Cloning and characterization of an insecticidal crystal protein gene from *Bacillus thuringiensis* subspecies *kenyae*

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Abstract

A sporulating culture of *Bacillus thuringiensis* subsp. *kenyae* strain HD549 is toxic to larvae of lepidopteran insect species such as *Spodoptera litura*, *Helicoverpa armigera* and *Phthorimaea operculella*, and a dipteran insect, *Culex fatigans*. A 1.9-kb DNA fragment, PCR-amplified from HD549 using *cryII*-gene-specific primers, was cloned and expressed in *E. coli*. The recombinant protein produced 92% mortality in first-instar larvae of *Spodoptera litura* and 86% inhibition of adult emergence in *Phthorimaea operculella*, but showed very low toxicity against *Helicoverpa armigera*, and lower mortality against third-instar larvae of dipteran insects *Culex fatigans*, *Anopheles stephensi* and *Aedes aegypti*. The sequence of the cloned crystal protein gene showed almost complete homology with a mosquitocidal toxin gene from *Bacillus thuringiensis* var. *kurstaki*, with only five mutations scattered in different regions. Amino acid alignment with different insecticidal crystal proteins using the MUTALIN program suggested presence of the conserved block 3 region in the sequence of this protein. A mutation in codon 409 of this gene that changes a highly conserved phenylalanine residue to serine lies in this block.

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Introduction

Subspecies of *Bacillus thuringiensis*, a sporulating bacterium, produce a wide range of insecticidal crystal proteins. These have been grouped on the basis of their toxicity spectrum for a class of insects (Hofte and Whiteley 1989; Schnepf *et al*. 1998). These proteins are encoded by *cry* genes. Many of these genes have been cloned and expressed in different organisms. Some of the genes are cryptic (Dankocsik *et al*. 1990) and have not been studied for their insecticidal properties. Sporulating cultures of *B. thuringiensis* subspecies *kenyae* (HD549), *B. thuringiensis* subspecies *kurstaki* (HD709) (Amonkar *et al*. 1985) and *B. thuringiensis* subspecies *kurstaki* (HD1) (Kronstad *et al*. 1983) have been tested for their insecticidal spectra. Compared to isolated crystal protein from HD1, proteins from HD549 and HD709 showed higher activity against larvae of *Spodoptera litura*, *Phthorimaea operculella* and *Helicoverpa armigera* (Amonkar *et al*. 1979, 1985; Kulkarni and Amonkar 1988a). The spores of HD549 were also shown to be toxic to mosquito larvae (Kulkarni and Amonkar 1988a). The crystal protein profiles of HD549 and HD1 were very similar but that of HD709 was different. The mosquitocidal property of different strains of *B. thuringiensis* was shown to be mainly due to presence of the *cryIV* or *cryIIA* (old nomenclature) genes, or both (Schnepf *et al*. 1998). X-ray structures of several insecticidal crystal proteins have been solved (Li *et al*. 1988; Li *et al*. 1991; Borisova *et al*. 1994; Grochulski *et al*. 1995; Galitsky *et al*. 2001). Amino acid alignment of different Cry proteins has revealed the presence of eight conserved blocks in the proteins of size 135–140 kDa but only the first five blocks in the proteins of size 60–75 kDa. Structural studies on these proteins

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have shown that the first five blocks are part of the N-terminal functional domains I, II and III, and the rest of the amino acid blocks lie in the C-terminal half of the proteins in the size range 135–140 kDa. The block 3-type conserved amino acid region is located between domains II and III of the protein (Schnepf *et al*. 1998). Block 3 has not been hitherto identified in Cry2 class of proteins. Here we report cloning and sequencing of a *cry2*-type gene from HD549, its expression in *E. coli* BL21 (DE3) pLysS, and the insecticidal profile of the synthesized protein against a few lepidopteran and dipteran insects. The gene sequence (GenBank accession number AF047038) is highly homologous to that of *cry2B2* (a mosquitocidal toxin gene) from *B. t.* var. *kurstaki* (Donovan *et al*. 1988), with only five base differences. Four of them change amino acids located in functional domains of the protein while the fifth is a silent mutation. Amino acid alignment of this protein with the block 3 residues of different Cry proteins suggested the presence of block 3 sequence (Schnepf *et al*. 1998) between domains II and III. A mutation that changes the conserved phenylalanine at position 409 to serine (F409S) lies within this block (Liang and Dean 1994).

Materials and methods

***Bacterial strains, plasmids and media*:** *B. thuringiensis* subspecies *kurstaki* (HD1) (Kronstad *et al*. 1983) was obtained from the United States Department of Agriculture (USDA). *B. thuringiensis* subspecies *kenyae* (HD549) and *B. thuringiensis* subspecies *kurstaki* (HD709) were isolated at the Bhabha Atomic Research Centre, Mumbai (Amonkar *et al*. 1985). The *E. coli* strain HB101 was used for maintaining the cloned gene and *E. coli* BL21 (DE3) pLysS was used for expression of the gene. The plasmid vectors pBluescript SK+ (Stratagene), pET3a (New England Biolabs) and pET28a+ (Novogen) were used. *E. coli* and *B. thuringiensis* were grown as batch cultures in LB broth (Miller 1972) with shaking at 37°C and 30°C respectively. *E. coli* harbouring pBluescriptSK+ and pET3a and their derivatives were grown in presence of ampicillin (100 μg/ml) and *E. coli* with pET28a+ and its derivatives were grown in presence of kanamycin (50 μg/ml).

***DNA isolation and Southern hybridization*:** Genomic DNA was prepared from actively growing cultures at 30°C following a procedure described earlier (Tuli *et al*. 1989). Large-scale and miniprep plasmid DNA from *E. coli* were prepared using protocols described in Sambrook *et al*. (1989). Total genomic DNA from different strains of *B. thuringiensis* was digested with restriction enzymes and the fragments were separated by electrophoresis on 0.8% agarose gel. The DNA fragments were transferred to nylon membrane, prehybridized for 2 h at 65°C in 6× SSC containing 100 μg/ml denatured herring sperm DNA, 5× Denhardt's solution and 0.5% SDS. The *cryII* gene fragment (25 ng) was labelled using the 'Multiprime labelling kit' from the Board of Radiation and Isotopes Technology, Department of Atomic Energy, India, and the labelled probe was added to the prehybridization mixture. Hybridization was carried out for a further period of 18 h. The membrane was washed and the autoradiogram was developed as described in Sambrook *et al*. (1989).

***PCR amplification*:** The 1.9-kb *cry* gene was amplified by PCR using total DNA from different strains of *B. thuringiensis* as templates and *cry*-specific primers (forward primer, 5′ GGA TCC ATA TGA ATA GTG TAT TGA AT 3′; and reverse primer, 5′ GTA CGG ATC CTA CTC AAA CCT TAA TAA 3′). PCR was carried out in a Thermo Hybaid thermal cycler in 100 μl reaction volume containing 50 ng DNA template, 0.5 μM of each primer, 100 μM deoxynucleoside triphosphate, 1× *Taq* DNA polymerase buffer and 2.5 U *Taq* DNA polymerase (Roche Molecular Biochemicals), and involved a denaturing step of 5 min at 94°C followed by 25 cycles of 2 min at 94°C, 1 min at 50°C and 2 min at 72°C.

***Cloning and expression in E. coli*:** The standard recombinant DNA techniques used in cloning were according to Sambrook *et al*. (1989). In brief, the ends of the PCR-amplified 1.9-kb DNA were recessed with T4 DNA polymerase and ligated at the *Sma*I site in pBluescript SK+ to yield pCryII. The 1.9-kb *Nde*I–*Bam*HI fragment of pCryII was subcloned at compatible sites in pET3a under the control of T7 promoter and the resultant recombinant plasmid was named pET*cry*II. The pET*cry*II was transferred to *E. coli* BL21 (DE3) pLysS for expression. For this an overnight culture of recombinant *E. coli* was diluted 1 : 100 in fresh 2× LB broth containing ampicillin (100 μg/ml) and grown at 37°C to A_{600} of 0.4. Isopropyl *b*-thio-D-galactopyranoside (IPTG, 0.5 mM) was added and growth continued for a further period of 5 h. The cells were then lysed and expression monitored by gel electrophoresis or bioassay.

***Bioassay of insecticidal activity*:** *E. coli* BL21 containing pET*cry*II was induced with IPTG. The cell pellet was washed thoroughly with feeding 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 soluble protein were tested for insecticidal activity against larvae of the lepidopterans *Spodoptera litura*, *Phthorimaea operculella* and *Helicoverpa armigera*. The toxicity against neonate larvae was tested either by incorporation in diet or by topical application on food substrates as

described in Ratnakar *et al*. (1994). In case of *S. litura* and *H. armigera*, the toxicity was scored on the basis of mortality of larvae exposed to cell extracts. *P. operculella* larvae were fed for a few hours on tubers surface-treated with cell extracts and after they had entered into the tuber they were further incubated to allow completion of full larval period in concealment; toxicity was scored on the basis of reduction in number of emerging adults. Cell-free extract of the culture of recombinant *E. coli* was used for testing mosquitocidal activity against three mosquito species *Culex fatigans*, *Anopheles stephensi* and *Aedes aegypti* as described in Menon *et al*. (1982). The assays were conducted in 100-ml glass beakers containing 25 ml tap water. In each test suspension 10 third-instar larvae were used, with three replicates for each treatment of 100 µg/ml, 50 µg/ml and 20 µg/ml of protein. Cell-free extracts of the culture of *E. coli* host BL21 containing no pET*cry*II and buffer alone prepared in a similar way as the test samples were used as controls. Mortality was recorded after 48 h of treatment.

***DNA sequencing*:** The 1.9-kb DNA fragment which was PCR-amplified using gene-specific primers was sequenced using an automated DNA sequencer (Applied Biosystems ABI50 version 3.0) at the Molecular Resource Facility of the University of Medicine and Dentistry of New Jersey, USA. The entire 1.9-kb gene was sequenced (GenBank accession number AF047038) using a series of internal primers. The translated amino acid sequence of the gene was submitted to *Bacillus* Genetic Stock Center (BGSC), Columbus, USA. The protein sequence was named Cry2Aa4 and the gene was reclassified as *cry2Aa4* according to the revised nomenclature (Crickmore *et al*. 1998).

Results

Cloning and expression of crystal protein gene from Bacillus thuringiensis HD549

Genomic DNA from strains HD1, HD549 and HD709 of *B. thuringiensis* was digested with *Eco*RI and *Hin*dIII and subjected to Southern hybridization using *cryIIA*-specific probes. The *cryII* gene was present in HD1 and HD549 and absent in HD709 (figure 1). This gene was PCR-amplified from genomic DNA of HD549 and HD1 using *cryII*-specific primers (Widner and Whiteley 1989). A single product of 1.9 kb was amplified from HD1 and HD549 genomic DNA (data not presented) and the PCR-amplified fragment from HD549 was first cloned in pBluescript SK+ and then subcloned in pET3a and pET28a+ for expression in *E. coli*. *E. coli* BL21 (DE3) pLysS was transformed with the recombinant plasmid pET*cry*II. Total protein was analysed on 8% SDS polyacrylamide gels. High level of synthesis of a protein of around 70 kDa in IPTG-induced cultures, which was absent in the control samples, was evident (figure 2). From densitometric scanning of the gel we estimate that this protein constitutes around 5% of the total protein in the host *E. coli*. The size of the protein agrees with the size of the gene (1.902 kb).

Host range of the cloned cryII (cry2Aa4) gene

Cell-free extract from IPTG-induced culture of BL21 (DE3) harbouring pET*cry*II was used to assess toxicity of the recombinant Cry2Aa4 protein against three lepidopteran and three dipteran insect species. The results (table 1) showed that the transformed *E. coli* made a biologically active insecticidal crystal protein. Lysates

Table 1. Toxicity of transgenic *E. coli* expressing *cry2Aa4* gene to three lepidopteran insect larvae.

Treatment	Amount of protein (mg)	Toxicity		
		S. litura[1] (% mortality)	*H. armigera*[2] (% mortality)	*P. operculella*[3] (% adult emergence)
No treatment	0	0	0	93
Sample buffer control	0	0	> 10	> 90
E. coli BL21 (DE3) pLysS	2.5	< 10	10	> 90
E. coli BL21 (DE3) pLysS	5.0	98.66	40	14
(pETcryII)	2.5	92.56	25	15
	1.0	47.75	0	70

The values given are averages of at least three replicates. The variations among the replicates were less than 10%. Per cent mortality in case of *S. litura* and *H. armigera* was calculated on the basis of the proportion of larvae killed upon treatment from the total larvae taken in the experiment. Per cent adult emergence for *P. operculella* is defined as percentile of the proportion of adult emergence from the treated samples with respect to control.
[1]Twentyfive first-instar larvae per replicate.
[2]Fifteen replicates of single second-instar larvae per experiment.
[3]Twentyfive eggs were taken for each of three replicates for both treated and control samples.

tested at 2.5 mg of total protein per replicate resulted in greater than 90% mortality of first-instar larvae of *Spodoptera litura* and 86% inhibition of adult emergence in *Phthorimaea operculella*. However, the protein was less toxic to *Helicoverpa armigera* with only a moderate effect up to 5 mg of total protein. Toxicity was insignificant with uninduced culture lysate and the feeding buffer controls. The protein was partially purified by hexahistidine affinity column chromatography and was tested for toxicity against all the three lepidopteran insect larvae. Qualitatively similar results were obtained though the amount of purified protein required to kill 50% of larvae was less. The amount of partially purified toxin required for 50% killing of these larvae was in the range of 100–500 µg while the amount of total protein from recombinant *E. coli* which showed significant toxicity was in the range of 2.5–5 mg for different lepidopteran larvae. Mosquitocidal toxicity results suggest that this protein is highly toxic to larvae of all the three mosquito species tested (figure 3).

DNA sequencing and sequences analysis

Both strands of the full-length PCR product were sequenced to get the sequence of 1937 nucleotides. Nucleotide sequence analysis using PCGENE software revealed an open reading frame of 1902 nucleotides coding for a protein of 633 amino acids with an estimated molecular mass of 71 kDa. Homology search revealed that this gene has very high similarity (99.74%) at DNA level with other reported mosquitocidal toxin genes, viz. *cry2Aa1* (Dankocsik *et al*. 1990) and *cry2Aa2* (Widner and Whiteley 1989). Alignment of the nucleotide sequence of *cry2Aa2* with our sequence (*cry2Aa4*) revealed five nucleotide differences at positions 8, 1226, 1515, 1622 and 1762, which correspond to codons 3, 409, 505, 541 and 588, respectively. The nucleotide position 1515 has a silent mutation. Hence there are differences at only four positions, viz. 3, 409, 541 and 588, in the primary sequence of Cry2Aa4 protein with respect to Cry2Aa2 and Cry2Aa1 proteins. Of the four altered amino acids, at least one, at position 409, is part of the host specificity determining region as defined in Cry2Aa1 toxin (Schnepf *et al*. 1998).

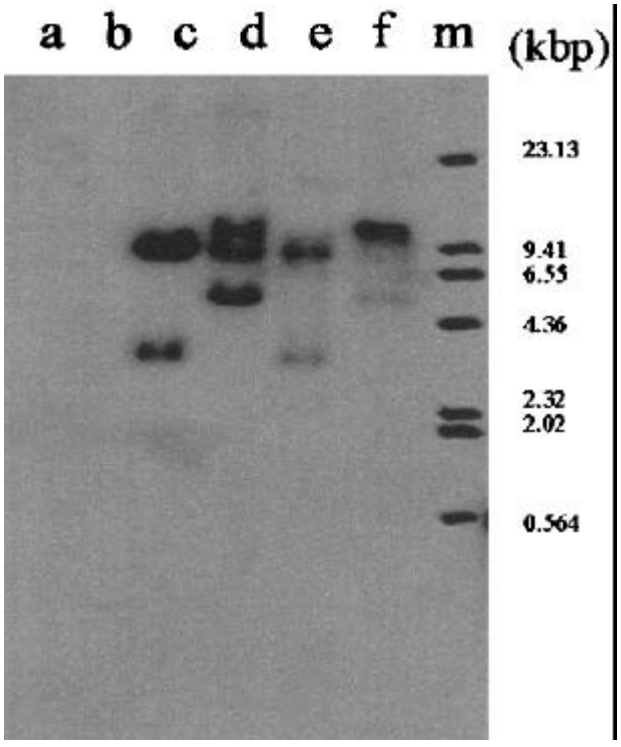

Figure 1. Southern hybridization of genomic DNA from different strains of *Bacillus thuringiensis*. Genomic DNA from HD709 (a and b), HD1 (c and d) and HD549 (e and f) was digested with *Eco*RI (a, c and e) and *Hin*dIII (b, d and f), electrophoresed on 0.8% agarose gel, and blotted on positively charged nylon membrane for hybridization with *cryII* gene probe. Lane m, molecular size markers.

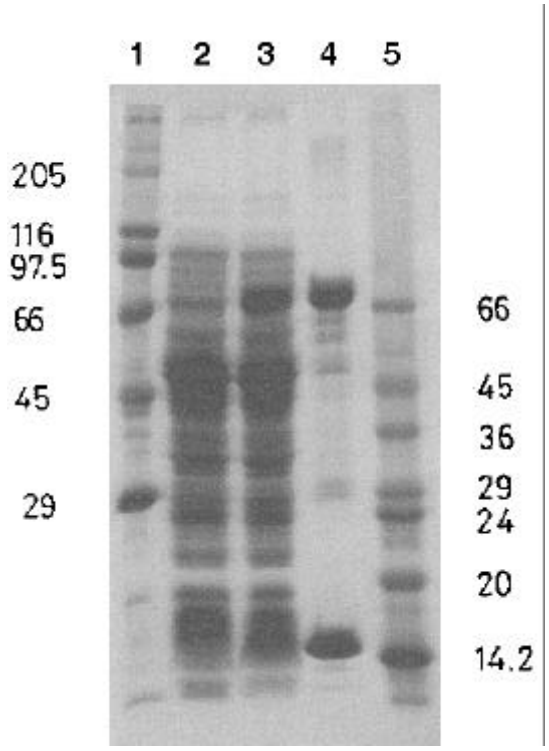

Figure 2. SDS polyacrylamide gel electrophoresis of total protein of *E. coli* containing recombinant plasmid bearing *cry2Aa4* gene from *Bacillus thuringiensis* HD549. The recombinant *E. coli* was induced with IPTG and total protein was extracted with SDS loading dye and separated on SDS polyacrylamide gel. Lane 1, molecular size markers, SDS-6H; lane 2, extract from uninduced recombinant cells; lane 3, extract from recombinant cells induced with IPTG; lane 4, partially purified recombinant protein; lane 5, molecular size markers, SDS-7.

Multiple alignment of amino acid blocks for identification of block 3

We used the MUTALIN program with the unite.dat symbol comparison table to obtain a dendrogram (figure 4A) of Cry2A-type proteins and Cry2Aa4. Our protein clusters with Cry2Aa type of proteins. Schnepf *et al*. (1998) have discussed structural and sequence similarities among insecticidal crystal toxins and identified conserved blocks of amino acids among different toxins. The block 3 conserved region was identified in Cry1 and Cry3 types of proteins and shown as a string of 50 amino acid residues lying between domains II and III of these proteins. In Cry3 these residues are located between positions 492 and 542 and in Cry1 they are present between positions 451 and 501. On alignment, these amino acids from Cry1 and Cry3 showed identity at about 50% of the residues and similarity at another 6%. They form *b*-pleated sheet in the three-dimensional structure (Li *et al*. 1988; Borisova *et al*. 1994). In Cry2A presence of block 3 remains ambiguous. In an effort to identify block 3 in Cry2Aa4 protein, we took the above block of 50 amino acids from Cry1 and Cry3 type proteins and aligned them with the amino acid sequence of the Cry2 protein using the risler.dat symbol comparison table. Risler.dat is an efficient scoring matrix for aligning distantly related sequences with similar structure and function (Risler *et al*. 1988). In the Cry2 protein the amino acid residues between positions 398 and 465 showed identity at 50% of the residues with the conserved amino acid sequence of block 3 from Cry1 protein and at 52% of residues with Cry3 protein. We suggest that the amino acids between positions 398 and 465, which lie between blocks 2 and 4, form block 3 in Cry2 proteins. The alignment of the amino acid residues of different Cry proteins for identification of block 3 conserved residues is shown in figure 4B.

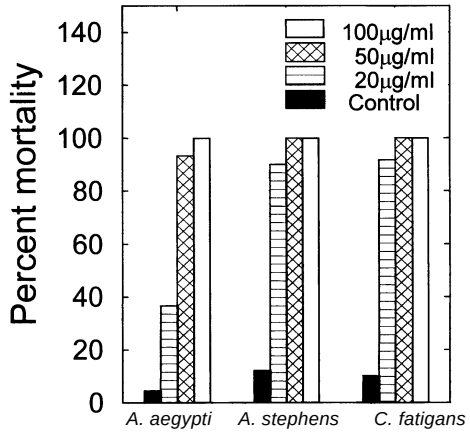

Figure 3. Larvicidal activity of recombinant *E. coli* expressing *cry2Aa4* gene from *Bacillus thuringiensis* HD549 against *Aedes aegypti*, *Anopheles stephensi* and *Culex fatigans* mosquitos. Cell-free extracts of cultures of transgenic and control *E. coli* were used for the toxicity assay. Three replicates were taken with 10 larvae each and the observations were recorded after 48 h of incubation under laboratory conditions as described in Materials and methods.

Discussion

We have reported identification of a *cryII* gene (*cry2Aa4*) from a local isolate (HD549) of *Bacillus thuringiensis* var. *kenyae*, its cloning and expression in *E. coli*, and its nucleotide sequencing. This gene is 1902 basepairs long and has 99.74% DNA sequence homology with *cry2Aa1* (Crickmore *et al*. 1998) (*cryIIA*), a mosquitocidal toxin from HD1. Therefore the *cry2A*-type gene from *B. t. kenyae* HD549 was expected to have toxicity against lepidopteran and dipteran insects. This was indeed the case and the toxicity level was higher than that reported for other members of this class. Widner and Whiteley (1989) had earlier cloned *cryIIA* and *cryIIB* genes from *B. thuringiensis* subspecies *kurstaki* HD1. They had shown that the CryIIA toxin kills both lepidopteran and dipteran larvae while the CryIIB toxin is specific only for lepidopterans. The lepidopteran and dipteran host specificity region in CryIIA was defined by generating chimaeric derivatives of *cryIIA* and *cryIIB* genes and testing the toxicity of the chimaeric toxin (Widner and Whiteley 1989; Liang and Dean 1994). Our toxicity

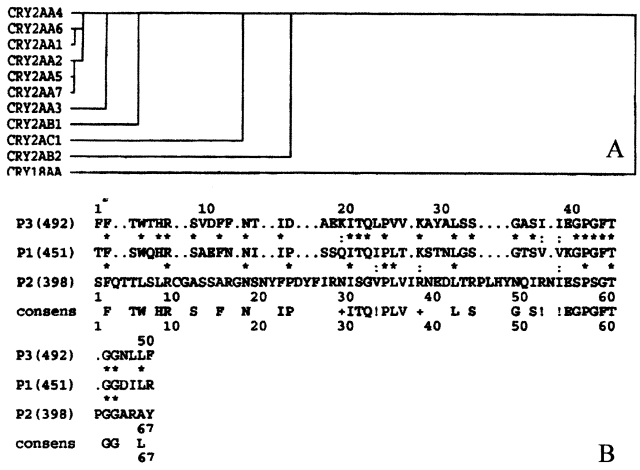

Figure 4. Identification of block 3 in amino acid sequence of Cry2Aa4. (A) Dendrogram showing the closeness in the deduced amino acid sequences of the Cry2A class (as classified in Crickmore *et al*. 1998) of proteins from different strains of *B. thuringiensis*. (B) Multiple alignment of amino acid residues from block 3 of Cry1 and Cry3 type proteins with the amino acid sequence of Cry2 protein using the risler.dat symbol comparison table with gap penalty of 5. The numbers in parentheses in each case indicate the position in the amino acid sequence. The GenBank accession numbers of the protein sequences used for multiple alignment are P3 (Cry3), accession number U31633; P2 (Cry2), accession number AF047038; and P1 (Cry1), accession number M11250.

bioassay for Cry2Aa4 showed high level of toxicity against two of the three lepidopterans and all the three dipterans examined. A sporulating culture of *B. thuringiensis* subspecies *kenyae* HD549, the host from which we cloned the gene, had shown toxicity against all the six insects tested in this study (Amonkar *et al*. 1979, 1985; Donovan *et al*. 1988). The lack of significant toxicity of the Cry2Aa4 protein against *H. armigera* suggests that the toxicity of the HD549 culture against this insect could be due to the combined effect of Cry2Aa4 and other toxins present in this strain. In an earlier study, the plasmid and crystal protein profiles of HD549 indicated that this strain is closely related to HD1 (Kulkarni and Amonkar 1988b; Kapila 1992). The differential activity of this protein against different lepidopteran insects may suggest that either the toxin has differential binding affinity with its target or its interaction with its target in different insects has differential signal transduction effects.

Alignment of the nucleotide sequences of *cry2Aa2* and *cry2Aa4* revealed five differences. Of these the mutation at nucleotide position 1226 is particularly interesting. This mutation, located in the host specificity determining region as defined in Cry2Aa1 toxin, changed the highly conserved phenylalanine at position 409 to serine (F409S). The other changes at nucleotide positions 8, 1622 and 1762 cause N3S, N541S and S588P mutations respectively in the primary structure of the protein. The dipteran and lepidopteran host specificity regions have been defined in CryIIA (Cry2Aa1) isolated from *B. t. k.* HD1 (Ge *et al*. 1991). Liang and Dean (1994) divided the toxicity domain into three host specificity regions in the CryIIA and CryIIB proteins from HD1. They generated different chimaeric CryII proteins and determined their toxicity against dipteran and lepidopteran insects. They concluded that the regions II and III, which correspond to the amino acids from 341 to 487, contribute to the lepidopteran toxicity and the regions I and II (from amino acids 278 to 412) are responsible for dipteran toxicity in CryIIA (Widner and Whiteley 1989; Liang and Dean 1994).

Matching the amino acid sequence of the Cry2Aa4 protein with block 3 conserved amino acids from Cry1 and Cry3 type of proteins tentatively identifies the block 3 in Cry2 proteins. The F409S mutation falls in the proposed block 3 region. A role for all these mutations, N3S, F409S, N541S and S588P, in enhancing the level of toxicity of this toxin for different insect larvae cannot be ruled out and further molecular and structural investigations may provide an answer to this question.

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