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Novel *Bacillus* Strains as Environmental Biopesticides.

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3. EXECUTIVE SUMMARY

Purpose of Project

The ultimate goal of this project proposal was to detect, evaluate and further develop existing and new *Bacillus* entomopathogens as urgently needed substitutes for the detrimental toxic chemicals toward integration into routine Integrated Biological Control (IBC).

Findings (accomplishments) over entire project period (in context of international development)

An extended multiplex PCR method was established to rapidly identify and classify *Bacillus thuringiensis* strains containing *cry* (crystal protein) genes toxic against Lepidopteran, Coleopteran and Dipteran species. The technique enriches current strategies and simplifies the initial stages of large-scale screening of *cry* genes by pinpointing isolates that contain specific genes or unique combinations of interest with potential insecticidal activities thus facilitating subsequent toxicity assays.

Six pairs of universal primers were designed to probe the highly conserved sequences and classify most genes known in the following groups: twenty *cry1* genes, three *cry2*, four *cry3*, two *cry4*, two *cry7*, three *cry8* and five *cry9* genes. DNA of each positive strain was probed by a set of specific primers, designed for 30 genes. Thirty five distinct *cry*-type profiles were identified from about 400 field-collected *B. thuringiensis* strains. **Several of them were found to be different from all published profiles.** Three standard and 27 field-collected strains were positive with universal but negative with specific primers for all five known genes of *cry7* and *cry8*. An alternative PCR analysis to screen for *cry7* and *cry8* genes was developed, based on the five conserved blocks of amino acids of *Bacillus thuringiensis* toxins and their encoding DNA sequences. A complete set of five specific primers was constructed, four direct and one reverse, yielding four specific amplicons. PCR analysis for *cry7* and *cry8* 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). None of the 27 field-collected isolates tested yielded amplicons with this same set of primers. These strains may serve as a potential pool for new genes from the *cry7* and *cry8* groups. Indeed, further screening for the presence of three *cry8* genes by their respective pairs for blocks 3 and

5 detected one that yielded an amplicon specific to *cry8Ba*. This same field-collected strain must be novel because it did not react with *cry8Ba*-specific pair of primers. **These field-collected strains seem to contain new gene(s) promising for biological control of insects and resistance management.**

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 was designed. PCR analyses were performed for *cry9* genes on 16 standard strains and 215 positive 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, but negative to all four specific primers for *cry9* genes. 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.**

Using this method we found seven distinct *cry*-type profiles and recorded their distributions in Israel, Usbekistan and Kazakhstan. Three new genes with potential insecticidal activity were identified. One of them seems to be a naturally-occurring recombinant, apparently composed of *cry1*- and *cry4*-type genes. The other two (apparently homologous to *cry8A* and *cry8B*) seem to include various combinations of either *cry1* or *cry4*.

Skills or awareness remains in developing country as a result of project

Skills were acquired by the collaborating laboratories' personnel in Uzbekistan and Kazakhstan on the methods and techniques of molecular biology and microbiology, in particular PCR analysis, through training workshops and several meetings where information was exchanged between the collaborating scientists and the PI's team in Ben Gurion University. Equipment and materials were also purchased within the framework of this project, enabling sustained investigations on novel Bt strains and genes in these laboratories after the termination of the funding period.

Context of international development:

The research project was designed in the context of collaboration between an Israeli team and Central Asian Republics: Uzbekistan and Kazakhstan. The collaborative research efforts strengthened their scientific research base by providing improved facilities and equipment. Through the exchange of information and know how the collaborating laboratories in these countries acquired skills in biological control of insect pests in general, in microbiology and in molecular biology, in particular. These skills were improved by training of scientists from Central Asian Republics (CAR) at the Center for Biological Control (CBC), BGU as well as mutual visits amongst the scientists of the participating laboratories. With the purpose to report the results of the ongoing project and plan for future activities, workshops were conducted both in Israel and in Uzbekistan. The workshops were conducted in the Russian language, with Israeli scientists Drs. Ben-Dov and Manasherob who are fluent in the Russian language and Prof. Margalith who has a fair command of the Russian language.

4. RESEARCH OBJECTIVES

Why project was conducted?

Over half a century of synthetic pesticide applications has led to the emergence and spread of resistance in agricultural pests and vectors of human diseases and to environmental degradation. The very properties that made these chemicals useful—long residual action and toxicity to a wide spectrum of organisms—have brought about serious problems (21, 25). An urgent need, therefore, has thus emerged for environment-friendly pesticides to reduce contamination and the likelihood of insect resistance (20, 25). Our project was conducted to find, evaluate and develop environmentally safe biological control agents for the protection of agricultural crops against insect pests and the abatement of vector-borne diseases.

What problem was addressed?

The specific objective of this project is the detection, isolation and characterization of new crystal-forming bacteria from insect cadavers, soil, food storages and other habitats.

Characterization of the bacterial isolates by a unique combination of microscopy, toxin protein analysis, gene characterization by Polymerase Chain Reaction (PCR) and insecticidal activity will lead to the identification of new important biopesticides.

The specific research objectives were:

1. To identify *B.t.* strains with **enhanced toxicity** against traditional target insects.
2. To discover *B.t.* strains with **novel *cry*-type genes** and **gene combinations**.
3. To find *B.t.* strains with **broader host spectra**
4. To detect **new toxin proteins** and their **genes** from crystals that show no homology to the known Cry proteins.

Why is problem important to development?

The multitude of insecticidal crystal proteins of *B. thuringiensis* subspecies has spurred their use as natural control agents with applications in agriculture, forestry, and human health (25). Recent trends suggest that biological control will become increasingly important, particularly as a part of strategies for integrated pest management (21, 22). Novel insecticidal bacteria, with an extended target spectrum, for

example, would enhance environmentally safe biocontrol practices and lead to increased food production and postharvest protection (25).

How project fits into on-going research by other scientists:

Recent trends suggest that biological control will become increasingly important, particularly as a part of strategies for integrated pest management (21, 22). Novel insecticidal bacteria, with an extended target spectrum, for example, would enhance environmentally safe biocontrol practices and lead to increased food production and postharvest protection (25). The genes coding for the insecticidal crystal proteins, which are normally associated with large plasmids, direct the synthesis of a family of related proteins that have been classified as CryI to - VI depending on the host specificity (Lepidoptera, Diptera, Coleoptera, and nematodes) and the degree of amino acid homology (12, 15, 20). PCR has been exploited to predict insecticidal activities (5), to identify *cry*-type genes (3, 6, 7, 13) and determine their distribution (8), and to detect new such genes (16, 18). To optimize identification of all reported *cry* genes, this methodology needs a complete PCR set of primers (17).

In line with the recent trends of research, we investigated and developed an enhanced strategy using PCR for extended multiplex rapid screening (3) of *B. thuringiensis* strains that harbor genes from seven classes. **This strategy has enriched the existing arsenal of insecticidal strains, identify novel toxin genes or new combinations of known genes, and predicted their toxicities.**

Innovative Aspects of Project:

Novel strains of Bt and novel genes and gene combinations were discovered during the course of this project, with potential use as biological control agents against a wide spectrum of insect pests in integrated biological control programs.

How other organizations supported the project?

Support from organizations of each of the collaborating researchers was provided in the form of existing laboratories, equipment and permanent staff not paid through grant money.

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.**

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) primers 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. 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; 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 µl 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	Mismatch of	Product
Current	Original	Access. #[a]	hybridized to primers[b]	primers[c]	size (bp)
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)	GGTTCACTTACATTGCCGGTTAGC	*cry9A*	1581-1604	1547
Un-9(r)	GTTTGAGC c GCTTCACAGCAATCC		3104-3127	
EB-9B(d)	GCAAATGCATTTAGCGCTGGTCAA	*cry9B*	1925-1948	701
Un-9(r)	GTTTGAGC c GCTTCACAGCAATCC		2602-2625	
EB-9C(d)	CCACCAGATGAAAGTACCGGAAG	*cry9C*	3473-3495	1232
Un-9(r)	GTTTGAGCCGCTTCACAGCAATCC		4681-4704	
EB-9D(d)	GCAATAAGGGTGTCGGTCACTGG	*cry9D*	1754-1776	938
Un-9(r)	GTTTGAGCCGCTTCACAGCAATCC		2668-2691	

[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

The results of our research within the framework of this project were published in several peer reviewed articles, chapters in collective volumes and presentations at international conferences (section 7 – Project Activites/Outputs). A summary of these results is provided below.

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. To optimize identification of all reported *cry* genes, this methodology needs a complete PCR set of primers. This newly designed set of primers complements the existing PCR methodology for most currently known *cry* genes. Here, a set of universal (Un9) and specific primers for multiplex rapid screening for all five known genes from the *cry9* group were designed (Tables 1 and 2). PCR analyses were performed for *cry9* genes on 16 standard strains and 411 field-isolates of *B. thuringiensis* (Table 5). 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* (Table 6, 7).

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 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*. A necessary condition for defining a new gene is either different interval(s) among the five conserved blocks, as found in *cry8* 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. PCR analyses were performed for *cry9* genes on 16 standard strains and 411 field-isolates of *B. thuringiensis*

Uzbekistan 1998-1999

colony no.	*cry1*	*cry2*	*cry3*	*cry4+11*	*cry7+8*	*cry9*
U-1/98	Ab, D	Ab, Ac	-	-	+	A, B
U-2/98	Aa, B, D, C	Ab	-	-	+	Un- not specif
U-3/98	Aa, Ab, D, C	Ab, Ac	-	-	+	A, B
U-4/98	Aa, Ab, Ac, D	Aa, Ab	-	-	+	A, B
U-5/98	Aa, Ac	Aa, Ab	-	-	-	-
U-6/98	B	Ab	-	-	Un-not specif	Un-not specif
U-7/98	-	-	-	-	+	Un-not specif
U-8/98	Aa, Ac	Aa, Ab	-	-	Un-not specif	-
U-9/98	Aa, Ac	Aa, Ab	-	-	Un-not specif	-
U-10/98	Ab, Ac	-	-	-	-	-
U-11/98	Ab, D, E	Aa, Ab	-	-	-	+
U-12/98	Ab, D, E	Ab	-	-	Un-not specif	A, B
U-13/98	-	Ab	-	-	+	-
U-14/98	-	Ab	-	-	-	Un-not specif
U-15/98	Aa, Ac	Aa, Ab	-	-	-	Sp-not specif
U-16/98	Aa, Ab, Ac, D	Aa, Ab	-	-	+	A, B
U-17/98	E	Aa, Ab	-	-	+	-
U-18/98	Aa, Ab	-	-	-	-	-
U-19/98	Ab	-	-	-	-	-
U-20/98	-	Ab	-	-	-	-
U-21/98	+	-	-	-	-	-
U-22/98	-	-	-	-	-	-
U-23/98	-	-	-	-	-	-
U-24/98	Aa, Ac	Aa, Ab	-	-	-	-
U-25/98	-	-	-	-	-	-
U-26/98	-	-	-	-	-	-
U-27/98	Ab	-	-	-	-	-
U-28/98	+	-	-	-	-	-
U-29/98	+	-	-	-	+	-
U-30/98	Aa, Ac	Ab	-	-	+	D
U-31/98	+	-	-	-	-	-
U-32/98	Aa, Ac	Aa, Ab	-	-	-	-
U-33/98	+	-	-	-	-	-
U-34/98	+	-	-	-	-	-
U-35/98	+	-	-	-	-	-
U-36/98	Ab, Da	-	-	-	-	+
U-37/98	Ab, Da	Ab	-	-	-	A, B
U-38/98	Ba	-	-	-	-	-
U-39/98	Aa, Ac	Aa, Ab	-	-	-	-
U-40/98	Aa, Ab, Ca, Da	Ab	-	-	+	E
U-41/98	+	-	-	-	-	-
U-42/98	-	-	-	4A, B, 11A	-	-
U-43/98	+	-	-	-	-	+
U-44/98	Ac	Aa, Ab	-	-	-	-
U-45/98	-	-	-	4A, B, 11A	-	-
U-46/98	Ac	Aa	-	-	-	-
U-47/98	+	-	-	-	-	-
U-48/98	Ac	Aa, Ab	-	-	-	-
U-49/98	+	Ab	-	-	-	-
U-50/98	+	Aa, Ab	-	-	-	-
U-51/98	+	-	-	-	-	-

colony no'	*cry1*	*cry2*	*cry3*	*cry4+11*	*cry7+8*	*cry9*
U-52/98	Ab	Ab	-	-	-	A, B
U-53/98	+	-	-	-	+	-
U-54/98	Ac			-		
U-55/98	+			-		
U-56/98	Ab, Da			-		
U-57/98	Ab, Da			-		
U-58/98						
U-59/98						
U-60/98	Ac					
U-61/98						
U-62/98	Ab					
U-63/98						
U-64/98						
U-65/98						
U-66/98						
U-67/98						
U-68/98						
U-69/98						
U-70/98						
U-71/98						
U-72/98						
U-73/98						
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U-75/98						
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U-77/98						
U-78/98						
U-79/98						
U-1/99						
U-2/99						
U-3/99						
U-4/99						
U-5/99						
U-6/99						
U-7/99						
U-8/99						
U-9/99						
U-10/99						
U-11/99						
U-12/99						
U-13/99						

Novosibirsk 1998-1999

colony no'	*cry1*	*cry2*	*cry3*	*cry4+11*	*cry7+8*	*cry9*
N-1/98	-	-	-	4A, B, 11A	-	-
N-2/98	-	-	-	4A, B, 11A	-	-
N-3/98	Ac	Aa, Ab	-	-	-	-
N-4/98	+	Aa	-	-	-	-
N-5/98	-	-	-	4A, B, 11A	-	-
N-6/98	-	-	-	4A, B, 11A	-	-
N-7/98	-	-	-	4A, B, 11A	-	-
N-8/98	-	-	-	4A, B, 11A	-	-
N-9/98	Ea	Aa	-	-	-	-
N-10/98	+	-	-	-	-	-
N-11/98	-	-	-	+	-	-
N-12/98	-	-	-	+	-	-
13N	+	-	-	-	-	-
14N	-	-	-	+	-	-
15N	Aa, Ac	+	-	-	-	-
16N	Ac	Aa	-	-	-	-
17N	-	-	-	-	-	-
18N	Ab	-	-	-	-	-
19N	Ab, D	-	-	-	-	-
20N	+	-	-	-	-	-
21N	+	-	-	-	-	-
22N	+	-	-	-	-	-
23N	Aa, Ac	-	-	-	-	-
24N	Ac	-	-	-	-	-
25N	Aa	-	-	-	-	-
26N	Ac	-	-	-	-	-
27N	Ac	-	-	-	-	-
28N	Ab, D	-	-	-	-	-
29N	Ac	Aa, Ab	-	-	-	-
30N	Ab, Da	-	-	-	-	-
31N	Ab, Da	-	-	-	-	-
32N	Ab, Da	-	-	-	-	-
33N	Ab, Da	-	-	-	-	-
34N	Ab, Ac	-	-	-	-	-
35N	Ac, Da	-	-	-	-	-
36N	Ac, Da	-	-	-	-	-
37N	Ac, Da	-	-	-	-	-
38N	Ac, Da	-	-	-	-	-
39N	-	-	-	+	-	-
40N	Ac, Da	-	-	-	-	-
41N	-	-	-	+	-	-
42N	Ac, Da	-	-	-	-	-
43N	+	-	-	-	-	-
44N	+	-	-	-	-	-
N-1/99						
N-2/99						
N-3/99						
N-4/99						
N-5/99						
N-6/99						
N-7/99						
N-8/99						
N-9/99						

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colony no'	*cry1*	*cry2*	*cry3*	*cry4+11*	*cry7+8*	*cry9*
K-1/98		-	-	4A, B, 11A	-	-
K-2/98	Un-not specif	-	-		-	+
K-3/98		-	-		+	-
K-4/98		-	-		+	-
K-5/98		-	-		+	-
K-6/98	Un-not specif	-	-		-	-
K-7/98	Un-not specif	-	-		Un-not specif	-
K-8/98		-	-		+	-
K-9/98		-	-		-	-
K-10/98		-	-	+	Un-not specif	-
K-11/98		-	-		-	-
K-12/98	Un-not specif	-	-		Un-not specif	+; Un-not spec
K-13/98						
K-14/98						
K-15/98						
K-16/98						
K-17/98						
K-18/98						
K-19/98						
K-20/98						
K-21/98						
K-22/98						
K-23/98		-	-		-	-
K-24/98		-	-		-	-
K-25/98		-	-	4A, B, 11A	+	-
K-26/98	+	-	-		-	-
K-27/98	+	Ab	-		-	D
K-28/98	-	-	-	4A, B, 11A	+	-
K-29/98	-	-	-		-	-
K-30/98	-	-	-		+	-
K-31/98	-	-	-	4A, B, 11A	-	-
K-32/98	-	-	-		-	-
K-33/98	-	-	-	4A, B, 11A	-	-
K-34/98	Un-not specif	-	-	+	-	-
K-35/98	-	-		-	-	-
K-36/98	-	-		-	-	-
K-37/98	-	-		-	-	-
K-38/98	-	-		-	-	-
K-39/98	-	-		+	Un-not specif	-
K-40/98	+	Aa, Ab		-	-	+A?
K-41/98	-	-		-	Un-not specif	-
K-42/98	+	Ab		-	-	+A?
K-43/98						
K-44/98						
K-45/98						
K-46/98						
K-47/98						
K-48/98						
K-49/98						
K-50/98						
K-51/98						

Table 6. 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 primer

Table 7. Distribution of *cry*-type gene profiles of *B. thuringiensis* isolates as analysed by PCR

cry-type gene profiles	Number of *Bt* isolates
cry1	58
cry2	3
cry3	0
cry4	41
cry7,8	6
cry9	4
cry1 + 2	51
cry1 + 7,8	3
cry1 + 9	5
cry1 + 2 + 7,8	4
cry1 + 2 + 9	4
cry1 + 2 + 7,8 + 9	13
Negative to PCR	4
Total	196

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. PCR analysis, and microbiological techniques as well as equipment and materials were introduced to the CAR laboratories, providing the appropriate resources for sustainable research activities in novel gene and protein identification, charaterization and development toward a new generation of environment-friendly biocontrol agents against insect pests of agricultural and public health importance.

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. A combined workshop and field trip were conducted in Uzbekistan between the dates August 23 to September 3, 1999. During this integrated workshop-field trip, results and activities under this project were discussed with all collaborating scientists. An additional workshop in May 3-6, 2001 was conducted in Almaty, Kazakhstan during which time final reporting and budgetary matters were discussed.

Four peer reviewed articles, 10 presentations in international conferences and 1 postdoc and 1 Ph.D. theses were produced as an outcome of the support provided in this project (Appendix – Full text of articles published).

Scientific Output	**published**
Papers in an International Journal	3
Papers in a National Journal *	1
Abstract in proceedings of a conference	11
Thesis (MSc, PhD, etc.) Postdoc	2
Chapter in collective volume	1

A. Refereed Articles in Scientific Journals

1. Manasherob, R., Ben-Dov, E., Margalit, J., Zaritsky, A. & Barak, Z. (1996). Raising activity of *Bacillus thuringiensis* var. *israelensis* against *Anopheles stephensi* larvae by encapsulation in *Tetrahymena pyriformis* (Hymenostomatida: Tetrahymenidae). J. Amer. Mosq. Control Assoc. **12:** 627-631.

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B. Chapter in Books

Margalith, Y. & Ben-Dov, E. (2000). Biological Control by *Bacillus thuringiensis* subsp. *israelensis.* Chapter **8**, pp. 243-301, in J. E. Rechcigl and N. A. Rechcigl (eds.), *Insect Pest Management: Techniques for Environmental Protection.* Lewis Publishers and CRC Press LLC, Boca Raton, FL.

C. Presentations in conferences

1. Manasherob, R., Margalit, J., Ben-Dov, E., Zaritsky, A. & Barak, Z. (1994). Raising larvicidal activity of *Bacillus thuringiensis* var. *israelensis*†δ-endotoxin against *Anopheles stephensi* by encapsulation in *Tetrahymena pyriformis.* Book of Abstracts, p. 30. VIII European Meet. Society for Vector Ecology. Barcelona, Spain.
2. Manasherob, R., Margalit, J., Ben-Dov, E., Zaritsky, A. & Barak, Z. (1994). Raising larvicidal activity of *Bacillus thuringiensis* var. *israelensis*†δ-endotoxin against *Anopheles stephensi* by encapsulation in *Tetrahymena pyriformis.* VIIth Conference Isr. Soc. Entomol., p. 27-28.

3. Ben-Dov, E., Dahan, E., Zaritsky, A., Sinai, R., Manasherob, R., Barak, Z., Khameraev, A., Troyetskaya, A., Dubitsky, A., Berezina, N. & Margalith, Y. (1996). Novel *cry*-type gene detected by extended PCR screening from field-collected strains of *Bacillus thuringiensis.* Second En Gedi Conference on Bacterial Control of Agricultural Insect Pests and Vectors of Human Diseases. (Abstract P#34), August 12-16, Shoresh, Israel.
4. Ben-Dov, E., Dahan, E., Zaritsky, A., Sinai, R., Manasherob, R., Barak, Z., & Margalith, Y. (1996). Extended screening by PCR for *cryI, cryII, cryIII* and *cryIV*from field-collected strains of *Bacillus thuringiensis.* 29th Annu. Meet. Soc. Invertebr. Pathol. and IIIrd International Colloquium on *Bacillus thuringiensis.* (Abstract, p 8), September 1-6, Cordoba, Spain.
5. Ben-Dov, E., Zaritsky, A., Manasherob, R., Barak, Z., & Margalith, Y. (1997). New *cry7,* and *cry8* from *Bacillus thuringiensis.* 30th Annu. Meet. Soc. Invertebr. PatholÆf(Abstract, p 8), August 24-29, Banff, Canada.
6. Ben-Dov, E., Zaritsky, A., Manasherob, R., Barak, Z., & Margalith, Y. (1997). New *cry7,* and *cry8* from *Bacillus thuringiensis.* 2nd International*f*Congress of Vector Ecology (Abstract P#36,
p 72), October 19-24, Orlando, USA.
7. Margalit, J. & Ben-Dov, E. (1998). Bti - present status and future trends. VIth Symposium on Biological Control. (Abstract-56, p 45), May 24-28, Rio de Janeiro, Brazil.
8. Ben-Dov, E., Wang, Q., Zaritsky, A., Manasherob, R., Barak, Z. & Margalith, Y. (1998). Multiplex PCR screening for detection *cry9* genes from *Bacillus thuringiensis.* XI European Meet. Society for Vector Ecology. Book of Abstracts, p. 30, October 13-17, Lisbon, Portugal.
9. Ben-Dov, E., Wang, Q., Zaritsky, A., Manasherob, R., Barak, Z. & Margalith, Y. (1998). Multiplex PCR screening for detection *cry9* genes from *Bacillus thuringiensis.* 2nd International Symposium on Biopesticides Book of Abstracts, p. 1-2. October 26-30, Wuhan, China.
10. Ben-Dov, E., Wang, Q., Zaritsky, A., Manasherob, R., Barak, Z. & Margalith, Y. (1998). Multiplex PCR screening for detection *cry9* genes from *Bacillus thuringiensis.* XI European Meet. Society for Vector Ecology. Book of Abstracts (Acta Parasitólogia Portuguesa, Vol.5, p. 41.), October 13-17, Lisbon, Portugal.
11. Margalith, Y., Zaritsky, A., Barak, Z., Manasherob, R., Schneider, B. & Ben-Dov, E. (2000). *Bacillus thuringiensis israelensis* (Bti) in integrated biological control (IBC) of mosquitoes and black flies – A global view. pp. 84-98. Proc. of the 13th European SOVE Meeting, Society for Vector Ecology. (Sept., 2000, Belek, Antalya Turkey).

8. PROJECT PRODUCTIVITY

We have accomplished our stated goals as follows:

1. *To identify B.t. strains with* ***enhanced toxicity*** *against traditional target insects.* We have identified ca. 200 new B.t strains with potentially novel activity against Coleoptera , Lepidoptera and Diptera
2. *To discover B.t. strains with* ***novel cry-type genes and gene combinations.*** About 70 isolates with novel *cry*-type genes and gene combinations were identified out of more than 400 field-collected samples.
3. *To find B.t. strains with* ***broader host spectra.*** Several Bt strains with novel gene combination have potentially broader host spetra.
4. *To detect* ***new toxin proteins*** *and their* ***genes*** *from crystals that show no homology to the known Cry proteins.* Some of the Bt strains negative to any of the primers used (universal and specific) formed crystal protein inclusions which are probably novel toxins with potential activity against new and existing insect pest targets.

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. All PCR products which reacted positively with universal and negatively with specific primers, are potentially new genes which could encode for new toxins, and may be effective against existing and new target pests. In order to

investigate this possibility the following investigations must be conducted: 1) isolate the gene by PCR amplification or by constructing a cloning library, 2) carry out gene sequencing, 3) conduct homology analyses with existing cry genes, 4) perform transformation of new genes to Bt acrystalliferous mutants in order to: a) learn about the expression of these genes on a level of transcription, translation and post translation (crystallization), b) determine the protein profile of interesting crystals by SDS-PAGE, c) determine the solubility of these novel proteins at different pHs.

Novel ICPs with interesting insecticidal properties will be digested with proteolytic enzymes for microsequence and their genes will be cloned by PCR.

Develop UV resistant strains.

A promising line of investigatioin is in the field of UV resistance since this is a key factor in the deactivatioin of Bt toxins under field conditions. Two lines of investigation are proposed:

1. Transfer or clone genes encoding for novel toxins into existing known UV resistance microorganisms.
2. Selection of UV resistant strains containing novel genes by artificial UV exposure over many generations.

Engineering transgenic microorganisms which combine several toxins with different modes of action in order to reduce the chance of resistance and enhance efficacy, e.g., Bacillus thuringiensis toxins in Photorhabdus luminescens.

We stand to benefit from continued research of novel genes and gene combinations for utilization in integrated biological control (IBC) programs for controlling insect pests of agricultural and public health importance. Continued research and development based on existing knowledge and gene bank could produce improved biocontrol agents in the near future.

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Multiplex PCR Screening To Detect *cry9* Genes in *Bacillus thuringiensis* Strains

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An extended PCR method was established to rapidly identify and classify *Bacillus thuringiensis* strains containing *cry* (crystal protein) genes toxic to lepidopteran, coleopteran, and dipteran pests (Ben-Dov et al., Appl. Environ. Microbiol. 63:4883–4890, 1997). To optimize identification of all reported *cry* genes, this methodology needs a complete PCR set of primers. In the study reported here, a set of universal (Un9) and specific primers for multiplex rapid screening for all four known genes from the *cry9* group was designed. PCR analyses were performed for *cry9* genes on 16 standard strains and 215 field isolates of *B. thuringiensis*. Among the standard strains, only *B. thuringiensis* subsp. *aizawai* HD-133, which harbors *cry1* and *cry2* genes, was positive with Un9 but negative to all four specific primers for *cry9* genes. DNA of 22 field-collected isolates was also found to be 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.

The soil bacterium *Bacillus thuringiensis* fulfills the requisites of a microbiological control agent against agricultural pests and vectors of diseases that lead to its widespread commercial application. It is a gram-positive, aerobic, endospore-forming saprophyte (1, 18). All known subspecies of *B. thuringiensis* produce large quantities of insecticidal crystal proteins (ICPs) which are segregated in parasporal bodies (also known as δ-endotoxins) (6). The genes encoding ICPs normally occur on large plasmids and direct the synthesis of a family of related proteins classified as *cry1-28* and *cyt1-2* groups according to their degree of amino acid homology (2a, 11).

Identifying novel *B. thuringiensis* isolates by bioassays is a long and exhaustive process which is impeded by repeated isolation of the same strains (18). Prediction of insecticidal activity of an unknown strain by serotyping seems impossible because it does not necessarily reflect the specific *cry* gene class(es) the strain(s) contains (1, 12). Alternatively, PCR requires minute amounts of DNA and allows quick, simultaneous screening of many *B. thuringiensis* samples, identification and classification of *cry* genes, and subsequent prediction of their insecticidal activities (3–5, 7–10, 13, 15, 16, 19, 21). Extended PCR methodology has recently been exploited to rapidly identify and classify *cry* genes of many groups (3, 5). A complete set of primers is required to optimize identification of all reported *cry* genes (18).

cry9 genes are promising tools for effective control (14, 26) and resistance management (22) of many agronomically important lepidopteran species of insect pests. For example, expression of Cry9Ca in transgenic corn protected the plant against the European corn borer (*Ostrina nubilalis*) (14). Cry9Ca is significantly more toxic to budworm (*Choristoneura fumiferana*) than the currently used Cry1A-F toxins (26) and displays high toxicity against *Plutella xylostella* (susceptible as well as resistant larvae), *Spodoptera exigua*, *Spodoptera littoralis*, *Heliothis virescens*, *Agrotis segetum*, and silkworm (*Bombyx mori*) (20, 26). Another toxin belonging to the Cry9 group is Cry9Aa, the major crystal component of *B. thuringiensis* subsp. *galleriae*, which exhibits unique toxicity toward *Galleria mellonella* larvae (25). The cryptic gene *cry9Ba* was found to be localized upstream of *cry9Aa* (23). The fourth protein in this group, Cry9Da, toxic to scarabaeid larvae of the order Coleoptera, was found in *B. thuringiensis* subsp. *japonensis* (2, 27).

In this study, we developed a new set of universal and spe-

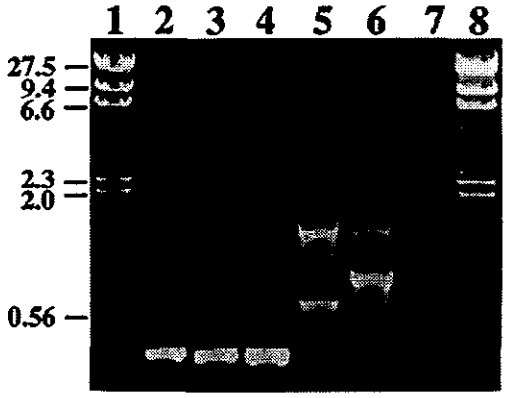

FIG. 1. Agarose gel (1%) electrophoresis of PCR products obtained with universal and specific primers for *cry9* genes. Lanes 1 and 8, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kilobases) indicated on left; lanes 2 to 4, respectively, DNA of field-collected isolates U-27, K-74, and *B. thuringiensis* subsp. *aizawai* HD-133 amplified with Un9; lanes 5 to 7, respectively, DNA of field-collected isolates U-27, K-74, and *B. thuringiensis* subsp. *aizawai* HD-133 amplified with a mixture of four specific primers and one reverse Un9(r).

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

Gene nomenclature		GenBank accession no.	Positions of nucleotides hybridized to primers[b]	Mismatch of primers[c]	Product size (bp)
Current	Original				
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] The sequences of the universal primers (d, direct; r, reverse) are as follows: Un9(d), 5′-CGGTGTTACTATTAGCGAGGGCGG-3′; Un9(r), 5′-GTTTGAGCC GCTTCACAGCAATCC-3′.
[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 sequences.

cific primers for multiplex rapid screening of *B. thuringiensis* strains that harbor any of the four currently known *cry9* genes.

B. thuringiensis strains were isolated as described previously (3) and selected for appearance of parasporal inclusions by phase-contrast microscopy. One pair of universal oligonucleotide primers (Un9) was selected from a highly conserved region present in the four *cry9* genes (extracted from the GenBank database in accordance with the method outlined in reference 11) to amplify a specific fragment from *cry9* genes by using the program Amplify 1.0 (Bill Engels, University of Wisconsin) (Fig. 1, lanes 2 to 4). Primer sequences, match and mismatch positions on each gene of the group, and the expected sizes of their amplicons are presented in Table 1. Sequences and match positions of the four specific primers, selected from highly variable regions in the known *cry9* genes, are presented in Table 2. A mixture of the four specific primers with the universal reverse primer [Un9(r)] was used for multiplex PCR screening to identify *cry9* genes by different sizes of their PCR products (Table 2; Fig. 1, lanes 5 to 7).

Amplification was carried out in a DNA MiniCycler (MJ Research, Inc., Watertown, Mass.) for 30 reaction cycles each. Reactions were routinely carried out in 25 μl; 1 μl of template DNA was mixed with reaction buffer, a 150 μM concentration of each deoxynucleoside triphosphate, a 0.2 to 0.5 μM concentration of each primer, and 0.5 U of *Taq* DNA polymerase (Appligene). Template DNA was denatured (1 min at 94°C) and annealed to primers (45 s at 56°C), and extensions of PCR products were achieved at 72°C for 50 s and 90 s for Un9 and specific primers, respectively. Each experiment was accompanied by a negative control (i.e., without DNA template).

Multiplex PCR screening for *cry9* genes was performed on 16 *B. thuringiensis* standard strains previously used by us (3), as well as on 215 *B. thuringiensis* field isolates. Among the standard strains, only *B. thuringiensis* subsp. *aizawai* HD-133 yielded an amplicon with Un9 (Fig. 1, lane 4), though it was negative to the specific primers (lane 7). This strain contains *cry2Ab* (in addition to the four *cry1* genes *-Aa*, *-Ab*, *-Ca*, and *-Da* [3, 10, 15]) and yielded a strong amplification product with universal primers for *cry7* and *cry8* (3). Another group (21) that claimed to have found *cry2Ab* in this strain only confirmed our previous finding (3). The low quality of their "degenerated family" primers for *cry7* and *cry8* (21) resulted in nonspecific amplicons (compare with reference 3). Another gene, *cry1I* (*cryV* in the old nomenclature), has also been found in this (21) and other *B. thuringiensis* subsp. *aizawai* strains (13, 24), and the product of this gene is known to be secreted into the medium in the early stationary phase (17).

A new gene, *cry9Ea*, has very recently been discovered in *B. thuringiensis* subsp. *aizawai* SSK-10 (2a) and should be recognized by Un9. Un9(d) hybridizes to nucleotides 2448 to 2471 with no mismatches, while Un9(r) hybridizes to nucleotides 2775 to 2798 with a single mismatch at residue 18. The resulting amplicon would be 351 bp in length. A new specific primer for *cry9Ea* should be designed to allow identification of *cry9Ea* in the strains that were positive to Un9 (see the Addendum in Proof).

Of the field-collected isolates, 22 yielded positive results with Un9 (Table 3). These were screened further for the presence of four *cry9* genes. Three different *cry9* gene profiles were found which contained also several combinations of *cry1* and *cry2* (Table 3). Fifteen isolates contained *cry9Aa* and *cry9Ba* (Fig. 1, lane 5) and two contained only *cry9Da* (lane 6), whereas five did not yield an amplicon by PCR with any of the four specific *cry9* primers tested. None of our field-collected isolates contained *cry9Ca*.

It is interesting to note that the specific primer EB-9B(d) nonspecifically amplified a *cry9Da* fragment of 1,534 bp (Fig. 1, lane 6). Alignment analysis discovered that it anneals with low binding strength to bases 1158 to 1181 in the *cry9Da* coding

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)	GGTTCACTTACATTGCCGGTTAGC	*cry9A*	1581–1604	1,547
Un9(r)	GTTTGAGCcGCTTCACAGCAATCC		3104–3127	
EB-9B(d)	GCAAATGCATTTAGCGCTGGTCAA	*cry9B*	1925–1948	701
Un9(r)	GTTTGAGCcGCTTCACAGCAATCC		2602–2625	
EB-9C(d)	CCACCAGATGAAAGTACCGGAAG	*cry9C*	3473–3495	1,232
Un9(r)	GTTTGAGCCGCTTCACAGCAATCC		4681–4704	
EB-9D(d)	GCAATAAGGGTGTCGGTCACTGG	*cry9D*	1754–1776	938
Un9(r)	GTTTGAGCCGCTTCACAGCAATCC		2668–2691	

[a] (d) and (r), direct and reverse primers, respectively.
[b] Bases that do not match appropriate sequences are shown in lowercase letters.
[c] Starting from the first base of the sequence (of the respective *cry* gene) in the GenBank database.

TABLE 3. Distribution of *cry9* gene profiles of *B. thuringiensis* field-collected isolates

cry9 gene profile	*cry*-type gene profile identified previously[b]	No. of isolate(s)
cry9D	*cry1Aa, -Ab + cry2Ab*	1
	cry1Aa, -Ab, -Ac + cry2Aa, -Ab	1
cry9A, -B	*cry1Ab, -D + cry2Ab*	4
	cry1Ab, -Ac, -D + cry2Ab	1
	cry1Ab, -D + cry2Ab, -Ac	10
cry9[a]	*cry1Ab, -D + cry2Ab, -Ac*	1
	cry1Aa, -Ab, -C, -D + cry2Ab	4

[a] *cry9* (without letter) indicates positive with universal and negative with specific primers.
[b] Ben-Dov et al. (3).

sequence. Increasing the temperature to 60 to 62°C can prevent this nonspecific annealing.

The recent report by Bravo et al. (5) on an expanded set of general and specific primers includes a set for detecting three genes of the *cry9* group (excluding *cry9Da*). At least one of these specific primers (spe-cry9C), corresponding to bases 1853 to 1868 (yielding an amplicon of 306 bp), is predicted to nonspecifically anneal also to bases 1961 to 1976 in *cry9Ca* (to amplify a fragment of 198 bp); it may thus interfere with amplification of the 306-bp fragment of *cry9Ca*. In addition, spe-cry9C is predicted to anneal nonspecifically both directly and in the reverse direction to *cry9Ca* and *cry9Aa*, thus giving rise to further nonspecific amplifications.

Bravo et al. (5) detected *cry9* genes in 2.6% of their *B. thuringiensis* strain collection, whereas we found them in 10.2% of our collection (Table 3). This apparent difference in frequencies may reflect a real difference in prevalence of *cry9* genes between the Latin American and Asian collections. It may however be due to the fact that in addition to a set of four specific primers we used a pair of universal primers (Un9) which amplifies all five *cry9* genes (and also potentially other unknown genes of this family).

Our screening procedure identified five field-collected *B. thuringiensis* isolates positive to Un9 but not to any of our specific primers for four *cry9* genes. This may indicate that these isolates contain new *cry9* genes. They may be potential biological control agents against insect pests.

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Gideon Raziel is gratefully acknowledged for producing the picture.

ADDENDUM IN PROOF

The new specific primer EB-9E(a), 5′-GCGGCTGGCTTT ACTTTACCGAG-3′, designed to identify *cry9Ea* by hybridization to nucleotides 1975 to 1977, amplified PCR product of 824 bp with Un9(r). *B. thuringiensis* subsp. *aizawai* HD-133 and four of the five field-collected isolates (positive to Un9) yielded an amplicon specific to *cry9Ea*.

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PCR Analysis of *cry7* Genes in *Bacillus thuringiensis* by the Five Conserved Blocks of Toxins

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Abstract. 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.

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 [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 [24, 29]. Known toxins kill subsets of insects among the Lepidoptera, Coleoptera, Diptera, and nematodes. The related toxins are 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, 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 [19, 27, 34].

Polymerase Chain Reaction (PCR) requires minute amounts of DNA and allows quick, simultaneous screening of many samples. This technique has been exploited to identify *cry* genes of *B. thuringiensis*, detect new such genes, and subsequently predict their insecticidal activities [2–5, 7–10, 14, 17, 18, 21, 25]. Extensive screening programs have considerably expanded the host range of strains available for pest control [2, 5].

The procedure used here is based on homologies to the five conserved blocks of the Cry proteins. As an example, we chose *cry7* and *cry8*, which are promising for effective control and resistance management of agronomically important coleopteran species [22, 26, 28, 31, 32]. Three *cry7* and three *cry8* genes are currently known: *cry7Aa*, *cry7Ab1*, and *cry7Ab2* in *B. thuringiensis* subsp. *galleriae* [22], *dakota* HD-511, and *kumamotoensis* HD-867 [1], respectively; *cry8Aa* and *cry8Ba* in *B. thuringiensis* subsp. *kumamotoensis* [1]; and *cry8Ca*, in subsp. *japonensis* [28, 31]. The four direct primers were designed to amplify four distinct amplicons with the single reverse primer and create a fingerprint specific to *cry7A* (Fig. 1A). A novel gene 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).

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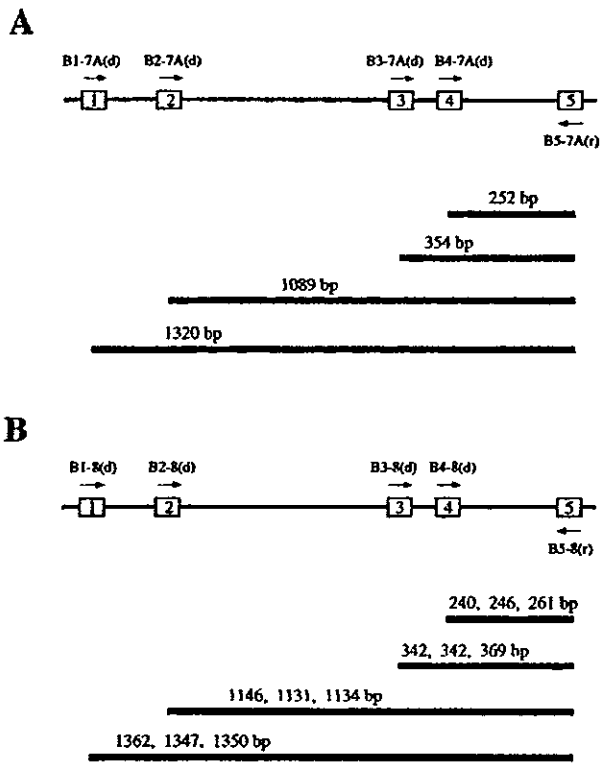

Fig. 1. Strategy for detecting *cry7* (A) and *cry8* (B) genes by PCR based upon the five conserved blocks of amino acids in toxins. Arrows above boxes (enclosing block number) indicate direction of primers (Tables 1 and 2). Predicted lengths (in bp) of the resultant amplicons (thick lines) are indicated.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

Materials and Methods

B. thuringiensis strains. *B. thuringiensis* subsp. *indiana* HD-521, *B. thuringiensis* subsp. *tochigiensis* HD-868, *B. thuringiensis* subsp. *dakota* HD-511, and *B. thuringiensis* subsp. *kumamotoensis* HD-867 were kindly supplied by D. R. Zeigler (Bacillus Genetic Stock Center, Columbus, Ohio). *B. thuringiensis* field strains were obtained from soil and insect cadavers, were isolated as described previously [2], and were selected for appearance of parasporal inclusions by phase-contrast microscopy.

Oligonucleotide primers and PCR analysis. Primer sequences, match and mismatch positions in each *cry7*, and expected sizes of their amplicons are presented in Table 1. 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, Madison, 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 2.

DNA templates, extracted from *B. thuringiensis* strains as described previously [2], served in the amplification reactions by a DNA MiniCycler (MJ Research, Inc., Watertown, MA, USA). Reactions (30 cycles each) were carried out in 25 µl: 1 µl of template DNA was mixed with reaction buffer, 250 µM of each dNTP, 0.2–0.5 µM of each primer, and 0.5 U of *Taq* DNA Polymerase (Appligene). Template DNA was denatured (1 min at 94°C) and annealed to primers (45 s at 50 to 54°C), and extensions of PCR products were achieved at 72°C for 30–90 s. Each experiment was accompanied by a negative (without DNA template) control. PCR amplicons of predicted sizes were easily identified by electrophoresis on 1% agarose gels.

Results and Discussion

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) [2]. 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 [1], yielded the four amplicons (Fig. 2) of the predicted sizes with the set of primers (Table 1). None of the 27 field-collected isolates tested yielded amplicons with this same set of primers. 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. Indeed, further screening for the presence of three *cry8* genes by their respective pairs for blocks 3 and 5 (Table 2) detected one that yielded an amplicon specific to *cry8Ba*. This same field-collected strain must be novel because it did not react with a *cry8Ba*-specific pair of primers [2].

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 [28, 29], or homology variation in conserved blocks. This method can be practical for a distinct *cry* group or for several groups by sets of degenerated primers.

Three additional conserved blocks have recently been identified in the C-termini of Cry protoxins [29]. 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*.

ACKNOWLEDGMENTS

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Table 1. 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)	GCTGTATTTCCT a TT t ATGACCC	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 2. 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*.

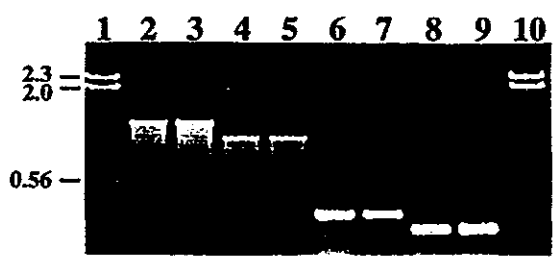

Fig. 2. Agarose gel (1%) electrophoresis of PCR products obtained with the direct primers for the four conserved blocks of *cry7* and the reverse primer of block 5 (Table 1). Lanes 2, 4, 6, and 8, template DNA from *B. thuringiensis* subsp. *dakota* HD-511 (with *cry7Ab1*); lanes 3, 5, 7, and 9 template 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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NOVEL *CRY*-TYPE GENES DETECTED BY EXTENDED PCR SCREENING FROM FIELD-COLLECTED STRAINS OF *BACILLUS THURINGIENSIS*

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ABSTRACT

A rapid method for identification of *Bacillus thuringiensis* strains was established by using extended multiplex PCR (Ben Dov et al., 1997). Using this method we found seven distinct *cry*-type profiles and recorded their distribution in Israel, Uzbekistan and Kazakhstan. Three new genes with potential insecticidal activity were identified. One of them seems to be a naturally occurring recombinant, apparently composed of *cryI*- and *cryIV*-type genes. The other two (apparently homologous to *cryIIIF* and *cryIIIG*) seem to include various combinations with either *cryI* or *cryIV*.

KEY WORDS: PCR screening, *Bacillus thuringiensis*, *cry* genes, biological control of pests.

INTRODUCTION

The very properties that rendered the chemical pesticides useful — long residual action and toxicity to a wide spectrum of organisms, have brought about serious environmental problems. The emergence and spread of insecticide resistance in many species of vectors, the concern with environmental pollution and the high cost of the new chemical insecticides made it apparent that vector control can no longer depend upon the use of chemicals. Thus, increasing attention has been directed toward biological agents that would be highly toxic to the target organism while being safe to nontarget organisms, able to being mass-produced on an industrial scale, have a long shelf life, can be applied using conventional application technology and being transportable (Carlton et al., 1990; Bernhard and Utz, 1993; Van Frankenhuyzen, 1993; Margalit et al., 1995). The unique capacity of insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis* (*Bt*) subspecies has spurred their use as natural control agents in agriculture, forestry and human health. The scientific benefits of the discovery of novel insecticidal bacteria will find their expression in environmentally safe bio-control practices and will lead to increased food production and post-harvest protection.

The use of *Bt* as a commercial insecticide is based on its remarkable ability to produce large quantities of insect-larvicidal proteins, that may reach as much as 20–30% of the dry cell mass. The insecticidal proteins, also known as δ-endotoxin, form crystalline inclusion bodies during

sporulation (Bulla et al., 1980; de Barjac and Sutherland, 1990). The ICP genes, normally associated with large plasmids, have been classified as *cryI-VI* (the old nomenclature) depending on the host specificity and the degree of amino acid homology. The *cryI* class is toxic to Lepidoptera, *cryII* — to Lepidoptera and Diptera, *cryIII* — to Coleoptera, *cryIV* — to Diptera, *cryV* — to Lepidoptera and Coleoptera and *cryVI* — to nematodes (Höfte and Whiteley, 1989; Feitelson et al., 1992; Margalit et al., 1995). The multitude of ICP genes directs the synthesis of related proteins to form either heterogeneous crystalline inclusions or separate crystals with distinct morphologies.

Isolation and identification of novel *Bt* isolates by bioassays is a long and complicated process, impeded by repeated isolation of the same strains. On the other hand serotypes of *Bt* strains do not directly reflect the specific *cry* gene class(es) in the corresponding bacteria. The Polymerase Chain Reaction (PCR), which is a highly sensitive method to detect rapidly and identify target DNA sequences (Saiki et al., 1988), requires minute amounts of DNA, and allows quick, simultaneous screening of many *Bt* samples, to classify them and predict their insecticidal activities. Several workers (Carozzi et al., 1991; Bourque et al., 1993; Kalman et al., 1993; Chak et al., 1994; Ceron et al., 1995; Kuo and Chak, 1996) have employed PCR in order to screen numerous *Bt* isolates: to predict insecticidal activity, to identify and determine distribution of *cry*-type genes and to detect new *cry*-type genes.

In this paper we used a PCR strategy for extended multiplex rapid screening of field-collected *Bt* strains that harbor genes from classes *cryI*, *cryII*, *cryIII* and *cryIV* (Ben-Dov et al., 1997). This strategy will enrich the existing arsenal of insecticidal strains, identify novel genes or new combinations of known genes and predict their insecticidal activities. Novel strains displaying unknown *cry* profiles (containing new genes or combinations) should be further characterized by bioassays.

MATERIALS AND METHODS

Bacterial strains

Standard *Bt* strains were kindly supplied by Dr. D.R. Zeigler (Bacillus Genetic Stock Center, Columbus, Ohio). The other *Bt* strains that we used were isolated from soil and cadaver samples collected in Israel, Kazakhstan and Uzbekistan.

Isolation of *Bt* from soil samples

Sporeforming strains were isolated according to the acetate selection protocol of Travers et al. (1987). An aliquot (0.5 g) of soil or insect cadavers was added to 10 mL of LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter), buffered with 0.25 M sodium acetate, which selectively inhibits the germination of *Bt* spores. The mixture was shaken (250 rpm) in a 125 mL flask at 30°C for 4 h. At the end of this period a 1.5 mL sample was taken, heat-shocked at 80°C for 3 min, plated on L broth solid medium and incubated overnight at 30°C. Random colonies were inoculated into 5 mL T3 medium (3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M sodium phosphate [pH 6.8] and 0.005 g $MnCl_2$ per liter) and allowed to sporulate overnight at 30°C. Cultures were checked microscopically for the presence of crystals, which were used as the criterion for *Bt* isolates. For storage, stocks of the liquid T3 cultures were kept at –70°C after addition of glycerol to 15%. Lyophilized stocks were also prepared from 96-h cultures that were washed twice with sterile distilled water.

Oligonucleotide PCR primers

To identify *cryI*, *cryII*, *cryIII* and *cryIV*-type genes we used five pairs of universal and 13 specific primers as previously designed (Ben-Dov et al., 1997). The 10 specific primers for identification of eight *cryI* genes were used as previously designed by Kalman et al. (1993). PCR products were easily identified by electrophoresis in agarose gels.

Preparation of DNA templates and PCR

Templates were prepared from 16–18 h cultures in L or in tryptic soy (Difco) 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 resuspended in 100 µL of lysis buffer (25% sucrose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA, 4 mg/mL lysozyme). The cell suspension was incubated at 37°C for 1 h. DNA extraction was performed according to Birnboim and Doly (1979).

PCR amplifications (30 reaction cycles each) were routinely carried out in a 25 µL reaction volume in a DNA MiniCycler (MJ Research, Inc., Watertown, Massachusetts, USA). 1 µL of template DNA was mixed with PCR reaction buffer, 150 µM of each dNTP, 0.1–0.5 µM of each primer, and 0.5U Taq DNA Polymerase (Appligene). PCR cycle conditions were: denaturation of template DNA at 94°C for 1 min, annealing of templates and primers at 54–60°C for 40–50 sec, and extension of PCR products at 72°C for 1–1.30 min. Each experiment was done with a negative (without DNA template) and a positive (with a standard DNA template) control. An aliquot of the PCR reaction products (7–9 µL) was analyzed by agarose gel (0.8–2.5%) electrophoresis, after staining with ethidium bromide, and visualized under UV light.

The reliability of the *cryI*, *-II*, *-III* and *-IV* oligonucleotide primers for detection of *cry*-type genes from *Bt* strains has been verified by use of the well known *Bt* strains: *Bt kurstaki* HD-1 for *cryI* and *cryII* classes, *Bt tenebrionsis* and *dakota* for *cryIII* class and *Bt israelensis* for *cryIV* class.

RESULTS AND DISCUSSION

Several hundred field-collected samples were isolated in Israel, Uzbekistan and Kazakhstan; of these, about two hundred spore-forming *Bt* isolates were identified by the *cry* universal (Un) primers and the positive 126 isolates were further specified by the appropriate specific primers (Ben-Dov et al., 1997). Based on their *cry*-type gene content (by Un primers), they can be divided into several groups with different potential larvicidal activities (Fig. 1). In Israel and Uzbekistan we found three distinct *cry* profiles from which only one (*cryIV*) was identical. In Kazakhstan we found six distinct *cry* profiles; two of these profiles (*cryI* and *cryII*) were unique to Kazakhstan. The different *cry*-type profiles of *Bt* strains isolated in the three countries may be explained by biological and environmental variations between the different sampling locations. Climatic variations, for example, were shown to affect the distribution of *cry*-type genes of *Bt* isolates (Chak et al., 1994.).

Profiles *cryI+cryII+cryIII* and *cryIV+cryIII* apparently contain new combinations of *cry* genes, with promising potential properties to be utilized as biological control agents against pests that are not susceptible to the known *Bt* strains. For example, the new combination of the endotoxins CryIA(b), CryIB and CryIIA in a *Bt* isolate were toxic to the common house fly, *Musca domestica* (Hodgman et al., 1993).

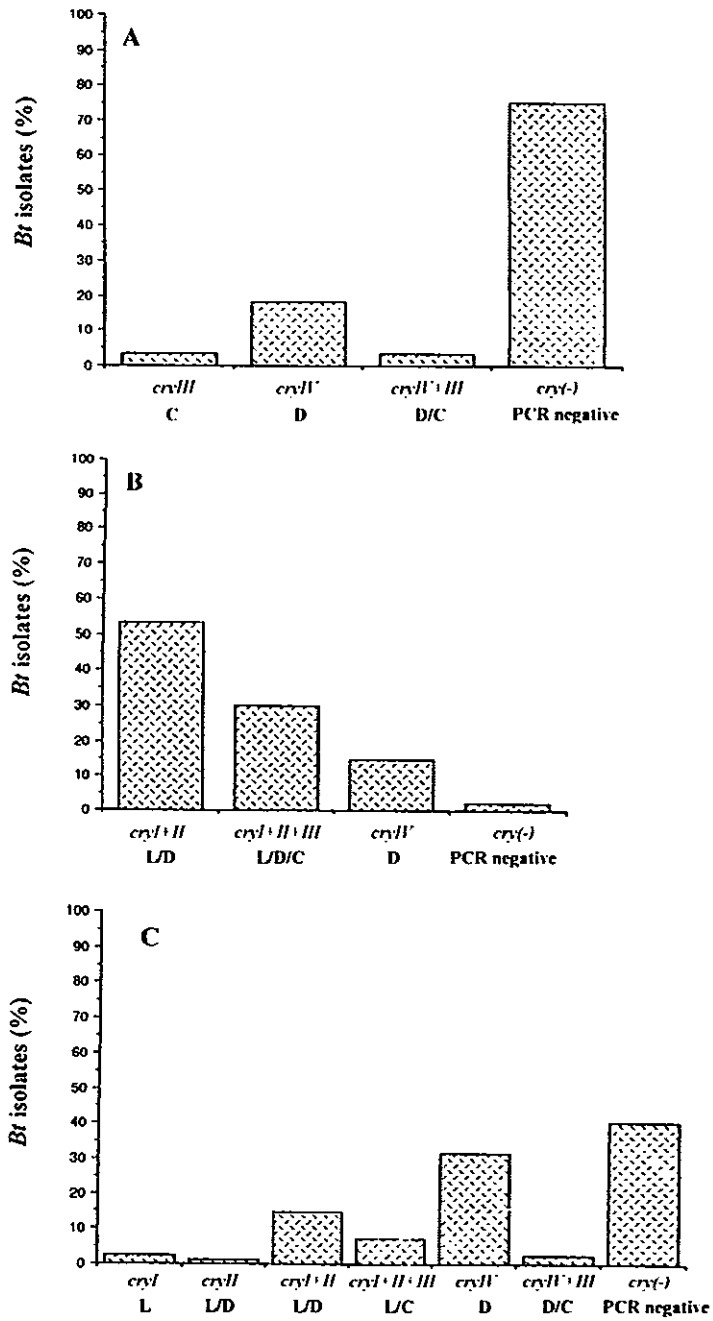

Fig. 1. Distribution of *cry*-type gene profiles identified by universal primers among the *Bt* isolates. A: in Israel; B: in Uzbekistan; C: in Kazakhstan. Predicted target organisms: L, Lepidoptera; D, Diptera; C, Coleoptera.

Many of the new *Bt* isolates did not give any PCR products with all of our universal primers. Some of these negative isolates may not harbor any toxin gene(s); however some may contain new Cry proteins with novel insecticidal activity and thus represent potentially new biological control agents.

We found no strain that harbors *cryIIIA*, *-B*, *-D* gene. We did, however, find several isolates that gave the appropriate DNA fragment with the universal primers Un7,8 for *cryIIIC*, *-E*, *-F*, *-G* genes. It is noteworthy that none of the latter *cryIII* isolates reacted with the specific primers of this *cry* group. Some of our *cryIII* positive isolates amplified either of two unexpected products (~700 or ~300 bp), using two direct specific primers EE-8B(d) and EE-8C(d) (Ben-Dov et al., 1997). These field-collected isolates apparently contain a new variant(s) of the *cryIII*-type gene(s).

One of our isolates (DL-1) produced a 1.8 kb fragment (Fig. 2) with a mixture of two pairs of the universal primers (Un1 and Un4; Ben-Dov et al., 1997). Further analysis revealed that it was obtained with the direct primers Un1(d) and Un4(d). This isolate also produced another, minor PCR product, of ~400 bp (Fig. 2), with the reverse primer Un4(r) alone. Further efforts to identify this crystal forming strain are now in progress.

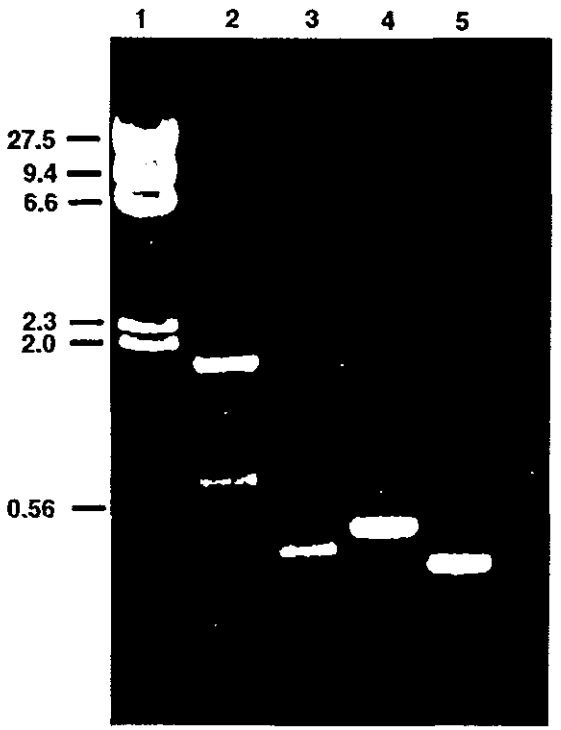

Fig. 2. Agarose gel electrophoresis analysis of PCR products from strain DL-1 obtained by using *cryI* and *cryIV* universal primers (Un). Line 1, molecular weight markers (λDNA cleaved by *Hin*dIII), with sizes (in kb) indicated on left; line 2, strain DL-1 with Un1(d) and Un4(d) primers; line 3, strain DL-1 with Un4(r) primer alone; line 4, *Bt israelensis* 4Q5 with Un4(d) and Un4(r) primers for *cryIV* genes; line 5, *Bt kurstaki* HD-1 with Un1(d) and Un1(r) primers for *cryI* genes.

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CHAPTER 8

Biological Control by *Bacillus thuringiensis* subsp. *israelensis*

Yoel Margalith and Eitan Ben-Dov

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8.1 INTRODUCTION

It is estimated that after nearly half a century of synthetic pesticide application, mosquito-borne epidemic diseases such as malaria, filariasis, yellow fever, dengue and encephalitis are still affecting over two billion people. Malaria remains one of the leading causes of morbidity and mortality in the tropics. An estimated 300 to 500 million cases of malaria each year result in about one million deaths, mainly children under five, in Africa alone (WHO, 1997).

The introduction of synthetic pesticides and prophylactics initially resulted in a drop in malaria cases. However, resistance of mosquitoes to synthetic insecticides, coupled with resistance developed by the malaria-causing pathogen, *Plasmodium* spp., to various anti-malaria drugs, resulted in a dramatic increase of malaria in the tropical world (Olliaro amd Trigg, 1995; WHO, 1997). The very properties that made chemical pesticides useful — long residual action and toxicity to a wide spectrum of organisms — have brought about serious environmental problems (Van Frankenhuyzen, 1993). The emergence and spread of insecticide resistance in many species of vectors, safety risks for humans and domestic animals, the concern with environmental pollution, and the high cost of developing new chemical insecticides, made it apparent that vector control can no longer depend upon the use of chemicals

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(Lacey and Lacey, 1990; Margalith, 1989; Mouchès et al., 1987; Wirth et al., 1990). An urgent need has thus emerged for environmentally friendly pesticides, to reduce contamination and the likelihood of insect resistance (Margalith et al., 1995; Van Frankenhuyzen, 1993).

Thus, increasing attention has been directed toward biological control agents, natural enemies such as predators, parasites, and pathogens. Unfortunately, none of the predators or parasites can be mass-produced and stored for long periods of time. They all must be reared *in vivo*. The ideal properties of a biological agent are: high specific toxicity to target organisms; safety to non-target organisms; ability to be mass produced on an industrial scale; long shelf life; and application using conventional equipment and transportability (Federici, 1995; Lacey and Lacey, 1990; Margalith, 1989; McClintock et al., 1995; Van Frankenhuyzen, 1993).

8.1.1 *Bacillus thuringiensis* (Bt) as an Environmentally Safe Biopesticide

Bacillus thuringiensis (Bt) fulfills the requisites of an "ideal" biological control agent better than all other biocontrol agents found to date, thus leading to its widespread commercial development. Bt is a gram-positive, aerobic, endospore-forming saprophyte bacterium, naturally occurring in various soil and aquatic habitats (Aronson, 1994; Kumar et al., 1996; Lacey and Goettel, 1995; Van Frankenhuyzen, 1993). Bt subspecies are recognized by their ability to produce large quantities of insect larvicidal proteins (known as δ-endotoxins) aggregated in parasporal bodies (Bulla et al., 1980; Kumar et al., 1996). These insecticidal proteins, synthesized during sporulation, are tightly packed by hydrophobic bonds and disulfide bridges (Bietlot et al., 1990). The transition to an insoluble state presumably makes the δ-endotoxins protease-resistant and allows them to accumulate inside the cell. The high potencies and specificities of Bt's insecticidal crystal proteins (ICPs) have spurred their use as natural pest control agents in agriculture, forestry and human health (Kumar et al., 1996; Van Frankenhuyzen, 1993). The gene codings for the ICPs, that are normally associated with large plasmids, direct the synthesis of a family of related proteins that have been classified as *cryI–VI* and *cytA* classes (the old nomenclature), depending on the host specificity (lepidoptera, diptera, coleoptera, and nematodes) and the degree of amino acid homology (see Table 8.1 and Feitelson et al., 1992; Höfte and Whiteley, 1989; Tailor et al., 1992). The current classification (*cry1–28* and *cyt1–2* group genes) is uniquely defined by the latter criterion(Crickmore et al.,1998; http://www.biols.susx.ac.uk/home/Neil_ Crickmore /Bt/index.html).

8.1.2 *Bacillus thuringiensis* subsp. *israelensis* (Bti)

Biological control of diptera in general and mosquitoes in particular has been the subject of investigation for many years. Biocontrol agents found to date which are active against diptera larvae include several species of larvivorous fish, mermithid nematode, fungi, protozoa, viruses, the bacteria, Bt, B. *sphaericus*, and *Clostridium bifermentis* (Delecluse et al., 1995a; Federici, 1995; Lacey and Goettel, 1995; Lacey and Lacey, 1990). *Bacillus thuringiensis* subsp. *israelensis* (Bti) was the first subspecies of Bt,

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Table 8.1 Current and Original Nomenclature of *cry* Genes and Host Specicfity

Original (based on host specificity and degree of amino acid homology)	Current (based solely on amino acid identity)	Host Specificity
cryI	*cry1, cry2, cry9, cry15*	Lepidoptera
cryII	*cry1, cry2*	Lepidoptera, Diptera
cryIII	*cry3, cry7, cry8, cry14, cry18, cry 23*	Coleoptera
cryIV	*cry4, cry10, cry11, cry16, cry17, cry19, cry20*	Diptera
cryV	*cry1*	Lepidoptera, Coleoptera
cryVI	*cry5, cry6, cry12, cry13, cry21*	Nematode
	cry5, cry22	Hymenoptera
cytA	*cyt1, cyt2*	Diptera; cytolitic *in vitro*

which was found to be toxic to diptera larvae. In the summer of 1976, as part of an ongoing survey for mosquito pathogens, we came across a small pond in a dried-out river bed in the north central Negev Desert near Kibbutz Zeelim (Goldberg and Margalith, 1977; Margalith, 1990). A dense population of *Culex pipiens* complex larvae were found dying, on the surface, in an epizootic situation. The etiological agent was later identified and designated by Dr. de Barjac of the Pasteur Institute of Paris (Barjac, 1978) as a new (H-14) serotype.

Bti was found to be much more effective against many species of mosquito and black fly larvae than any previously known biocontrol agent. Bti in addition to being biologically effective, possesses all of the desirable properties of an "ideal" biocontrol agent as mentioned above (Becker and Margalith, 1993; Federici et al., 1995). Bti has been shown to be completely safe to the user and the environment. Extensive mammalian toxicity studies clearly demonstrate that the tested isolates are not toxic or pathogenic (McClintock et al., 1995; Murthy, 1997; Siegel and Shadduck, 1990). The extensive laboratory studies, coupled with no reported cases of human or animal disease after more than 15 years of widespread use, clearly argue for the safety of this active microbial biocontrol agent (McClintock et al., 1995; Siegel and Shadduck, 1990). Due to its high specificity, Bti is remarkably safe to the environment; it is non-toxic to non-target organisms (except for a few other nematocerous Diptera and only when exposed to much higher than recommended rates of application) (Margalith et al., 1985; Mulla, 1990; Mulla et al., 1982; Painter et al., 1996; Ravoahangimalala et al., 1994). No resistance has been detected to date toward Bti in field populations of mosquitoes despite 15 years of extensive field usage (Becker and Ludwig, 1994; Georghiou et al., 1990; Becker and Margalith, 1993; Margalith et al., 1995). Bti has been proven over the years to be a highly successful control agent against mosquito and black fly larvae and has been integrated into vector control programs at the national and international levels.

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8.1.3 Mosquitocidal Bt and Other Microbial Strains

Recent extensive screening programs (Ben-Dov et al., 1997; Ben-Dov et al., 1998; Prieto-Samsonov et al., 1997) have expanded the number of novel microbial strains active against diptera. The current status of microbial mosquitocidal strains which harbor diptera-specific Cry toxins fall into three groups of Bt and one other group of *Clostridium*, based on the classification suggested by Delécluse et al., 1995a.

1. Bt strains which demonstrate larvicidal activity as potent as Bti and contain all four major Bti toxins Cry4A, Cry4B, Cry11A and Cyt1Aa, but belong to different serotypes (Delecluse et al., 1995a; Lopez-Meza et al., 1995; Ragni et al., 1996); Bt *kenyae* (serotype H4a, 4c), Bt *entomocidus* (serotype H6), Bt *morrisoni* (serotype H8a, 8b), Bt *canadensis* (serotype H5a, 5c), Bt *thompsoni* (serotype H12), Bt *malaysiensis* (serotype H36), Bt AAT K6 and Bt AAT B51 (two last autoagglutinated strains that cannot be serotyped). These results demonstrate that the 125 kb transmissible plasmid (Gonzalez and Carlton, 1984) bearing these insecticidal genes occurs in ecologically diverse habitats as well as in different subspecies of Bt. Moreover, the latter finding in conjunction with previous studies shows further that the serotype/subspecies designation used to classify isolates of this bacterium is not a definitive indicator of the insecticidal spectrum of activity.
2. Bt strains producing different toxins nearly as active as Bti (Delecluse et al., 1995b; Kawalek et al., 1995; Orduz et al., 1996, 1998; Rosso and Delecluse, 1997a; Thiery et al., 1997); Bt *jegathesan* (H28a, 28c) and Bt *medellin* (H30).
3. Bt strains synthesizing different toxins but displaying weak activity (Drobniewski and Ellar, 1989; Held et al., 1990; Ishii and Ohba, 1997; Lee and Gill, 1997; Ohba et al., 1995; Smith et al., 1996; Yomamoto and McLaughlin, 1981; Yu et al., 1991); Bt *kurstaki* (H3a 3b), Bt *fukuokaensis* (H3a, 3d, 3e), Bt *canadensis* (serotype H5a, 5c), Bt *aizawai* (H7), Bt *darmstadiensis* (H10a, 10b), Bt *kyushuensis* (H11a, 11c), and Bt *higo* (H44).
4. Anaerobic bacterium which produce mosquitocidal toxins; *Clostridium bifermentas* subsp. *malaysia* (CH18), *C. bifermentas* subsp. *paraiba*, *C. septicum* strain 464 and *C. sordelli* strain A1 (Barloy et al., 1996; Barloy et al., 1998; Delecluse et al., 1995a; Seleena et al., 1997). Existence of *cry* genes associated with transposable elements may indicate that transfer of these genes occurs from one bacterial species to another and suggests that cry-like genes are widely distributed between bacterial species (Barloy et al., 1998).

A second *Bacillus* species, *B. sphaericus*, has potential as a mosquito larvicide. Bs contains binary toxin and Mtx toxins, but its host range is considerably narrower, being toxic mostly against *Culex* species (Porter et al., 1993). Resistance has recently been demonstrated to *B. sphaericus* in a laboratory colony of *Culex quinquefasciatus* (Rodcharoen and Mulla, 1996) and under natural conditions (Silva-Filha et al., 1995). Production costs are higher for *B. sphaericus* than for Bti since carbohydrates cannot be utilized as a carbon source, and production relies upon more expensive amino acids. Recently, a third crystal forming *Bacillus* species, *Bacillus laterosporus*, has been found to be effective against *Aedes aegypti*, *Anopheles stephensi* and *Culex pipiens* (Orlova et al., 1998).

Among the above mosquitocidal isolates, Bti remains the most potent against the majority of the mosquito species. Microbial agents in groups 2, 3, 4 (see above) and Bs are not as toxic as Bti, but produce toxins related to those found in Bti, and therefore these toxic genes may prove useful for recombinant strain improvement for overcoming potential problems associated with resistance (Lee and Gill, 1997).

It has recently been reported that Bt strains, such as Bti (HD567), Bt *kurstaki* (HD1) and Bt *tenebrionis* (NB-125), which were isolated from various food items and are used commercially for insect pest management (Damgard et al., 1996) demonstrated enterotoxin activity very similar to that of *B. cereus* FM1 (Asano et al., 1997). However, these Bt strains have been used for decades as insecticides, and have been applied on a large scale to food crops and unlike *B. cereus* (which contains enterotoxin-causing diarrhea in higher animals); there is no report that substantiates the human health problem caused by Bt (McClintock et al., 1995).

8.1.4 Expanded Host Range of Bti

Horak et al. (1996) recently demonstrated that the water-soluble metabolite of Bti (M-exotoxin, which belongs to same class as β-exotoxin, but has shown no activity in animal tests) was toxic to aquatic snails, including *Biomphalaria glabrata* and on cercariae of seven trematode species including a human parasitic species, *Schistosoma mansoni* and an avian parasite, *Trichobilharzia szidati*.

An expanded host range of Bti was recently found by several investigators: larvicidal activity was demonstrated against *Tabanus triceps* (Thunberg) (Diptera: Tabanidae) (Saraswathi and Ranganathan, 1996), Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae) (Robacker et al., 1996), fungus gnats, *Bradysia coprophila* (Diptera: Sciaridae) (Harris et al., 1995), *Rivellia angulata* (Diptera: Platystomatidae) (Nambiar et al., 1990) and root-knot nematode, *Meloidogyne incognita* on barley (Sharma, 1994). Recently, Bti has been used for the control of nuisance chironomid midges (Ali, 1996; Kondo et al., 1995a; Kondo et al., 1995b).

8.1.5 Limited Application of Bti

Application of Bti for mosquito control is limited by short residual activity of current preparations, under field conditions (Becker et al., 1992; Eskils and Lovgren, 1997; Margalith et al., 1983; Mulla, 1990; Mulligan et al., 1980). The major reasons for this short residual activity are: (a) sinking to the bottom of the water body (Rashed and Mulla, 1989); (b) adsorption onto silt particles and organic matter (Margalith and Bobroglo, 1984; Ohana et al., 1987); (c) consumption by other organisms to which it is nontoxic (Blaustein and Margalith, 1991; Vaishnav and Anderson, 1995); and (d) inactivation by sunlight (Cucchi and Sanchez de Rivas, 1998; Hoti and Balaraman, 1993; Liu et al., 1993). In order to overcome these disadvantages, efforts are being made to improve effectiveness of Bti by prolonging its activity as well as targeting delivery of the active ingredient in the feeding zone of the larvae. These improvements are being facilitated by development of new formulations utilizing conventional and advanced tools in molecular biology and genetic engineering.

Originally isolated from a temporary pond with *Cx. pipiens* larvae (Goldberg and Margalith, 1977), Bti seems able to reproduce and survive under natural conditions, but the actual reproduction cycle is still a mystery. Recycling of ingested spores in the carcasses of mosquito larvae (Aly et al., 1985; Barak et al., 1987; Khawaled et al., 1988; Zaritsky and Khawaled, 1986) and pupae (Khawaled et al., 1990) was demonstrated for Bti in the laboratory. Manasherob et al. (1998b) recently described a new possible mode of Bti recycling in nature by demonstrating that, at least under laboratory conditions, the bacteria can recycle in climate protozoan *Tetrahymena pyriformis* food vacuoles. Recycling is thus not restricted to carcasses of its target organisms: *B. thuringiensis* subsp. *israelensis* can multiply in non-target organisms as well.

8.2 STRUCTURE OF TOXIN PROTEINS AND GENES

The family of related ICPs, encoded by genes that are normally associated with large plasmids (Lereclus et al., 1993), have been classified as *cryI–VI* and *cytA* classes on the basis of their host specificity (lepidoptera, diptera, coleoptera and nematodes; the old nomenclature) (Feitelson et al., 1992; Höfte and Whiteley, 1989) and depending on the degree of amino acid homology as *cry1–22* and *cyt1–2* classes (the current classification) (Crickmore et al., 1998; http://www.biols.susx.ac.uk/home/ Neil_Crickmore/Bt/index.html). The ICPs of Bt strains contains two classes of toxins Cry: insecticidal and the Cyt, cytolytic δ-endotoxins. Cyt δ-endotoxins are found only in Dipteran-specific Bt strains. Although these toxins are not related structurally, they are functionally related in their membrane-permeating activities.

8.2.1 The Polypeptides and Their Genes

The larvicidal activity of Bti is localized in a parasporal, proteinaceous crystalline body (δ-endotoxin) synthesized during sporulation (Porter et al., 1993) and is composed of at least four major polypeptides (δ-endotoxins), with molecular weights of about 27, 72, 128 and 135 kDa (as calculated from the derived amino acid sequences of the genes), encoded by the following respective genes: *cyt1Aa*, *cry11A*, *cry4B* and *cry4A* (see Table 8.2 and Federici et al., 1990; Höfte and Whiteley, 1989). The specific mosquitocidal properties are attributed to complex, synergistic interactions between the four proteins, Cry4A, Cry4B, Cry11A and Cyt1Aa, but still the whole crystal is much more toxic than combination of these four proteins (Crickmore et al., 1995; Federici et al., 1990; Poncet et al.,1995; Tabashnik, 1992). In addition, the Bti parasporal body contains at least three minor polypeptides: Cry10A, Cyt2Ba, and 38 kDa protein (Table 8.2) which might contribute to the overall toxicity of Bti (Guerchicoff et al., 1997; Lee et al., 1985; Thorne et al., 1986). Expression in recombinant bacteria and sequence determinations yielded the following information:

1. Cry4A protoxin is encoded by a sequence of 3543 bp (1180 amino acids) and determined by SDS-PAGE as 125 kDa (Sen et al., 1988; Ward and Ellar,1987) Cry4A toxin (48 to 49 kDa) is toxic to the larvae of all three mosquito species: *Ae. aegypti*, *An. stephensi* and *Cx. pipiens* (Angsuthanasombat et al., 1992; Poncet

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Table 8.2 δ-endotoxin Proteins of *B. thuringiensis* subsp. *israelensis* Parasporal Inclusion Body

Major Toxins and % in a Crystal[a]	Predicted Mol Mass (kDa)	Predicted No. of Amino Acids	Raning by SDS-PAGE (kDa)	Activated Toxin (kDa)	Transcriptional σ-Factors	Toxicity (function)[b]
Cry4A (12–15%)	134.4	1180	125	48–49	σ^H, σ^E, σ^K	Cx > Ae > An Synergistic
Cry4B (12–15%)	127.8	1136	135	46–48	σ^H, σ^E	An > Ae > Cx[c] Synergistic
Cry11A (20–25%)	72.4	643	65–72	30–40	σ^H, σ^E, σ^K	Ae > Cx > An Synergistic
Cyt1Aa (45–50%)	27.4	248	25–28	22–25	σ^E, σ^K	Ae > Cx > An (in high con.) Highly synergistic; Suppress resistance; Haemo and cytolytic *in vitro*
Minor Toxins						
Cry10A	77.8	675	58	?	ND[d]	Ae > Cx[c] Synergistic
Cyt2Ba	29.0	263	25	22.5	σ^E	Haemolytic; Potentially synergistic
38 kDa			38	ND	ND	Non-toxic to Ae larvae

[a] Six genes encoding these polypeptides are located on a plasmid 125 kb (75 MDa; see Figure 8.1). Gene encoding the 38 kDa protein is located on a 66 MDa plasmid (Purcell and Ellar, 1997).
[b] Toxicity of δ-endotoxin proteins against *Cx, Culex pipiens*; *Ae, Aedes aegypti* and *An, Anopheles stephensi.*
[c] Both polypeptides Cry4B and Cry10A are needed for the toxicity against *Cx. pipiens.*
[d] Not determined.

et al.,1995). The gene, *cry4A*, is carried on a 14 kb-*Sac*I fragment, which contains two insertion sequences (ISs) — namely IS240A and B — lying in opposite orientations and forming a composite transposon-like structure (Bourgouin et al., 1988). The ISs of 865 bp, each differing in six bases only, contain 16 bp of identical terminal inverted repeats and an open-reading-frame (Orf), encoding 235 amino acids of putative transposase (Delecluse et al., 1989). Six copies of ISs were found on the 125 kb plasmid, the Orfs of which differ in five amino acids only (Bourgouin et al., 1988; Rosso and Delecluse, 1997b).

2. Cry4B is encoded by a sequence of 3408 bp (1136 amino acids) and determined by SDS-PAGE as 135 kDa (Chungiatupornchai et al., 1988; Sen et al., 1988). Its gene, *cry4B*, is found on a 9.9 kb-*Sac*I (Bourgouin et al., 1988) or on 9.6 kb-*Eco*RI fragment. Two Orfs: Cry10A (58 to 65 kDa, Orf1) and Orf2 (56 kDa) (Thorne et al., 1986; Delecluse et al., 1988) are found 3 kb downstream from *cry4B*. Cry4B is a protoxin, which is cleaved by proteolysis in the gut of the mosquito larva to polypeptides (46 to 48 kDa) having high larvicidal activity against *Ae. aegypti* and *An. stephensi*, and very low activity against *Cx. pipiens* (Delecluse et al., 1988; Angsuthanasombat et al., 1992). Both Cry4B and Cry10A are needed for the toxicity against *Cx. pipiens* (Delecluse et al., 1988). There is a high level of homology (40%) between the carboxylic ends of Cry4A and Cry4B, while the amino acid identity is only 25% in their amino end (Sen et al., 1988).
3. Cry10A is encoded by a sequence of 2025 bp (675 amino acids) and determined by SDS-PAGE as 58 to 65 kDa (Thorne et al., 1986). The sequence of Cry10A differs markedly from that of Cry4A and Cry4B. Cry10A shows a 65% homology to Cry4A only in the first 58 amino acids on the amino end (Delecluse et al., 1988). Cry10A contains two potential trypsin cleavage sites. The first site is homolgous to that of Cry4A, whereas it is identical in only two amino acids in Cry4B. The second site is homologous in all three proteins. The *orf2* is located 66 bp downstream from *cry10A* (Thorne et al., 1986) and is highly homologous (over 65%) to sequences at the carboxylic end of Cry4A and Cry4B (Delecluse et al., 1988; Sen et al., 1988). There is a theory that *cry10A* (*orf1*) and *orf2* are modifications of the *cry4* genes (Delecluse et al., 1988). When Cry10A is produced in a recombinant *B. subtilis*, *Escherichia coli* or in a Bti mutant without the 125 kb plasmid, it is converted to a 58 kDa toxin, (probably as a result of proteolysis) and demonstrate low mosquitocidal activity (Thorne et al., 1986). The 53 to 58 kDa polypeptide is also found in minor amounts in Bti crystals (Garguno et al., 1988; Lee et al., 1985)
4. Cry11A is encoded by a sequence of 1929 bp (643 amino acids) and determined by SDS-PAGE as 65 to 72 kDa (Donovan et al., 1988). It is found on a 9.7 kb-*Hin*dIII fragment. Cry11A is cleaved by proteolysis into two small fragments of about 30 kDa, both of which are needed for full toxicity (Dai and Gill, 1993). This polypeptide is not highly homologous to the other toxic Bti polypeptides; it rather shows some homolgy to the Cry2-type polypeptides (Höfte and Whiteley, 1989; Porter et al., 1993). The 72 kDa protein isolated from the crystal has the highest larvicidal activity against *Ae. aegypti*, *Cx. pipiens* and less against *An. stephensi* (Poncet et al., 1995).
5. Cyt1Aa is encoded by a sequence of 744 bp (248 amino acids), localized on a 9.7 kb-*Hin*dIII fragment (Waalwijck et al., 1985). It is toxic to some vertebrate and invertebrate cells and causes lysis of mammalian erythrocytes (Thomas and Ellar, 1983a). The cytotoxicity seems to derive from an interaction between its hydrophobic segment and phospholipids in the membrane, which is thus perforated. Recombinant *E. coli* cells expressing *cyt1Aa* lose viability, probably as a result of an immediate inhibition of DNA synthesis (Douek et al., 1992). Cyt1Aa has low

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Table 8.3 Sequence Alignment of the Cyt1Aa1 from Bti to Cytolitic Toxins from Different Bt Strains[a]

Cyt-type Toxin	Seq. Similarity to Cyt1Aa (%)	Seq. Identity to Cyt1Aa (%)	Bt Strains and Their Serotypes
Cyt1Aa3	99.6	99.6	Bt *morrisoni* (H14)
Cyt1Ab1	90.7	86.3	Bt *medellin* (H30)
Cyt1Ba1	74.5	65.0	Bt *neoleoensis* (H24)
Cyt2Aa1	53.9	46.1	Bt *kyushuensis* (H11a, 11c), *darmstadiensis* (H10a, 10b)
Cyt2Ba1	50.8	43.5	Bt *israelensis* (H14)
Cyt2Bb1	51.1	42.1	Bt *jegathesan* (H28a, 28c)
CytC not sequenced			Bt *fukuokaensis* (H3a, 3d, 3e)

[a] Alignment and comparisons of amino acid sequences of cytolitic toxins were performed with the Genetic Computer Group package (BestFit program; creates an optimal alignment of the best segment of similarity between two sequenses). GenBank accession number of Cyt sequences were as follows: X03182 for Cyt1Aa1; Y00135 for Cyt1Aa3; X98793 for Cyt1Ab1; U37196 for Cyt1Ba; Z14147 for Cyt2Aa; U52043 for Cyt2Ba; and U82519 for Cyt2Bb.

larvicidal activity, but in combination with Cry4A, Cry4B and/or Cry11A toxins, a synergistic effect is achieved. This synergistic effect is greater than that obtained by a combination of three Cry polypeptides only (Crickmore et al., 1995; Wirth et al., 1997). The sequence of Cyt1Aa does not show any homology to genes encoding other δ-endotoxin polypeptides (Porter et al., 1993) but play a critical role in delaying the development of resistance to Bti's Cry proteins (Georghiou and Wirth, 1997; Wirth and Georghiou, 1997; Wirth et al., 1997). To date, seven cytolitic, mosquitocidal specific toxins from different Bt strains are known (see Table 8.3 and Cheong and Gill, 1997; Drobniewski and Ellar, 1989; Earp and Ellar, 1987; Guerchicoff et al., 1997; Koni and Ellar, 1993; Thiery et al., 1997; Yu et al., 1997). These toxins demonstrate cytolitic activity *in vitro* and highly specific mosquitocidal activity *in vivo* which imply a specific mode of action. Moreover, these Cyt toxins contain several conserved regions observed in loop regions as well as in α-helices and β-strands (Cheong and Gill, 1997; Thiery et al., 1997).

6. A new gene, *cyt2Ba* encoding for the 29 kDa (263 amino acids) cytolytic toxin and run by SDS-PAGE as 25 kDa, has recently been detected in Bti and other mosquitocidal subspecies (Guerchicoff et al., 1997). It is found on a 10.5 kb-*Sac*I about 1 kb upstream from *cry4B*. The toxin, Cyt2Ba, was found at very low concentrations in their crystals. Cyt2Ba is highly homologous (67.6%) to the Cyt2Aa toxin from Bt subsp. *kyushuensis*. In addition, a stabilizing sequence at the 5′ mRNA of *cyt2Ba*, which resembled that described for *cry3* genes, was found (Guerchicoff et al., 1997). Truncated 22.5 kDa Cyt2Ba (by *Ae. aegypti* gut extract) was shown to be hemolytic against human erythrocytes. A synergistic effect was demonstrated when Cyt2Ba was combined with Cry4A, Cry4B, and Cry11A, respectively; therefore, Cyt2Ba may also contribute to the overall toxicity of Bti (Purcell and Ellar, 1997).
7. A gene encoding a 38 kDa protein is located on a 66 MDa plasmid (and not on 75 MDa which contains all other δ-endotoxin genes). This protein is found in the Bti inclusion body (Lee et al., 1985; Purcell and Ellar, 1997) and its function is still unknown (38 kDa protein alone was not toxic to *Ae. aegypti* larvae) (Lee et al., 1985).

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8.2.2 Accessory Proteins (P19 and P20)

Large ICPs (130 to 140 kDa) have conserved C-terminal halves participating in spontaneous crystal formation via inter and intra-molecular disulphide bonds (Bietlot et al., 1990; Couche et al., 1987). The smaller ICPs, which do not possess the conserved C-terminal domain, may require assistance in crystal formation. Cry2A, Cry11A and Cyt1Aa indeed require the presence of accessory proteins for assembly of an inclusion body (Adams et al., 1989; Crickmore and Ellar, 1992; McLean and Whiteley, 1987; Visick and Whiteley, 1991; Wu and Federici, 1995) and the genes of two former proteins are organized in operons; they are co-transcribed with genes not involved in toxicity *orf1/orf2* and *p19/p20*, respectively (Agaisse and Lereclus, 1995; Baum and Malvar, 1995; Widner and Whiteley, 1989).

At least two accessory proteins (P19 and P20) seem to be involved in Bti's δ-endotoxin production, as follows:

1. The 20 kDa product of *p20* stabilizes both Cyt1Aa and Cry11A in recombinant *E. coli* and Bt by a post-transcriptional mechanism (Adams et al., 1989; McLean and Whiteley, 1987; Visick and Whiteley, 1991; Wu and Federici, 1993; Wu and Federici, 1995). Substantially more Cry11A was produced in recombinant *E. coli* carrying the 20 kDa protein gene than in those without it (Visick and Whiteley, 1991). Induction of *cry11A* alone in *E. coli* resulted in no larvicidal activity, but when expressed together with 20 kDa protein gene, some toxicity was obtained (Ben-Dov et al., 1995). Cry11A is thus apparently degraded in *E. coli*, and partially stabilized by the 20 kDa regulatory protein. The combination of Cry11A and 20 kDa protein was larvicidal in *B. megaterium* but not in *E. coli* (Donovan et al., 1988; Chang et al., 1992). Cry11A alone was produced and formed parasporal inclusions in an acrystalliferous Bt species, but higher levels were observed in the presence of the 20 kDa protein (Chang et al., 1992; Chang et al., 1993; Wu and Federici, 1995).

   Expression of *p20* (in *cis* or in *trans*) significantly increases the amount of Cyt1Aa in *E. coli*, but not of its mRNA, implying that the effect of P20 is exerted after transcription (Adams et al., 1989; Visick and Whiteley, 1991). Expression of *cyt1Aa* alone in acrystalliferous strains of Bt was poor and no obvious inclusions were observed, but in the presence of the 20 kDa protein relatively large (larger than those of wild-type Bt) ovoidal, lemon-shaped inclusions of Cyt1Aa were produced (Crickmore et al., 1995; Wu and Federici, 1993). In the absence of P20, recombinant cells of *E. coli* and of an acrystalliferous Bt *kurstaki* lost its colony-forming ability (Douek et al., 1992; Wu and Federici, 1993). Expression of *cyt1Aa* in the presence of P20, however, preserved cell viability (Manasherob et al., 1996a; Wu and Federici, 1993). Proteolysis of Cyt1Aa in *E. coli* occurs during its synthesis or before completing its tertiary stable structure. The protein-protein interaction between P20 and Cyt1Aa occurs while Cyt1Aa is synthesized. P20 therefore protects unfolded and nascent peptide from proteolysis (Adams et al., 1989; Visick and Whiteley, 1991). These results suggest that the 20 kDa protein promotes crystal formation, perhaps by chaperoning Cyt1Aa molecules during synthesis and crystallization, concomitantly preventing them from a lethal interaction with the host.

   A chimera of *cry4A* with Δ*lacZ* (on a high copy number pUC-type plasmid) in *E. coli* when expressed with the 20 kDa protein gene *in trans* (on another compat-

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ible low copy number pACYC-type plasmid) resulted in an increased production of the fused Cry4A (Yoshisue et al., 1992). However, other researchers who cloned *cry4A* and *p20 in cis* on the high copy number plasmid (so that *cry4A* was expressed under a strong promoter and *p20* with its own promoter) in *E. coli*, did not obtain increased toxicity (Ben-Dov et al., 1995). Likewise, inclusion formation of Cry4A was not induced in acrystalliferous Bti in the presence of *p20* (Crickmore et al., 1995). Low levels of expression of the *p20* were more effective than high levels in assisting the production of Cyt1Aa (Adams et al., 1989). The balance of intracellular concentrations of the Cyt1Aa and P20 proteins could thus be important. It is conceivable that P20 increases production of the major crystal components such as Cyt1Aa and Cry11A to a greater extent than that of the minor components such as Cry4A (Yoshisue et al., 1992).

It has recently been shown that expression of *p20* could increase the rate of production of heterogenous truncated Cry1C proteins in acrystalliferous Bt *kurstaki*, and that this is apparently due to protection from endogenous proteases (Rang et al., 1996). A new finding has been reported of a P21 protein from Bt subsp. *medellin* (located upstream of *cyt1Ab* and transcribed in the same direction) which has 84% similarity to the P20 and may potentially have same chaperone-like activity (Thiery et al., 1997).

2. P19 may play a role in protein-protein interactions (as another chaperone; 11.7% of its amino acids are cysteine residues) necessary for assembly of the crystal (Dervyn et al., 1995) and stabilization by disulfide bonds (Gill et al., 1992). If P19 is involved in the crystallization process of Cyt1Aa, it is predicted to protect host cells from the lethal action of Cyt1Aa, as does P20 (Manasherob et al., 1996a; Wu and Federici, 1993). When *p19* was cloned in a pairwise combination with *cyt1Aa* using inducible expression vectors in *E. coli*, P19 did not prevent lethal action as predicted (Manasherob et al., 1996a).

   P19 and Orf1 from Bt *kurstaki* are homologous (33%), but their roles in crystallization are not known yet. The electrophoretic mobility of the expression product of cloned *p19* in *E. coli* and acrystalliferous Bti corresponds to a molecular mass of about 30 kDa rather than 19 kDa (Manasherob et al., 1997a), as predicted from the coding sequence. The same slow migration anomaly was also demonstrated with Orf2 (29 kDa) from Bt *kurstaki* which has an electrophoretic mobility corresponding to a molecular mass of 50 kDa (Widner and Whiteley, 1989). This phenomenon is known to occur in small spore-coat proteins of *B. subtilis* (Zhang et al., 1993) and may shed light on the nature of P19 and its function.

8.2.3 Extra-Chromosomal Inheritance

Bti harbors eight circular plasmids, ranging in size from 5 to 210 kb (3.3 to 135 MDa) and a linear replicon of approximately 16 kb. One of the largest plasmids (125 kb) contains all genetic information for mosquitocidal activity (Gonzalez and Carlton, 1984; Sekar, 1990). The genes encoding toxic proteins have been cloned and expressed, their sequences deciphered and toxicities examined, yielding much information (see below Section 8.5, and Sekar, 1990). Toxic proteins are produced during sporulation, but the plasmid is not required for the sporulation process.

A partial restriction map was constructed and all currently known genes located (Figure 8.1) (Ben-Dov et al., 1996). The two linkage groups (with sizes of about 56 and 76 kb) have recently been aligned and full circularity proved

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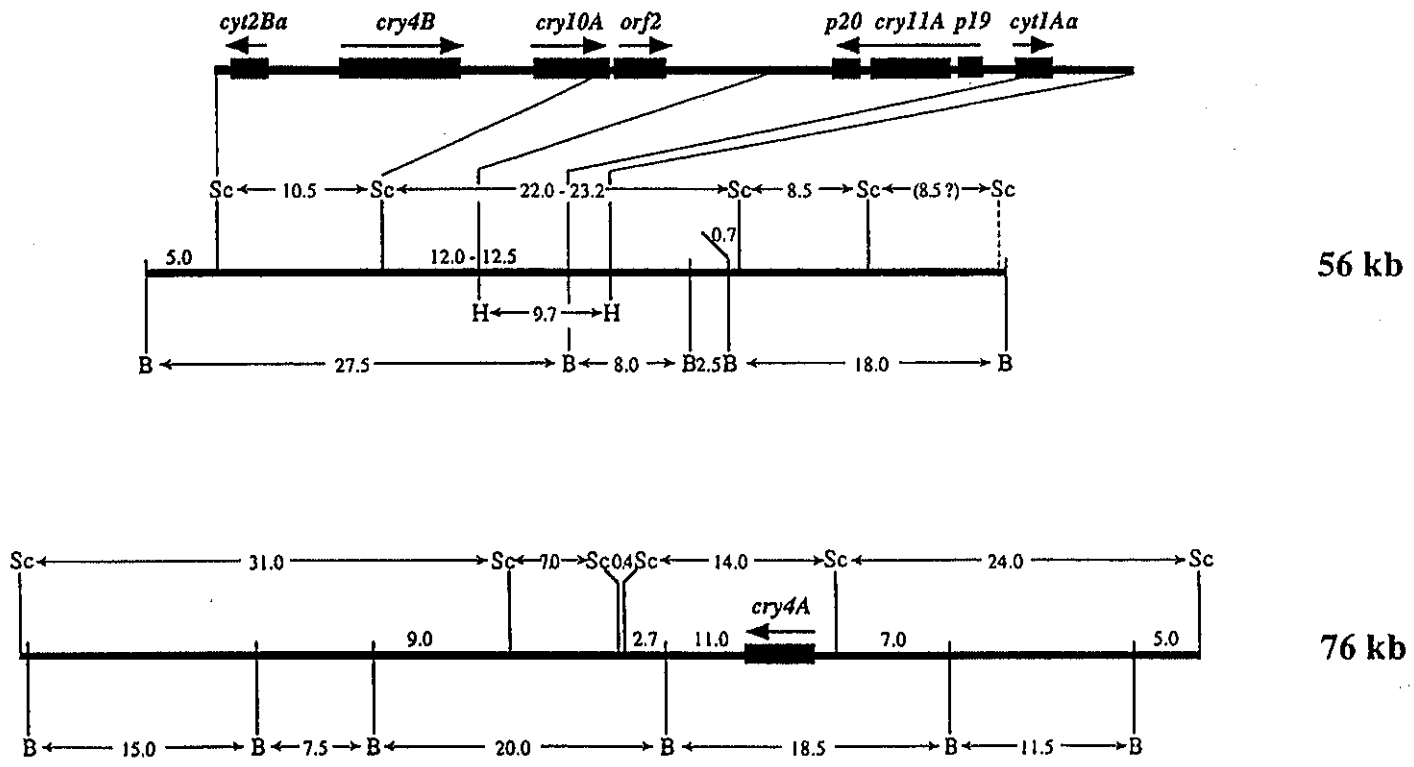

Figure 8.1 Partial restriction map of the *B. thuringiensis* subsp. *israelensis* 125 kb plasmid. Numbers indicate sizes of the relevant fragments, some of which (*Bam*HI [B], *Sac*I [Sc], and one *Hin*dIII [H]) are enclosed by double-headed, thin arrows and fragments of BamHI-SacI are on black thick line. Genes are indicated by black boxes and their transcription direction by thick arrows. The 26 kb (*Sac*I-HindIII) region with most of the known genes is enlarged about 2.5-fold. Based on Ben-Dov et al., 1996.

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(http://www.bgu.ac.il/life/zaritsky.html; Ben-Dov et al., 1999). Five δ-endotoxin genes (*cry4B*, *cry10A*, *cry11A*, *cyt1Aa* and *cyt2Ba*), two regulatory genes (*p19* and *p20*) and another gene with an unknown function (*orf2*) were localized on a 23 kb stretch of the plasmid; however, without *cyt1Aa*, they are placed on a single 27 kb *Bam*HI fragment (Figure 8.1). This convergence enables sub-cloning of δ-endotoxin genes (excluding *cry4A*, localized on the other linkage group) as an intact natural fragment (Ben-Dov et al., 1996). The two accessory protein genes (*p19* and *p20*) are linked to *cry11A* on an operon (organized as a single transcriptional unit; Dervyn et al., 1995). *p19* is the first, *cry11A* is the second and the last, *p20*, is located 281 bp downstream from *cry11A*. *p20* is located 4 kb upstream from *cyt1Aa* and is transcribed in opposite orientation (Adams et al., 1989). All four genes occupy 5.2 kb on a single 9.7 kb *Hind*III fragment. Four additional genes (*cyt2Ba*, *cry4B*, *cry10A* and *orf2*) occupy about 11.5 kb (Ben-Dov et al., 1996; Guerchicoff et al., 1997). Several insertion sequences (IS*231*F, V, W and IS*240*A and B) have been found on the plasmid, which seem to allow transposition, duplication, rearrangement, and modification of the genes for the crystal polypeptides (Ben-Dov et al., 1999; Mahillon et al., 1994). The coding information on this plasmid, known to date, accounts for less than 20% its length. The role of the remaining 80% of the genetic information on this plasmid is still unknown and its elucidation will contribute to the understanding of the genetic interactions important for developing mosquitocidal crystal proteins.

The 125 kb plasmid can be mobilized naturally to acrystalliferous recipient strains (Cry^-) (Gonzalez and Carlton, 1984) converting them to Cry^+ strains. Andrup et al. (1993), distinguished between two phenotypes of aggregation, Agr^+ and Agr^-, which depend on the presence of a conjugative plasmid in Bti and is expressed after mixing cells of both phenotypes in exponential phase in liquid medium. Transfer of small plasmids from the Agr^+ to the Agr^- cells of Bti is accompanied by formation of aggregates between donor and recipient cells (Andrup et al., 1993; Andrup et al., 1995). The genetic basis of this aggregation system and Agr^+ phenotype is associated with the presence of the large 135 MDa self-transmissible plasmid (Andrup et al., 1998; Jensen et al., 1995; Jensen et al., 1996). Furthermore, the large plasmid is efficient in mobilizing the small "nonmobilizable" plasmids. It was suggested that this is a new mobilization mechanism of the aggregation-mediated conjugation system of Bti (Andrup et al., 1996; Andrup et al., 1998).

8.2.4 Three-Dimensional Structure of Bt Toxins

8.2.4.1 Cry δ-endotoxins

Basic studies of genetic structure and mode of action of δ-endotoxins and him receptors are very important for future development of biopesticides and for combating insect resistance mechanisms. The structure and mode of action has been studied in some depth only for the lepidoptera- and coleoptera-active toxins belonging to the Cry1 and Cry3 classes and, to a lesser extent, for the lepidoptera- and diptera-specific Cry2 and Cry4 classes. However, because the mosquitocidal proteins, particularly Cry4A, Cry4B and Cry10A, show significant amino acid sequence

and secondary-structure homology with Cry1 and Cry3, and they all contain five conserved sequence blocks, it is likely that their mechanisms of action and tertiary conformations are similar (Porter et al., 1993). A major advance toward the understanding of the three-dimensional structure of Bt crystal proteins (Cry3A) was achieved by Li et al. (1991) and recently those results were complemented by Grochulski et al. (1995), who determined the tertiary structure of the Cry1Aa. The structure of Cry toxins consists of three distinct domains (I to III) which are from N- to C-terminal:

a). Domain I consists of seven-α helix bundle (for Cry1Aa, eight-α helices) (hydrophobic and amphipatic helices) arranged in an α5-helix in the center and clearly adapted for pore formation in the insect membrane (Dean et al., 1996).
b) Domain II consists of three anti-parallel β-sheets arranged in common "Greek key" motifs (eleven β-strands) packed around a hydrophobic core (α-helix) and three surface-exposed loops at the apex of the domain. Domain II is responsible for receptor binding and host specificity determination (Dean et al., 1996).
c) Domain III consists of two anti-parallel sheets packed in a β-sandwich (twelve β-strands) and two loops which provide the interface for interactions with Domain I. It may be essential for maintaining the structural integrity of the toxins (Li et al., 1991; Nishimoto et al., 1994), and may play a role in regulation of pore-forming activity by conductance effect (Wolfersberger et al., 1996). Domain III also may contribute to the initial, specific reversible binding to the receptors (Aronson et al., 1995; Dean et al., 1996; Flores et al., 1997).

Three domains are closely packed due to van der Waals, hydrogen bond (salt bridges), and electrostatic interactions, where the largest number of interactions occur between Domains I and II (Li et al., 1991; Grochulski et al., 1995).

The crystal structure of a representative Cry toxin consists three domains, including a helix bundle able to function in pore formation and a β-sheet prism whose apical loops are probably responsible for receptor binding (Li et al., 1991). The structure of a Cyt δ-endotoxin, however, is entirely distinct from this three-domain mode (Li et al., 1996).

8.2.4.2 Cyt δ-endotoxins

The structure and function of Cyt δ-endotoxin has recently been investigated by a number of researchers. The crystal structure of Cyt2Aa (CytB) toxin was determined by isomorphous replacement using heavy-atom derivatives (Li et al., 1996). The three dimensional structure of Cyt2Aa has a single pore-forming domain, composed of two outer layers of α-helix hairpins, wrapped around mixed β-sheets (Li et al., 1996). Due to the high similarity (70% in their amino acid sequences) between Cyt1Aa and Cyt2Aa (the existence and positioning of α-helices and β-sheets in Cyt1Aa was predicted from the alignment sequences of these two genes), it was supposed that Cyt1Aa would show a similar folding pattern (Li et al., 1996; Gazit et al., 1997).

8.3 MODE OF ACTION

Early studies investigating the mode of action of Bti toxicity revealed that the primary target is the midgut epithelium, where the enzymatic systems transforms the protoxin into an active toxin under alkaline conditions. After liberation of crystal proteins by dissolution, proteolytic enzymes cleave the four major protoxins Cyt1Aa, Cry11A, Cry4A, and Cry4B to yield the active δ-endotoxin polypeptides of 22 to 25 kDa, 30 to 40 kDa, 48 to 49 kDa and 46 to 48 kDa, respectively (Al-yahyaee and Ellar, 1995; Dai and Gill, 1993; Anguthanasombat et al., 1992). These toxins act coordinately and synergistically to disrupt the epithelial cells of the larval gut (midgut cells vacuolize and lyse) (Lahkim-Tsror et al., 1983). The symptoms caused by the Cry and Cyt toxins of Bti are similar to those caused by toxins in other Bt strains, i.e., larvae become paralyzed and die within a short time.

In fact, Cry polypeptides of Bti and Cyt1Aa are not structurally related, and inevitably form pores with different structures; however, they are functionally related in their membrane-permeating ability. They also differ in their requirement of essential membranal components; the Cry toxins of Bti bind to membranal proteins (receptors) while Cyt1Aa binds to the unsaturated phospholipids acting as "binding sites" (Federici et al., 1990; Feldmann et al., 1995; Gill et al., 1992; Gazit et al., 1997; Porter et al., 1993).

8.3.1 Cry δ-endotoxins

Basically, a two-step model was proposed for the mode of action of Bt toxins by Knowles and Ellar (1987). This model consists of the δ-endotoxin binding to a cell receptor and subsequent pore formation. The δ-endotoxin is released as protoxin, which is solubilized in the midgut of insects and activated by gut proteases. It is assumed that the trigger for the insertion of the pore-forming domain (Domain I) into the epithelial cell membrane is a conformational change in the toxin. This change occurs when Domain II of the toxin binds to a receptor present on the brush-border membranes (Dean et al., 1996; Flores et al., 1997). Binding involves two steps: reversible and irreversible binding to a receptor. The irreversible binding occurs when Domain I is inserted into the plasma membrane of the cell, leading to pore formation, and is more critical than reversible binding for determining ICP specificity (Chen et al., 1995; Flores et al., 1997; Ihara et al., 1993; Rajamohan et al., 1995). Gazit and Shai (1998) recently demonstrated that only helices α4 and α5 (Domain I) of Cry3A insert into the membrane as a helical hairpin in an antiparallel manner, while the other helices lie on the membrane surface like ribs of an umbrella (the "umbrella model" Li et al., 1991), and α7 serves as a binding sensor to initiate the structural rearrangement of the pore-forming domain (Gazit et al., 1994; Gazit and Shai 1995; Gazit and Shai 1998). It was recently demonstrated that unfolding of the Cry1Aa protein around a hinge region linking Domain I and II is a necessary step for pore formation, and that membrane insertion of α4 and α5 helices (Domain I) plays a critical role in the formation of a functional pore (Schwartz et al., 1997). The suggested role for the α5 helix is consistent with the recent finding that the

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cleavage site of Cry4B protoxin (cut by exposure to gut enzymes *in vitro*) was found in an inter-helical loop between α5 and α6 and is extremely important for its larvicidal activity (Angsuthanasombat et al., 1993). The α4 helix (Domain I) of the Cry4B δ-endotoxin was recently demonstrated to play a crucial role in membrane insertion and pore formation. The substitution of glutamine 149 by proline in the center of helix 4 resulted in a nearly complete loss of toxicity against *Ae. aegypti* mosquito larvae (Uawithya et al., 1998).

The production of truncated proteins was achieved by sequential deletions of *cry4A* and *cry4B* genes, which resulted in minimum 75 kDa and 72 kDa active proteins, respectively (Yoshida et al., 1989a; Pao-intara et al., 1988). However, Cry4A and Cry4B protoxins digested by mosquito gut extracts were truncated to active toxins sized 48 to 49 kDa and 46 to 48 kDa, respectively (Anguthanasombat et al., 1992). Specific toxicity *in vitro* was dependent on the type of gut extract used to activate the protoxin. For example, Cry4B toxin was very toxic to *Ae. aegypti* cells when activated by gut extract from *Ae. aegypti* and was non-toxic to the same cells when treated with *Culex* gut proteases (Angsuthanasombat et al., 1992).

Mechanism of action of the Cry11A is significantly different than Cry4A and Cry4B. Cry11A has a specific pattern of proteolytic cleavage into two small fragments of about 30 kDa, which occurs even prior to solubilization, whereas proteolytic products of the solubilized protein were 40 and 32.5 kDa. The 40 kDa N-terminal fragment then further degraded to 30 kDa (Dai and Gill, 1993). It was demonstrated that cleaved Cry11A toxin has a somewhat higher toxicity than uncleaved solubilized toxin; however, the N- and C-terminal moieties of the cleaved toxin have none or very marginal larvicidal activity when applied individually. It was proposed that the N- and C-terminal fragments of cleaved Cry11A toxin probably held together as aggregate in conformation, resulting in slightly greater toxicity than the intact Cry11A polypeptide (Dai and Gill, 1993). Ligand-blotting experiments on dipteran brush border membrane vesicles (BBMVs) showed binding of Cry11A to 148 kDa and 78 kDa protein in *An. stephensi* and *Tipula oleracea*, respectively (Feldmann et al., 1995). The specific receptors for Cry4A and Cry4B still remain to be determined.

8.3.2 Cyt1Aa δ-endotoxin

Histopathological and biochemical studies investigating the mode of action of activated toxin on cultured insect cells have provided evidence that the cellular targets of the 27 kDa cytolytic toxin are the plasma-membrane liposomes containing phospholipids (Thomas and Ellar, 1983b). Toxin binding leads to a detergent-like rearrangement of the bound lipids, resulting in hypertrophy, disruption of membrane integrity, and eventually cytolysis. The binding affinity of the crystalline polypeptides to lipids containing unsaturated fatty acids is higher than that to lipids with saturated fatty acids. Incubation of the Cyt1Aa with lipids extracted from *Ae. albopictus* larvae neutralized its activity, while incubation with *B. megaterium* membranes, which do not contain suitable unsaturated phospholipids, did not neutralize toxin activity (Thomas and Ellar, 1983b). The mechanism of Cyt1Aa toxicity begins with primary binding of Cyt1Aa, as a monomer, followed after a time lag by aggregation of several

molecules of Cyt1Aa which are produced in the membrane of the epithelium cells; pores are formed and, finally, cytolysis occurs (Gill et al., 1992).The pores that Cyt1Aa forms (1 to 2 nm in diameter) are selective channels to cations as K^+ and Na^+ in the phosphatidyl-ethanolamine planar bilayer with fast cooperative opening and closing. Equilibrium of these ions across the insect cell membrane results in an influx of water which leads to a colloid osmotic lysis (Knowles et al., 1989). Alkali soluble Cyt1Aa (27 kDa) is active *in vitro* against mosquito cell lines and erythrocytes, but proteolytic cleavage by trypsin and proteinase K, as well as endogenous proteases from both the N and C-termini to polypeptides of 22 to 25 kDa, enhances toxicity (Al-yahyaee and Ellar, 1995; Gill et al., 1987). Recent studies demonstrate that both Cyt1Aa and its proteolytically active form (24 kDa) are very effective in membrane permeabilization of unilamellar lipid vesicles. The 24 kDa form was about three times more effective than the protoxin (Butko et al., 1996). At least 311 and 140 aggregate-forming molecules of protoxin and Cyt1Aa activated toxin, respectively, must bind to unilamellar lipid vesicles which subsequently lose their contents via the "all-or-none mechanism." This suggests that the effect of Cyt1Aa is a general, detergent-like, perturbation of membrane rather than creation of ion-specific proteinaceous channels (Butko et al., 1996; Butko et al., 1997). Recently contradictory results were reported by Gazit et al. (1997) who demonstrated that membrane permeability of unilamellar vesicles induced by the Cyt1Aa is via formation of distinct trans-membrane pores rather than by a detergent-like effect. It is still possible that cation-selective channels and detergent-like effect in permeabilization of the membrane occur at different steps in the mode of action.

Recent studies of membrane permeation experiments suggest that Cyt1Aa toxin (with four major helices A to D and seven β1 to β7 strands) exerts its activity by aggregation of several toxin monomers (Gazit et al., 1997). Furthermore they suggest that Cyt1Aa toxin self-assembles within phospholipid membranes, and helices A and C are major structural elements involved in the membrane interaction (strong membrane permeating agents). Helices A and C, but not the β-strands and helix D, caused a large increase in the fluorescence of membrane-bound fluorescein-labeled Cyt1Aa, whereas helix B had only a slight effect. These results demonstrate that helices A and C interact specifically with Cyt1Aa and suggest that they both serve as structural elements in the oligomerization process. Intermolecular aggregation of several toxin monomers may have a direct role in the formation of pores by Cyt1Aa toxin (Gazit and Shai, 1993; Gazit et al., 1997).

In vitro binding of Bti toxins to midgut cells of *An. gambiae* larvae by immunodetection demonstrate that Cry4A, Cry4B, Cry11A, and Cyt1Aa were detected on the apical brush border of midgut cells (rich in specific receptors), in the gastric caecae and posterior stomach. Cyt1Aa was also detected in anterior stomach cells which could be related to the ability of the toxin to induce pores without requiring the participation of any specific receptor (Ravoahangimalala and Charles, 1995). A relatively higher proportion of unsaturated phospholipids in dipteran insects (as compared to other insects) can be expected to lead to a greater affinity of the Cyt δ-endotoxins to their cell membranes and activity *in vivo*. This implies a specific mode of action; however, an insect-specific protein receptor may still be essential for this toxin specificity (Koni and Ellar, 1993; Li et al., 1996). Furthermore, spec-

ificity of Cyt1Aa to certain cells may be enhanced, for example, by linking Cyt1Aa to insulin. This insulin-Cyt1Aa conjugate was toxic to cells bearing an insulin receptors (Al-yahyaee and Ellar, 1996).

8.3.3 Synergism

The insecticidal activity of Bti derives from a parasporal proteinaceous inclusion body (δ-endotoxin) which is synthesized during sporulation. The δ-endotoxin proteins differ qualitatively and quantitatively in their toxicity levels and against different species of mosquitoes (Table 8.2) (Federici et al., 1990; Poncet et al., 1995). The crystal is much more toxic than each of the polypeptides alone. The toxic activity of Cry11A, Cry4B and Cry4A is much greater than that of Cyt1Aa (Crickmore et al., 1995; Delecluse et al., 1991), but this alone does not explain the high larvicidal activity of the crystal. Different combinations of these four proteins display synergistic effects. For example, Cry4A and Cry4B display a synergistic effect against *Culex*, *Aedes*, and *Anopheles* mosquito larvae (Anguthanasombat et al., 1992; Delecluse et al., 1993; Poncet et al., 1995). Mixtures of purified Cry4A and Cry11A display significant synergy against three mosquito species (Poncet et al., 1995). Furthermore, the combination of *cry4A* and *cry11A* cloned into *E. coli* demonstrate a synergistic activity, seven-fold higher than that of *cry4A* alone, against *Ae. aegypti*, probably due to cross-stabilization of the polypeptides (Ben-Dov et al., 1995). Contradictory results regarding the synergistic activity between Cry4B and Cry11A have been reported. Crickmore et al. (1995) reported synergism between these proteins against *Ae. aegypti*, while no synergism against *Ae. aegypti* and simple additive effect against *Cx. pipiens* were reported by Poncet et al. (1995). These differences may be explained by recombinant strains used, methods of purification of inclusions, different proportions of combined toxins, and mosquito-larvicidal bioassays. Mixtures of three Cry4A, Cry4B, and Cry11A protoxins display expanded synergism against mosquito species (Crickmore et al.,1995; Poncet et al., 1995).

Cyt1Aa is the least toxic of the four δ-endotoxin proteins, but is the most active synergist with any of the other three polypeptides and their combinations (Crickmore et al.,1995; Tabashnik, 1992; Wirth et al., 1997; Wu and Chang, 1985; Wu et al., 1994). This may be related to the possible differences in the mechanism of action of Cyt1Aa and the Cry toxins. Moreover, different binding behavior of Cyt1Aa was demonstrated when it was used alone or in combination with Cry toxins of Bti, apparently due to different conformations of Cyt1Aa in the presence of Cry toxins of Bti (Ravoahangimalala and Charles, 1995; Ravoahangimalala et al., 1993). Cyt1Aa preferentially bind in the same region as the Cry toxins and this might explain the mechanism of synergism between Cry and Cyt1Aa toxins. It has already been suggested that Cyt1Aa may synergize the activity of Cry11A by facilitating the interactions between Cry11A and the target cell or the translocation of the corresponding toxic fragment (Chang et al., 1993). The mechanism responsible for synergism has not yet been clarified; however, it may be due to hydrophobic interactions between different toxins, cooperative receptor binding, and/or formation of hybrid pores, allowing a more efficient membrane permeability breakdown (Poncet et al., 1995). Because whole crystals demonstrate insecticidal activity greater even than the combinations

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of the four major polypeptides, several additional factors associated with the native crystal for example, minor components like Cry10A, Cry2Ba, and 38 kDa protein may induce the overall toxicity, and affect ingestion and solubilization of the whole crystal.

Recently it was demonstrated that *cry11A* and *cyt1Aa* cloned into Bt field strain with dual activity against lepidoptera and diptera, are stably expressed. Diptera toxicity was enhanced by a synergistic effect between introduced and resident crystal proteins (Park et al., 1995).

8.3.4 The Properties of Inclusions and Their Interactions

Crystallization of δ-endotoxin into the inclusion body and its solubility is a main characteristic of Bt and is an important factor in susceptibility. Amount of toxin within the cell and the particular combinations of toxins depend on the following factors: availability of accessory proteins (see accessory proteins above, 8.2.2); intra- and inter-toxin bonding as disulfide bonds and salt bridges; host strain effects; sporulation dependent proteases; and growth and storage conditions of the product. These all affect the production of the crystal, proteolytic stability and its resulting solubility profile (Angsuthanasombat et al., 1992; Aronson et al., 1991; Ben-Dov et al., 1995; Bietlot et al.,1990; Chilcott et al., 1983; Couche et al., 1987; Delecluse et al., 1993; Donovan et al., 1997; Kim and Ahn, 1996; Kraemer-Schafhalter and Moser, 1996; Li et al., 1991).

For example, inclusion bodies were not formed when *cry4A* was weakly expressed, but formed when expressed at a high level or with Cry4B which could promote crystallization of Cry4A (Delecluse et al., 1993). Cry4B produced inclusions when *cry4B* was cloned on a low-copy-number plasmid in a crystal-negative strain (4Q7) of Bti (Delecluse et al., 1993); however, not as a native crystal protein body, but as a large loosely amorphous inclusion (Panjaisee et al., 1997).

The δ-endotoxins of Bt closely packed by several types of bonding like van der Waals, hydrogen bond, electrostatic interactions, and covalent disulfide bonds can affect the solubility of an inclusion body (Grochulski et al., 1995; Couche et al., 1987). Solubilization of Cry4A and Cry4B proteins (3.24 disulfide bonds per 100 kDa) occurs at pH 11.25 or higher required disulfide cleavage, where the disulfide bonds are responsible for the biphasic solubility properties of the crystal (Couche et al., 1987). Cyt1Aa protein contains two cysteine residues and interchain disulfide bonds responsible for 52 kDa Cyt1Aa dimer even after solubilization at pH 12 (Couche et al., 1987). Alkali-solubilized Bti δ-endotoxins contained both intra- and interchain disulfide bonds which have structural significance; it is unlikely that disulfide bonds participate in larvicidal activity (Couche et al., 1987).

Cry4A and Cry4B inclusions had different solubility when synthesized in Bti acrystalliferous strain. Solubility of Cry4A was also dependent on acrystalliferous host strain; in Bt *kurstaki* it was two-fold higher than in Bti (Angsuthanasombat et al., 1992). The combination of toxins present can affect the solubility profile of an inclusion body; for example, the absence of a Cry1Ab toxin in Bt *aizawai* has been shown to dramatically affect the solubility of inclusions, but the solubility and toxicity properties of the inclusions were restored upon reintroduction of *cry1Ab*

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gene (Aronson et al., 1991; Aronson, 1995). During sporulation, Bti synthesizes proteolytic enzymes (Chilcott et al., 1983; Hotha and Banik, 1997; Reddy et al., 1998) and a certain percentage of crystal proteins are susceptible to degradation by neutral protease A. In neutral protease A-deficient strains, this susceptible protein improved stability and is detected as increased full-length crystal protein (Donovan et al., 1997).

Differential solubility of distinct toxins may be used for partial separation of the toxins. Cry4A, Cry4B, and Cyt1Aa are soluble at pH 9.5 to 11, while the Cry11A toxin requires a pH of 12 (Gill et al., 1992). Furthermore, the processing of Cry11A is affected both by the physical configuration and the pH. At pH 10, (the pH of mosquito midguts), solubilization of the Cry11A parasporal crystal proceeds slowly, but proteolytic cleavage occurs simultaneously in the midgut of mosquito larvae (Dai and Gill, 1993; Feldmann et al., 1995). Different mechanisms in toxin processing in the gut are affected by pH and protease activity and may therefore explain the differences in specificity and level of toxicity against mosquito species. These differential toxin processing mechanisms may also imply a synergistic mode of action for the whole crystal.

8.4 REGULATION OF SYNTHESIS AND TARGETING

Crystal formation involves accumulation of toxin proteins. Accumulation of toxin proteins is achieved in Bt by gene expression with a strong promoter in non-dividing cells, thus avoiding protein dilution by cell division. A *Bacillus* endospore develops in a sporangium consisting of two cellular compartments, mother cell and forespore. In *B. subtilis,* the process is temporally and spatially regulated at the transcriptional level by successive activation of 5 σ factors, σ^H, σ^F, σ^E, σ^G and σ^K, respectively (σ^A is active in vegetative cells only); σ^H functions primarily during stationary phase, prior to septation (Baum and Malvar, 1995; Errington, 1993; Haldenwang, 1995). Transcription of genes within the forespore compartment required for early and late prespore development depends upon σ^F and σ^G, respectively, while early (mid-sporulation) and late (late-sporulation) transcription in the mother cell are controlled by σ^E and σ^K, respectively (Agaisse and Lereclus, 1995; Baum and Malvar, 1995). This timing and compartmentalization of σ activities in *B. subtilis* ensures precise control over gene expression during spore development (Errington, 1993; Haldenwang, 1995).

Many of the proteins that regulate sporulation in *B. subtilis* are present and appear to function similarly in Bt, including σ^E (homologous to σ^{35} of Bt) and σ^K (homologous to σ^{28} of Bt) (Adams et al., 1991; Agaisse and Lereclus, 1995; Baum and Malvar, 1995; Bravo et al., 1996). The production of ICPs in Bt normally coincides with sporulation, resulting in the appearance of parasporal crystalline inclusions within the mother cell. The dependence of δ-endotoxin gene transcription on σ^E and σ^K links its expression to sporulation to the mother-cell compartment and ensures its production throughout much of the sporulation process which contributes to the large amounts of ICP produced by Bt (Agaisse and Lereclus, 1995). The promoters of most gene codings for ICPs are dual, including one (proximal) strong

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σ^E-dependent promoter, and another (distal) weak σ^K-dependent promoter (Yoshisue et al., 1997). Some of the genes are preceded only by σ^E-dependent promoters (Baum and Malvar, 1995).

To date, sporulation-specific ICP genes of Bti appear to be transcribed generally by either or both of the σ^E and σ^K forms of RNA polymerase (Table 8.2). The genes *p19, cry11A, p20* (three-gene operon) and *cyt1Aa* are under σ^E and σ^K transcriptional control (Dervyn et al., 1995; Baum and Malvar, 1995). Analysis of the promoter region for *cry4A* found that the gene transcribed by RNA polymerases contains σ^H, σ^E (with overlapping consensus sequences), and σ^K (Yoshisue et al., 1993a; Yoshisue et al., 1995; Yoshisue et al., 1997). While *cry4B* and *cyt2Ba* have only σ^E-dependent transcription (Guerchicoff et al., 1997; Yoshisue et al., 1993b; Yoshisue et al., 1995). Recently, it was demonstrated that *cry4B* and *cry11A* are also expressed during the transition phase by RNA polymerases associated with the σ^H, but were weaker than the *cry4A* gene (Poncet et al., 1997a). The σ^H-specific promoters for *cry4A*, *cry4B*, and *cry11A* overlap with σ^E-specific promoters. The 38 kDa protein begins to be synthesized during the first hour after onset of sporulation (sigma factors used still unknown) and the polypeptide accumulates as small "dot" inclusions (Lee et al., 1985). Both Cyt1Aa and Cry11A which form rounded large and bar-shaped inclusions, respectively, are synthesized during middle and late stages of sporulation, whereas Cry4A and Cry4B, which form hemispherical to spherical body, are synthesized during midsporulation (Lee et al., 1985; Federici et al., 1990). These differences apparently indicate that the quantitative accumulation of Cry protoxins in the parasporal body of Bti, which are synthesized and assimilated in a stepwise manner, depend more on promoter strength and less on the number of promoters existing.

8.5 EXPRESSION OF BTI δ-ENDOTOXINS IN RECOMBINANT MICROORGANISMS

Expression of Bt δ-endotoxins in recombinant microorganisms is used to evaluate the toxicities of the individual proteins and to study their structure-function relationships. In addition this tool can be used to improve toxin stability in the environment, enhance expression levels, increase reproduction levels under field conditions, improve toxicity, and expand host spectrum.

The Bti toxin genes have already been expressed, in previous studies, individually or in combinations in *E. coli* (Adams et al., 1989; Angsuthanasombat et al., 1987; Ben-Dov et al., 1995; Bourgouin et al., 1986; Bourgouin et al., 1988; Chungiatupornchai et al., 1988; Delecluse et al., 1988; Donovan et al., 1988; Douek et al., 1992; McLean and Whiteley, 1987; Thorne et al., 1986; Visick and Whiteley, 1991; Ward and Ellar, 1988; Yoshisue et al., 1992), *B. subtilis* (Thorne et al., 1986; Ward et al., 1986; Ward et al., 1988; Ward and Ellar, 1988; Yoshida et al., 1989b) *B. megaterium* (Donovan et al., 1988; Sekar and Carlton 1985), *B. sphaericus* (Bar et al., 1991; Poncet et al., 1994; Poncet et al., 1997b; Servant et al., 1999; Trissicook et al., 1990), *B. thuringiensis* (Angsuthanasombat et al., 1992; Angsuthanasombat et al., 1993; Chang et al., 1992; Chang et al., 1993; Crickmore et al., 1995; Delecluse et al., 1993; Panjaisee et al., 1997; Park et al., 1995; Roh et al., 1997; Wu and Federici, 1993;

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Wu and Federici, 1995), different Cyanobacteria (Angsuthanasombat and Panyim, 1989; Chungjatupornchai, 1990; Murphy and Stevens, 1992; Soltes-Rak et al., 1993; Soltes-Rak et al., 1995; Stevens et al., 1994; Wu et al., 1997), *Caulobacter crescentus* (Thanabalu et al., 1992; Yap et al., 1994a), *Ancylobacter aquaticus* (Yap et al., 1994b), Baculoviruses (Pang et al., 1992), *Bradyrhizobium* (Nambiar et al., 1990), and *Rhizobium* spp (Guerchicoff et al., 1996). Moreover, an attempt was made to obtain a broader spectrum of activity against mosquito larvae, using Bti as a heterologous host for *B. sphaericus* binary toxin genes, but without success (Bourgouin et al., 1990). Crystal negative strains of Bti can also be used as a host for expressing mosquitocidal toxin genes from other sources; for example, *cryIIBb* gene encoding the 94 kDa toxin from Bt. *medellin* was cloned and expressed in such a strain (Orduz et al., 1998; Restrepo et al., 1997). Recently, it was demonstrated that efficient synthesis of mosquitocidal toxins (binary toxin of *B. sphaericus*) in *Asticcacaulis excentricus* gram-negative bacteria has potential for mosquito control. Genetically engineered *A. excentricus* has potential advantages as a larvicidal agent especially with regard to persistence in the larval feeding zone, resistance to UV light, lack of toxin-degrading proteases, and low production costs (Liu et al., 1996).

The amount of active heterologous protein expressed depends on various factors including: regulation of replication (plasmid copy number); transcription (promoter strength, tandem promoters and σ factors); translation (efficiency of ribosomal binding site, U-rich sequence and codon usage); and mRNA stability (stem-loop structure at the 3′ end, and 5′ mRNA stabilizer) (Agaisse and Lereclus, 1995; Baum and Malvar, 1995; Chandler and Pritchard, 1975; Dong et al., 1995; Ikemura, 1981; Nordström, 1985; Soltes-Rak et al., 1995; Studier and Moffatt, 1986; Vellanoweth and Rabinowitz, 1992; Yap et al., 1994a).

8.5.1 Expression of Bti δ-endotoxins in *Escherichia coli*

Toxicity of the recombinant *E. coli* in contrast with the recombinant *Bacillus* spp, is usually poor due to weak expression of Bti δ-endotoxin genes (Bti's promoters for *cry* genes are weakly expressed in *E. coli*), low stability and proteolytic cleavage of polypeptides, and nonformation or malformation of crystals. Furthermore, the expression of *cytIAa* into *E. coli* and acrystalliferous Bt *kurstaki* kills the cells by a lethal interaction of CytIAa molecules with the host (Douek et al., 1992; Wu and Federici, 1993) and/or spore formation in latter bacteria was aberrant (Chang et al., 1993). The *cry4B* gene, however, was efficiently expressed in *E. coli* and form phase-bright insoluble inclusions which were highly toxic to *Ae. aegypti* larvae (Angsuthanasombat et al., 1987; Chungiatupornchai et al., 1988; Delecluse et al., 1988; Ward and Ellar, 1988). The best expression and highest toxicity in recombinant *E. coli* was achieved when the combination *cry4A* + *cryIIA*, with or without the 20 kDa protein gene was cloned under a stronger resident promoter (Ben-Dov et al., 1995).Values of LC_{50} against third instar *Ae. aegypti* larvae for these clones were about $3 \cdot 10^5$ cells ml^{-1} after 4 h induction.

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8.5.2 Expression of Bti δ-endotoxins in Cyanobacteria

To overcome the low efficacy and short residual activity in nature of current formulations of Bti, and to create more stable and compatible agents for toxin delivery, toxin genes should be cloned into alternative hosts that are eaten by mosquito larvae and multiply in their habitats. Photosynthetic cyanobacterial species are attractive candidates for this purpose (Boussiba and Wu, 1995;, Boussiba and Zaritsky, 1992; Porter et al., 1993; Zaritsky, 1995): they are ubiquitous, float in the upper water layer and resist adverse environmental conditions. They are used as natural food sources for mosquito larvae (Avissar et al., 1994; Merritt et al., 1992; Stevens et al., 1994), can be cultured on a large scale (Boussiba, 1993), and are genetically manipulatable (Elhai, 1993; Elhai and Wolk, 1988; Shestakov and Khyen, 1970; Wolk et al., 1984; Wu et al., 1997). Several attempts have been made during the last decade to produce transgenic mosquito larvicidal cyanobacteria (Angsuthanasombat and Panyim, 1989; Chungjatupornchai, 1990; Murphy and Stevens, 1992; Soltes-Rak et al., 1993; Soltes-Rak et al., 1995; Stevens et al., 1994; Tandeau de Marsac et al., 1987; Wu et al., 1997; Xudong et al., 1993). Some success has been achieved in expressing single *cry* genes in unicellular species, but larvicidal activity was limited. For example, recombinant cyanobacterium *Agmenellum quadruplicatum* PR-6, bearing *cry11A*,with its own strong phycocyanin promoter (P_{cpcB}) had very limited mosquitocidal activity against *Cx. pipiens* larvae (Murphy and Stevens, 1992). Transgenic *A. quadruplicatum* PR-6 expressing *cry4B* under the same (P_{cpcB}) promoter produced a maximum of 45% mortality against second instar *Ae. aegypti* larvae after 48 h exposure (Angsuthanasombat and Panyim, 1989). When *cry4B* was expressed in *Synechocystis* PCC 6803 from P_{psbA}, levels of the toxic polypeptide were very low and whole cells were not mosquitocidal at $4 \cdot 10^8$ cell ml^{-1} (Chungjatupornchai, 1990). Using tandem promoters for expression of *cry4B* (its own and P_{lac}) in *Synechococcus* PCC 7942 slightly improved mosquitocidal activity against first instar larvae of *Cx. restuans*, but was still insufficient (Soltes-Rak et al., 1993).

Very high mosquito larvicidal activities were achieved in the cyanobacterium *Anabaena* PCC 7120 when *cry4A* + *cry11A*, with and without *p20*, were expressed by the dual constitutive and very efficient promoters P_{psbA} and P_{A1} (Wu et al., 1997). An additional reason that high activities were obtained is because codon usage of *Anabaena* resembles that of the four *cry* genes of Bti. The LC_{50} of these clones against third instar *Ae. aegypti* larvae is ca. $9 \cdot 10^4$ cells ml^{-1}, which is the lowest reported value for engineered cyanobacterial cells with Bti toxin genes (Wu et al., 1997). In addition, the recombinant plasmids are stable inside the transgenic *Anabaena* PCC 7120; the constitutive expression of Bti Cry toxins is apparently not harmful to the host cells. Preliminary results indicate that toxicities of these clones were retained following irradiation by high doses of UV-B (at wavelengths of 280 to 330 nm), which is an important asset for Bti formulations (Manasherob et al., 1998a). These transgenic strains are thus of high potential value and have recently been patented (Boussiba et al., 1997; Boussiba et al., 1998).

8.5.3 Expression of Bti δ-endotoxins in Photoresistant *Deinoccocus radiodurans*

Commercial Bti preparations undergo rapid deactivation by sunlight in the field (Hoti and Balaraman, 1993; Liu et al., 1993); therefore, it was recently proposed to clone Bti δ-endotoxin genes into the extremely photoresistant bacterium *Deinoccocus radiodurans* R1 (Manasherob et al., 1997b). The species *Deinoccocus radiodurans* is extremely resistant to ionizing and UV radiation (Battista, 1997; Moseley, 1983) and desiccation (Mattimore and Battista, 1995). It is a gram-positive, non-sporulating and nonpathogenic diplococcus containing a red pigment. Its resistance is acquired by an exceedingly efficient DNA repair mechanism, which extends to resident plasmids with a similar efficacy (Daly et al., 1994). The characteristic pigmentation of *D. radiodurans* may play a role in resistance on the protein level by the free radical scavenging potential of its carotenoids (Carbonneau et al., 1989), which might exert protection on heterologous proteins. Additional factors which may contribute to the extreme radiation protection of proteins are: its unusually complex cell wall (Battista, 1997), UV screening by high concentrations of sulfhydryl groups, and unique lipids (Reeve et al., 1990). Indeed, cells of *D. radiodurans* R1 were found to be much more photoresistant to UV-B (280 to 330 nm) than spores of Bti (Manasherob et al., 1997b). Cloning Bti's mosquito larvicidal genes for expression in *D. radioduran* R1 is thus expected to protect them as well as their products from the harmful affects of sunlight.

8.5.4 Molecular Methods for Enhancing Toxicity of Bti

Despite the fact that no resistance has been detected to date toward Bti in field populations, laboratory-reared *Cx. quinquefasciatus* develop different levels of resistance to individual Bti toxins under heavy selection pressure (Georghiou and Wirth, 1997). Various approaches that utilize the tools of molecular biology and genetic engineering will be developed to lessen the chance of resistance development in the future. Engineered toxins with improved efficacy by differing modes of action or receptor-binding properties may be used for recombinant cloning. For example, Cry4B mutant toxin inclusion (site-directed mutagenesis of *cry4B* for replacement of arginine-203 by alanine) was twice more toxic to *Ae. aegypti* larvae than the wild-type toxin inclusion (Angsuthanasombat et al., 1993), and toxicity of Cyt1Aa mutant (lysine124 replaced by alanine) increased cytolytic activity *in vitro* by threefold (Ward et al., 1988). Recently, hyper-toxic mutant strains of Bti were isolated by mutagenising the parent strain which produce more toxin (6- to 25-fold) than the parent (Bhattacharya, 1995).

On the other hand, co-expression of natural toxins from different origins by unique combinations of their genes, chimeric toxins, or replacement of one gene on another more potent gene in the same bacterial strain may enhance larvicidal activity by a synergistic effect between them. In addition, it can delay or prevent the development of resistance and expand the host spectrum. Mosquitocidal strains Bt *medellin* and Bt *jegathesan* are less potent than Bti, but they harbor the CryIIBb and Cry11Ba toxins, respectively, which are more toxic than any of the individual Bti

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toxins (Delecluse et al., 1995b; Orduz et al., 1996, 1998) making good candidates for use in genetic improvement efforts.

8.6 RESISTANCE OF MOSQUITOES TO BTI δ-ENDOTOXINS

Resistance to microbial insecticides was detected in several species in the laboratory as well as in field strains of diamondback moth *Plutella xylostella* and mosquito species *Cx. quinquefasciatus* and *Cx. pipiens*. A knowledge of resistance mechanisms in insect pests is therefore very important for developing resistance management programs (Georghiou, 1994).

Resistance mechanisms include: toxin receptors, binding characteristics, competition aspects of different toxins, and physiological changes. Altered and/or slower protoxin activation by midgut proteinases, lack of major gut proteinase that activate Bt protoxins, or decreased solubility (by change in pH) in resistant subspecies (Dai and Gill, 1993; McGaughey and Whalon, 1992; Oppert et al., 1994; Oppert et al., 1997) are potential mechanisms for insect resistance to Bt toxins. The characterization of some lepidopteran-active Bt toxin receptors previously showed that the mechanism of resistance was based on reduction in binding affinity to the membrane receptor and/or decrease in receptor concentration (Ferre et al., 1991; Tabashnik et al., 1994; Van Rie et al., 1990). However, in some cases no difference in affinity of toxin to receptor was observed in susceptible and resistant larvae. The hypothesis is that the insect could attain resistance by altering toxin binding without eliminating toxin binding proteins (Gould et al., 1992; Luo et al., 1997). Other aspects of the possible mechanisms of resistance remain to be explored, including post-binding events such as membrane insertion and pore formation (Marrone and MacIntosh, 1993). In addition, it was recently demonstrated that spores of Bt increased the toxicity of Bt δ-endotoxins to both resistant and susceptible larvae of *P. xylostella*. The role of spores, therefore, may also be helpful for understanding and managing pest resistance to Bt (Liu et al., 1998).

The phenomenom of cross-resistance to Bt has been recorded in several studies (Gould et al., 1992; Tabashnik, 1994; Tabashnik et al., 1993; Tang et al., 1997). Cross-resistance may develop against Bt toxins that are similar or differ in structure and activity. Cross-resistance in the former case may be due to several Bt toxins with the same binding site, and in the latter case receptors which bind multiple toxins. However, in some cases resistance to Cry1C is inherited independently and differently than resistance to Cry1Ab and cross-resistance is not conferred (Luo et al., 1997). Multiple toxin genes with different modes of action or receptor-binding properties may reduce the chances of insect developing resistance (Tabashnik, 1994). To date, there have been no reported cases of cross-resistance between Bt toxins and synthetic insecticidal pesticides (Georghiou, 1994; Marrone and MacIntosh, 1993).

Partially or fully recessive inheritance of resistance by an autosomal trait (primarily controlled by one or few genes) to Bt was reported (Marrone and MacIntosh, 1993; McGaughey, 1985; Tabashnik, 1994; Tang et al., 1997). Recently the partially dominant inheritance of resistance (Gould et al., 1992; Liu and Tabashnik, 1997) was observed. Selection for resistance by partial dominance is affected by either

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high or low concentrations of toxin, depending on the Cry protein. In addition, other environmental factors may affect resistance inheritance and complicate resistance management programs (Gould et al., 1992; Liu and Tabashnik, 1997).

Significant levels of resistance and cross-resistance to different strains of *B. sphaericus* which harbor binary toxin, have already been demonstrated for *Cx. quinquefasciatus* and *Cx. pipiens* larval populations, both in the laboratory and in the field (Nielsen-LeRoux et al., 1995; Nielsen-LeRoux et al., 1997; Rodcharoen and Mulla, 1996; Silva-Filha et al., 1995). Some cases of resistance are related to a loss of the crystal toxin's binding ability (Nielsen-LeRoux et al., 1995; Rodcharoen and Mulla, 1996). One possible explanation for this is that the functionality of the receptor has been altered, and another is that a reduction in active receptor sites occurred. In other cases of resistance, the binding step remains unchanged (Nielsen-LeRoux et al., 1997; Silva-Filha et al., 1995; Silva-Filha et al., 1997). In both cases, the inheritance of the resistance was recessive, and due to a single gene (Nielsen-LeRoux et al., 1995; Nielsen-LeRoux et al., 1997). No competition between the *B. sphaericus* binary toxin and the δ-endotoxin of Bti on binding-site was observed in resistant *Cx. quinquefasciatus* populations indicating the involvement of different specific receptors (Nielsen-LeRoux et al., 1995; Rodcharoen and Mulla, 1996). The fact that the *B. sphaericus* crystal toxin binds to a single type of receptor (Nielsen-LeRoux and Charles, 1992; Nielsen-LeRoux et al., 1995), means that it is possible to obtain quick development of mosquito resistance, while this is not the case for Bti (Cheong et al., 1997; Georghiou and Wirth, 1997; Wirth et al., 1997; Wirth and Georghiou, 1997).

No resistance has been detected to date toward Bti in field populations of mosquitoes despite 15 years of extensive field usage (Becker and Ludwig, 1994; Georghiou, 1990; Becker and Margalith, 1993; Margalith et al., 1995). Selection attempts in the laboratory with natural Bti toxins have produced no resistance in *Ae. aegypti* (Goldman et al., 1986) and negligible levels of resistance in *Cx. quinquefasciatus* under heavy selection pressure (Georghiou, 1990). Resistance of *Cx. quinquefasciatus* was obtained, however, by selection to the polypeptides Cry4A, Cry4B, and Cry11A alone or in combination (Georghiou and Wirth, 1997; Wirth et al., 1997). These strains retained their original wild-type sensitivity levels to the above polypeptide combinations in the presence of moderate concentrations of Cyt1Aa, thus resistance was completely suppressed by Cyt1Aa (Wirth et al., 1997). Moreover, cross-resistance was observed between resistant strains; for example, a strain resistant to Cry11A demonstrated significant cross-resistance to Cry4A + Cry4B, and vice versa (Wirth and Georghiou, 1997). Extremely low resistance was obtained to the toxin mixture (Cry4A, Cry4B, Cry11A plus Cyt1Aa) but moderate cross-resistance levels were detected toward individual Cry toxins or their combinations (Georghiou and Wirth, 1997; Wirth and Georghiou, 1997). Despite the presence of resistance and cross-resistance to Cry proteins, all of the selected strains remained sensitive to the three Cry toxin mixture plus Cyt1Aa. In addition, resistant laboratory strains of *Cx. quinquefasciatus* to single or multiple toxins of Bti, showed cross-resistance to Cry11Ba from Bt *jegathesan* (Wirth et al., 1998). In the same study, it was found that Cyt1Aa combined with Cry11Ba can suppress most of the cross-resistance to Cry11Ba in the resistant strains. Cyt1Aa has been shown to be

toxic to the Cottonwood Leaf beetle, *Chrysomela scripta*, and it also suppressed high levels of resistance to Cry3Aa found in Bt *tenebrionis* (Federici and Bauer, 1998). All the above findings suggest that the Cyt1Aa toxin may play a critical role in suppressing resistance to the Cry toxins and may be useful in managing resistance to bacterial insecticides (Cheong et al., 1997; Federici and Bauer, 1998; Wirth and Georghiou, 1997; Wirth et al., 1998).

Recently, the gene encoding cytolytic Cyt1Ab protein from Bt *medellin* (Thiery et al., 1998), Cry11A from Bti and Cry11Ba from Bt *jegatheson* (Servant et al., 1999) were introduced into *B. sphaericus* toxic strains. Production of these proteins in *B. sphaericus* partially restored susceptibility of resistant mosquito populations to the binary toxin (Servant et al., 1999; Thiery et al., 1998). Furthermore, to date several Cyt toxins have been identified (all of them host specific against mosquito larvae, Table 8.2).

Bti contains a unique natural Cry protein complex of δ-endotoxin which confers an effective defense against the development of resistance in the target organisms, and which ensures successful biological control over many years to come.

8.7 USE OF BTI AGAINST VECTORS OF DISEASES

Bti is a highly selective biological larvicide used to control mosquitoes and black fly larvae. Because of its selective activity, Bti does not harm mosquito and black fly predators such as fish, frogs, insects and crustaceans that contribute significantly to larval control. Bti is also non-hazardous to human, livestock, pets, and other forms of beneficial organisms (McClintock et al., 1995; Murthy, 1997; Siegel and Shadduck, 1990). One other significant edge for this biological larvicide is the absence of field resistance to Bti products, even when used repeatedly for over 15 years (Becker and Ludwig, 1994; Becker and Margalith, 1993; Margalith et al., 1995).

Many environmental factors affect control performance of Bti, such as water quality, solar radiation, high organic content, suspended material, water current, and weather conditions, as well as larval age and mosquito species (surface or bottom feeder). Bti has a short residual activity, often only 1 to 2 days due to adsorption to particles and sinking to the bottom, out of the feeding zone of larval mosquitoes and black flies (Blaustein and Margalith, 1991; Liu et al., 1993; Vaishnar and Anderson, 1995). Denaturation of the crystal by sunlight and engulfment of Bti by filter feeding fauna are also factors in reducing the control efficacy of Bti. Since water filters out much of the UV radiation, sunlight is much less important in aqueous habitats, where adsorption to particles and settling are the main factors in efficacy reduction (Ignoffo et al., 1981).

Presently, Bti is used in all continents. Over 300,000 liters of Bti are applied annually in Western Africa against black flies — vectors of onchocerciasis (Margalith et al., 1995). In Europe, along the Upper Rhine Valley, over 100 communities with a population of over 2.5 million people are protected through mosquito abatement programs utilizing Bti (Becker and Margalith, 1993). The number of abatement districts using Bti is rising steadily in the U.S. and Canada. With the development of appropriate formulations, effective and economic control of mosquitoes and black

flies is now generally possible. Worldwide usage of Bti is increasing year by year. It is estimated that over 1000 metric tons (mt) of Bti preparations are used annually (Keller et al., 1994). So far, there have been no negative effects. In suitable formulations, this microbial agent is a useful supplement to, or replacement for, broad-spectrum chemicals in larval mosquito control programs. Further improvements, particularly to extend their residual activity and to enhance *Anopheles* control, will increase Bti consumption still further. In temperate regions, Bti offers an ecologically defensible compromise between the desire of man to protect himself from troublesome mosquitoes or black flies and the requirements of current environmental policies to protect sensitive ecosystems by the use of selective methods.

8.7.1 Formulations

Many different formulations of Bti have been developed since its establishment as a commercially viable and promising alternative to conventional pesticides several years after its discovery in 1976 (Goldberg and Margalith, 1977). Differently formulated products are required for mosquito and black fly larvae of different feeding types and habitats. Preparations, which persist at the bottom of a water container, are suited for the control of so-called bottom-feeding larvae like *Aedes* spp. The larvae of anophelines, on the other hand, are controlled most effectively by granules, which float on the water surface and release the toxin slowly. Black fly larvae live in fast-moving water courses and are controlled by pouring bacterial suspensions into the water at consecutive points, from which they are carried downstream. Development of effective formulations suited to the biology and habitats of the target organisms is the basic requirement for the successful use of Bti (Ali et al., 1994; Becker et al., 1991; Ravoahangimalala et al., 1994).

The major limiting factor in the further development of Bti is its short residual activity (Becker et al., 1992; Blaustein and Margalith, 1991; Eskils and Lovgren, 1997; Margalith and Bobroglo, 1984; Margalith et al., 1983; Mulla, 1990; Mulligan et al., 1980; Ohana et al., 1987; Rashed and Mulla, 1989). To increase environmental stability of the Bti, several studies were carried out by encapsulation with latex beads, emulsions, polyethylene, and starch-based matrices (Cheung and Hammock,1985; Margalith et al., 1984; Schnell et al., 1984).

Efforts are being made not only to improve the efficacy of Bti by prolonging its residual effect, but also targeting delivery of the active ingredient in the feeding zone of the larvae. These improvements are primarily based on development of a variety of formulations (Ali et al., 1994). To date several commercial formulations are available: liquid concentrates, wettable powders, granules, pellets, dunks and briquets, tablets, polymer matrix, and ice granules.

8.7.1.1 Production Process

Liquid concentrate represents the most widely used and largest volume of formulation. Bti, the naturally occurring bacterium, is grown commercially in fermenting vats. During the production process, variables such as nutrients, temperature and supply of oxygen can affect bacterial growth, sporulation, and yield of crystal toxins

(Kim and Ahn, 1996; Kraemer-Schafhalter and Moser, 1996). Once the desired insecticidal activity is achieved, the bacterial cells are allowed to lyse. Spores and insecticidal crystalline proteins are harvested after approximately 24 h of fermentation time by precipitation, centrifugation, or ultrafiltration. At the end of this process, preservatives and dispersing agents are added to obtain the final liquid concentrate formulation product.

Primary powder ("technical powder") of Bti is produced by spray drying of the concentrated culture medium. *Wettable powder* formulations are produced by adding dispersing and stabilizing inert ingredients, such as bentonites and diatomes to the primary powder. Primary powder is the active ingredient used in production of granules, pellets, briquets, tablets, polymer matrix (Culigel®), and ice formulations.

Granules consist of 1 to 3 mm particles of ground corn cob impregnated with Bti.

Sand granules consist of primary powder mixed with fire-dried quartz and vegetable oil as a binding agent.

Pellets consist of ground corn cob mixed with Bti and compressed into 5 to 10 mm pellets.

Ice granule formulations have recently been developed and produced by the German Mosquito Control Association (KABS) (Becker and Mercatoris, 1997). An aqueous suspension of primary powder is sprayed into special ice machines, which transforms the Bti water suspension into ice granules using liquid nitrogen. When applied in breeding sites, melting ice granules gradually release Bti into the feeding zone of mosquito larvae, allowing a more cost-effective control operation.

Culigel®, a granulated controlled release formulation, has been developed by Lee County Mosquito Abatement District, U.S. It consists of crosslinked polyacrylamide superabsorbant matrix, capable of absorbing over ×100 to *ca* ×5000 their weight of water-containing primary powder. This formulation is reported to be active for well over one month under field conditions (Burges and Jones, 1998; Levy, 1989). These air-dried granules of 4 to 5 mm diameter contain up to 50% technical bacterial powder and about 50% Culigel® polymer.

Tablets of 1 cm diameter, containing Bti primary powder, have been developed in Germany by KABS in order to provide a suitable formulation for controlling *Ae. aegypti* and *Cx. pipiens* mosquitoes which breed in containers around households. A long-term effect of about 30 days was achieved in trials in Jakarta, Indonesia (Becker, 1996).

Briquets and dunks were designed by Summit Chemical Co., Baltimore, MD, U.S., as floating, sustained-release larvicides for long-term control of mosquito larvae. They consist of ground cork particles mixed with primary powder and are compressed into donut-shaped dunks measuring 5 cm in diameter with a hole in the center for anchoring. These formulations are designed to release effective levels of Bti for a period of 30 days or more (Kase and Branton, 1986).

8.7.1.2 Application Methods

For the use of Bti against larval black flies in turbulent and flowing waters, highly concentrated liquid formulations had to be produced that float as long as possible in the river current.

Microbial control formulations have been developed to improve the efficacy of Bti by prolonging its activity as well as targeting delivery of the active ingredient in the feeding zone of the larvae.

Liquid concentrate (LC) and aqueous suspensions of wettable powder (WP) are sprayed in open breeding habitats, in situations where dense weed or tree canopy does not obstruct delivery of the active ingredient. In these situations, the active biocontrol agent can easily reach the feeding zone of the target larval mosquitoes. These formulations can be applied effectively with ground application equipment such as conventional back pack; truck or tractor mounted sprayers; or by aerial application with helicopter and fixed winged aircraft. Bti formulations are applied at the rate of 2.5 liter of LC per hectare or 1kg of WP per hectare, suspended in 400 to 500 liters water.

The corn cob granules, pellets, granular sand formulations, as well as the recently developed ice granules ("IcyCube") are dropped through tree canopy and dense vegetation, penetrating to the obstructed breeding habitats, using specially devised applicators either mounted on trucks and tractors, or helicopters and fixed winged aircraft.

Briquets, dunks, tablets and Culigel® are designed to provide prolonged residual activity, usually 30 days or more. Briquets and dunks can be used in ponds, cisterns, ditches, and other breeding places of limited dimensions. Tablets are suitable for small containers such as drinking jars, barrels, and other breeding places in and around the household. Culigel® has been designed to be used in most of the above described habitats.

Ultralow volume (ULV) technology has been utilized with considerable success in a recent effort to develop a more effective technique in dispersing Bti against larval mosquitoes of several *Aedes* species — potential vectors of diseases — that breed in confined habitats such as crevices along the tidal zones and in containers, drinking jars and tires, in and around the household, mostly in tropical and subtropical countries (Lee et al., 1996; Tidwell et al., 1994). Successful mosquito control operations under canopies using ULV aerial sprays have been reported as well (Cyanamid, 1992). Lim and Poorani (1998) have successfully used microdroplet ULV application techniques with Bti, using cold fog ULV generators and mist blowers — substantially enhancing the effectiveness and coverage of Bti, especially in the control of dengue vectors. They also applied an aqueous suspension mixture of Bti with organophosphate insecticides in order to control both larval and adult stages in a single application.

8.7.1.3 Encapsulation

Encapsulated formulations have been developed to provide protection against destructive UV radiation and to provide sustained release of the active ingredient for prolonged control efficacy. Several types of encapsulated products have been developed: matrices, microencapsulation, lipid droplets, and bioencapsulation. Margalith et al., 1984 made two types of capsules: 1) by stirring a mixture of Bti and dried yeast in a solution of low density polyethylene in cyclohexane; and 2) by stirring the bacteria into a slowly cooling fine emulsion of a fatty acid (decanoic,

palmitic, or stearic). Both types increased the flotation coefficient and improved the insecticidal activity against *Culex* and *Aedes* larvae in glass containers with mud on the bottom.

Bti was encapsulated in alginate microcapsules in an attempt to develop durable formulations resistant to detrimental environmental conditions (Elçin, 1995). A mixture of Bti spores and crystals was also encapsulated in an insolubilized carboxymethylcellulose (CMC)-aluminum matrix to obtain a controlled-release system. This system could improve stability of the δ-endotoxin to environmental factors during and following application (Cokmus and Elçin, 1995).

Cheung and Hammock (1985) formed lipid capsules of different buoyancy. These microcapsules of 3 to 12 μm in diameter remained in the upper water column at the depth of 0.2 to 12 inches up to 24 h. Efficacy improved two to three times against *Culex* and *Aedes* larvae and twenty times against *Anopheles*.

An innovative approach has recently been devised, to concentrate and deliver Bti and its δ-endotoxin to the larvae by a ciliate protozoan, *T. pyriformis* (Manasherob et al., 1994; Manasherob et al., 1996b; Zaritsky et al., 1991; Zaritsky et al., 1992). Spores and δ-endotoxin of Bti are not destroyed during the digestion process in the food vacuoles of the protozoan *T. pyriformis*; in fact, spores germinate in excreted food vacuoles, develop to vegetative bacteria and complete a full sporulation cycle in them (Ben-Dov et al., 1994; Manasherob et al., 1998b). This approach was termed "bioencapsulation" and proved efficient under laboratory conditions (Zaritsky et al., 1991), but its usefulness in the field is still to be explored.

8.7.1.4 Standardization

Standardized methods have been developed to determine the LC_{50} values using standard formulations for comparative purposes (e.g., IPS-82 produced by Institut Pasteur).The active ingredient may be stated in several ways: 1) in International Toxic Units (ITUs); and 2) in AA units, with AA standing for *Aedes aegypti*. The original measure of Bti activity was the ITU, which was established several years ago by Pasteur Institut in France and the World Health Organization (deBarjac, 1983). A product's ITU is established by conducting mosquito bioassays using *A. aegypti* as the test species. Each AA unit (based on tests using larval instar II), however, represents less activity than an ITU (based on tests using larval instars III and IV), differing by a factor of 2.5. Dividing the AA unit value by 2.5 gives the ITU equivalent (Wassmer, 1995). Potency is usually expressed in ITU and is calculated according to the following formula:

$$\text{Potency sample (ITU/mg)} = \frac{LC_{50}\ \text{standard}}{LC_{50}\ \text{sample}} \times \text{potency standard (ITU/mg)}$$

8.7.2 Worldwide Use of Bti Against Mosquitoes and Black Flies

Numerous strains of Bt have been isolated and tested against mosquito larvae; however, the discovery of Bti availed an agent that was highly effective against mosquito species under field conditions (Mulla, 1990). Bti is used for mosquito and black fly control on a large scale worldwide; at present, more than 1000 mt are used annually. The dramatic increase in utilization of this biopesticide is due to its properties, e.g., efficacious, safe to the environment and users, simple to produce and apply, availability of formulations, and no build-up of resistance in mosquito populations (Becker and Ludwig, 1994; Becker and Margalith, 1993; Margalith, 1989).

8.7.2.1 U.S.

Larviciding with Bti is an important component of most U.S. mosquito control programs.

Based on a U.S. EPA pesticide usage survey for 1987 though 1996, an average of 1.5 million acres of surface water had been treated annually with Bti throughout the U.S., compared to approximately 2.1 million acres of agricultural land treated with Bt. Production quantities of Bti in the U.S. were generally not avalilable since these data are confidential business information (CBI).

The number of abatement districts using Bti is rising steadily in the U.S. and Canada. Two examples illustrate Bti usage: 1) in Massachusetts, approximately 70 mt of Bti corn cob granules were applied by helicopter in one week during August 1993 to 20,000 acres of surface water, mostly against *Ae. vexans*, one of the suspected zoonotic vectors of Eastern Equine Encephalitis (EEE) (Margalith, unpublished data); 2) in Florida, ca. 800 kg of active ingredient (primary powder) was used in 1995.

8.7.2.2 Germany

Due to public concern and growing awareness of environmental protection in Europe, microbial agents are being used increasingly in integrated control programs. Among the European countries, Germany leads the way in controlling mosquitoes with Bti.

In Germany, 89 cities and municipalities along a 300 km stretch of the Upper Rhine River have joined forces to form an agency whose purpose is to control mosquitoes, mainly *Ae. vexans* over a breeding area of over 500 square km. The responsible agency, German Mosquito Control Association (GMCA), has set up a program to address all aspects of mosquito control with emphasis on environmental protection. The control of *Aedes* mosquitoes is based solely on the use of Bti products. The program involves mapping of all mosquito breeding places, monitoring water flow in the Rhine and in the flood plains, sampling mosquito populations to determine success of control measures, documenting the environmental impact, and monitoring resistance to Bti (Becker and Ludwig, 1983; Becker and Margalith, 1993).

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In the past years, approximately 50 mt of Bti powder and 25 mt of Bti liquid concentrates have been used for treating over 700 square km of breeding area. Since the widespread application of Bti, initiated in 1981, more than 90% of the *Ae. vexans* population has been successfully controlled each year. Despite extreme flooding in the past few years, mass occurrences of mosquitoes have successfully been averted. Environmental impact is minimal since all other invertebrates are unaffected and *Aedes* mosquitoes form a negligible part of the food chain. No resistance to Bti has been detected over the past 10 years (Becker and Ludwig, 1994).

All studies carried out to date have shown that since the introduction of Bti in the mosquito control program in Germany, mosquitoes are successfully controlled with minimal impact on the environment with no foreseeable resistance development (Margalith et al., 1995).

8.7.2.3 People's Republic of China

The Hubei Province, with a population of more than 20 million people on both sides of the Yangtze River is threatened by malaria, caused by *Plasmodium vivax*. Control of the main vector, *An. sinensis*, with insecticides has become increasingly difficult, due to developing resistance and ecological and toxicological risks. In routine treatments, liquid formulations of microbial control agents are applied using high pressure sprayers attached to a 600-liter tank pulled by a mini-tractor. In the years 1986–1990, about 25 tons of microbial agents (Bti and *B. sphaericus*, local strains) have been produced annually (using locally available culture media) in the Hubei Province, which was enough to treat approximately 12,000 hectares of mosquito breeding sites. The impact of the treatments was recorded by measuring the density of adult mosquitoes and the incidence of malaria before and after the campaign. Both the mosquito population density and the malaria incidence were reduced by more than 90%, from 8.2 cases per 10,000 people in 1986 to 0.8 per 10,000 in 1989 (Becker, 1996). The great success of this campaign is based on an adequate infrastructure and defined breeding sites inside or near the human settlements.

8.7.2.4 Peru, Ecuador, Indonesia, and Malaysia

In these countries, the potential of Bti to control malaria vectors such as *An. albimanus*, *An. rangeli*, *An. nigerrimus* and *An. sundaicus* was evaluated in field studies. Adult mosquito density (measured by bites per person per hour on human baits) was reduced by more than 70% using Bti at 1 to 2 kg/hectare at weekly intervals (Becker, 1996).

In Indonesia, pellets were introduced by Becker (1995, personal communication) in household water containers such as barrels, concrete cisterns, etc. where *Aedes* spp dengue vectors breed. Nearly a 100% larval mortality was obtained. In Malaysia, Lim and Poorani (1998) have successfully used microdroplet ULV application techniques with Bti in an urban situation, using cold fog ULV generators and mist blowers — substantially enhancing the effectiveness and coverage of Bti, especially in the control of dengue vectors.

8.7.2.5 Israel

Mosquito control in Israel is directed against five major mosquito species. Although malaria had been eradicated from Israel 50 years ago, two species are vectors of the West Nile Fever disease and others are a public nuisance. The growing awareness of environmental pollution, as well as development of resistance to synthetic pesticides, have been the motivating force, as in many other countries, to develop integrated pest control methods which rely more on selective biological pesticides. Bti has successfully been used in Israel to control populations of nuisance mosquitoes, allowing the continued regulation by natural enemies. A typical use pattern of Bti against mosquitoes in Israel includes a series of 45 consecutive applications at 10-day intervals. The first spray usually reduces mosquito populations by ca. 90%, the remaining treatments prevent population build-up, and from that point natural enemies suffice in keeping populations below threshold levels. Bti is used predominantly in nature reserves and other ecologically sensitive breeding sites (Margalith et al., 1995).

8.7.2.6 West Africa

The onchocerciasis control program (OCP) — Human onchocerciasis, also called river blindness, is a parasitic disease caused by a filarial worm, *Onchocerca volvulus*. In West Africa, this disease is transmitted by adult black fly females belonging to the *Simulium damnosum* complex (Diptera: Simuliidae). The larvae of these species breed in the rapids of small to large rivers. In the 1960s, human onchocerciasis was identified as a concern for public health and as a major obstacle to social and economic development in West Africa, since it prevented the settlement of fertile river valleys (Samba, 1994). International commitment led to the creation of the Onchocerciasis Control Programme in West Africa (OCP) in 1974, with the objective of eliminating this threat to health and development (Kurtak, 1986). The funds contributed by donor countries and agencies are administered by the World Bank, and WHO is the executing agency (Samba, 1994). The initial area of OCP (654,000 km^2) has been gradually expanded and now covers 11 countries of West Africa, for a total area of 1,235,000 km^2.

Strategy — Over most of this area, the main strategy of the OCP has been to interrupt transmission of the disease by controlling vector populations for a period longer than the lifespan of the adult worm in man, now known to be approximately 12 years. Vector control operations consisted of weekly aerial treatments with insecticides of river rapids where the black fly larvae breed (Agoua et al., 1991). In addition to vector control, ivermectin (a microfilaricide) is now distributed yearly to nearly two million people within the OCP area; this very safe drug provides relief from onchocerciasis symptoms, prevents the development of blindness, and consolidates the results obtained by vector control.

Between 1974 and 1979, OCP exclusively used temephos, an organophosphorous (OP) compound, for black fly control. Resistance appeared in rivers of southern Côte d'Ivoire, and in 1980 a second OP insecticide, chlorphoxim, was introduced, to

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which resistance appeared as well the following year (Guillet et al., 1990; Kurtak, 1986). In the absence of suitable or ecologically acceptable alternative chemicals, Bti was identified as a potential candidate for OCP operations (Guillet et al., 1990). Based on successful experimental treatments in 1980 (Lacey et al., 1982), Bti was first used on a large scale by OCP in 1981 (Akpoboua et al., 1989), when 8000 L of a commercial formulation produced by Sandoz (Teknar WDC) were applied in the Bandama River basin (southern Côte d'Ivoire). This water-dispersible formulation had to be mixed with a minimum of 20% of water, and it was applied at the rate of 1.2 liters per m^3/s of river discharge (1.2 L/m^3s). This dosage was eight times higher than that of temephos, which put a heavy burden on aerial operations, but OP-resistant black fly populations were effectively controlled by Bti. Based on this first success and due to the spread of resistance to temephos and chlorphoxim in southern Côte d'Ivoire, the use of Teknar WDC increased up to 233,000 L in 1982, and to 310,000 L in 1983. In 1984, it decreased to 257,000 L, due to an increase in chlorphoxim use and because of river discharges too high for Bti treatments.

Since 1985, the annual use of Bti by OCP has varied between 210,000 L and 406,500 L. This wide range has been due to several factors including the introduction of more potent Bti formulations: shifts in the susceptibility of *S. damnosum s.l.* to OPs; changes in the area under vector control; and introduction of new insecticides. In 1985, Sandoz produced a more potent formulation, Teknar HPD, which could be used without dilution at the dosage rate of 0.72 L/m3/s. Concerning chemical insecticides, OCP introduced in 1985 two additional compounds (permethrin and carbosulfan) for the treatment of rivers at high discharges. This marked the onset of the rotation strategy still used by OCP. In 1987, OCP started using Vectobac 12AS, a formulation of Bti equivalent to Teknar HPD, which could be used at the same dosage rate; Vectobac 12AS has been used exclusively by OCP since 1990. The number of insecticides used by OCP has now increased from five to seven, with the introduction of pyraclofos (an OP compound) in 1990, the replacement of chlorphoxim by phoxim in 1991, and the introduction of etofenprox (a pseudo-pyrethroid) in 1994.

Success of program — OCP is now considered a great success of international cooperation. Onchocerciasis has been eliminated from the initial area, enabling the settlement of 13 million hectares of arable land, and with the more recent extensions an additional 10 million hectares have been opened to settlement. Due to OCP, 1.5 million people have recovered from early onchocerciasis symptoms and sight impairment, and it is estimated that 265,000 cases of blindness have been avoided since 1974. Thirty million people are now protected from onchocerciasis, and the 10 million children born since the start of the program have been spared the risk of contracting the disease. These major achievements are largely the result of an efficient vector control, of which Bti has been a major tool. Bti has been used mostly along the upstream rivers and rivulets, the tributaries in the Volta River basin, preserving all the non-target hydrofuana which was able to replenish the high discharge downstream rivers where the aquatic fauna had been exterminated by OP chemical pesticides.

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It is now scheduled that OCP will draw to an end around 2002, when blinding onchocerciasis will have been eliminated as a public health concern and as an obstacle to social and economic development in West Africa.

8.7.2.7 Temperate Climate Zones

In those regions of the world with temperate climate, where black flies do not transmit pathogens that affect human health, these insects cause considerable nuisance (Molloy, 1990), to man and cattle. Temperate regions subject to these problems generally are ecologically sensitive as, for example, mountain resorts. The quantity of Bti to be applied in mountain streams varies with discharge rate of the water, profile of the stream, and degree of vertical mixing, turbidity and presence of pollutants, water temperature and pH, settling due to presence of pools, and characteristics of the substrate. Although the ideal particle size seems to be about 35 μm, the rate of application varies several-fold depending on these characteristics (Molloy et al., 1984). Larger particles settle faster than smaller particles. This provides liquid formulations with a distinct advantage over powders; the small particles in a liquid formulation carry better, a crucial factor in the treatment of fast-moving streams. Some 5 to 30 ppm of Bti per minute generally provide satisfactory control of larval black flies over a span of 50 to 250 m in moderate sized streams (Knutti and Beck, 1987).

The non-target effects of Bti in these sites are minimal. Of the wide variety of organisms present, only filter-feeding chironomids were found to be sensitive to Bti, and at very high rates of application. Bti is thus the sole larvicide that can be used in such sensitive ecosystems.

8.8 CONTROL OF OTHER DIPTERA

Due to the increasing eutrophication of many rivers and lakes, aquatic midges such as *Chironomus* species often find optimal developmental conditions in aquatic sediments. Although the large-scale occurrence of flying insects can be very unpleasant (Ali, 1996; Mulla et al., 1990), chironomids occupy an important position in the food chain because of their large biomass. Furthermore, they play a critical role in self-cleaning of waterways, leading to reduction in the degree of eutrophication. Chironomids are neither vectors of diseases nor biting pests. Their ecological value far outweighs the disadvantages of their occurrence and, therefore, one should generally refrain from controlling them. Recently, Bti has been used for the control of filter feeding nuisance chironomid midges deriving from eutrophication of inland, urban, and suburban natural and man-made aquatic ecosystems (Ali, 1996; Kondo et al., 1995a; Kondo et al., 1995b).

In Germany, studies have shown that larval *C. plumosus* and *C. annularius*, which occur in aquatic sediments, are only affected by Bactimos at doses of above 4 ppm, approximately 10 times the dosage used in mosquito control. Mulla et al. (1990) achieved similar results in California, where they found that *C. decorus* could be controlled successfully with a dosage of 5 kg Vectobac TP or 10 liters Vectobac 12 AS per hectare.

Because of their ecological value, chironomids and similar insects are protected in Germany. Due to their low sensitivity towards Bti and occurrence mostly in permanent waters with silty sediments, in which *Aedes* mosquitoes generally do not occur, such protection is easily achieved. Additionally, other species of midges, such as *Smittia*, *Procladius* and *Tanypus*, are unaffected by Bti; as a result of this selective activity, a rich and diverse aquatic fauna remain as prey for predators, birds, and other higher trophic-level organisms after application.

Psychodidae, particularly *Psychoda alternata*, can occasionally become troublesome in sewage treatment plants. In laboratory experiments, 1 ppm of Bactimos was sufficient to kill larval *P. alternata*. In the field, however, a dose of 100 ppm was required to achieve nearly 100% mortality. Economic control of *Psychoda* therefore seems unfeasible. Even if Bti was active against the related phlebotomine vectors of Leishmaniasis, similar problems in their control could be anticipated (de Barjac and Larget, 1981).

Tipulid larvae (e.g., *Tipula paludosa* and *T. oleracea)* are the main insect pests in grasslands in Europe. Experiments in the laboratory, greenhouses, and in the field showed that first instar tipulid larvae could be successfully controlled by spraying Bti (Feldmann et al., 1995; Smits and Vlug, 1990). To avoid the high doses of Bti which are usually necessary for tipulid control, food baits containing high concentrations of Bti can be used as an alternative method.

8.9 FUTURE PROSPECTS

Numerable challenges lie ahead in the further development and commercialization of Bti as an environmentally safe and economically feasible alternative to chemical pesticides for the control of mosquitoes and black flies. During the past two decades, since the discovery of Bti, increasing environmental awareness has gained tremendous momentum, providing impetus to the exponential growth in scientific and technological advancements in the fields of fermentation, formulation, microbiology, and genetic engineering.

Various approaches that utilize the tools of molecular biology and genetic engineering will be further developed to lessen the chance of resistance development in the future. As genetically engineered and formulated products become more acceptable, biotechnology will continue to provide new and improved products. Engineered toxins with improved efficacy, achieved by differing modes of action or receptor-binding properties, may be used for recombinant cloning. Mutagenic studies will also be used to produce more toxic Bti strains. Co-expression of natural toxins from different origins by unique combinations of their genes, chimeric toxins, or replacement of one gene with another more potent gene in the same bacterial strain may enhance larvicidal activity by a synergistic effect, delay or prevent the development of resistance, and expand the host spectrum.

Advances in fermentation technology and downstream processes in producing primary powder and the entire range of formulations will reduce production costs, as well as improve the quality of Bti-related products. Formulations of aqueous suspensions avoid the cost of drying; however, they have a shorter shelf life than

the powder and granular formulations. Shelf life of dry products is comparable with chemical pesticides, giving them a competitive edge over liquid formulations.

Bti has a unique mode of action which prevents the development of resistance. This property derives from four major and three minor proteins which are different in homology and mode of action, especially with regard to the Cyt1Aa protein which exhibits a very high degree of synergism with the other toxin proteins, which has also been found to almost completely suppress resistance development. This finding has significant implications for future resistance management programs.

Since the gram-positive, non-sporulating, and non-pathogenic diplococcus *Deinoccocus radiodurans* is extremely resistant to ionizing and UV radiation and desiccation, it would be advisable to attempt to clone Bti δ-endotoxin genes into this extremely photoresistant bacterium to protect them as well as their products from the harmful effects of sunlight.

New formulations and new isolates which can overcome the short residual activity will allow its future utilization in a variety of habitats and conditions. Development of effective formulations suited to the biology and habitats of the target organisms is the basic requirement for the successful use of Bti. Encapsulation technology, including microencapsulation and bioencapsulation which is continuously undergoing improvement, will be utilized in the development of durable slow release formulations, resistant to detrimental environmental conditions with improved residual activity.

Success in meeting these challenges provides opportunities for controlling insect vectors of human and animal diseases and nuisance pests of public health importance.

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microsporidia is found in a different region of Bulgaria, suggestive of competitive exclusion.

Tuesday, 10:45

A new baculovirus from the mosquito *Culex nigripalpus*

James J. BECNEL, Susan White, Bettina Moser, Tokuo Fukuda and Margaret A. Johnson

Center for Medical, Agricultural and Veterinary Entomology. U. S. Department of Agriculture, Agricultural Research Service, Gainesville, Florida 32604

A new Dipteran baculovirus was isolated from the larval stages of the mosquito *Culex nigripalpus*. This virus infects and destroys the larval midgut causing death 72 - 96 hours post-exposure. Microscopic observations confirm the existence of occlusion bodies containing up to 8 singly enveloped nucleocapsids. This pathogen has high infectivity for larval mosquitoes with up to 60% of field populations naturally infected. Laboratory bioassay has verified that the presence of an activator is essential for transmission and the presence of low concentrations of an inhibitor prevents transmission. These findings represent a significant breakthrough on the mechanisms of transmission of mosquito baculoviruses and can explain, in part, the occurrence or absence of epizootics in natural populations of mosquitoes.

Friday, 11:00, SYMPOSIUM

Overview of Microsporidia in Insects

James J. BECNEL

Center for Medical, Agricultural and Veterinary Entomology. U. S. Department of Agriculture, Agricultural Research Service, Gainesville, Florida 32604

The microsporidia are a large and ubiquitous group of obligate, intracellular parasites that infect nearly all major animal groups from protozoa to man but are especially common in arthropods. Currently, there are 142 genera of microsporidia with 69 of these having insects as type hosts. Approximately 600 of the 1000 named species of microsporidia are reported from insects. Most of these species have been investigated to exploit them as manipulated biological control agents.

Microsporidia are transmitted by spores that are usually small (1-15 μ), possess a spore wall and contain a coiled polar filament (tube). The spore, when stimulated, everts the polar filament and the germ cell travels through the hollow tube and is inoculated into the target cell. Transmission is either horizontal (usually via the gut) or vertical (from parent to progeny). The life cycles of entomogenous microsporidia range from relatively simple to the extremely complex. Microsporidia described as having a simple life cycle are usually characterized by only one sporulation sequence that involves only one host individual. Microsporidia with complex life cycles usually have multiple sporulation sequences that involve more than one generation of the host and sometimes an intermediate host. Examples of various microsporidian life cycles will be presented with particular emphasis on microsporidia in fire ants.

Tuesday, POSTER BP13

SCREENING BY PCR FOR DETECTION OF *cry9* GENES FROM *Bacillus thuringiensis*

BEN-DOV, E.,[1] Wang, Q.,[1] Zaritsky, A.,[1] Manasherob, R.,[1] Barak, Z.,[1] Schneider, B.,[1] Khamraev, A.,[2] Baizhanov, M.,[3] Glupov, V.,[4] and Margalith, Y.[1]

Department of Life Sciences, Ben-Gurion University of the Negev, Be'er-Sheva 84105, Israel[1]; Institute of Zoology, Uzbek Academy of Science, Tashkent, Uzbekistan[2]; Institute of Zoology, Kazakhstan Academy of Science, Almaty, Kazakhstan[3]; and Institute of Systematic and Animal Ecology, Siberian Branch of Academic Science Novosibirsk, Russia[4]

Insecticidal *cry9* genes are promising tools for effective control and resistance management of many agronomically important lepidopteran species. For example, expression of *cry9Ca* protects transgenic corn against the European corn borer (*Ostrina nubilalis*). Cry9Ca is significantly more toxic to budworm (*Choristoneura fumiferana*) than the currently used Cry1A-F toxins, and displays high toxicity against *Plutella xylostella*, *Spodoptera exigua*, *S. littoralis*, *Heliothis virescens*, *Agrotis segetum* and *Bombyx mori*. Another toxin belonging to the same group is Cry9Aa, the major crystal component of *B. thuringiensis* subsp. *galleriae*, exhibiting unique toxicity toward *Galleria mellonella* larvae. The cryptic gene *cry9Ba* was found to be localized upstream of *cry9Aa*. The fourth protein in this group, Cry9Da, toxic to scarabaeid larvae of the order Coleoptera, was found in *B. thuringiensis* subsp. *japonensis*. Cry9E, the toxicity of which is still unknown, has very recently been discovered in *B. thuringiensis* subsp. *aizawai* SSK-10.

We developed a new set of universal and specific primers for multiplex rapid screening of *B. thuringiensis* strains that harbor all five known genes from the *cry9* group. Universal primers (Uns) were selected from a highly conserved sequence whereas five specific primers were selected from highly variable regions in the respective genes, each producing a unique-size PCR product. PCR analyses were performed for *cry9* genes on 16 standard strains and 215 field-isolates of *B. thuringiensis*. Among the standard strains, only subsp. *aizawai* HD-133, which harbors *cry1* and *cry2*, was positive to Un9, but negative to all five specific primers for the *cry9* genes. 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.

Thursday, POSTER BP25

Ingested particles protect *Aedes aegypti* larvae from *Bacillus thuringiensis* subsps. *israelensis* toxicity

BEN-DOV, E., Wang, Q.-F., Saxena, D., Manasherob, R., Boussiba, S., and Zaritsky, A.

Department of Life Sciences and Microalgal Biotechnology Laboratory, Ben-Gurion University of the Negev, POB 653, Be'er-Sheva 84105, Israel, and
The Research Institute, The College of Judea and Samaria, Ariel 44837, Israel

Toxicity of various *Bacillus thuringiensis* (Bt) subspecies is affected by variations in food composition and consumption. Starved larvae of susceptible species are more sensitive to Bt than fed larvae, probably due to dilution of the pathogen and feeding inhibition in the presence of particles. We propose that ingested particles protect midgut epithelial cells by covering their surface thus preventing approach of the toxin to gut receptors. To test this hypothesis, toxicity of Bt subsp. *israelensis* (Bti) was determined before, during and following feeding of *Aedes aegypti* larvae with nutritious (Pharmamedia) and inert (coal and carmine dye) particles.

Program and Abstracts

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P35. CONTROLLED DELIVERY OF BIORATIONAL PESTICIDES FROM MATRICAP™ COMPOSITIONS: PEST-MANAGEMENT APPLICATIONS. R. Levy, M. A. Nichols, and W. R. Opp, Lee County Mosquito Control District, P. O. Box 60005, Ft. Myers, FL 33906 USA.

An encapsulation system was developed for controlled delivery of bioactive agents from solid carrier matrices. The efficacy of several encapsulation techniques and polymer or nonpolymer coating-complex formulations in regulating the controlled-delivery duration and profile of the biopesticides *Bacillus thuringiensis* var. *israelensis* or *Bacillus sphaericus*, or the insect growth regulators (IGRs) methoprene or pyriproxyfen from Biodac® or corn cob granules was evaluated against larvae of the mosquitoes *Aedes taeniorhynchus*, *Anopheles albimanus*, or *Culex quinquefasciatus*. Bioassays against mosquito larvae in a variety of water qualities suggested that the controlled-delivery compositions could be used to direct the biopesticides or IGRs to specific surface and/or subsurface areas of a water column from submerged granules to target the feeding zones and/or orientation patterns of each type of mosquito for prolonged periods. Preliminary ground and aerial field trials were also conducted to test the validity of the bioassays. The data suggested that the controlled-release profiles for each bioactive agent encapsulated on Biodac® or corn cob granules were directly related to the type, concentration, specific gravity, solubility, rate of hydrolysis, and rate of biodegradation of each coating utilized in a coating complex, as well as to habitat water quality and the concentration of a bioactive agent encapsulated on a granular matrix. Matricap™ technology was also applicable to terrestrial pest control. Polymer-base bait stations formulated with the IGR pyriproxyfen were developed for prolonged control of the German cockroach *Blattella germanica*. Bioassays indicated that the solid bait stations were effective against multiple populations of German cockroach nymphs for at least one year.

P36. NEW *cry7* AND *cry8* GENES FROM *Bacillus thuringiensis*. Eitan Ben-Dov, Arieh Zaritsky, Ze'ev Barak, Robert Manasherob, and Yoel Margalith, Life Sciences Department, Ben-Gurion University of the Negev, P. O. Box 653, Be'er-Sheva 84105 ISRAEL.

An enhanced strategy using PCR for extended multiplex rapid screening of *B. thuringiensis* strains with genes from seven *cry* groups will enrich the existing arsenal of insecticidal strains, identify novel genes or new combinations of known genes and predict their toxicities. Universal primers (Uns) were selected from a highly conserved sequence in 34 genes of *cry-1*, *-2*, *-3*, *-4*, *-7* and *-8*. DNA of each strain which reacted with at least one pair of Uns was identified with specific primers for 20 of these genes and 1 *cry11*, which produce each a unique-size PCR product. DNAs of subsps. *aizawai* HD-133, *indiana* HD-521 and *tochigiensis* HD-868, which reacted with Un7,8, were negative with all specific primers for known *cry7* and *-8*. Thirty-five field-collected strains with other *cry* genes were positive to Un7,8 but not identified by any specific primer of *cry7* and *-8*; they are promising for biological control of insects and resistance management. Sequences of the PCR products (423 bases) obtained with Un7,8 from subsps. *indiana* HD-521 and *tochigiensis* HD-868 show about 80 and 90% homology to *cry7A*, respectively, and 67% to all three *cry8* genes. The 80% homology between themselves confirms that both are novel. Similarly, the field-collected strain R1 contains a novel gene: the homology to *cry8B* of its PCR (423 bp) product with Un7,8 was 98.8%, but it did not react with the specific primer to *cry8B* at 54°C annealing. This specific fragment was however amplified under less stringent conditions (43°C). SDS-polyacrylamide gel electrophoresis of sporulating culture of R1 yielded two large polypeptides, one of about 140 kDa and another of about 100 kDa, one of which may be the new postulated gene.

P37. APPLICATION OF *BACILLUS SPHAERICUS* REDUCES RISK OF FILARIASIS TRANSMISSION IN INDIA. A. P. Daash, Deputy Director and Head, Division of Medical Entomology and Parasitology, Regional Medical Research Centre, Chandrasekharpur Bhubaneswar 751 016, Orissa, INDIA.

The Orissa State of India has been an endemic home for lymphatic filariasis. Lymphatic filariasis due to *Wuchereria bancrofti* Cobbold accounts for 90% of the infection and is transmitted by the ubiquitous mosquito *Culex quinquefasciatus* Say. Vector Control continues to be an important tool in curtailing the transmission of lymphatic filariasis. *Bacillus sphaericus* is increasingly tried against *Cx. quinquefasciatus*, the vector of bancroftian filariasis. The advantage of this biocide is its residual activity over other larvicides used in the control operations. A large scale field evaluation of *B. sphaericus* against filariasis transmission in Khurdha (Orissa, India) area with 40,000 population was undertaken and the results were compared with an identical isolated comparison areas with 35,000 population. Application (5 g/m^2) twice a year in May and October significantly reduced the vector densities and immediately brought down the transmission to zero level. The risk of infection index and annual transmission index was reduced to zero after one year of intervention and to 0.2 and 234, respectively, after second year of intervention. The biocide also drastically reduced the transmission parameters like the biting infection and infective rates. Exploratory analysis revealed some interesting patterns in the data. Mosquito density could be predicted with high accuracy based on environmental information. The same was true of biting rate, and to a lesser extent, infectivity rate.

P38. ZOONOSES AND ENVIRONMENT. E. I. Korenberg, Gamaleya Institute for Epidemiology and Microbiology, Academy of Medical Sciences, Gamaleya Str. 18, Moscow, RUSSIA.

Most zoonoses, particularly those with natural focality, spread depending on the structure and functioning of ecosystems to which their pathogens belong as normal biocenotic components. Natural foci of zoonoses and their epidemic manifestation are under the cumulative effect of at least three processes that develop in parallel and are difficult to distinguish. These are urbanization, economic development of nonurbanized territories, and, as a consequence, a change in the frequency of human contact with pathogens. A basic scheme proposed to model the epidemiological situation for zoonoses in relation to changes in the environment is illustrated by the example of the dynamics of Lyme borreliosis and tick-borne encephalitis morbidity in Russia. The conditions develop that promote the formation of new pathways of pathogen transmission and human infection.

XIth European SOVE Meeting

Unidade de Entomologia Médica - Instituto de Higiene e Medicina Tropical (UNL), Lisboa, Portugal, 13 -17 October 1998.

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MULTIPLEX PCR SCREENING FOR DETECTION *cry9* GENES FROM *Bacillus thuringiensis*

Ben-Dov, E., Wang, Q., Zaritsky, A., Manasherob, R., Barak, Z. & Margalith, Y.

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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. We developed a new set of universal and specific primers for multiplex rapid screening of *B. thuringiensis* strains that harbor all four known genes from *cry9* group. Universal primers (Uns) were selected from a highly conserved sequence of *cry9* whereas four specific primers (EB) were selected from highly variable regions in the respective genes which each produce a unique-size PCR product. PCR analysis for *cry9* genes, never previously reported, was performed on 16 standard *B. thuringiensis* strains and among 215 field-isolated *B. thuringiensis* strains, where the DNA of 22 field-collected strains were found as positive to Un9 primers. We found three different *cry9* gene profiles which contain in addition several other *cry1* and *cry2* genes;

i. Fifteen independent isolates were positive to *cry9A* and *cry9B*.
ii. Two field-collected strains were positive to *cry9D*.
iii Five field-collected were positive with Un9 but not identified by any specific primer of our four *cry9*.

None of our field-collected strains was positive to *cry9C*. All of our standards were negative to *cry9* genes, except one standard *B. thuringiensis aizawai* HD-133 strain which was positive with universal primers only.

The multiplex PCR screening for detection *cry9* genes enriches existing PCR strategies for screening most currently known *cry* genes by developing expanded PCR sets of universal and specific primers.