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

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

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

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

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

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

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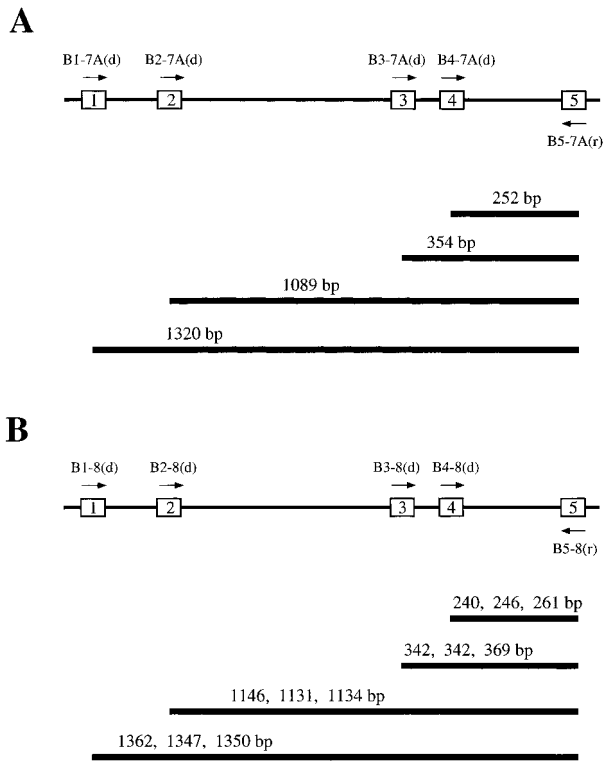

Fig. 1. Strategy for detecting *cry7* (A) and *cry8* (B) genes by PCR based upon the five conserved blocks of amino acids in toxins. Arrows above boxes (enclosing block number) indicate direction of primers (Tables 1 and 2). Predicted lengths (in bp) of the resultant amplicons (thick lines) are indicated.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

Materials and Methods

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

Oligonucleotide primers and PCR analysis. Primer sequences, match and mismatch positions in each *cry7*, and expected sizes of their amplicons are presented in Table 1. They were selected from regions coding for the five conserved blocks to amplify specific fragments using Amplify 1.0 program (Bill Engels, University of Wisconsin, Madison, USA). A partial set of primers to detect the conserved blocks 3 and 5 specific for the three known *cry8* was similarly examined as well (Fig. 1B); their sequences, match positions, and expected sizes of resultant amplicons are presented in Table 2.

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

Results and Discussion

PCR analysis for *cry7* genes was performed on four *B. thuringiensis* standard strains, as well as on 27 *B. thuringiensis* field isolates, which have previously been found positive to a pair of universal primers (Un7,8 for *cry7* and *cry8* groups) [2]. Among the standard strains, only *B. thuringiensis* subsp. *dakota* HD-511 and *B. thuringiensis* subsp. *kumamotoensis* HD-867, known to harbor *cry7Ab1* and *cry7Ab2*, respectively [1], yielded the four amplicons (Fig. 2) of the predicted sizes with the set of primers (Table 1). None of the 27 field-collected isolates tested yielded amplicons with this same set of primers. This result could be a consequence of an unfortunate mismatch of the reverse primer (even of the terminal 3′-nucleotide), but these strains may serve as a potential pool for new genes from the *cry7* and *cry8* groups. Indeed, further screening for the presence of three *cry8* genes by their respective pairs for blocks 3 and 5 (Table 2) detected one that yielded an amplicon specific to *cry8Ba*. This same field-collected strain must be novel because it did not react with a *cry8Ba*-specific pair of primers [2].

A new gene will be detected by propagating a specific amplicon(s) differing from the standard pattern for *cry7* (Fig. 1A). A necessary condition for defining a new gene is either different interval(s) among the five conserved blocks, as found in *cry8* (Fig 1B) and in other *cry* genes [28, 29], or homology variation in conserved blocks. This method can be practical for a distinct *cry* group or for several groups by sets of degenerated primers.

Three additional conserved blocks have recently been identified in the C-termini of Cry protoxins [29]. They may be exploited for extended PCR screening of *cry* genes, alone or together with the original five conserved blocks. For example, a set of four primer pairs each for a tandem pair of the eight conserved blocks can be designed and used in a mixture for a single reaction. This reaction will yield four major amplicons from a standard *cry* created by matching the respective primers to pairs of adjacent blocks, and several additional minor amplicons between primers of distinct pairs, creating together a fingerprint specific to each *cry*.

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Table 1. Characteristics of primers specific for *cry7Aa*, *cry7Ab1*, and *cry7Ab2*

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

[a] (d) and (r), direct and reverse primers, respectively.
[b] Lowercase letters in B2-7A(d) and B4-7A(d) are of bases that do not match the sequences of *cry7Aa* and *cry7Ab*, respectively.
[c] Starting from the first base (A) of the start codon of the respective *cry*. GenBank accession numbers of *cry7* sequences: M64478 for *cry7Aa1*; U04367 for *cry7Ab1*; U04368 for *cry7Ab2*.

Table 2. Characteristics of primers specific for *cry8*

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

[a] (d) and (r), direct and reverse primers, respectively.
[b] Starting from the first base (A) of the start codon of the respective *cry*. GenBank accession numbers of *cry8* gene sequences: U04364 for *cry8Aa*; U04365 for *cry8ABa*; U04366 for *cry8Ca*.

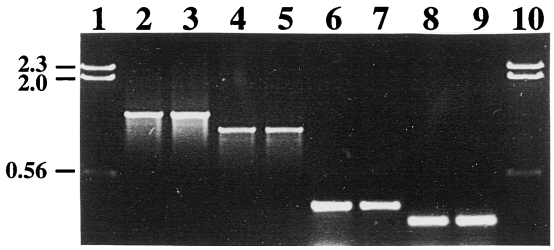

Fig. 2. Agarose gel (1%) electrophoresis of PCR products obtained with the direct primers for the four conserved blocks of *cry7* and the reverse primer of block 5 (Table 1). Lanes 2, 4, 6, and 8, template DNA from *B. thuringiensis* subsp. *dakota* HD-511 (with *cry7Ab1*); lanes 3, 5, 7, and 9 template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

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