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PCR-Based Approach for Detection of Novel *Bacillus thuringiensis cry* Genes

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A two-step strategy, named exclusive PCR or E-PCR, has been developed to overcome the main limitation of PCR, which is the detection of already-known sequences only. This strategy allows the ability to detect and further clone and sequence genes for which no specific primers are available and in which a variable region exists between two conserved regions. This approach has been applied to *Bacillus thuringiensis cryI* genes by the use of mixtures of degenerate and specific primers recognizing well-known sequences. The first step allows the accurate identification of already-characterized *cryI* genes by the use of three primers. During the second step, the same sets of primers are used to exclude known sequences and to positively detect *cryI* genes unrecognized by any specific primer. The method, as well as its application to detect, clone, and sequence a novel *cryIB* gene, is described in this article.

Bacillus thuringiensis is a spore-forming bacterium producing upon sporulation a parasporal crystal toxic to some invertebrates, mostly insects and nematodes (11, 13). The parasporal inclusion body is composed of proteins, or δ-endotoxins, varying in quantity and type depending on the strain. Each type of crystal protein is characterized by a specific host range, and based upon differences in sequence and specificity, insecticidal crystal δ-endotoxins have been classified into several groups of proteins, designated Cry (8, 11, 13).

This entomopathogenic bacterium is the most important biopesticide sold worldwide (3), and its share of the world market of pesticides is expected to rise in the coming years. *B. thuringiensis*-based products are, however, limited with respect to the diversity of strains used in commercial products, and more toxins are needed to target other insect pests and to manage the emerging problem of insect resistance (12, 24). Large screening programs, leading to important collections of isolates, have been conducted. The need for novel crystal proteins has prompted the development of molecular approaches to quickly and easily characterize toxin genes present in *B. thuringiensis* isolates. In the last few years, several PCR-based methodologies, mostly multiplex PCR, which allowed the accurate determination of families of *cry* genes (5) or specific δ-endotoxin genes have been proposed (4, 6, 7). Although powerful, PCR approaches are limited to the detection of already-known genes and fail to detect and identify novel *cry* genes even though various strategies have been proposed to increase their efficiency (15, 17).

We report here a PCR-based two-step approach, which we named exclusive PCR or E-PCR after the amplicon exclusion process in the second step, which allows both the identification of known *cry* genes present in *B. thuringiensis* isolates and the detection and identification of *cryI*-related sequences unrecognized by specific primers. A sequence and a probe for gene cloning and characterization can be obtained from the PCR product specific to the *cry*-related unknown gene. E-PCR is used in this study on *cryI* genes due to the complexity of this family of *cry* genes, which makes it a good candidate for demonstration. The approach, however, is fully applicable to other families of *cry* genes. The detection of a novel *cryIB*-related gene by use of E-PCR is reported.

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MATERIALS AND METHODS

Bacterial strains and *cry* genes. HD-1 (16, 18), HD-73 (1), HD-110 (13), and HD-133 (2, 18) were used as standard *B. thuringiensis* strains. Other *B. thuringiensis* strains were isolated as described previously (25) from Africa, the South Pacific Islands, and southern France (14). The following *cry* genes, cloned in *Escherichia coli*, were used as standards: *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* from HD-1 and *cryID* from HD-133 (kindly provided by L. Masson, BRI-NRC, Montréal, Canada), *cryIC* from strain 4F1 and *cryIE* from *B. thuringiensis* subsp. *entomocidus* 60.5 (26, 27), *cryIG* from *B. thuringiensis* subsp. *galleriae* (23), *cryIIA* from NRD-12 (19), and *cryIIIA* from *B. thuringiensis* subsp. *tenebrionis* (22). Strain 19 was isolated from soil samples collected in southern Spain. The host range and H serotype of strain 19 have not yet been investigated. For convenience, the older classification of *B. thuringiensis* insecticidal crystal proteins (13) was used throughout the article, except in Fig. 5, where the proposed new nomenclature (8) was used. This change in nomenclature was done to allow an easy comparison of the tree proposed in Fig. 5 with those currently presented in the literature based on the new nomenclature.

DNA extraction. High-purity DNA was obtained from *B. thuringiensis* as described previously (9). A fast DNA extraction procedure was adapted from various techniques (4, 15). A 5-ml *B. thuringiensis* culture was incubated overnight at 30°C in LB medium with vigorous shaking. Five milliliters of LB medium was inoculated with 0.1 ml of the overnight culture and incubated at 30°C for 3 h with vigorous shaking. Cells were pelleted by centrifugation for 5 min at 14,000 × *g* and resuspended in 100 μl of sterile double-distilled water. Cells were disrupted by two cycles of incubation for 10 min each in a dry ice-alcohol bath immediately followed by a 10-min incubation in boiling water. Cell debris was removed by centrifugation for 5 min at 14,000 × *g*, and the supernatant was used directly for PCRs. Plasmid DNA from *E. coli* was extracted by the standard alkaline lysis procedure (21).

Primer design. Nucleotide sequences of *cryI* genes available from GenBank were aligned by use of the Megalign program of the DNAStar software package. Two highly conserved regions among all *cryI* genes were selected, and two 20-mer 5′-degenerate primers, or family primers I(+) and I(−), were designed to match any of the known *cryI* genes. Primers are presented in Table 1. By use of a similar computer analysis, type primers, or specific primers (i.e., primers specific to a given type of *cryI* gene), were designed to specifically match the hypervariable region of *cryIA(a)*, *cryIA(b)*, *cryIA(c)*, *cryIA(d)*, *cryIB*, *cryIC*, *cryID*, *cryIE*, *cryIF*, and *cryIG*. Type primers are also presented in Table 1.

TABLE 1. Design and position of primers

Type of primer	Primer	Gene recognized	Position	Size of band	Primer sequence
Family[a]	I(−)	All *cryI* genes	2268[b]		5′MDATYTCTAKRTCTTGACTA3′
	I(+)	All *cryI* genes	726[b]	1.5–1.6 kb[c]	5′TRACRHTDDBDGTATTAGAT3′
Type[d]	IA's	*cryIA*	567	1,720 bp[e]	5′CAATAGTCGTTATAATGATT3′
	IAa	*cryIA(a)*	1023	1,286 bp[e]	5′TTCCCTTTATTTGGGAATGC3′
	IAb	*cryIA(b)*	940	1,371 bp[e]	5′CGGATGCTCATAGAGGAGAA3′
	IAc	*cryIA(c)*	1452	844 bp[e]	5′GGAAACTTTCTTTTTAATGG3′
	IAd	*cryIA(d)*	1057	1,212 bp[e]	5′ACCCGTACTGATCTCAACTA3′
	IB	*cryIB*	1063	1,323 bp[e]	5′GGCTACCAATACTTCTATTA3′
	IC	*cryIC*	1160	1,176 bp[e]	5′ATTTAATTTACGTGGTGTTG3′
	ID	*cryID*	1126	1,138 bp[e]	5′CAGGCCTTGACAATTCAAAT3′
	IE	*cryIE*	1155	1,137 bp[e]	5′TAGGGATAAATGTAGTACAG3′
	IF	*cryIF*	1302	967 bp[e]	5′GATTTCAGGAAGTGATTCAT3′
	IG	*cryIG*	1300	1,128 bp[e]	5′GGTTCTCAAAGATCCGTGTA3′

[a] Family primers have been designed to recognize all currently known *cryI* genes. They are degenerate primers, and their sequence is given according to the degenerate DNA genetic code: B = C, G, or T; D = A, G, or T; H = A, C, or T; K = G or T; M = A or C; R = A or G; Y = T or C. Numbering starts at the A of the first ATG in the holotype gene.

[b] Positions are those of 3′-end of primers I(+) and I(−) on the *cryIA(a)* holotype gene.

[c] The family band corresponds to the amplification product obtained with both family primers, I(+) and I(−), which recognize two highly conserved regions among all *cryI* genes. The variation in size is due to slight differences in the length of *cryI* genes.

[d] Type primers have been designed to recognize only one type of *cryI* gene. Whenever possible, they have been designed to match all closely related variants of a given type of *cryI* gene.

[e] The type band is the PCR product obtained with the I(−) family primer and one type primer selected to recognize specifically only one known *cryI* gene. Primer orientation and relative positions are shown in Fig. 2.

PCRs. Identification of known *cryI* genes was conducted for 250 ng of total *B. thuringiensis* DNA with 2.5 U of *Taq* DNA polymerase (Eurobio), 200 nM each deoxynucleoside triphosphate, 1 μM reverse primer I(−) and 0.5 μM each forward primer [specific type primer and primer I(+)], and 3 mM $MgCl_2$ in a final volume of 50 μl. Amplification was done in a Perkin-Elmer Cetus thermal cycler under the following conditions: 5 min of denaturation at 94°C followed by 25 cycles of amplification with a 1-min denaturation at 94°C, 45 s of annealing at 45°C, and 2 min of extension at 72°C. An extra extension step of 10 min at 72°C was added after completion of the 25 cycles. PCR products were analyzed by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer (21). PCRs for elimination of known family bands were conducted as described above except for a few parameters which were modified as follows. The concentration of family primer I(+) was increased to 3.5 μM, the annealing temperature was raised to 47°C, the $MgCl_2$ concentration was increased to 4 mM, and the concentration of each of the four deoxynucleoside triphosphates was decreased to 100 mM.

Miscellaneous techniques. Standard recombinant DNA techniques were performed as described by Sambrook et al. (21). PCR fragments were cloned into a pGEM-T vector (Promega). DNA sequencing was performed by the chain termination technique with an Applied Biosystems 370A nucleotide sequence analyzer. DNA and protein sequence alignments and distance calculations were obtained through the Clustal method with the Megalign software from the DNAStar package.

RESULTS

Determination of the *cry* gene contents of *B. thuringiensis* isolates. Degenerate family primers I(+) and I(−) and type primers (Table 1) were tested as triplets (i.e., both family primers and one type primer for each PCR) for specificity and accuracy by using *cry* genes cloned in *E. coli* [i.e., *cryIA(a)*, *cryIA(b)*, *cryIA(c)*, *cryIC*, *cryID*, *cryIE*, *cryIG*, *cryIIA*, and *cryIIIA*] as templates. *cryIB* was not available as a cloned gene and was thus amplified from high-purity total DNA from strain HD-551. Amplification of a family band of about 1.5 to 1.6 kb was observed for all of the *cryI* genes, whereas no PCR products were detected for the *cryII* and *cryIII* templates (Fig. 1A). *cryIA(a)*, *cryIA(b)*, *cryIA(c)*, *cryIB*, *cryIC*, *cryID*, *cryIE*, and *cryIG* were also specifically identified (Fig. 1A; Table 1). PCR products corresponded to the expected sizes according to the positions of the primers in the holotype genes (Table 1). All possible combinations of primers were tested with all the different *cryI*, *cryIIA*, and *cryIIIA* templates available, and no cross-reaction was detected (Fig. 1B). The efficiency of the PCR approach and that of DNA extraction procedures were evaluated by the use of *B. thuringiensis* strains of known *cry* gene content (i.e., HD-1 [16, 18], HD-73 [1], HD-110 [13], and HD-133 [2, 18]) as templates. High-purity total DNA and DNA extracted by the fast extraction procedure were used as templates under the same PCR conditions, and identical results were obtained with both sets of DNA (data not shown). The *cry* gene contents of these selected strains was found to be in full accordance with already published data. A typical result is illustrated with strain HD-133 as a template (Fig. 1C). Four *cryI* genes, *cryIA(a)*, *cryIA(b)*, *cryIC*, and *cryID*, which correspond to previous reports of strain HD-133 *cryI* gene content, were clearly detected (2, 18).

Rationale for the detection of *cryI*-related sequences. If a *cryI* gene different from those already known were present in a *B. thuringiensis* strain, it would remain undetected by the type primers since these primers have been designed to match a variable region specific to a particular *cryI* gene. However, degenerate primers I(+) and I(−) are able to direct the amplification of any *cryI* gene present in a *B. thuringiensis* strain. The family band of 1.5 to 1.6 kDa detected when multiple *cryI* genes are present in a single strain (Fig. 1C) should therefore contain PCR products from all of the *cryI* genes present in that strain regardless of their detection by a specific type primer. Isolation of PCR products related to *cryI* genes undetected by type primers would then be possible if they could be physically separated from those corresponding to the *cryI* genes identified by the type primers. E-PCR was therefore based on the exclusion from the family band of PCR products corresponding to genes previously detected by type primers (Fig. 2).

The detection of two PCR products (i.e., family and type bands) is a consequence of a competition between the I(+) primer and the type primer (sense primers) for extension with respect to the I(−) primer (antisense primer). In such a competition, the following two parameters must be considered: (i) the concentration of the I(−) primer which limits the overall amount of PCR products (family and type bands), and (ii) the

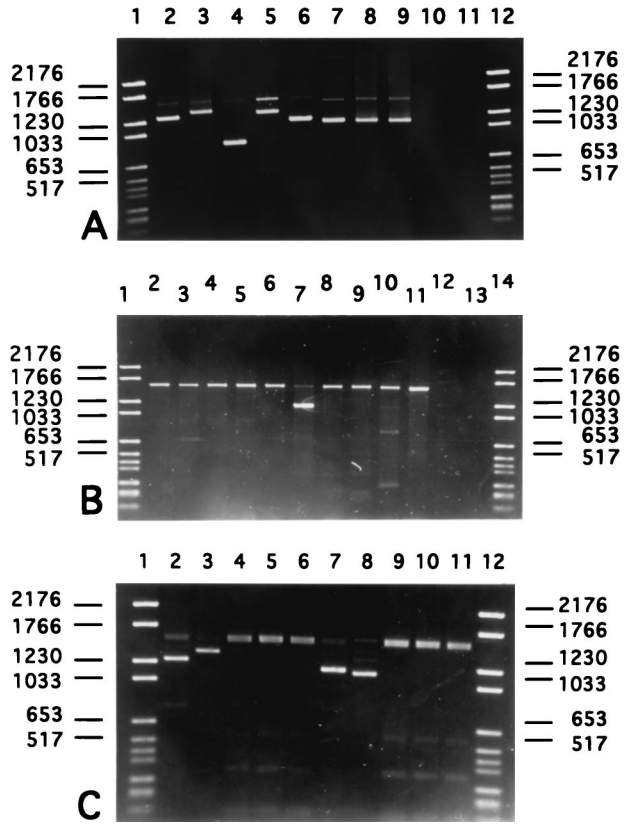

FIG. 1. Assessment of the specificity of family and type primers. (A) Assessment of triplet specificity against various DNA templates. Lanes: 1, molecular weight marker VI (Boehringer); 2, *cryIA(a)*; 3, *cryIA(b)*; 4, *cryIA(c)*; 5, *cryIB*; 6, *cryIC*; 7, *cryID*; 8, *cryIE*; 9, *cryIG*; 10, *cryIIA*; 11, *cryIIIA*; 12; molecular weight marker VI (Boehringer). DNA templates were amplified with family primers I(+) and I(−) to test their specificity. (B) Assessment of the specificity of type primers. A cloned *cryIC* gene was used as a template and amplified with all of the type primers used in this work. All lanes, except 1 and 14, contain a type primer present in a triplet with I(+) and I(−). Lanes: 1, molecular weight marker VI (Boehringer); 2, primer IAa; 3, primer IAb; 4, primer IAc; 5, primer IAd; 6, primer IB; 7, primer IC; 8, primer ID; 9, primer IE; 10, primer IF; 11, primer IG; 12, primer IIA; 13, primer IIIA; 14, molecular weight marker VI (Boehringer). Similar controls for the absence of cross-reactions were conducted by testing all triplets on each of the DNA templates illustrated in panel A. (C) Determination of the *cryI* gene content of strain HD-133. All lanes, except 1 and 12, contain a type primer present in a triplet with I(+) and I(−). Lanes: 1, molecular weight marker VI (Boehringer); 2, primer IAa; 3, primer IAb; 4, primer IAc; 5, primer IAd; 6, primer IB; 7, primer IC; 8, primer ID; 9, primer IE; 10, primer IF; 11, primer IG; 12, molecular weight marker VI (Boehringer). Molecular weights of markers are indicated on the right and left.

ratio between the I(+) primer and the type primer which will orient the PCR to yield preferentially either the family band or the type band. In the case of multiplex PCR products of different lengths but similar sequences, there is a preferential amplification of the shortest product which may be related to a limited processivity or impaired amplification of the longer PCR product by the shorter one when primers anneal on the same strand (10, 20). Orienting the yield of a multiplex PCR towards the production of the shorter amplicon (type band) will ultimately cause the longer product (family band) to disappear. Indeed, the simultaneous amplification of the family band and the type band by use of a triplet of primers frequently resulted in a lower intensity of the family band when the type primer annealed on the template DNA (Fig. 1). This would lead to the removal from the family band of PCR products related to known genes when a multiplex PCR is conducted with a mixture of both family primers and all of the type primers corresponding to previously detected *cryI* genes. If all the *cryI* genes present in the isolate have been identified by use of triplets, this competition will cause the family band to disappear (Fig. 2). If *cryI* genes undetected by the type primers are present in the family band, the competition will still result in the presence of a family band of 1.5 to 1.6 kb (Fig. 2). This band will then contain essentially an amplicon related to the

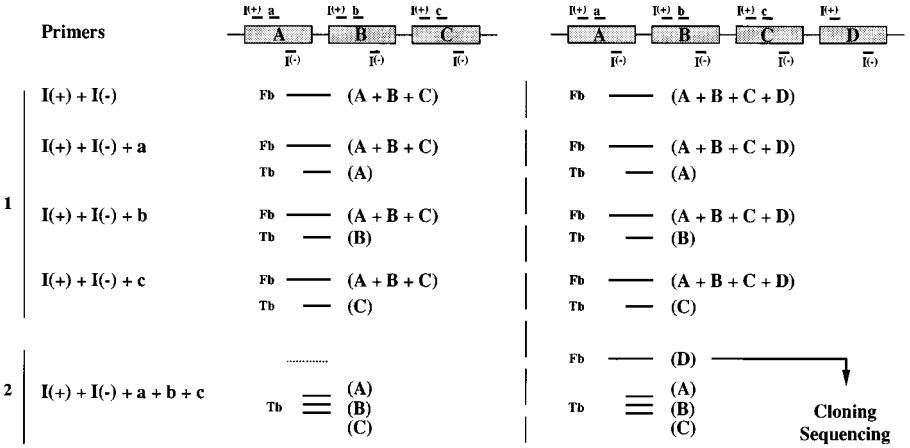

FIG. 2. Rationale of gene detection by E-PCR. (A) Products expected when all the *cryI* genes present in a strain have been identified by use of triplets of primers. (B) Products expected when a *cryI* gene remains undetected by the type primers available. Combinations of primers are listed on the left of the figure. Uppercase letters (A, B, C, and D) refer to the *cryI* genes, whereas lowercase letters (a, b, c, and d) refer to the type primers specific to these genes. Family primers are labelled I(+) and I(−). Capital letters in parentheses refer to genes represented in the PCR product. Shaded boxes represent the *cryI* genes. 1, First step of the strategy (gene detection with triplets); 2, second step of the strategy (identification of previously undetected *cryI* gene). Abbreviations: Fb, family band; Tb, type band. The presence of only primers I(−) and I(+) on gene D (far right end of section B of the figure) is intended to illustrate the situation where no complementary sequence is recognized by any type primer. In this case, only the family primers can anneal and the PCR product will be detected as a family band corresponding to a potentially novel *cryI* gene which will be further cloned and sequenced.

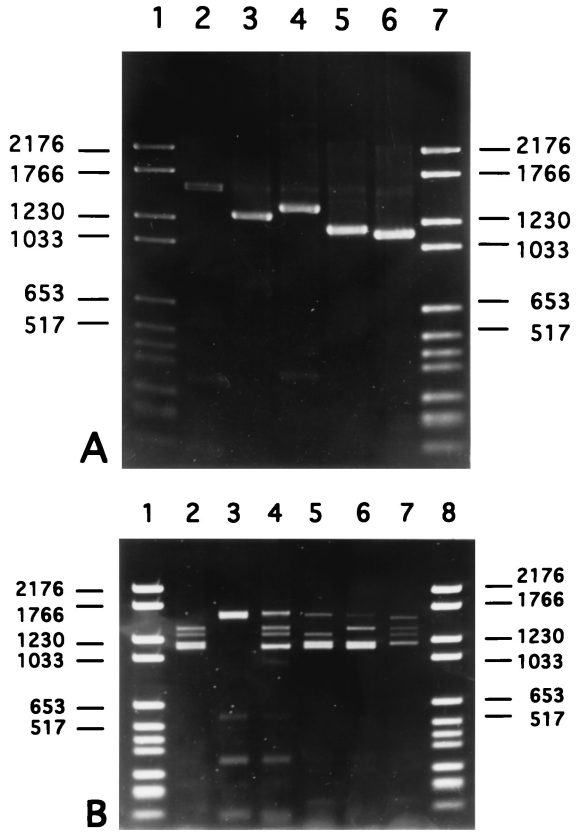

FIG. 3. Detection of *cryI* sequences unrelated to the type primers. (A) Identification of the *cryI* genes represented in the family band from HD-133. PCRs were conducted by use of the family band from HD-133 as a template. Reactions were performed with doublets of primers, i.e., primer I(−) and one of the four type primers which yielded a positive response as shown in Fig. 2A. All lanes, except 1, 2, and 7, contain a type primer mixed with I(−). Lanes: 1, molecular weight marker VI (Boehringer); 2, family band from HD-133 obtained with primers I(+) and I(−) which was used as the template; 3, primer IAa; 4, primer IAb; 5, primer IC; 6, primer ID; 7, molecular weight marker VI (Boehringer). (B) Sequential detection of *cryI* genes unrelated to the type primers. Multiplex PCRs were conducted with DNA from strain HD-133 as a template by use of mixtures of several type primers and the family primers. All lanes, except 1, 3, and 8, contain type primers mixed with primers I(+) and I(−). Lanes: 1, molecular weight marker VI (Boehringer); 2, primers IAa, IAb, IC, and ID; 3, primers I(+) and I(−); 4, primers IAa, IAb, and ID; 5, primers IAa, IC, and ID; 6, primers IAb, IC, and ID; 7, primers IAa, IAb, and IC; 8, molecular weight marker VI (Boehringer).

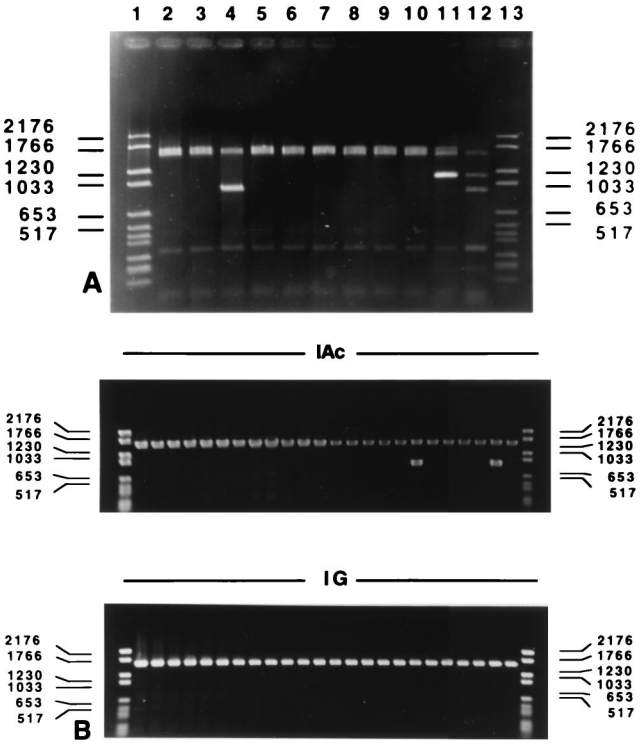

FIG. 4. Detection of a novel *cryI* gene from strain 19 by E-PCR. (A) Identification of the *cryI* genes present in strain 19 and the determination of a putative new *cryI* sequence. Triplets of primers were used to identify the *cryI* gene contents of strain 19. All lanes, except 1 and 13, contain a type primer mixed with primers I(+) and I(−). Lanes: 2, primer IAa; 3, primer IAb; 4, primer IAc; 5, primer IAd; 6, primer IB; 7, primer IC; 8, primer ID; 9, primer IF; 10, primer IE; 11, primer IG. Since strain 19 reacted positively only with primers IAc and IG, an E-PCR was conducted with primers I(+), I(−), IAc, and IG, and as is visible in lane 12, a family band of 1.5 to 1.6 kb as well as two bands corresponding to the *cryIAc* and *cryIG* genes resulted. Lanes 1 and 13 contain molecular weight markers VI (Boehringer). (B) PCR analysis of clones bearing the family bands amplified from strain 19. PCRs were conducted with 24 clones obtained after cloning the remaining family band revealed by E-PCR assay on strain 19 (Fig. 4A, lane 12). Clones were assayed against triplets containing both I(+) and I(−) family primers and the type primers IAc and IG in the upper and lower gels, respectively. Positive reactions with the type primer IAc were observed only with clones 18 and 23 (lanes 19 and 24 in the upper gel), whereas no positive reaction was obtained with type primer IG. All other clones reacted only with both family primers as shown by the presence of a family band.

undetected *cryI*-related gene. PCR conditions and primer ratios were thus modified as described in Materials and Methods to exclude from the family band PCR products corresponding to *cryI* genes detected by type primers.

The use of E-PCR to detect the presence of a *cryI* gene undetected by specific PCR primers is illustrated with HD-133 as a template (Fig. 3). The family band obtained with primers I(+) and I(−) was excised from an agarose gel and used as a template for a subsequent multiplex PCR with primer I(+), IAa, IAb, IC, or ID. The four expected type bands were detected by agarose gel analysis, showing that the family bands contained PCR products from all the genes present in strain HD-133 (Fig. 3A). A PCR was conducted with a mixture of type primers IAa, IAb, IC, and ID and family primers I(+) and I(−) (Fig. 3B, lane 2). This PCR yielded amplification products for *cryIA(a)*, *cryIA(b)*, *cryIC*, and *cryID*, whereas the 1.5- to 1.6-kb family band was no longer visible (Fig. 3B, lane 2), indicating that, as expected, the family band was challenged by specific primers and disappeared. Similar PCRs conducted on the same template DNA and in which one type primer (i.e., IAa, IAb, IC, or ID) was omitted one at a time showed the presence of only three type bands and one family band (Fig. 3B, lanes 4 to 7). The patterns of type bands in lanes 4 to 7 were different, indicating that the type band corresponding to the missing primer was lacking (Fig. 3B). A slight difference in the size of the remaining family band is visible in Fig. 3B, lanes 4 and 7, indicating that a different PCR product remained as a family band. Family bands in lanes 5 and 6 are too close in size for a difference to be clearly visible (Fig. 3B). Cloning and sequencing the PCR product remaining in the family band confirmed that it corresponded to the expected gene. This approach was applied with similar results to the other strains considered in this work as well as to a collection of 250 naturally occurring strains. The latter analysis led to the characterization of the *cry* gene contents with respect to the *cryI*, *cryII*, and *cryV* gene families (data not shown).

Detection of a novel *cryI* gene. Naturally occurring strains were also tested for the presence of *cryI* genes undetected

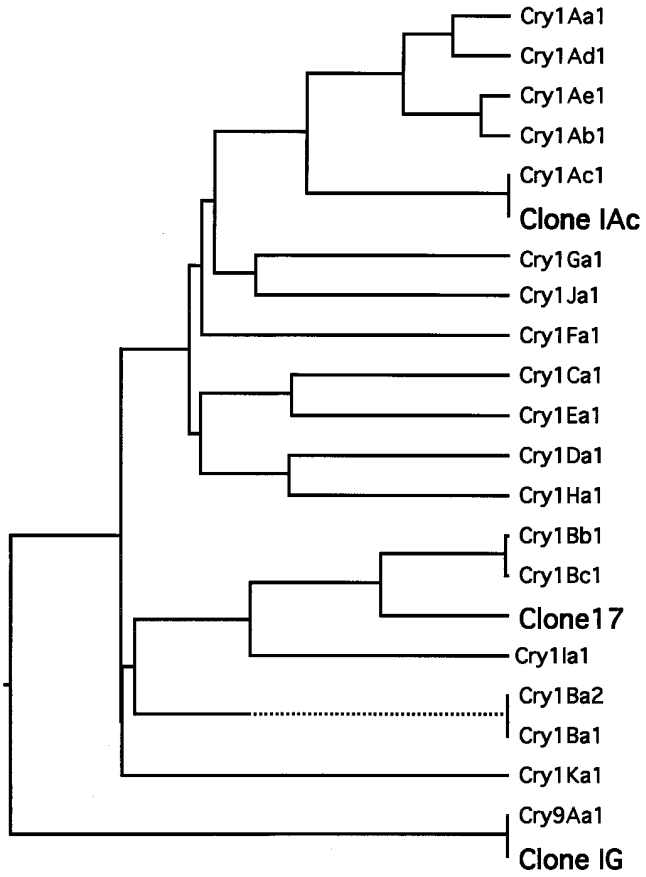

FIG. 5. Relatedness of protein sequences deduced from strain 19 amplicons to Cry proteins. Protein sequence alignments and divergence calculations were conducted by comparing sequences of all existing CryI proteins with those deduced from the various PCR products obtained from strain 19. Only the protein sequence corresponding to the gene region between the annealing sites of primers I(+) and I(−) on all *cryI* genes was considered for the alignment. Except for the Cry protein groups related to the translation products of *cryI* genes detected in strain 19, i.e., CryIA, CryIG, and CryIB, only one representative of each group is shown. In contrast to the nomenclature used in the remainder of this paper, the novel nomenclature proposed by Crickmore et al. (8) was used in this figure to allow easy retrieval and comparison with data from data banks. In this figure, Cry proteins deduced from genes identified in strain 19 were modified as follows: CryIA(c) was changed to Cry1Ac1 and CryIG was changed to Cry9Aa1.

when specific type primers were used, and analysis of the *cryI* gene contents of strain 19 is shown as an example. Two *cryI* genes, *cryIA(c)* and *cryIG*, were first detected in this strain (Fig. 4A, lanes 4 and 11). E-PCR was then conducted on DNA extracted from strain 19 by the simultaneous use of primers I(+), I(−), IAc, and IG (Fig. 4A, lane 12). Two type bands corresponding to *cryIA(c)* and *cryIG* as well as a remaining family band of about 1.5 kb were observed (Fig. 4A, lane 12). This family band was extracted from the gel and cloned. Of 24 clones analyzed by PCR, two corresponded to *cryIA(c)*, whereas 22 clones showed a clear amplification of the family band but no type band (Fig. 4B). The 1.5-kb insert present in clone 17, a random-selected representative of the group of 22 clones, was sequenced and compared to all *cryI* sequences available from GenBank. Alignments of both DNA and protein sequences showed that this clone contained a gene related to *cryIB* (Fig. 5). PCR products related to the *cryIA(c)* and *cryIG* genes were also sequenced and were similar to the holotype genes (Fig. 5).

Aligning DNA-deduced protein sequences from all known members of the CryIB protein group showed that the protein sequence from clone 17 differed from the closest relative, known under the new nomenclature as Cry1Bb1 (8), by 16.1%. This makes it a novel member of the CryIB group (Fig. 5) with respect to the latest nomenclature of *B. thuringiensis* insecticidal crystal proteins (8). Since the sequenced 1.5-kb PCR product ranged from base 845 to 2509, including the most variable region of the *cryI* gene family, the overall divergence of the new sequence should be somewhat less than 16.1%. Complete sequencing will be required to determine the exact divergence.

DISCUSSION

The results presented here demonstrate that a new PCR-based approach, exclusive PCR or E-PCR, can be used for systematic, large-scale screening of *B. thuringiensis* isolates to identify known *cry* genes and, more importantly, to detect and identify novel *cry* genes by use of the same initial set of primers. The use of triplets allowed the specific detection in a *B. thuringiensis* isolate of any known *cryI* gene for which a type primer was available. Like previously reported PCR-based strategies, however, it failed to detect novel sequences or variants without resorting to sophisticated mixtures of a large numbers of primers. Such approaches were shown to be efficient (15) but are limited to a single type of *cry* gene and are not suitable for the systematic detection of variants or novel *cryI* genes. E-PCR provides a means for isolating variants or novel *cry* sequences which are characterized by the presence of a 1.5- to 1.6-kb PCR product which can be easily detected, cloned, and sequenced. This yields valuable information on the relatedness of this *cryI*-related gene with respect to already published sequences, since the amplified and sequenced region corresponds to the most variable, thus specific, region of a *cryI* gene. Since the lack of detection by a type primer will result in the presence of a visible family band, no *cryI*-related sequence can be missed. If the annealing of the type primer is impaired by a mutation, i.e., a mismatch at the 3′ end of the type primer, this *cry* sequence will be visible as a 1.5- to 1.6-kb PCR fragment which will be considered a putative novel gene and subsequently cloned and sequenced to determine its relatedness to other *cry* genes. The ca. 1.5-kb fragment corresponding to the putative novel sequence also represents a well-characterized DNA probe which can be produced in large amounts after cloning and used to easily locate the gene in a DNA library.

The novel *cryIB* gene identified in strain 19 differs by 16.1% from other members of the *cryIB* group with respect to the DNA-deduced protein sequence. Although clearly different, this gene will be submitted for consideration in the *B. thuringiensis cry* gene nomenclature only after cloning, full-length sequencing, and expression in a recombinant host strain to confirm the toxicity of the protein. Although presented only for *cryI* genes, the approach described herein has been tested also on *cryII* and *cryV* genes and gave results similar to those for *cryI* genes, indicating that it can be applied to other families of *cry* genes. The possibility of detecting sequences unrecognized by available type primers, illustrated here by the detection of a novel *cryIB* gene, is a clear demonstration of the potential of the E-PCR method for identification of novel *cry* genes and for an easy and systematic screening of collections of *B. thuringiensis* strains. Aside from the identification of novel *cry* genes, E-PCR may also be potentially applicable to any multigene family for which a highly variable domain is flanked by two conserved regions. This would extend the range of application of E-PCR beyond *B. thuringiensis* and insecticidal proteins to

other fields of investigation for which extensive screening for members of such multigene families is of interest.

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REFERENCES

1. **Adang, M., J. Staver, T. Rocheleau, J. Leighton, R. Baker, and D. Thompson.** 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. Gene **36:**289–300.
2. **Aronson, A. I., E. S. Han, W. McGaughey, and D. Johnson.** 1991. The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects. Appl. Environ. Microbiol. **57:**981–986.
3. **Bernhard, K., and R. Utz.** 1993. Production of *Bacillus thuringiensis* insecticides for experimental and commercial uses, p. 256–267. *In* P. F. Entwistle, J. S. Cory, M. J. Bailey, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmetal biopesticide: theory and practice. John Wiley & Sons, Inc. Chichester, England.
4. **Bourque, S. N., J. Valero, M. Mercier, C. Lavoie, and R. C. Levesque.** 1993. Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis*. Appl. Environ. Microbiol. **59:** 523–527.
5. **Carozzi, N. B., V. C. Kramer, G. W. Warren, S. Evola, and M. Koziel.** 1991. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl. Environ. Microbiol. **57:**3057–3061.
6. **Cerón, J., L. Covarrubias, R. Quintero, A. Ortiz, M. Ortiz, E. Aranda, L. Lina, and A. Bravo.** 1994. PCR analysis of the *cryI* insecticidal crystal family genes from *Bacillus thuringiensis*. Appl. Environ. Microbiol. **60:**353–356.
7. **Cerón, J., A. Ortiz, R. Quintero, L. Guereca, and A. Bravo.** 1995. Specific PCR primers to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. Appl. Environ. Microbiol. **61:**3826–3831.
8. **Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, B. Lambert, D. Lereclus, C. Gawron-Burke, and H. D. Dean.** 1995. Revision of the nomenclature for the *Bacillus thuringiensis cry* genes, p. 14. *In* Program and abstracts of the 28th Annual Meeting of the Society for Invertebrate Pathology. Society for Invertebrate Pathology, Bethesda, Md.
9. **Delécluse, A., J. F. Charles, A. Klier, and G. Rapoport.** 1991. Deletion by in vivo recombination shows that the 28-kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. J. Bacteriol. **173:**3374–3381.
10. **Edwards, M. C., and R. A. Gibbs.** 1994. Multiplex PCR: advantages, development and applications. PCR Methods Appl. **3:**S65–S75.
11. **Feitelson, J. S., J. Payne, and L. Kim.** 1992. *Bacillus thuringiensis*: insects and beyond. Bio/Technology **10:**271–275.
12. **Ferré, J., B. Escriche, Y. Bel, and J. Van Rie.** 1995. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis* insecticidal crystal proteins. FEMS Microbiol. Lett. **132:**1–7.
13. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
14. **Itoua-Apoyolo, C., L. Drif, J. M. Vassal, H. DeBarjac, J. P. Bossy, F. Leclant, and R. Frutos.** 1995. Isolation of multiple subspecies of *Bacillus thuringiensis* from a population of the European sunflower moth, *Homeosoma nebulella*. Appl. Environ. Microbiol. **61:**4343–4347.
15. **Kalman, S. H., L. Kiehne, J. L. Libs, and T. Yamamoto.** 1993. Cloning of a novel *cryIC*-type gene from a strain of *Bacillus thuringiensis* subsp. *galleriae*. Appl. Environ. Microbiol. **59:**1131–1137.
16. **Kronstad, W. S., and H. R. Whiteley.** 1986. Three classes of homologous *Bacillus thuringiensis* crystal-protein genes. Gene **43:**2940.
17. **Kuo, W. S., and K. F. Chak.** 1996. Identification of novel *cry*-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA. Appl. Environ. Microbiol. **62:**1369–1377.
18. **Masson, L., G. Préfontaine, G. L. Péloquin, P. C. K. Lau, and R. Brousseau.** 1989. Comparative analysis of the individual protoxin components in P1 crystals of *Bacillus thuringiensis* subsp. *kurstaki* isolates NRD-12 and HD-1. Biochem. J. **269:**507–512.
19. **Moar, W. J., J. T. Trumble, R. H. Hice, and P. A. Backman.** 1994. Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf-colonizing strain of *Bacillus cereus*. Appl. Environ. Microbiol. **60:**896–902.
20. **Repp, R., S. Rhiel, K. H. Heermann, S. Schaefer, C. Keller, P. Ndumbe, F. Lambert, and W. H. Gerlich.** 1993. Genotyping by multiplex polymerase chain reaction for detection of endemic hepatitis B virus transmission. J. Clin. Microbiol. **31:**1095–1102.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Sekar, V., D. V. Thompson, M. J. Maroney, R. G. Bookland, and J. M. Adang.** 1987. Molecular cloning and characterization of the insecticidal crystal protein of *Bacillus thuringiensis* var. *tenebrionis*. Proc. Natl. Acad. Sci. USA **84:**7036–7040.
23. **Smulevitch, S. V., A. L. Osterman, A. B. Shevelev, S. V. Kaluger, A. I. Karasin, R. M. Kadyrov, O. P. Zagnitko, G. G. Chestukhina, and V. M. Stepanov.** 1991. Nucleotide sequence of a novel delta-endotoxin gene *cryIG* of *Bacillus thuringiensis* ssp. *galleriae*. FEBS Lett. **293:**25–28.
24. **Tabashnik, B.** 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. **39:**47–79.
25. **Vassal, J. M., H. De Barjac, R. Frutos, and B. A. Federici.** 1993. Isolation of *Bacillus thuringiensis* subsp. *israelensis* from diseased field-collected larvae of the saturniid moth *Hylesia metabus*, in French Guiana. FEMS Microbiol. Lett. **107:**199–204.
26. **Visser, B., T. Salm, W. van der Brink, and G. Folkers.** 1988. Genes from *Bacillus thuringiensis entomocidus* 60.5 coding for insect-specific crystal proteins. Mol. Gen. Genet. **212:**219–224.
27. **Visser, B., E. Musterman, A. Stoker, and G. Dirkse.** 1990. A novel *Bacillus thuringiensis* gene encoding a *Spodoptera exigua*-specific crystal protein. J. Bacteriol. **172:**6783–6788.