0273-2289/02/102–103/0213/$13.50

Phenotypic and Genetic Diversity of *Bacillus thuringiensis* Strains Isolated in India Active Against *Spodoptera litura*

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Abstract

Bacillus thuringiensis strains isolated from different agroclimatic regions of India were found to harbor *cry1* family genes. Of 831 strains 18 that were found to produce 130- and 68-kDa mol wt proteins in sodium dodecyl sulfate polyacrylamide gel electrophoresis were subjected to bioassay against second instar larvae of *Spodoptera litura*. According to the time response curve, while the highest toxic activity against *S. litura* was observed in PBT-782 with an LT_{50} of 25.46 h, strains PBT-372, PBT-574, PBT-801, and PBT-716 in descending order of merit had LT_{50} values of 36.81, 48.18, 50.35, and 73.53 h. The results of the field experiment testing the efficacy of different *B. thuringiensis* strains in controlling *S. litura* larvae infecting peanut plants showed that the chemical insecticide chlorpyriphos was the most effective in controlling *S. litura* throughout the study period. However, among *B. thuringiensis* strains, PBT-372 was superior. All the *B. thuringiensis* strains except PBT-689 were found to contain *cry1Ac1*-type gene. However, only nine strains contained *cry1Aa1* gene. While *cry1Ab1* was present only in PBT-372 and PBT-689, *cry1Ca1* was present in PBT-574, PBT-688, PBT-689, and PBT-695. *cry1Da1* was detected only in PBT-688 and PBT-692. None of the strains contained *cry1Ba1* and *cry1Ea1* genes. When polymerase chain reaction analysis using *cry1Ca1* primer was performed, PBT-695 produced an unexpected 739-bp product, which showed 33% homology with *cry1Ca1* gene between nucleotides 1819 and 2107. Our results indicated that among the field-collected *B. thuringiensis* strains, PBT-372 harbors multiple *cry*-type genes and could be employed for biological control of insects.

Index Entries: *Bacillus thuringiensis*; Indian isolates; polymerase chain reaction; *cry* genes; *Spodoptera litura*; bioassays.

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Introduction

Bacillus thuringiensis is a rod-shaped Gram-positive bacterium normally producing parasporal crystal inclusions during sporulation. These inclusions consist of toxin protein known as pesticidal crystal proteins (PCPs), which are toxic to a variety of lepidopteran, dipteran, and coleopteran insects.

The parasporal crystals comprise approximately two polypeptide chains each with an estimated molecular mass of 130 kDa *(1)*. On ingestion by the insect larvae, the high mol wt 130-kDa toxic protein is cleaved to a low mol wt 68-kDa protein by the combined effects of proteases *(2)* and alkaline pH *(3)* in the insect gut. Consequently, the brush border membrane vesicles develop lesions or pores leading to ion leakage *(4)* and adenosine triphosphate hydrolysis *(5)*, which ultimately lead to death of the larvae. Recently, Estruch et al. *(6)* have described a class of specific proteins called vegetative insecticidal proteins, which are expressed during vegetative growth of *B. thuringiensis* unlike the PCPs that are produced only during sporulation.

B. thuringiensis strains have been isolated from many habitats including soil, dust, insects, and leaves *(7–9)*. The remarkable diversity of *B. thuringiensis* strains and toxins is owing at least in part to a high degree of genetic plasticity. However, novel *B. thuringiensis* strains with an expanded host range or toxicity are highly sought after for the development of new and advanced microbial insecticides and formulations *(10)*. Notwithstanding the variability of cry proteins described up until now, it is still necessary to search for novel toxins since a sizable number of insects are not controlled by the available cry proteins. Further, it is also important to cope with the problem of development of resistance in insects.

Different methods, flagellar H-antigen serotyping for subspecies identification *(11)*, Southern blot analysis in search of known homologous genes *(12)*, analysis of reactivity to monoclonal antibodies, and electrophoretic analysis of polymerase chain reaction (PCR) products using specific primers, have been widely employed to isolate, identify, and characterize novel *cry* genes bearing *B. thuringiensis* from Taiwan *(13)* and Mexico *(14)*. Using PCR-based methodologies, attempts have been made to identify different *cry* genes in *B. thuringiensis* strains *(15)*. However, with the advent of new nomenclature *(16)*, the *cry* gene test is increasing and novel PCR primers are needed to identify some of the recently described *cry* genes.

In this article, we describe the isolation and characterization of a battery of *B. thuringiensis* strains from different ecologic regimes of the Indian subcontinent. The strategy used was based on multiplex PCR analysis with specific oligonucleotide primers meant for detection of *cry1Aa1*, *cry1Ab1*, *cry1Ac1*, *cry1Ba1*, *cry1Ca1*, *cry1Da1*, and *cry1Ea1* genes *(15)*. Although PCR analysis is considered the best choice, because it allows rapid determination of the presence or absence of a sequence and is highly sensitive, relatively fast, and can be easily used on a routine basis *(17)*, Carozzi et al. *(18)*

were of the opinion that the PCR method of screening for novel *B. thuringiensis* delta endotoxin genes is not without risk and should be used for different screening goals. We found *B. thuringiensis* strains containing a wide range of *cry* genes. In addition, one of the strains (PBT-695) contained an altered version of a gene of known subclass (*cry1Ca1*) whose product was significantly larger (739 bp) than the predicted PCR product size (288 bp). The field experiment to test the efficacy of different *B. thuringiensis* strains bearing different *cry* genes revealed that the strain PBT-372 was superior in controlling *Spodoptera litura* larvae infecting peanut crop.

Since LT_{50} values directly reflect the time taken for knockdown effects on the insect larvae, in our study the strain PBT-782 proved to be the best. However, when applied in the field, the strain PBT-372 was found to be superior next only to chlorpyriphos, a chemical insecticide. To our best knowledge, the information provided herein is the first of its kind correlating the presence or absence of specific *cry* subgroup genes in different *B. thuringiensis* strains on the LT_{50} values and their efficacy in controlling *S. litura* under field conditions.

Materials and Methods

B. thuringiensis standard strains were kindly provided by D. Zeigler (BGSC, Ohio State University) and Dr. M. Lecadet (Institut Pasteur, France).

PCR primers, reagents and cloning vectors were procured from Life Technologies, M/s. Bangalore Genei (India) (Bangalore), and Promega (Madison, WI). Media compounds and biochemicals were purchased from Sigma (St. Louis, MO) and Hi-Media (India).

Sample Collection

Soil sample collection kits, consisting of numbered sterile polyethylene bags, forms for recording the type and origin of the samples along with self-addressed envelopes, and an accompanying letter were sent to various contacts in India and received in reinforced envelopes provided by the senders. Instructions to avoid collection of soil samples from places where *B. thuringiensis* was sprayed were also sent. Moribund lepidopteran larvae collected in sterile microfuge tubes from crop fields in and around Pondicherry were transported to the laboratory and stored frozen at –20°C until further use.

Isolation of B. thuringiensis

Ten grams of soil sample was suspended in 25 mL of sterile distilled water in conical flasks and shaken vigorously for 2 min. After incubation at 60°C for 10 min, the suspension was serially diluted and spread on Katznelsons tryptose agar plates *(19)* and incubated for 3 d at 30°C *(7,20)*. The strains were observed after amido black (10%) staining under a microscope for the presence of crystal protein inclusions. After growth in Katznelsons tryptose agar, the strains were examined periodically under a

phase contrast microscope until at least 95% of the population had lysed releasing spores and crystals.

From each sample up to 20 colonies were microscopically examined for such inclusions to be classified as *B. thuringiensis*. Only colonies from each soil sample showing different morphology, crystal shape, or size were stored in 50% glycerol at –20°C for further study. For convenience, spore-impregnated filter paper discs were also prepared in sterile vials.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The spore-crystal mixture was washed thrice with 0.5 *M* NaCl, followed by three washings in cold sterile water, and resuspended in sterile water containing phenylmethylsulfonyl fluoride at a concentration of 1 m*M (21)*. These preparations were stored at –20°C until further use *(22)*. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis was carried out according to the procedure of Laemmli *(23)*. The crystal preparations (100 µg of protein) were mixed with loading dye, boiled for 3 min, and electrophoresed on a 10% gel using Mini Protean II Electrophoresis cell (Bio-rad, Hercules, CA). Gels were stained using Coomassie Brilliant Blue (R-250) stain.

Bioassays

Field-collected larvae of *S. litura* were reared on castor leaves in a sterile culture room with environmental simulation of 27 ± 1°C, relative humidity of 60 ± 5%, and a 16- to 8-h scoot/photophase regime. Castor leaves were thoroughly washed and dried under shade or a laminar flow and then coated with 2 µg/cm^2 of spore-crystal mixture *(24)*. After proper drying in shade, the leaves were placed in a sterile 10-mm-diameter Petri dish and 3-d-old (II instar) larvae of the test insect were released at 10/dish. To avoid drying of the feed material, a thin layer of 1% agar was poured up to 3–5 mm thick before placing the leaves. Thirty such insects were subjected to test for each strain, and mortality levels were recorded at 12-h intervals. Mortality data were recorded up to 5 d of exposure period, and whenever needed, the old leaves were replenished with fresh ones.

Median lethal time (LT_{50}) was calculated for differential time of the *B. thuringiensis* strain, and the regression equations were also derived with χ^2 tests. Among all the strains tested, those strains that crossed 50% mortality alone were used to estimate LT_{50} values based on Probit analysis *(25)*. Appropriate corrections were made where necessary *(26)*. The data given are the mean of two independent experiments.

PCR Analysis

Total genomic DNA isolation was done as per the method of Kronstad and Whiteley *(27)*, which was used as template for all PCR experiments. Identification of *cry*1-type genes from collected *B. thuringiensis* cultures was done by using primers suggested by Bravo et al. *(14)* and Kalman et al.

Table 1
Primer Sequences Used

cry gene	Primer	Sequence	PCR product size (bp)
cry1 (f)	gra1-cry1(d)	TGGATTTACAGGTGGGGATAT	560–590
cry1 (r)	gra1-cry1(r)	TGAGTCGCTTCGCATATTTGACT	
cry1Aa1	TY1AA	GAGCCAAGCAGCTGGAGCAGTTTACACC	724
cry1Ac1	TY1AC	TCACTTCGGATCGACATCTACC	487
cry1Ba1	TY1B	GTCAACCTTATGAGTCACCTGGGCTTC	830
cry1Ca1	TY1C	CAACCTCTATTTGGTGCAGGTTC	288
cry1Da1	TY1D	GGTACATTTAGATATTCACAGCCAC	414
cry1Ea1	TY1E	CTTAGGGATAAATGTAGTACAG	883
cry1 (r1)	TY1UNI12	ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC	—
cry1 (r2)	TY6	GGTCGTGGCTATATCCTTCGTGTCACAGC	—
cry1Ab	TY14	GAATTGCTTTCATAGGCTCCGTC	238

(15). The reaction mixture employed for identifying known *cry* genes in a PCR reaction contained the following: 200 ng of template DNA, 0.1 µ*M* each of forward and reverse primers, 1.5 m*M* $MgCl_2$, 1.25 m*M* dNTPs, and 0.5 U of *Taq* DNA polymerase in a final volume of 50 µL. PCR assays were performed with a Mastercycler® (Eppendorf) using *cry1* family primers with an initial 4-min denaturation at 94°C followed by 30 cycles of amplification consisting of a 1-min denaturation at 94°C, 1-min annealing at 52°C, and 1-min extension at 72°C. After 30 cycles, an extra extension step of 10 min at 72°C was added. The conditions for the PCRs done with other primers were similar, except that the annealing temperatures were set at 60°C with 25 cycles. The primer sequences used are given in Table 1. After the completion of the PCR reaction, the products were separated and analyzed on an agarose gel (2%).

DNA Sequence Analysis

Computer analysis was performed using a Gene Tool Lite 1.0 (Advanced Bioinformatics Solution, Biotools) package. The partial nucleotide sequence of the *cry1Cax* gene of strain PBT-695 has been submitted to GeneBank and given accession no. AF354640.

Field Performance

To evaluate the efficacy of native *B. thuringiensis* against *S. litura*, a field trial was conducted in peanut (VRI-2) at Vanur Taluk of Villupuram district (Tamilnadu) during Kharif 1999. The trial was done in randomized block design with a plot size of 5 × 4 m (20 m^2). The treatments comprising *B. thuringiensis* strains PBT-372, PBT-574, PBT-716, PBT-782, PBT-801, and standard HD-1 along with untreated control were replicated thrice. Triton X-100 was added at 0.001% as surfactant to all treatments. *B. thuringiensis*

Table 2
Probit Analysis of Time-Mortality Response
of *S. litura* to *B. thuringiensis* Strains

Strain	No. of larvae	χ^2 (n – 2)	b	LT_{50}	Feducial limit	
					Upper	Lower
PBT-372	30	1.95	7.41	36.81	32.98	40.69
PBT-574	30	18.15	8.97	48.18	34.78	59.63
PBT-716	30	8.30	9.06	73.53	69.86	77.28
PBT-782	30	1.70	5.44	25.46	22.09	28.94
PBT-801	30	9.63	8.75	50.35	42.12	59.22
HD-1	30	1.88	5.80	28.69	26.04	31.58

strains were applied as high-volume spray using a hand-operated knapsack sprayer. Three sprays were given at weekly intervals and observations were made thereafter. The number of live larvae present in 10 plants, selected randomly, in each replication on the third and seventh day after spraying was recorded.

Results and Discussion

Of a total of 831 entomopathogenic *B. thuringiensis* strains obtained from different soils under cultivation in different agroclimatic regions of the Indian subcontinent, one strain came from the moribund larvae of plume moth (*Exelastis atomosa*). Based on the size and shape of the crystal-producing *B. thuringiensis (7)*, 18 strains that produced distinct crystals and protein of mol wt 130 and 68 kDa (data not shown) were selected for further studies. As to the wider distribution of *B. thuringiensis* in different environments, various researchers have reported isolation from soil, water, sawdust, and so on *(9,14)*. With a view to screen, identify, and characterize the toxin-producing *B. thuringiensis* strains, all 18 strains were subjected to single-dose assays against second instar larvae of castor cut worm (*S. litura*). The data relating to bioassay of five *B. thuringiensis* strains, which alone were found to be effective in killing at least 50% of the laboratory-reared insect population, are given in Table 2.

Estimates of median lethal time (LT_{50}) calculated by probit analysis against *S. litura* ranged between 24.46 and 73.53 h. According to the time response curve, while the highest toxic activity against *S. litura* was observed in PBT-782 with an LT_{50} of 25.46 h, the other strains, PBT-372, PBT-574, PBT-801, and PBT-716 in the descending order of merit, had LT_{50} values of 36.81, 48.18, 50.35, and 73.53 h, respectively. The strain PBT-782 was found to be as good as standard *B. thuringiensis* HD-1.

Barbara et al. *(1)* were of the opinion that insect-specific factors of the gut juice such as pH and digestive enzymes, which decide the degree of solubilization of the crystal, vis-à-vis the generation of appropriate degradation products, were responsible for differences in insect mortality.

Helgason et al. *(28)* indicated that the stability of the crystal, which could vary from strain to strain, might be responsible for the differences in activities.

The results of the field experiment testing the effect of different *B. thuringiensis* strains in controlling *S. litura* larvae infecting peanut crop are given in Table 3. For comparative analysis, chlorpyriphos, a commonly used chemical insecticide, and standard *B. thuringiensis* HD-1 were employed in the field trial. The data in Table 3 show that by far chlorpyriphos was superior in controlling *S. litura* larvae throughout the period of observation compared to treatments receiving *B. thuringiensis* spray. The fluctuation in the larval population prior to and after spraying with chlorpyriphos was reported to be a direct reflection on the stability of the chemical insecticide sprayed *(29,30)*. All the *B. thuringiensis* strains were effective, although to varying levels under field conditions, in controlling *S. litura* larvae throughout the trial period. In the treatments, whether chemical spray or *B. thuringiensis* application, a conspicuous and statistically significant initial knockdown effect causing mortality of insect larvae was observed. The buildup in the larval population, in other words, larval resurgence, more so in *B. thuringiensis* sprays, after the initial spray could be owing to a decrease in concentration of active ingredients in the sprayed chlorpyriphos or inactivation of *B. thuringiensis* by sunlight. Because the spraying of the chemical insecticide or *B. thuringiensis* had been undertaken at shorter and frequent intervals there was a low possibility of development of resistance. However, since the larval mortality was studied only up to 44 d in the field trial and the development of resistance, if any, was not monitored, it is not known whether the field population of insect larvae had become resistant to chlorpyriphos or *B. thuringiensis* strains.

Another significant finding that emerged from the field trial was that the native *B. thuringiensis* strain PBT-372 was comparable with the international standard *B. thuringiensis* HD-1. Although the initial knockdown effect with respect to chlorpyriphos is undoubtedly superior, and this effect was maintained throughout the experimental period, the health-associated problems, environmental hazards, likelihood of this chemical insecticide entering into the food chain, biomagnification, and so forth restricts its use as a safe insecticide. It appears that *B. thuringiensis* PBT-372, with all the added advantages, offers the best scope for exploitation to serve as an alternative to the chemical insecticide.

Although *B. thuringiensis* strains were found to be effective in controlling lepidopteran insect larvae, in light of the observations that *B. thuringiensis* strains contain many subgroup *cry* genes *(16)* showing specific activity against insects, it is desirable to ascertain the preponderance and distribution of specific *cry* genes in the *B. thuringiensis* strains employed in the present study. Therefore, using PCR as a tool and employing the primers designed by Bravo et al. *(14)*, the pattern of *cry* gene distribution in the *B. thuringiensis* strains was determined; the results have clearly revealed that all the *B. thuringiensis* strains contained *cry1* genes corresponding to 560–590 bp (data not shown).

Table 3
Field Efficacy of *B. thuringiensis* Strains Against *S. litura* in Peanut[a]

Treatment	Pretreatment	First spray		Second spray		Third spray		
		3DAS	7DAS	3DAS	7DAS	3DAS	7DAS	14DAS
Control	18.7 (4.37)*	19.0 (4.41)†	18.7 (4.37)§	18.3 (4.33)‖	17.7 (4.26)‖	16.7 (4.14)‖	17.0 (4.18)§	17.7 (4.26)¶
Chlorpyriphos at 1.5 mL/L	18.3 (4.33)*	5.3 (2.37)*	7.3 (2.79)*	5.0 (2.33)*	7.3 (2.79)*	3.3 (1.95)*	4.3 (2.19)*	4.3 (2.19)*
PBT-372	17.7 (4.25)*	7.7 (2.85)*,‡	9.3 (3.13)‡	5.7 (2.48)*,‡	8.7 (3.20)*,‡	4.7 (2.26)*,‡	5.3 (2.41)*,‡	5.7 (2.48)‡
PBT-574	17.0 (4.18)*	8.3 (2.97)‡	9.7 (3.18)‡	6.0 (2.53)*,‡	9.3 (3.13)‡	5.7 (2.47)‡,†,¶	6.3 (2.60)‡,†	6.7 (2.67)‡
PBT-716	16.3 (4.10)*	8.7 (3.02)‡	13.0 (3.66)¶	8.3 (2.97)¶	12.3 (3.58)¶	7.0 (2.73)†,¶	9.0 (3.07)¶	12.0 (3.53)¶
PBT-782	18.7 (4.37)*	8.7 (3.02)‡	10.3 (3.29)‡	6.3 (2.61)‡,†	9.3 (3.13)‡	6.3 (2.61)†,¶	7.3 (2.79)†,¶	7.0 (2.73)‡,†
PBT-801	17.0 (4.17)*	9.0 (3.07)‡	12.0 (3.53)†,¶	7.3 (2.79)†,¶	11.3 (3.43)†,¶	5.7 (2.48)‡,†,¶	7.7 (2.85)†,¶	8.3 (2.97)†
HD-1	16.3 (4.10)*	8.3 (2.97)‡	10.7 (3.89)‡,†	5.3 (2.41)*,‡	9.7 (3.18)‡,†	5.0 (2.33)‡,†	5.3 (2.41)*,‡	6.7 (2.67)‡

[a]Numbers in parentheses are arc-sine transformed values. In a column, means followed by a common symbol do not differ significantly by DMRT ($p = 0.05$). DAS, days after spraying; DMRT, Duncan's multiple range test.

Table 4
Distribution of *cry*-Type Genes in *B. thuringiensis* Isolates

Location	Isolate no.	Gene types
Pondicherry	PBT-372	*Cry1Aa1, Cry1Ab1, Cry1Ac1*
	PBT-558	*Cry1Ac1*
Kanpur	PBT-569	*Cry1Aa1, Cry1Ac1*
	PBT-574	*Cry1Aa1, Cry1Ac1, Cry1Ca1*
Jodhpur	PBT-688	*Cry1Ac1, Cry1Ca1, Cry1Da1*
	PBT-689	*Cry1Aa1, Cry1Ab1, Cry1Ca1*
	PBT-690	*Cry1Ac1*
Nagpur	PBT-692	*Cry1Ac1, Cry1Da1*
	PBT-695	*Cry1Ac1, Cry1Ca1*[a]
	PBT-705	*Cry1Ac1*
Calicut	PBT-709	*Cry1Ac1*
	PBT-711	*Cry1Aa1, Cry1Ac1*
	PBT-716	*Cry1Aa1, Cry1Ac1*
Barrackpore	PBT-733	*Cry1Ac1*
	PBT-738	*Cry1Ac1*
Karnal	PBT-782	*Cry1Aa1, Cry1Ac1*
	PBT-790	*Cry1Aa1, Cry1Ac1*
Bangalore	PBT-801	*Cry1Aa1, Cry1Ac1*

[a]See text.

The presence or absence of a specific subgroup of *cry* genes in *B. thuringiensis* strains would reflect the range and spectrum of activity of these strains against lepidopteran insects.

Therefore, to delineate further the subgroup toxin genes present in different *B. thuringiensis* strains, seven specific forward and two reverse oligonucleotide primers, as suggested by Kalman et al. *(15)*, were employed to amplify specific DNA fragments. The DNA-banding pattern in all 18 strains except PBT-689 confirmed the presence of *cry1Ac1* gene (Fig. 1, Table 4). However, only nine strains contained *cry1Aa1* gene. While *cry1Ab1* was present only in PBT-372 and PBT-689 (Fig. 1, lanes 2 and 7) and *cry1Ca1* was present only in PBT-574, PBT-688, PBT-689, and PBT-695 (Fig. 1, lanes 5, 6, 7, and 10). *Cry1Da1* was detected only in PBT-688 and PBT-692 (Fig. 1, lanes 6 and 9). Surprisingly, none of the strains contained *cry1Ba1* and *cry1Ea1* genes. Various researchers *(13,14,18)* have described the presence or absence of *cry1* family genes in different *B. thuringiensis* strains collected from different countries. The predominant presence of *cry1Ac1* and *cry1Aa1* in the majority of the *B. thuringiensis* strains obtained from soil samples collected from agriculturally active zones may indicate that these genes are widely distributed among the Indian *B. thuringiensis* strains and may play an important ecologic role in the Indian subcontinent. Data on the presence of various *cry* genes in different regions of the country would be valuable *(7)*.

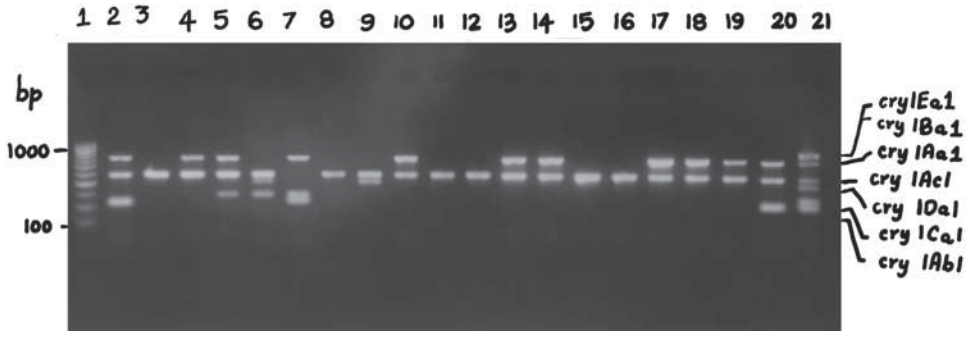

Fig. 1. PCR amplification profile of *cry*-type genes in *B. thuringiensis* strains. The PCR reactions were performed separately for each *cry* type of gene using different primers as described in Materials and Methods. Individual PCR reactions corresponding to each strain were mixed before loading on the 2% agarose gel. Lane 1, molecular weight marker; lanes 2–20, PBT-372, PBT-558, BT-569, PBT-574, PBT-688, PBT-689, PBT-690, PBT-692, PBT-695, PBT-705, PBT-709, PBT-711, PBT-716, PBT-733, PBT-738, PBT-782, PBT-790, PBT-801, and standard HD-1, respectively; lane 21, PCR-amplified products of control templates.

The astronomic increase in the number of recent submissions to the *B. thuringiensis* gene database bears testimony to the unrelenting concerted efforts made by different researchers worldwide in their endeavor to search for new and novel *cry1*-bearing *B. thuringiensis* strains *(31)*. When PCR analysis using *cry1Ca1* primers was performed, PBT-695 produced an unexpected 739-bp product, which misled us to believe that it was *cry1Aa1* product (724 bp) in multiplex PCR analysis. Subsequently, when simple PCRs were performed using *cry1Ca1*- and *cry1Ac1*-specific primers in comparison with appropriate controls, the presence of *cry1Cax* and the absence of *cry1Aa1* in PBT-695 was confirmed. This high molecular weight DNA (739 bp) against the expected 288 bp was suspected to be a putative novel gene.

Masson et al. *(32)* have suggested that altered versions of a gene of a known subclass can be detected if the PCR product is either significantly smaller or larger than the predicted size. In conformity with this suggestion, it is likely that strain PBT-695 carried some *cry1C*-specific sequences but not the subgroup *cry1Ca1* gene. Therefore, to further identify and precisely understand the locations of insertions in the *cry1Cx* gene, the 739-bp-long DNA was cloned into pGEM-T vector and sequenced. Sequence homology analysis of the 739-bp PCR product done with BLAST search (www.ncbi.nlm.nih.gov) showed 33% homology with *cry1Ca6* gene between nucleotides 1819 and 2107. Several cry proteins in the database also showed <33% homology, which is near the secondary level of divergence according to the classification proposed by Schnepf et al. *(2)*. This partial nucleotide sequence of the *cry1Cx* gene has been submitted to GeneBank (AF354640). The *Cry1C* types of genes confer insecticidal activ-

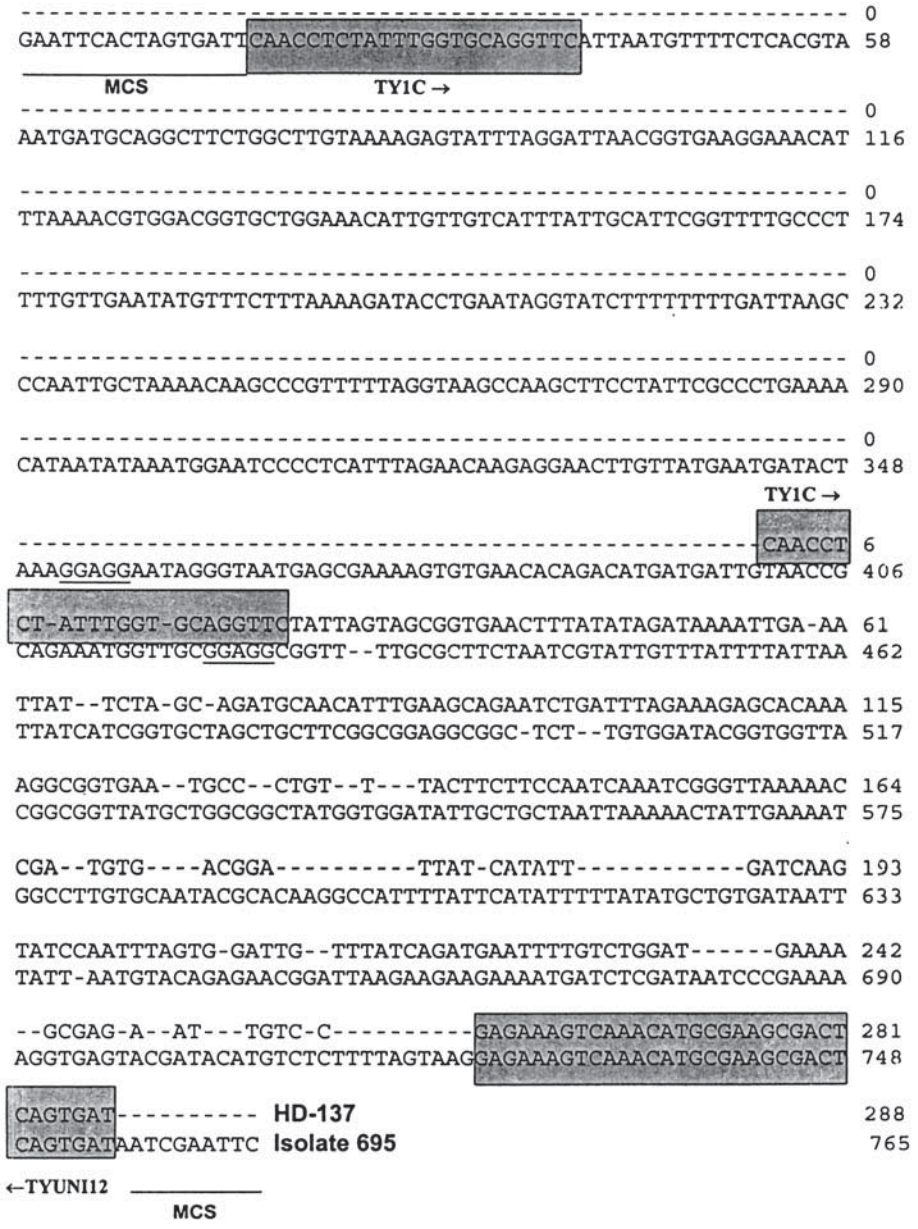

Fig. 2. Nucleotide sequence alignment of cry1Ca1 PCR products of *B. thuringiensis* standard HD137 and strain PBT-695. Shaded boxes are forward and reverse primers. RBSs are underlined.

ity against *S. exigua (30)*, *S. littorallis (17)*, and *S. litura (33)*. PCR products of various *B. thuringiensis* strains in the present study (*cry* genes) revealed that the strain PBT-695 contained *cry1Cx* and *cry1Ac* genes. Typical ribosomal-binding sites (RBSs) GGAGG *(34)* were also found at two positions, 351 and 420 (Fig. 2).

In the assay for high-level primary insecticidal activity undertaken with promising *B. thuringiensis* strains against castor cut worm (*S. litura*), we found that strain PBT-372 containing *cry1Aa1*, *cry1Ab1*, and *cry1Ac1* exhibited maximum larvicidal activity throughout the period of observation compared with PBT-716, PBT-782, and PBT-801 containing only *cry1Ac1* and *cry1Aa1* genes. Interestingly, the strain PBT-574 contained *cry1Ca1* gene besides *cry1Ac1* and *cry1Aa1* genes. However, the results of bioassays performed to determine the LT_{50} values revealed that PBT-782 had the minimum LT_{50} value (quicker toxicity) compared with PBT-372, which had an LT_{50} of 36.81 h. We do not know why PBT-782, which carried *cry1Ac1* and *cry1Aa1*, was less toxic to *S. litura* under field conditions. It is possible that the level of expression of toxin genes within the subgroup family would decide the degree of toxicity vis-à-vis larval mortality under a given set of conditions *(35)*. In conformity, Van Frankenhuyzen et al. *(36)* were of the view that even when toxin gene composition is the same, the relative expression of those genes might differ among strains. Further, Masson et al. *(37)* reported that HD-1 and NRD-12 differed in contents of the *cry1Aa2* and *cry1Ab1* toxins and raised the possibility that differences in gene expression occur within a strain under various fermentation conditions. They also postulated that such differences translate into differential toxicity toward some species but not to others, depending on the combination and ratio of toxins required for optimum toxicity of each *cry1Ax* toxin to gypsy moth and spruce budworm.

Based on the level of sensitivity of different species of *Spodoptera* to various *cry* genes, Bai *(38)* have reported that the first instar larva of *S. littoralis* was highly sensitive to *cry1Cx*, *cry1Ex*, and *cry1Abx*. By contrast, the first instar larvae were only slightly sensitive to *cry1Bx*, *cry1Dx*, *cry1Aax*, and *cry1Acx*. In the present study, all five strains employed contained *cry1Aa1* and *cry1Ac1* genes. It is likely that the presence of *cry1Ab1* and *cry1Ca1* in PBT-372 and PBT-574, respectively, led to the observed synergistic effect on the level of toxicity to *S. litura* larvae.

Differential toxicities of various *cry* genes to different *Spodoptera* spp. were also observed by Van Rie et al. *(39)*. Apparently, PCP susceptibility patterns differ for different *Spodoptera* spp. and the activity of a given PCP against a particular S*podoptera* spp. therefore cannot be extrapolated. It is not known whether the effects of sunlight and environment would have direct adverse effects on the product of *cry1Ab1* gene, thus reducing the efficacy of the strain PBT-782 against *S. litura* infecting peanut crop. It is also possible that some other *cry*-type genes that have not been detected in our PCRs could have synergistic effects with other *cry*-type genes present in our *B. thuringiensis* strains, thus exhibiting differential toxicities against *S. litura*. In conclusion PBT-372 is more toxic to *S. litura* even when compared to the standard *B. thuringiensis* HD-1 culture under field conditions and holds promise for development and use as a biopesticide.

Acknowledgments

We are grateful to the All India Council for Technical Education (no. 302/97-98/320) and the Department of Biotechnology, Government of India (no. BT/MP/01/004/93), for their financial support. We also gratefully acknowledge the Dr. K. S. Krishnan Research fellowship provided to SRP by the Department of Atomic Energy (Government of India).

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