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PCR Analysis of the *cryI* Insecticidal Crystal Family Genes from *Bacillus thuringiensis*

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A method allowing rapid and accurate identification of different subgroups within the insecticidal crystal CryI protein-producing family of *Bacillus thuringiensis* strains was established by using PCR technology. Thirteen highly homologous primers specific to regions within genes encoding seven different subgroups of *B. thuringiensis* CryI proteins were described. Differentiation among these strains was determined on the basis of the electrophoretic patterns of PCR products. *B. thuringiensis* strains, isolated from soil samples, were analyzed by PCR technology. Small amounts of bacterial lysates were assayed in two reaction mixtures containing six to eight primers. This method can be applied to rapidly detect the subgroups of CryI proteins that correspond with toxicity to various lepidopteran insects.

Bacillus thuringiensis is characterized by the production of parasporal crystals composed of protein molecules known as δ-endotoxins or insecticidal crystal proteins (ICP) that are toxic to the larvae of various insects.

It has been estimated that up to 15% of crops worldwide are lost because of insect damage alone (2). ICPs have been used as biopesticides during the last 30 years; different commercial products are now available and used in agricultural fields. Furthermore, some ICP genes have been introduced into the plant genome, with a high rate of protection against insect attack (11, 12). The use of *B. thuringiensis* as a microbial insecticide offers several advantages over chemical control agents: ICPs have a highly specific host range that renders them harmless to nontarget insects and vertebrates and the environment. Recently isolated strains with demonstrated toxic potential against various organisms (5) have raised interest in isolating new *B. thuringiensis* strains with specificities for a much broader range of pests, extending the use of *B. thuringiensis* of a pesticide beyond traditional markets.

Numerous ICP genes have been cloned, sequenced, and classified on the basis of their homology and specificity (9). CryI toxins are proteins toxic to lepidopteran insects. To date, seven subgroups of *cryI* genes and four subgroups belonging to the *cryIA* group have been characterized and identified. Each CryI toxin has a narrow range of toxicity against different lepidopteran insects (7), which are some of the most devastating pests in agricultural fields. Therefore, our main goal is to isolate and characterize *B. thuringiensis* strains harboring *cryI* genes.

One of the most important aspects about establishing a *B. thuringiensis* collection is to have a method which allows for rapid and accurate characterization. This is especially important if the different δ-endotoxin genes carried by a certain strain are critical to its specificity and toxicity. Analysis of these genes by bioassay has proved to be an exhaustive, time-consuming process, since it is necessary to screen all target insect isolates. Different methods have been developed in an effort to reduce the number of bioassays, such as (i) Southern blot analysis in search of known homologous genes (10), (ii) analysis of reactivity to different monoclonal antibodies (8), or (iii) electrophoretic analysis of PCR products using specific primers (4). From the above-mentioned methods, PCR analysis is considered to be the best choice, because it allows rapid determination of the presence or absence of a sequence and it is highly sensitive, relatively fast, and can easily be used on a routine basis. Carozzi et al. (4) performed PCR analysis to identify different δ-endotoxins and reported the sequences of 12 PCR primers that distinguished three major classes of ICP genes (*cryI*, *cryIII*, and *cryIV*). Nevertheless, CryI proteins belonging to different subgroups could exert different insecticidal effects on lepidopteran pests (7). PCR analysis of three *cryIA* subgroups was recently reported (3); however, this analysis did not identify the remaining *cryI* gene subgroups. In order to characterize our *B. thuringiensis* collection, in this study we present a method designed to identify the specific *cryI* genes carried by a particular strain.

Isolation of *B. thuringiensis* strains. To isolate *B. thuringiensis* crystal-producing strains, 148 soil samples were analyzed as described previously (4). A total of 651 crystal-producing strains were isolated, as judged by microscopic observations. *B. thuringiensis* strains were found primarily in soils from active agricultural zones.

These strains were prescreened by an enzyme-linked immunosorbent assay (ELISA) using polyclonal antisera against the toxic fragments of CryIA(b), CryIIIA, and CryIV toxins. A total of 241 strains were characterized by the ELISA. In the group of strains identified as CryI-type producing (47 strains), some strains were isolated from one soil sample and may be sibling strains. In order to identify individual strains, we performed total-protein electrophoresis on the strains isolated from one soil sample (data not shown).

Designing oligonucleotides to identify CryI subgroups. Specific *cryI* genes carried by each strain were identified by

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TABLE 1. Characteristics of primers used

Primer pair[a]	Positions[b]	Gene(s) recognized	Predicted size (bp) of PCR product	Sequence
CJ1, reg 5′	1105–1125	*cryIA(a)*,	246	5′TTATACTTGGTTCAGGCCC3′
CJ2, reg 3′	1332–1351	*cryIA(d)*		5′TTGGAGCTCTCAAGGTGTAA3′
CJ3, reg 5′	1181–1198	*cryIA(d)*	171	5′CAGCCGATTTACCTTCTA3′
CJ2, reg 3′	1332–1351			5′TTGGAGCTCTCAAGGTGTAA3′
CJ4, reg 5′	1133–1153	*cryIA(b)*,	216	5′AACAACTATCTGTTCTTGAC3′
CJ5, reg 3′	1328–1348	*cryIA(c)*		5′CTCTTATTATACTTACACTAC3′
CJ6, reg 5′	1495–1514	*cryIA(c)*	180	5′GTTAGATTAAATAGTAGTGG3′
CJ7, reg 3′	1656–1674			5′TGTAGCTGGTACTGTATTG3′
CJ8, reg 5′	1007–1025	*cryIB*	367	5′CTTCATCACGATGGAGTAA3′
CJ9, reg 3′	1355–1374			5′CATAATTTGGTCGTTCTGTT3′
CJ10, reg 5′	1306–1325	*cryIC*	130	5′AAAGATCTGGAACACCTTT3′
CJ11, reg 3′	1416–1436			5′CAAACTCTAAATCCTTTCAC3′
CJ12, reg 5′	837–856	*cryID*	290	5′CTGCAGCAAGCTATCCAA3′
CJ13, reg 3′	1107–1127			5′ATTTGAATTGTCAAGGCCTG3′

[a] reg, refers to the coding strand.
[b] The A of the first ATG is numbered 1.

using PCR technology. Oligonucleotides were designed by computer analysis with a GeneWork 2 program that allows for the simultaneous alignment of several gene sequences. The primers were selected from the highly variable region of the *cryI* genes. Table 1 shows the sequence of each primer, the *cryI* gene subgroup identified, and the predicted size of the PCR product. Table 1 also shows the locations of the primers within the DNA sequence of each gene.

With the use of this set of primers, we were able to identify *cryIA* to *cryID* gene subgroups, including subdivisions inside *cryIA* genes. The selected primers were synthesized in a Microsyn 1450A (Systec Inc.), using the manufacturer's reagents and conditions.

Identification of *cryI* genes on Mexican *B. thuringiensis* isolates. *B. thuringiensis* strains were grown for 12 h on a nutrient agar plate. A loop of cells from a single colony was transferred to 0.1 ml of H_2O, and the mixture was boiled for 10 min to lyse the cells. The resulting cell lysate was briefly spun (10 s, 10,000 rpm [Eppendorf model 5415C]), and 15 μl was transferred to a microcentrifuge tube containing 0.5 to 2.5 U of *Taq* DNA polymerase enzyme from Boehringer Mannheim, 0.1 to 0.5 μM each of the primers used in the reaction, and 2.5 mM each of the four deoxynucleoside triphosphates in a total volume of 50 μl.

HD1 and HD137 *B. thuringiensis* strains were used as control strains to test the set of primers. The HD1 strain contains the *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* genes, while the HD137 strain contains the *cryIA(a)*, *cryIB*, *cryIC*, and *cryID* genes. PCR with two reaction mixtures containing the *cryI* primers was performed on both control strains. Reaction mixture A contains primers CJ1 to CJ7 and this reaction detects *cryIA* genes [from *cryIA(a)* to *cryIA(d)*] while reaction mixture B contains primers CJ8 to CJ13 and this reaction detects *cryIB*, *cryIC*, and *cryID* genes. Amplification was done in the DNA thermal cycler (Omnigene HB-TR3-CM; Hybaid Limited, Teddington, Middlesex, United Kingdom) with the step cycle program set for 25 cycles (with a cycle consisting of denaturation at 92°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min); an extra step of extension at 72°C during 10 min was then performed. The reaction proved to be highly specific, because after electrophoretical analysis, the PCR products corresponded to the predicted products (Fig. 1). We observed that the HD1 strain did not yield PCR products when

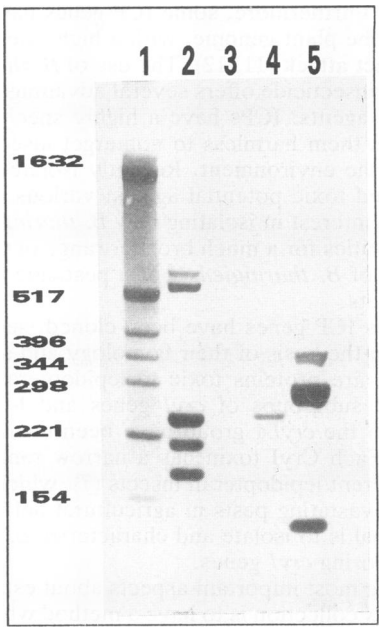

FIG. 1. Agarose (3%) gel electrophoresis of nucleic acid amplification products from *B. thuringiensis* HD1 and HD137 control strains. Lane 1, molecular weight markers (pBR322 digested with *Hin*fI); lanes 2 and 3, PCR products obtained with HD1 strain lysate as the template; lanes 4 and 5, PCR products obtained with HD137 strain lysate as the template. Reaction mixtures A (lanes 2 and 4) and B (lanes 3 and 5) were used. The HD1 strain codes for CryIA(a), CryIA(b), and CryIA(c) toxins, while the HD137 strain codes for CryIA(a), CryIB, CryIC, and CryID toxins. The numbers to the left of the gel are molecular weights.

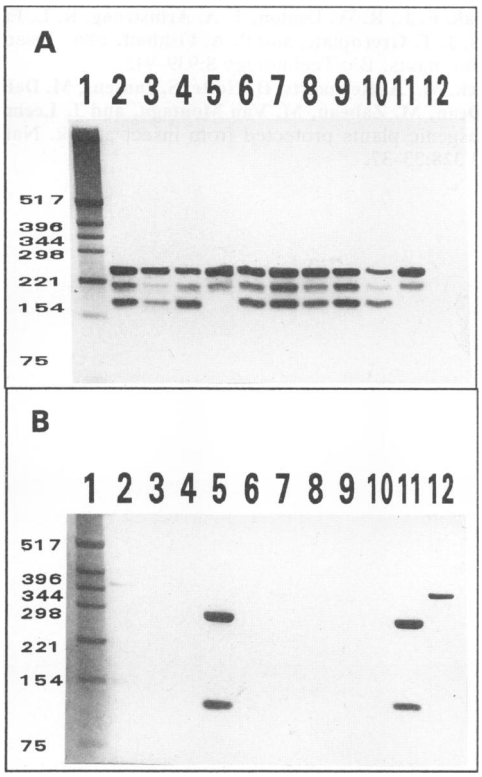

FIG. 2. Agarose (3%) gel electrophoresis of nucleic acid amplification products of representative *B. thuringiensis* strains isolated from soil samples. PCR products obtained with reaction mixture A (A) or B (B) are shown. Lane 1, molecular weight markers (pBR322 digested with *Hin*fI); lane 2, IBT10 strain; lane 3, IBT12 strain; lane 4, IBT13 strain; lane 5, IBT17 strain; lane 6, IBT23 strain; lane 7, IBT37 strain; lane 8, IBT42 strain; lane 9, IBT44 strain; lane 10, IBT60 strain; lane 11, IBT98 strain; lane 12, IBT99 strain. The numbers to the left of the gel are molecular weights.

CryIB, CryIC, or CryID primer was used. This result was very important because this strain did not contain these genes. Strain HD137 did not yield PCR products when CryIA(b) or CryIA(c) primer was used.

PCR analysis of the *cryI* genes present in different strains was finally carried out on 11 Mexican *B. thuringiensis* isolates. Figure 2 shows the products obtained after PCR with the *cryI* set of primers; all strains reacted with at least one pair of *cryI* primers. Table 2 summarizes the *cryI* gene contents of the 11 strains. Nine strains harbor the *cryIA(a)* and *cryIA(b)* genes. Seven strains also had a PCR product identified as the *cryIA(c)* gene. Strain IBT60 had only the PCR products identified as *cryIA(a)* and *cryIA(c)* genes. Of the remaining strains, two contained *cryIC* and *cryID* genes, besides the *cryIA(a)* and *cryIA(b)* genes. Strain IBT99 contained only the *cryIB* gene. We focused these data on IBT17 and IBT98 strains, because both harbor the *cryIC* and *cryID* genes, which have been reported to be highly toxic to *Spodoptera* insects. In order to determine whether these strains showed increased toxicity to the insect, we performed a bioassay on first-instar *Spodoptera frugiperda* larvae. A freshly prepared artificial diet (1) 1 ml was dispensed into 2-cm^2 vials (Nunc Multidish-4). Five dilutions of the samples were prepared in buffer solution (10 mM K_2HPO_4 [pH 7.4] with 0.15 M NaCl). A sample dilution was applied uniformly to the food surface in each vial and allowed to dry. Four larvae were placed in each vial; 24 larvae were used per sample dilution. Mortality counts were made after 6 days, and the toxicity data were analyzed by probit analysis (6). Table 2 also shows the toxicity of each strain on this insect, indicating that strains which contain the *cryIC* and *cryID* genes pose the highest toxic threat to this insect larvae. These strains were isolated from soil samples collected from agricultural active zones (maize crops). The fact that most of the analyzed strains were found to harbor the *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* genes may indicate that these genes are widely distributed among the Mexican *B. thuringiensis* strains and may play an important ecological role in Mexico. Data on the presence of various *cry* genes in different regions of the country would be valuable.

TABLE 2. Identification of *cryI* genes present in different *B. thuringiensis* strains and their toxicity against *S. frugiperda* larvae

Strain	Gene(s) identified by PCR analysis	LD_{50}[a] (ng/cm^2)	CI_{95}[b] of LD_{50}
HD1	*cryIA(a)*, *cryIA(b)*, *cryIA(c)*	969	651–1,363
HD137	*cryIA(a)*, *cryIB*, *cryIC*, *cryID*	43	32–71
IBT10	*cryIA(a)*, *cryIA(b)*, *cryIA(c)*	ND[c]	
IBT12	*cryIA(a)*, *cryIA(b)*, *cryIA(c)*	587	284–795
IBT44	*cryIA(a)*, *cryIA(b)*, *cryIA(c)*	ND	
IBT13	*cryIA(a)*, *cryIA(b)*, *cryIA(c)*	ND	
IBT23	*cryIA(a)*, *cryIA(b)*, *cryIA(c)*	ND	
IBT37	*cryIA(a)*, *cryIA(b)*, *cryIA(c)*	790	590–1,250
IBT60	*cryIA(a)*, *cryIA(c)*	787	527–1,089
IBT42	*cryIA(a)*, *cryIA(b)*, *cryIA(c)*	283	172–372
IBT17	*cryIA(a)*, *cryIA(b)*, *cryIC*, *cryID*	47	35–76
IBT98	*cryIA(a)*, *cryIA(b)*, *cryIC*, *cryID*	43	32–70
IBT99	*cryIB*	ND	

[a] LD_{50}, 50% lethal dose.
[b] CI_{95}, 95% confidence interval.
[c] ND, not determined.

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