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Novel *Bacillus* Strains as Environmental Biopesticides.

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Center for Biological Control (CBC)
Department of Life Sciences,
Ben-Gurion University of the Negev, Beer-Sheva, Israel.

Second Year Report

Covering May 1999 – April 2000

Investigators
Prof. Y. Margalith*, P.I.
Prof. Z. Barak, Co-Investigator
Prof. A. Zaritsky, Co-Investigator
Department of Life Sciences Ben-Gurion
Univ. of the Negev, Beer Sheva 84105
Israel
Tel. 972-07- 646-1340
Fax. 972-07- 647-2963
E-mail yoelm@bgumail.bgu.ac.il

Dr. A. Khamraev, Co-P.I.
Dr. E. N. Troitskaya, Co-Investigator
Head , Entomology Department
Inst. of Zoology and Parasitology, Usbek
Academy of Sciences
1, A.Niyazov st. Tashkent 700095
Republic of **Uzbekistan**

Dr. M. Baizhanov, Co-P.I.
Dr. N. Berezina, Co-Investigator
Institute of Zoology and Genofound of Animals
Kazakhastan Academy of Sciences
Alma Ata 480032 **Kazakhastan**

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3. EXECUTIVE SUMMARY

The ultimate goal of this project proposal is to evaluate and further develop existing and new *Bacillus* entomopathogens as urgently needed substitutes for the detrimental toxic chemicals toward commercialization and integration into routine Integrated Biological Pest Management (IBPM). The specific research objectives are: 1) to identify *B.t.* strains with enhanced toxicity against traditional and non-traditional target insects, and 2) to discover *B.t.* strains with novel *cry*-type genes and gene combinations.

An extended PCR method was established to rapidly identify and classify *Bacillus thuringiensis* strains containing *cry* (crystal protein) genes toxic against lepidopteran, coleopteran and dipteran pests (Ben-Dov *et al.*, 1997. Appl. Environ. Microbiol. 63:4883-4890). To optimize identification of all reported *cry* genes, this methodology needs a complete PCR set of primers. Here, a set of universal (Un9) and specific primers for multiplex rapid screening for all five known genes from the *cry9* group were designed (Table 1 and 2). PCR analyses were performed for *cry9* genes on 16 standard strains and 411 field-isolates of *B. thuringiensis.* Among the standard strains, only *B. thuringiensis* subsp. *aizawai* HD-133 , which harbors *cry1* and *cry2* genes, was positive to Un9, and was found positive to *cry9Ea.* DNA of 22 field-collected isolates was also found positive with Un9. These isolates were classified into three *cry9* profiles using specific primers; all of them harbor *cry1* and *cry2.* This newly designed set of primers complements the existing PCR methodology for most currently known *cry* genes.

An alternative PCR analysis to screen for *cry7* genes is proposed, based on the five conserved blocks of amino acids of *Bacillus thuringiensis* toxins and their encoding DNA sequences. A complete set of five primers was constructed, four direct and one reverse, yielding four specific amplicons. Modified profiles can identify new *cry* genes.

BACKGROUND

Subspecies of the gram-positive, aerobic, endospore-forming bacterium *Bacillus thuringiensis* are recognized by their ability to produce during sporulation large quantities of insect larvicidal proteins (δ-endotoxins) aggregated in parasporal crystalline bodies of various forms (e.g., 6, 20). The high potencies and specificities of *B. thuringiensis* insecticidal crystal (Cry) proteins have spurred their use as natural pest control agents in agriculture, forestry and human health. Known toxins kill subsets of insects among the Lepidoptera, Coleoptera, Diptera, and nematodes (24, 29). The related toxins have recently been classified by their degree of amino acid homology (1, 11).

Five highly conserved blocks exist in the toxic core of most known Cry protoxins, which are important for their activities and specificities (16). They are arranged in three distinct-domains (I-III, from N- to C-termini) (15, 23). Block 1, encompassing the central helix α5 of domain I, has been implicated in pore formation, a role that might explain its highly conserved nature (13). Block 2 includes the C-terminal half of helix α6 and all of α7 of domain I, and the first β-strand of domain II. Helix α7 serves as a binding sensor to initiate the structural rearrangement of the pore-forming domain (12, 13). Residues within block 2 are involved in formation of salt bridges, which could be considerable in conformational changes upon binding of the toxin to receptor or for maintaining the protein in globular form (29). Block 3 contains the last β-strand of domain II and the N-terminal segment of domain III, the latter forming the interface with domains I and II (15). Block 4 corresponds to the second β-strand of domain III that affects the structural integrity of the protein molecule, oligomeric aggregation and the appropriate function of the ion channels (29, 30, 33). The highly conserved block 5 in domain III is at the C-terminus of the activated toxin, and is another major element that stabilizes the mature toxin molecules (19, 27, 34).

4. RESEARCH OBJECTIVES

The ultimate aim of this project is to protect crops from harvest to the time it reaches the consumer. The objectives of the second year were to design **specific primers**, for *cry7* and *cry8* group genes based on 5 conserved blocks of Cry toxins and their encoding DNA sequences. A novel *cry7* may thus be discovered by altered profiles such as different amplicon(s) size(s) (modified interval(s) between blocks) or absence of at least one amplicon (homology variation in a conserved block). and their encoding DNA sequences.

5. METHODS AND RESULTS

A. Methods

a. Isolation of microbial pathogens.

New potent entomotoxic *Bacilli* were isolated by the methods developed at the Center for Biological Control (CBC), Ben Gurion University (Brownbridge and Margalith, 1986, 1987). **Comparison of LC_{50} values of the new isolates with those of the standard strains will give us an indication of their relative toxicity.** We utilized the fast screening methods for the isolation of microbial pathogens that were developed during the previous stages of our collaborative research. The protocol for isolation is selective for spore-forming bacteria. These methods enable the isolation of *Bacillus* spp. from the field-collected samples, avoiding problems inherent in enrichment culture. Using these processes we were able to isolate desired bacteria at levels of less than 100 spores per gram of field-collected samples (soil, litter, insect cadavers) and to eliminate unwanted bacteria.

B.t. strains were isolated from soil using the following protocol: 0.5g soil were added to L broth buffered with 0.25 M sodium acetate to selectively inhibit germination of *B.t.* spores (Travers *et al.*, 1987). After a short incubation a 1.5 ml sample was heat shocked at 80°C for 3 min to eliminate vegetative cells. The sample was plated on L agar plates and incubated overnight at 30°C to allow spore gemination. Random colonies are picked onto T3 medium (agar dots) and allowed to sporulate overnight at

30°C. Cultures were checked for the presence of crystals, to confirm the isolation of *B.t.*. The candidate isolates were cultured on LB slants and stored at 4°C. Samples were preserved as powders by lyophillization. The powders were stored under dessication at -20°C. Samples of the liquid cultures were stored frozen at -70°C in 15% glycerol. *B.t.* isolates were further characterized according to the **morphology** of their **purified crystals** from sporulating cultures using light microscopy.

b. Gene identification by PCR.

Oligonucleotide primers. One pair of **universal** (Un) were selected from a region that is highly conserved in all four *cry9* genes (extracted from GeneBank database) to amplify a specific fragment: Their sequences and match (as well as mis-match) positions on each gene of the group, and the expected sizes of their PCR products, are displayed in Table 1.

Four **specific** primers (were selected from highly variable regions in the genes) with one universal was designed to detect four genes from *cry9* group by different size of their PCR products.The sequences and match positions of all specific primers, and the expected sizes of their PCR products, are displayed in Table 2.

The oligonucleotide primers were obtained from Ransom Hill Bioscience, Inc. (Ramona, Calif.); each pair was highly specific and yielded a PCR product of predicted size that was easily identified by electrophoresis in agarose gels (0.8-2.5%).

Here, this approach was tested to detect specifically *cry7* genes by a set of PCR primers homologous to the five conserved blocks (Fig. 1A). The four direct primers were designed to amplify four distinct amplicons with the single reverse primer and create a fingerprint specific to *cry7A* genes. A novel *cry7* may thus be discovered by altered profiles such as different amplicon(s) size(s) (modified interval(s) between blocks) or absence of at least one amplicon (homology variation in a conserved block). Primer sequences, match and mismatch positions in each *cry7*, and expected sizes of their amplicons are presented in Table 3. They were selected from regions coding for the five conserved blocks to amplify specific fragments using Amplify 1.0 program (Bill Engels, University of Wisconsin, USA). A partial set of primers to detect the conserved blocks 3 and 5 specific for the three known *cry8* was similarly examined as well (Fig.

1B); their sequences, match positions and expected sizes of resultant amplicons are presented in Table 4.

c. DNA templates and PCR analysis.

Templates were prepared from 16-18 h cultures in LB or tryptic soy broth enriched with 0.3% (w/v) yeast extract. Aliquots of 3-4.5 ml were harvested by centrifugation, washed once in TES (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl), and the pellets were resuspended in 100 □l of lysis buffer (25% sucrose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 4 mg/ml lysozyme). The cell suspension was incubated 1 h at 37°C. Further DNA extraction was performed as described by Sambrook *et al*, 1989.

Amplification was carried out in a DNA MiniCycler (MJ Research, inc., Watertown, MA, USA) for 30 reaction cycles each. Reactions were routinely carried out in 25 µl; 1 µl of template DNA was mixed with reaction buffer, 150 µM of each dNTP, 0.2-0.5 µM of each primer, and 0.5 U Taq DNA Polymerase (Appligene). Template DNA was denatured (1 min at 94°C) and annealed to primers (40-50 sec at 54-60°C), and extensions of PCR products were achieved at 72°C for 50-90 sec. Each experiment was associated with negative (without DNA template) and positive (with a standard template) controls.

TABLE 1. Characteristics of universal primers for *cry9* group genes.

Gene nomenclature			Nucleotide positions hybridized to primers[b]	Mismatch of primers[c]	Product size (bp)
Current	Original	Access. #[a]			
Sequences of *cry9* universal primers: Un9(d)[d]: 5' CGGTGTTACTATTAGCGAGGGCGG-3' Un9(r)[d]: 5'- GTTTGAGCCGCTTCACAGCAATCC-3'					
cry9A	*cryIG*	X58120	2774-2797, 3104-3127	4, 16(d); 9(r)	354
cry9B	*cryIX*	X75019	2272-2295, 2602-2625	4, 16(d); 9(r)	354
cry9C	*cry9C*	Z37527	4354-4377, 4681-4704	0(d); 0(r)	351
cry9D	-	D85560	2338-2361, 2668-2691	0(d); 0(r)	354

[a] In GenBank database.

[b] Starting from the first base of the sequence (of the respective *cry* gene) in the GenBank database.

[c] Numbers indicate bases from 5' of primers that do not match to the respective sequence.

[d] (d) and (r), direct and reverse primers, respectively.

TABLE 2. Characteristics of specific primers for *cry9* genes.

Primer Pair[a]	Sequence of primers[b]	Gene Recognized	Positions[c]	Product size (bp)
EB-9A(d) Un-9(r)	GGTTCACTTACATTGCCGGTTAGC GTTTGAGC c GCTTCACAGCAATCC	*cry9A*	1581-1604 3104-3127	1547
EB-9B(d) Un-9(r)	GCAAATGCATTTAGCGCTGGTCAA GTTTGAGC c GCTTCACAGCAATCC	*cry9B*	1925-1948 2602-2625	701
EB-9C(d) Un-9(r)	CCACCAGATGAAAGTACCGGAAG GTTTGAGCCGCTTCACAGCAATCC	*cry9C*	3473-3495 4681-4704	1232
EB-9D(d) Un-9(r)	GCAATAAGGGTGTCGGTCACTGG GTTTGAGCCGCTTCACAGCAATCC	*cry9D*	1754-1776 2668-2691	938

[a] (d) and (r), direct and reverse primers, respectively.

[b] Bases that do not match appropriate sequences are shown by lowercase letters.

[c] Starting from the first base of the sequence (of the respective *cry* gene) in the GenBank database.

TABLE 3. Characteristics of primers specific for *cry7Aa, cry7Ab1* and *cry7Ab2*

Primer pair[a]	Sequence of primers[b]	Positions[c]	Product size (bp)
B1-7A(d)	CATCTAGCTTTATTAAGAGATTC	583-605	1320
B5-7A(r)	GATAAATTCGATTGAATCTAC	1882-1902	
B2-7A(d)	GCTGTATTTCCTaTTtATGACCC	814-836	1089
B5-7A(r)	GATAAATTCGATTGAATCTAC	1882-1902	
B3-7A(d)	GGGCCTGGATTTACAGGTGG	1549-1568	354
B5-7A(r)	GATAAATTCGATTGAATCTAC	1882-1902	
B4-7A(d)	GTTAGAGTTCGATACGC t AC	1651-1670	252
B5-7A(r)	GATAAATTCGATTGAATCTAC	1882-1902	

[a] (d) and (r), direct and reverse primers, respectively.

[b] Lowercase letters in B2-7A(d) and B4-7A(d) are of bases that do not match the sequences of *cry7Aa* and *cry7Ab*, respectively.

[c] Starting from the first base (A) of the start codon of the respective *cry*. GenBank accession numbers of *cry7* sequences: M64478 for *cry7Aa1*; U04367 for *cry7Ab1*; U04368 for *cry7Ab2*.

TABLE 4. Characteristics of primers specific for *cry8*

Primer Pair[a]	Sequence of primers	Gene Recognized	Positions[b]	Product size (bp)
B3-8A(d)	GGTCCTGGATTTACAGGAGGAGAT	*cry8Aa*	1636-1659	342
B5-8A(r)	GATGAATTCGATTCGGTCTAT		1957-1977	
B3-8B(d)	GGGCGTGGTTATACAGGGGGAGAC	*cry8Ba*	1624-1647	342
B5-8B(r)	GATGAATTCGATTCGGTCTAA		1945-1965	
B3-8C(d)	GAAGGTCTATATAATGGAGGAC	*cry8Ca*	1600-1621	369
B5-8C(r)	AATAAATTCAATTCTATCAAT		1948-1968	

[a] (d) and (r), direct and reverse primers, respectively..

[b] Starting from the first base (A) of the start codon of the respective *cry*. GenBank accession numbers of *cry8* gene sequences: U04364 for *cry8Aa*; U04365 for *cry8ABa*; U04366 for *cry8Ca*.

B. Results and Discussion

An extended PCR method was established to rapidly identify and classify *Bacillus thuringiensis* strains containing *cry* (crystal protein) genes toxic against lepidopteran, coleopteran and dipteran pests (Ben-Dov *et al.*, 1997. Appl. Environ. Microbiol. 63:4883-4890). To optimize identification of all reported *cry* genes, this methodology needs a complete PCR set of primers. Here, a set of universal (Un9) and specific primers for multiplex rapid screening for all five known genes from the *cry9* group were designed (Table 1 and 2). PCR analyses were performed for *cry9* genes on 16 standard strains and 411 field-isolates of *B. thuringiensis.* Among the standard strains, only *B. thuringiensis* subsp. *aizawai* HD-133 , which harbors *cry1* and *cry2* genes, was positive to Un9, and was found positive to *cry9Ea*. DNA of 22 field-collected isolates was also found positive with Un9. These isolates were classified into three *cry9* profiles using specific primers; all of them harbor *cry1* and *cry2*. This newly designed set of primers complements the existing PCR methodology for most currently known *cry* genes.

PCR analysis for *cry7* genes was performed on four *B. thuringiensis* standard strains, as well as on 27 *B. thuringiensis* field-isolates, which have previously been found positive to a pair of universal primers (Un7,8 for *cry7* and *cry8* groups). Among the standard strains, only *B. thuringiensis* subsp. *dakota* HD-511 and *B. thuringiensis* subsp. *kumamotoensis* HD-867, known to harbor *cry7Ab1* and *cry7Ab2*, respectively yielded the four amplicons (Fig. 2) of the predicted sizes with the set of primers (Table 3). None of the 27 field-collected isolates tested yielded amplicons with this same set of primers, hence were further screened for the presence of three *cry8* genes by their respective pairs for blocks 3 and 5 (Table 4). Of these, only one yielded an amplicon specific to *cry8Ba* and none to the other *cry8*. This result could be a consequence of an unfortunate mismatch of the reverse primer (even of the terminal 3'-nucleotide), but these strains may serve as a potential pool for new genes from the *cry7* and *cry8* groups. A new gene will be detected by propagating a specific amplicon(s) differing from the standard pattern for *cry7* (Fig. 1A). A necessary condition for defining a new gene is either different interval(s) among the five conserved blocks, as found in *cry8* (Fig 1B) and in other *cry* genes, or homology variation in conserved blocks. This method can be practical for a distinct *cry* group or several groups by sets of degenerated primers.

Three additional conserved blocks have recently been identified in the C-termini of Cry protoxins. They may be exploited for extended PCR screening of *cry* genes, alone or together with the original five conserved blocks. For example, a set of four primer pairs each for a

tandem pair of the eight conserved blocks can be designed and used in a mixture for a single reaction. This reaction will yield four major amplicons from a standard *cry* created by matching the respective primers to pairs of adjacent blocks, and several additional minor amplicons between primers of distinct pairs, creating together a fingerprint specific to each *cry*.

Several hundred field-collected samples were isolated in Israel, Uzbekistan and Kazakhstan. Distribution of potential insecticidal activity based on *cry* gene identification (194 *cry* positive B.t.) is described by 11% potentially active against Lepidoptera and Diptera; 50% Lepidoptera; 14% Lepidoptera and Coleoptera; 4% Coleoptera and 21% Diptera. Some of these isolates represent a promising potential for new biological control agents effective against Lepidotera, Coleoptera and Diptera. Thirty-five distinct *cry*-type profiles were identified from these field-collected *B. thuringiensis* strains. Several of them were found to be different from all published profiles. These field-collected strains seem to contain new gene(s) promising for biological control of insects and resistance management.

TABLE 5. Distribution of cry-type gene profiles of *B. thuringiensis* isolates collected in Israel, Kazakhstan and Uzbekistan

Cry-type gene profile[a]	Number of isolate(s)	Predicted insecticidal activity
1. *cry1*	26	Lep.
2. *cry1* + *cry2Aa*	1	Lep.
3. *cry1* + *cry2Aa, -Ab*	1	Lep. + Dip.
4. *cry1* + *cry2Ab* + *cry9D*	1	Lep.
5. *cry1*+ *cry7, -8*	2	Lep. + Col.
6. *cry1*+ *cry9*	3	Lep
7. *cry1Aa*	1	Lep.
8. *cry1Aa, -Ac*	7	Lep.
9. *cry1Aa, - Ac* + *cry2*	1	Lep.
10. *cry1Aa, -Ac* + *cry2Aa, -Ab*	17	Lep. + Dip.
11. *cry1Aa, -Ac* + *cry2Ab,* + *cry7, -8* + *cry9D*	1	Lep. + Col.
12. *cry1Aa, -Ab, -C, -D* + *cry2Ab, -Ac* + *cry7, -8* + *cry9E*	12	Lep. + Col.
13. *cry1Aa, -Ab, -Ac, -D* + *cry2Aa, -Ab* + *cry7, -8* + *cry9A, -B*	2	Lep. + Col.
14. *cry1Aa, -B, -C, -D* + *cry2Ab* + *cry7, -8*	3	Lep. + Col.
15. *cry1Ab*	2	Lep.
16. *cry1Ab* + *cry2Ab* + *cry9A, -B*	5	Lep.
17. *cry1Ab, -Ac*	2	Lep.
18. *cry1Ab, -D*	12	Lep.
19. *cry1Ab, -D* + *cry9*	5	Lep.
20. *cry1Ab, -D* + *cry2Ab* + *cry9A, -B*	3	Lep.
21. *cry1Ab, -D* + *cry2Ab, -Ac*+ *cry7, -8* + *cry9A, -B*	6	Lep. + Col.
22. *cry1Ab, -D, -E* + *cry2Aa, -Ab* + *cry9A, -B*	3	Lep. + Dip.
23. *cry1Ac*	7	Lep.
24. *cry1Ac*+ *cry2Aa*	2	Lep.
25. *cry1Ac*+ *cry2Aa, -Ab*	3	Lep.
26. *cry1Ac, -D*	6	Lep.
27. *cry1B*	1	Lep.
28. *cry1B* + *cry2Ab*	1	Lep.
29. *cry1E* + *cry2Aa*	1	Lep. + Dip.
30. *cry1E* + *cry2Aa, -Ab* + *cry7, -8*	1	Lep. + Col.
31. *cry2Ab*	3	Lep.
32. *cry4*	1	Dip.
33. *cry4A, -B, cry11A*	40	Dip.
34. *cry7, -8*	8	Col.
35. *cry9*	4	Lep.

[a] *cry1, cry2, cry4, cry7, cry8* and *cry9* (without letter) indicate positive with universal and negative with specific primers.

We have amassed a collection of *B.t.* isolates some of which represent new *cry* toxin gene combinations. These are now awaiting screening against a variety of agricultural and public health pest insects.

6. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

This project strengthens the participating laboratories by advancing their scientific capabilities through training and requisition of needed equipment. Equipment such as autoclaves, shakers, computers, fax machines and Xerox machines were purchased for the CAR collaborators. Six collaborating scientists from Uzbekistan and Kazakhstan were trained for 3 weeks in the PI's Center for Biological Control at BGU. They exchanged and coordinated information on isolation and screening technologies and coordinated future joint work. Efforts should be made to introduce the PCR analysis technique to the CAR laboratories.

7. PROJECT ACTIVITIES/OUTPUTS

Collaboration among the participating scientists was achieved by exchange of visits in each other's laboratories. During the past years scientists from Uzbekistan and Kazakhstan stayed in the Center for Biological Control of the Ben-Gurion University and the Israeli PI visited in the Academies of Sciences of the collaborating countries. Professional and technical capability of the participating laboratories were advanced through the equipment purchased and training conducted during the course of this study. Collaboration among the participating scientists was achieved by exchange of visits in each other's laboratories. A workshop was conducted in Ben Gurion University of the Negev on the dates April 26 – May 3, 1999 (see attached workshop schedule) in which results, research activities and future collaborative work was discussed.

8. PROJECT PRODUCTIVITY

Scientific Output	published	in press/accepted
Paper in an International Journal	1	
Paper in a National Journal *		1
Abstract in proceedings of a conference	2	
Thesis (MSc, PhD, etc.) Postdoc	1	
Patent		
Oral Presentation, Public Lecture	1	

Scientific papers, presentation & patents

Ben-Dov, E., Q. Wang, A. Zaritsky, R. Manasherob, Z. Barak, B. Schneider, A. Khamraev, M. Baizhanov, V. Glupov and Y. Margalith (1999) Multiplex PCR screening to detect *cry9* genes in *Bacillus thuringiensis* strains. Appl. and Environ. Microbiol. **65**: 3714-3716

Ben-Dov, E., R. Manasherob, A. Zaritsky, Z. Barak and Y. Margalith (submitted). PCR analysis of *cry7* genes in *Bacillus thuringiensis* by the five conserved blocks of toxins. Submitted to Appl. and Environ. Microbiol..

Ben-Dov, E. Q. Wang, A. Zaritsky, R. Manasherob, Z. Barak and Y. Margalith (1998) Multiplex PCR screening to detect *cry9* genes in *Bacillus thuringiensis*. Acta Parasitologica Portuguesa 5(1): 41. Xith European SOVE Meeting 13-17 October 1998.

Ben-Dov, E., Q. Wang, A. Zaritsky, R. Manasherob, Z. Barak, B. Schneider, A. Khamraev, M. Baizhanov, V. Glupov and Y. Margalith (1999). Screening by PCR for detection of *cry9* genes from *Bacillus thuringiensis*. Society for Invertebrate Pathology 32nd Annual Meeting, Irvine California, 23-27 August 1999.

9. FUTURE WORK

Microbial insecticides in current use, particularly *B.t.*, are effective against a limited range of insect pests. We have detected novel combinations of toxin proteins and recombinant *cry*-toxin genes in new isolates of *B.t.* (Table 5). These are being tested against pests belonging to "traditional" and other orders, such as orthopterans. The multifaceted analysis of strains collected from a wide geographic and climatic area based on a combination of microscopy, protein analysis, and gene characterization by PCR, will lead to the identification of useful microbial pesticides.

Detection of new toxin proteins and their genes from crystals that show no homology to the known Cry proteins.

Several of our spore-forming field isolates did not give PCR products. Their crystal morphology will be studied by microscopy to determine their size, shape and number of inclusions (Bulla *et al.*, 1980). The protein profile of interesting crystals will be analysed by SDS-PAGE. The solubility of these novel proteins at different pHs will be investigated. Novel ICPs with interesting insecticidal properties will be digested with proteolytic enzymes for microsequence and their genes will be cloned by PCR. This will lead to the identification of new toxin genes.

We plan to design PCR primers based on the sequence of the five conserved boxes among additional *cry* genes, as we have done for *cry7* and *cry8* during his reporting period.. *Cry* genes contain either five or one conserved boxes (Juarez-Perez et al., 1994). The detection of new combinations of these boxes may infer the

new natural recombinant *cry* genes in our field samples. Strains with novel insecticidal activity will provide new genes for the development of recombinant organisms.

Recently Kuo and Chak (1996) suggest that restriction fragment length polymorphism (RFLP) following PCR amplification, can employed to identify the origin of the *cry*-type genes. The PCR-RFLP typing system is a facile method to detect both known and novel cry genes existing in a B.t. strains. All these plans are presented in great detail in the submitted follow-on project proposal.

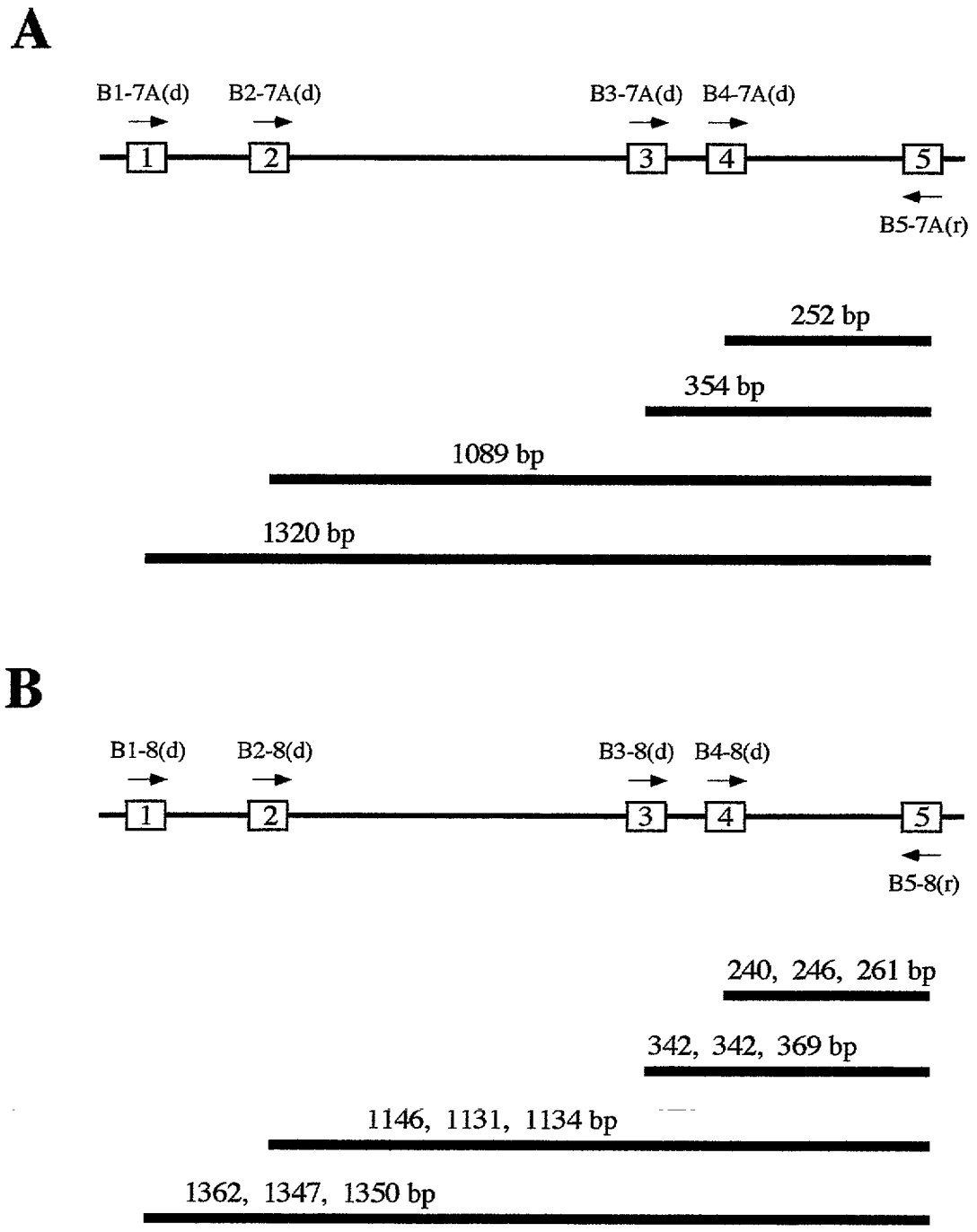


Figure 1. Strategy for detecting *cry7* (A) and *cry8* (B) genes by PCR based upon the five conserved blocks of amino acids in toxins. Arrows above boxes (enclosing block number) indicate direction of primers (Tables 1 and 2). Predicted lengths (in bp) of the resultant amplicons (thick lines) are indicated. The three sizes correspond to *cry8Aa*, *cry8Ba* and *cry8Ca* variants. The three *cry7* variants result in identical amplicon.

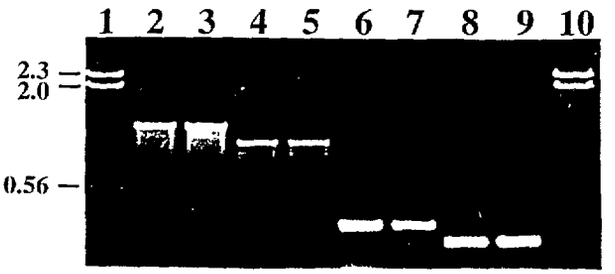
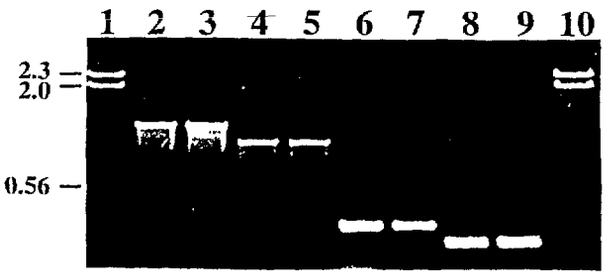


Figure 2. Agarose gel (1%) electrophoresis of PCR products obtained with the direct primers for the four conserved blocks of *cry7* and the reverse primer of block 5 (Table 1). Lanes 2, 4, 6 and 8, template DNA from *B. thuringiensis* subsp. *dakota* HD-511 (with *cry7Ab1*); lanes 3, 5, 7 and 9,template DNA from *B. thuringiensis* subsp *kumamotoensis* HD-867 (with *cry7Ab2*). Lanes 2 and 3, amplification performed with the direct primer for block one (B1); lanes 4 and 5, with that for B2; lanes 6 and 7, with that for B3, lanes 8 and 9, with that for B4. Lanes 1 and 10, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII); sizes (in kb) indicated on left.



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