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Profile of Coleopteran Specific *cry* Genes in Native *Bacillus thuringiensis* Isolates

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The characterization of the strains containing Coleopteran specific and also putative novel *cry* genes in 993 native *Bacillus thuringiensis* collection is presented. Characterization was based on PCR analysis using specific primers for *cry1I*, *cry1B*, *cry3*, *cry7,8*, *cry14*, *cry18*, *cry23*, *cry26*, *cry28*, *cry34*, *cry35*, *cry36*, *cry37* and *cry55* genes, protein band patterns as well as their insecticidal activity on *Tribolium castraneum*. Strains containing *cry26* and *cry3* were the most abundant and represent 6.15% and 6.03% of the isolates respectively, whereas *cry1I*, *cry7,8*, *cry14*, *cry18*, *cry23*, *cry26*, *cry28*, *cry34*, *cry35*, *cry36*, *cry37* and *cry55* were less abundant. None of the isolates were amplified for *cry1B*. Two strains CFE3(1) and CFE 43(1) containing Coleopteran active *cry* genes showed higher activity against *Tribolium castraeneum* than reference strains. Cloning and sequencing of the amplicons allowed both the identification of known *cry* genes and detection of putative novel *cry* sequences .

Key words: *Bacillus thuringiensis*, PCR, Coleoptera, *Tribolium castraeneum*, *cry*

Bacillus thuringiensis(*Bt*), a gram positive, rod shaped, spore forming bacterium produces crystal (Cry) proteins that are toxic to insects [1].The lethality of *Bacillus thuringiensis* to insects is largely attributed to the Cry proteins produced during the sporulation stage of *Bt* growth cycle [2]. The Cry proteins are protoxins which can be converted to active toxin upon ingestion by a susceptible insect and possess lethal toxicity towards a wide range of insects, belonging to the Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera and Mallophaga, as well as some invertebrates[3] .

These toxins can be grouped according to the degree of amino acid homology. More than 400 different genes encoding toxins classified as *cry1* to *cry72* , *cyt1*, *cyt2* and *cyt3* have been identified in different *Bacillus thuringiensis* isolates[4] .The discovery of the insecticidal properties of *Bacillus thuringiensis* toxins in the 20th century was of considerable significance for plant protection against pest insects. Intensive screening programs leading to important collection of isolates have been conducted in last decades. The need of novel Cry proteins with toxic potential against different organisms with specificity for a much broader range of pests or to provide alternatives after the appearance of insect resistance has resulted in a continuous search for new experimental approaches in order to expand the host ranges of the strains available [3].

Most of the *Bacillus thuringiensis* strains show activity on Lepidoptera and those that show insecticidal activity against Dipteran and Coleopteran pests are rare. In our laboratory, we have a *Bacillus thuringiensis* collection comprising more than 5000 isolates isolated from different regions of India . In the present study, we report the characterization and distribution of Coleopteran

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specific and putative novel *cry* genes among the collection, and also the toxicity of isolates containing Coleopteran active *cry* genes against a Coleopteran pest, *Tribolium castraeneum.*

MATERIALS AND METHODS

Bacterial isolates

993 native *Bacillus thuringiensis* strains already available at the Institute of Agri Biotechnology, University of Agricultural Sciences, Dharwad were used. Cultures of *Bacillus thuringiensis* strains were grown at 28ºC in Luria broth with vigorous shaking.

Oligonucleotide primers and PCR analysis

For detection of Coleopteran specific *cry* gene i.e., *cry1I*, *cry1B*, *cry3*, *cry7,8*, *cry14*, *cry18*, *cry23*, *cry26*, *cry28*, *cry34*, *cry35*, *cry36*, *cry37* and *cry55*, specific primers from regions of related genes were synthesized as previously described [5] . Oligonucleotides were synthesized at the Sigma Life Science, Bangalore, India.

Total DNA was extracted and purified following the method described by Sambrook and Russell, 2001 (16). PCR was conducted for 250 ng of total *Bacillus thuringiensis* DNA with 5 U of Taq DNA polymerase, 1 mM each NTP, 5 pM each primer, 25 mM $MgCl_2$ in a final volume of 10 µl. Amplification was done in an Eppendorf thermal cycler under the following conditions. 5 min of denaturation at 94ºC followed by 25 cycles of amplification with a 1 min denaturation at 94ºC, 1 min of annealing at different temperatures, and 2 min of extension at 72ºC. An extra extension step of 10 min at 72 ºC was added after completion of 25 cycles . PCR products were analysed on 1% agarose gel.

Characterization of parasporal inclusions and protein

Sporulating cultures of *B.thuringiensis* strains were produced in the T3 medium (Travers *et al*., 1987) at 28ºC for 72 hours when more than 90% of the cells is expected to be lysed, releasing spores and crystals. Single colony was inoculated into 5ml T3 broth and incubated in a rotary shaker, maintained at 28ºC at 200 rpm for nearly 72 hours. The sporulated broth culture was transferred to 4ºC, at least half an hour before harvesting. The T3 broth containing spore crystal mixture was centrifuged for 10 min at 10000 rpm at 4 ºC in 2 ml eppendorf tube. The pellet was washed once with 2 ml of ice cold $T_{10}E_1$ and once with 2ml of ice cold 0.1M NaCl followed by two more washes with 2 ml of $T_{10}E_1$ centrifuging at the same speed and time. Finally, the spore crystal pellet was suspended in 100 µl of Laemmli s buffer (4X) and then stored in -20ºc until further use. The protein content of spore crystal mixtures of the strains containing Coleopteran specific active *cry* genes was determined by Seifinejad *et al*., 2008[6].

Bioassay

The pellet of *Bt* in falcon tubes was mixed with distilled water and centrifuged at 3000 rpm for 5 min. This centrifugation was repeated thrice in centrifugation tubes till all the media was washed away and the supernatant was discarded .Three concentrations of 0.5, 1.0, 1.5 g of *B. thuringiensis* pellet were added in each vial containing one gram diet. Twenty larvae of *T. castaneum* were placed in each vial and mortality was observed after 24, 48, and 72 h. The same concentrations were made for newly emerged adults of *T. castaneum* in separate glass vials. Results were observed after every 24, 48 and 72 h at 30°C [7].

Identification of putative novel *cry* type genes

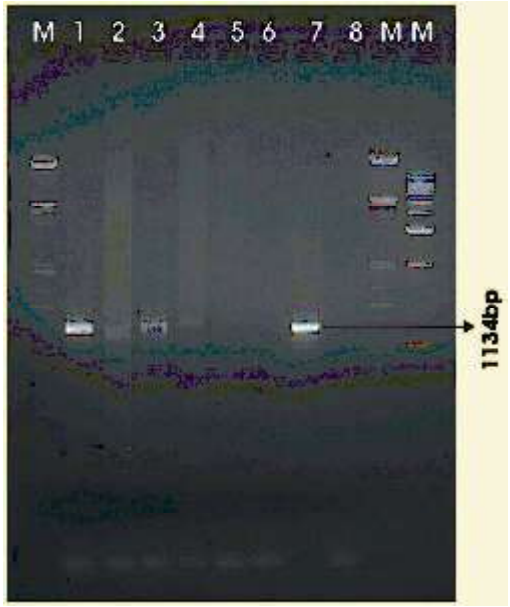
In the present and previous studies[6,8], when isolates were assayed for different *cry* genes , some isolates showed different size bands from that were expected . These results showed that these isolates may contain novel *cry* gene. PCR reactions for these isolates and genes were repeated as previously described by Juarez perez *et al*.,1997 [9]. PCR amplified products were ligated to the TA cloning vector pTZ57R/T , which was used for transforming *E. coli* DH5± . Finally, the cloned fragments were sequenced at Blast X (version 2.6)[10]. Known *cry* sequences were obtained from the nonredundant protein database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov.in) .The sequences of more than 50 Cry proteins were used for sequence alignments.

RESULTS

In this study, the distribution of Coleopteran effective genes, among 993 native *Bacillus thuringiensis* strains was evaluated, and finally based on the PCR and bioassays, the most Coleopteran and effective strains were selected

Table 1. Characteristics of specific primers for Coleopteran active *cry* type gene

S.No.	Gene	Sequence	Size(bp)	Accession No.
1.	*cry1I*	FP: ACAATTTACAGCTTATTAAG RP: CTACATGTTACGCTCAATAT	1134 bp	X62821
2.	*cry3*	FP:CGTTATCGCAGAGATGACATTAC RP:CATCTGTTGTTTCTGGAGGCAAT	1.45 Kb	M30503
3.	*cry7,8*	FP: CCCTTTAGCAAACGATCAAACG RP:ATTGGGCGGTACGTGTCACCTGAC	641 bp	AB089299
4.	*cry14*	FP: ATAATGCGCGACCTACTGTTGT RP: TGCCGTTATCGCCGTTATT	456 bp	U13955
5.	*cry18*	FP: CCGAGGCGATTTGGATAGAT RP: TGCCGGTGTAAACAAAGAAGG	419 bp	X99049
6.	*cry26*	FP: CGCGCTGTTCAATTATCAAGTGC RP:ATATGGAAAGAAAAGGCGTGTA	362 bp	AF122897
7.	*cry28*	FP: TACAGTCGCTGTAGTAAGCGCA RP:TGACAGCCAAGTAAATAGCCCTG	862 bp	AF285775
8.	*cry34*	FP: ATGTCAGCTCGCGAAGTACA RP: TATCTCCTGATCCGCTTTGAG	287 bp	AY016411
9.	*cry35*	FP: AGTCTTGATGATTCAGGTGTTA RP:CAAGGTACTAATGTCCATCCCAT	479 bp	AY016411
10.	*cry36*	FP: CTTGTGGATGTGGTTGCCAGCAA RP:CCTCCAAATGTTTGAGCAGCTGA	1399 bp	AY036012
11.	*cry23*	FP: CTGTATCGTTCACATGGACGGAA RP: AATGCTTCGCAAGCCTTGTGCA	476 bp	AF038048
12.	*cry37*	FP: AAGTAGCGACACTGGTTCCCCTA RP: CAAGTCGTACTGTTACACCAGG	140 bp	AF038049
13.	*cry55*	FP: AGCTCAAACGTTCTAGTCCCAG RP: TTGGATCAGGTGTTTGAGTGC	805 bp	EU121522



PCR screening of *cry1I* (50.9 ° C) Lane 1,2 ,3 and 4 : CFE 20(3), CFE21(2), CFE64(2), CFE25(2) isolates.Lane7: positive control (HD1)M1 : » DNA/ *Eco*RI/*Hind*III Double digest M2 : 1 kb marker .

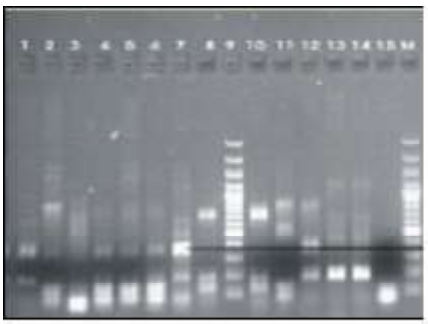
for further cloning and expression studies.

The PCR methodology described here utilized 14 primers (Table1) to detect 14 different Coleopteran active *cry* genes, including *cry1*(*cry1B*, *cry1I*), *cry3*, *cry8*, *cry14*, *cry18*, *cry23*, *cry26*, *cry28*, *cry34*, *cry35*, *cry36*, *cry37* and *cry55* described to codify for proteins active against Coleoptera. Fig1.shows some PCR products obtained with strains CFE 20(3), CFE21(2), CFE64(2), CFE25(2) isolates which possess *cry1I*, DBT2638, DBT2639, CFE11(1), CFE34(1), CFE39(1), CFE52(2), CFE53(1), CFE22(2), CFE64(2), DBT1269, DBT1279, DBT1308 which possess *cry26*, DBT2575, DBT2576, DBT2593, DBT2630, CFE4(1), CFE42(1), CFE43(2), CFE49(2), CFE58(2), CFE16(2), CFE19(2), CFE19(3) which possess *cry28* and CFE43(1) which possess *cry34* and *cry35*. Known strains *i.e.,* HD1 amplified for *cry1I*, *cry18*, *cry26*, *cry28*, *cry23*, 4AA1 amplified for *cry3*; 4AT1 amplified for *cry7,8*; *cry8* and *cry9*; 4E2 amplified for *cry14* served as references for Coleopteran specific *cry* genes showed the expected PCR

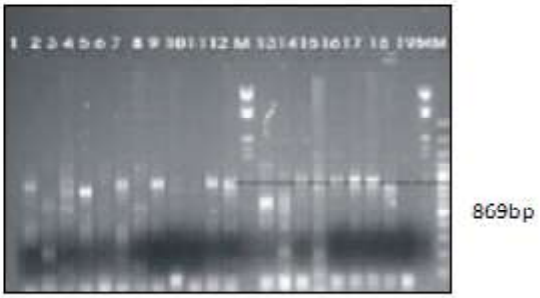
products with primers for expected *cry* genes.

Strains carrying *cry26* and *cry3* were the most abundant and representing 6.16 and 6.03% of the isolates respectively (Fig2). *Bt* strains harboring *cry14*(4.14%) and *cry18*(4.18%) were also abundant whereas *cry28*, *cry11*, *cry7,8*, *cry23*, *cry36*, *cry34* and *cry35*, *cry37* and *cry55* were less abundant and found in 3.48, 3.36, 3.13, 2.09, 2.78, 2.20, 2.20, 0.1 and 0.34% of the strains respectively. None of the isolates were positive for *cry1B*.

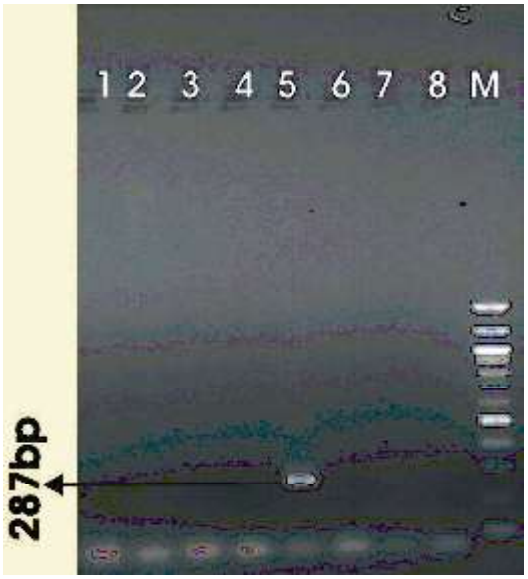
The isolate containing Coleopteran specific *cry* genes that produce crystals were later analyzed by SDS-PAGE to estimate the molecular weight of the Cry proteins (Fig 3). Isolates



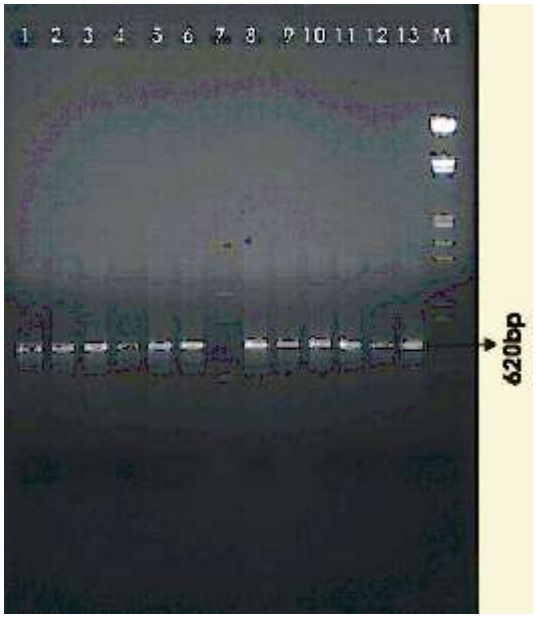
PCR screening of *cry26* (50.9 p C)
Lane 1-12 represents the DBT2638, DBT2639, CFE11(1), CFE34(1), CFE39(1), CFE52(2), CFE53(1), CFE22(2), CFE64(2), DBT1269, DBT1279, DBT1308 isolates.
M : 100 bp marker.



PCR screening of *cry28* (45.4 p C)
Lane 1,3, 4 ,6,8, 11, 12 15, 16, 17, 18 and 19 : DBT2575, DBT2576, DBT2593, DBT2630, CFE4(1), CFE42(1), CFE43(2), CFE49(2), CFE58(2), CFE16(2), CFE19(2), CFE19(3) isolates .
M : λ DNA/*Eco*RI/*Hind*III Double digest
M1 : 100 bp marker .



PCR screening of *cry34* (44.2 p C)
Lane 5 :CFE43(1) isolate .
M :100 bp marker.



PCR screening of *cry35* (44.0 p C)
Lane 1-13 : gradient PCR for *cry*35 gene (40 - 50 °C) .
M : λ DNA/*Eco*RI/*Hind*III double digest

Fig. 1. Agarose gel electrophoresis of PCR products amplified from the native *B. thuringiensis* strains

presented ′ endotoxins with molecular weight between 20 and 135kDa, but the most common pattern was composed of pattern was composed of proteins with molecular weights between 60-135kDa.

Fifty PCR products obtained for different *cry* gene were cloned to a TA cloning vector pTZ57R/T and sequenced. The obtained sequences were aligned and compared with *cry* gene sequences in NCBI. Comparisons showed that only sequence hit/ shows homology to the *cry1I*, *cry 34 /35* and *cry3*. Comparisons showed that the other sequences were not related to *cry* or *vip* genes and did not show any homology with these genes but showed homology to the *Bacillus thuringiensis* genome indicating non specific amplification. Efforts for isolation and cloning of whole sequence of these putative novel genes for

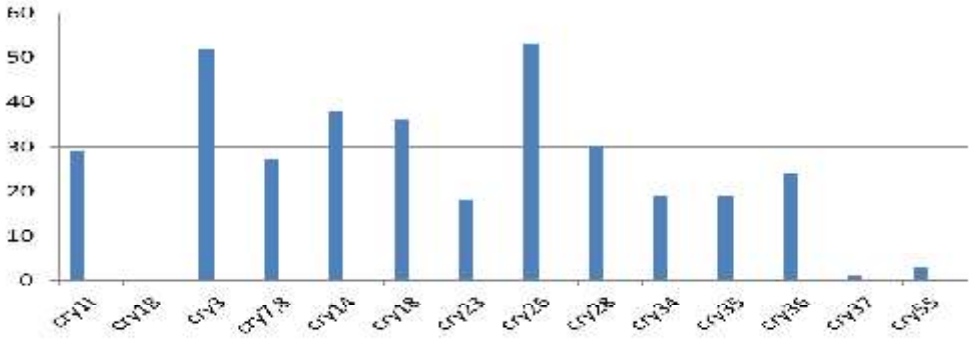
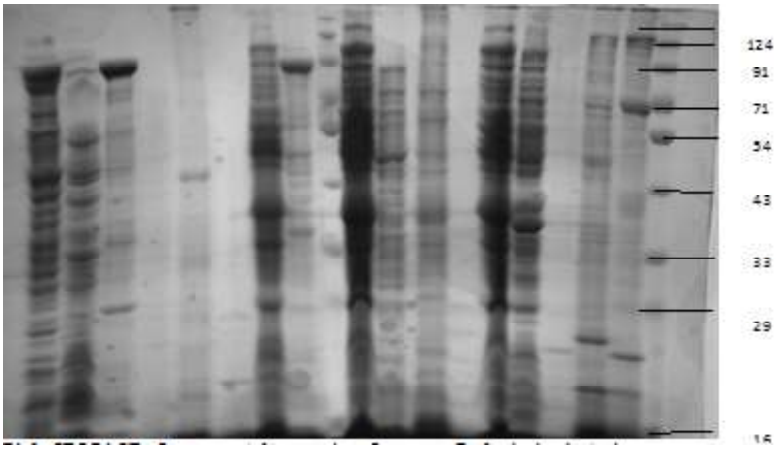


Fig. 2. Distribution of *cry* gene among the 993 *Bt* isolates analyzed



Lane 1 to 17 represents CFE43(1), CFE3(1), CFE1(1), CFE51(2), CFE26(2), CFE64(1), CFE21(2), CFE17(4), CFE51(3), CFE49(1), CFE6(1), CFE45(2), CF50(2), DBT730, DBT4440, DBT1010 and CFE20(3) respectively . M represents prestained protein ladder .

Fig. 3. SDS PAGE of spore crystal suspensions from some *B. thuringiensis* strains

more details, identification and detection of other putative novel toxin genes in the collection will be continued.

The sequence of the *cry1I* showed a 98% homology to the available *cry1I* sequence in the NCBI database and query coverage was 96% (Accession no: JN226100.1). The sequence obtained for the *cry3* gene showed 87% homology to the sequence available in the database and the query coverage was 12% (Accession No. EU332160.1). The sequence obtained for the *cry34/35* showed 99% homology to the binary insecticidal crystal protein present in the database and the query coverage was 76% (Accession no: AY53689.7).

Based on the *cry* profile, isolates were

selected for bioassays. A preliminary bioassay with highly concentrated spore crystal suspensions of selected isolates was performed on third instar larvae of *Tribolium castraenum*. The selected strains (CFE1(1), CFE3(1), CFE20(3), CFE43(1)) showed different toxicity levels of 0 to 30 %[11].

DISCUSSION

The results presented here demonstrate that the PCR based approach can be used for systematic, large scale screening of *B.thuringiensis* isolates to characterize toxicity, identify known *cry* genes and more importantly to detect and identify novel *cry* genes. The results obtained in our studies were used for production of toxicity of the isolates on *Tribolium castraneum* based on PCR results.

In the present study, the characterization and distribution of Coleopteran specific and putative novel *cry* genes in native *B.thuringiensis* were studied. Characterization was based on PCR analysis using specific primers for all *cry* genes encoding proteins active against Coleoptera, protein band patterns as well as their insecticidal activity on *Tribolium castraneum* adult and larvae. These studies were useful for understanding the distribution of *cry* genes and is expected to lead to the identification of the effective isolates for application in biological control of pests and novel candidate genes for bioengineered crop protection.

According to the Nazarian *et al.,* 2009, based on universal primers, strains containing *cry18* and *cry26* genes were the most abundant and represent 27.1% and 24% of the isolates, respectively, whereas *cry14*, *cry3*, *cry28*, *cry34*, c*ry35*, *cry7,8* genes were less abundant found in 14.2, 12.5, 10, 7, 7 and 5.6% of the strains respectively. Based on specific primers isolates containing *cry1I* were the most abundant (48.5%) [5].According to Mahadeva *et al.,* 2011 *cry1I* and *cry7,8* were found to be predominant[12]. In our study, strains carrying *cry26* and *cry3* were the most abundant and representing 6.16 and 6.03% of the isolates respectively. *cry1I* and *cry7,8* were present in 3.36 and 3.13 % of the isolates respectively. According to the Bravo *et al*., 1998, *cry3* readed the second highest frequency[13]. Similarly, we found *cry3* to be second abundant i.e., 6.03 % of the isolates. Arrieta *et al*., 2004 found high frequency of *cry3*, *cry7,8* and low frequency of *cry1B* in their *Bt* collection[14]. But in our study, none of the isolates amplified for *cry1B*. Similarly, in study conducted by Nazarian *et al*., 2009, none of the *B*t isolates were positive for *cry1B*.

The diversity of the *cry* gene profiles, molecular weights of the ´-endotoxins observed in the SDS-PAGE and different shapes of crystals, suggest that there are strains expressing different shapes of crystal, suggest that there are strains expressing different *cry* proteins that could be toxic against Coleopterans, Dipterans and Lepidopterans as were shown previously .Isolate CFE 20(3) positive for *cry1I*, shows a protein band pattern of 60kDa. Nazarian *et al*., 2009 also had found the most common pattern was composed of proteins with molecular weight between 60-135 kDa. According to Arrieta *et al*., 2004 , 44 kDa protein band was present in isolate positive for *cry34*. In our study, isolate CFE43(1) positive for *cry34* and *cry35*, which showed a protein band pattern of 14kDa and 44kDa respectively.

In this research, we studied the effect of the strains containing Coleopteran active *cry* genes on *Tribolium castraneum*. Isolates CFE1(1), CFE3(1), CFE20(3) and CFE43(1) showed the most toxicity as compared to reference strains HD1 and 4AA1. According to Yilmaz, S. *et al*., 2012 Bt SY49.1 showed 62 % mortality against *Tribolium castraenum*[15]. In our study,the maximum toxicity was 30% by selected strains (CFE1(1), CFE3(1), CFE20(3), CFE43(1)) .The discovery of the novel *B.thuringiensis* toxins is likely to continue at least into the near future, because need for new strains containing new *cry* genes with high toxicity and wide scope of control of different order of insects will be increased.

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