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

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

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

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

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

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

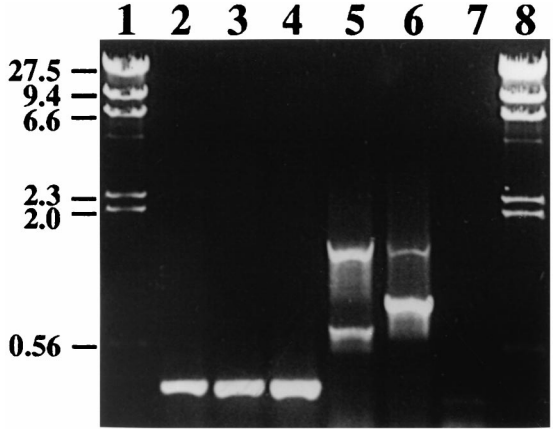

FIG. 1. Agarose gel (1%) electrophoresis of PCR products obtained with universal and specific primers for *cry9* genes. Lanes 1 and 8, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kilobases) indicated on left; lanes 2 to 4, respectively, DNA of field-collected isolates U-27,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

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

Gene nomenclature		GenBank accession no.	Positions of nucleotides hybridized to primers[b]	Mismatch of primers[c]	Product size (bp)
Current	Original				
cry9A	*cryIG*	X58120	2774–2797, 3104–3127	4, 16(d); 9(r)	354
cry9B	*cryIX*	X75019	2272–2295, 2602–2625	4, 16(d); 9(r)	354
cry9C	*cry9C*	Z37527	4354–4377, 4681–4704	0(d); 0(r)	351
cry9D		D85560	2338–2361, 2668–2691	0(d); 0(r)	354

[a] The sequences of the universal primers (d, direct; r, reverse) are as follows: Un9(d), 5′-CGGTGTTACTATTAGCGAGGGCGG-3′; Un9(r), 5′-GTTTGAGCC GCTTCACAGCAATCC-3′.
[b] Starting from the first base of the sequence (of the respective *cry* gene) in the GenBank database.
[c] Numbers indicate bases from 5′ of primers that do not match to the respective sequences.

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

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

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

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

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

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

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

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

Primer pair[a]	Sequence of primers[b]	Gene recognized	Positions[c]	Product size (bp)
EB-9A(d)	GGTTCACTTACATTGCCGGTTAGC	*cry9A*	1581–1604	1,547
Un9(r)	GTTTGAGCcGCTTCACAGCAATCC		3104–3127	
EB-9B(d)	GCAAATGCATTTAGCGCTGGTCAA	*cry9B*	1925–1948	701
Un9(r)	GTTTGAGCcGCTTCACAGCAATCC		2602–2625	
EB-9C(d)	CCACCAGATGAAAGTACCGGAAG	*cry9C*	3473–3495	1,232
Un9(r)	GTTTGAGCCGCTTCACAGCAATCC		4681–4704	
EB-9D(d)	GCAATAAGGGTGTCGGTCACTGG	*cry9D*	1754–1776	938
Un9(r)	GTTTGAGCCGCTTCACAGCAATCC		2668–2691	

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

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

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

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

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

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

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

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

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Gideon Raziel is gratefully acknowledged for producing the picture.

ADDENDUM IN PROOF

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

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