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Specific PCR Primers Directed To Identify *cryI* and *cryIII* Genes within a *Bacillus thuringiensis* Strain Collection

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In this paper we describe a PCR strategy that can be used to rapidly identify *Bacillus thuringiensis* strains that harbor any of the known *cryI* or *cryIII* genes. Four general PCR primers which amplify DNA fragments from the known *cryI* or *cryIII* genes were selected from conserved regions. Once a strain was identified as an organism that contains a particular type of *cry* gene, it could be easily characterized by performing additional PCR with specific *cryI* and *cryIII* primers selected from variable regions. The method described in this paper can be used to identify the 10 different *cryI* genes and the five different *cryIII* genes. One feature of this screening method is that each *cry* gene is expected to produce a PCR product having a precise molecular weight. The genes which produce PCR products having different sizes probably represent strains that harbor a potentially novel *cry* gene. Finally, we present evidence that novel crystal genes can be identified by the method described in this paper.

The main focus in studies of *Bacillus thuringiensis* strains is the production of insecticidal crystal inclusions by these organisms during sporulation. These crystals are composed of proteins called δ-endotoxins or Cry proteins. Workers have described different Cry proteins that exhibit toxic activity against larvae of very different insects (lepidopterans, dipterans, and coleopterans), as well as other organisms, including platyhelminths, nematodes, and protozoans (10, 16). The use of *B. thuringiensis* as a microbial insecticide has several advantages over the use of chemical control agents; *B. thuringiensis* strains are highly specific for certain hosts and are not toxic to other insects, plants, and vertebrates.

Cry proteins are synthesized as protoxins, which must be solubilized and activated through protease action in the insect midgut. The activated toxin binds to its specific binding site, which is located in the apical microvilli of susceptible larval midgut epithelial cells (14, 26, 27). After binding, the toxin inserts itself into the cell plasma membrane (25) and forms a pore or lesion that allows net uptake of ions and water, leading to midgut cell swelling and eventual lysis (3, 18).

Some *cry* genes have been cloned and sequenced. These genes have been organized into six different groups on the basis of their sequence similarities and ranges of specificity (10, 16). The CryI, CryII, CryIII, CryIV, and CryV proteins are toxic to lepidopteran, lepidopteran and dipteran, coleopteran, dipteran, and nematode larvae, respectively. The CryVI proteins are also toxic to nematodes, but their origins seem to be different from the origins of the other Cry proteins (10).

The order Coleoptera and the order Lepidoptera contain some of the most devastating crop pests that have been described. Within the CryI protein group (toxic to lepidopterans) there are 10 different subclasses (4, 8, 12, 16, 22, 28). Each subclass of CryI proteins has a specific range of activity against different lepidopteran insects. Within the CryIII protein group (toxic to coleopterans) five subclasses have been described (1, 9, 11, 19, 20, 23). Integrated insect control requires information concerning the toxins that are most active against selected crop pests.

The search for and characterization of novel *B. thuringiensis* genes is a worldwide project. The resulting knowledge should provide novel alternatives for the control of different insects and for coping with the problem of resistance. Because of this it is important to have a screening method which can be used to identify the known *cry* genes.

The PCR method has proven to be a powerful tool for identification of the specific insecticidal genes carried by different *B. thuringiensis* strains (2, 5–7, 13). In a previous paper, we described the sequences of PCR primers designed to identify some lepidopteran-active genes (*cryIA* to *cryID*) present in soil isolates of *B. thuringiensis* (6). The PCR method also has potential for identifying new *cry* genes, as reported by Kalman et al. (17) and Chak et al. (7), who used it to identify novel *cryIC* genes.

In this paper we describe a PCR strategy designed to identify strains that harbor any of the known *cryI* or *cryIII* genes. General primers for these genes were selected from a region that is highly conserved in the *cryI* and *cryIII* genes. Strains with unique PCR product profiles can be easily characterized by performing additional PCR with the specific primers described previously (6) and the novel specific primers described in this paper. Using this method, we identified 10 different *cryI* genes and five different *cryIII* genes. One feature of this screening method is that each *cry* gene should produce a PCR product having a unique molecular weight. Strains that produce products of different sizes probably contain novel genes. We describe evidence which suggests that novel *cry* genes can be identified by the PCR method.

MATERIALS AND METHODS

Bacterial strains. Known *B. thuringiensis* strains were provided by the *Bacillus* Genetic Stock Center, Ohio State University, Columbus. *B. thuringiensis* strains that express the different CryIII proteins were kindly supplied by M. Peferoen,

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TABLE 1. Characteristics of *cryI* and *cryIII* general primers used in primer mixture D

Primer pair	Sequence	Gene recognized	Positions	Product size (bp)	Accession no.
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryIAa*	3263-3285	272	D17518
CJI-2	5′TATCGGTTTCTGGGAAGTA		3515-3534		
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryIAb*	3180-3202	284	X54939
CJI-2	5′TATCGGTTTCTGGGAAGTA		3444-3463		
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryIAc*	3186-3208	272	M73248
CJI-2	5′TATCGGTTTCTGGGAAGTA		3438-3457		
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryIB*	3321-3343	290	X06711
CJI-2	5′TATCGGTTTCTGGGAAGTA		3591-3610		
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryIC*	3210-3232	284	M73251
CJI-2	5′TATCGGTTTCTGGGAAGTA		3474-3493		
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryID*	3401-3423	284	X54160
CJI-2	5′TATCGGTTTCTGGGAAGTA		3665-3684		
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryIEa*	3165-3187	275	M73252
CJI-2	5′TATCGGTTTCTGGGAAGTA		3420-3439		
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryIEb*	3159-3181	290	M73253
CJI-2	5′TATCGGTTTCTGGGAAGTA		3429-3448		
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryIF*	3165-3187	284	M73254
CJI-2	5′TATCGGTTTCTGGGAAGTA		3429-3448		
CJI-1	5′TGTAGAAGAGGAAGTCTATCCA	*cryIFa*	3642-3664	284	M63897
CJI-2	5′TATCGGTTTCTGGGAAGTA		3906-3925		
CJIII20	5′TTAACCGTTTTCGCAGAGA	*cryIIIA*	794-813	703	Y00420
CJIII21	5′TCCGCACTTCTATGTGTCCAAG		1474-1496		
CJIII20	5′TTAACCGTTTTCGCAGAGA	*cryIIIB*	821-840	709	X17123
CJIII21	5′TCCGCACTTCTATGTGTCCAAG		1507-1529		
CJIII20	5′TTAACCGTTTTCGCAGAGA	*cryIIIC*	797-816	709	M89794
CJIII21	5′TCCGCACTTCTATGTGTCCAAG		1483-1505		
CJIII20	5′TTAACCGTTTTCGCAGAGA	*cryIIIC-gall*	767-786	694	M64478
CJIII21	5′TCCGCACTTCTATGTGTCCAAG		1438-1460		
CJIII20	5′TTAACCGTTTTCGCAGAGA	*cryIIICb*	767-786	694	U04367
CJIII21	5′TCCGCACTTCTATGTGTCCAAG		1438-1460		
CJIII20	5′TTAACCGTTTTCGCAGAGA	*cryIIICc*	767-786	694	U04368
CJIII21	5′TCCGCACTTCTATGTGTCCAAG		1438-1460		
CJIII20	5′TTAACCGTTTTCGCAGAGA	*cryIIID*	1019-1038	718	X59797
CJIII21	5′TCCGCACTTCTATGTGTCCAAG		1715-1736		
CJIII20	5′TTAACCGTTTTCGCAGAGA	*cryIIIF*	803-822	652	U04366
CJIII21	5′TCCGCACTTCTATGTGTCCAAG		1486-1508		
CJIII20	5′TTAACCGTTTTCGCAGAGA	*cryIIIG*	803-822	733	U04365
CJIII21	5′TCCGCACTTCTATGTGTCCAAG		1513-1535		

Plant Genetic Systems, Ghent, Belgium. The other *B. thuringiensis* strains that we used were collected during 1991 to 1994 from Mexican soil samples (6). All bacterial strains were maintained on nutrient agar (Difco).

Oligonucleotide PCR primers. The general primers used to detect *cryI* or *cryIII* genes were selected from highly conserved regions by using a simultaneous alignment of all previously described sequences. This survey was done by performing a computer analysis with the Geneworks 2.3 program (Intelligenetics). Table 1 shows the sequences of the four general primers, their locations in the sequences, and the expected sizes of the PCR products.

The specific primers used to identify specific *cryI* and *cryIII* genes were selected from highly variable regions in the genes. The *cryIE*-, *cryIF*-, and *cryIG*-specific primers were designed to be used in the same reaction mixture. Table 2 shows the sequence of each primer and the location and size of its expected PCR product. In order to identify *cryIII* type genes, a single directed primer and five reversed primers were used together in one reaction (Table 2).

The oligonucleotides were synthesized with a DNA synthesizer (model Microsyn 1450A; Systec, Inc.) by using the reagents and conditions recommended by the manufacturer. Each pair of primers was highly specific and gave a PCR product of known size that was easily identified by electrophoresis in agarose gels.

Sample preparation and PCR. *B. thuringiensis* strains were grown for 12 h on nutrient agar plates. A loopful of cells from a single colony was transferred to 0.1

TABLE 2. Characteristics of *cryI*- and *cryIII*-specific primers used in reaction mixtures C and E

Primer mixture	Primer pair	Sequence	Gene recognized	Positions	Product size (bp)	Accession no.
C	CJ14 CJ15	5′GGAACCAAGACGAACTATTGC 5′GGTTGAATGAACCCTACTCCC	*cryIEa*	1029-1050 1154-1175	147	M73252
C	CJ14 CJ15	5′GGAACCAAGACGAACTATTGC 5′GGTTGAATGAACCCTACTCCC	*cryIEb*	1026-1047 1151-1172	147	M73253
C	CJ16 CJ17	5′TGAGGATTCTCCAGTTTCTGC 5′CGGTTACCAGCCGTATTTCG	*cryIF*	813-834 969-989	177	M73254
C	CJ16 CJ17	5′TGAGGATTCTCCAGTTTCTGC 5′CGGTTACCAGCCGTATTTCG	*cryIFa*	1290-1311 1446-1466	177	M63897
C	CJ16 CJ17	5′TGAGGATTCTCCAGTTTCTGC 5′CGGTTACCAGCCGTATTTCG	*cryIFb*	1295-1316 1451-1471	177	Z22512
C	CJ18 CJ19	5′ATATGGAGTGAATAGGGCG 5′TGAACGGCGATTACATGC	*cryIG*	1778-1797 1994-2012	235	X58120
E	CJIIIcte 22 CJIIIA23	5′CAATCCCAGTGTTTACTTGGAC 5′CCCCGTCTAAACTGAGTGT	*cryIIIA*	1460-1482 1725-1744	285	Y00420
E	CJIIIcte 22 CJIIIB24	5′CAATCCCAGTGTTTACTTGGAC 5′AACGAAAGATTCTGCTCC	*cryIIIB*	1493-1515 1911-1929	437	X17123
E	CJIIIcte 22 CJIIIC25	5′CAATCCCAGTGTTTACTTGGAC 5′CCTATTCTTTCATTTTGACC	*cryIIIC*	1670-1692 2184-2204	535	M89794
E	CJIIIcte 22 CJIIICg26	5′CAATCCCAGTGTTTACTTGGAC 5′AGTGGAGAGTTTACGGTAGCC	*cryIIIC-gall*	1424-1446 1613-1634	211	M64478
E	CJIIIcte 22 CJIIICg26	5′CAATCCCAGTGTTTACTTGGAC 5′AGTGGAGAGTTTACGGTAGCC	*cryIIICb*	1424-1446 1613-1634	211	U04367
E	CJIIIcte 22 CJIIICg26	5′CAATCCCAGTGTTTACTTGGAC 5′AGTGGAGAGTTTACGGTAGCC	*cryIIICc*	1424-1446 1613-1634	211	U04368
E	CJIIIcte CJIIID27	5′CAATCCCAGTGTTTACTTGGAC 5′CGAAATACGAAATACTATGAG	*cryIIID*	1700-1722 1990-2011	312	X59797
E	CJIIIE28 CJIIIE29	5′TGACAAGTACTGGATTCTGCAA 5′GTTGTTGATGAGGTTCCCCTT	*cryIIIE*	944-966 1316-1337	394	U04364

ml of H_2O, and the mixture was boiled for 10 min to lyse the cells. The resulting cell lysate was centrifuged briefly (10 s at 10,000 rpm; Eppendorf model 5415C centrifuge), and 15 μl of the supernatant was used as the DNA sample in the PCR mixture. PCR mixtures were prepared as described previously (6). Primer mixture C was used to identify *cryIE*, *cryIF*, and *cryIG* genes and contained primers CJ14 to CJ19; primer mixture D contained the four general primers (primers CJI-1, CJI-2, CJIII20, and CJIII21). Primer mixture E contained specific primers designed to identify the *cryIII* genes (primers CJIII22 to CJIII29). Amplification was performed with a DNA thermal cycler (model Omnigene HB-TR3; Hybaid, Teddington, Middlesex, United Kingdom) by using a single denaturation step (2 min at 95°C), followed by a 30-cycle program, with each cycle consisting of denaturation at 95°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min; a final extension step (72°C for 5 min) was also used. A total of 15 μl of each PCR mixture was electrophoresed on a 3% agarose gel in 0.5× Tris-borate buffer at 250 V for 30 to 35 min and stained with ethidium bromide.

Crystal protein purification. Crystalline inclusions were purified from spores and cell debris by centrifugation in discontinuous sucrose gradients as described previously (24). The following sucrose gradient was used: 67, 72, 79, 84, and 90%. The crystal inclusion bodies were solubilized, activated, and purified as described previously (15).

Electrophoresis and immunoblotting. Proteins were analyzed by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% polyacrylamide gels. A two-dimensional gel analysis was performed by the method of O'Farrel (21) by using pH 4 to 10 ampholytes. For the dot blot assay 10- and 20-μg portions of purified toxins were spotted onto two nitrocellulose strips. Both strips were incubated for 5 min in phosphate-buffered saline (PBS) containing 2% Tween 20 and then washed with PBS–0.05% Tween 20. One strip was incubated for 1 h with monoclonal antibody $16A_5E_4$ (1/5,000) specific for CryIE, which was kindly supplied by M. Peferoen, and the second strip was incubated for 1 h with monoclonal antibody $6A_5C_1$ (1/5,000) specific for CryIIIA, which was also supplied by M. Peferoen. After the strips were rinsed twice for 5 min with 0.05% Tween 20 in PBS, they were incubated for 1 h with the secondary antibody (1/1,000), horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Sigma). Diaminobenzidine (25 mg/100 ml) and H_2O_2 were used as the peroxidase substrates.

RESULTS

Specificity of the *cryI* and *cryIII* general primers. PCR was used to examine a number of *B. thuringiensis* strains for their *cry* gene contents. Primer pairs (Table 1) were used to detect strains that harbor either *cryI* genes or *cryIII* genes. The expected sizes of the PCR products of the *cryI* genes range from 272 to 290 bp, while the expected sizes of the PCR products of the *cryIII* genes range from 652 to 769 bp. Figure 1 shows the results obtained with different *cryI* control strains (Fig. 1A) and *cryIII* control strains (Fig. 1B) when primer mixture D was used. In this analysis rapid cell lysates were used as the DNA samples; this allowed us to analyze several samples at the same time and reduced the possibility of sample contamination. Nevertheless, there was intrinsic variation in the quantity of

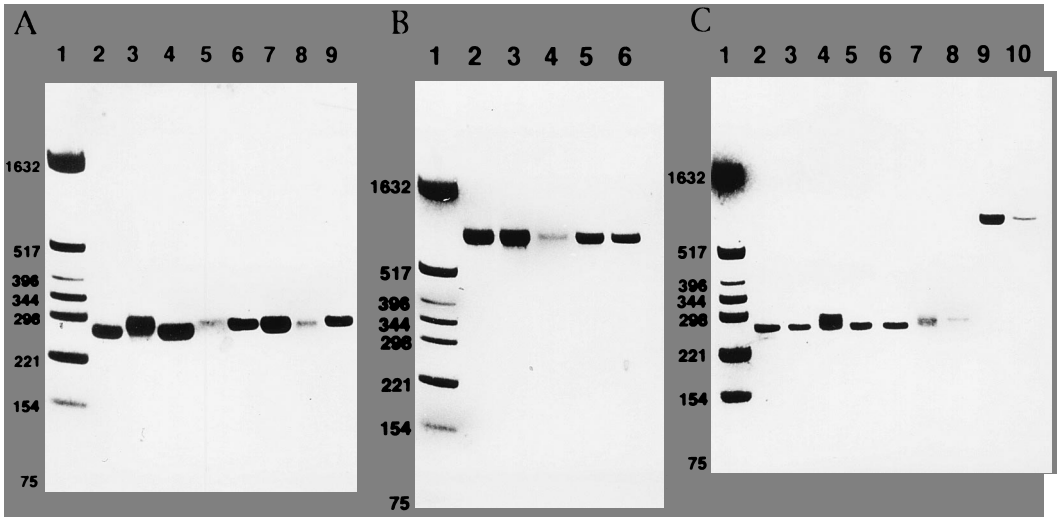

FIG. 1. Agarose gel electrophoresis analysis of PCR products obtained by using the *cryI* and *cryIII* general primer pairs. In panels A and B PCR were performed with the four PCR primers in mixture D. In panel C the *cryI* general primers (CJI-1 and CJI-2) were used for lanes 2 through 8 and the *cryIII* general primers (CJIII20 and CJIII21) were used for lanes 9 and 10. (A) Lane 1, molecular weight markers; lane 2, strain HD1 (*cryIA* genes); lane 3, strain HD73 (*cryIAc*); lane 4, strain HD137 (*cryIAa cryIC cryID*); lane 5, strain IB43 (*cryIB*); lane 6, *Escherichia coli cryIC* strain; lane 7, *E. coli cryID* strain; lane 8, strain HD125 (*cryIE*); lane 9, strain PS81 (*cryIAd cryIC cryID cryIF*). (B) Lane 1, molecular weight markers; lane 2, *B. thuringiensis cryIIIA*; lane 3, *B. thuringiensis cryIIIB*; lane 4, *B. thuringiensis cryIIIC*; lane 5, *B. thuringiensis cryIIID*; lane 6, *B. thuringiensis cryIIIE*. (C) Analysis of soil strains. Lane 1, molecular weight markers; lane 2, strain IB127; lane 3, strain IB129; lane 4, strain IB144; lane 5, strain IB148; lane 6, strain IB152; lane 7, strain IB153; lane 8 and lane 10, strain IB31; lane 9, strain IB17.

the PCR product because of the lack of a standardized amount of DNA in the PCR mixture. In order to avoid false-negative results, some PCR were performed at least three times. Regardless of the intensity of the band, the presence of a PCR product of the expected size indicated that the corresponding gene was present. Figure 1C shows the PCR products obtained when the *cryI* and *cryIII* primer pairs were used separately with different native *B. thuringiensis* strains isolated from different areas (6). Strain IB31 contained both PCR products, suggesting that this strain may harbor *cryI* and *cryIII* genes.

Identification of specific *cryI* and *cryIII* genes from soil samples. An additional PCR survey was performed with 181 selected strains. The following three primer mixtures were used in this survey: previously described primer mixtures A and B, which amplify specific regions from the *cryIA* genes to *cryID* (6), and primer mixture C, which is described in this paper (Table 2) and amplifies specific regions from *cryIE*, *cryIF*, and/or *cryIG*. This analysis allowed us to identify specific *cryI* genes present in a particular strain on the basis of the sizes of the PCR products. We found six different *cryI* gene profiles in our collection. Profile 1 consisted of *cryIAa* and *cryIAb*; profile 2 consisted of *cryIAa*, *cryIAb*, and *cryIAc*; profile 3 consisted of *cryIAa*, *cryIAb*, *cryIAc*, and *cryID*; profile 4 consisted of *cryIAa*, *cryIAb*, *cryIC*, and *cryID*; profile 5 consisted of *cryIC* and *cryID*; and profile 6 consisted of *cryIB*. We did not find any strain that harbors *cryIE*, *cryIG*, or *cryIF*.

We identified a strain (strain IB31) that produced an unexpected 302-bp product when it was assayed with the primer mixture C (Fig. 2A, lane 4), suggesting that this strain may harbor a unique *cryI* gene. Further analysis revealed that this PCR product was obtained with primers CJ15 (reverse primer specific for the *cryIE* gene) and CJ18 (directed primer specific for the *cryIG* gene).

The *cryIII* gene types present in 14 selected strains were analyzed (Fig. 2B), and all of the strains tested contained a *cryIIIA* gene. Only strain IB31 contained a different *cryIII* gene since it produced a PCR product of unexpected size, 224 bp. This fragment was produced when the directed constant primer was used with the reversed *cryIIIC-gall* primer.

Analysis of the crystal proteins from strain IB31. The crystal inclusions produced by strain IB31 are bipyramidal crystals, but they are extremely large and long. These crystals are composed of a single 105-kDa protein. Trypsin digestion produced

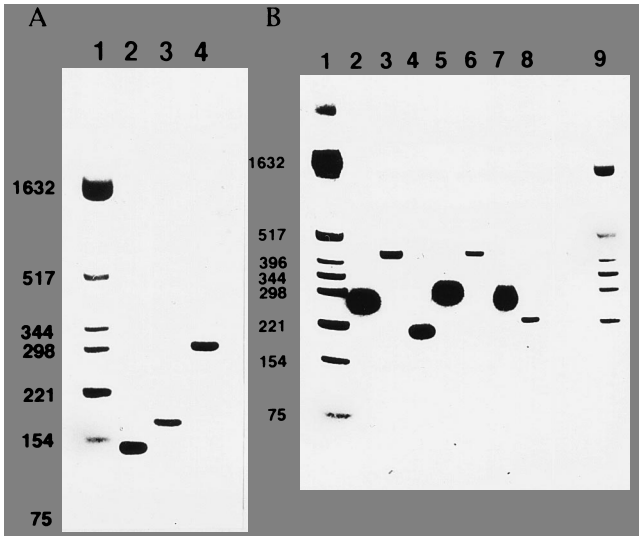

FIG. 2. Agarose gel electrophoresis of PCR products obtained with specific primers. (A) Analysis in which primer mixture C specific for the *cryIE*, *cryIF*, and *cryIG* genes was used. Lane 1, molecular weight markers; lane 2, strain HD125 (*cryIE*); lane 3, strain PS81 (*cryIAd cryIC cryID cryIF*); lane 4, strain IB31. (B) Analysis in which *cryIII*-specific primers (primer mixture E) were used. Lanes 1 and 9, molecular weight markers; lane 2, *B. thuringiensis cryIIIA* strain; lane 3, *B. thuringiensis cryIIIB* strain; lane 4, *B. thuringiensis cryIIIC-gall* strain; lane 5, *B. thuringiensis cryIIID* strain; lane 6, *B. thuringiensis cryIIIE* strain; lane 7, strain IB17; lane 8, strain IB31. Strains IB17 and IB31 are representative *cryIII* strains.

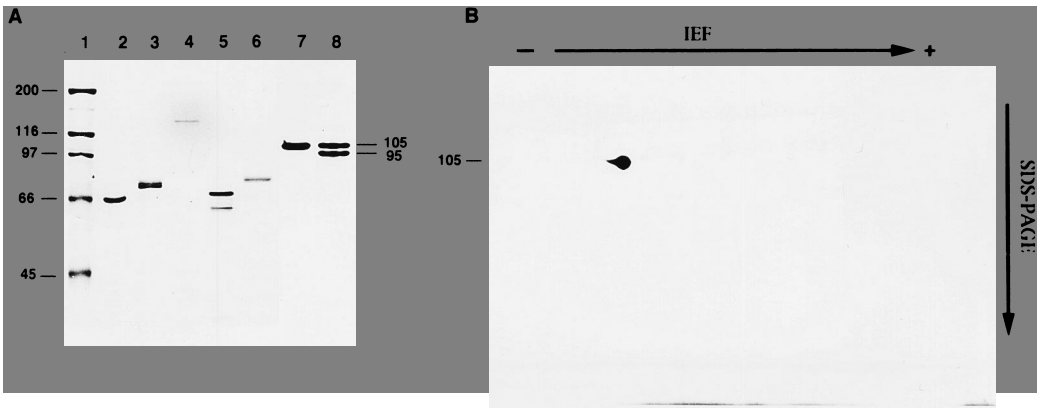

FIG. 3. Analysis of the crystal protein present in strain IB31. (A) Polyacrylamide gel electrophoresis of crystal inclusions purified from different *B. thuringiensis* strains. Lane 1, molecular weight markers; lane 2, *B. thuringiensis* CryIIIA; lane 3, *B. thuringiensis* CryIIIB; lane 4, *B. thuringiensis* CryIIIC; lane 5, *B. thuringiensis* CryIIID; lane 6, *B. thuringiensis* CryIIIE; lane 7, strain IB31 protoxin; lane 8, strain IB31 toxin. (B) Two-dimensional electrophoresis of the crystal protein from strain IB31. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

a 95-kDa polypeptide (Fig. 3A). Two-dimensional electrophoresis of the crystal protein revealed a single band in the acidic region (Fig. 3B), suggesting that these crystals are composed of a single protein.

Immunoblotting experiments revealed that strain IB31 produced a crystal inclusion protein that cross-reacted with two specific monoclonal antibodies raised against purified CryIE or CryIIIA toxins (Fig. 4). Bioassays were performed with different lepidopteran insects (*Spodoptera frugiperda*, *Diatraea grandiosella*, *Diatraea saccharalis*, *Heliothis virescens*) and with one coleopteran insect (*Epilachna varivestis*), but no insecticidal activity was found.

DISCUSSION

Workers have described different PCR screening methods which were designed to predict the protoxin gene contents of previously uncharacterized *B. thuringiensis* strains (2, 5, 6, 17). However, these methods were not complete, since they did not identify all of the *cryI* and *cryIII* genes that have been described. The orders Coleoptera and Lepidoptera contain some of the most devastating insect crop pests known. It is important to search for novel insecticidal proteins that will help control these pests. In this paper we describe a PCR screening method to determine which specific lepidopteran- and coleopteran-active genes are present in a particular strain. The screening procedure described in this paper is rapid and highly specific.

We identified the most common *cryI* and *cryIII* gene profiles present in *B. thuringiensis* strains isolated in Mexico. Strains that harbor the three *cryIA* genes were the most abundant strains (48% of the population). Strains that harbor the *cryIB* gene were also very abundant (30% of the population). Strains that harbor both *cryIC* and *cryID* genes are the most toxic strains for larvae of *S. frugiperda* (data not shown). We found some strains that carried only the *cryID* gene, and these strains were also highly toxic to *S. frugiperda* larvae. We did not find any *cryIE*, *cryIG*, or *cryIF* gene-containing strains, suggesting that *B. thuringiensis* strains that harbor these genes are not abundant in Mexico. Also, *cryIII* gene-containing strains were not observed frequently.

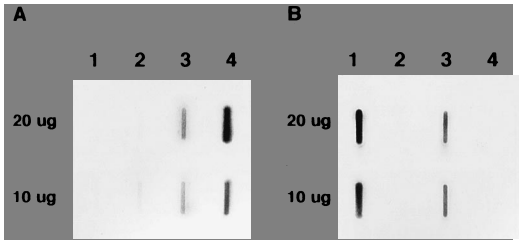

FIG. 4. Immunological characterization of the crystal protein from strain IB31. (A) Analysis performed with monoclonal antibody $16A_5E_4$ specific for CryIE toxin. (B) Analysis performed with monoclonal antibody $6A_5C_1$ specific for CryIIIA toxin. Lane 1, *B. thuringiensis cryIIIA* strain; lane 2, *B. thuringiensis cryIIIC-gall* strain; lane 3, strain IB31; lane 4, strain HD125.

By using this screening procedure, we identified *B. thuringiensis* IB31, a strain that contains a novel and very different crystal protein gene. This strain produced the two expected PCR products when it was assayed with the *cryI* general primers (CJI-1 and CJI-2) and the *cryIII* general primers (CJII20 and CJII21). These data suggest that this strain harbors one or more genes related to the *cry* family of genes. We also found another *B. thuringiensis* strain (strain IB68) that produced both PCR products when it was assayed with the four general primers in mixture D. Further PCR analysis revealed that this strain harbors the *cryIAa*, *cryIAb*, and *cryIIIA* genes (data not shown). However, when strain IB31 was assayed with the *cryI*- and *cryIII*-specific primers, we obtained PCR products with unexpected molecular weights. The unexpected PCR products were obtained when reaction mixture C and *cryIII*-specific primer mixture E were used (Fig. 2A and B). These data indicate that this strain may harbor one or two different *cry* genes. The PCR product produced with the *cryI*-specific primers was obtained with primers CJ15 (reverse primer specific for the *cryIE* gene) and CJ18 (directed primer specific for the *cryIG* gene). Directed primer CJ18 is located at positions 1778 to 1797, while reverse primer CJ15 is located at positions 1151 to 1172. These two primers were not able to amplify a sequence from *cryIE* or *cryIG* genes. We propose that the PCR product obtained with reaction mixture C in this strain may be an artifact produced by nonspecific hybridization of at least one primer. The fact that the crystal protein from this strain cross-reacted with the monoclonal antibody specific for CryIE toxin (Fig. 4) and the fact that an aberrant PCR product was obtained with the *cryIE* reverse primer suggest that the crystal protein from this strain may be related to the *cryIE* toxin.

Strain IB31 also produced an unexpected PCR product when it was assayed with the *cryIII*-specific primers. This PCR

product was obtained with the directed constant primer and the *cryIIIC*-specific primer. The PCR product obtained in this reaction was 13 bp larger that the PCR product obtained from a *B. thuringiensis* CryIIIC-producing strain, and it was obtained with the same PCR primers, suggesting that the crystal protein from this strain is also related to the CryIIIC protein. It is important to note that both strains produced bipyramidal crystals (19), but strain IB31 produced very long crystals that were at least twice as long as the crystals from the CryIIIC-producing strain. Two important differences between the two crystals are (i) that the CryIIIC crystal protein has a molecular mass of 130 kDa, while the crystal protein from strain IB31 has a molecular mass of 105 kDa (Fig. 3A), and (ii) that the crystal protein from strain IB31, but not the crystal protein from the CryIIIC-producing strain, cross-reacts with the monoclonal antibodies against both the CryIE and CryIIIA toxins (Fig. 4).

Our results showed that a *B. thuringiensis* strain collection can be easily characterized by the PCR method and that it is possible to identify some novel *cry* genes. However, our results did not provide direct information about the insecticidal properties of the putative novel *cry* genes or about the identity of the protein that exhibits the highest level of toxic activity against a selected pest. We know that this method cannot be used to identify all new genes in a *B. thuringiensis* collection. One great limitation is that if the novel gene does not have any of the primer sequences, no PCR product will be produced and the gene will be missed. Also, if the novel gene has an identical sequence in the region between the primers, but a different sequence in other regions important for specificity, it would not be detected. After the screening procedure described in this paper, selected strains could be examined to try to find novel genes with the PCR footprint described by Kalman et al. (17), who used a collection of primers through the sequence of the *cryIC* gene; this has proved to be an effective way to identify novel *cryIC* genes.

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