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.

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: cryA(a), cryA(b), and cryA(c) from HD-1 and cryD from HD-133 (kindly provided by L. Masson, BRI-NRC, Montréal, Canada), cryIC from strain 4F1 and cryE from B. thuringiensis subsp. entomocidus 60.5 (26, 27), cryIG from B. thuringiensis subsp. galleriae (23), cryIA from NRD-12 (19), and cryIIA 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 F(+) and F(−), 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 regions of cryA(a), cryA(b), cryA(c), cryA(d), cryIB, cryIC, cryID, cryIE, cryIF, and cryIG. These primers are also presented in Table 1.
TABLE 1. Design and position of primers

<table>
<thead>
<tr>
<th>Type of primer</th>
<th>Primer</th>
<th>Gene recognized</th>
<th>Position</th>
<th>Size of band</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familya</td>
<td>I(−)</td>
<td>All cryl genes</td>
<td>2268b</td>
<td>1.5–1.6 kb</td>
<td>5′MDATYTCTAKRTCTTGACTA3′</td>
</tr>
<tr>
<td></td>
<td>I(+)</td>
<td>All cryl genes</td>
<td>726c</td>
<td></td>
<td>5′TRACRHTDDDGTATAGTATTAGT3′</td>
</tr>
<tr>
<td>Typefd</td>
<td>LA’s</td>
<td>cryLA</td>
<td>567</td>
<td>1.720 bp</td>
<td>5′CATAGTGCGTTAAAAATGATTT3′</td>
</tr>
<tr>
<td></td>
<td>Ia</td>
<td>cryLA(a)</td>
<td>1023</td>
<td>1.286 bp</td>
<td>5′TTCCCTATTATTGGGAAATGC3′</td>
</tr>
<tr>
<td></td>
<td>Ia</td>
<td>cryLA(b)</td>
<td>940</td>
<td>1.371 bp</td>
<td>5′CGGATGCTCATAGGAGAGAA3′</td>
</tr>
<tr>
<td></td>
<td>Ia</td>
<td>cryLA(c)</td>
<td>1452</td>
<td>0.844 bp</td>
<td>5′GGAACATTCCTTTTTATGG3′</td>
</tr>
<tr>
<td></td>
<td>Ia</td>
<td>cryLA(d)</td>
<td>1057</td>
<td>1.212 bp</td>
<td>5′ACCCGTACTGTCCTCAAAAT3′</td>
</tr>
<tr>
<td></td>
<td>IB</td>
<td>cryIB</td>
<td>1063</td>
<td>1.323 bp</td>
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<tr>
<td></td>
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<td>cryIC</td>
<td>1160</td>
<td>1.176 bp</td>
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<td></td>
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<td>1.138 bp</td>
<td>5′CAGGCTTGACAAATTTCAAT3′</td>
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<tr>
<td></td>
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<td></td>
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<td>1302</td>
<td>0.967 bp</td>
<td>5′GATTTCAGAGATTGATATT3′</td>
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<td></td>
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<td>1300</td>
<td>1.128 bp</td>
<td>5′GGTTCTCAAGATCCGCTG3′</td>
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</table>

a Family primers have been designed to recognize all currently known cryl 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 cryLA(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 cryl genes. The variation in size is due to slight differences in the length of cryl genes.

d Type primers have been designed to recognize only one type of cryl gene. Whenever possible, they have been designed to match all closely related variants of a given type of cryl 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 cryl gene. Primer orientation and relative positions are shown in Fig. 2.

PCRs. Identification of known cryl genes was conducted for 250 ng of total R. 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 MgCl₂ 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 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 cryl 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., cryLA(a), cryLA(b), cryLA(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-55. Amplification of a family band of about 1.5 to 1.6 kb was observed for all of the cryl genes, whereas no PCR products were detected for the cryII and cryIII templates (Fig. 1A). cryLA(a), cryLA(b), cryLA(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 in the holotype genes (Table 1). All possible combinations of primers were tested with 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 cryl 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 cryl 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 cryl genes, cryLA(a), cryLA(b), cryIC, and cryID, which correspond to previous reports of strain HD-133 cryl gene content, were clearly detected (2, 18).

Rationale for the detection of cryl-related sequences. If a cryl 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 cryl gene. However, degenerate primers I(+) and I(−) are able to direct the amplification of any cryl gene present in a B. thuringiensis strain. The family band of 1.5 to 1.6 kDa detected when multiple cryl genes are present in a single strain (Fig. 1C) should therefore contain PCR products from all of the cryl genes present in that strain regardless of their detection by a specific type primer. Isolation of PCR products related to cryl genes undetected by type primers would then be possible if they could be physically separated from those corresponding to the cryl 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).
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 ampiclon (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 ampiclon related to 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, cryIC; 10, cryID; 11, cryIAA; 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 IIB; 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.
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 were different, indicating that the type band corresponding to the cryI gene contents with respect to the cryI Wilcox gene family. The latter analysis led to the characterization of the cryI 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. 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, 5, and 8, contain type primers mixed with primers I(+) and I(−). Lanes: 1, molecular weight marker VI (Boehringer); 2, family band from HD-133 obtained with primers I(+) and I(−); 3, primers IAa, IAb, IC, and ID; 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. Triples 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.
CryIA(c) was changed to Cry1Ac1 and CryIG was changed to Cry9Aa1. Proteins deduced from genes identified in strain 19 were modified as follows: allow easy retrieval and comparison with data from data banks. In this figure, Cry is shown. In contrast to the nomenclature used in the remainder of this paper, the Cry protein groups related to the translation products of cryI genes were 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(+) and 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 gene (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.

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.

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Aligning DNA-deduced protein sequences from all known

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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 cryI 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 cryI 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 cryI 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 cryI 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 cryI 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 cryI 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


