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EXPERIMENTAL ARTICLES

Screening of New Isolates of *Bacillus thuringiensis* for *cry1* Genes and Testing of Toxicity against *Dichocrocis punctiferalis* (Family: *Pyralidae*, Order: *Lepidoptera*)[1]

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Abstract—In order to identify the more toxic novel *cry* gene, the *cry1* gene was screened in six indigenous isolates of Bt by PCR with degenerate primers showed amplification in all the Bt isolates. Subsequent screening of *cry1* subfamily gene(s) by gene specific primer showed amplification of *cry1A* gene in the five Bt isolates, three out of the six *cry1* positive isolates showed the presence of *cry1Aa* gene. One of the six Bt isolates showed the presence of *cry1Ab* gene. Five Bt isolates showed amplification for *cry1Ac* gene and a variation in size of amplification was observed in one of the Bt isolates Bt, T27. Further, SDS-PAGE analysis of a spore crystal mixture isolated from new isolates of Bt, T27 showed a single band of ~135 kDa indicating presence of *cry1Ac* gene. The toxicity analysis of Bt strain T27 against *Dichocrocis punctiferalis* showed 100 per cent mortality on the fifth day after treatment. The varied ~925 bp amplicon of *cry1Ac* gene of Bt, T27 was amplified and cloned in a T/A vector. Comparison of nucleotide sequence data generated from the *cry1Ac* (~925 bp) gene showed 99 percent homology and two amino acid variation when comparison with its holotype sequence of Cry1Ac1.

Keywords: Bacillus thuringiensis, *cry1Ac* gene, *Dichocrocis punctiferalis*

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INTRODUCTION

The *Bacillus thuringiensis* (Bt) Berliner is considered as one of the most versatile microbial insecticides. It is a gram-positive spore-forming soil bacterium. The insecticidal activity is based on the ability of the bacterium to produce large quantity of larvicidal proteins known as delta-endotoxins (Cry proteins). The Bt has been used as a successful biological insecticide for more than 40 years and is a uniquely specific, safe, and effective tool for the control of a wide variety of insect pests (Nester et al., 2002). The Cry toxins constitute a family of related proteins that can kill insects belonging to the *Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera*, and *Mallophaga*, as well as other invertebrates (Feitelson et al., 1999; Schnepf et al., 1998). These crystal (Cry) proteins are sequestered as protoxins in crystalline inclusions. After sporulation they mediate specific pathogenecity against insects (Schnepf et al., 1998). Molecular potency of Bt toxins is high compared to the chemical pesticides, i.e., 300 times higher than synthetic pyrethroids and 80,000 times stronger than organophosphates (Feitelson et al., 1999).

Continuous exposure of insect pests to a single kind of Bt toxin can lead to development of resistance in insects (Kranthi et al., 2006). Several approaches to reduce or circumvent the development of resistance to Cry proteins in natural insect populations are currently being employed. These approaches include the isolation of new strains of Bt that produce novel toxins, as well as the use of recombinant DNA technology to construct novel bacterial strains engineered to produce combinations of wild-type toxins along with toxins engineered for improved efficacy (Swiecicka et al., 2008). The PCR method is proven to be a powerful tool which allows quick, simultaneous screening of many Bt samples, identification of specific insecticidal genes carried by different Bt strains, classification of *cry* genes, and subsequent prediction of their insecticidal activities (Carozzi et al., 1991; Ben-Dov, 1997; Juarez-Perez et al., 1997).

The amplification, subsequent toxicity analysis and cloning of the *cry* genes from new isolates of Bt are important because they may provide new gene sequences encoding more active toxins which could be used for developing better versions of transgenic crop plants. Till date more than 770 different *cry* genes were identified and classified into 74 groups (Cry1–Cry74) (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). So, the present study was undertaken by screening of new isolates of Bt by PCR for *cry1* genes, toxicity analysis of Bt Cry pro-

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Table 1. Primers used for screening of *cry1* genes

S. No.	Type of primer	Primer name	Primer sequence (5'→3')	Amplicon size, bp
1	*cry1* Family	J1R	MDATYTCTAKRTCTTGACTA	~1500–1600
2		J1F	TRACRHTDDBDGTATTAGAT	
Subfamily *cry1A* gene, s				
3	*cry1* Subfamily gene(s)	J1AF	CAATAGTCGTTATAATGATT	~1720
4		J1AaF	TTCCCTTTATTTGGGAATGC	~1286
5		J1AbF	CGGATGCTCATAGAGGAGAA	~1371
6		J1AcF	GGAAACTTTATTTTTAATGG	~844

B = C, G or T; D = A, G or T; H = A, C or T; K = G or T; M = A or C; R = A or G and Y = T or C. (Source: Juarez-Perez et al., 1997).

tein against *D. punctiferalis* and cloning of DNA fragment of *cry1Ac* from a novel isolate of Bt, T27.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. thuringiensis* strains used in this study were obtained from the Bt Biotechnology laboratory, Centre for Plant Molecular Biology and Biotechnolgy. Tamil Nadu Agricultural University, Coimbatore, India. *E. coli* vector pTZ57R\T obtained from Fermentas INC.

Screening of new isolates of Bt by PCR for cry1 family genes. Total genomic DNA isolated from six indigenous isolates of Bt strains was used as template for PCR screening of *cry1* gene by using a set of degenerate *cry1* family gene primer (JF and JR) described by Juarez et al. (1997). These primers are expected to amplify fragments of ~1.6 kb of the *cry1* family genes. The *cry1* sub family gene(s) were further screened by combination of degenerate reverse primer along with specific forward primer of sub family gene (JR and J1AF or J1AaF or J1AbF or J1AcF) (Table 1).

The PCR was accomplished using an Eppendorf thermal cycler in 25 µL reaction volume containing total genomic DNA of Bt (30 ng), 2.5 µL of 10× PCR buffer (10 mMTris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 0.5 Units of *Taq* DNA polymerase. The PCR was performed for 30 cycles as follows: 94°C for 1 min, 43°C for 45 s, 72°C for 2 min and the final extension was performed for 7 min at 72°C. The genomic DNA of reference strain of Bt, HD1 was used as positive control.

Preparation of spore-crystal mixture from *B. thuringiensis* isolates. Single colony of *B. thuringiensis* strains was inoculated into 5 mL T3 broth and incubated in a rotatory shaker, maintained at 30°C at 200 rpm. After overnight growth, 1 per cent (250 µL) inoculum was added to 250 mL flask containing 25 mL of T3 broth and incubated at 30°C in a rotatory shaker (Orbitek, India) maintained at 200 rpm for nearly 48–60 h, and the bacterial sporulation was monitored through phase contrast microscope. When more than 90 per cent of cells lysed, the sporulated broth culture was transferred to 4°C, at least half-an-hour before harvesting. The T3 broth containing spore-crystal mixture was centrifuged for 10 min at 10000 rpm at 4°C. The pellet was resuspended in ice cold 25 mL of (Tris-EDTA buffer Tris 10 mM, EDTA 1mM, pH 8.0 with 1mM phenyl methyl sulphonyl fluoride (PMSF)) and washed once with 25 mL of ice-cold 0.5 M NaCl followed by two washes with 25 mL of Tris-EDTA buffer with 0.5 mM PMSF and centrifuged at the same speed and time. Finally, the spore-crystal pellet was suspended in 500 µl of sterile distilled water containing 1 mM PMSF and stored in –20°C.

Establishment and Maintenance of Insect Culture of D. Punctiferalis

Culture establishment. Second instar larvae of *D. punctiferalis* were collected from the Tapioca and Caster Research Station (TCRS), TNAU, Yethapur (Salem district, Tamil Nadu, India) to establish the stock culture. Field collected larvae were reared and closely watched for any disease symptoms. Larvae with disease symptoms, and slow growth as well as malformed pupae were destroyed by autoclaving. The chickpea based semi-synthetic diet was developed based on the formulations of Patel et al. (1968) with addition of three per cent ginger (*Gingiber officinale*) as a supplement. Growth and survival of *D. punctiferalis* on semi-synthetic diet were observed.

Preparation of semi-synthetic diet. Agar shreds were melted in 360 mL of water in a pan. Chickpea seeds (Kabuli type) soaked overnight in water were cooked in 360 mL of water and transferred to a blender. Ginger was added while blending. The molten agar was added to the chickpea seeds and mixed thoroughly. To this Wesson's salt mixture, methylparahydroxy benzoate (dissolved in alcohol), sorbic acid and yeast granules were added and homogenized for 5 min. After the diet has cooled to about 60°C the vitamin mix, ascorbic

acid, choline chloride, carbendazim, formalin and streptomycin sulphate were added and mixed well for 5 min. While in semi-solid condition, the diet was dispensed in sterile vials (5 ml) at 3 mL/vial in a laminar air flow chamber. The dispensed diet was allowed to cool and solidify for 45 min.

Cloning of partial cry1Ac gene fragment from Bt isolate, T27. The gel eluted PCR products of partial *cry1Ac* gene (~925) from Bt isolate, T127 was ligated into T/A vector (pTZ57R\T Fermentas INC) as per the manufacturer's instruction. The ligated mixture was transformed into *E. coli* as per the standard procedure (Sambrook et al., 1989). The transformed colonies of *E. coli* were screened by colony PCR with M13F and M13R primers for checking the presence of insert.

Nucleotide sequencing of recombinant plasmids. The plasmid DNA was isolated from the *E. coli* transformants containing *cry1Ac* gene of Bt isolate, T27 and nucleotide sequence of recombinant plasmids was carried out by automated sequencing (Ist Base, Singapore). The sequence data was subjected to homology search through Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnological Information (NCBI). The deduced amino acid sequence was generated by BioEdit software.

RESULTS

Screening of new isolates of Bt for cry1 genes. Total genomic DNA isolated from six new isolates of Bt (T15, T16, T21, T25, T27 and T29) were used as template for screening of *cry1* family genes by PCR using degenerate primers (J1F and J1R) specific to *cry1* genes. Amplification of expected size (~1.6 kb) of PCR products were obtained from all the six new isolates of Bt (Fig. 1a). Further screening of *cry1* sub family gene(s) by using combination of degenerate reverse primer and forward of *cry1A* sub family specific primer showed five out of the six new Bt isolates positive for *cry1A* gene, in case of *cry2Aa* gene positive for three out of six Bt isolates. Similarly, *cry1Ab* gene was obtained one out of six. Screening of *cry1Ac* gene showed amplification in five out of six Bt isolates. But one of the isolate, T27 showed variation in their amplification of expected size (Fig. 1b, lane *6*) (Table 2).

SDS-PAGE analysis of Bt protein from the new isolate, T27. Based on variation in amplicon size for *cry1Ac* gene during PCR screening, T27 was selected for SDS-PAGE analysis. Prominent protein band of ~135 kDa was observed in the new Bt isolate, T27 as that in the case of reference strain of Bt, HD73 (Fig. 2).

Bioassay of Bt strains, for toxicity against *D. punctiferalis*. Chickpea based semi-synthetic diet was used with and without ginger (*Gingiber officinale*) (3 per cent). Second instar larvae of *D. punctiferalis* was grown well in the diet contain ginger and also the larva had entered pupation and emerged into adults but in case of without gingers based diet the larva was not attained pupation. The bioassay of standard strain of Bt, HD73 showed 80 per cent mortality, whereas all

Table 2. Screening of new isolates of Bt with primers specific to *cry1A* family and subfamily genes

S. No.	Bt strains	*cry1* gene	*cry1* genes			
			cry1A	*cry1Aa*	*cry1Ab*	*cry1Ac*
1	T15	+	+	+	–	+
2	T16	+	+	+	+	+
3	T21	+	–	–	–	–
4	T25	+	+	–	–	+
5	T27	+	+	–	–	+
6	T29	+	+	+	–	+
7	HD1 (Reference strain)	+	+	+	+	+

+, Positive; –, Negative.

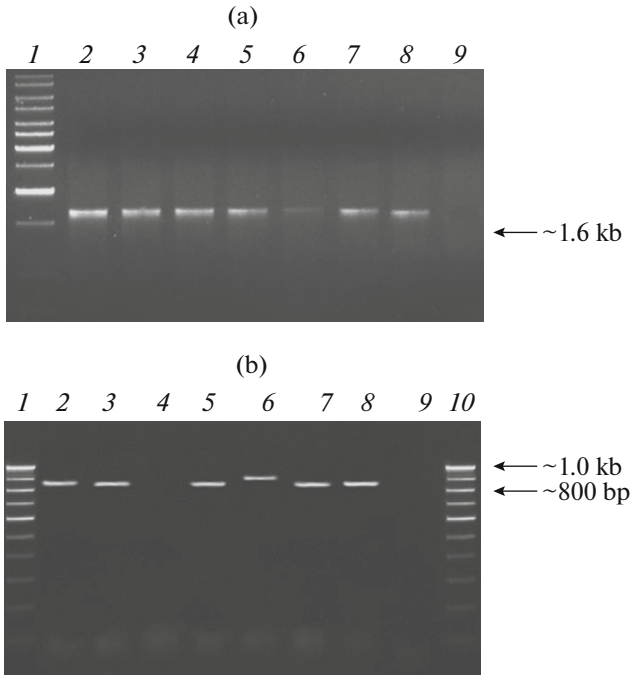

Fig. 1. Screening of *cry1* gene from test isolates of Bt. (a) A 1.6 kb of internal sequence of *cry1* gene was amplified by PCR from the DNA isolated from indigenous isolates of Bt. Lane *1*: 1.0 kb ladder, lane *2* to *7*: test isolates of Bt (T15, T16, T21, T25, T27 and T29), lane *8*: positive control (HD1), lane *9*: negative control (water). (b) A 844 bp of internal sequence of *cry1Ac* gene was amplified by PCR from the DNA isolated from indigenous isolates of Bt. Lane *1*&*10*: 1.0 kb ladder, lane *2* to *7*: test isolates of Bt, lane *8*: positive control, HD1.

Table 3. Analysis of Bt strains for toxicity against caster capsule borer (*Dichocrocis punctiferalis*)

S. No.	Treatment	Percent mortality as indicated days after treatment (DAT)							Cumulative mortality, %
		1	2	3	4	5	6	7	
1	HD73	–	20	–	20	20	10	10	80
2	T27	–	30	40	20	10	–	–	100
3	4Q7	–	–	–	–	–	–	–	–
4	Control	–	–	–	–	–	–	–	–

20 second instar larvae were used for each treatment; HD73 is used for positive control and it also known for the presence of *cry1* gene; T27 is a test isolate used for our study; control is a diet without any treatment of crystal protein; DAT: days after treatment.

the larvae (20 numbers) on the control and acrystalliferous strain (4Q7) treatment were alive till seven days after treatment. Interestingly the insecticidal activity of new isolate of Bt, T27 recorded as 100 per cent mortality at five days after treatment (Fig. 3, Table 3).

Cloning and sequence analysis of cry1Ac gene from Bt isolate, T27. DNA fragments of about ~925 bp were amplified by PCR (Fig. 4a). The DNA fragment of Bt isolate, T27 was eluted from agarose gel and ligated to T/A vector (pTZ57R/T). The ligation mixture was cloned in *E. coli* vector. The recombinant clones (white colonies) were selected on LB agar containing X-gal, IPTG and ampicillin. The *E. coli* recombinants were screened by colony PCR with M13F and M13 primers showed expected size of 1.1 kb (including vector size of M13 primers) (Fig. 4b). The similar result was found in one of the isolated recombinant plasmid DNA. To determine the nucleotide sequence of the *cry1Ac* gene cloned from Bt strain T27. Sequence data generated with M13F and M13R primers showed 99 per cent homology with its holotype *cry1Ac* gene. The Cry1Ac amino acid sequences of Bt strain, T27 deduced from the nucleotide sequences generated by M13F and M13R showed two amino acid variation with that of CrycAc1 holotype (Fig. 5).

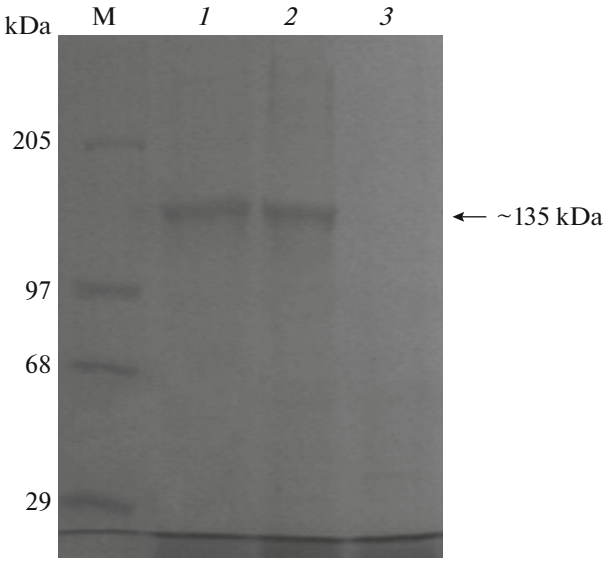

Fig. 2. SDS-PAGE analysis of spore-crystal mixture of Bt strains. M: Genei Protein marker (Higher Range #105977). Lanes *1*: test isolate t27, lane *2*: reference strain hd1, lane *3*: acrystalliferous Bt 4Q7.

DISCUSSION

PCR is a method that has been popularly used for characterization genes coding for Cry proteins and for analysis of *B. thuringiensis* collections (Nester et al., 2002). The technique was first introduced to identify *cry* genes in order to predict insecticidal activity. Over the last decade, PCR methods for screening *cry* genes present in *B. thuringiensis* collections, have been used to identify strains that harbor genes coding for known *cry* genes. Undoubtedly, the use of PCR has greatly improved *cry* gene detection, however, this method is mostly limited to members of previously described gene families and requires a large number of primers (Ceron et al., 1995; Kuo et al., 2000). Hence, uses of degenerate primers were designed to amplify all the members of different subfamilies of *cry* genes (Carozzi et al., 1991; Bourque et al., 1993; Ben-Dov et al., 1997; Juarez-Perez et al., 1997; Bravo et al., 1998; Porcar et al., 2002; Thammasittirong and Attathom, 2008). Although the use of these degenerate oligonucleotides increases the probability of amplifying novel genes,

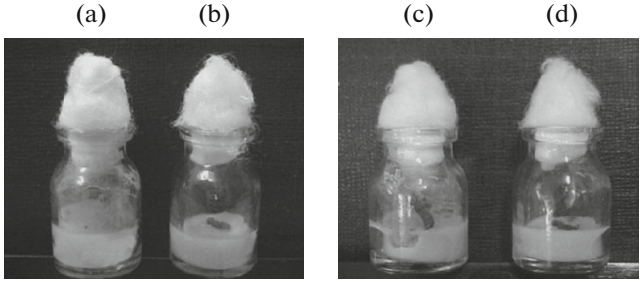

Fig. 3. Testing of Bt proteins for toxicity against *Dichocrocis puntiferalis*. (a) Control (diet without any treatment of Bt protein),(b) Bt protein from new isolate T27, (c) Bt protein from 4Q7 (acrystalliferous), (d) Bt protein from HD73.

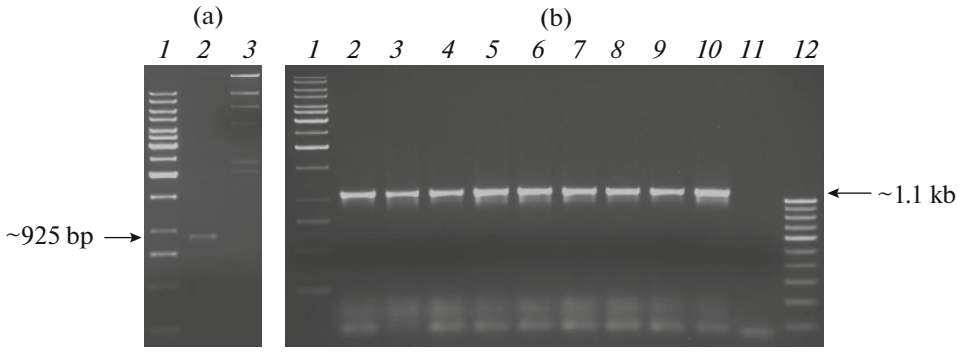

Fig. 4. Cloning of *cry1Ac* gene from indigenous isolates of Bt T27. (a) Amplification of *cry1Ac* gene from the test isolates of Bt, T27. Lane *1*: 1 kb ladder, lane *2*: test isolate T27, lane *3*: λ/*Hin*dIII marker. (b) Screening of recombinant *E. coli* colonies by M13F and M13R primers. Lane *1*: 1kb ladder, lane *2* to *9*: recombinants of *E. coli* colonies, lane *10*: positive control, lane 11: negative control, lane 12: 100 bp ladder.

the efficiency is restricted to detection of closely related genes in the same group (Juarez-Perez et al., 1997).

In the present study, six new isolates of Bt were screened for the presence of *cry1* family genes through PCR. All the six isolates indicated the presence of *cry1* family genes. The six isolates were further screened for the presence of *cry1A* subfamily, *cry1Aa, cry1Ab* and *cry1Ac* type genes. The *cry1A* subfamily gene(s) were present in five isolates, *cry1Aa* gene was present in three isolates, *cry1Ab* gene was present in the isolate, T16 alone and *cry1Ac* gene was present in five isolates. The DNA fragment amplified from the new isolate, T27 showed the variation in size of ~925 instead of expected size of ~845 bp while screening for the presence of *cry1Ac* gene. Based on this variation, it was selected for toxicity analysis. Porcar and Juarez-Perez (2003) suggested that strains yielding unusual PCR products could be selected for further analyses leading to the identification and characterization of hypothetical novel *cry* genes. Identification of novel Bt isolates by insect bioassays is very important to know their toxic potency of Cry protein.

The SDS-PAGE of crystal protein inclusions isolated from broth cultures of new Bt isolate, T27 showed a clear band of ~135 kDa, similar to that of the reference strain, HD73. The ~135 kDa protein of Bt strain HD73 is characterized as Cry1Ac protein. In the present study, the new isolate of Bt, T27 is also having ~135 kDa protein suggesting the presence of *cry1* genes.

For optimization of the diet, chickpea based semi-synthetic diet was used with and without supplementation of ginger (*Gingifer officinale*). Second instar larvae of *D. punctiferalis* released on both diets. The larvae released on the diet without ginger did not survive well, whereas the larvae released on diet containing ginger had grown well pupated and emerged into adults.

Since, ginger is one of the host plants for *D. punctiferalis*, addition of ginger in semi-synthetic diet might have acted as an attractant for feeding by the larvae of *D. punctiferalis*. Ginger up to 3 per cent sup-

Score: 432 bits(1110), Expect: 4e-147, Method: Compositional matrix adjust,
Identities: 209/212 (99%), Gaps: 0/212 (0%)

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Query   1    LMDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFLPGAGFVLG  60
Sbjct   5    CMDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLG  64

Query   61   LVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEA  120
Sbjct   65   LVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEA  124

Query   121  DPTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFG  180
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Query   181  QRWGFDAATINSRYNDLTRLIGNYTDYAVRWY  212
Sbjct   185  QRWGFDAATINSRYNDLTRLIGNYTDYAVRWY  216

Query   : Bt isolate T27
Subject : Holotype cry1AC1
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Fig. 5. Comparison of deduced amino acid sequence of Bt isolate T27 and its holotype Cry1Ac1.

ported the successful development of *D. punctiferalis*. Vanderzant (1974) also pointed that addition of host plant material may be required for some insects to satisfy the unknown nutrient or chemical feeding stimulant. Bt strain HD73 that is known to produce Cry1Ac protein alone, was used in this study as comparison for the toxicity of Cry1Ac protein of new isolate of Bt, T27 against *D. punctiferalis*. The new Bt isolate, T27 showed 100 cent per cent morality within five days after treatment, whereas the reference strain of Bt, HD73, showed 80 per cent mortality after seven days. This preliminary result showed difference between the isolates of Bt concerning their toxicity against *D. punctiferalis*. This may be related to different crystal protein genes of two strains, which resulted in various degrees of toxicity. The toxins within the Cry1A subfamily exhibit variation in toxicity and specificity due to minor amino acid substitutions (Tounsi et al., 1999; Xue et al. 2008) reported cloning of novel *cry1Ah* gene and its protein was more toxic to lepidopteran insect, Asian corn borer. Based on this the new isolate of Bt, T27 showed variation in the size of *cry1Ac* amplification while screening by PCR. Therefore, the DNA fragments amplified by *cry1Ac* primers from Bt strain, T27 were cloned in *E. coli* vector. PCR screening of recombinant plasmid by M13F and M13R primers also showed the amplification of expected size (~1.1 kb). The nucleotide sequence analysis of *cry1Ac* gene of new Bt isolate, T27 showed 99 per cent similarity to the already reported *cry1Ac1* holotype sequence. Deduced amino acid sequence of Cry1Ac of the new Bt isolate, T27 showed variation of amino acid at two position in the C-terminal region in comparison to the Cry1Ac1. Therefore variation of a single amino acid can significantly influence the level of toxicity in Cry proteins (Manikandan et al., 2015; Udayasuriyan 1994). Further studies on cloning of complete orf (open reading frame) of *cry1Ac* gene from new isolate of Bt, T27 will be useful to know about the novelty of the sequence and insecticidal potency of its protein.

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REFERENCES

Ben-Dov, E., Zaritsky, A., Dahan, E., Barak, Z., Sinai, R., Manasherob, R., Khamraev, A., Troitskaya, E., Dubitsky, A., Berezina, N., and Margalith, Y., Extended screening by PCR for seven *cry* group genes from field-collected strains of *Bacillus thuringiensis, Appl. Environ. Microbiol.,* 1997, vol. 63, pp. 4883–4890.

Bourque, S.N., Valero, J.R., Mercier, J., Lavoie, M.C., and Levesque, R.C., Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis, Appl. Environ. Microbiol.,* 1993, vol. 59, pp. 523–527.

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., Characterization of *cry* genes in a Mexican Bt strain collection, *Appl. Environ. Microbiol.*, 1998, vol. 64, pp. 4965–4972.

Carozzi, N.B., Kramer, V.C., Warren, G.W., Evola, S., and Koziel, M.G., Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles, *Appl. Environ. Microbiol.*, 1991, vol. 57, pp. 3057–3061.

Ceron, J., Ortiz, A., Quintero, R., Guereca, L., and Bravo, A., Specific PCR primers directed to identify *cryI* and *cryIII* genes within a *B. thuringiensis* strain collection, *Appl. Environ. Microbiol.*, 1995, vol. 61, pp. 3826–3831.

Feitelson, J.S., Payne, J., and Kim, L., *Bacillus thuringiensis*: insects and beyond, *Bio/Technology*, 1999, vol. 10, pp. 271–275.

Juarez-Perez, V.M., Ferrandis, M.D., and Frutos, R., PCR-based approach for detection of novel *Bacillus thuringiensis cry* genes, *Appl. Environ. Microbiol.*, 1997, vol. 63, pp. 2997–3002.

Kranthi, K.R., Dhawad, C.S., Naidu, S.R., Mate, K., Behere, G.T., Wadaskar, R.M., and Kranthi, S., Inheritance of resistance in Indian *Helicoverpa armigera* (Hubner) to Cry1Ac toxin of *Bacillus thuringiensis, Crop Prot.*, 2006, vol. 25, pp. 119–124.

Kuo, W.S., Lin, J.H., Tzeng, C.C., Kao, S.S., and Chak, K.F., Cloning of two new *cry* genes from *Bacillus thuringiensis* subsp. *wuhanensis* strain, *Curr. Microbiol.*, 2000, vol. 40, pp. 227–232.

Nester, E.W., Thomashow, L.S., Metz, M., and Gordon, M., *100 Years of Bacillus thuringiensis*: *A Critical Scientific Assessment,* Washington: Amer. Soc. Microbiol., 2002.

Manikandan, R., Ramalakshmi, A., Balasubramani, V., and Udayasuriyan, V., Characterization and cloning of *cry2A* gene from Indian isolate of *Bacillus thuringiensis, Mol. Biol.*, 2015, vol. 49, pp. 520–526.

Patel, R.C., Patel, J.K., Patel, P.B., and Singh, R., Mass breeding of *Heliothis armigera* (H.), *Indian J. Entomol.*, 1968, vol. 30, pp. 272–280.

Porcar, M., and Juarez-Perez, V., PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes, *Microbiol. Rev.*, 2003, vol. 26, pp. 419–432.

Porcar, M., Juarez-Perez, V., Delecluse, A., Isolation and characterization of a strong promoter from *Bacillus sphaericus* strain 2297, *J. Invertebr. Pathol.*, 2002, vol. 81, pp. 57–58.

Sambrook, J.E., Fritsch, F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor, N.Y.: Cold Spring Harbour Lab. Press, 1989.

Schnepf, E., Crickmore, N., Van Rie, J., Lerecurs, D., Baum, J., Feitelson, J., Zeigler, J.D.R., and Dean, D.H.,

B. thuringiensis and its pesticidal crystal proteins, *Microbiol. Mol. Biol. Rev.,* 1998, vol. 62, pp. 775–806.

Swiecicka, I.D., Bidesh, K., and Ferderici, B.R., Novel isolate of *Bacillus thuringiensis* that produce a quasicuboidal crystal of Cry2Ab21 toxic to larva of *Trichoplusia ni, Appl. Environ. Microbiol.*, 2008, vol. 74, pp. 923–930.

Thammasittirong, A., and Attathom, T., PCR-based method for the detection of *cry* genes in local isolates of *Bacillus thuringiensis* from Thailand, *J. Invertebr. Pathol.*, 2008, vol. 98, pp. 121–126.

Tounsi, S., Mal, J., Zouari, A., and Jaoua, N., Cloning and nucleotide sequence of a novel *cry1Aa*-type gene from *Bacillus thuringiensis* subsp. *Kurstaki, Biotechnol. Lett.*, 1999, vol. 21, pp. 771–775.

Udayasuriyan, V., Nakamura, A., Mori, A., Masaki, H., and Uozumi, T., Cloning of a new *crylA*(a), gene from *B thuringiensis* strain FU-2-7 and analysis of chimeric *cry1A*(a) proteins of toxicity, *Biosci. Biotechnol. Biochem.*, 1994, vol. 58, pp. 830–835.

Vanderzant, E.S., Development, significance and application of artificial diets for insects, *Annu. Rev. Entomol.*, 1974, vol. 19, pp. 139–160.

Xue, J., Gemei, N., Crickmore, H., Li1, K., Hel, F., Song, X., Feng, D., Huang, J., and Zhang, H., Cloning and characterization of a novel Cry1A toxin from *Bacillus thuringiensis* with high toxicity to the Asian corn borer and other lepidopteran insects, *Microbiol. Lett.*, 2008, vol. 280, pp. 95–101.