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Cloning of a Novel *cryIC*-Type Gene from a Strain of *Bacillus thuringiensis* subsp. *galleriae*

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A novel *cryIC*-type gene was isolated from a strain of *Bacillus thuringiensis* subsp. *galleriae*. A new polymerase chain reaction (PCR) technique with a set of several oligonucleotide primer pairs specific to the *cryIC* gene was used to screen a number of *B. thuringiensis* strains. PCR amplified several DNA fragments ranging from 100 bp to 1 kb for *B. thuringiensis* strains containing a *cryIC* gene. PCR fragments amplified from the *Bacillus thuringiensis* subsp. *galleriae* HD29 DNA differed from the fragments amplified from other *cryIC*-containing strains, indicating strain HD29 contained a novel *cryIC*-type gene. To isolate crystal genes homologous to *cryIC*, an HD29 gene library was probed with a 984-bp fragment of the amino-terminal coding region of the *cryIC* gene cloned from *Bacillus thuringiensis* subsp. *aizawai* HD229. A putative toxin gene was isolated from a phage that hybridized strongly to the *cryIC* probe. Translation of the putative toxin DNA sequence revealed an open reading frame of 1,176 amino acids whose predicted molecular mass was 132.8 kDa. Comparisons of the toxin gene sequence with sequences of other *cry* genes indicated that this gene is a subclass of *cryIC*. We propose to designate this gene *cryIC(b)*. In *Escherichia coli*, the *cryIC(b)* gene produced a protein of approximately 130 kDa toxic to *Spodoptera exigua* and *Trichoplusia ni*.

Bacillus thuringiensis is a gram-positive, spore-forming bacterium that produces an insecticidal parasporal crystal. *B. thuringiensis* strains that produce crystal toxins active against lepidopteran, dipteran, and coleopteran insects have been found. *B. thuringiensis* crystal protein genes have been designated *cry* because of the crystal formation phenotype (Cry). Those genes which encode bipyramidal proteins active only against Lepidoptera are designated *cryI* (7).

Spodoptera exigua is an agronomically important pest that is relatively insensitive to *B. thuringiensis* crystal toxins. The CryIC protein from *B. thuringiensis* subsp. *entomocidus* and *B. thuringiensis* subsp. *aizawai* is the only CryI toxin significantly active against *S. exigua* and other *Spodoptera* species (20). For this reason, we are interested in finding additional crystal proteins active against *S. exigua*.

Screening a large number of *B. thuringiensis* isolates for high *Spodoptera* activity is labor intensive and time-consuming and may not be the most effective way to identify genes encoding highly active toxins. Some *B. thuringiensis* strains contain "silent" crystal toxin genes (5) which are carried on the genome but not expressed. So, some *B. thuringiensis* strains that do not show high insecticidal activity may carry toxin genes that encode insecticidal proteins with high specific activities. Because of this, we are interested in screening *B. thuringiensis* strains at the molecular level for their gene content.

In this study, we used the polymerase chain reaction (PCR) as a rapid means for determining the *cryI* gene content of a number of *B. thuringiensis* strains. Additionally, we designed a PCR method to search for *cryIC*-type genes. Using the method, we identified a novel *cryIC*-related gene in *B. thuringiensis* subsp. *galleriae* strain HD29, a strain that showed no significant *Spodoptera* activity. We isolated the gene from HD29 by DNA-DNA hybridization and found it encoded a *Spodoptera*-active crystal protein. We propose to designate the novel gene *cryIC(b)* because its predicted product is highly homologous to CryIC.

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MATERIALS AND METHODS

Bacterial strains. *B. thuringiensis* strains were obtained from the U.S. Department of Agriculture strain collection in Peoria, Ill. *Escherichia coli* DH5α was obtained from Bethesda Research Laboratories (BRL) and used for cloning and expression of crystal toxins, and *E. coli* P2392 from Stratagene was used for screening the Lambda DASH II library.

Isolation and modification of DNA. Total *B. thuringiensis* DNA was isolated as follows. Cultures were grown in 2× YT (5 g of yeast extract, 5 g of tryptone, and 2.5 g of NaCl per liter) to an optical density at 600 nm of 0.8. The cells were harvested by centrifugation, washed once in TES (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl), and resuspended in 25% sucrose–25 mM Tris-HCl (pH 8.0)–25 mM EDTA–1 mg of lysozyme per ml. The cell suspension was incubated at 37°C for 1 h. The cells were solubilized by addition of sodium dodecyl sulfate (SDS) to a final concentration of 2%, and the solution was incubated at 50°C for 15 min. NaCl was added to a final concentration of 1 M, and the solution was incubated at 50°C for 5 min and then at 4°C overnight. The solution was centrifuged, and DNA in the supernatant was precipitated with ethanol. The DNA was resuspended in 10 ml of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 1 M NaCl, 10 μg of RNase per ml, and 0.6 mg of proteinase K per ml and incubated at 37°C for 30 min. The mixture was extracted with phenol, phenol-chloroform (1:1), and chloroform, and the DNA was precipitated with ethanol. LambdaSorb phage adsorbent from Promega was used to isolate lambda phage DNA according to the manufacturer's instructions.

PCR-amplified *cryIC* gene fragments used for Southern probes were excised from a 0.8% agarose gel, electroeluted in 0.5× TBE (13), and purified with an Elutip-d column obtained from Schleicher & Schuell. Twenty-five nanograms

TABLE 1. Oligonucleotides

Primer	Sequence	Gene or vector	nt[a]
cryI-specific primers for PCR screening			
TYIAA	GAGCCAAGCAGCTGGAGCAGTTTACACC	*cryIA(a)*	1837–1864
TYIAC	TCACTTCCCATCGACATCTACC	*cryIA(c)*	1941–1962
TYIB	GTCAACCTTATGAGTCACCTGGGCTTC	*cryIB*	1292–1318
TYIC	CAACCTCTATTTGGTGCAGGTTC	*cryIC*	1820–1842
TYID	GGTACATTTAGATATTCACAGCCAC	*cryID*	1839–1863
TYIE	CTTAGGGATAAATGTAGTACAG	*cryIE*	1263–1284
TYIF	CCGGTGACCCATTAACATTCCAATC	*cryIF*	2126–2150
TYIUNI2[b]	ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC	*cryI*	2528–2560
TY6	GGTCGTGGCTATATCCTTCGTGTCACAGC	*cryI*	3599–3626
TY14	GAATTGCTTTCATAGGCTCCGTC	*cryIA(b)*	3352–3376
cryIC-specific primers for PCR screening			
TY54	CTCTGTCACTTGTTCAG	*cryIC*	159–175
TY55	GAGAAGGGCCAACTATTCCCC	*cryIC*	237–256
TY56	GAATGGGAAGAAGATCC	*cryIC*	389–405
TY57	GGCCGCTTGAGCATAAACGG	*cryIC*	519–538
TY58	GGGGATTGACAACGATAAATG	*cryIC*	588–608
TY59	GGAAAGAAAGCGGCGATATC	*cryIC*	667–786
TY60	GGAGATATCCAATTCAGCCAG	*cryIC*	801–821
TY61	GATATTACTCGATGTCCTCCCC	*cryIC*	1011–1032
TY62	CCTTATAGGAGGTGGTAAC	*cryIC*	1036–1054
TY63	GGTGTTCCAGATCTTTGAAC	*cryIC*	1349–1368
TY64	GGGGGGGCACCTCTGTC	*cryIC*	1482–1498
TY65	CCGCTACTAATAGAACCTGCACC	*cryIC*	1831–1854
Cloning			
GALP1	CCACAGTTACAGTCTGTAGCTCAATTACC	*cryIC*	871–899
GALP2	CCGCTACTAATAGAACCTGCACCA	*cryIC*	1831–1854
KK3	TGCTTCCACATATATATTGA	*cryIC(b)*	316–335
KK5R	ACAGACTGTAACTGTGG	*cryIC(b)*	825–841
M13UNIV	GTTTTCCCAGTCACGAC	pUC18/19	
MI13REV	AACAGCTATGACCATG	pUC18/19	
SK13	AGTGGAGGGAACCCATGGAG	*cryIC(b)*	282–301
TY7	CCACGCTATCCACGATGAATGTTCCTTC	*cryI*	4007–4034
TY8	CGGAGGTATTCCATGGAGGAAAATAATC	*cryIC*	34–61
TY9	GGTAATTGAGCTACAGACTGTAACTGTGG	*cryIC*	871–899

[a] Location where primer hybridizes to *cryI* holotype (5, 7, 19) or *cryIC(b)*.
[b] For primers that hybridize to more than one gene, the hybridization position is given for *cryIA(a)*.

of the PCR-generated fragment was labeled with [α-^{32}P]dCTP with the Random Primers DNA Labeling System from BRL. The hybridization temperature for gene detection, phage isolation, and locating the N-terminal coding region of the crystal gene was 45°C. The hybridization temperature used for locating the C-terminal coding regions of the toxin gene within the lambda phages was 65°C.

Lambda DASH II library construction. *B. thuringiensis* subsp. *galleriae* HD29 total DNA was restricted with *Eco*RI and subjected to electrophoresis on a 0.6% agarose gel. The *Eco*RI fragments of approximately 9 to 11 kb were purified from the agarose gel as described above and ligated to Lambda DASH II *Eco*RI arms obtained from Stratagene. The ligation mixture was packaged into phage particles with the GigaPack Gold packaging extract from Stratagene.

PCR. Oligonucleotide primers were synthesized with an Applied Biosystems model 391 DNA synthesizer and are listed in Table 1. To identify *cryI*-type genes, eight forward primers (TYIAA, TY6, TYIAC, TYIB, TYIC, TYID, TYIE, and TYIF) and two reverse primers (TYIUNI2 and TY14) were used together in one reaction. TY6 and TY14 were specific to *cryIA(b)*, and all other *cryI* genes were amplified between specific forward primers and the universal primer (TYIUNI2). To detect *cryIC*-type genes, six primer pairs based on the *cryIC* gene sequence were synthesized and used together in one reaction. The six *cryIC*-specific primer pairs (TY54 to TY65 [Table 1]) encompass almost the entire region of the CryIC toxin (the first 620 amino acid residues of CryIC protoxin).

The 984-bp probe for identification of *cryIC*-type sequences and phage isolation was generated by PCR with primers GALP1 and GALP2 with template DNA prepared from *B. thuringiensis* subsp. *aizawai* HD229. For locating the coding regions within the lambda phage inserts, the N-terminal probe was generated with primers TY8 and GALP2 with HD229 total DNA as a template, and the C-terminal probe was generated with primers TY6 and TY7 and an HD29 total DNA template.

Concentration of genomic DNA in PCRs was 1 to 5 μg/ml, and the concentration of plasmid DNA was 0.1 to 0.5 μg/ml. In reactions containing multiple primers, the concentration of each primer was 1 μM. AmpliTaq polymerase of Perkin-Elmer Cetus was used for all PCRs, and concentrations of other components were as recommended by Perkin-Elmer Cetus.

Isolation of crystal proteins. *E. coli* cells expressing *cryIC*

or *cryIC(b)* were grown in 2× YT for 48 h at 37°C and shaking at 300 rpm. The cells were harvested by centrifugation at 5,000 × *g*, washed in 10 mM Tris-HCl (pH 8.0) containing 0.25% Tween 20, and resuspended in a 1/50 volume of 10 mM Tris-HCl (pH 8.0) containing 10 mM EDTA. The cells were disrupted by a French press, centrifuged at 15,000 × *g*, washed twice in 0.5 M NaCl containing 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid]-NaOH (pH 10.5) and 1 mM EDTA, washed once with water, and resuspended in 10 mM Tris-HCl (pH 8.0) containing 10 mM EDTA. A 6-ml aliquot of the suspension was mixed with 120 μl of β-mercaptoethanol and 500 mM phenylmethylsulfonyl fluoride on ice. The crystal protein was solubilized by addition of 2 N NaOH to pH 10.5, the sample was centrifuged at 15,000 × *g*, and the supernatant was loaded on a Sephacryl S-300 HR column (32 by 1,000 mm). The column was eluted with 10 mM CAPS-NaOH (pH 10.5) containing 50 mM NaCl and 1 mM EDTA. The crystal protein was precipitated at pH 4.4 by addition of HCl and dissolved in TE.

Bioassay. Twofold serial dilutions made from the purified crystal protein were mixed with 9 volumes of molten insect diet at 50°C and aliquoted into trays (3 ml per well). Ten second-instar *Spodoptera exigua* and *Trichoplusia ni* larvae were infested in each dilution, and mortality was recorded after 4 days. Percent mortality was plotted as a function of concentration.

Western blotting (immunoblotting). *E. coli* strains carrying the cloned crystal protein genes were grown in Luria-Bertani medium (13) for 48 h, concentrated fivefold, and analyzed by SDS-polyacrylamide gel electrophoresis on 8% gels obtained from Novex. Electrophoretic transfer of proteins to nitrocellulose and immunological detection of crystal proteins was by the method of Towbin et al. (17). The primary antibody was a 1:100 dilution of antisera raised against the crystal of *B. thuringiensis* subsp. *aizawai* HD229, and the secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad).

DNA sequencing. The T7Sequencing Kit from Pharmacia LKB was used with synthetic oligonucleotide primers according to the manufacturer's instructions. Previously synthesized primers based on the *cryIC* sequence were used when they hybridized to *cryIC(b)*, as determined by PCR. Twenty-two additional primers were synthesized to obtain the *cryIC(b)* sequence on both strands.

Nucleotide sequence accession number. The nucleotide sequence of *cryIC(b)* is assigned GenBank accession number M97880.

RESULTS

PCR screening. The PCR was used to survey a number of *B. thuringiensis* strains for their crystal protein gene content. One set of eight primer pairs was used to detect, in one reaction, eight published *cryI*-type genes, from *cryIA(a)* to *cryIF* (5, 7, 19). PCR with these primers and a total DNA template generated a uniquely sized fragment for each *cryI*-type gene. Therefore, the sizes of PCR products indicated the presence of particular crystal protein genes. Previous reports concerning the occurrence of crystal genes were confirmed by this PCR technique as follows. Among the strains shown in Fig. 1, only *B. thuringiensis* subsp. *aizawai* (HD137) carried the *cryIC* gene (18). The *cryIE* gene was detected in HD136 of *B. thuringiensis* subsp. *kenyae* (19) as well as in HD125 (*B. thuringiensis* subsp. *tolworthi*) and HD147 (*B. thuringiensis* subsp. *darmstadiensis*). Three

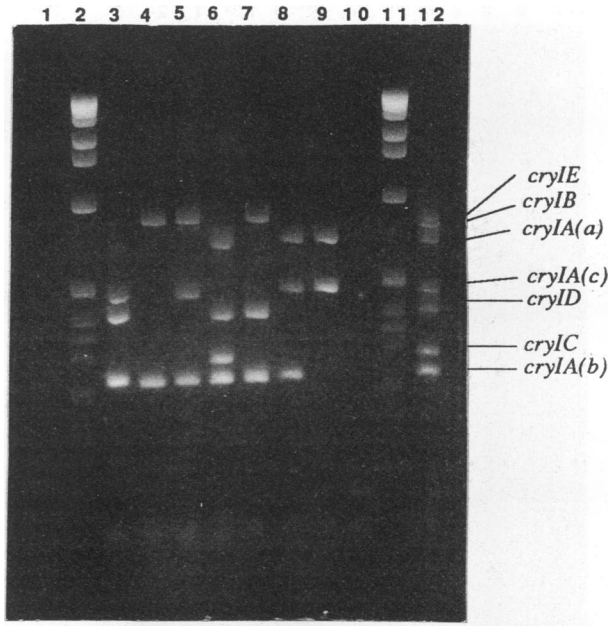

FIG. 1. PCR survey of *B. thuringiensis* strains for their *cryI* contents. Total DNA samples from seven *B. thuringiensis* strains were analyzed by PCR with a mixture of *cryI*-specific primers. Lane 1, no sample; lane 2, BRL 1-kb size ladder; Lane 3, HD29 (*B. thuringiensis* subsp. *galleriae*); lane 4, HD125 (*B. thuringiensis* subsp. *tolworthi*); lane 5, HD136 (*B. thuringiensis* subsp. *kenyae*); lane 6, HD137 (*B. thuringiensis* subsp. *aizawai*); lane 7, HD147 (*B. thuringiensis* subsp. *darmstadiensis*); lane 8, HD203 (*B. thuringiensis* subsp. *kurstaki*); lane 9, HD245 (*B. thuringiensis* subsp. *kurstaki*); lane 10, PCR with no template; lane 11, BRL 1-kb size ladder; lane 12, PCR with *cryI* gene mixture (excluding *cryIF*). Expected sizes of PCR fragments were as follows: *cryIA(a)*, 724 bp; *cryIA(b)*, 238 bp; *cryIA(c)*, 487 bp; *cryIB*, 830 bp; *cryIC*, 288 bp, *cryID*, 414 bp; *cryIE*, 883 bp; and *cryIF*, 368 bp.

cryIA-type genes (10) were found in HD203 of *B. thuringiensis* subsp. *kurstaki*. The PCR products of *B. thuringiensis* subsp. *galleriae* HD29 indicated that this strain contained *cryIA(b)*, *cryIA(c)*, and *cryID*.

To search for a novel *cryIC*-type gene, an additional PCR survey was performed. Six pairs of primers described in Materials and Methods extending almost the entire length of the toxic region of *cryIC* based on the published *cryIC* sequence were synthesized (8). PCR with these six primer pairs and a *cryIC* template produced six major fragments from priming between immediate primer pairs and other minor fragments because of priming between oligonucleotides from different pairs, creating a fingerprint specific to *cryIC*. Three strains that were previously shown to contain *cryIC* (HD137, HD198, and HD229) showed identical PCR product profiles with the *cryIC*-specific primer pairs (Fig. 2). HD29 had a pattern substantially different from the pattern of the *cryIC*-containing strains; only four of the six major PCR products appeared to be present. There were no fragments of the size expected from primer pairs TY62/TY63 and TY64/TY65. None of the other strains examined in Fig. 2 gave PCR products with the *cryIC*-specific primers, indicating the high specificity of the primer mixture. This result clearly indicated that strain HD29 carried some *cryIC*-specific sequences but not the *cryIC* gene.

Crystal gene isolation. Southern hybridization was per-

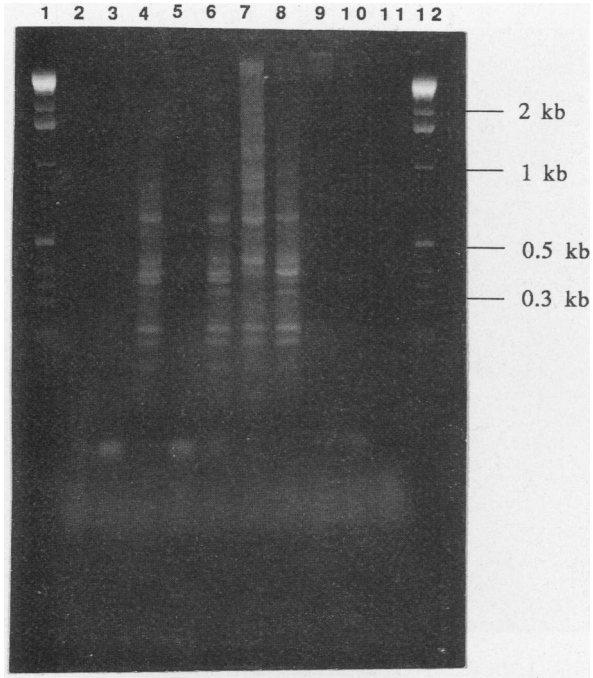

FIG. 2. PCR analysis of *cryIC*-type genes in selected *B. thuringiensis* strains. Total DNA samples from nine selected *B. thuringiensis* strains were analyzed by PCR with a mixture of *cryIC*-specific primers. Lane 1, BRL 1-kb size ladder; lane 2, HD12 (*B. thuringiensis* subsp. *morrisoni*); lane 3, HD136 (*B. thuringiensis* subsp. *kenyae*); lane 4, HD198 (*B. thuringiensis* subsp. *entomocidus*); lane 5, HD203 (*B. thuringiensis* subsp. *kurstaki*); lane 6, HD229 (*B. thuringiensis* subsp. *aizawai*); lane 7, HD29 (*B. thuringiensis* subsp. *galleriae*); lane 8, HD137 (*B. thuringiensis* subsp. *aizawai*); lane 9, HD125 (*B. thuringiensis* subsp. *tolworthi*); lane 10, HD147 (*B. thuringiensis* subsp. *darmstadiensis*); lane 11, no template; lane 12, BRL 1-kb size ladder. The respective fragment sizes of PCR products were as follows: TY54 plus TY55, 99 bp; TY56 plus TY57, 150 bp; TY58 plus TY59, 190 bp; TY60 plus TY61, 232 bp; TY62 plus TY63, 333 bp; and TY64 plus TY65, 373 bp.

formed to investigate whether restriction fragments from HD29 hybridized to *cryIC*. The hybridization probe was a 984-bp PCR-generated fragment from *B. thuringiensis* subsp. *aizawai* HD229 that spanned amino acids 276 to 603 of CryIC, the region sharing the least homology with *cryIA(a)*. Southern hybridization under low-stringency conditions (45°C) showed a 10-kb *Eco*RI fragment contained sequences that hybridized to the 984-bp probe (not shown).

To isolate the 10-kb *Eco*RI fragment from HD29 that hybridized to the *cryIC* probe, a subgenomic library of 9- to 11-kb *Eco*RI fragments in the Lambda Dash II vector was screened with the 984-bp *cryIC* probe under low-stringency hybridization conditions (45°C). Approximately 7,800 phage were screened, and 45 positive phage were identified. Twelve positive phage were plaque purified and divided into two classes according to the strength of their hybridization signals. Three phage hybridized weakly to the hybridization probe, while nine phage hybridized strongly under the stringency conditions used. An *Eco*RI digest was performed on phage DNA from the twelve positive phage to separate the HD29 insert from the lambda arms. All twelve phage contained an insert of approximately 10 kb, and three phage carried additional, smaller inserts. One weakly hybridizing

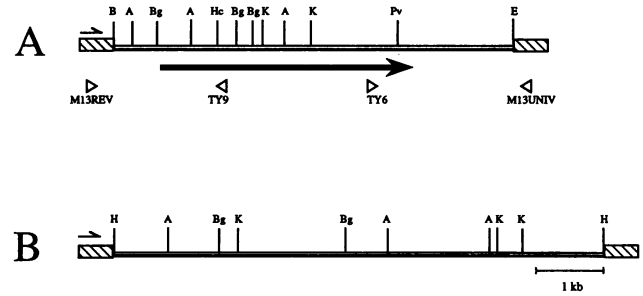

FIG. 3. Restriction maps of pSB204 (A) and pSB205 (B) inserts. *cryIC(b)* is indicated with a thick black arrow. Flanking pUC vector sequences are cross-hatched. The direction of *lacZ* is shown with a half-arrow above the vector sequences. Oligonucleotide primers situated below the pSB204 restriction map were used to orient the *cryIC(b)* coding region. Abbreviations: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Hc, *Hin*cII; H, *Hin*dIII; K, *Kpn*I; Pv, *Pvu*II.

phage, number 34, and one strongly hybridizing phage, number 42, were chosen for further study.

A Southern blot was performed to locate the N- and C-terminal coding regions of the *cry* gene within each phage. The N-terminal probe was an 1,820-bp fragment that spanned the first 600 residues of CryIC, and the 446-bp C-terminal probe spanned amino acids 1021 to 1170. A 5.5-kb *Bam*HI-*Eco*RI fragment from phage 42 and a 7.1-kb *Hin*dIII fragment from phage 34 hybridized to both probes. The 5.5-kb *Bam*HI-*Eco*RI fragment from phage 42 was subcloned in pUC19 and designated plasmid pSB204. The 7.1-kb *Hin*dIII fragment from phage 34 in pUC18 was designated pSB205. Restriction maps of pSB204 and pSB205 did not match those of any crystal toxin genes described in the literature (Fig. 3).

PCR was used to orient the putative toxin gene within plasmid pSB204. Primers that hybridized to pUC vector sequences were used with primers that hybridized to the coding region of *cryI* genes. PCR on plasmid pSB204 with the M13REV and TY9 primers gave a 1.55-kb product, indicating that the start codon of the toxin gene was approximately 0.7 kb from the *Bam*HI site (Fig. 3A). The M13UNIV and TY6 primers gave a 1.7-kb product, indicating that the stop codon was approximately 1.3 kb from the *Eco*RI site (Fig. 3A). Although primers TY6 and TY7 hybridized to pSB205, PCR with TY6 and TY7 in combination with the M13REV and M13UNIV primers failed to give PCR products. Since pSB205 carried a 7.1-kb insert, the distances between the crystal gene and the vector sequences may have been too long to generate a PCR product.

PCR was performed on plasmids pSB204 and pSB205 with the *cryIC*-specific primers. The pSB204 template gave five of the six *cryIC*-specific fragments (the product expected from primers TY64 and TY65 was absent), indicating that this plasmid carried a gene homologous to *cryIC* (not shown). Plasmid pSB205 gave no *cryIC*-specific fragments, demonstrating that it did not carry a gene closely related to *cryIC*.

DNA sequence. The DNA sequence of 4,106 bp from plasmid pSB204 was determined on both strands and revealed an open reading frame encoding a protein of 1,176 residues whose predicted molecular mass was 132.8 kDa (Fig. 4). Sequences presumably involved in the transcription from the BtI promoter (2) and the BtII promoter (3) of *cryIA(a)* were found upstream from the putative start codon (nucleotides [nt] 196 to 224 and 176 to 220 in Fig. 4). A putative ribosome binding site (14) lay just upstream from

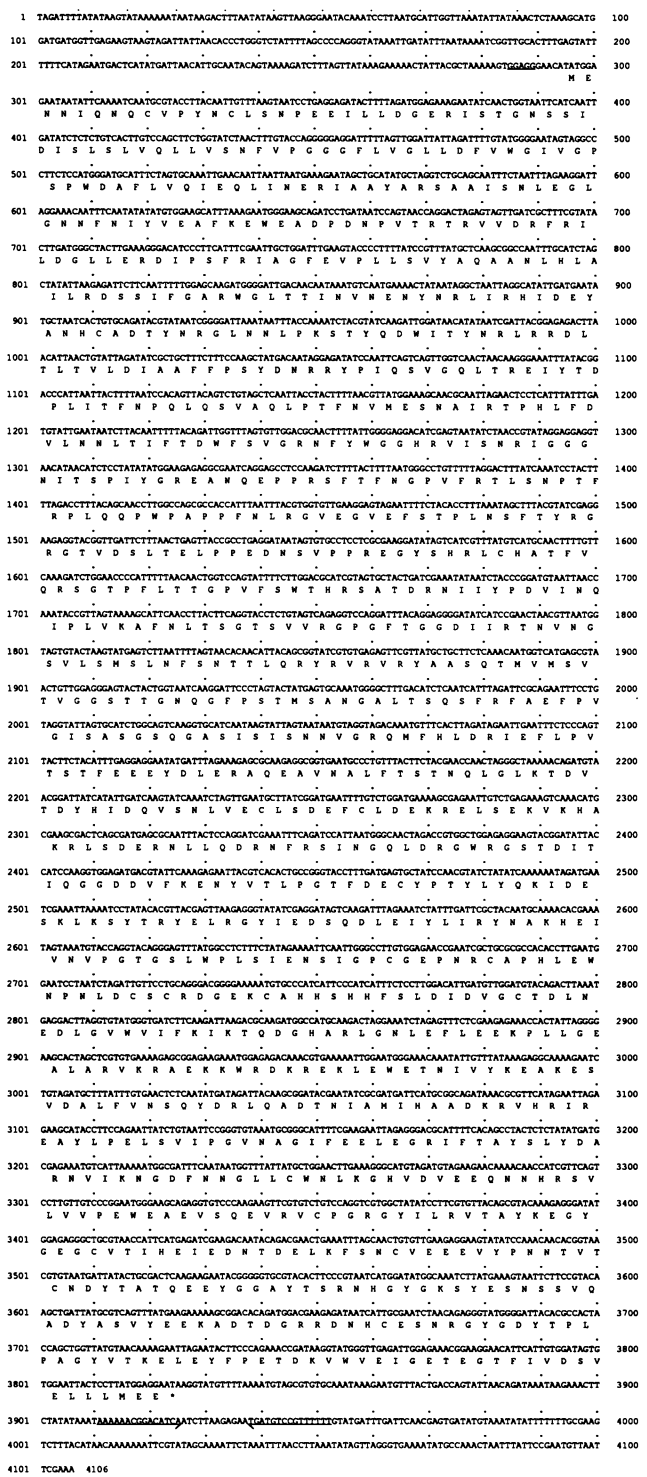

FIG. 4. Nucleotide sequence and predicted amino acid sequence of CryIC(b) including 5′ and 3′ flanking sequences. The proposed ribosome binding site is underlined. A region of dyad symmetry downstream from the *cryIC(b)* coding sequence is indicated by arrows.

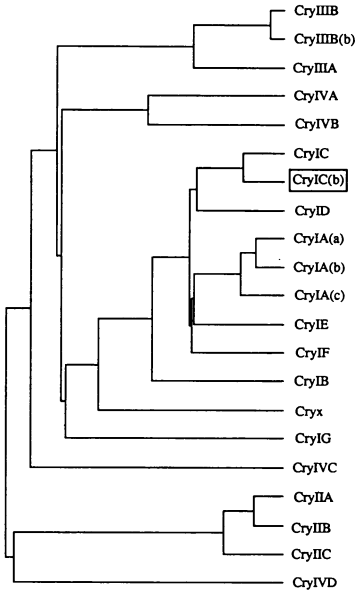

FIG. 5. Dendrogram comparing the amino acid sequences of the crystal proteins (20). Cryx was named CryIIIC (11) but shows greater amino acid sequence identity to CryI proteins than CryIII proteins. Analysis was done by the Pileup program in the Genetics Computer Group Sequence Analysis Software Package (6).

the presumed start codon. A transcript through a region of dyad symmetry located downstream from the *cryIC(b)* stop codon (nt 3911 to 3952 in Fig. 4) has the potential to form a stem-loop and may act as a transcription terminator.

The novel toxin gene from HD29 cloned in pSB204 was most similar to *cryIC* (Fig. 5). Because of this similarity, we propose to designate the gene *cryIC(b)*. CryIC(b) was 92% identical to CryIC in the first 450 amino acids and in residues 606 to 1177 but was only 44% identical to CryIC in residues 451 through 605. Because CryIC(b) diverged from CryIC between amino acids 450 and 605, this region of CryIC(b) was compared with the same region in other crystal proteins (6). Between residues 451 and 605, CryIC(b) shared slightly more identity with CryIF than CryIC (48 versus 44%).

Insecticidal activity of CryIC(b). Western blotting showed that *E. coli* DH5α carrying plasmid pSB204 produced a protein of approximately 130 kDa that cross-reacted with polyclonal antisera raised against purified crystals from *B. thuringiensis* subsp. *aizawai* HD229. No protein was observed from *E. coli* carrying pSB205.

To compare the insecticidal activities of CryIC(b) and CryIC against *T. ni* and *S. exigua*, crystal proteins were purified from *E. coli* cells carrying either plasmid pSB204 or plasmid pSB607. Plasmid pSB607 contained the *cryIC* gene cloned from HD229 whose nucleotide sequence was identical to the sequence of *cryIC* from *B. thuringiensis* subsp. *entomocidus* reported by Honée et al. (8). Serial dilutions were incorporated into insect diet, and mortality was scored after 4 days. CryIC and CryIC(b) had comparable toxicities against *T. ni* larvae; their 50% lethal concentration (LC_{50}) values were 7 and 8 ppm, respectively. However, CryIC was somewhat more toxic to *S. exigua* larve than was CryIC(b). The LC_{50} of CryIC was 7 ppm, while CryIC(b) had an LC_{50} of 34 ppm.

DISCUSSION

Carozzi et al. (4) used PCR screening to predict the insecticidal activity of previously uncharacterized *B. thuringiensis* strains. Their method determines whether a given strain contains genes likely to be active against lepidopteran, coleopteran, or dipteran insects. In contrast, our PCR screening method determines which lepidopteran-active genes are present. Primers used in our PCR were very specific, as they were intended to detect *cryI* genes in *B. thuringiensis* DNA samples. As many as eight *cryI* genes were detected in one reaction, allowing a rapid screening of a large number of *B. thuringiensis* strains. PCR analyses with *cryI*-specific primers showed that *B. thuringiensis* subsp. *galleriae* HD29 carried *cryIA(b)*, *cryIA(c)*, and *cryID* but not *cryIC*.

The second PCR technique in this study utilized a set of *cryIC*-specific primers to search for novel *cryIC*-type genes. This PCR amplified several DNA fragments with different sizes and quantities. When the fragments were analyzed by agarose gel electrophoresis, they created a fingerprint specific to the target gene sequence. PCR screening with *cryIC*-specific primers suggested that HD29 contained a *cryIC*-type gene that differed from the *cryIC* gene commonly occurring in *B. thuringiensis* subsp. *aizawai* and *B. thuringiensis* subsp. *entomocidus*. Southern blotting revealed that *cryIC*-type sequences were present on a 10-kb *Eco*RI fragment of HD29 DNA. We cloned the *cryIC*-type gene in *E. coli*, and the nucleotide sequence was determined.

DNA sequencing showed that the novel toxin gene was notably homologous to *cryIC* cloned from *B. thuringiensis* subsp. *entomocidus* 60.5 (8). A slightly different *cryIC* gene was cloned from *B. thuringiensis* subsp. *aizawai* HD137 (16). We cloned a *cryIC* gene from *B. thuringiensis* subsp. *aizawai* HD229 but have failed to confirm the sequence diversity found in the HD137 *cryIC*; the sequence of *cryIC* from strain HD229 was identical to that of the *B. thuringiensis* subsp. *entomocidus cryIC*. Bosse et al. (1) cloned a *cry* gene from *B. thuringiensis* subsp. *kenyae* and proposed to designate the gene *cryIC(b)*. However, the sequence of the *B. thuringiensis* subsp. *kenyae* gene had some mistakes, and after the correction was made, it was identical to *cryIE* cloned by Visser et al. (19). We compared the sequence of our novel gene from HD29 to all other *cry* genes simultaneously and found that it was indeed a subclass of the *cryIC* gene (Fig. 5). Therefore, we propose to designate this gene *cryIC(b)*.

The amino acid sequence of CryIC(b) diverged from CryIC between residues 451 and 605 (44% identity in this region). We compared residues 451 through 605 to the same region in other *cryI* genes, and it did not appear to be derived from any other *cryI* gene. Recently, Li et al. (12) determined the tertiary structure of CryIIIA. Assuming that CryIC has a tertiary structure similar to that of CryIIIA, the region containing the amino acid differences between CryIC and CryIC(b) corresponds mostly to domain III of the CryIIIA crystal structure.

CryIC(b) and CryIC expressed in *E. coli* showed similar toxicities against *T. ni*, but the LC_{50} of CryIC(b) against *S. exigua* was higher than that observed for CryIC. Although CryIC was more toxic to *S. exigua* than was CryIC(b), the activity of CryIC(b) against *S. exigua* was quite significant relative to the toxicities of other CryI proteins against *Spodoptera* species. CryIA(a), CryIA(b), CryIA(c), CryIB, and CryID show no toxicity toward *Spodoptera littoralis* (7). CryIE and CryIF were reported to possess some toxicity toward *S. exigua* (5, 19); however, we have not directly compared their activities with those of CryIC and CryIC(b).

The differential spectra of CryIC and CryIC(b) must be due to the primary sequence differences between the two proteins. The residues in domain III of CryIC(b) may cause this protein to be less stable in the *S. exigua* midgut than CryIC. Alternatively, CryIC and CryIC(b) may have the same receptor binding regions for *T. ni*, whereas the amino acids involved in receptor binding for *S. exigua* might differ. Honée et al. (9) proposed that CryIC has a *Spodoptera* specificity determinant in residues 258 to 646. However, since CryIC(b) and CryIC both show *Spodoptera* activity and since these two proteins diverge significantly after residue 450, we believe the *Spodoptera* specificity determinant lies between residues 258 and 450. Residues 258 to 450 lie in domain II of the CryIIIA tertiary structure, which is considered the receptor binding domain (12).

It appears that strain HD29 contains a diversity of crystal toxins. Nicholls et al. (15) isolated a 49-kDa P2 (CryII) crystal toxin from HD29 and showed that it was active against *Pieris brassicae*, *Aedes aegypti*, and *Anopheles gambiae*. This group also showed by SDS-polyacrylamide gel electrophoresis that HD29 contained more than one type of P1 protein of 130 to 140 kDa (CryI).

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