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

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An extended multiplex PCR method was established to rapidly identify and classify *Bacillus thuringiensis* strains containing *cry* (crystal protein) genes toxic to species of Lepidoptera, Coleoptera, and Diptera. The technique enriches current strategies and simplifies the initial stages of large-scale screening of *cry* genes by pinpointing isolates that contain specific genes or unique combinations of interest with potential insecticidal activities, thus facilitating subsequent toxicity assays. Five pairs of universal primers were designed to probe the highly conserved sequences and classify most (34 of about 60) genes known in the following groups: 20 *cry1*, 3 *cry2*, 4 *cry3*, 2 *cry4*, 2 *cry7*, and 3 *cry8* genes. The DNA of each positive strain was probed with 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 for *cry2Ac*. Three standard and 38 field-collected strains were positive by universal primers but negative by specific primers for all five known genes of *cry7* and *cry8*. These field-collected strains seem to contain a new gene or genes that seem promising for biological control of insects and management of resistance.

Over half a century of synthetic pesticide applications has led to the emergence and spread of resistance in agricultural pests and vectors of human diseases and to environmental degradation. The very properties that made these chemicals useful—long residual action and toxicity to a wide spectrum of organisms—have brought about serious problems (21, 25). An urgent need has thus emerged for environment-friendly pesticides to reduce contamination and the likelihood of insect resistance (20, 25). The use of *Bacillus thuringiensis* as a commercial insecticide is based on its remarkable ability to produce large quantities of larvicidal proteins (known as δ-endotoxin) that form crystalline inclusion bodies during sporulation (4). The multitude of insecticidal crystal proteins of *B. thuringiensis* subspecies has spurred their use as natural control agents with applications in agriculture, forestry, and human health (25). Recent trends suggest that biological control will become increasingly important, particularly as a part of strategies for integrated pest management (21, 22). Novel insecticidal bacteria, with an extended target spectrum, for example, would enhance environmentally safe biocontrol practices and lead to increased food production and postharvest protection (25).

The genes coding for the insecticidal crystal proteins, which are normally associated with large plasmids, direct the synthesis of a family of related proteins that have been classified as CryI to -VI (the old nomenclature), depending on the host specificity (Lepidoptera, Diptera, Coleoptera, and nematodes) and the degree of amino acid homology (12, 15, 20). The current classification is uniquely defined by the latter criterion (9).

Identification of novel *B. thuringiensis* isolates by bioassays is a long and exhaustive process, impeded by repeated isolation of the same strains (17). Prediction of the insecticidal activity of an unknown strain by serotyping seems impossible, because it does not necessarily reflect the specific *cry* gene classes that the strains contain (1, 11). PCR, which is a highly sensitive method of rapidly detecting and identifying 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, 5–8, 13, 16, 18). Being faster and more efficient than serotyping, PCR analysis provides a valuable preliminary tool preceding 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, 18). To optimize identification of all reported *cry* genes, this methodology needs a complete PCR set of primers (17). We describe here an enhanced strategy using PCR for extended multiplex rapid screening (3) of *B. thuringiensis* strains that harbor genes from seven classes. This strategy will enrich the existing arsenal of insecticidal strains, identify novel toxin 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 the GenBank database, aided by reference 9), and specific primers were designed to identify eight different *cry1* genes, three *cry2* genes, three *cry3* genes, two *cry4* genes, one *cry7* gene, three *cry8* genes, and one *cry11* gene. DNAs of strains which reacted to at least one pair of the universal primers were characterized by amplification with specific primers. The preliminary screening by universal primers saves effort by sorting 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.

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

Gene nomenclature		GenBank accession no.	Nucleotide positions hybridized to primers[b]	Mismatch of primers[c]	Product size (bp)
Current	Original				
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
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
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
cry4A2	*cryIVA*	D00248	3324–3347, 3738–3762	0(d), 0(r)	439
cry4B4	*cryIVB*	D00247	3259–3282, 3673–3697	0(d), 0(r)	439
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] The sequences of the universal primers (d, direct; r, reverse) are as follows: *cry1*, Un1(d), 5′CATGATTCATGCGGCAGATAAAC-3′; Un1(r), 5′-TTGTGACA CTTCTGCTTCCCATT-3′; *cry2*, Un2(d), 5′-GTTATTCTTAATGCAGATGAATGGG-3′; Un2(r), 5′-CGGATAAAATAATCTGGGAAATAGT-3′; *cry3*, Un3(d), 5′-CGTTATCGCAGAGAGATGACATTAAC-3′; Un3(r), 5′-CATCTGTTGTTTCTGGAGGCAAT-3′; *cry4*, Un4(d), 5′-GCATATGATGTAGCGAAACAAGCC3′; Un4(r), 5′-GCGTGACATACCCATTTCCAGGTCC-3′; and *cry7* and *cry8*, Un7,8(d), 5′-AAGCAGTGAATGCCTTGTTTAC-3′, and Un7,8(r), 5′-CTTCTAAACCTT GACTACTT-3′.

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

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

MATERIALS AND METHODS

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

B. thuringiensis cells were enriched from the isolates by growth in Luria-Bertani medium containing 0.25 M acetate, which selectively inhibits germination of their spores and not that of other sporeformers, and were plated on Luria-Bertani agar following a heat shock (24). Single colonies were grown in liquid T3 medium and selected for the appearance of parasporal inclusions by phase-contrast microscopy. Samples (after 96 h of growth) were frozen at −70°C with 15% glycerol or lyophilized after being washed with sterile distilled water by centrifugation.

Oligonucleotide PCR primers. One pair of universal primers (e.g., Un1 direct and reverse primers) for each of the five *cry* homology groups (9) was designed to amplify a specific fragment by simultaneous alignment with all previously described genes in that group by using the Amplify 1.0 program (Bill Engels, University of Wisconsin, Madison). Their sequences and match (as well as mismatch) 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 universal primer 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* (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 the predicted size that was easily identified by electrophoresis in agarose gels (0.8 to 2.5%).

DNA templates and PCR analysis. Templates were prepared from 16- to 18-h cultures in Luria-Bertani medium or tryptic soy broth enriched with 0.3% (wt/vol) yeast extract. Aliquots of 3 to 4.5 ml were harvested by centrifugation and 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 of lysozyme per ml). The cell suspension was incubated for 1 h at 37°C. Further DNA extraction was performed as described by Sambrook et al. (23).

Amplification was carried out in a DNA MiniCycler (MJ Research, Inc., Watertown, Mass.) for 30 reaction cycles each. Reactions were routinely carried out in 25 μl; 1 μl of template DNA was mixed with reaction buffer, 150 mM (each) deoxynucleoside triphosphate, 0.2 to 0.5 μM (each) primer, and 0.5 U of *Taq* DNA polymerase (Appligene). Template DNA was denatured (1 min at 94°C) and annealed to primers (40 to 50 s at 54 to 60°C), and extensions of PCR products were achieved at 72°C for 50 to 90 s. Each experiment was associated

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

Primer pair[a]	Sequence of primer[b]	Gene recognized	Position[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	1,103
EE-3Ba(r)	ACGAAAGATTCTGCTCCTAT		1909–1928	
Un3(d)	CGTTaTCGCAGAGAgATGACaTTAAC	*cry3Bb1*	1003–1028	1,103
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	1,529
Un4(r)	GCGTGACATACCCATTTCCAGGTCC		3738–3762	
EE-4B(d)	GAGAACACACCTAATCAACCAACT	*cry4B4*	1747–1770	1,951
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 the 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.

with negative (without DNA template) and positive (with a standard template) controls.

The reliability of the primers was verified with the following *B. thuringiensis* reference strains: *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *aizawai* HD-133, *B. thuringiensis* subsp. *galleriae* HD-155, *B. thuringiensis* subsp. *kenyae* HDB-23, *B. thuringiensis* subsp. *tolworthi* HDB-8, and *B. thuringiensis* subsp. *thuringiensis* HD-2 for the *cry1* and *cry2* classes; *B. thuringiensis* subsp. *tenebrionis*, *B. thuringiensis* subsp. *kumamotoensis* (EG4961) NRRL B-18533, and NRRL B-18655 (EG5144) for the *cry3* class; *B. thuringiensis* subsp. *israelensis* ONR60A for the *cry4* and *cry11* classes; and *B. thuringiensis* subsp. *dakota* HD-511, *B. thuringiensis* subsp. *tochigiensis* HD-868, *B. thuringiensis* subsp. *indiana* HD-521, and *B. thuringiensis* subsp. *kumamotoensis* HD-867 for the *cry7* and *cry8* classes (see Results and Table 3).

RESULTS

Identification by universal primers. Five pairs (direct and reverse) of universal primers were designed to detect genes by the sizes of their PCR products (Table 1). The DNA of each *B. thuringiensis* isolate served as the template in three reactions (Fig. 1). (i) Reaction 1 was done with a mixture of two pairs, Un1 and Un4 (to detect 20 genes from the *cry1* group and 2 genes from the *cry4* group). (ii) Reaction 2 was done with a mixture of two other pairs, Un2 and Un3 (to detect three genes from the *cry2* group and four genes from the *cry3* group. (iii) Reaction 3 was done with Un7,8 (to detect two and three genes from the *cry7* and *cry8* groups, respectively).

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

Among 215 field-isolated *B. thuringiensis* strains, the DNA of 89 strains did not amplify the universal primers. The rest were grouped in seven *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 seven *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*, and *-F*) of the 20 known genes that are identified by Un1 (Table 1 and Fig. 3). We found nine different *cry1* gene profiles (profiles 3 to 16 [Table 4]); an additional *cry1* profile (profiles 1 and 2) was not identified by any specific primer of *cry1*.

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

TABLE 3. Distribution of *cry*-type gene profiles of *B. thuringiensis* strains as analyzed by PCR

Strain	Result for[a]:				
	cry1	*cry2*	*cry3*	*cry4* and *cry11A*	*cry7* and *cry8*
B. thuringiensis subsp. *aizawai* HD-133	*-Aa*, *-Ab*, *-Ca*, *-Da*	*-Ab*	−	−	+
B. thuringiensis subsp. *galleriae* HD-155	*-Aa*, *-Ab*, *-Ba*	*-Aa*, *-Ab*	−	−	−
B. thuringiensis subsp. *kenyae* HDB-23	*-Ab*, *-Ac*, *-Ea*	*-Aa*, *-Ab*	−	−	−
B. thuringiensis subsp. *tolworthi* HDB-8	*-Ab*	*-Aa*, *-Ab*	−	−	−
B. thuringiensis subsp. *thuringiensis* HD-2	*-Ab*, *-Ba*	−	−	−	−
B. thuringiensis subsp. *kurstaki* HD-1	*-Aa*, *-Ab*, *-Ac*	*-Aa*, *-Ab*	−	−	−
B. thuringiensis subsp. *israelensis* ONR60A	−	−	−	*-A*, *-B*, *-11A*	−
B. thuringiensis subsp. *tenebrionis*	−	−	*-Aa*	−	−
B. thuringiensis NRRL B-18533	−	−	*-Bb*	−	−
B. thuringiensis NRRL B-18655	−	−	*-Bb*	−	−
B. thuringiensis subsp. *dakota* HD-511	−	−	−	−	*-7Aa*
B. thuringiensis subsp. *indiana* HD-521	−	−	−	−	+
B. thuringiensis subsp. *tochigiensis* HD-868	−	−	−	−	+
B. thuringiensis subsp. *kumamotoensis* HD-867	−	−	−	−	*-7Aa*
B. thuringiensis subsp. *japonensis* 4AT1	−	−	−	−	−
B. thuringiensis subsp. *kyushuensis* HD-541	−	−	−	−	−

[a] −, negative by universal primers; +, positive by universal primers.

positive to *cry2Ac*, but all field-collected strains that contained *cry2Ac* were positive for *cry2Ab* as well (profile 16 [Table 4 and Fig. 4]).

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

Three genes, *cry4A*, *cry4B*, and *cry11A*, which code for Diptera-specific polypeptides (15), were identified by a set of specific primers (Table 2 and Fig. 6). This set yielded three different profiles, and all included *cry11A*: one with *cry4A* (profile 19), one with *cry4B* (profile 20), and one with both (profiles 21 and 22 [Table 4]).

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

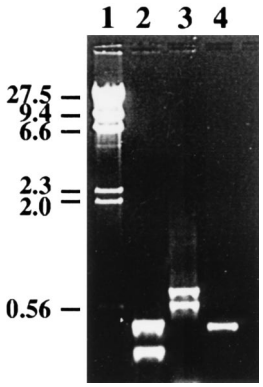

FIG. 1. Agarose gel (1.2%) electrophoresis of PCR products amplified from standard strains of *B. thuringiensis* with universal primers. Lanes: 1, molecular weight markers (λ DNA cleaved by *Hin*dIII), with sizes (in kilobases) indicated on the left; 2 to 4, PCR products obtained from mixed DNA of *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *kurstaki* HD-1 with Un1 and Un4 (lane 2), mixed DNA of *B. thuringiensis* subsp. *kurstaki* HD-1 and *B. thuringiensis* subsp. *tenebrionis* with Un2 and Un3 (lane 3), and DNA of *B. thuringiensis* subsp. *dakota* HD-511 with Un7,8 (lane 4). The expected sizes of the products are indicated in Table 1.

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 with the first group of primers (universal primers [Table 1 and Fig. 1 and 2]), and the positive 126 isolates were identified by the

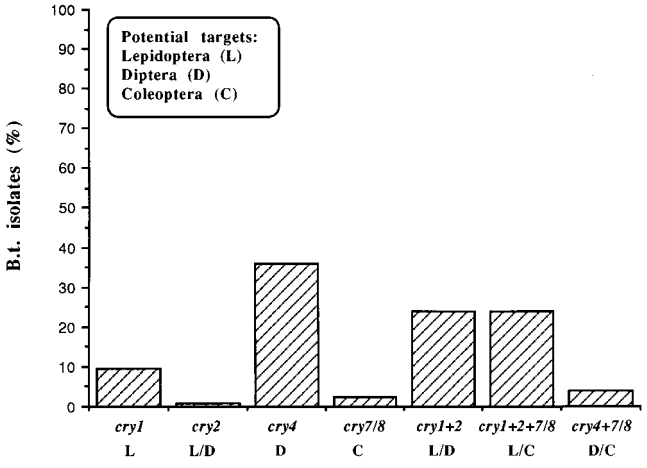

FIG. 2. Distribution of *cry*-type gene profiles from 126 field-collected strains of *B. thuringiensis* (B.t.) identified by universal primers.

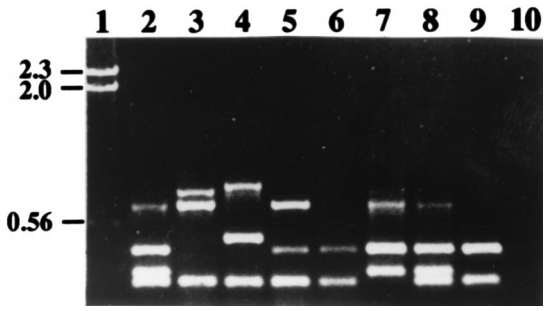

FIG. 3. Agarose gel (1.2%) electrophoresis of PCR products obtained with specific primers for *cry1* genes. Lanes: 1, molecular weight markers (λ DNA cleaved by *Hin*dIII), with sizes (in kilobases) indicated on the left; 2 to 4, DNA of *B. thuringiensis* subsp. *aizawai* HD-133 (lane 2), *B. thuringiensis* subsp. *galleriae* HD-155 (lane 3), and *B. thuringiensis* subsp. *kenyae* HDB-23 (lane 4); 5 to 9, DNA of field-collected strains U-12 (profile 9 [lane 5]), U-25 (profile 10 [lane 6]), U-35 (profile 14 [lane 7]), U-38 (profile 15 [lane 8]), and U-27 (profile 16 [lane 9]); 10, negative control of PCR mixture (without template). The expected sizes of the products are indicated in Table 2.

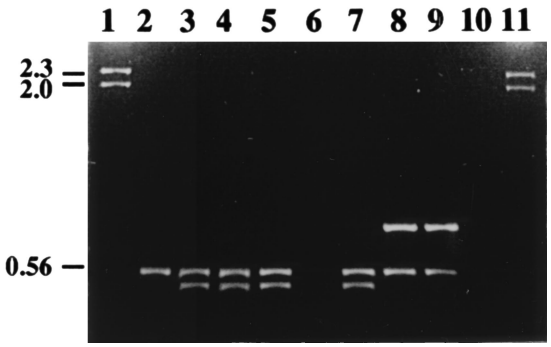

FIG. 4. Agarose gel (2.5%) electrophoresis of PCR products obtained with specific primers for the *cry2Aa*, *-Ab*, and *-Ac* genes. Lanes: 1 and 11, molecular weight markers (λ DNA cleaved by *Hin*dIII), with sizes (in kilobases) indicated on the left; 2 to 7, DNA of *B. thuringiensis* subsp. *aizawai*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

second, specific primers (Tables 2 and 4 and Fig. 3 to 7). The profiles of all products were compared with those of 16 standard strains (Table 3 and Fig. 3 to 7). Twenty-two different profiles were observed (Table 4), 7 of which (including 36 independent isolates) were novel (profiles 9, 10, 14, 16, 17, 18, and 22); the latter strains may be promising for new biological control agents. Twelve independent isolates (profiles 1 and 2) were positive with Un1 but not identified by any specific primer of our eight *cry1* genes. They may contain at least 1 of 12 other *cry1* genes (amplified by Un1 [Table 1]) or a new *cry1* gene or genes. Three additional profiles (19, 20, and 21, found in 45 isolates) contained genes of *B. thuringiensis* subsp. *israelensis*. The remaining 33 isolates were distributed in 10 different profiles (profiles 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 six different profiles which were identical to those of standard strains as follows (Table 3) (1, 3, 8, 15, 16): profiles 3 and 5 were identical to *B. thuringiensis* subsp. *entomocidus*, profiles 4 and 7 were identical to *B. thuringiensis* subsp. *alesti*, profile 6 was identical to *B. thuringiensis* subsp. *sotto*, profiles 8 and 13 were identical to *B. thuringiensis* subsp. *kurstaki*, profiles 11 and 12 were identical to *B. thuringiensis* subsp. *galleriae*, and profile 15 was identical to *B. thuringiensis* subsp. *aizawai.*

New toxic specificities may stem from new combinations of known genes, such as in the strains displaying profiles 9 and 10. For example, *B. thuringiensis* YBT-226 (14) 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 the toxicity of Cry2Aa is low (14).

TABLE 4. Distribution of *cry*-type gene profiles of *B. thuringiensis* isolates collected in Israel, Kazakhstan, and Uzbekistan

Profile no.	*cry*-type gene profile[a]	No. of isolates	Predicted insecticidal activity
1	*cry1*	10	Lepidoptera
2	*cry1* + *cry2Ab*	2	Lepidoptera
3	*cry1Aa*	1	Lepidoptera
4	*cry1Aa*, *-Ac*	1	Lepidoptera
5	*cry1Aa*, + *cry2Aa*, *-Ab*	2	Lepidoptera + Diptera
6	*cry1Aa*, *-Ab*, + *cry2Ab*	1	Lepidoptera
7	*cry1Aa*, *-Ac*, + *cry2Aa*, *-Ab*	8	Lepidoptera + Diptera
8	*cry1Aa*, *-Ab*, *-Ac*, + *cry2Aa*, *-Ab*	6	Lepidoptera + Diptera
9	*cry1Aa*, *-Ab*, *-D* + *cry2Ab*	1	Lepidoptera
10	*cry1Ab*, *-D* + *cry2Ab*	4	Lepidoptera
11	*cry1Ab*, *-Ac*, *-D* + *cry2Ab*	2	Lepidoptera
12	*cry1Ab*, *-Ac*, *-D* + *cry2Aa*, *-Ab*	1	Lepidoptera + Diptera
13	*cry1Ac* + *cry2Aa*, *-Ab*	3	Lepidoptera + Diptera
14	*cry1Aa*, *-C*, *-D* + *cry2Ab* + *cry7*, *cry8*	8	Lepidoptera + Coleoptera
15	*cry1Aa*, *-Ab*, *-C*, *-D* + *cry2Ab* + *cry7*, *cry8*	8	Lepidoptera + Coleoptera
16	*cry1Ab*, *-D* + *cry2Ab*, *-Ac* + *cry7*, *cry8*	14	Lepidoptera + Coleoptera
17	*cry2Aa*, *-Ab*	1	Lepidoptera + Diptera
18	*cry7*, *cry8*	3	Coleoptera
19	*cry4A*, *cry11A*	7	Diptera
20	*cry4B*, *cry11A*	2	Diptera
21	*cry4A*, *-B*, *cry11A*	36	Diptera
22	*cry4A*, *-B*, *cry11A* + *cry7*, *cry8*	5	Diptera + Coleoptera

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

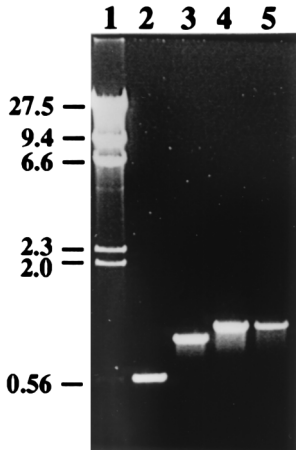

FIG. 5. Agarose gel (1%) electrophoresis of PCR products obtained with primers for *cry3* genes. Lanes: 1, molecular weight markers (λ DNA cleaved by *Hin*dIII), with sizes (in kilobases) indicated on the left; 2, DNA of *B. thuringiensis* subsp. *tenebrionis* amplified with Un3; 3 to 5, DNA of *B. thuringiensis* subsp. *tenebrionis* (lane 3), NRRL B-18533 (containing *cry3Bb1* [lane 4]), and NRRL B-18655 (containing *cry3Bb2* [lane 5]) amplified with specific primers. The expected sizes of the products are indicated in Tables 1 and 2.

To the best of our knowledge, PCR screening by primers to *cry2* genes has never been performed before. We found that all but one (*B. thuringiensis* subsp. *thuringiensis* HD-2) of the standard strains with at least one *cry1* gene (Table 3 and Fig. 4) also contained *cry2Ab*. All of these except one (*B. thuringiensis* subsp. *aizawai* HD-133) also contained *cry2Aa*. None of the six standard strains that we screened responded to primers specific to *cry2Ac*. This gene was discovered in *B. thuringiensis* S_1 (26), with toxicity against Lepidoptera. All fourteen isolates containing *cry2Ac* displayed a single profile (profile 16 [Table 4 and Fig. 4]), which was also positive to *cry2Ab*. The other 47 field strains with at least one *cry2* gene can be divided into two types (Table 4): 21 which contain *cry2Aa* and *cry2Ab* (displaying six different profiles), and 26 containing *cry2Ab* alone (displaying seven different profiles). Strains containing either *cry2Aa*, *cry2Ac*, or 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, *B. thuringiensis* subsp. *tolworthi* contains *cry1Ab*, *cry1E*, *cry2*, and *cry3B* (1). According to Kuo and Chak (18), *cry1*-containing *B. thuringiensis* subsp. *wuhanensis* HD-525 and *B. thuringiensis* subsp. *morrisoni* HD-12 contain an apparently novel *cry7A* gene. Strain BTI109P of *B. thuringiensis* subsp. *kurstaki* contains *cry3C* (19). DNA from *B. thuringiensis* 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 of the specific primers of these groups. This observation is consistent with that of Kuo and Chak (18), reporting that the same strain was not identified as *cry7A*. Since Kuo and Chak (18) did not test for any of the *cry8* group genes, this observation may imply that *B. thuringiensis* subsp. *aizawai* HD-133 contains a new gene of the *cry8* group.

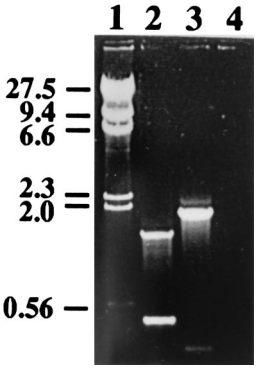

FIG. 6. Agarose gel (1%) electrophoresis of PCR products from *B. thuringiensis* subsp. *israelensis*. Lanes: 1, molecular weight markers (λ DNA cleaved by *Hin*dIII), with sizes (in kilobases) indicated on the left; 2 and 3, results obtained with specific primers for *cry4A* plus *cry11A* and for *cry4B*, respectively; 4, negative control without template.

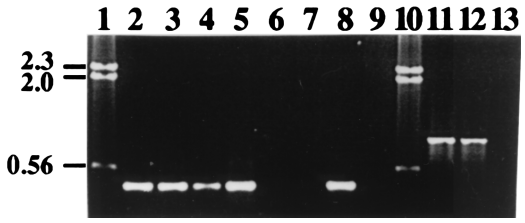

FIG. 7. Agarose gel (1%) electrophoresis of PCR products obtained with universal and specific primers for *cry7* and *cry8* genes. Lanes: 1 and 10, molecular weight markers (λ DNA cleaved by *Hin*dIII), with sizes (in kilobases) indicated on the left; 2 to 8, respectively, DNA of *B. thuringiensis* subsp.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

B. thuringiensis subsp. *dakota* HD-511 and *B. thuringiensis* subsp. *kumamotoensis* HD-867 responded similarly to our specific primers for *cry7A* (Fig. 7). Kuo and Chak (18) recently indeed reported that the same *B. thuringiensis* subsp. *kumamotoensis* strain and a closely related *B. thuringiensis* subsp. *dakota* strain (HD-932) yielded (by the PCR-restriction fragment length polymorphism method [18]) "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* subsp. *indiana* HD-521 and *B. thuringiensis* subsp. *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 (18), the partial nucleotide sequence of the product from the same *B. thuringiensis* subsp. *indiana* strain was similar to that of the *cry7*-type gene, but *B. thuringiensis* subsp. *tochigiensis* HD-868 was not identified. Indeed, the nucleotide sequences of the PCR products (423 bases long, obtained with Un7,8) from these two subspecies show about 80 and 90% homology to *cry7A*, respectively, and 67% homology to all three *cry8* genes (to be extensively described elsewhere); in addition, they are about 80% homologous, thus confirming that they are two novel genes. The sequence of the same 423-base PCR product obtained with a field strain (R1 of profile 18 [Table 4]), positive to Un7,8 only, was found to be 67% homologous to *cry7A* and 98.8% homologous 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]). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a sporulating culture of strain R1 yielded two large polypeptides, one with a size of about 140 kDa

(typical size of Cry7 and Cry8 polypeptides), and one with a size of 100 kDa, which may be the newly postulated gene (unpublished data).

Thirty of our field strains (exhibiting profiles 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 the *cry7* group or the *cry8* group. The eight isolates with profile 15 are identical (by our criteria) to *B. thuringiensis* subsp. *aizawai* HD-133. The remaining 22 isolates (with profiles 14 and 16) seem to contain a new *cry* gene or genes and thus have the potential to be effective biopesticides of insects for which biological control agents do not exist.

Five new isolates (displaying profile 22), which contain the three genes of *B. thuringiensis* subsp. *israelensis* (*cry4A* and *-B* and *cry11A* [toxic to Diptera]), seem to contain a new *cry7* or *cry8* gene or genes (i.e., were positive with Uns7,8) and may thus be useful as new biological control agents for larvae of both Coleoptera and Diptera. A single subspecies (*B. thuringiensis* subsp. *morrisoni*, serotype 8a 8b [1]) is known to contain these Diptera-toxic genes in combination with *cry3A* (toxic to Coleoptera). It is interesting 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 19 to 22 [Table 4]).

The absence of PCR products when the DNA of each of the 89 *B. thuringiensis* isolates was challenged with all of our universal primers does not necessarily imply that these strains are devoid of genes coding for insecticidal polypeptides. Genes from known strains which have not been sequenced yet may not be discovered by this method (5). Novel toxins may be recognized among these isolates by characterization of the proteins detected on gels or microscopically by the shape of their crystals, but cryptic genes that are not expressed (1) will not be discovered by any other known method. On the other hand, a newly discovered gene does not necessarily possess known insecticidal activity: an example of such a nontoxic field-collected strain (IB31) has recently been shown by PCR to contain a gene homologous to *cry1*, while its product cross-reacted with two antibodies specific to Cry1E and Cry3A (7). The two isolates *B. thuringiensis* subsp. *kyushuensis* HD-541 and *B. thuringiensis* subsp. *japonensis* 4AT1 can serve as examples for the limitation of the PCR method: they include crystals and represent distinct serotypes (11, 17), but no *cry* gene or genes have been identified with our set of primers (Table 3) or those of others (18). In addition, a strain may contain a novel gene with sequences annealing to the primers for known genes but different sequences in other regions defining a new insecticidal activity. This limitation can be resolved, at least partially, by a set of specific primers through the sequence of a particular gene (16).

Specificity of oligonucleotide primers for PCR. Ceron et al. (7) have recently prepared one pair of universal primers (CJIII20 and 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 into 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 of these genes and low variability in the sizes of the PCR products for each group. The numbers of mismatches were from 0 to 3 bases, and the size ranges of the 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 of four mismatched bases with primer CJIII21 to *cry3C*) and weakly to *cry8B* and *cry8C*, with maxima of five (CJIII20) and six (CJIII21) mismatches, respectively. This pair of universal primers, moreover, cannot amplify *cry8A*. The range of fragment sizes (39 bases) produced by this pair (CJIII20 and CJIII21) was also much higher.

Three genes, *cry4A*, *cry4B*, and *cry11A*, which code for Diptera-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, because of 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 to 60°C for all except Un7,8 and their four 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 (except for extension time [1.5 min] with EE-4A and EE-4B, which amplify fragments with sizes of 1,529 and 1,951 bases, respectively).

To minimize the effort in screening, three reactions with universal primers were performed for each isolate (see Results) with two mixtures of two pairs (Un1 with Un4 and Un2 with Un3) and with one pair for both the *cry7* and *cry8* groups (Un7,8). To further raise efficiency, it should be possible to design five pairs (or more for more extensive 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 nonspecific 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 in order to direct them for subsequent toxicity assays against Lepidoptera, Coleoptera, and Diptera. This method enriches existing PCR strategies for screening most currently known *cry* genes by improving and developing expanded PCR sets of universal and specific primers. 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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