirmed through restriction digestion with EcoR I enzyme.

III. Results

To isolate and identify the gene of interest, the total genomic DNA was isolated by the method of Kronstad et al., (1983), (fig 1).

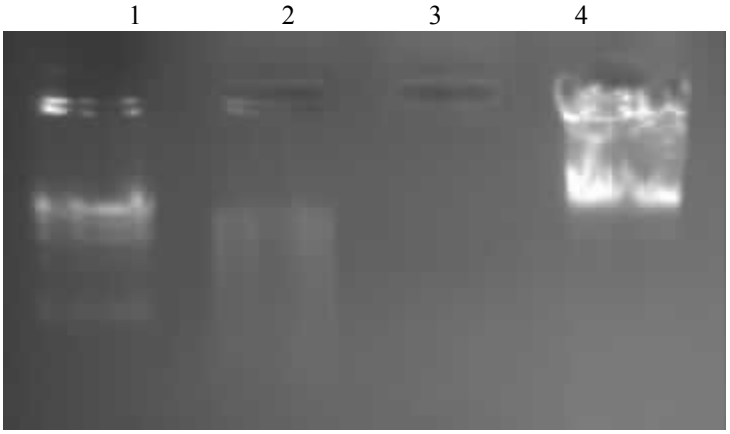

Fig 1.: Isolation of total genomic DNA from the locally isolated Bt strain 30382. Lane 1. :λ/ hind III marker, Lane 2; genomic DNA subjected to the restriction , Lane3;-ve control, Lane 4; +ve control.

To amplify the gene of interest, Polymerase Chain reaction was done by a modification by saiki et.al., (1988).

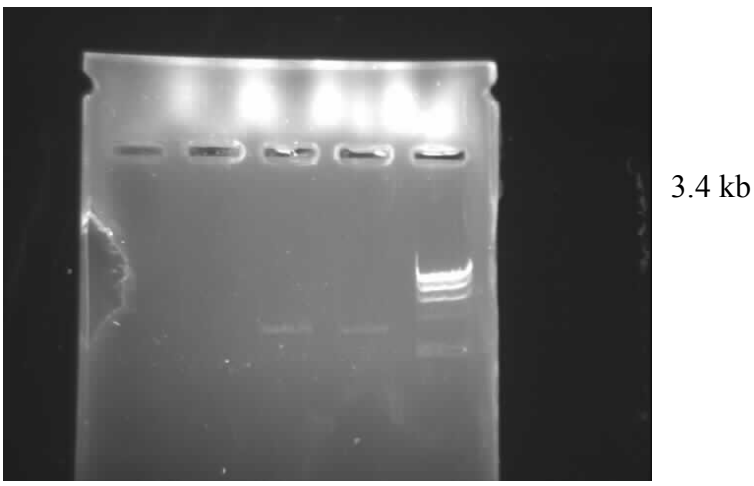

Figure 2: PCR amplification of cry3 gene, using specific primers, from the isolated genomic DNA..
Lane1 & 2: amplified 3.4 kb cry3 gene. Lane3: λ/Hind III DNA Marker.

For cloning of the delta- endotoxin gene a complete coding sequence including initiations as well as termination codons was amplified. A number of PCR reactions were performed. The PCR product was run through agarose gel. The 3.4 kb fragment was eluted from gel using DNA purification kit (QIAGEN). PCR product was cloned in pGEMT vector (Promega) (Fig3), specially designed for cloning of the PCR products with A-overhanging at 5' terminal. Ligation was followed by transformation through electroporation in *E.coli* strain DH5α. Transformants were selected on LB agar plates which contained ampicillin (100ug/ml), X-gal (40ug/ml), and IPTG (40ug/mg), for blue /white colony selection.

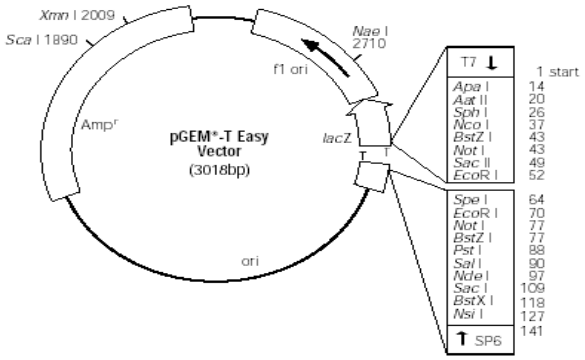

Fig: 3. Restriction map of pGEM-T vector, showing restriction sites for EcoR1.

The positive clones were also confirmed through PCR amplification using forward and reverse primers. The positive clones were also confirmed through restriction digestion with *EcoR I* enzyme (fig 4).White colonies analyzed contained recombinant plasmids. Positive clones were confirmed by PCR amplification of the gene cloned, using reverse and forward primers (Fig 4). The positive clones were also confirmed through restriction digestion with *EcoR1.*

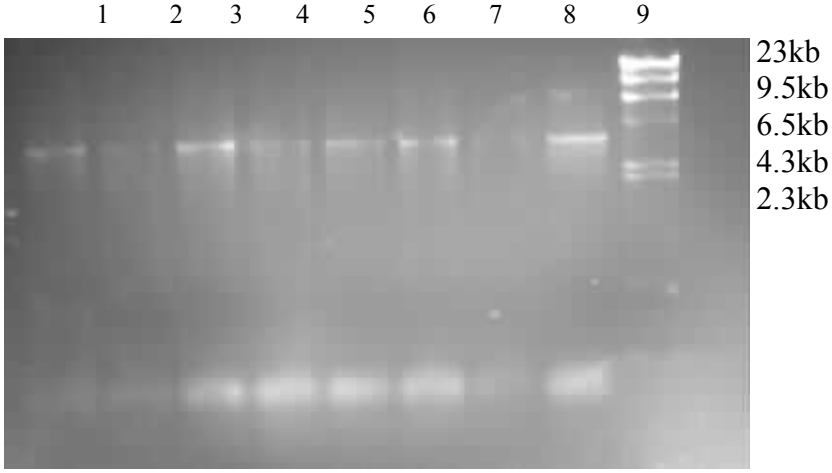

Fig 4: Confirmation of cry3 gene within the clones through PCR, using specific primers. Lane 1-8, amplified 3.4 kb cry3 gene, Lane 9:λ/ hind III marker

The DNA from a selected clone was used as a template with 2 oligonucleotide primers for the full length sequencing of the gene through automated DNA sequencing system. The nucleotide sequence comparison of the cloned gene with cry3f gene sequence present in gene data bank (Gene bank Accession number U04366) showed homology between two genes with some significant nucleotide changes. Homology study studies of the sequencing data with the gene sequences present in gene data bank showed that it was a variant of a novel CryIII protein gene reported by Sato *et al*. (1994).

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POF1: 1                taatcaaaatgagtatgaaattatagatgctttatcacccacttctgta      50
                       ||||||||||||||||||||||||||||||||||||||||||||||||
U04366 1    atgagtccaaataatcaaaatgagtatgaaattatagatgctttatcacccacttctgta      60
 PCF1: 51    tccgataattctattagatatcctttagcaaacgatcaaacgaacacattacaaaacatg     110
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366 61   tccgataattctattagatatcctttagcaaacgatcaaacgaacacattacaaaacatg     120
 PCF1:  111  aattataaagattatctgaaaatgaccgaatcaacaaatgctgaattgtctcgaaatccc     170
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366 121 aattataaagattatctgaaaatgaccgaatcaacaaatgctgaattgtctcgaaatccc     180
 PCF1: 171   gggacatttattagtgcgcaggatgcggttggaactggaattgatattgttagtactata     230
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366 181 gggacatttattagtgcgcaggatgcggttggaactggaattgatattgttagtactata     240
 PCF1:  231   ataagtggtttagggattccagtgcttggggaagtcttctcaattctgggttcattaatt     290
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366 241  ataagtggtttagggattccagtgcttggggaagtcttctcaattctggqttcattaatt     300
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PCF1:  291   ggcttattgtggccgtcaaataatgaaaatgtatggcaaatatttatgaatcgagtggaa   350
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
004366 301   ggcttattgtggccgtcaaataatgaaaatgtatggcaaatatttatgaatcgagtggaa   360
PCFl:   351       gagctaattgatcaaaaaatattagattctgtaagatcaagagccattgcagatttagct   410
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366  361       gagctaattgatcaaaaaatattagattctgtaagatcaagagccattgcagatttagct   420
PCF1:   411       aattctagaatagctgtagagtactatcaaaatgcacttgaagactggagaaaaaaccca 470
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U004366  421      aattctagaatagctgtagagtactatcaaaatgcacttgaagactggagaaaaaaccca 480
PCF1:   471       cacagtacacgaagcgcagcacttgtaaaggaaagatttggaaatgcagaagcaatttta        530
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366  481       cacagtacacgaagcgcagcacttgtaaaggaaagatttggaaatgcagaagcaatttta        540
PCF1:   531       cgtactaacatgggttcattttctcaaacgaattatgagactccactcttacccacatat   590
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366  541       cgtactaacatgggttcattttctcaaacgaattatgagactccactcttaccoacatat   600

PCF1: 591         gcacaggccgcctctctgcatttgcttgtaatgagggatgttcaaatttacgggaaggaa 650
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366  601       gcacaggccgcctctctgcatttgcttgtaatgagggatgttcaaatttacgggaaggaa 660
                                ★                    ★
U04366: 651  tggggatatcctct**t**aaaatgatattgacctattttataaa**c**aacaagtatcttatacggct   710
             ||||||||||||  ||||||||||||||||||||||  ||||||||||||||||||
U04366 661   tggggatatcctc**a**aaaatgatattgacctattttataaa**t**aacaagtatcttatacggct   720
PCF1:   711       agatattccgatcattgcgtocaatgqtacaatgctggtttaaataaattaagaggaacg   770
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366  721       agatattccgatcattgcgtocaatggtacaatgctggtttaaataaattaagaggaacg   780
PCF1:
U04366  781       ggtgctaagcaatgggtggattataatcgtttccgaagagaaatgaatgtgatggtattg   840
PCF1:
004366 841        gatctagttgcattatttccaaactacgatgcgcgtatatatccactggaaacaaatgca   900
PCF1:
U04366 901        gaacttacaagagaaattttcacagatcctgttggaagttacgtaactggacaatcgagt   960
PCF1:
004366 961        acccttatatcttggtacgatatgattccagcagctcttccttcattttcaacgctcgag   1020
PCF1:
U04366 1021       aacctacttagaaaacctgatttctttactttgctgcaagaaattagaatgtatacaagt   1080
PCF1:
U04366 1081       tttagacaaaacggtacgattgaatattataattattggggaggacaaaggttaaccctt   1140
PCF1:
U04366 1141       tcttatatctatggttcctcattcaataaatatagtggggttcttgccggtgctgaggat   1200
PCF1:
U04366 1201       attattcctgtgggtcaaaatgatatttacagagttgtatggacttatataggaaggtac   1260
PCF1:
U04366 1261       acgaatagtctgctaggagtaaatccagttacttttttacttcagtaataatacacaaaaa   1320
PCF1:
004366 1321       acttattogaagccaaaacaattcgcgggtggaataaaaacaattgattccggcgaagaa   1380
PCF1:
U04366 1381       ttaacttacgaaaattatcaatcttatagtcacagggtaagttacattacatcttttgaa   1440
PCF1:
U04366 1441       ataaaaagtaccggtggtacagtattaggagtagttcctatatttggttggaogcatagt   1500
PCF1:
U04366 1501       agtgccaqtcgcaataactttatttacgcaacaaaaatctcacaaatcccaatcaataaa   1560
PCF1:
U04366 1561       gcaagtagaactagcggtggagcggtttggaatttccaagaaggtctatataatggaqga   1620
PCF1:
U04366 1621       cctgtaatgaaattatctgggtctggttcccaagtaataaacttaagggtcgcaacaqat   1680
PCF1:
U04366 1681       gcaaagggagcaagtcaaagatatcgtattagaatcagatatgcctctgatagagcgggt   1740
PCF1:
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U04366 1741 aaatttacgatatcttccagatctccagagaatcctqcaacctattcagcttctattgct 1800
PCF1:
U04366 1801 tatacaaatactatgtctacaaatgcttctctaacgtatagtacttttgcatatgcagaa 1860
PCF1:
004366 1861 tctggccctataaacttagggatttcgggaagttcaaggacttttgatatatctattaca 1920
PCF1:
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PCF1:
U04366 1981 ttatttgaagcagaagaagacctagatgtggcaaagaaagctgtgaatggcttgtttacg 2040
PCF1:
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PCF1:
U04366 2101 ttaatagaatgcctatccgatgagttatacccaaatgaaaaacgaatgttatgggatgca 2160
PCF1:
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PCF1:
U04366 2221 aggattaatggagaaaacggatggacgggaagtacgggaatcgaggttgtggaaggagat 2280

PCF1:
U04366 2281 gttctgtttaaagatcgttcgcttcgtttgacaagtgcgagagagattqatacagaaaca 2340
PCF1:
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U04366 2821 aaacgtgcagcatcagaaaaaacatattatgcagcaaagcaagccattgatcgtttattc 2880
★ ★
PCF1: 2866 gcagatta-tcaagaccaaaaacttaattctggtgtcgaaatgtcagatttgttggcagcc 292
|||||||| |||||||||||||||||||||
U04366 2881 gcagattatcaagaccaaaaacttaattctggtgta—gaaatgtcagatttgttggcagc 294
PCF1: 2926 caaaaccttgtacagtccattccttacgtatataatgatgcgttaccggaaatccctgga 2985
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U04366 2941 caaaaccttgtacagtccattccttacgtatataatgatgcgttaccggaaatccctgga 3000
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PCF1: 2986 atgaactatacgagttttacagagttaacaaatagactccaacaagcatggaatttgtat 3045
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PCF1: 3046 gatcttcaaaacgctataccaaatggagattttcgaaatggattaagtaattggaatgca 3105
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366 3061 gatcttcaaaacgctataccaaatggagattttcgaaatggattaagtaattggaatgca 3120
F1: 3106 acatcagatgtaaatgtgcaacaactaagcgatacatctgtccttgtcattccaaactgg 3165
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366 3121 acatcagatgtaaatgtgcaacaactaagcgatacatctgtccttgtcattccaaactgg 3180
PCF1: 3166 aattctcaagtgtcacaacaatttacagttcaaccgaattatagatatgtgttacgtgtc 3225
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366 3181 aattctcaagtgtcacaacaatttacagttcaaccgaattatagatatgtgttacgtgtc 3240

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PCF1: 3226      acagcgagaaaagagggagtaggagacggatatgtgatcatccgtgatggtgcaaatcaq      3285
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366  3241    acaqcgagaaaagagggagtaggagacggatatgtgatcatccqtqatggtgcaaatcag      3300
PCF1: 3286      acagaaacactcacatttaatatatgtgatgatgatacaggtgttttatctactgatcaa    3345
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U043663301      acagaaacactcacatttaatatatgtgatgatgatacaggtgttttatctactgatcaa      3360
PCF1: 3346      actagctatatcacaaaaacagtggaattcactccatctacagagcaagtttggattgac  3405
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U04366 3361     actagctatatcacaaaaacagtggaattcactccatctacagagcaagtttggattgac  3420
PCF1: 3406      atgagtgagaccqaaggtgtattcaacatagaaagtgtagaactcgtgttagaagaagag      3465
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
004366 3421     atgagtgagaccgaaggtgtattcaacatagaaagtgtagaactcgtgttagaagaagag      3480
PCF1:   3481 taa  3483
                |||
U04366  3481    taa  3483
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Figure 15: Comparison of *cry* gene cloned from local Bt. isolate 30382 with the published sequence of *cryIIIF* gene (Gene bank Accession#U04366)

IV. Discussion

According to Adang *et al*. (1993), over 60 cry genes encoding 26 distinct insecticidal crystal proteins have been sequenced, whereas Baum and Malver (1995) found that over 90 ICP genes have now been cloned and sequenced. Thompson *et al*; (1995) compared the primary sequence of 50 full length toxins and produced a computer-generated dendrogram of possible evolutionary relatedness.

A number of toxin genes effective against lepidopteran, coleopteran and diapteran larvae from different strains of Bt. have been cloned & expressed in *E. coli*, *Bacillus subtilis,* Pseudomonas, clavibacter, Baculovirus, and nuclear polyhederosis virus (Haider *et. al*., 1988).

Screening for novel activities through biotoxicity assays alone may not be the most effective approach, although in practice worldwide. Since certain cry genes are poorly expressed (or even silent) in their native hosts (Lee and Aronson, 1991; Aronson *et al.,* 1991; Sanchis *et al*., 1989; Chaambers *et al*., 1991). Alternative screening methods include southern blotting in search of known homologous genes (Kronstad and Whiteley *et al*., 1986), analysis of reactivity to different monoclonal antibodies (Hofte *et al*., 1998) and electrophoretic analysis of PCR products using specific primers (Carozi *et al.,* 1991).

The availability of toxin genes permitted bioassays of individual crystal proteins to study their spectrum of insecticidal without interference from other toxins or other pathogenecity factors (spores, beta exotoxins etc).

The *Bacillus thuringiensis* strain 30382 was analyzed through gene cloning and sequencing. From the amino acid sequence of that gene, a set of primers was designed to amplify the full length cry gene. The amplified gene was cloned in a cloning vector, pGEM-T. Restriction analysis of the cloned fragment also confirmed the presence of cry3 gene within the clones. Southern blotting of the total DNA from isolate 30382 digested with Hind III enzyme showed that a cry3 type gene was present on approximately 4 kb fragment.Therefore southern hybridized 4 kb fragment might be the full length cry3f gene containing promoter, ribosomal binding sites other than regulatory sequences and 5′& 3′ flanking sequences from isolate 30382. Homology studies of the sequences present in the gene data bank showed it a variant of a novel *Cry III* protein gene reported by Sato *et al.,* (1994). Homology studies between two nucleotide sequences showed two changes at nucleotide 676 and 701 from 5' end. This change in nucleotide level was not affecting the protein sequence of the gene due to codon degeneracy at amino acid phenylalanine and proline. Two other significant deletion and insertion of nucleotides at position 2888 and 2909 of holotype cry F gene (Genebank Accession # U04366) near 3' end were observed. These changes were causing change in stretch of amino acids from valine, tyrosine, serine, glutamine, in holotype cry3 F to glycine, phenylalanine, alanine, asparginine respectively in the clone from CAMB isolate 30382. This change may have a drastic effect on the toxicity spectrum on the cry protein isolated from the local 30382, in comparison with the cry protein from strain *Buibui.* serovar *japonesis*. The cry3f gene has been reported to encode proteins of 129 kDa, which occurs in Bt. serovar *japonesis* (Ogiwara *et al*., 1995). The nucleotide sequencing of the gene cloned in present study was of more interest, hence it may provide more insight into the structure and function of this class of genes, because very small differences in crystal structure, including the presence or absence of protease processing sites, can have deleterious or beneficial effects on the toxicity of molecules (Ward *et al*., 1988; Haider and Eller 1989; Wu and Aronson1992). Newly found variations may further be helpful to extend the host spectrum of the cryIIIF type gene.

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