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Protection of Post Harvest Grains by New Isolates of Spore-Forming Bacteria

PI: Yoel Margalith Ph.D.
Center for Biological Control (CBC)
Department of Life Sciences
Ben-Gurion University of the Negev
Israel

Collaborators: Allovuddin Khameraev Ph.D.
Institute of Zoology
Uzbek Academy of Science
Uzbekistan

Anatolij M. Dubitskij Ph.D.
Institute of Zoology
Kazakhstan Academy of Science
Kazakhstan

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Protection of Post Harvest Grains by New Isolates of Spore-Forming Bacteria

3. EXECUTIVE SUMMARY

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

Several hundred field-collected samples of spore forming bacteria were isolated in Israel, Uzbekistan and Kazakhstan. Distribution of potential insecticidal activity based on genes encoding the specific protein toxins of *Bacillus thuringiensis (B.t.)* insect pathogens is described. These genes are designated cry (crystal) genes. A total of 126 cry positive *B.t.* genes were identified, 24.6% potentially active against Lepidoptera and Diptera; 9.5% Lepidoptera; 23.8% Lepidoptera and Coleoptera; 2.4% Coleoptera and 35.7% Diptera. In addition 4.0% of the isolates contain genes which are potentially active against Diptera and Coleoptera (Fig. 2). Some of these isolates represent a promising potential for new biological control agents effective against Lepidotera, Coleoptera and Diptera.

Twenty two distinct *cry*-type profiles were identified from 126 field-collected *B. thuringiensis* strains. Several of them were found to be different from all published profiles. Some of the field-collected strains but none of the 16 standard strains were positive to *cry2Ac*. 38 field-collected strains were positive with universal but negative with specific primers for all five known genes of *cry7* and *cry8*. These field-collected strains seem to contain new gene(s) promising for biological control of insects and resistance management.

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

Collaboration among the participating scientists was achieved by exchange of visits in each other's laboratories. In these mutual visits field collected entomopathogens were exchanged, training was conducted and uniform working procedure and protocol were compared and designed. During the past three years 6 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 was advanced through the equipment purchased and training conducted during the course of this study.

4. RESEARCH OBJECTIVES

The ultimate aim of this project is to protect crops from harvest to the time it reaches the consumer. To this end the objective for the first year was to develop fast screening methods for the **isolation** of field-collected pathogens. The objectives of the second year were to design **universal primers** for each gene class and identify general gene profiles using these primers.

The objectives of the third year were to design **specific primers**, identify the specific profile of *cry* genes in the general classes of *cry1, cry2, cry3, cry4, cry7, cry8* and *cry11*, and develop methodology based on PCR techniques, for predicting insecticidal activity of new isolates needed to substitute the toxic chemicals and facilitate *B.t.* toxin resistance management .

5. METHODS AND RESULTS

A. Methods

a. Isolation of microbial pathogens.

We utilized the fast screening methods for the isolation of microbial pathogens from the field collected soil samples, dead insects, fragmented insect litter in grain storages and from other such environs. These methods were described by Brownbridge and Margalith (1986, 1987) and Travers (1987). Cultures were checked for the presence of crystals to confirm the isolation of *B.t.* and later prepared for PCR analysis for the specific cry gene identification.

b. Gene identification by PCR.

To identify the *cry* genes PCR analysis was used. One pair of universal primers (Uns) for each of the five *cry* homology groups (Crickmore *et al,* 1995) was designed to amplify a specific fragment by simultaneous alignment to all previously described genes in that group using Amplify 1.0 program (Bill Engels, University of Wisconsin, USA). 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.

To identify *cry1* (Kalman *et al,* 1993), *cry2, cry3, cry4, cry7* and *cry8*, a single Un and several specific primers for each *cry* class (selected from their highly variable regions) were used together in one reaction. Two specific primers were designed for *cryIAb* (Kalman *et al,* 1993) and *cry11A*. 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%).

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 mM 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.

Reliability of the primers was verified using the following *B. thuringiensis* reference strains: *kurstaki* HD-1, *aizawai* HD-133, *galleriae* HD-155, *kenyae* HDB-23, *tolworthi* HDB-8 and HD-2 for *cry1* and *cry2* classes, *tenebrionis*, NRRL B-18533, NRRL B-18655 for *cry3* class, *israelensis* ONR60A for *cry4* and *cry11* clases and *dakota* HD-511, *tochigiensis* HD-868, *indiana* HD-521 and *kumamotoensis* HD-867 for *cry7* and *cry8* clases (see RESULTS, Table 3).

TABLE 1. Characteristics of universal primers for *cry1*, *cry2*, *cry3*, *cry4*, *cry7* and *cry8* group genes.

Gene nomenclature			Nucleotide positions	Mismatch of primers[c]	Product
Current	Original	Access. #[a]	hybridized to primers[b]		size (bp)
Sequences of *cry1* universal primers: Un1(d)[d]: 5'CATGATTCATGCGGCAGATAAAC-3' Un1(r)[d]: 5'-TTGTGACACTTCTGCTTCCCATT-3'					
cry1Aa5	*cryIA(a)*	D17518	2858-2880, 3112-3134	0(d); 0(r)	277
cry1Ab9	*cryIA(b)*	X54939	2775-2797, 3029-3051	1(d); 0(r)	277
cry1Ac5	*cryIA(c)*	M73248	2781-2803, 3035-3057	0(d); 0(r)	277
cry1Ad	*cryIA(d)*	M73250	2778-2800, 3032-3054	1(d); 10(r)	277
cry1Ae	*cryIA(e)*	M65252	2861-2883, 3115-3137	11(d); 0(r)	277
cry1Ba	*cryIB*	X06711	2919-2941, 3170-3192	0(d); 0(r)	274
cry1Bb	*cryI ET5*	L32020	3000-3022, 3251-3273	0(d); 0(r)	274
cry1Ca1	*cryIC*	X07518	2851-2873, 3105-3127	1(d); 16(r)	277
cry1Cb	*cryIC(b)*	M97880	3061-3083, 3315-3337	1(d); 4, 10(r)	277
cry1Da	*cryID*	X54160	2996-3018, 3250-3272	1(d); 16(r)	277
cry1Db	*prtB*	Z22511	2973-2995, 3227-3249	0(d); 0(r)	277
cry1Ea3	*cryIE*	M73252	2760-2782, 3014-3036	1(d); 0(r)	277
cry1Eb	*cryIE(b)*	M73253	2754-2776, 3008-3030	1, 21(d); 0(r)	277
cry1Fa2	*cryIF*	M73254	2760-2782, 3014-3036	0(d); 0(r)	277
cry1Fb	*prtD*	Z22512	3239-3261, 3493-3515	1(d); 0(r)	277
cry1G	*prtA*	Z22510	2802-2824, 3056-3078	1(d); 0(r)	277
cry1H	*prtC*	Z22513	3283-3305, 3537-3559	0(d); 0(r)	277
cry1Hb	-	U35780	3472-3494, 3726-3748	0(d); 7(r)	277
cry1Ja	*cryI ET4*	L32019	2840-2862, 3091-3113	1(d); 7, 10, 15(r)	274
cry1K	-	U28801	3318-3340, 3569-3591	1, 4, 7, 12(d); 0(r)	274
Sequences of *cry2* universal primers: Un2(d): 5'-GTTATTCTTAATGCAGATGAATGGG-3' Un2(r) 5'-CGGATAAAATAATCTGGGAAATAGT-3'					
cry2Aa1	*cryIIA*	M31738	726-750, 1402-1426	0(d); 0(r)	701
cry2Ab2	*cryIIB*	X55416	1444-1468, 2120-2144	9(d); 3(r)	701
cry2Ac	*cryIIC*	X57252	2695-2719, 3359-3383	3, 6(d); 22(r)	689
Sequences of *cry3* universal primers: Un3(d) 5'-CGTTATCGCAGAGAGATGACATTAAC-3' Un3(r) 5'-CATCTGTTGTTTCTGGAGGCAAT-3'					
cry3A6	*cryIIIA*	U10985	1367-1392, 1933-1955	0(d); 0(r)	589
cry3Ba1	*cryIIIB*	X17123	826-851, 1398-1420	5, 15(d); 17, 20(r)	595
cry3Bb1	*cryIIIBb*	M89794	1003-1028, 1575-1597	5, 15, 21(d); 17, 20(r)	595
cry3C	*cryIIID*	X59797	1024-1049, 1605-1627	0(d); 12, 13(r)	604
Sequences of *cry4* universal primers: Un4(d) 5'- GCATATGATGTAGCGAAACAAGCC-3' Un4(r) 5'- GCGTGACATACCCATTTCCAGGTCC-3'					
cry4A2	*cryIVA*	D00248	3324-3347, 3738-3762	0(d); 0(r)	439
cry4B4	*cryIVB*	D00247	3259-3282, 3673-3697	0(d); 0(r)	439
Sequences of *cry7* and *cry8* universal primers: Un7,8(d): 5'-AAGCAGTGAATGCCTTGTTTAC-3' Un7,8(r): 5'-CTTCTAAACCTTGACTACTT-3'					
cry7Aa	*cryIIIC*	M64478	2135-2156, 2535-2554	5, 12(d); 9, 11(r)	420
cry7Ab1	*cryIIICb*	U04367	1952-1973, 2352-2371	5, 12(d); 9, 11(r)	420
cry8A	*cryIIIE*	U04364	2027-2048, 2430-2449	0(d); 1, 8(r)	423
cry8B	*cryIIIG*	U04365	2015-2036, 2418-2437	0(d); 1, 8(r)	423
cry8C	*cryIIIF*	U04366	2018-2039, 2421-2440	5, 13(d); 1, 2, 9(r)	423

[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.

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TABLE 2. Characteristics of specific primers for *cry2, cry3, cry4, cry7* and *cry8* and *cry11* genes.

Primer pair[a]	Sequence of primers[b]	Gene recognized	Positions[c]	Product size (bp)
Un2(d)	GTTATTCTTAATGCAGATGAATGGG	*cry2Aa1*	726-750	498
EE-2Aa(r)	GAGATTAGTCGCCCCTATGAG		1203-1223	
Un2(d)	GTTATTCTtAATGCAGATGAATGGG	*cry2Ab2*	1444-1468	546
EE-2Ab(r)	TGGCGTTAACAATGGGGGGAGAAAT		1965-1989	
Un2(d)	GTtATtCTTAATGCAGATGAATGGG	*cry2Ac*	2695-2719	725
EE-2Ac(r)	GCGTTGCTAATAGTCCCAACAACA		3396-3419	
Un3(d)	CGTTATCGCAGAGAGATGACATTAAC	*cry3A6*	1367-1392	951
EE-3Aa(r)	TGGTGCCCCGTCTAAACTGAGTGT		2294-2317	
Un3(d)	CGTTaTCGCAGAGAgATGACATTAAC	*cry3Ba1*	826-851	1103
EE-3Ba(r)	ACGAAAGATTCTGCTCCTAT		1909-1928	
Un3(d)	CGTTaTCGCAGAGAgATGACaTTAAC	*cry3Bb1*	1003-1028	1103
EE-3Ba(r)	ACGAAAGATTCTGCTCCTAT		2086-2105	
Un3(d)	CGTTATCGCAGAGAGATGACATTAAC	*cry3C*	1024-1049	461
EE-3C(r)	ATTTTGGTACCTCCTGTACCCACC		1461-1484	
EE-4A(d)	GGGTATGGCACTCAACCCCACTT	*cry4A2*	2234-2256	1529
Un4(r)	GCGTGACATACCCATTTCCAGGTCC		3738-3762	
EE-4B(d)	GAGAACACACCTAATCAACCAACT	*cry4B4*	1747-1770	1951
Un4(r)	GCGTGACATACCCATTTCCAGGTCC		3673-3697	
EE-7Aa(d)	GCGGAGTATTACAATAGAATCTATCC	*cry7Aa*	1639-1664	916
Un7,8(r)	CTTCTAAAcCtTGACTACTT		2535-2554	
EE-7Aa(d)	GCGGAGTATTACAATAGAATCTATCC	*cry7Ab1*	1456-1481	916
Un7,8(r)	CTTCTAAAcCtTGACTACTT		2352-2371	
EE-8A(d)	GAATTTACTCTATACCTTGGCGAC	*cry8A*	1771-1794	679
Un7,8(r)	cTTCTAAaCCTTGACTACTT		2430-2449	
EE-8B(d)	GACCGCATCGGAAGTTGTGAG	*cry8B*	1663-1683	775
Un7,8(r)	cTTCTAAaCCTTGACTACTT		2418-2437	
EE-8C(d)	GGTGCTGCTAACCTTTATATTGATAG	*cry8C*	1930-1955	511
Un7,8(r)	ctTCTAAAcCTTGACTACTT		2421-2440	
EE-11A(d)	CCGAACCTACTATTGCGCCA	*cry11A1*	111-130	445
EE-11A(r)	CTCCCTGCTAGGATTCCGTC		536-555	

[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.

B. Results and Discussion

Identification by universal primers. Five pairs (Direct and Reverse) of Uns (Table 1) were designed to detect genes by their following PCR products: Un1 for 20 *cry1* genes with products of 274 or 277 bp; Un2 for 3 *cry2* genes with products of 689 or 701 bp; Un3 for 4 *cry3* genes with products' size range between 589-604 bp; Un4 for 2 *cry4* genes with size 439 bp; Un7,8 for 2 *cry7* and 3 *cry8* genes with products of 420 and 423 bp, respectively.

DNA of each *B. thuringiensis* isolate served as template in three reactions (Figure 1):

(i) with a mixture of two pairs, of Un1 and Un4 (to detect genes from *cry1* and *cry4* groups);

(ii) with a mixture of two other pairs, of Un2 and Un3 (to detect genes from *cry2* and *cry3* groups);

(iii) with Un7,8 (to detect genes from *cry7* and *cry8* groups).

Such an extended PCR analysis for *cry*-type genes, never previously reported, was performed on 16 standard *B. thuringiensis* strains with universal (as well as specific; see below) primers; results are summarized in Table 3. Indeed, some of these well-known strains contain additional *cry*-type genes. For example, subsp. *aizawai* HD-133 reacted positively to Un7,8 by producing a fragment of 420 bp. Two subsps., *kyusushensis* HD-541 and *japonensis* 4AT1, however, were not identified with our primers.

Among 215 field-isolated *B. thuringiensis* strains, the DNA of 89 did not amplify the Uns. The rest were grouped in 7 *cry*-type gene profiles, as presented in Fig. 2.

Identification of specific genes. The specific primers (labeled EE, except for *cry1*), designed to identify 21 genes from the 7 *cry*-groups, were selected from highly variable regions in the respective genes (Table 2). Our PCR analysis for the *cry1* group, based on the primers' design by Kalman *et al.*, was thus limited to identify 8 (*Aa, -Ab, -Ac, -B, -C, -D, -E, -F*) of the 20 known genes that are identified by Un1 (Table 1; Fig. 3). We found 9 different *cry1* gene profiles (numbers 3 to 16 in Table 4); an additional *cry1* profile (numbers 1 and 2) was not identified by any specific primer of *cry1*.

All except two standard *B. thuringiensis* strains containing *cry1* were found to also contain both *cry2Aa* and *cry2Ab*: subsp. *thuringiensis* HD-2 was negative to *cry2*, and *aizawai* HD-133 was positive to *cry2Ab* only (Table 3; Figure 4). None of our standard strains was positive to *cry2Ac*, but those field-

collected strains that were positive contained *cry2Ab* as well (profile number 16, Table 4; Figure 4).

None of our field-collected strains was positive to *cry3*. Three *B. thuringiensis* standard strains (subsps. *tenebrionis*, NRRL B-18533 and NRRL B-18655) were positive controls (Fig. 5, Table 3).

Three genes, *cry4A*, *cry4B* and *cry11A*, which code for Dipteran-specific polypeptides (15), were identified by a set of specific primers (Table 2; Fig. 6). This set yielded 3 different profiles, all included cry11A: one with *cry4A* (profile number 19), one with *cry4B* (number 20) and one with both (numbers 21/22).

Despite the positive reactions with Un7,8, DNAs of subsps. *aizawai* HD-133, *indiana* HD-521 and *tochigiensis* HD-868 were negative with all specific primers for genes of *cry7* and *cry8*; subsps. *dakota* HD-511 and *kumamotoansis* HD-867, on the other hand, were identified as containing *cry7A* (Table 3; Fig. 7). Thirty of the field strains with a combination of *cry1* and *cry2* (profiles number 14, 15 and 16; Table 4) and 5 with *cry4* and *cry11* (profile 22; Table 4) were positive to Un7,8 but not identified by any specific primer of *cry8* and *cry7*. Three strains (profile 18; Table 4) were positive only to Un7,8. Ten of these 38 isolates produced either one of two unexpected PCR products, of ~700 and ~300 bp, by two direct specific primers (EE-8B and EE-8C, of *cry8B* and *cry8C*, respectively; Table 2).The 126 field strains examined here displayed 22 distinct profiles (Table 4), reflecting different *cry* combinations, some of which are novel (profiles 9, 10, 14, 16, 17, 18 and 22).

TABLE 3. Distribution of *cry*-type gene profiles of *B. thuringiensis* strains as analysed by PCR[a]

B. thuringiensis strains	*cry1*	*cry2*	*cry3*	*cry4* and *11A*	*cry7* and *8*
aizawai HD-133	*Aa, Ab, Ca, Da*	*Ab*	-	-	+
galleriae HD-155	*Aa, Ab, Ba*	*Aa, Ab*	-	-	-
kenyae HDB-23	*Ab, Ac, Ea*	*Aa, Ab*	-	-	-
tolworthi HDB-8	*Ab*	*Aa, Ab*	-	-	-
thuringiensis HD-2	*Ab, Ba*	-	-	-	-
kurstaki HD-1	*Aa, Ab, Ac*	*Aa, Ab*	-	-	-
israelensis ONR60A	-	-	-	*A, B, 11A*	-
tenebrionis	-	-	*Aa*	-	-
NRRL B-18533	-	-	*Ba*	-	-
NRRL B-18655	-	-	*Ba*	-	-
dakota HD-511	-	-	-	-	*7Aa*
indiana HD-521	-	-	-	-	+
tochigiensis HD-868	-	-	-	-	+
kumamotoensis HD-867	-	-	-	-	*7Aa*
japonensis 4AT1	-	-	-	-	-
kyusushensis HD-541	-	-	-	-	-

[a] -, negative with universal primers; +, positive with universal primers.

***cry* profiles of PCR products from standard and field-collected strains**. Several hundred field-collected samples were isolated in Israel, Uzbekistan and Kazakhstan; of these, about 215 spore-forming *B. thuringiensis* isolates were analyzed by the first group of primers (Uns; Table 1; Figs. 1 and 2), and the positive 126 were identified by the second, specific primers (Tables 2 and 4; Figs. 3-7). The profiles of all were compared with those of 16 standard strains (Table 3; Figs. 3-7). Twenty two different profiles were observed (Table 4), 7 of which (including 36 independent isolates) were novel (profiles number 9, 10, 14, 16, 17, 18 and 22); these latter strains may be promising for new biological control agents. Twelve independent isolates (profiles number 1 and 2) were positive with Un1 but not identified by any specific primer of our 8 *cry1*. They may contain at least one of 12 other *cry1* genes (amplified by Un1; Table 1) or new *cry1* gene(s). Three additional profiles (number 19, 20 and 21, found in 45 isolates) contained genes of subsp. *israelensis*. The remaining 33 isolates were distributed in 10 different profiles (numbers 3, 4, 5, 6, 7, 8, 11, 12, 13 and 15), with various combinations of genes from groups *cry1*, *cry2*, and *cry7* or *cry8* (Table 4). When challenged with specific *cry1* primers only, they produced 6 different profiles which were identical to standard strains as follows (Table 3 and (1, 3, 8, 15, 16)): profile numbers 3 and 5 to subsp. *entomocidus*, profile numbers 4 and 7 to *alesti*, number 6 to *sotto*, 8 and 13 to *kurstaki*, 11 and 12 to *gallerie*, and 15 to *aizawai*.

New toxic specificities may stem from new combinations of known genes, such as in the strains displaying profiles 9 and 10. An interesting case in point is a *B. thuringiensis* strain YBT-226 isolated in China (Hodgman *et al.,* 1993), which produces Cry1Ab and Cry1Ba (toxic to Lepidoptera) as well as Cry2Aa (toxic to both, Lepidoptera and Diptera). This strain is highly toxic to the common housefly, *Musca domestica*, despite the fact that toxicity of Cry2Aa is low (Hodgman *et al.,* 1993).

To the best of our knowledge, PCR screening by primers to *cry2* genes has never been performed before. We found that all but one (subsp. *thuringiensis* HD-2) standard strains with at least one *cry1* gene (Table 3; Figure 4) also contained *cry2Ab*. All of these except one (subsp. *aizawai* HD-133) also contained *cry2Aa*. None of the 6 standard strains we screened responded to primers specific to *cry2Ac*. This gene was discovered in *B. thuringiensis* S_1) (Wu *et al.,* 1991), with toxicity against Lepidoptera. All 14 isolates containing *cry2Ac* displayed a single profile (number 16, Table 4; Figure 4), which was also positive to *cry2Ab*. The other 47 field strains with at least one *cry2* gene can be divided in two types (Table 4): 21 which contain *cry2Aa* and *cry2Ab* (displaying 6 different profiles) and 26 containing *cry2Ab* alone (displaying 7

TABLE 4. 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*	10	Lep.
2. *cry1* + *cry2Ab*	2	Lep.
3. *cry1Aa*	1	Lep.
4. *cry1Aa, -Ac*	1	Lep.
5. *cry1Aa,* + *cry2Aa, -Ab*	2	Lep. + Dip.
6. *cry1Aa, -Ab,* + *cry2Ab*	1	Lep.
7. *cry1Aa, -Ac,* + *cry2Aa, -Ab*	8	Lep. + Dip.
8. *cry1Aa, -Ab, -Ac,* + *cry2Aa, -Ab*	6	Lep. + Dip.
9. *cry1Aa, -Ab, -D* + *cry2Ab*	1	Lep.
10. *cry1Ab, -D* + *cry2Ab*	4	Lep.
11. *cry1Ab, -Ac, -D* + *cry2Ab*	2	Lep.
12. *cry1Ab, -Ac, -D* + *cry2Aa, -Ab*	1	Lep. + Dip.
13. *cry1Ac* + *cry2Aa, -Ab*	3	Lep. + Dip.
14. *cry1Aa, -C, -D* + *cry2Ab* + *cry7, cry8*	8	Lep. + Col.
15. *cry1Aa, -Ab, -C, -D* + *cry2Ab* + *cry7, cry8*	8	Lep. + Col.
16. *cry1Ab, -D* + *cry2Ab, -Ac* + *cry7, cry8*	14	Lep. + Col.
17. *cry2Aa, -Ab*	1	Lep. + Dip.
18. *cry7, cry8*	3	Col.
19. *cry4A, cry11A*	7	Dip.
20. *cry4B, cry11A*	2	Dip.
21. *cry4A, -B, cry11A*	36	Dip.
22. *cry4A, -B, cry11A* + *cry7, cry8*	5	Dip. + Col.

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

different profiles). *cry2Aa*, *cry2Ac* and the two combinations between them (with and without *cry2Ab*) were not found.

Several known strains which contain a *cry1* gene (toxic to Lepidoptera) have been found to also include a gene from either the *cry3* or the *cry7* group (toxic to Coleoptera); for example, subsp. *tolworthi* contains *cry1Ab, cry1E, cry2* and *cry3B* (1). According to Kuo and Chak 1996, *cry1*-containing subsps. *wuhanensis* HD-525 and *morrisoni* HD-12 contain apparent novel *cry7A*. Strain BTI109P of subsp. *kurstaki* contains *cry3C* (Lambert *et al.,* 1992). DNA from subsp. *aizawai* HD-133, known to contain four genes from the *cry1* group (Chak *et al.,* 1994) as well as *cry2Ab* (Table 3), was amplified with Un7,8 (Fig. 7) but not with all specific primers of these groups. This observation is consistent with that of Kuo and Chak 1996, reporting that the same strain was not identified as *cry7A*. Since Kuo and Chak 1996, did not test for any of the *cry8* group, it may imply that subsp. *aizawai* HD-133 contains a new gene of the *cry8* group.

B. thuringiensis subsps. *dakota* HD-511 and *kumamotoansis* HD-867 responded similarly to our specific primers for *cry7A* (Fig. 7). Kuo and Chak 1996, recently indeed reported that the same *kumamotoansis* strain and a close *dakota* strain (HD-932) yielded (by the PCR-RFLP method; (Kuo and Chak 1996,)) "typical" but not identical restriction patterns of *cry7A*; in their words "partial nucleotide sequence of the PCR products confirmed that this predicted *cry7*-type gene is novel".

Both *B. thuringiensis* subsps. *indiana* HD-521 and *tochigiensis* HD-868 responded positively to Un7,8, but not to any of our specific primers to genes of these groups (*cry7A*, *cry8A, cry8B* and *cry8C*). Consistently (Kuo and Chak 1996), the partial nucleotide sequence of the product from the same *indiana* strain was similar to that of the *cry7*-type gene but *tochigiensis* HD-868 was not identified. Indeed, nucleotide sequences of the PCR products (423 bases long, obtained with Un7,8) from these two subsps. show about 80 and 90% homology to *cry7A*, respectively, and 67% to all three *cry8* genes (to be extensively described elsewhere); in addition, they are about 80% homologous between themselves, thus confirming that they are two novel genes. The sequence of the same 423-bases PCR-product obtained with a field strain (R1 of profile number 18; Table 4), positive to Un7,8 only, was found to be 67% homologous to *cry7A* and 98.8%—to *cry8B*. This gene must be novel (despite this high homology) because it did not react with the specific primer to *cry8B* (which was designed to anneal to the variable region; Table 2). SDS-polyacrylamide gel electrophoresis of sporulating culture of strain R1 yielded two large polypeptides, one of about 140 kDa (typical size of a Cry7 and Cry8 polypeptides) and one of 100 kDa, which may be the new postulated gene (unpublished data).

Thirty of our field strains (exhibiting profiles number 14, 15 and 16; Table 4) were positive with Un1, Un2 and Un7,8 but were not identified with specific primers to genes from either *cry7* or *cry8* group. The 8 isolates with profile number 15 are identical (by our criteria) to *B. thuringiensis* subsp. *aizawai* HD-133. The remaining 22 isolates (with profiles number 14 and 16) seem to contain new *cry* gene(s), thus have the potential to be effective biopesticides of insects for which biological control agents do not exist.

Five new isolates (displaying profile number 22), which contain the 3 genes of subsp. *israelensis* (*cry4A, -B*, *cry11A*; toxic to Diptera), seem to contain new *cry7* or *cry8* gene(s) (were positive with Uns7,8), and may thus be useful as new biological control agents for larvae of both Coleoptera and Diptera. A single subsp. (*morrisoni*, serotype 8a 8b; (Aronson 1994)) is known to contain these Dipteran-toxic genes in combination with *cry3A* (toxic to Coleoptera). It is interesting to note that all 48 isolates which were positive with Un4 (contain *cry4A* or *cry4B* (or both)) were consistently positive with the specific primers to *cry11A* (profiles number 19-22; Table 4).

The absence of PCR products when DNA of 89 *B. thuringiensis* isolates was challenged with all our universal primers does not necessarily imply that these strains are devoid of genes coding for anti-insecticidal polypeptides. Novel toxins may be recognized among these isolates by characterizing the proteins detected on gels or microscopically by the shape of their crystals.

The extended multiplex PCR screening is a rapid method for detecting and differentiating (by their PCR product profiles) *B. thuringiensis* field strains and for predicting their insecticidal activities thus to direct them for subsequent toxicity assays. Our isolates displaying new profiles (containing apparent new genes) should be characterized and further developed for integration with other control measures.

B

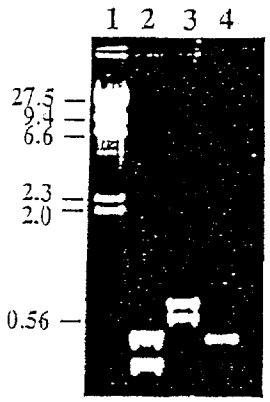

Figure 1. Agarose gel (1.2%) electrophoresis of PCR products amplified from standard strains of *B. thuringiensis* with universal primers. Lane 1, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lanes 2-4, PCR products obtained from: lane 2, mixed DNA of subsps. *israelensis* and *kurstaki* HD1 with Un1 and Un4; lane 3, mixed DNA of subsps. *kurstaki* HD1 and *tenebrionis* with Un2 and Un3; lane 4, DNA of subsp. *dakota* HD-511 with Un7,8. Expected sizes of products indicated in Table 1.

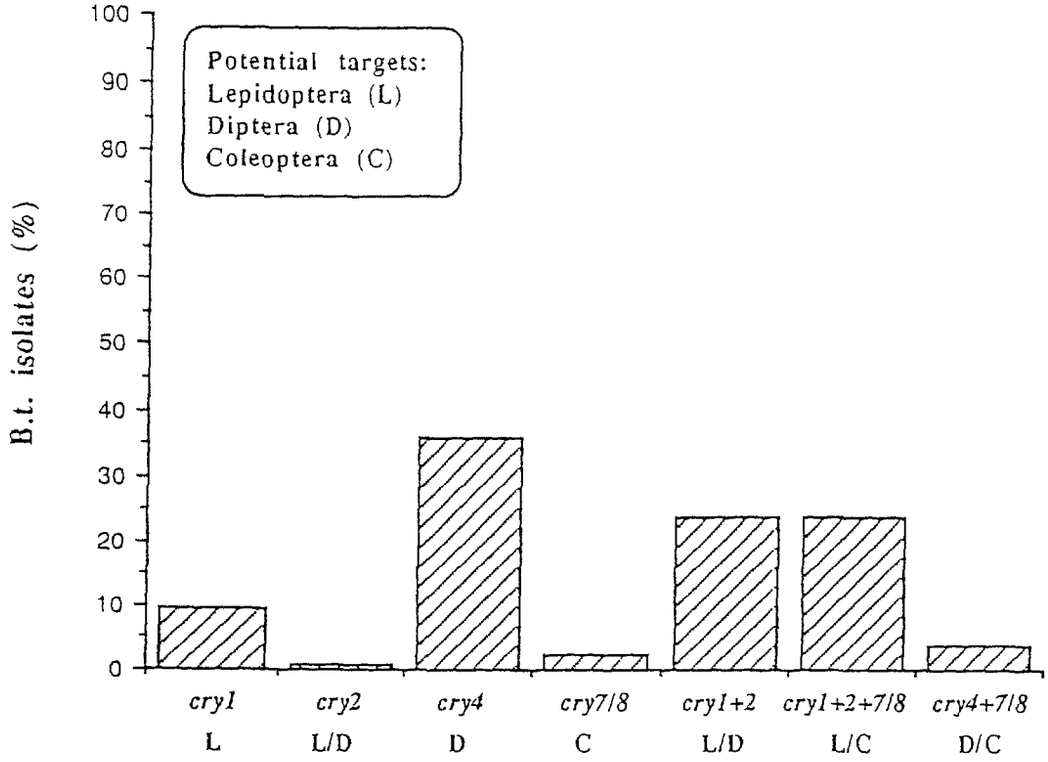

Figure 2. Distribution of *cry*-type gene profiles from 126 field-collected strains of *B. thuringiensis* identified by universal primers.

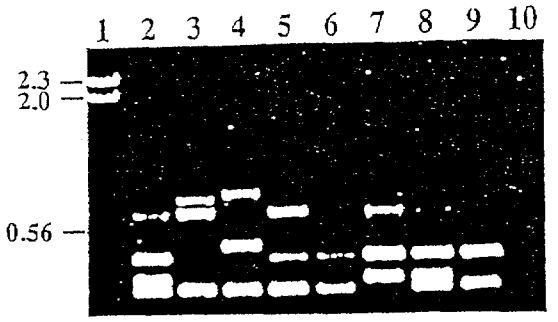

Figure 3. Agarose gel (1.2%) electrophoresis of PCR products obtained with specific primers for *cry1* genes. Lane 1, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lanes 2-4, DNA of *B. thuringiensis* subsp. *aizawai* HD-133 (lane 2), *galleriae* HD-155 (lane 3), *kenyae* HDB-23 (lane 4); lanes 5-9, DNA of field-collected strain U-12 (with profile number 9; lane 5), strain U-25 (profile 10; lane 6), strain U-35 (profile 14; lane 7), strain U-38 (profile 15; lane 8), strain U-27 (profile 16; lane 9); lane 10, negative control of PCR reaction (without template). Expected sizes of products indicated in Table 2.

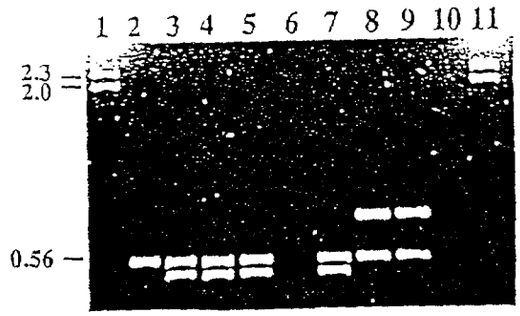

Figure 4. Agarose gel (2.5%) electrophoresis of PCR products obtained with specific primers for *cry2Aa, -Ab, -Ac* genes. Lanes 1 and 11, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lane 2-7, DNA of *B. thuringiensis* subsp. *aizawai* HD-133 (lane 2), *galleriae* HD-155 (lane 3), *kenyae* HDB-23 (lane 4), *tolworthi* HDB-8 (lane 5), *thuringiensis* HD-2 (lane 6), *kurstaki* HD-1 (lane 7); DNA of field-collected strains U-21 (lane 8) and U-27 (lane 9) (both with profile number 16); lane 10, negative control without template. Expected sizes of products indicated in Table 2.

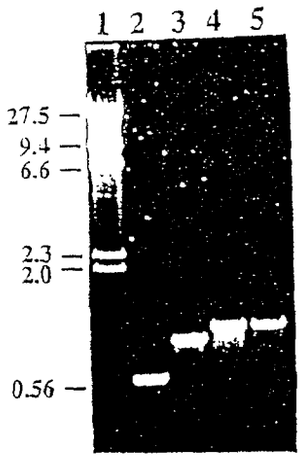

Figure 5. Agarose gel (1%) electrophoresis of PCR products obtained with primers for *cry3* genes. Lane 1, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lane 2, DNA of *B. thuringiensis* subsp. *tenebrionis* amplified with Un3; lanes 3-5, DNA of *B. thuringiensis* subsp. *tenebrionis* (lane 3), NRRL B-18533 (containing *cry3Bb1*; lane 4) and NRRL B-18655 (containing *cry3Bb2*; lane 5) amplified with specific primers. Expected sizes of products indicated in Tables 1 and 2.

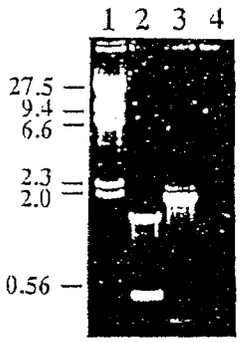

Figure 6. Agarose gel (1%) electrophoresis of PCR products from *B. thuringiensis* subsp. *israelensis*. Lane 1, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lanes 2 and 3, obtained with specific primers for *cry4A* plus *cry11A*, and for *cry4B*, respectively.

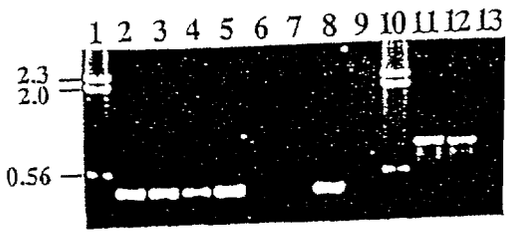

Figure 7. Agarose gel (1%) electrophoresis of PCR products obtained with universal and specific primers for *cry7* and *cry8* genes. Lanes 1 and 10, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lanes 2-8, respectively, DNA of *B. thuringiensis* subsp. *dakota* HD-511, *indiana* HD-521, *tochigiensis* HD-868, *kumamotoensis* HD-867, *japonensis* 4AT1, *kyusushensis* HD-541 and *aizawai* HD-133, amplified with Un7,8; lane 11-12, DNA of *B. thuringiensis* subsp. *dakota* HD-511 and *kumamotoensis* HD-867, with specific primers; lanes 9 and 13, negative controls without template, with Un7,8 and with a mixture of specific primers for *cry7* and *cry8* genes, respectively.

6. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

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

7. PROJECT ACTIVITIES/OUTPUTS

1) Collaboration activities are based on initial planning at the outset of the project and parallel and complimentary work of all the parties during the past three years. Isolation work of field collected material was carried out by all three participating laboratories. The studies in molecular biology took place in the Center for Biological Control under the direction of the PI. PCR analysis has been conducted by the post doctoral student Dr. Eitan Ben-Dov and the graduate student Edith Dahan. The isolation work was carried out by the graduate student Roza Sinai. Professors Zaritsky and Barak provided their generous advice. During their visit to CBC the collaborating scientists received target pest insects and protocols for rearing them as practiced in CBC-BGU.

2) The following travel and training took place during the second year of this project: 3 scientists: Drs. Khamraev, Azimov and Troyetskaya from Uzbekistan and 3 from Kazakhstan: Drs. Dubitsky, Berezina and Auezova, stayed for three weeks in April 1994 at the CBC-BGU. Each team consisted of entomologists and microbiologists. The training consisted of actual work and processing of the field collected material in CAR and in Israel as well as of field trips to storage facilities of food products in Beduin settlements and kibbutzim. During the course of this project numerous graduate and under-graduate students of BGU were trained at the Laboratory for Biological Control.

In September 1995 the PI stayed for 10 days in Almati conducting field trips, collecting material as well as working in the laboratory. The same activity took place for one week in Uzbekistan following the trip to Kazakhstan.

On the same trip in September 1995 the PI visited three microbiology laboratories of the Russian Academy of Sciences maintaining collections of B.t. strains, and one major fermentation facility in Siberia:

1) Institute for Agricultural Microbiology - Leningrad - Pushkin;

2) Laboratory of Microbiology (Institute of Systematic Animal Ecology) Novosibirsk;

3) Berdsk Biological Products Plant. This is a major fermentation facility with over 2 million liters fermentation capacity. The purpose of this visit was to evaluate the possibility of mass production of various B.t. preparations deriving from isolates in Uzbekistan and Kazakhstan.

4) The most significant visit was paid to the Institute of Genetics and Collection of Industrial Microorganisms of the Martinovsky Institute for Medical Parasitology and Tropical Medicine in Moscow headed by Prof. R. R. Azizbekyan. This laboratory has a very valuable and sizeable collection of *Bacillus* entomopathogens consisting of hundreds of potent strains including unique pathogens active against important pest insects. Dr. Azizbekyan has expressed his interest in joining our present reseatch with CAR, or, initiating a new project with us.

In August of 1995 the participating scientist from each of the collaborating countries visited CBC-BGU to be trained in aspects of molecular biology-PCR technology with the purpose of transferring this technology to the respective countries. The last six month segment of this project was dedicated, in addition to regular research activities, to the conclusions and summary report writing and preparation of joint publications. Oral and poster presentations were contributed by the participating scientists in numerous international conferences.

5) Publication list:

1.Eitan Ben-Dov, Arieh Zaritsky, Edith Dahan, Ze'ev Barak, Rosa Sinai, Robert Manasherob, Allovuddin Khamraev , Eugenia Troitskaya, Anatoly Dubitsky, Natasha Berezina, and Yoel Margalith. 1997. Extended Screening by PCR for Seven *cry*-group genes from Field-Collected Strains of *Bacillus thuringiensis*. Submitted to Appl. Environ. Microbiol.

2.Eitan Ben-Dov, Arieh Zaritsky, Edith Dahan, Ze'ev Barak, Rosa Sinai, Robert Manasherob, Allovuddin Khamraev , Eugenia Troitskaya, Anatoly Dubitsky, Natasha Berezina, and Yoel Margalith. 1997. Novel cry-type genes detected by extended PCR screening from field-collected strains of *Bacillus thuringiensis*. Submitted to Isr. J. Entomol.

3.Eitan Ben-Dov, Arieh Zaritsky, Edith Dahan, Ze'ev Barak, Rosa Sinai, Robert Manasherob, Allovuddin Khamraev , Eugenia Troitskaya, Anatoly Dubitsky, Natasha Berezina, and Yoel Margalith. 1997. Novel cry-type genes detected by extended PCR screening from field-collected strains of *Bacillus thuringiensis*. Abstract No. P#34 Second En Gedi Conference on Bacterial Control of Agricultural Pests and Vectors of Human Diseases. Shoresh, Israel August 12-16, 1996.

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8. PROJECT PRODUCTIVITY

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

Collaboration among the participating scientists was achieved by exchange of visits in each other's laboratories. During the past years 6 scientists from Uzbekistan and Kazakhstan stayed in the Laboratory 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 was advanced through the equipment purchased and training conducted during the course of this study.

All participating scientists in this project have presented the results of their studies at International Conferences (please see Attached abstracts). Copies of two recently submitted manuscripts are appended.

Small scale field trials using new isolates identified as part of this project are planned in the framework of a future collaborative project (submitted). These plans include devising formulations of the new isolates by linking up with the fermentation industry.

Several hundred field-collected samples were isolated in Israel, Uzbekistan and Kazakhstan. Distribution of potential insecticidal activity based on *cry* gene identification (126 *cry* positive B.t.) is described by 24.6% potentially active against Lepidoptera and Diptera; 9.5% Lepidoptera; 23.8% Lepidoptera and Coleoptera; 2.4% Coleoptera and 35.7% Diptera. In addition 4.0% of the isolates contain genes which are potentially active against Diptera and Coleoptera (Fig. 2). Some of these isolates represent a promising potential for new biological control agents effective against Lepidotera, Coleoptera and Diptera.

Twenty two distinct *cry*-type profiles were identified from 126 field-collected *B. thuringiensis* strains. Several of them were found to be different from all published profiles. Some of the field-collected strains but none of the 16 standard strains were positive to *cry2Ac*. **38 field-collected strains were positive with universal but negative with specific primers for all five known genes of *cry7* and *cry8*. These field-collected strains seem to contain new gene(s) promising for biological control of insects and resistance management.**

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

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 3). 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.

About 40% of our spore-forming field isolates did not give PCR products. Their crystal morphology will be studied by microscopy to determine their size, shape and number of inclusions (Bulla *et al.,* 1980). The protein profile of interesting crystals will be analysed by SDS-PAGE. The solubility of these novel proteins at different pHs will be investigated. Novel ICPs with interesting insecticidal properties will be digested with proteolytic enzymes for microsequence and their genes will be cloned by PCR. This will lead to the identification of new toxin genes.

In addition we plan to design PCR primers based on the sequence of the five conserved boxes among *cry* genes. *Cry* genes contain either five or one conserved boxes (Juarez-Perez et al., 1994). The detection of new combinations of these boxes may infer the existance of new genes. This methodology should be useful for finding new natural recombinant *cry* genes in our field samples. Strains with novel insecticidal activity will provide new genes for the development of recombinant organisms.

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

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Extended Screening by PCR for Seven *cry*-group genes from Field-Collected Strains of *Bacillus thuringiensis*

EITAN BEN-DOV,[1] ARIEH ZARITSKY,[1] EDITH DAHAN,[1] ZE'EV BARAK,[1] ROSA SINAI,[1] ROBERT MANASHEROB,[1] ALLOVUDDIN KHAMRAEV,[2] EUGENIA TROITSKAYA,[2] ANATOLY DUBITSKY,[3] NATASHA BEREZINA,[3] AND YOEL MARGALITH[1]*

Department of Life Sciences, Ben-Gurion University of the Negev
P.O. Box 653, Be'er-Sheva 83105, Israel,[1]
Institute of Zoology, Uzbek Academy of Science, Uzbekistan,[2]
and
Institute of Zoology, Kazakhstan Academy of Science, Kazakhstan[3]

*Corresponding author: Phone: 972-7-6461.340 or 972-7-6472.642
Fax.: 972-7-6472.890
email: yoelm@bgumail.bgu.ac.il

Running title: PCR SCREENING OF *B. thuringiensis cry* GENES

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ABSTRACT

A rapid method for identification of *cry*-containing *Bacillus thuringiensis* strains was established by using extended multiplex PCR. Five pairs of universal primers were designed to probe the highly conserved sequences of most (34 from about 60) genes known in the following groups: 20 *cry1* genes, 3 *cry2*, 4 *cry3*, 2 *cry4*, 2 *cry7* and 3 *cry8* genes. DNA of each positive *B. thuringiensis* strain was probed by a set of specific primers, designed for 20 of these genes and for *cry11A*. Twenty two distinct *cry*-type profiles were identified from 126 field-collected *B. thuringiensis* strains. Several of them were found to be different from all published profiles. Some of the field-collected strains but none of the 16 standard strains were positive to *cry2Ac*. Three standard and 38 field-collected strains were positive with universal but negative with specific primers for all five known genes of *cry7* and *cry8*. These field-collected strains seem to contain new gene(s) promising for biological control of insects and resistance management.

INTRODUCTION

Over half a century of synthetic pesticide applications has led to 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 organism—have brought about serious problems (20, 24). An urgent need has thus emerged for environment-friendly pesticides, to reduce contamination and the likelihood of insect resistance (19, 24). The use of *Bacillus thuringiensis* as a commercial insecticide is based on its remarkable ability to produce large quantities of insect larvicidal proteins (1). Also known as δ-endotoxin, they form crystalline inclusion bodies during sporulation (4). The multitude of insecticidal crystal proteins (ICPs) of *B. thuringiensis* subspecies has spurred their use as natural control agents in agriculture, forestry and human health (24). Recent trends suggest that biological control will become increasingly important, particularly as part of strategies for integrated pest management (20, 21). Novel insecticidal bacteria, with extended host spectrum for example, would enhance environmentally safe bio-control practices and lead to increased food production and post-harvest protection (24).

The genes coding for the ICPs, normally associated with large plasmids, direct the synthesis of a family of related proteins that have been classified as *cryI - VI* (the old nomenclature) depending on the host specificity (lepidoptera, diptera, coleoptera and nematodes) and the degree of amino acid homology (12, 15, 19). The current classification is uniquely defined by the latter criterion (9).

Identifying novel *B. thuringiensis* isolates by bioassays is a long and exhaustive process, impeded by repeated isolation of the same strains. Prediction of insecticidal activity of an unknown strain by serotyping seems impossible because it does not necessarily reflect the specific *cry* gene classe(s) the strain(s) contain (1, 11). The Polymerase Chain Reaction (PCR), which is a highly sensitive method to rapidly detect and identify target DNA sequences, requires minute amounts of DNA, and allows quick, simultaneous screening of many *B. thuringiensis* samples, to classify them and to predict their insecticidal activities (3, 5, 6, 7, 8, 13, 16,17). Being faster and more efficient than serotyping, PCR analysis provides a valuable preliminary tool preceeding bioassays of newly isolated *B. thuringiensis* strains.

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 (16,17). We describe here an enhanced

strategy using PCR for extended multiplex rapid screening (3) of field-collected *B. thuringiensis* strains that harbor genes from classes *cry1, cry2, cry3, cry4, cry7, cry8* and *cry11*. This strategy will enrich the existing arsenal of insecticidal strains, identify novel genes or new combinations of known genes and predict their toxicities. Universal primers were selected from a region that is highly conserved in 20 *cry1*, 3 *cry2*, 4 *cry3*, 2 *cry4*, 2 *cry7* and 3 *cry8* genes (extracted from GeneBank database, aided by reference 9). DNA of strains which reacted to at least one pair of the universal primers were characterized by performing additional PCR with specific primers, by which 8 different *cry1*, 3 *cry2*, 3 *cry3*, 2 *cry4*, 1 *cry7*, 3 *cry8* and 1 *cry11* genes were identified. The preliminary screening by universal primers saves efforts by assorting the strains for the specific screening, which then produces a PCR product with a unique size for each *cry* gene. Novel strains yielding unknown *cry* profiles (containing new genes or combinations thereof) should be further characterized by bioassays.

MATERIALS AND METHODS

***B. thuringiensis* strains.** Known strains (10) that served as references (see below, Table 3) were kindly supplied by Drs. D. R. Zeigler (Bacillus Genetic Stock Center, Columbus, Ohio) and B. Sneh (Tel-Aviv University) and by the USDA, Agricultural Research Service (Peoria, Illinois). Others were obtained from soil and insect cadavers collected in Israel, Kazakhistan and Uzbekistan.

B. thuringiensis were enriched from the isolates by acetate, which selectively inhibits germination of their spores and not of other sporeformers, and plated on LB agar following a heat shock (23). Single colonies were grown in liquid T3 medium and selected for appearance of parasporal inclusions by phase-contrast microscopy. Samples (after 96 h growth) were frozen at -70°C with 15% glycerol, or lyophilized after washing with sterile distilled water by centrifugation.

Oligonucleotide PCR primers. One pair of universal primers (Uns) for each of the five *cry* homology groups (9) was designed to amplify a specific fragment by simultaneous alignment to all previously described genes in that group using Amplify 1.0 program (Bill Engels, University of Wisconsin, USA). 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.

To identify *cry1* (16), *cry2*, *cry3*, *cry4*, *cry7* and *cry8*, a single Un and several specific primers for each *cry* class (selected from their highly variable regions) were used together in one reaction. Two specific primers were designed for *cry1Ab* (16) and *cry11A*. 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%).

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. (22).

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 mM 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.

Reliability of the primers was verified using the following *B. thuringiensis* reference strains: *kurstaki* HD-1, *aizawai* HD-133, *galleriae* HD-155, *kenyae* HDB-23, *tolworthi* HDB-8 and HD-2 for *cry1* and *cry2* classes, *tenebrionis*, NRRL B-18533, NRRL B-18655 for *cry3* class, *israelensis* ONR60A for *cry4* and *cry11* clases and *dakota* HD-511, *tochigiensis* HD-868, *indiana* HD-521 and *kumamotoensis* HD-867 for *cry7* and *cry8* clases (see RESULTS, Table 3).

RESULTS

Identification by universal primers. Five pairs (Direct and Reverse) of Uns (Table 1) were designed to detect genes by their following PCR products: Un1 for 20 *cry1* genes with products of 274 or 277 bp; Un2 for 3 *cry2* genes with products of 689 or 701 bp; Un3 for 4 *cry3* genes with products' size range between 589-604 bp; Un4 for 2 *cry4* genes with size 439 bp; Un7,8 for 2 *cry7* and 3 *cry8* genes with products of 420 and 423 bp, respectively.

DNA of each *B. thuringiensis* isolate served as template in three reactions (Figure 1):
(i) with a mixture of two pairs, of Un1 and Un4 (to detect genes from *cry1* and *cry4* groups);
(ii) with a mixture of two other pairs, of Un2 and Un3 (to detect genes from *cry2* and *cry3* groups);
(iii) with Un7,8 (to detect genes from *cry7* and *cry8* groups).

Such an extended PCR analysis for *cry*-type genes, never previously reported, was performed on 16 standard *B. thuringiensis* strains with universal (as well as specific; see below) primers; results are summarized in Table 3. Indeed, some of these well-known strains contain additional *cry*-type genes. For example (and see in Discussion), subsp. *aizawai* HD-133 reacted positively to Un7,8 by producing a fragment of 420 bp. Two subsps., *kyusushensis* HD-541 and *japonensis* 4AT1, however, were not identified with our primers.

Among 215 field-isolated *B. thuringiensis* strains, the DNA of 89 did not amplify the Uns. The rest were grouped in 7 *cry*-type gene profiles, as presented in Fig. 2.

Identification of specific genes. The specific primers (labeled EE, except for *cry1*), designed to identify 21 genes from the 7 *cry*-groups, were selected from highly variable regions in the respective genes (Table 2). Our PCR analysis for the *cry1* group, based on the primers' design by Kalman et al. (16), was thus limited to identify 8 (*Aa, -Ab, -Ac, -B, -C, -D, -E, -F*) of the 20 known genes that are identified by Un1 (Table 1; Fig. 3). We found 9 different *cry1* gene profiles (numbers 3 to 16 in Table 4); an additional *cry1* profile (numbers 1 and 2) was not identified by any specific primer of *cry1*.

All except two standard *B. thuringiensis* strains containing *cry1* were found to also contain both *cry2Aa* and *cry2Ab*: subsp. *thuringiensis* HD-2 was negative to *cry2*, and *aizawai* HD-133 was positive to *cry2Ab* only (Table 3; Figure 4). None of our standard strains was positive to *cry2Ac*, but those field-collected strains that were positive contained *cry2Ab* as well (profile number 16, Table 4; Figure 4).

None of our field-collected strains was positive to *cry3*. Three *B. thuringiensis* standard strains (subsps. *tenebrionis*, NRRL B-18533 and NRRL B-18655) were positive controls (Fig. 5, Table 3).

Three genes, *cry4A*, *cry4B* and *cry11A*, which code for Dipteran-specific polypeptides (ref), were identified by a set of specific primers (Table 2; Fig. 6). This set yielded 3 different profiles, all included cry11A: one with *cry4A* (profile number 19), one with *cry4B* (number 20) and one with both (numbers 21/22).

Despite the positive reactions with Un7,8, DNAs of subsps. *aizawai* HD-133, *indiana* HD-521 and *tochigiensis* HD-868 were negative with all specific primers for genes of *cry7* and *cry8*; subsps. *dakota* HD-511 and *kumamotoansis* HD-867, on the other hand, were identified as containing *cry7A* (Table 3; Fig. 7). Thirty of the field strains with a combination of *cry1* and *cry2* (profiles number 14, 15 and 16; Table 4) and 5 with *cry4* and *cry11* (profile 22; Table 4) were positive to Un7,8 but not identified by any specific primer of *cry8* and *cry7*. Three strains (profile 18; Table 4) were positive only to Un7,8. Ten of these 38 isolates produced either one of two unexpected PCR products, of ~700 and ~300 bp, by two direct specific primers (EE-8B and EE-8C, of *cry8B* and *cry8C*, respectively; Table 2).

The 126 field strains examined here displayed 22 distinct profiles (Table 4), reflecting different *cry* combinations, some of which are novel (profiles 9, 10, 14, 16, 17, 18 and 22).

DISCUSSION

***cry* profiles of PCR products from standard and field-collected strains.** Several hundred field-collected samples were isolated in Israel, Uzbekistan and Kazakhstan; of these, about 215 spore-forming *B. thuringiensis* isolates were analyzed by the first group of primers (Uns; Table 1; Figs. 1 and 2), and the positive 126 were identified by the second, specific primers (Tables 2 and 4; Figs. 3-7). The profiles of all were compared with those of 16 standard strains (Table 3; Figs. 3-7). Twenty two different profiles were observed (Table 4), 7 of which (including 36 independent isolates) were novel (profiles number 9, 10, 14, 16, 17, 18 and 22); these latter strains may be promising for new biological control agents. Twelve independent isolates (profiles number 1 and 2) were positive with Un1 but not identified by any specific primer of our 8 *cry1*. They may contain at least one of 12 other *cry1* genes (amplified by Un1; Table 1) or new *cry1* gene(s). Three additional profiles (number 19, 20 and 21, found in 45 isolates) contained genes of subsp. *israelensis*. The remaining 33 isolates were distributed in 10 different profiles (numbers 3, 4, 5, 6, 7, 8, 11, 12, 13 and 15), with various combinations of genes from groups *cry1*, *cry2*, and *cry7* or *cry8* (Table 4). When challenged with specific *cry1* primers only, they produced 6 different profiles which were identical to standard strains as follows (Table 3 and (1, 3, 8, 15, 16)): profile numbers 3 and 5 to subsp. *entomocidus*, profile numbers 4 and 7 to *alesti*, number 6 to *sotto*, 8 and 13 to *kurstaki*, 11 and 12 to *gallerie*, and 15 to *aizawai*.

New toxic specificities may stem from new combinations of known genes, such as in the strains displaying profiles 9 and 10. An interesting case in point is a *B. thuringiensis* strain YBT-226 isolated in China (14), which produces Cry1Ab and Cry1Ba (toxic to Lepidoptera) as well as Cry2Aa (toxic to both, Lepidoptera and Diptera). This strain is highly toxic to the common housefly, *Musca domestica*, despite the fact that toxicity of Cry2Aa is low (14).

To the best of our knowledge, PCR screening by primers to *cry2* genes has never been performed before. We found that all but one (subsp. *thuringiensis* HD-2) standard strains with at least one *cry1* gene (Table 3; Figure 4) also contained *cry2Ab*. All of these except one (subsp. *aizawai* HD-133) also contained *cry2Aa*. None of the 6 standard strains we screened responded to primers specific to *cry2Ac*. This gene was discovered in *B. thuringiensis* S_1) (25), with toxicity against Lepidoptera. All 14 isolates containing *cry2Ac* displayed a single profile (number 16, Table 4; Figure 4), which was also positive to *cry2Ab*. The other 47 field strains with at least one *cry2* gene can be divided in two types (Table 4): 21

which contain *cry2Aa* and *cry2Ab* (displaying 6 different profiles) and 26 containing *cry2Ab* alone (displaying 7 different profiles). *cry2Aa*, *cry2Ac* and the two combintions between them (with and without *cry2Ab*) were not found.

Several known strains which contain a *cry1* gene (toxic to Lepidoptera) have been found to also include a gene from either the *cry3* or the *cry7* group (toxic to Coleoptera); for example, subsp. *tolworthi* contains *cry1Ab*, *cry1E*, *cry2* and *cry3B* (1). According to Kuo et al. (17), *cry1*-containing subsps. *wuhanensis* HD-525 and *morrisoni* HD-12 contain apparent novel *cry7A*. Strain BTI109P of subsp. *kurstaki* contains *cry3C* (18). DNA from subsp. *aizawai* HD-133, known to contain four genes from the *cry1* group (8) as well as *cry2Ab* (Table 3), was amplified with Un7,8 (Fig. 7) but not with all specific primers of these groups. This observation is consistent with that of Kuo et al. (17), reporting that the same strain was not identified as *cry7A*. Since Kuo et al. did not test for any of the *cry8* group, it may imply that subsp. *aizawai* HD-133 contains a new gene of the *cry8* group.

B. thuringiensis subsps. *dakota* HD-511 and *kumamotoansis* HD-867 responded similarly to our specific primers for *cry7A* (Fig. 7). Kuo et al. (17) recently indeed reported that the same *kumamotoansis* strain and a close *dakota* strain (HD-932) yielded (by the PCR-RFLP method; (17)) "typical" but not identical restriction patterns of *cry7A*; in their words "partial nucleotide sequence of the PCR products confirmed that this predicted *cry7*-type gene is novel".

Both *B. thuringiensis* subsps. *indiana* HD-521 and *tochigiensis* HD-868 responded positively to Un7,8, but not to any of our specific primers to genes of these groups (*cry7A*, *cry8A*, *cry8B* and *cry8C*). Consistently (17), the partial nucleotide sequence of the product from the same *indiana* strain was similar to that of the *cry7*-type gene but *tochigiensis* HD-868 was not identified. Indeed, nucleotide sequences of the PCR products (423 bases long, obtained with Un7,8) from these two subsps. show about 80 and 90% homology to *cry7A*, respectively, and 67% to all three *cry8* genes (to be extensively described elsewhere); in addition, they are about 80% homologous between themselves, thus confirming that they are two novel genes. The sequence of the same 423-bases PCR-product obtained with a field strain (R1 of profile number 18; Table 4), positive to Un7,8 only, was found to be 67% homologous to *cry7A* and 98.8%—to *cry8B*. This gene must be novel (despite this high homology) because it did not react with the specific primer to *cry8B* (which was designed to anneal to the variable region; Table 2). SDS-polyacrylamide gel electrophoresis of sporulating culture of strain R1 yielded two large polypeptides, one

of about 140 kDa (typical size of a Cry7 and Cry8 polypeptide) and one of 100 kDa, which may be the new postulated gene (unpublished data).

Thirty of our field strains (exhibiting profiles number 14, 15 and 16; Table 4) were positive with Un1, Un2 and Un7,8 but were not identified with specific primers to genes from either *cry7* or *cry8* group. The 8 isolates with profile number 15 are identical (by our criteria) to *B. thuringiensis* subsp. *aizawai* HD-133. The remaining 22 isolates (with profiles number 14 and 16) seem to contain new *cry* gene(s), thus have the potential to be effective biopesticides of insects for which biological control agents do not exist.

Five new isolates (displaying profile number 22), which contain the 3 genes of subsp. *israelensis* (*cry4A*, *-B*, *cry11A*; toxic to Diptera), seem to contain new *cry7* or *cry8* gene(s) (were positive with Uns7,8), and may thus be useful as new biological control agents for larvae of both Coleoptera and Diptera. A single subsp. (*morrisoni*, serotype 8a 8b; (1)) is known to contain these Dipteran-toxic genes in combination with *cry3A* (toxic to Coleoptera). It is interesting to note that all 48 isolates which were positive with Un4 (contain *cry4A* or *cry4B* (or both)) were consistently positive with the specific primers to *cry11A* (profiles number 19-22; Table 4).

The absence of PCR products when DNA of 89 *B. thuringiensis* isolates was challenged with all our universal primers does not necessarily imply that these strains are devoid of genes coding for anti-insecticidal polypeptides. Novel toxins may be recognized among these isolates by characterizing the proteins detected on gels or microscopically by the shape of their crystals.

Specificity of oligonucleotide primers for PCR. Ceron et al. (7) have recently prepared one pair of universal primers (CJIII20, CJIII21) to detect all genes currently known to code for toxins uniquely specific against Coleoptera (*cryIII*, in the old nomenclature; (15)). This specificity group is now divided in three homology groups (*cry3*, *cry7* and *cry8*; (9)). We designed and prepared two pairs of universal primers (Un3 and Un7,8) with high stability for all these genes and low variability in sizes of the PCR products for each group. The number of mismatches were from 0 to 3 bases, and the size ranges of fragments were 15 bases for *cry3* and only 3 bases for *cry7* and *cry8* (Table 1). The single pair of universal primers of Ceron et al. (7) anneals well to *cry3* and *cry7* (maximum 4 mismatch bases with primer CJIII21 to *cry3C*) and weakly to *cry8B* and to *cry8C* with maximum of 5 (CJIII20) and 6 (CJIII21) mismatches, respectively. This pair of universal primers, moreover, cannot amplify *cry8A*. The range of fragment sizes produced by this pair (39 bases) was also much higher.

Three genes, *cry4A*, *cry4B* and *cry11A*, which code for Dipteran-specific polypeptides and are located on the same plasmid in *B. thuringiensis* subsp. *israelensis* (2), were identified together by one set of specific primers (Table 2). However, due to competition between *cry4A* (1,529 base fragment) and *cry4B* (1,951 base fragment), the latter (weakly amplified in the mixture) was detected separately (Fig. 6).

One of the major limitations of PCR is nonspecific amplification. To minimize this limitation, we maximized the annealing temperature of our designed primers. We used 58-60°C for all except Un7,8 and their 4 specific primers (54°C), and for the specific *cry1* primers as recommended (54°C; 8). In addition, annealing and extension times were shorter than 1 min (exept extension time (1.30 min) with EE-4A and EE-4B, which amplify fragments of 1,529 and 1,951 bases, respectivly).

To minimize the effort in screening, three reactions with universal primers were performed for each isolate (see in Results), two mixtures of two pairs (Un1 with Un4 and Un2 with Un3) and one pair for both *cry7* and *cry8* groups (Un7,8). To further raise efficiency, it should be possible to design 5 pairs (or more, for more extended analyses) that can be used in a mixture for a single reaction. To this end, one should be aware of possible difficulties due to interactions between the primers themselves, for example. To prevent non-specific amplification, each pair (which is specific to one group of genes) must not interact with genes from other groups.

The extended multiplex PCR screening is a rapid method for detecting and differentiating (by their PCR product profiles) *B. thuringiensis* field strains and for predicting their insecticidal activities thus to direct them for subsequent toxicity assays. Our isolates displaying new profiles (containing apparent new genes) should be characterized and further developed for integration with other control measures.

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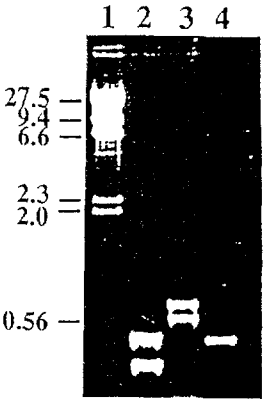

Figure 1. Agarose gel (1.2%) electrophoresis of PCR products amplified from standard strains of *B. thuringiensis* with universal primers. Lane 1, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lanes 2-4, PCR products obtained from: lane 2, mixed DNA of subsps. *israelensis* and *kurstaki* HD1 with Un1 and Un4; lane 3, mixed DNA of subsps. *kurstaki* HD1 and *tenebrionis* with Un2 and Un3; lane 4, DNA of subsp. *dakota* HD-511 with Un7,8. Expected sizes of products indicated in Table 1.

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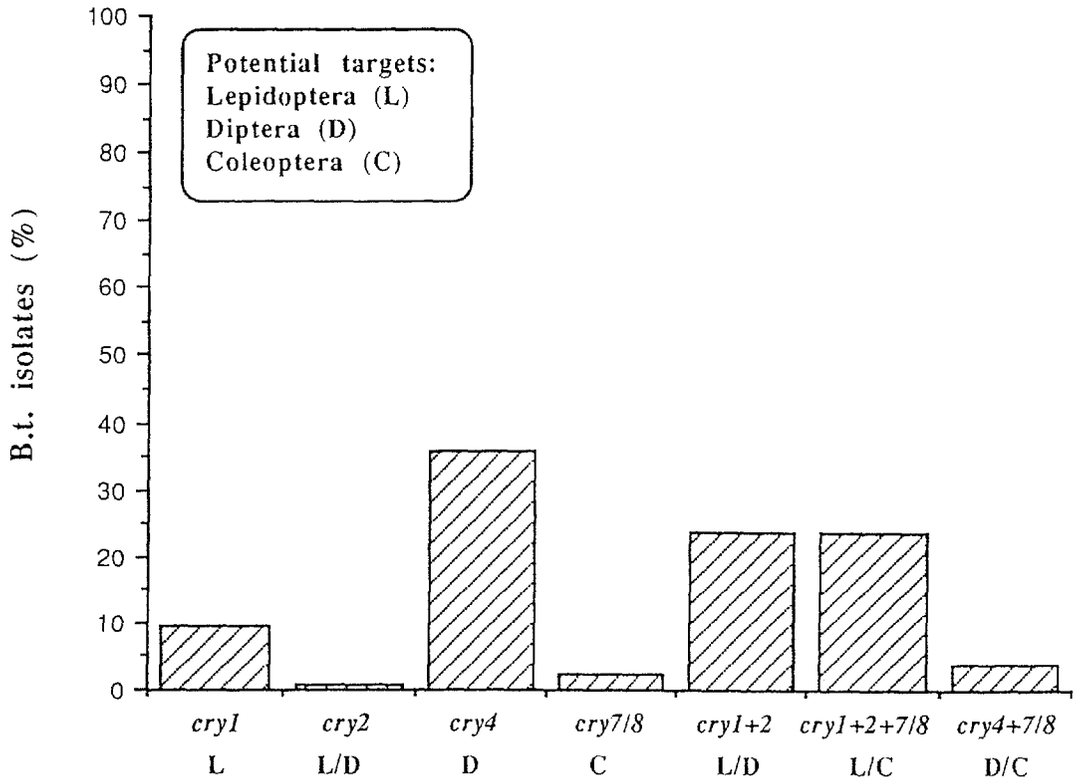

Figure 2. Distribution of *cry*-type gene profiles from 126 field-collected strains of *B. thuringiensis* identified by universal primers.

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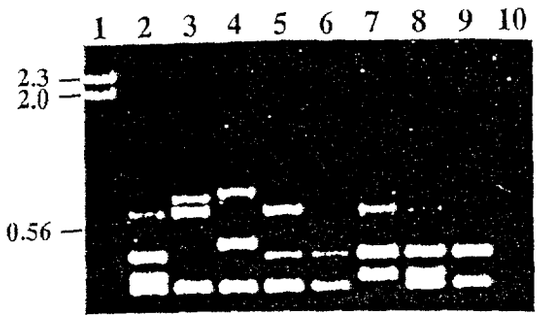

Figure 3. Agarose gel (1.2%) electrophoresis of PCR products obtained with specific primers for *cry1* genes. Lane 1, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lanes 2-4, DNA of *B. thuringiensis* subsp. *aizawai* HD-133 (lane 2), *galleriae* HD-155 (lane 3), *kenyae* HDB-23 (lane 4); lanes 5-9, DNA of field-collected strain U-12 (with profile number 9; lane 5), strain U-25 (profile 10; lane 6), strain U-35 (profile 14; lane 7), strain U-38 (profile 15; lane 8), strain U-27 (profile 16; lane 9); lane 10, negative control of PCR reaction (without template). Expected sizes of products indicated in Table 2.

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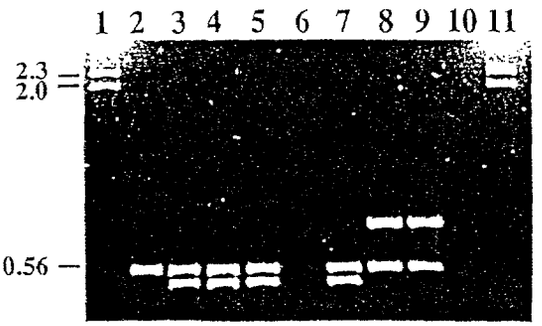

Figure 4. Agarose gel (2.5%) electrophoresis of PCR products obtained with specific primers for *cry2Aa, -Ab, -Ac* genes. Lanes 1 and 11, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lane 2-7, DNA of *B. thuringiensis* subsp. *aizawai* HD-133 (lane 2), *galleriae* HD-155 (lane 3), *kenyae* HDB-23 (lane 4), *tolworthi* HDB-8 (lane 5), *thuringiensis* HD-2 (lane 6), *kurstaki* HD-1 (lane 7); DNA of field-collected strains U-21 (lane 8) and U-27 (lane 9) (both with profile number 16); lane 10, negative control without template. Expected sizes of products indicated in Table 2.

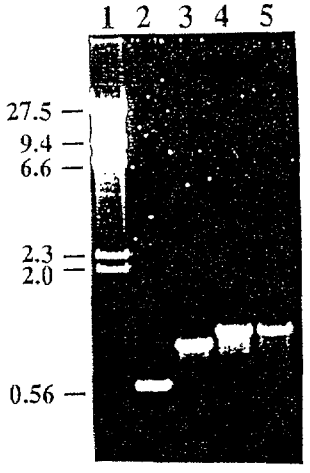

Figure 5. Agarose gel (1%) electrophoresis of PCR products obtained with primers for *cry3* genes. Lane 1, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lane 2, DNA of *B. thuringiensis* subsp. *tenebrionis* amplified with Un3; lanes 3-5, DNA of *B. thuringiensis* subsp. *tenebrionis* (lane 3), NRRL B-18533 (containing *cry3Bb1*; lane 4) and NRRL B-18655 (containing *cry3Bb2*; lane 5) amplified with specific primers. Expected sizes of products indicated in Tables 1 and 2.

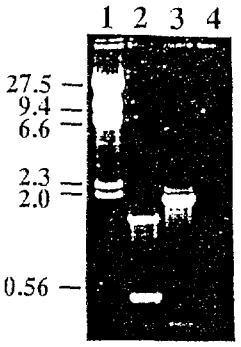

Figure 6. Agarose gel (1%) electrophoresis of PCR products from *B. thuringiensis* subsp. *israelensis*. Lane 1, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lanes 2 and 3, obtained with specific primers for *cry4A* plus *cry11A*, and for *cry4B*, respectively.

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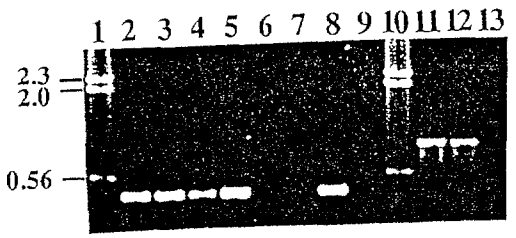

Figure 7. Agarose gel (1%) electrophoresis of PCR products obtained with universal and specific primers for *cry7* and *cry8* genes. Lanes 1 and 10, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lanes 2-8, respectively, DNA of *B. thuringiensis* subsp. *dakota* HD-511, *indiana* HD-521, *tochigiensis* HD-868, *kumamotoensis* HD-867, *japonensis* 4AT1, *kyusushensis* HD-541 and *aizawai* HD-133, amplified with Un7,8; lane 11-12, DNA of *B. thuringiensis* subsp. *dakota* HD-511 and *kumamotoensis* HD-867, with specific primers; lanes 9 and 13, negative controls without template, with Un7,8 and with a mixture of specific primers for *cry7* and *cry8* genes, respectively.

TABLE 1. Characteristics of universal primers for *cry1*, *cry2*, *cry3*, *cry4*, *cry7* and *cry8* group genes.

Gene nomenclature			Nucleotide positions	Mismatch of primers[c]	Product
Current	Original	Access. #[a]	hybridized to primers[b]		size (bp)
Sequences of *cry1* universal primers: Un1(d)[d]: 5'CATGATTCATGCGGCAGATAAAC-3' Un1(r)[d]: 5'-TTGTGACACTTCTGCTTCCCATT-3'					
cry1Aa5	*cryIA(a)*	D17518	2858-2880, 3112-3134	0(d); 0(r)	277
cry1Ab9	*cryIA(b)*	X54939	2775-2797, 3029-3051	1(d); 0(r)	277
cry1Ac5	*cryIA(c)*	M73248	2781-2803, 3035-3057	0(d); 0(r)	277
cry1Ad	*cryIA(d)*	M73250	2778-2800, 3032-3054	1(d); 10(r)	277
cry1Ae	*cryIA(e)*	M65252	2861-2883, 3115-3137	11(d); 0(r)	277
cry1Ba	*cryIB*	X06711	2919-2941, 3170-3192	0(d); 0(r)	274
cry1Bb	*cryI ET5*	L32020	3000-3022, 3251-3273	0(d); 0(r)	274
cry1Ca1	*cryIC*	X07518	2851-2873, 3105-3127	1(d); 16(r)	277
cry1Cb	*cryIC(b)*	M97880	3061-3083, 3315-3337	1(d); 4, 10(r)	277
cry1Da	*cryID*	X54160	2996-3018, 3250-3272	1(d); 16(r)	277
cry1Db	*prtB*	Z22511	2973-2995, 3227-3249	0(d); 0(r)	277
cry1Ea3	*cryIE*	M73252	2760-2782, 3014-3036	1(d); 0(r)	277
cry1Eb	*cryIE(b)*	M73253	2754-2776, 3008-3030	1, 21(d); 0(r)	277
cry1Fa2	*cryIF*	M73254	2760-2782, 3014-3036	0(d); 0(r)	277
cry1Fb	*prtD*	Z22512	3239-3261, 3493-3515	1(d); 0(r)	277
cry1G	*prtA*	Z22510	2802-2824, 3056-3078	1(d); 0(r)	277
cry1H	*prtC*	Z22513	3283-3305, 3537-3559	0(d); 0(r)	277
cry1Hb	-	U35780	3472-3494, 3726-3748	0(d); 7(r)	277
cry1Ja	*cryI ET4*	L32019	2840-2862, 3091-3113	1(d); 7, 10, 15(r)	274
cry1K	-	U28801	3318-3340, 3569-3591	1, 4, 7, 12(d); 0(r)	274
Sequences of *cry2* universal primers: Un2(d): 5'-GTTATTCTTAATGCAGATGAATGGG-3' Un2(r) 5'-CGGATAAAATAATCTGGGAAATAGT-3'					
cry2Aa1	*cryIIA*	M31738	726-750, 1402-1426	0(d); 0(r)	701
cry2Ab2	*cryIIB*	X55416	1444-1468, 2120-2144	9(d); 3(r)	701
cry2Ac	*cryIIC*	X57252	2695-2719, 3359-3383	3, 6(d); 22(r)	689
Sequences of *cry3* universal primers: Un3(d) 5'-CGTTATCGCAGAGAGATGACATTAAC-3' Un3(r) 5'-CATCTGTTGTTTCTGGAGGCAAT-3'					
cry3A6	*cryIIIA*	U10985	1367-1392, 1933-1955	0(d); 0(r)	589
cry3Ba1	*cryIIIB*	X17123	826-851, 1398-1420	5, 15(d); 17, 20(r)	595
cry3Bb1	*cryIIIBb*	M89794	1003-1028, 1575-1597	5, 15, 21(d); 17, 20(r)	595
cry3C	*cryIIID*	X59797	1024-1049, 1605-1627	0(d); 12, 13(r)	604
Sequences of *cry4* universal primers: Un4(d) 5'- GCATATGATGTAGCGAAACAAGCC-3' Un4(r) 5'- GCGTGACATACCCATTTCCAGGTCC-3'					
cry4A2	*cryIVA*	D00248	3324-3347, 3738-3762	0(d); 0(r)	439
cry4B4	*cryIVB*	D00247	3259-3282, 3673-3697	0(d); 0(r)	439
Sequences of *cry7* and *cry8* universal primers: Un7,8(d): 5'-AAGCAGTGAATGCCTTGTTTAC-3' Un7,8(r): 5'-CTTCTAAACCTTGACTACTT-3'					
cry7Aa	*cryIIIC*	M64478	2135-2156, 2535-2554	5, 12(d); 9, 11(r)	420
cry7Ab1	*cryIIICb*	U04367	1952-1973, 2352-2371	5, 12(d); 9, 11(r)	420
cry8A	*cryIIIE*	U04364	2027-2048, 2430-2449	0(d); 1, 8(r)	423
cry8B	*cryIIIG*	U04365	2015-2036, 2418-2437	0(d); 1, 8(r)	423
cry8C	*cryIIIF*	U04366	2018-2039, 2421-2440	5, 13(d); 1, 2, 9(r)	423

[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.

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TABLE 2. Characteristics of specific primers for *cry2*, *cry3*, *cry4*, *cry7* and *cry8* and *cry11* genes.

Primer pair[a]	Sequence of primers[b]	Gene recognized	Positions[c]	Product size (bp)
Un2(d)	GTTATTCTTAATGCAGATGAATGGG	*cry2Aa1*	726-750	498
EE-2Aa(r)	GAGATTAGTCGCCCCTATGAG		1203-1223	
Un2(d)	GTTATTCTtAATGCAGATGAATGGG	*cry2Ab2*	1444-1468	546
EE-2Ab(r)	TGGCGTTAACAATGGGGGGAGAAAT		1965-1989	
Un2(d)	GTtATtCTTAATGCAGATGAATGGG	*cry2Ac*	2695-2719	725
EE-2Ac(r)	GCGTTGCTAATAGTCCCAACAACA		3396-3419	
Un3(d)	CGTTATCGCAGAGAGATGACATTAAC	*cry3A6*	1367-1392	951
EE-3Aa(r)	TGGTGCCCCGTCTAAACTGAGTGT		2294-2317	
Un3(d)	CGTTaTCGCAGAGAgATGACATTAAC	*cry3Ba1*	826-851	1103
EE-3Ba(r)	ACGAAAGATTCTGCTCCTAT		1909-1928	
Un3(d)	CGTTaTCGCAGAGAgATGACaTTAAC	*cry3Bb1*	1003-1028	1103
EE-3Ba(r)	ACGAAAGATTCTGCTCCTAT		2086-2105	
Un3(d)	CGTTATCGCAGAGAGATGACATTAAC	*cry3C*	1024-1049	461
EE-3C(r)	ATTTTGGTACCTCCTGTACCCACC		1461-1484	
EE-4A(d)	GGGTATGGCACTCAACCCCACTT	*cry4A2*	2234-2256	1529
Un4(r)	GCGTGACATACCCATTTCCAGGTCC		3738-3762	
EE-4B(d)	GAGAACACACCTAATCAACCAACT	*cry4B4*	1747-1770	1951
Un4(r)	GCGTGACATACCCATTTCCAGGTCC		3673-3697	
EE-7Aa(d)	GCGGAGTATTACAATAGAATCTATCC	*cry7Aa*	1639-1664	916
Un7,8(r)	CTTCTAAAcCtTGACTACTT		2535-2554	
EE-7Aa(d)	GCGGAGTATTACAATAGAATCTATCC	*cry7Ab1*	1456-1481	916
Un7,8(r)	CTTCTAAAcCtTGACTACTT		2352-2371	
EE-8A(d)	GAATTTACTCTATACCTTGGCGAC	*cry8A*	1771-1794	679
Un7,8(r)	cTTCTAAaCCTTGACTACTT		2430-2449	
EE-8B(d)	GACCGCATCGGAAGTTGTGAG	*cry8B*	1663-1683	775
Un7,8(r)	cTTCTAAaCCTTGACTACTT		2418-2437	
EE-8C(d)	GGTGCTGCTAACCTTTATATTGATAG	*cry8C*	1930-1955	511
Un7,8(r)	ctTCTAAAcCTTGACTACTT		2421-2440	
EE-11A(d)	CCGAACCTACTATTGCGCCA	*cry11A1*	111-130	445
EE-11A(r)	CTCCCTGCTAGGATTCCGTC		536-555	

[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. Distribution of *cry*-type gene profiles of *B. thuringiensis* strains as analysed by PCR[a]

B. thuringiensis strains	*cry1*	*cry2*	*cry3*	*cry4* and *11A*	*cry7* and *8*
aizawai HD-133	*Aa, Ab, Ca, Da*	*Ab*	-	-	+
galleriae HD-155	*Aa, Ab, Ba*	*Aa, Ab*	-	-	-
kenyae HDB-23	*Ab, Ac, Ea*	*Aa, Ab*	-	-	-
tolworthi HDB-8	*Ab*	*Aa, Ab*	-	-	-
thuringiensis HD-2	*Ab, Ba*	-	-	-	-
kurstaki HD-1	*Aa, Ab, Ac*	*Aa, Ab*	-	-	-
israelensis ONR60A	-	-	-	*A, B, 11A*	-
tenebrionis	-	-	*Aa*	-	-
NRRL B-18533	-	-	*Ba*	-	-
NRRL B-18655	-	-	*Ba*	-	-
dakota HD-511	-	-	-	-	*7Aa*
indiana HD-521	-	-	-	-	+
tochigiensis HD-868	-	-	-	-	+
kumamotoensis HD-867	-	-	-	-	*7Aa*
japonensis 4AT1	-	-	-	-	-
kyusushensis HD-541	-	-	-	-	-

[a] -, negative with universal primers; +, positive with universal primers.

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TABLE 4. 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*	10	Lep.
2. *cry1 + cry2Ab*	2	Lep.
3. *cry1Aa*	1	Lep.
4. *cry1Aa, -Ac*	1	Lep.
5. *cry1Aa, + cry2Aa, -Ab*	2	Lep. + Dip.
6. *cry1Aa, -Ab, + cry2Ab*	1	Lep.
7. *cry1Aa, -Ac, + cry2Aa, -Ab*	8	Lep. + Dip.
8. *cry1Aa, -Ab, -Ac, + cry2Aa, -Ab*	6	Lep. + Dip.
9. *cry1Aa, -Ab, -D + cry2Ab*	1	Lep.
10. *cry1Ab, -D + cry2Ab*	4	Lep.
11. *cry1Ab, -Ac, -D + cry2Ab*	2	Lep.
12. *cry1Ab, -Ac, -D + cry2Aa, -Ab*	1	Lep. + Dip.
13. *cry1Ac + cry2Aa, -Ab*	3	Lep. + Dip.
14. *cry1Aa, -C, -D + cry2Ab + cry7, cry8*	8	Lep. + Col.
15. *cry1Aa, -Ab, -C, -D + cry2Ab + cry7, cry8*	8	Lep. + Col.
16. *cry1Ab, -D + cry2Ab, -Ac + cry7, cry8*	14	Lep. + Col.
17. *cry2Aa, -Ab*	1	Lep. + Dip.
18. *cry7, cry8*	3	Col.
19. *cry4A, cry11A*	7	Dip.
20. *cry4B, cry11A*	2	Dip.
21. *cry4A, -B, cry11A*	36	Dip.
22. *cry4A, -B, cry11A + cry7, cry8*	5	Dip. + Col.

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

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Novel *cry*-Type Genes Detected by Extended PCR Screening from Field-Collected Strains of *Bacillus thuringiensis*

E. BEN-DOV,[1] E. DAHAN,[1] A. ZARITSKY,[1] Z. BARAK,[1] R. SINAI,[1] R. MANASHEROB,[1] A. KHAMERAEV,[2] A. TROYETSKAYA,[2] A. DUBITSKY,[3] N. BEREZINA,[3] AND Y. MARGALITH[1]

Department of Life Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Be'er-Sheva 83105, Israel,[1]

Institute of Zoology, Uzbek Academy of Science, Uzbekistan,[2]

and

Institute of Zoology, Kazakhstan Academy of Science, Kazakhstan[3]

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ABSTRACT

A rapid method for identification of *Bacillus thuringiensis* strains was established by using extended multiplex PCR. Five pairs of universal oligonucleotide primers were designed to probe the most conserved sequences of most known *cryI-IV* gene classes. The classes include, respectively, 20 *cryI* genes, 3 *cryII*, 4 *cryIII*, 5 newly-discovered *cryIII*, and 2 *cryIV* genes. DNA of each positive *B. thuringiensis* isolate was probed by a set of specific primers, designed for 20 genes. Twenty distinct *cry*-type profiles were identified from 110 field-collected *B. thuringiensis* strains. Several of them were found to be different from all published *cry*-type profiles. The screening procedure resulted in at least 3 new genes, promising for biological control of insects and resistance management. 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 of either *cryI* or *cryIV*.

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INTRODUCTION

Over half a century of synthetic pesticide applications has led to 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 environmental problems. An urgent need has thus emerged for environment-friendly pesticides, to reduce contamination and the likelihood of insect resistance to widely used synthetic chemicals [1, 15, 18]. The unique capacity of insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis* (*B.t.*) subspecies has spurred their use as natural control agents in agriculture, forestry and human health. Recent trends suggest that biological control will become increasingly important, particularly as part of strategies for integrated pest management [5]. The market share of *B.t.* is expected to reach 5-10% of the total insecticide market by the year 2,000 [1]. The scientific benefits of the discovery of novel insecticidal bacteria will be to enhance environmentally safe bio-control practices and will lead to increased food production and post-harvest protection.

The use of *B.t.* as a commercial insecticide is based on its remarkable ability to produce large quantities of insect larvicidal proteins, that may achieve 20-30% of the dry cell mass. The insecticidal proteins, also known as δ-endotoxin, form crystalline inclusion bodies during sporulation [4, 9]. 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[10, 12, 15]. The multitude of ICP genes direct the synthesis of related proteins to form either heterogeneous crystalline inclusion or separate crystals with distinct morphologies.

Isolation and identification of novel *B.t.* isolates by bioassays is a long and complicated process, impeded by repeated isolation of the same strains. On the other hand serotypes of *B.t.* 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, requires minute amounts of DNA, and allows quick, simultaneous screening of many *B.t.* samples, to classify them and predict their insecticidal activities [6, 16]. Several workers [3, 6, 7, 8, 13, 14] have employed PCR in order to screen numerous *B.t.* 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 describe a PCR strategy for extended multiplex rapid screening of field-collected *B.t.* strains that harbor genes from classes *cryI, cryII, cryIII* and *cryIV*. This strategy will enrich the existing arsenal of insecticidal strains, identify novel genes or new combinations of known genes and predict their insecticidal activities. Universal primers were selected from a region that is highly conserved in 20 *cryI*, 3 *cryII*, 9 *cryIII* and 2 *cryIV* genes. Strains with unique PCR product profiles were characterized by performing additional PCR with specific primers described in this paper. Using this method we identified 10 different *cryI*, 3 *cryII*, 8 *cryIII* and 3 *cryIV* genes. Screening by universal primers preliminary assorts the strains for a further screening by specific primers, which produces a PCR product with a unique size for each *cry* gene. Novel strains display unknown *cry* profiles (containing new genes or combinations) should be further characterized by bioassays.

MATERIALS AND METHODS

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

Isolation of *B.t.* from soil samples. Sporeforming strains were isolated according to the acetate selection protocol of Travers *et al.* [17]. An aliquot (0.5 g) of dirt or insect cadavers was added to 10 ml of L broth (per liter: 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl), buffered with 0.25 M sodium acetate which selectively inhibits the germination of *B.t.* spores. The mixture was shaken (250 rpm) in 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 agar plates and grown overnight at 30°C. Random colonies were inoculated into 5 ml T3 medium (per liter: 3 g of tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M sodium phosphate [pH 6.8] and 0.005 g of MnCl) and allowed to sporulate overnight at 30°C. Cultures were checked microscopically for the presence of crystals, which were used as the criterion for *B.t.* 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. One pair of universal (Un) primers was designed for each *cry* class. The primers were selected from conserved regions found by alignment of all the previously described sequences in each *cry* class. For this purpose we used the computer program Amplify 1.0,

that also enabled to test the degree of specificity of each pair of primers to its appropriate *cry* class. Table 1 shows the sequences of the five pairs of universal primers, their locations in each of the gene sequences and the expected sizes of the PCR products.

The specific primers for identification of each *cryI* [13], *cryII*, *cryIII* and *cryIV* were selected from highly variable regions in the genes. Table 2 shows the sequences of the specific primers, their locations in the sequences and the expected sizes of the PCR products. Identification of each of the *cry* genes in a *cry* group was determined by the specific size of the PCR product after amplification with one universal and a mixture of specific primers. To identify *cryIAb* (13) and *cryIVD*, two specific (direct and reversed) primers were required (Table 2). 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 ml 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 & Doly [2].

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 mM 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) controls. 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 *B.t.* strains has been verified by use of the well known *B.t.* strains: *B.t. kurstaki* HD-1 for *cryI* and *cryII* classes, *B.t. tenebrionsis* and *dakota* for *cryIII* class and *B.t. israelensis* for *cryIV* class.

TABLE 1. Characteristics of universal primers for the different *cry* gene groups.

Gene nomenclature			Nucleotide positions	Product
Current	Original	Access. #	hybridized to primers[a]	size (bp)
cry1 universal primers: Un1-D[b]: 5'CATGATTCATGCGGCAGATAAAC-3' Un1-R[b]: 5'-TTGTGACACTTCTGCTTCCCATT-3'				
cry1Aa5	*cryIA(a)*	D17518	2858-2880, 3112-3134	277
cry1Ab9	*cryIA(b)*	X54939	2775-2797, 3029-3051	277
cry1Ac5	*cryIA(c)*	M73248	2781-2803, 3035-3057	277
cry1Ad	*cryIA(d)*	M73250	2778-2800, 3032-3054	277
cry1Ae	*cryIA(e)*	M65252	2861-2883, 3115-3137	277
cry1Ba	*cryIB*	X06711	2919-2941, 3170-3192	274
cry1Bb	*cryI ET5*	L32020	3000-3022, 3251-3273	274
cry1Ca1	*cryIC*	X07518	2851-2873, 3105-3127	277
cry1Cb	*cryIC(b)*	M97880	3061-3083, 3315-3337	277
cry1Da	*cryID*	X54160	2996-3018, 3250-3272	277
cry1Db	*prtB*	Z22511	2973-2995, 3227-3249	277
cry1Ea3	*cryIE*	M73252	2760-2782, 3014-3036	277
cry1Eb	*cryIE(b)*	M73253	2754-2776, 3008-3030	277
cry1Fa2	*cryIF*	M73254	2760-2782, 3014-3036	277
cry1Fb	*prtD*	Z22512	3239-3261, 3493-3515	277
cry1G	*prtA*	Z22510	2802-2824, 3056-3078	277
cry1H	*prtC*	Z22513	3283-3305, 3537-3559	277
cry1Hb	-	U35780	3472-3494, 3726-3748	277
cry1Ja	*cryI ET4*	L32019	2840-2862, 3091-3113	274
cry1K	-	U28801	3318-3340, 3569-3591	274
cry2 universal primers: Un2-D: 5'-GTTATTCTTAATGCAGATGAATGGG-3' Un2-R: 5'-CGGATAAAATAATCTGGGAAATAGT-3'				
cry2Aa1	*cryIIA*	M31738	726-750, 1402-1426	701
cry2Ab2	*cryIIB*	X55416	1444-1468, 2120-2144	701
cry2AC	*cryIIC*	X57252	2695-2719, 3359-3383	689
cry3 universal primers: Un3-5' 5'-CGTTATCGCAGAGAGATGACATTAAC-3' Un3-3' 5'-CATCTGTTGTTTCTGGAGGCAAT-3'				
cry3Aa6	*cryIIIA*	U10985	1367-1392, 1933-1955	589
cry3Ba1	*cryIIIB*	X17123	826-851, 1398-1420	595
cry3Bb1	*cryIIIBb*	M89794	1003-1028, 1575-1597	595
cry3C	*cryIIID*	X59797	1024-1049, 1605-1627	604
cry4 universal primers: Un4-D 5'- GCATATGATGTAGCGAAACAAGCC-3' Un4-R 5'- GCGTGACATACCCATTTCCAGGTCC-3'				
cry4A2	*cryIVA*	D00248	3324-3347, 3738-3762	439
cry4B4	*cryIVB*	D00247	3259-3282, 3673-3697	439
cry7 and *cry8* universal primers: Un7,8-D: 5'-AAGCAGTGAATGCCTTGTTTAC-3' Un7.8-R: 5'-CTTCTAAACCTTGACTACTT-3'				
cry7Aa	*cryIIIC*	M64478	2135-2156, 2535-2554	420
cry7Ab1	*cryIIICb*	U04367	1952-1973, 2352-2371	420
cry8A	*cryIIIE*	U04364	2027-2048, 2430-2449	423
cry8B	*cryIIIG*	U04365	2015-2036, 2418-2437	423
cry8C	*cryIIIF*	U04366	2018-2039, 2421-2440	423

[a] The number 1 indicates the first base of the sequences as it appears in GenBank database.

[b] D and R indicate direct and reverse primers respectively.

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TABLE 2. Characteristics of specific primers for each of the different genes.

Primer pair	Sequence	Gene recognized	Positions[a]	Product size (bp)
Un2- D	GTTATTCTTAATGCAGATGAATGGG	*cry2Aa1*	726-750	498
EE-2Aa	GAGATTAGTCGCCCCTATGAG		1203-1223	
Un2- D	GTTATTCTTAATGCAGATGAATGGG	*cry2Ab2*	1444-1468	546
EE-2Ab	TGGCGTTAACAATGGGGGGAGAAAT		1965-1989	
Un2- D	GTTATTCTTAATGCAGATGAATGGG	*cry2AC*	2695-2719	725
EE-2Ac	GCGTTGCTAATAGTCCCAACAACA		3396-3419	
Un3- D	CGTTATCGCAGAGAGATGACATTAAC	*cry3Aa6*	1367-1392	951
EE-3Aa	TGGTGCCCCGTCTAAACTGAGTGT		2294-2317	
Un3- D	CGTTATCGCAGAGAGATGACATTAAC	*cry3Ba1*	826-851	1103
EE-3Ba	ACGAAAGATTCTGCTCCTAT		1909-1928	
Un3- D	CGTTATCGCAGAGAGATGACATTAAC	*cry3Bb1*	1003-1028	1103
EE-3Ba	ACGAAAGATTCTGCTCCTAT		2086-2105	
Un3- D	CGTTATCGCAGAGAGATGACATTAAC	*cry3C*	1024-1049	461
EE-3C	ATTTTGGTACCTCCTGTACCCACC		1461-1484	
EE-7Aa	GCGGAGTATTACAATAGAATCTATCC	*cry7Aa*	1639-1664	916
Un7,8-R	CTTCTAAACCTTGACTACTT		2535-2554	
EE-7Aa	GCGGAGTATTACAATAGAATCTATCC	*cry7Ab1*	1456-1481	916
Un7,8- 3'	CTTCTAAACCTTGACTACTT		2352-2371	
EE-8A	GAATTTACTCTATACCTTGGCGAC	*cry8A*	1771-1794	679
Un7,8-R	CTTCTAAACCTTGACTACTT		2430-2449	
EE-8B	GACCGCATCGGAAGTTGTGAG	*cry8B*	1663-1683	775
Un7,8-R	CTTCTAAACCTTGACTACTT		2418-2437	
EE-8C	GGTGCTGCTAACCTTTATATTGATAG	*cry8C*	1930-1955	511
Un7,8-R	CTTCTAAACCTTGACTACTT		2421-2440	
EE-4A	GGGTATGGCACTCAACCCCACTT	*cry4A2*	2234-2256	1529
Un4-R	GCGTGACATACCCATTTCCAGGTCC		3738-3762	
EE-4B	GAGAACACACCTAATCAACCAACT	*cry4B4*	1747-1770	1951
Un4-R	GCGTGACATACCCATTTCCAGGTCC		3673-3697	
EE-11A-D	CCGAACCTACTATTGCGCCA	*cry11A1*	111-130	445
EE-11A-R	CTCCCTGCTAGGATTCCGTC		536-555	

[a] The number 1 indicates the first base of the sequences as it appears in GenBank database.

RESULTS AND DISCUSSION

Several hundreds field-collected samples were isolated in Israel, Uzbekistan and Kazakhstan; of these, about 190 spore forming *B.t.* isolates were identified by the *cry* universal (Un) primers (Table 1). 110 *cry* containing isolates were further specified by the appropriate specific primers (Table 2). They displayed 20 distinct *cry*-type gene profiles (Table 3). Based on their *cry*-type gene content, they can be divided into 7 groups with different potential larvicidal activities (Fig. 1). Several of the isolates (i.e. profile No. 7, 8, 12, 14 and 20) contain new combinations of *cry* genes, with promising potential properties to be utilized as biological control agents. These new isolates have the potential to be effective biopesticides of insects for which biological control agents do not exist. For example, Hodgman *et al.*, (11) have isolated a strain of *B.t.* toxic to the common housefly, *Musca domestica*. That strain produces CryIA(b), CryIB and CryIIA endotoxins.

TABLE 3. Distribution of *cry*-type gene profiles of *B.t.* isolates collected in Israel, Kazakhstan and Uzbekistan

cry-type gene profile	Number of isolate(s)	Predicted insecticidal activity
1. *cryIAa*	2	Lep.
2. *cryIAa, -Ac*	1	Lep.
3. *cryIAa, + cryIIA, -B*	2	Lep. + Dip.
4. *cryIAa, -Ab, + cryIIB*	1	Lep.
5. *cryIAa, -Ac, + cryIIA, -B*	7	Lep. + Dip.
6. *cryIAa, -Ab, -Ac, + cryIIA, -B*	5	Lep. + Dip.
7. *cryIAa, -Ab, -D + cryIIB*	1	Lep.
8. *cryIAb, -D + cryIIB*	4	Lep.
9. *cryIAb, -Ac, -D + cryIIB*	2	Lep.
10. *cryIAb, -Ac, -D + cryIIA, -B*	1	Lep. + Dip.
11. *cryIAc + cryIIA, -B*	3	Lep. + Dip.
12. *cryIAa, -C, -D + cryIIB + cryIII*	8	Lep. + Col.
13. *cryIAa, -Ab, -C, -D + cryIIB + cryIII*	8	Lep. + Col.
14. *cryIAb, -D + cryIIB, -C + cryIII*	14	Lep. + Col.
15. *cryIIA, -B*	1	Lep. + Dip.
16. *cryIII*	2	Col.
17. *cryIVA, -D*	6	Dip.
18. *cryIVB, -D*	2	Dip.
19. *cryIVA, -B, -D*	36	Dip.
20. *cryIVA, -B, -D + cryIII*	4	Dip. + Col.

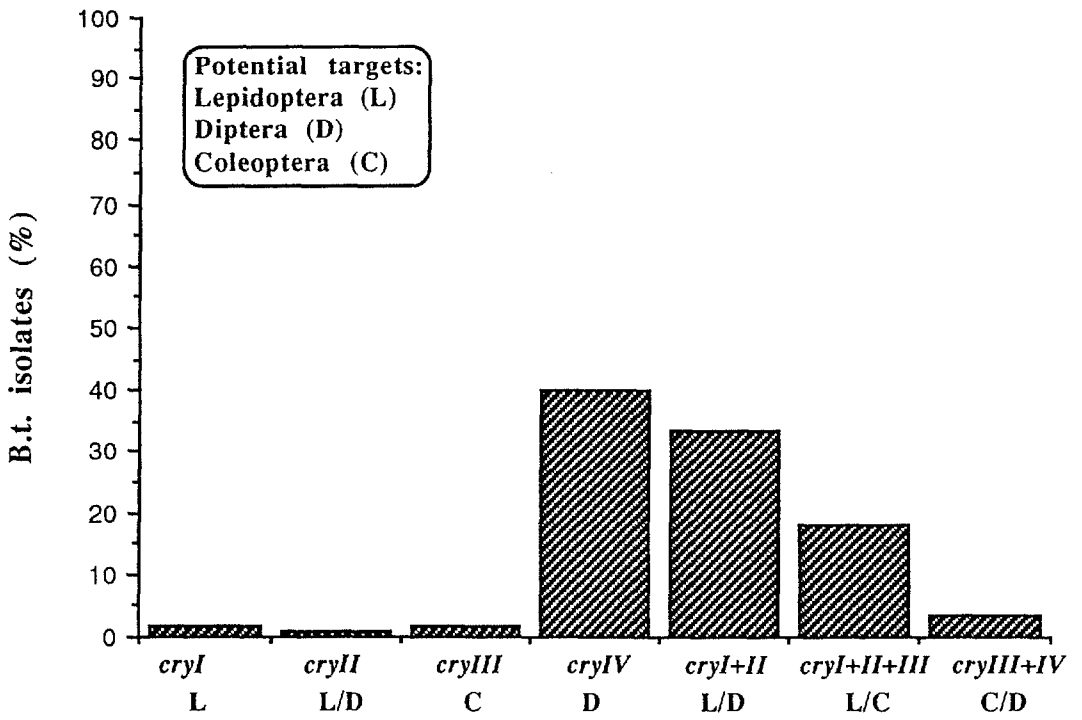

Figure 1. Distribution of *cry*-type gene general profiles of *B.t.* isolates which identified by universal primers.

We did not find any strain that harbors *cryIIIA, -B, -D* gene. However, we did 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 and EE-8C (Table 2). 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 univeral primers (Un1 and Un4). Further analysis revealed that it was obtained with the direct primers Un1-D and Un4-D (Table 1). This isolate also produced another, minor PCR product, of ~400 bp (Fig. 2), with the reverse primer Un4-R (Table 2) alone. Further efforts to identify this crystal forming strain are now in process.

Figure 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 (l-*Hin*dIII); line 2, strain DL-1 (Un1-D and Un4-D primers); line 3, strain DL-1 (Un4-R primer alone); line 4, B.t. *israelensis* 4Q5 (Un4-D and Un4-R primers of *cryIV* genes); line 5, B.t. *kurstaki* HD-1 (Un1-D and Un1-R primers of *cryI* genes);

Eighty of our new spore and crystal forming isolates, did not contain any known *cry* genes. The absence of PCR products might be indicative of a novel strain of *B.t.* with an unusual toxin gene(s). Such isolates should be further analyzed for homology to known toxin genes by using additional primers. However, crystal forming bacteria do not necessarily harbor any toxin gene(s) and thus are of no interest for biological control of pests.

Novel toxins may be recognized in our new isolates by comparing the PCR products, that identify known genes, with the size and number of the proteins detected on gels. Novel toxins might also be identified microscopically by the presence of a new shape(s) of crystal(s).

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 3). These are being tested against pests belonging to "traditional" and other orders, such as orthopterans.

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Novel *cry*-Type Gene Detected by Extended PCR Screening from Field-Collected Strains of *Bacillus thuringiensis*

E. Ben-Dov[1], E. Dahan[1], A. Zaritsky[1], R. Sinai[1], R. Manasherob[1], Z. Barak[1], A. Khameraev[2], A. Troyetskaya[2], A. Dubitsky[3], N. Berezina[3] and Y. Margalit[1]

[1] Department of Life Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Be'er-Sheva 83105, [2]Institute of Zoology, Uzbek Academy of Science, Uzbekistan and [3]Institute of Zoology, Kazakhstan Academy of Science, Kazakhstan

The Polymerase Chain Reaction (PCR) allows quick screening of many samples of *Bacillus thuringiensis* (B.t.), to classify them and predict their insecticidal activities. It can be used to identify new combinations of genes and discover strains containing novel genes.

One pair of **universal** (Un) primers was prepared for each *cry* class:

(i) cryI-A(a), -A(b), -A(c), -A(d), -A(e), -B, -C, -C(b), -D, -E, -E(b), -F, -ET4, -ET5, -prtA, -prtB, -prtC, -prtD, which amplifies about 275 bp each;

(ii) cryII-A, -B, -C-about 700 bp each;

(iii) cryIII-A, -B, -B(b), -D - about 600 bp each;

(iv) cryIII-C, -C(b), -E, -F and *-G* - about 420 bp each;

(v) *crylV-A, -B* - 439 bp each.

Positive isolates were screened by a set of **specific** primers, each generating a unique-size fragment for each of the known *cry* genes (of groups *I-IV*). The size(s) of the PCR product(s) indicate presence of particular crystal protein gene(s).

Several hundred field-collected samples from soil, dust, dead insects and store products were isolated in and around storage facilities in Israel, Uzbekistan and Kazakhstan; of these, about 150 spore forming B.t. isolates were identified by the first group of primers, and specified by the second. They displayed 18 distinct *cry*-type gene profiles.

One B.t. isolate (DL-1) produced an unexpected PCR product of about 1,800 bp with a mixture of two pairs of the universal primers *(cryI* and *cryIV)*. Further analysis revealed that it was obtained with primers Un1-5' (primer for *cryI)* and Un4-5' (primer for *cryIV)*. In addition, this isolate was positive with *cryIVD* (a pair of specific primers which amplify 445 bp). The novel gene discovered has been isolated; it will be sequenced and its encoded protein characterized.

The new gene and its product may have a promising potential for biological control of susceptible insects and resistance management.

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APPENDIX

APPROVED

Professor D.A.Azimov, Director
INSTITUTE OF ZOOLOGY
Uzbek Academy of Sciences
Corresponding Member of Uzbek
Academy of Sciences

Date 08 August 1996

FINAL TRIENNIAL REPORT

PROTECTION OF POST-HARVEST GRAINS BY NEW ISOLATES OF SPORE-FORMING BACTERIA

within the co-operative American-Israeli-Uzbek program for 1993-1996

Head of the project for Uzbekistan:
Dr. Khamraev A.Sh., Dr.Biol.

Investigators:
Dr. Khamraev A.Sh., Dr.Biol.
Prof. Troitskaya E.N.

GOALS. The goals of the study are obtaining cultures of entomopatogenic sporous bacteria, assessment of a possibility to use them for the biological control of the pests of grain and products made of it when stored, and revealing of a genetic marker of pathogenecity (toxitype) of the cultures for various pest species.

To that end, we:

1) surveyed the sites of mass breeding and development of the insect pests of stored food in Uzbekistan;

2) collected samples of the biological material in the form of sick, dead and healthy pest individuals and the samples of the food stored;

3) identified the species composition of the insect pests;

4) isolated the cultures of the sporous bacteria of various species from the samples of the biological material;

5) identified their belonging to the bacteria of the group *Bacillus thuringiensis;*

6) studied on the collection of isolated entomopathogenic bacteria of the group *Bacillus thuringiensis* and preserved it using various techniques.

7) the investigators of the Ben-Gurion University of the Negev (Beer-Sheva) made a genetic analysis of isolated cultures to identify the range of the toxicity on the insects belonging to various species, and identified the genetic markers of the toxins *Bacillus thuringiensis.*

I. THE CONTENT OF THE WORK DONE

1. Introduction (theoretical rationale)

Big supplies of grain and other products made of it annually stored in elevators and storages are heavily affected by noxious insects, bacteria, fungi, rodents, etc. The most dangerous of them are as follows: *Sitophilus granarius L., S. oryzae L., Sitotroga cerealella Oliv., Corcyra cephalonica Staint., Ephestia elutella Hbn., Ephestia kuchniella Zell., Pyralis farinalis L., Rizopertha dominica F.,* etc. Due to high ferundity and quite short development time of one generation, they reach high numbers in a shot period of time.

The losses inflicted by the pests of stored food are extremely high and can reach 50-80% (1).

Protection of stored food from pests is extremely burning problem. The attempts to resolve it by using chemicals do not produce desirable and stable results due to high natural and acquired resistability of the insect pests to the insecticides used. Besides, repeated application of the chemical drugs contaminates environments and creates the danger of their accumulation in food and severely aggravates sanitary conditions.

The descrease in the efficacy of the drugs, accumulation of the drugs in the stored food, environmentel pollution, increase in the hazard of human and animal intoxication make specialists of varions countries look for new, ecologically friendly means of pest control.

And one of such methods is the biological one, based on application of the entomopathogenic bacteria, viruses, fungi protozoans and other predatory and parasitic organisms. The application of these organisms enables controlling the numbers of the pests on economically safe levels.

In thes connection, the goals of our studies are revealing of cultures of the entomopathogenic sporeus bacteria as posible agents of the microbiological control of the stored-grain pests and identification of toxitypes of the isolated strains based on the genetic analysis of their chromosomes.

II. SURVEY OF LITERATURE

As ealy as ancient times humans encountered insects affecting stored food. Excavations of ancient tombs has shown that insects affected grain as early 3 century B.C. (2). However, it was Linney (3) who first described insect pests. He described the insect body, way of feeding, nature of harm done and gave latin names to more than 30 species of pests.

In the following two a half centures the knowledge of species compasition, ecology, biology, harmfulness of the pest has been enriched by thousands of works in the various parts of the globe.

According to FAO the annual losses of grain caused by pests reach 65 million tonnes. This amount of the grain resourses is enough to feed, e.g, the population of Africa in a year's time (4). In England, over 10% of grain is lost after harvesting due to pest and the quality deteriorates. In India, pests destroy 60-65% of wheat and 45-55% of rice (6).

The data of literature document that in 27 states of the USA infestation of wheat by pests reaches 20-26%, maize, 78-82%, oats, 56-66%. In Canada, infestation of crops by pests in farms and elevators reaches 42 and 52%, respectively (7), Studies conducted by American entomologists of agricultural service of the US Department of Agriculture witness that upon heavy infestation of wheat and oats by *Sitophylus oryzae L.* and *Rhizopertha Dominica Fabricius* and up to 80% of these crops can

be destroyed within two months (8). Similar results were obtained in the former Soviet Union (4, 9, 11, 18, 25, 27, 19, 30, 34 etc.).

In the Phillippines, the losses due to pests reached 42,5% in central grain storages within a year. In China and Thailand the losses constitute 24 and 13%, respectively (10).

Taking into consideration that losses inflicted by pests increase southwards, its preservation of the grain in Centrl Asian states is of special importance, since climatic factors - many days with high temperatures, etc. - promote the development of the parasites. Besides, the grain contaninated with excrements and larvae skin cause malfunction of human organs, changes in blood composition and other pathological changes.

In spite of heavy losses in Uzbekistan, many questions connected with the pests remain unstudied, as yet. The earliest records of the pest study in Uzbekistan date back to 1930 (11). Much attention has been paid to the study on species composition, level of harmfulness and means of mechanocal and chemical control, as wel as the biology of individual species (17, 18, 47).

As a result it had been established by 1980 that 110 pest species belonging to 59 genera and 22 families were encountered in the storages of Central Asia. Twelve species are new to science and 48 spp. were for the first time recorded in the region. Thus, the studies of demonstrated that the present-day fauna insects and mites affecting stored grain is diverse. Many species, being hihly plastic, inhabit not only storages, but natural habitats. Most dangerous of them are as follows: *Acarus sizo L.*, *Tyrophagus putrescentiae (Schrank)*, *T. asiaticus Volgin in litt.*, *Caloglyphus rodionovi Zachv.*, *Acotylodon turanica A.Z.*, *Gohieria fusca Oudms.*, *Niptus hololeucus Fold.*, *Stegobium paniceum L.*, *Ptinus fur L.*, *Dermestes frischi Kugel.*, *Trogoderma Versicolor G.*, *Tenebrioides mauritanicus L.*, *Oryzaephilus surinamensis L.*, *Tribolium castaneum Herbst.*, *Sitophilus granarius L.*, *S. oryzae L.*, *Nemapogon granellus L.*, *Ephestia elutella Hb.*, *Plodia interpunctella Hb.* and *Pyralis farinalis L.* Acaroid mites also cause heavy losses to grain (11). Their species composition and biology are studied in-depth in Uzbekistan (25, 26, 27, 28, 29). Also, about 30 insect species which inhabit storages were not found to be able to affect grain,

however their excrements and larvae skim deteriorate the quality of grain. It is belived that these species were introduced into Central Asia from the northern region of the former Soviet Union.

A comparative assessment of the grain biocenoses in both storages and the envirenments is provided; ecological and faunistic complexes are revealed and the biology of the most important insect pests and mites is studied; a seasonal dynamics of the pest numbers upon different way of grain storing is clarified and their harmfulness analysed.

The biological consists of prophylacties and destruction of the pests (30, 31), but the development of the biological method of control is impossible without the study or the range of the agent of biocontrol in all areas of the pests. This is why it is recessary to set up specialized stations on identification of the species composition and the dynamics of pest development in various types of storages. No such stations are available in Uzbekistan at present.

Based on the publications available, one may have an impression that the qualitative and quantitative composition of the pests has not changed for years, but, actually, the value of the species composition and numbers of the pests constantly change both due to the type of the food stored and to the changes of the ecological situation. Therefore, there is a necessity for the storages to be surveyed by qualified specialists who have a thorough knowledge of the species composition, biological peculiarities of the pests, and a complex of factors affecting heir growth and breeding.

Research stations located all over the world could play a rather important role in amassing information and exchanging agents of the biocontrol, entomophages and entomopathogenic microorganisms.

For surveys, it would be desirable to select storages most typical for specific regions where complete screening should be made.

The experiense of the work of such station will be highly valuable in that the amassment of long-term data on the species composition of the pests, their location, peculiarities of biology and dynamics of accumulation of the most noxious species, etc., would enable conducting systematic work using veterinary, sanitary and biological methods and developing systems of their

application.

In the past decades, in Uzbekistan and other Central Asia states, there is is an increased interest to alternative forms of plant protection. The method that holds mush promise is biopesticidal used as insecticides, herbicides or fungicides, in which the active egent is a microorganism or a substance of its vital activit. However, in the past, the biopesticides often were unreliable and expensive. Besides, there were difficulties with obtaining necessary amounts of high-quality drugs. As present, there are three key points which can enable improvement of the biopesticides and making them a real alternative to chemical plant protection. These are : (i) a growing onterest of leading chemical concerns, such as Abbot Sandoz, ICI, Shell, development of new biopesticides; (ii) emerging of new markets where new biopesticides can be sold; and (iii) currently, new echnologies and research activities are based on appearance of new scientific development, lines and methods. For example, genetic engineering enable, obtaining microorganisms (bacteria, fungi and viruses) withpreliminary set properties, hence improving their potential activity, and obtaining plants resistant to insects, etc. Besides new methods ofproduction of preparations with the aim of their improvement have been developed.

Discussed are the problems of the production of pesticides further development of their production and the problems of their marketing (32, 33).

In the light of above-mentioned material, the work on the collection of specific agents of biocontrol should be conducted taking into consideration resistability of the pests to them, i.e. agent that can overcome the resistance of the pests. Finding of an active agent that well develops in specific ecological conditions wich enables elimination of the harm done to stored food. Thi new line opens a broad avenue, but, however, it should not be overestimated, since the range of entomopathogenic microorganisms is smaller than the range of entomophages, parsites and predators of insects. The application of endemic entomophages is also worthypaying attention to, and this question has never been studied on, as yet, in connection with storage pests.

It is known that introduction of specific natural enemies (entomophages and entomopathogenic microorganisms) from the

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natural habitats more frequently provides successful control. It appears that for successful inhibition of the storage pests it is necessary to use the, whole complex of specific entomophages and entomopathogenic microorganisms, whose interrelation imroves their controlling effect on the psts. Besides, domination of certain species in the complex and their efficacy may vary in adverse ecological situations.

Skilled specialists, such as virologists, protozoologists, entomologists and others should take part in the search for entomopathogenic microorganisms and entomophages.

The most noxious pests of stored food are now widespread all over the globe and the development therefore of the methods of biological control is a relevant problem everywhere in the world.

III. MATERIAL AND METHODS

The studies were conducted at the Departmen of General and Experimental Entomology of the Institute of Zoology, Uzbek Academy of Sciences from August 1993 to August 1996. The material was collected as described by Veizer (45) and Evlakhova et Shvetsova (36) including preservation, labelling and trasporting of sick and dead pests. The biological material was collected after repeated surveys of mass breeding sites of the pests of stored grain and products made of it. The surveys took place in various plants connected with grain processing and in the farms located in different regions of Uzbekistan and in Tashkent. Surveyed were elevators, milling plants, mills, race-dryers, storges belonging to collective and state farms, as well as individual storages, etc. Sick, dead insects of various species, samples of stored producte, spilled grain and flour, soil, pices of scraped surface of walls, wooden and iron constructions were collected. All the surveys connected with infection of the products with noxious insects were conducted according with the guidelines of the State Committee for Supplies. The methods by Krashkevich and Gubergrits (34) were used while taring samples. The levels of infection of grain and other products made of it were identified using State Standard 10841-64 (35).

Identification of the pests was made using routine techniques (11, 42, 43) on the basis of the morphological and biological traits of the pests. Isolation of the microorganism cultures was conducted using common methods (37, 38, 39). Bacteriological studies were conducted as follows:

1. Visual survey of the insects, grain and other products made of it with the aim of revealling of diseases (or dead insects);

2. Examination of the specimens using a magnifier and decsription of external signs of diseases;

3. Preparation of homogenates from dead individuals;

4. Preparation and microscopy of native and stained smears trom the insect homogenates;

5. Plating of the homogenates ti the Petri dishes containing nutritive media, incubation of the dishes in thermostate at 28-32°C for 48-72 hours (beginning from 1995, the method by Margalit et Brownbridge (40, 41) has been used in isolatio of *Bacillus thuringiensis*);

6. Replating of the monocultures into tubes with slant Peptone or beef-extraet agar.

7. Study on the morphological and cultural-biochemical properties of the isolates;

8. Conservation of th pathogenic strains in a collection using periodical replatings and stripes of the filter paper.

Isolation of sprous entomopathogenic bacteria of the group *Bacillus thuringiensis* was conducted using two methods - the one as described by Brownbrige et Margalit (40, 41) and the other as described by Troitskaya (43). To isolate of the spore-forming bacteria, the homogenates of the insects processed by the methods of Brownbrige et Margalit (1986, 1987) were plated on the nutritive agar and after incubating in thermostate were used in obtaining the monocultures *Bacillus thuringiensis*. To isolate spore-forming bacteria from the grain stored, washings of the grain and flour mixtures were also plated into the Petri dishes containing nutritive medium.

According to the technique by Troitskaya (43), the homogenate of sick and dead insects was without preliminary incubation with the solution of PBS was plated on the surface of the nutritive agar and the plating was then incubated in the thetmoctate at 28-30°C for 48-72 hours. After the incubation the

Petri dishes were incubated and the smears were made from the colonies whish were closes in morphology to *Bacillus thuringiensis*. If the crystal (endotoxin) was available, the culture from the colony was then replated to the slat nutrient agar and used in further studies.

The studies on the collected cultures were conducted using routne techniques (46). After the cultures had been replated to the fresh nutritive agar, the morphology of the culture and changes taking place in the course of maintenance and replating were recorded.

IV. RESULTS OF THE STUDIES

Over 50 visits to the sites of mass breeding of stared-grain pests were launched and 142 surveus of the storages, grain storages, milling plants, mills, grain crushers, rice driers, elevators and other habitats of the pests were undertaken (Table 1).

Table 1. **Data on the number of surveys of the sites of mass breeding of the storage pests.**

Areas	*Number of surveys*						
	Stora-ges	*Eleva-tor*	*Mill*	*Grain stora-ge*	*Rice drier*	*Mil-ling plant*	*Grain cru-sher*
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Tashkent region	15	-	7	7	5	-	4
Tashkent	13	6	-	11	5	6	5
Bukhara region	14	-	1	8	4	-	-
Kashkadarya region	11	-	6	9	1	-	-
Israel (Lakit El Sana) ***	-	-	-	4	-	-	-
T o t a l:	**53**	**6**	**14**	**39**	**15**	**6**	**9**

As a resuly, collected are 147 samples of the biological consisting of sick, dead and healthy insects, as well as the samples of the sored grain.

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4.1. Survey of the Fauna of Insect in Storages of grain and food made of it.

The fauna of insects in the storages of grain and products made of it in the ity of Tashkent, Bukhara and Kashkadarya regions has been studied by us since 1993. The analysis of the material collected during the surveys of the abovementioned habitats of the insect pests (Table 1) has demonstrated that most insects belong to 13 species belonging to 8 families, namely, *Curcutionidae, Tenebriodidae, Cucujidae, Anobiidae, Bruchidae, Gelechiidae, Tineidae* and *Pyralididae* (Table 2).

Table 2. **The fauna of insect pests of grain by types of habitats**

Families and species	*Ditribution over habitats*						
	Grain storages	*Mills*	*Grain crushers*	*Rice driers*	*Elevators*	*Milling plants*	*Home kept storages*
1	2	3	4	5	6	7	8
I. Curculionidae							
1. *Sitophilus granarius L.*	++++	+++	++	+	+++	++	++++
2. *S. oryzae L.*	+++						
II. Tenebrionidae							
3. *Tribolium confusum Duv.*		+++			++	++++	++++
4. *Tenebrio molitor L.*	+++	++	++		++++	++	++
III. Cucujidae							
5. *Laemothocus testaceus F.*		+				+++	+++
IV. Anobiidae							
6. *Stigobium paniceum L.*	++++	+++	++	++	+++	+	++++
V. Bruchidae							
7. *Bruchis pisorum L.*	+++		++				+++
8. *Acanthoscelides obtestus S.G.*	+++		++				+++

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1	2	3	4	5	6	7	8
VI. Gelechiidae							
9. *Sitotroga cerealella Oliv.*	++++	++++					
VII. Tineidae							
10. *Nemapogon granellus L.*	+++				++	+++	++
VIII. Pyralididae							
11. *Ephestia kuchniella*	+++	++	+	++	+	++	+++
12. *Pyralis farinalis L.*							
13. *Plodia interpunctella Hb.*	+++	++	++	++	++	+++	++++

Notes: ++++ - abundant species
+++ - spread species
++ - less spread species
+ - sole species

The analysis of the material collected in sites of mass breeding of insects has shown that individual species reach high numbers (abundant specis), whereas other species are less abundant, or rare.

4.2. Bacteriological analysis of the biological material.

Of 847 samples of the biological material collected in the sites of mass breeding of the pests, 272 samples were used in the bacteriological studies (Table 3).

Table 3. **Data on the material processed and collected**

Samples	*Total collected*	*Processed*
Ephestia kuchniella	62	17
Sitotroga cerealella	48	16
Acanthocelidus obtestus	31	12
Sitophillus granarius	74	27
Bruchus pisorium	35	11
Sitophillus oryzae	51	18
Stigobium paniceum	69	19
Nemapogon granellus	53	18
Tribolium confusum	42	15
Lacmothoeus testaceus	81	9

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Samples	*Total collected*	*Processed*
Plodia interpunctella	59	21
Pyralis farinalis	35	12
Tenebrio molitor	37	11
Home-kept flour	29	6
Wheat	50	12
Rice	17	10
Peas	19	10
Haricot	15	9
Oats	17	6
Millet	10	5
Spilt products (Israel)	11	4
Dry bread	2	2
T o t a l:	**847**	**272**

As a result of bacteriological studies, isolated are 50 cultures of the entomopathogenic crystal-formig bacteria of the group *Bacillus thuringigensis* (Table 4).

Table 4. **Sources of isolation of the sporous bacteria of the group *Bacillus thuringigensis*.**

Name of sample	*Number of strain*
Sitophilus granarius L.	5, 12, 13, 14, 31, 33, 41
S. oryza L.	4, 32, 15, 44
Tribolium confusum Duv.	9, 17, 18, 50
Tenebrio molitor L.	-
Laemothoeus testaceus F.	-
Stigobium paniceum L.	19, 20, 21, 41
Bruchus pisorum L.	-
Acanthoscelides obtestus Say	27, 42
Sitotroga cerealella Oliv.	1, 2, 34, 43
Nemapogon granellus L.	3, 6, 7, 8, 25
Ephestia kuchniella Zll.	10, 11, 22, 23, 26, 28, 29, 46, 47
Pyralis farinalis L.	16, 36, 37
Plodia interpunctella Hb.	24, 38, 48
Spilt products from a Beduin settlement	30, 35
Washings of peas	40, 45, 49
Washings of wheat	39
T o t a l:	**50 strains**

The data of the Table 4 also show that from the different insect species and samples different numbers of the strains of *Bacillus thuringiensis* were isolated. However, these data have

only a relative character, since the inoculation of the insect homogenates and washings of the food samples produced much more colonies of *Bacillus thuringiensis* and, while sifting cultures from some colonies, we were quided by the presence of the morphological features of the colonies or cultures (size and form of the vegetative cells, spores and crystals, rate of the sporulation beginning, presence of involutionary cells, cellular detritus, etc.). The data analysis also shows a different natural level of infection of the pests of stored food by the bacterion of the group *Bacillus thuringiensis* (Table 5). A comparative analysis of the results of isolation of *Bacillus thuringiensis* strains from various species of insects and stored food has demonstrated that they belong to different toxitypes.

Table 5. **A Comparative Analysis of the Samples of Biological Material by their level of infection by the Bacteria of the Group *Bacillus thuringiensis***

Name of sample	Number of samples		
	processed	containing *Bacillus thuringiensis*	% level of infection
Sitophilus granarius	27	8	33,7
Sitophilus orizae	18	4	22,2
Tribolium confusum	15	4	26,6
Tenebrio molitor	11	-	-
Laemothoeus testoceus	9	-	-
Stigobium paniceum	19	4	21,0
Bruchus pisorium	11	-	-
Acanthoscelides obtestus	10	-	-
Sitotroga cerealella	16	2	12,5
Nemapogon granellus	18	5	27,8
Ephestia kuchniella	17	7	47,0
Pyralis farinalis	12	-	-
Plodia interpunctella	21	1	4,7
Home-kept flour	6	-	-
Wheat	12	1	8,3
Rice	10	-	-
Peas	10	3	30,0
Haricot	9	-	-
Oats	6	1	16,6
Millet	5	-	-
Spilt products (Israel)	4	2	50,0
Dry bread	2	-	-
Dust samples (Israel)	1	-	-
T o t a l:	**272**	**40**	**14,3**

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The data obtained have shown that samples containing the highest numbers of *Bacillus thuringiensis* belong to *Sitophilus granarius*, *Nemapogon pranellus*, *Ephestia kuchniella* and *Agobium paniceum*, as well as the samples of oats and dust in a storage of the Beduin settlement. In other samples, namely, *Sitophillus oryzae*, *Tribolium confuzum*, *Sitotroga cerealella*, *Plodia interpunctella*, wheat and peas, the number crystal-forming bacteria was lower (5,2 to 20%). No bacteria *Bacillus thuringiensis* were isolated from *Tenebrio*, *Laemothloens testaceus*, *Bruchus pisorium*, *Acanthocelides optestus*, *Pyralis farinalis*, as well as from the samples of flour, race and haricot in the period of studies.

A comparative analysis of the methods of *Bacillus thuringiensis* isolation conducted in Israel by Brownbrige et Margalith (1986) and that conducted in Uzbekistan by Troitskaya (1983) was made upon isolation of the spore-forming entomopathogenic bacteria. This showed that direct inoculation of the homogenates on the plates with nutrient agar produces greater variety of biotypes and toxitypes of *Bacillus thuringiensis* (Table 6).

Table 6. **The Conposition of the Biotypes and Toxitypes of *Bacillus thuringiensis* upon different methods of isolation**

Methods tested	*Characteristic of cultures isolated*	
	biotypes	*toxitypes*
Brownbrige et Margalit (1986)	-	CryIII, CryIV
Troitskaya (1983)	Lez^+ $Sach^+$ Gl^+ Man^+, Sal^+,.... Lez^- $Sach^-$ Gl^- Man^-, Sal^-,....	CryI, CryII, CryIII, CryIV

Note: Toxitypes were determined at Ben-Gurion University of the Negev, Israel. Strains isolated in Uzbekistan within the 1st and 2nd toxitypes have about 10 variants of the combination of toxin fragment (Table 7).

Table 7. **Combination of fragments* within 1st and 2nd toxitypes of Uzbek strains**

Number of strains *Bacillus thuringigensis*	toxitypes			
	Cry-I	Cry-II	Cry-III	Cry-IV
1	2	3	4	5
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 21, 23, 27	+AbD	+B, C	+	-
12, 26	+Aa, Ab, D	+B	+	-
13	+Aa, Ac, D	+A, B	+	-
15, 16, 19, 24, 25	+AC	+A, B	-	-
20, 22	+Ab, D	+B	-	-
28, 29, 30, 32, 34, 35, 39, 40	+Aa, C, D	+B	+	-
31		+B, C	-	-
36, 37, 38, 41	Aa, Ab, C, D	+B	+	-
**41, 42, 43, 44, 45, 46, 47, 48				+A, B, D

Note: * Data of the fragmentry composition of the crystals of the 1st and 2nd toxitypes were obtained from BGU, Israel. The data on the fragmentary analysis of the 3rd and 4th toxitypes are not available.

** Strains N 41 to 48 were isolated in Israel from soil samples collected in Uzbekistan in 1995.

The results of the genetic analysis of the cultures isolated by us in the corse of our study demonstrates that among above - mentioned strains all currently known toxitypes are available, whereas the isolates obtained using the other above - mentioned technique bilong to the 3rd and 4th toxitypes. However, there is no reason to state that the cause for the diversity of toxitypes isolated from the biological materials collected in Uzbekistan is in the use of a different method of isolation. To reveal the cause for such a result, it is necessary to conduct special studies.

All the isolated cultures of the crystal-forming entomopathogenic bacteria of the group *Bacillus thuringiensis* are preserved in the collection using periodical reinoculations on

stripes of filter paper (the method by Pasteur Institute, Paris) and on dry slants nutrient agar. These were made once six months. After 96 hors of incubation at 28-30°C, all the cultures were studied under microscopy and their state discribed; presence of spores, crystal toxins involutionary cells, etc. It was found out that 1,2% of the cultures, upon storing using periodical reinoculations, completely or partly lose their ability to form crystals of endotoxin and 0,3% of the cultures begin forming involutionary forms as elongated worm-like cells, and very thin or inflated cells.

At present, while implementing the studies on the grant "Protection of post harvest grain by new isolates of spore forming bacteria" we submitted to professor J.Margalith 50 cultures of *Bacillus thuringiensis* isolated from the pests of grain and products made of it, as well as from the samples of products kept in the storanges of Uzbekistan and 10 samples of the biologycal material being the source for obtaining *Bacillus thuringiensis* strains. The biological material comprises pests of storages such as Agrotis segetum Den et Schiff and Helicoverpa armigera Hb. and other specimens.

The strains were submithed in the state of vegetetive growth and sporulation on the slant triptose agar. The microscopy of the smears demonstrated absence of foreign microflora and involutionary forms.

While on the business trip to BGU, Israel we prepared 26 cultures for the study on the insecticidal activity and DNA structure upon the determination of toxitypes. We are grateful to the specialists of BGU for their expert advice (handling of 26 cultures is described in the scientific report of 1995).

On the basis of the genetic analysis of the crystals of Uzbek isolates of *Bacillus thuringiensis* obtained by the investigators of BGU, Beer-Sheva, Israel, we think it reasonable to patent Uzbek isolates as having a wider range of activity on the insect pests belonging to various species after identification of biotypes and obtaining a registration number in one of the collections of microorganisms (e.g.m Moscow, Paris, Israel, etc.).

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Conclusions

1. As a resul of 50 visits and 142 surveys of stored grain places, 847 samples of the biological material comprising dead and sick insects pests, samples of stored food, spilt products and dust of storages, etc;

2. In Uzbekistan, the most abundant insect pests of stored grain belong to eight families: *Curculionidae, Tenebrionidae, Cucujidae, Anobiidae, Bruchiidae, Gelechiidae, Tineidae* and *Pyralididae*;

3. Stored grain pests inhabit grain storages, milling plants, elevators, rice-driers and ither grain-processing facilities; and outside them, in home storages, in subsidiary premises of shops, canteens, etc. These and natural habitats may prove a permanent source for contamination of grain and other products made of it upon storing;

4. Insect pests found are poliphages and pests of most plantproducts stored.

5. As a result of isolation of 50 strains of entomopathogenic bacteria *Bacillus thuringiensis*, a difference in spread of this microorganism among various pests. *Bacillus thuringiensis* is most frequent *Sitophilus granarius, Stigobium paniceum, Ephestia kuchmiella and Nemapogon granellus.*

6. Most of the insects which died in natural habitats (grain storages) have been found to be infected by the bacteria of the group *Bacillus thuringiensis.*

7. The crystal-forming bacteria, wide spead in grain, staw and dust of storages, belong to all know toxitypes, namely, CryI, CryII, CryIV.

8. While kept in collection, 1,2% of *Bacillus thuringiensis* lose their ability to form crystals of endotoxin and 0,3% start forming involutionary forms.

9. Upon identification of Uzbek isolates with known biotypes of *Bacillus thuringiensis*, provided that the most active cultures are included into a collection for otaining a registration number, we find it reasonable to patent Uzbek strains of *Bacillus thuringiensis* as possible agents of microbial control possessing a broader range of activity than known cultures.

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THE STRAINS BACILLUS THURINGIENSIS FROM INSECT PESTS OF STORED GRAIN

Evgenia Troitskaja[2], Aloviddin Khamraev[2]

2. The Institute of Zoology, Uzbek Academy of Sciences A.Niyazova 1, Tashkent 700095, Uzbekistan

ABSTRACT

From the insect pests of stored grain, as well as other insects, isolated are 50 strains of entomopathogenic crystal-forming bacteria of the group *Bacillus thuringiensis*, the agents that hold promise in microbial control of the insects the families *Lepidoptera, Coleoptera* and *Diptera*. Their interrelations with different pests and stored grain, as well as their pathogenecity for test insects from the above families are studied. The results of the studies on the collected cultures of *Bac.thuringiensis* are presented.

Key words: *Bacillus thuringiensis*, pests of stored grain, storage, pathogenecity, collection.

INTRODUCTION

Big supplies of grain and other products made of it annually stored in elevators and storages are heavily affected by noxious insects, bacteria, fungi, rodents, etc. The most dangerous of them are as follows: *Sitophilus granarius L., S. oryzae L., Sitotroga cerealella Oliv., Corcyra cephalonica Staint., Ephestiaelutella Hbn., Ephestia kuchniella Zell., Pyralis farinalis L., Rizopertha dominica F.*, etc. Due to high fecundity and quite short development time of one generation, they reach high numbers in a short period of time. The losses inflicted by the pests of stored food are extremely high and can reach 50-80% .

Protection of stored food from pests is an extremely burning problem. The attempts to resolve it by using chemicals do not produce desirable and

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stable results due to high natural and acquired resistability of the insect pests to the insecticides used. Besides, repeated application of the chemical drugs contaminates environments and creates the danger of their accumulation in food and severely aggravates sanitary conditions.

The decrease in the efficacy of the drugs, accumulation of the drugs in the stored food, environmental pollution, increase in the hazard of human and animal intoxication make specialists of varions countries look for new, ecologically friendly means of pest control. And one of such methods is the biological one, based on application of the entomopathogenic bacteria, viruses, fungi protozoans and other predatory and parasitic organisms. The application of these organisms enables controlling the numbers of the pests on economically safe levels.

In this connection, the goals of our studies are revealing of cultures of the entomopathogenic sporous bacteria as possible agents of the microbiological control of the stored-grain pests and identification of toxitypes of the isolated strains based on the genetic analysis of their chromosomes.

MATERIAL AND METHODS

The studies were conducted at the Departmen of General and Experimental Entomology of the Institute of Zoology, Uzbek Academy of Sciences from August 1993 to August 1996. The material was collected as described by Veizer (3) and Evlakhova et Shvetsova (4) including preservation, labelling and trasporting of sick and dead pests. The biological material was collected after repeated surveys of mass breeding sites of the pests of stored grain and products made of it. The surveys took place in various plants connected with grain processing and in the farms located in different regions of Uzbekistan and in Tashkent.Surveyed were elevators, milling plants, mills, rice-dryers, storges belonging to collective and state farms, as well as individual storages, etc. Sick, dead insects of various

species, samples of stored products, spilled grain and flour, soil, pieces of scraped surface of walls, wooden and iron constructions were collected. All the surveys connected with infection of theproducts with noxious insects were conducted according with the guidelines of the State Committee for Supplies. The methods by Krashkevich and Gubergrits (5) were used while storing samples. The levels of infection of grain and other products made of it were identified using State Standard 10841-64 .

Identification of the pests was made using routine techniques (13, 8) on the basis of the morphological and biological traits of the pests. Isolation of the microorganism cultures was conducted using common methods (9, 6, 7).

Isolation of sporous entomopathogenic bacteria of the group Bacillus thuringiensis was conducted using two methods - the one as described by Brownbrige et Margalit (1, 2) and the other as described by Troitskaya (8). To isolate of the spore-forming bacteria, the homogenates of the insects processed by the methods of Brownbrige et Margalit (1986, 1987) were plated on the nutritive agar and after incubating in thermostate were used in obtaining the monocultures *Bacillus thuringiensis*. To isolate spore-forming bacteria from the grain stored, washings of the grain and flour mixtures were also plated into the Petri dishes containing nutritive medium. According to the technique by Troitskaya (8), the homogenate of sick and dead insects was without preliminary incubation with the solution of PBS was plated on the surface of the nutritive agar and the plating was then incubated in the thermostate at 28-30° C for 48-72 hours. After the incubation the Petri dishes were incubated and the smears were made from the colonies which were closest in morphology to *Bacillus thuringiensis*. If the crystal (endotoxin) was available, the culture from the colony was then replated to the slant nutrient agar and used in further studies.

The studies on the collected cultures were conducted using routine techniques (10). After the cultures had been replated to the fresh nutritive

agar, the morphology of the culture and changes taking place in the course of maintenance and replating were recorded.

As a result of bacteriological studies, from 272 samples, isolated are 50 cultures of the entomopathogenic crystal-formig bacteria of the group *Bacillus thuringigensis* (Table 1).

Table 1. Sources of isolation of the sporous bacteria of the group *Bacillus thuringigentsis.*

Name of sample	Number of strain
Sitophilus granarius L.	5, 12, 13, 14, 31, 33, 41
S. oryza L.	4, 32, 15, 44
Tribolium confusum Duv.	9, 17, 18, 50
Tenebrio molitor L.	-
Laemothoeus testaceus F.	-
Stigobium paniceum L.	19, 20, 21, 41
Bruchus pisorum L.	-
Acanthoscelides obtestus Say	27, 42
Sitotroga cerealella Oliv.	1, 2, 34, 43
Nemapogon granellus L.	3, 6, 7, 8, 25
Ephestia kuchniella Zll.	10, 11, 22, 23, 26, 28, 29, 46, 47
Pyralis farinalis L.	16, 36, 37
Plodia interpunctella Hb.	24, 38, 48
Spilt products from a Beduin settlement	30, 35
Washings of peas	40, 45, 49
Washings of wheat	39
Total :	**50 strains**

The data of the Table 1 also show that from the different insect species and samples different numbers of the strains of *Bacillus thuringiensis* were isolated. However, these data have only a relative character, since the inoculation of the insect homogenates and washings of the food samples produced far more colonies of *Bacillus thuringiensis* and, while sifting cultures from some colonies, we were guided by the presence of the morphological features of the colonies or cultures (size and form of the vegetative cells, spores and crystals, rate of the sporulation beginning, presence of involutionary cells, cellular (*Hetritus*, etc.). The data analysis

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also shows a different natural level of infection of the pests of stored food by the bacterion of the group Bacillus thuringiensis. A comparative analysis of the results of isolation of Bacillus thuringiensis strains from various species of insects and stored food has demonstrated that they belong to different toxitypes.

The data obtained have shown that samples containing the highest numbers of *Bacillus thuringiensis* belong to *Sitophilus granarius, Nemapogon pranellus, Ephestia kuchniellaя and Agobium paniceum*, as well as the samples of oats and dust in a storage of the Beduin settlement. In other samples, namely, *Sitophillus oryzae, Tribolium confuzum, Sitotroga cerealella, Plodia interpunctella*, wheat and peas, the number crystal-forming bacteria was lower (5, 2 to 20%). No bacteria *Bacillus thuringiensis* were isolated from *Tenebrio, Laemothloens testaceus, Bruchus pisorium, Acanthocelides optestus, Pyralis farinalis*, as well as from the samples of flour, race and haricot in the period of studies.

A comparative analysis of the methods of *Bacillus thuringiensis* isolation conducted in Israel by Brownbrige et Margalith (1986) and that conducted in Uzbekistan by Troitskaya (1983) was made upon isolation of the spore-forming entomopathogenic bacteria. This showed that direct inoculation of the homogenates on the plates with nutrient agar produces greater variety of biotypes and toxitypes of *Bacillus thuringiensis* (Table 2).

Table 2. The Conposition of the Biotypes and Toxitypes of Bacillus thuringiensis upon different methods of isolation

Methods tested	*Characteristic of cultures isolated*	
	biotypes	*toxitypes*
Brownbrige et Margalit (1986)	-	CryIII, CryIV
Troitskaya (1983)	Lez^+, $Sach^+$, Gl^+, Man^+, Sal^+, Lez^-, $Sach^-$, Gl^-, Man^-, Sal^-,	CryI, CryII, CryIII, CryIV

Note: Toxitypes were determined at Ben-Gurion University of the Negev, Israel. Strains isolated in Uzbekistan within the 1st and 2nd toxitypes have about 10 variants of the combination of toxin fragment (Table 3).

Table 3. Combination of fragments* within 1st and 2nd toxitypes of Uzbek strains

Number of strains *Bacillus thuringigensis*	toxitypes			
	Cry-I	Cry-II	Cry-III	Cry-IV
1	2	3	4	5
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 21, 23, 27	+AbD	+B,C	+	-
12, 26	+Aa,Ab, D	+B	+	-
13	+Aa,Ac, D	+A,B	+	-
15, 16, 19, 24, 25	+AC	+A,B	-	-
20, 22	+Ab,D	+B	-	-
28, 29, 30, 32, 34, 35, 39, 40	+Aa,C,D	+B	+	-
31		+B,C	-	-
36, 37, 38, 41	Aa,Ab,C, D	+B	+	-
**41, 42, 43, 44, 45, 46, 47, 48				+A,B,D

Note: * Data of the fragmentry composition of the crystals of the 1st and 2nd toxitypes were obtained from BGU, Israel. The data on the fragmentary analysis of the 3rd 5and 4th toxitypes are not available.

** Strains N 41 to 48 were isolated in Israel from soil samples collected in Uzbekistan in 1995.

The results of the genetic analysis of the cultures isolated by us in the course of our study demonstrates that among above-mentioned strains all currently known toxitypes are available, whereas the isolates obtained using the other above-mentioned technique belong to the 3rd and 4th toxitypes.

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However, there is no reason to state that the cause for the diversity of toxitypes isolated from the biological materials collected in Uzbekistan is in the use of a different method of isolation. To reveal the cause for such a result, it is necessary to conduct special studies.

All the isolated cultures of the crystal-forming entomopathogenic bacteria of the group Bacillus thuringiensis are preserved in the collection using periodical reinoculations on stripes of filter paper (the method by Pasteur Institute, Paris) and on dry slants nutrient agar. It was found out that 1,2% of the cultures, upon storing using periodical reinoculations, completely or partly lose their ability to form crystals of endotoxin and 0,3% of the cultures begin forming involutionary forms as elongated worm-like cells, and very thin or inflated cells.

CONCLUSION

1. In Uzbekistan, the most abundant insect pests of stored grain belong to eight families: *Curculionidae, Tenebrionidae, Cucujidae, Anobiidae, Bruchiidae, Gelechiidae, Tineidae and Pyralididae;*

2. As a result of isolation of 50 strains of entomopathogenic bacteria *Bacillus thuringiensis*, a difference in spread of this microorganism among various pests. *Bacillus thuringiensis* is most frequent *Sitophilus granarius, Stigobium paniceum, Ephestia kuchmiella and Nemapogon granellus.*

3. Most of the insects which died in natural habitats (grain storages) have been found to be infected by the bacteria of the group *Bacillus thuringiensis.*

4. The crystal-forming bacteria, wide spead in grain, staw and dust of storages, belong to all know toxitypes, namely, CryI, CryII, CryIV.

5. While kept in collection, 1,2% of *Bacillus thuringiensis* lose their ability to form crystals of endotoxin and 0,3% start forming involutionary forms.

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