Characterization of two genes encoding Bacillus thuringiensis insecticidal crystal proteins toxic to Coleoptera species.

W P Donovan, M J Rupar, A C Slaney, T Malvar, M C Gawron-Burke and T B Johnson

Appl. Environ. Microbiol. **1992, 58(12):3921.**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1992, p. 3921–3927
0099-2240/92/123921-07$02.00/0

Vol. 58, No. 12

Characterization of Two Genes Encoding *Bacillus thuringiensis* Insecticidal Crystal Proteins Toxic to *Coleoptera* Species

WILLIAM P. DONOVAN,* MARK J. RUPAR, ANNETTE C. SLANEY, THOMAS MALVAR, M. CYNTHIA GAWRON-BURKE, AND TIMOTHY B. JOHNSON

Ecogen Inc., 2005 Cabot Boulevard West, Langhorne, Pennsylvania 19047

Received 13 May 1992/Accepted 4 October 1992

Bacillus thuringiensis **EG2838 and EG4961 are highly toxic to Colorado potato beetle larvae, and only strain EG4961 is toxic to southern corn rootworm larvae. To investigate the cause of the different insecticidal activities of EG2838 and EG4961, *cryIII*-type genes toxic to coleopterans were cloned from each strain. The *cryIIIB* gene, cloned as part of an 8.0-kb *Eco*RI fragment of EG2838 DNA, encoded a crystal protein (CryIIIB) of 74,237 Da. The *cryIIIB2* gene, cloned as part of an 8.3-kb *Pst*I-*Asp*718 fragment of EG4961 DNA, encoded a crystal protein (CryIIIB2) of 74,393 Da that was 94% identical to CryIIIB. Analysis of the transcriptional start sites showed that *cryIIIB* and *cryIIIB2* were initiated from a conserved region located within 130 nucleotides upstream from the translation start sites of both genes. Although the CryIIIB and CryIIIB2 proteins were similar in sequence, they displayed distinct insecticidal activities: CryIIIB was one-third as toxic as CryIIIB2 to Colorado potato beetle larvae, and CryIIIB2, but not CryIIIB, was toxic to southern corn rootworm larvae. Genes encoding crystal proteins of approximately 32 and 31 kDa were located adjacent to the *cryIIIB* and *cryIIIB2* genes, respectively. The 32- and 31-kDa crystal proteins failed to enhance the insecticidal activities of CryIIIB and CryIIIB2.**

During the process of spore formation, *Bacillus thuringiensis* synthesizes large amounts of certain proteins, referred to as crystal proteins, that aggregate within the mother cell to form stable, protease-resistant crystals. The sequences of more than 22 crystal proteins, which were deduced from the sequences of cloned crystal protein genes (*cry* genes), have been reported, and most of these crystal proteins are specifically toxic to lepidopteran insect larvae (caterpillars) (for a review, see reference 14). Compared with the number of characterized proteins that are toxic to lepidopterans, crystal proteins that are toxic to coleopteran insects (beetles) are rare. Among the few known strains of *B. thuringiensis* that are toxic to coleopterans, three similar strains of *B. thuringiensis* (subsp. *morrisoni* and subsp. *tenebrionis*) have been reported. Each of these strains contains the *cryIIIA* gene, which encodes a crystal protein (CryIIIA) of 73 kDa that is toxic to coleopterans (9, 13, 19, 30). A fourth strain toxic to coleopterans, *B. thuringiensis* subsp. *tolworthi*, contains the *cryIIIB* gene, which encodes a crystal protein (CryIIIB) of 74 kDa that is toxic to coleopterans (31). Cidaria et al. (3) have also reported the discovery of a strain of *B. thuringiensis* subsp. *tolworthi* that is toxic to coleopterans, although the sequence of the gene that is toxic to coleopterans was not determined. The CryIIIA and CryIIIB proteins share 69% sequence identity, and both proteins are primarily toxic to Colorado potato beetle (CPB) larvae (9, 13, 31).

We have previously described two novel strains toxic to coleopterans, *B. thuringiensis* EG2838 and EG4961, which were isolated from dust samples taken from crop storage areas (28). Strains EG2838 (*B. thuringiensis* subsp. *tolworthi*) and EG4961 (*B. thuringiensis* subsp. *kumamotoensis*) were shown to produce crystal proteins of approximately 70 kDa that reacted with anti-CryIIIA antibodies, and each strain produced smaller crystal proteins of approximately 30 kDa that were not related to the 70-kDa proteins (28). Sporulated cultures containing both spores and crystal proteins of strains EG2838 and EG4961 were toxic to CPB larvae, and EG4961 displayed a moderate level of toxicity to southern corn rootworm (SCR) larvae, a rare and perhaps unique activity among the *B. thuringiensis* strains that have been reported. Here, we describe the isolation, sequencing, and transcription start site mapping of genes *cryIIIB* and *cryIIIB2* of strains EG2838 and EG4962, respectively, which are toxic to coleopterans. We also show that the gene products display distinct insecticidal activities. Furthermore, we partially characterize two genes that encode crystal proteins of approximately 32 and 31 kDa that are adjacent to the *cryIIIB* and *cryIIIB2* genes, respectively.

MATERIALS AND METHODS

Strains, growth media, and plasmids. *Escherichia coli* HB101, DH5α, and GM2163 (23) were grown at 37°C in LB medium (1% Difco tryptone, 0.5% Difco yeast extract, 0.5% NaCl, pH 7.0) plus 50 μg of ampicillin ml^{-1} where appropriate. *B. thuringiensis* EG2838 and EG4961, and *B. thuringiensis* HD73-26 harboring recombinant plasmids, were grown at room temperature (21 to 24°C) in DSM medium [0.4% (wt/vol) Difco nutrient broth, 25 mM K_2HPO_4, 25 mM KH_2PO_4, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 μM $FeSO_4$, 10 μM $MnCl_2$, 0.5% (wt/vol) glucose], plus 5 μg of chloramphenicol ml^{-1} or 10 μg of tetracycline ml^{-1} where appropriate, until sporulation and cell lysis had occurred (approximately 5 days). HD73-26 is a crystal-negative *B. thuringiensis* subsp. *kurstaki* strain (11). Plasmids pBR322 and pUC18 were used for cloning and subcloning in *E. coli*. Plasmid pNN101 (Tet^r Cam^r [27]) was used as a replication vector for the expression of cloned DNA in *B. thuringiensis*.

Construction of *E. coli-B. thuringiensis* shuttle plasmids and *B. thuringiensis* electroporation. *B. thuringiensis* vector pNN101 was ligated into the unique *Bam*HI or *Sph*I sites of pBR322 and pUC18 that contained various subclones of *B. thuringiensis* DNA, as described in Results, resulting in *E.*

* Corresponding author.

coli-B. thuringiensis shuttle plasmids. All plasmid constructions were done in *E. coli* HB101 or DH5α. Constructed shuttle plasmids were isolated from HB101 or DH5α and transformed into *E. coli* GM2163 (*dcm-6 dam-13*::Tn*9 hsdR2*) (23). Shuttle plasmids were isolated from GM2163 and transformed into *B. thuringiensis* HD73-26 by electroporation as described by Macaluso and Mettus (22) by using a Bio-Rad gene pulser apparatus set at 2,500 V and 25 μF.

Colony blot hybridization. *E. coli* colonies containing plasmid libraries of *B. thuringiensis* DNA were constructed as previously described (8) by ligating size-selected *Eco*RI restriction fragments of EG2838 DNA or size-selected *Pst*I-*Asp*718 restriction fragments of EG4961 DNA into pBR322 or pUC18. A 2.0-kb *Hin*dIII-*Xba*I fragment containing the *cryIIIA* gene (9) and a 2.4-kb *Ssp*I fragment containing the *cryIIIB* gene were radioactively labeled with [α-^{32}P]dATP by random primer extension (10) and used as probes in colony blot (12) and DNA blot (33) hybridizations. Hybridizations were carried out at 65°C in 3× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)–10× Denhardt's solution (1× Denhardt's solution is 0.02% [wt/vol] bovine serum albumin, 0.02% [vol/vol] Ficoll, and 0.02% [wt/vol] polyvinylpyrrolidone)–200 μg of heparin ml^{-1}–0.1% [wt/vol] sodium dodecyl sulfate (SDS).

DNA sequencing. DNA fragments containing the *cryIIIB* and *cryIIIB2* genes were cloned into M13 vectors mp18 and mp19 as described in Results. A total of 14 *cryIIIB*-specific and 12 *cryIIIB2*-specific 17-mer primers (synthesized on an Applied Biosystems model 380B DNA synthesizer) and one M13-specific primer (supplied by Bethesda Research Laboratories) were used to determine the complete DNA sequences of both strands of the *cryIIIB* and *cryIIIB2* genes by the dideoxy method (29). DNA sequence and deduced protein sequence homologies were identified by use of the computer program of Korn and Queen (18).

Extraction and quantification of crystal protein. Crystal proteins were solubilized from *B. thuringiensis* cultures that had been grown to sporulation in DSM medium plus antibiotic where appropriate, and the proteins were fractionated by SDS-polyacrylamide gel electrophoresis as described previously (4). Fractionated proteins were quantified by densitometer tracing of Coomassie-stained protein gels by using a Molecular Dynamics model 300A computing densitometer. Purified CryIIIA crystal protein served as a reference standard during densitometer tracing. The CryIIIA protein was purified by NaBr solubilization and recrystallization as described by Slaney et al. (32), and the concentration of the purified protein was determined by the bicinchoninic acid kit assay method as recommended by the manufacturer (Pierce Chemicals).

Mapping transcription start sites. Total RNA was isolated from cells grown overnight in DSM medium. RNA isolation was conducted as described by Zuber et al. (39). Primer extension reactions and oligonucleotide kinase reactions were performed by using the primer extension system (Promega, Madison, Wis.) exactly as described by the manufacturer. Approximately 5 μl of the RNA solution was used for the primer extension reaction.

Insect bioassays. First-instar larvae of *Diabrotica undecimpunctata howardi* (SCR) and *Leptinotarsa decemlineata* (CPB) were bioassayed via surface contamination of artificial diets (24, 28). Each bioassay consisted of eight doses, with 32 larvae tested per dose. Bioassay results were obtained by scoring CPB larva mortality after 3 days and SCR larva mortality after 7 days.

Nucleotide sequence accession number. The sequence of the *cryIIIB2* gene has been deposited in the GenBank data base under accession number M89794.

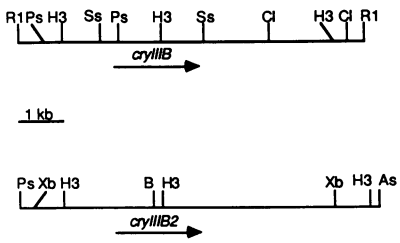

FIG. 1. Restriction maps of the *cryIIIB* and *cryIIIB2* genes of strains EG2838 and EG4961, respectively. Abbreviations: R1, *Eco*RI; Ps, *Pst*I; H3, *Hin*dIII; Ss, *Ssp*I; Cl, *Cla*I; Xb, *Xba*I; B, *Bam*HI; As, *Asp*718.

RESULTS

Isolation and characterization of *cryIIIB* and *cryIIIB2*. The radiolabeled *cryIIIA* gene (9) was used as a hybridization probe in colony blot experiments (see Materials and Methods) to isolate a *cryIIIA*-hybridizing 8.0-kb *Eco*RI DNA fragment of strain EG2838 (Fig. 1). Portions of the 8.0-kb DNA fragment (a 2.5-kb *Hin*dIII fragment and a 3.3-kb *Cla*I-*Pst*I fragment) were subcloned into M13 vectors and sequenced. DNA sequencing showed that the 8.0-kb DNA fragment contained an open reading frame of 651 codons that was 75% identical to the sequence of the *cryIIIA* gene and 100% identical to the sequence of the *cryIIIB* gene toxic to coleopterans reported by Sick et al. (31). Thus, strain EG2838 contained the *cryIIIB* gene, and the gene was located approximately in the middle of the cloned 8.0-kb *Eco*RI DNA fragment (Fig. 1).

DNA sequencing showed that the complete coding region of the *cryIIIB* gene was contained within a 2.4-kb *Ssp*I DNA fragment (Fig. 1). The 2.4-kb *Ssp*I DNA fragment was used as a radiolabeled probe in colony blot experiments to isolate a *cryIIIB*-hybridizing 8.3-kb *Pst*I-*Asp*718 restriction fragment of strain EG4961 DNA (Fig. 1). DNA sequencing of portions of the 8.3-kb DNA fragment (a 2.4-kb *Hin*dIII DNA fragment and a 4.0-kb *Bam*HI-*Xba*I DNA fragment) showed that the 8.3-kb DNA fragment contained an open reading frame, designated *cryIIIB2*, of 652 codons. The *cryIIIB2* gene was 96% identical to the *cryIIIB* gene and 75% identical to the *cryIIIA* gene. The sequences of the *cryIIIB* and *cryIIIB2* genes as well as the deduced amino acid sequences of the encoded proteins, CryIIIB and CryIIIB2, are shown in Fig. 2. Nucleotide differences between the *cryIIIB* and *cryIIIB2* genes appeared to occur randomly throughout the two genes, and gaps were not required to achieve maximum homology (Fig. 2). A comparison of the upstream noncoding regions showed that the *cryIIIB* upstream region lacked eight nucleotides that were found between positions −87 and −98 of the *cryIIIB2* upstream region (Fig. 2). The deduced amino acid sequence of the CryIIIB protein (651 residues; 74,237 Da) of strain EG2838 was found to be 94% identical to the deduced amino acid sequence of the CryIIIB2 protein (652 residues; 74,393 Da) of strain EG4961. The CryIIIB and CryIIIB2 proteins were 68 and 69% identical, respectively, to the CryIIIA crystal protein (644 residues), which is toxic to coleopterans.

The *cryIIIB* and *cryIIIB2* genes utilize similar transcription start sites. A 17-mer oligonucleotide that was homologous to both the *cryIIIB* and *cryIIIB2* genes from nucleotides −3 to

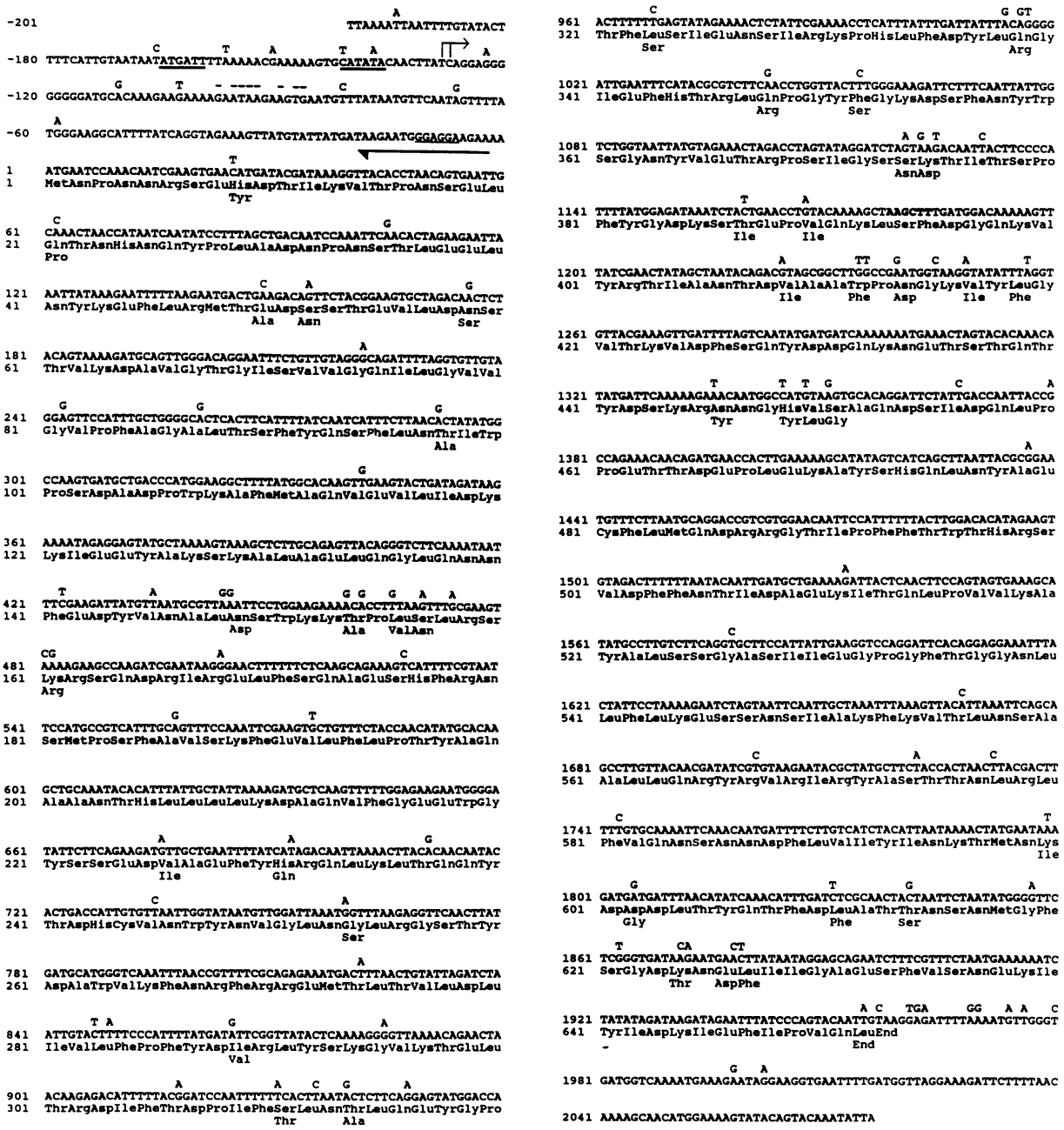

FIG. 2. DNA sequence and deduced amino acid sequence of the *cryIIIB2* gene. The *cryIIIB2* DNA and deduced amino acid sequences are presented in their entirety. These sequences were aligned with the *cryIIIB* gene sequence and the sequence of its predicted gene product; only the differences in the *cryIIIB* DNA and amino acid sequences are shown. The putative ribosome binding sites are indicated by a thin underline. The transcription start sites and the −10 and −35 regions are indicated by a double arrow and thick underlines, respectively. Gaps in the nucleotide sequence (indicated by dashes) were created in the *cryIIIB* upstream sequence to permit optimum alignment with the *cryIIIB2* sequence. The arrow at nucleotides −3 to −19 indicates the 17-mer primer that was used in primer extension experiments.

−19 (Fig. 2) was used to generate reverse transcripts from EG2838 (*cryIIIB*$^+$) and EG4961 (*cryIIIB2*$^+$) total RNA. Primer extension analysis of RNA isolated from strain EG2838 (*cryIIIB*$^+$) yielded two reverse transcripts (Fig. 3) that corresponded to transcription start sites at positions −120 (cytosine) and −121 (thymine) relative to the translation start site of the *cryIIIB* gene (Fig. 2). The two *cryIIIB* transcription start sites were used with roughly equal frequencies, as indicated by the similar intensities of the transcripts (Fig. 3). A similar analysis of RNA isolated from EG4961 (*cryIIIB2*$^+$) yielded two reverse transcripts of similar intensities (Fig. 3) that corresponded to transcription start sites at positions −128 (cytosine) and −129 (thymine) relative to the translation start site of the *cryIIIB2* gene (Fig. 2). Additional experiments indicated that transcription of the *cryIIIA* gene of strain EG2158 was initiated at positions −128 (cytosine) and −129 (thymine) relative to the translation start site of the *cryIIIA* gene (result not shown).

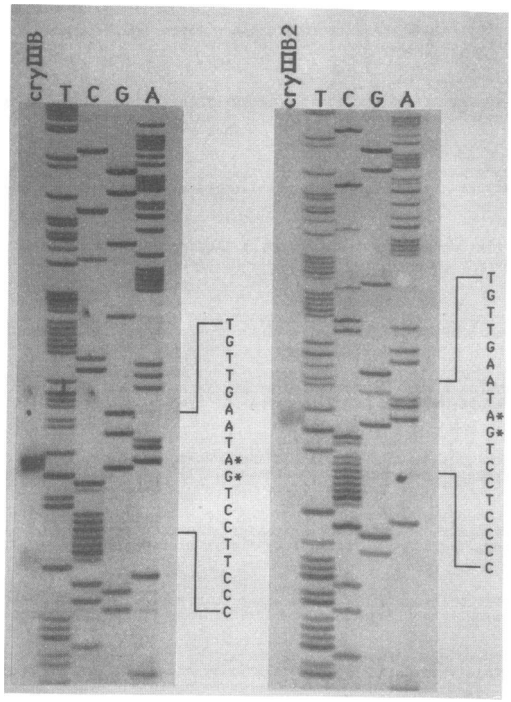

FIG. 3. Transcription start sites of the *cryIIIB* and *cryIIIB2* genes. Lanes cryIIIB and cryIIIB2 contain total RNA of strains EG2838 (cryIIIB) and EG4961 (cryIIIB2), respectively, which was reverse transcribed with a radioactively labeled 17-mer primer homologous to nucleotides −3 to −19 of the *cryIIIB* and *cryIIIB2* genes (Fig. 2). Lanes T, C, G, and A contain the DNA sequence of the *cryIIIB* and *cryIIIB2* genes and were generated with the same 17-mer primer used for the reverse transcription reaction. Asterisks indicate transcription start sites.

The *cryIIIB* and *cryIIIB2* genes are highly expressed in recombinant *B. thuringiensis* strains. We have found that cloned *B. thuringiensis* crystal protein genes are poorly expressed in *E. coli* cells but are usually highly expressed in *B. thuringiensis* host cells (unpublished results). The 8.0-kb *Eco*RI *cryIIIB* restriction fragment of EG2838 and the 8.3-kb *Pst*I-*Asp*718 *cryIIIB2* restriction fragment of EG4961 were subcloned onto *E. coli-B. thuringiensis* shuttle plasmids (see Materials and Methods), and the resulting plasmids, designated pEG242 (*cryIIIB*$^+$) and pEG260 (*cryIIIB2*$^+$), were transformed by electroporation into crystal-negative *B. thuringiensis* HD73-26. HD73-26 cells harboring pEG242 (*cryIIIB*$^+$) and pEG260 (*cryIIIB2*$^+$) produced minor amounts of proteins of approximately 74 kDa and large amounts of proteins of approximately 70 kDa (Fig. 4). The 74- and 70-kDa proteins reacted with anti-CryIIIA antibodies (results not shown) and corresponded to the expected sizes of the full-length (74-kDa) and processed (70-kDa) forms of the CryIIIB and CryIIIB2 proteins (28). In addition to the CryIIIB and CryIIIB2 proteins, HD73-26 cells harboring pEG242 and pEG260 produced crystal proteins of approximately 32 and 31 kDa, respectively, which appeared identical in size to the 32- and 31-kDa proteins produced by strains EG2838 and EG4961 (Fig. 4). The 32- and 31-kDa proteins failed to react with anti-CryIIIA antibodies (results not shown), a result which indicated that the 32- and 31-kDa proteins were not degradation products of the CryIIIB and CryIIIB2 proteins.

To determine the location of the gene for the 32-kDa protein, subclones of the 8.0-kb *cryIIIB* DNA fragment were generated. HD73-26 cells harboring plasmid pEG262, which contained a 4-kb *Pvu*II-*Hpa*I subclone of the 8.0-kb DNA fragment, produced the 70-kDa CryIIIB protein but failed to produce the 32-kDa protein (Fig. 4). HD73-26 cells harboring plasmid pEG289, which contained a 3.5-kb *Hin*dIII subclone of the 8.0-kb DNA fragment, produced the 32-kDa protein but did not produce the CryIIIB protein (Fig. 4). These results demonstrated that the gene for the 32-kDa protein, which we refer to as *32kD*, was located within a 3-kb region of DNA immediately downstream from the *cryIIIB* gene.

To determine the location of the gene for the 31-kDa protein, subclones of the 8.3-kb *cryIIIB2* DNA fragment were generated. HD73-26 cells harboring pEG269, which contained a 5-kb *Sau*3A subclone of the 8.3-kb DNA fragment, produced the 70-kDa CryIIIB2 protein but failed to produce the 31-kDa protein (Fig. 4). A minor band of approximately 29 kDa can be seen on the protein gel in Fig. 4, lane pEG269. The appearance of this band was erratic in that it was not observed on all gels. Although the exact nature of this band was unknown, the band did not appear to be a truncated form of the 31-kDa protein since it did not react with anti-31-kDa-protein antibodies (result not shown). HD73-26 cells harboring pEG286, which contained a 4-kb *Hin*dIII subclone of the 8.3-kb DNA fragment, produced significant amounts of the 31-kDa protein but did not produce the 70-kDa CryIIIB2 protein (Fig. 4). These results demonstrated that the gene for the 31-kDa crystal protein, referred to as the *31kD* gene, was located within an approximately 3-kb region of DNA immediately downstream from the *cryIIIB2* gene.

The CryIIIB and CryIIIB2 proteins possess distinct insecticidal specificities which are not enhanced by the 32- or 31-kDa protein. With clones of the *cryIIIB*, *cryIIIB2*, *32kD*, and *31kD* genes, we were able to quantify the insecticidal activities of the individual crystal proteins. A sporulated culture of HD73-26(pEG262) that produced only the CryIIIB protein and a sporulated culture of HD73-26(pEG269) that produced only the CryIIIB2 protein were assayed for toxicity to CPB and SCR larvae. The CryIIIB protein was found to be one-third as toxic to CPB larvae as the CryIIIB2 protein (Table 1). A more striking difference was seen with regard to SCR toxicity: the CryIIIB protein displayed no measurable toxicity, as quantified by 50% lethal doses, to SCR larvae, which is in contrast to the CryIIIB2 protein, which had a significant toxicity to SCR larvae (Table 1). However, the toxicity of CryIIIB2 to SCR larvae was relatively low in comparison to its toxicity to CPB larvae (Table 1). The CryIIIB and CryIIIB2 proteins displayed no toxicity to the lepidopteran insects *Spodoptera exigua*, *Heliothis virescens*, and *Plutella xylostella* (results not shown). Sporulated cultures of recombinant *B. thuringiensis* cells that produced only the 32-kDa protein [HD73-26(pEG289)] or only the 31-kDa protein [HD73-26(pEG286)] were not toxic to CPB or SCR larvae (Table 1).

The wild-type strain EG2838 produced both the CryIIIB protein and the 32-kDa protein (Fig. 4), and we hypothesized that the 32-kDa protein may function to enhance the activity of the CryIIIB protein. A sporulated culture which contained only the CryIIIB protein [HD73-26(pEG262)] had a toxicity to CPB larvae similar to that of a mixture of sporulated cultures [HD73-26(pEG262) plus HD73-26(pEG289)] which contained a 2:1 (wt/wt) ratio of CryIIIB and 32-kDa proteins (Table 1). Thus, the 32-kDa protein did not enhance the toxicity of the CryIIIB protein to CPB larvae.

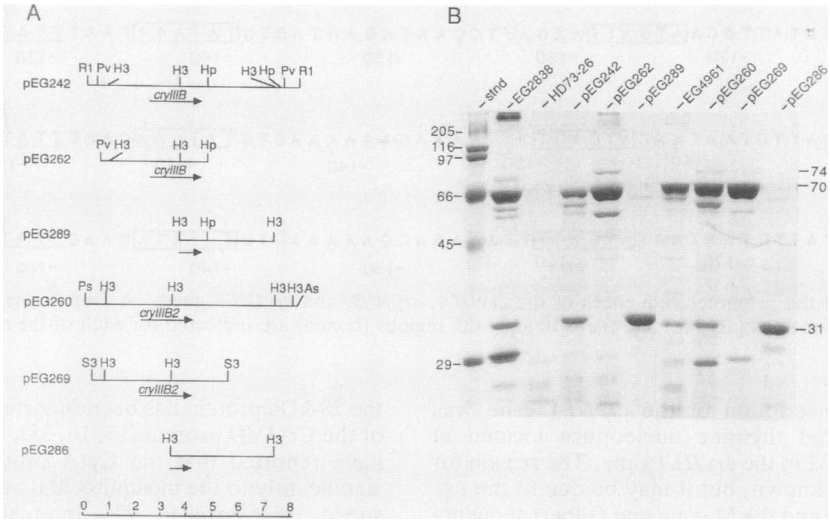

FIG. 4. Synthesis of crystal proteins by *B. thuringiensis* strains harboring the *cryIIIB* and *cryIIIB2* genes. (A) Cloned restriction fragments are shown along with the designations of plasmids (pEG) that contain the fragments. (B) Coomassie-stained acrylamide gel containing size-fractionated proteins extracted from sporulated cultures of various *B. thuringiensis* strains. Lanes: EG2838, strain EG2838; HD73-26, the crystal-negative strain HD73-26; pEG242, pEG262, pEG289, pEG260, pEG269, and pEG286, strain HD73-26 harboring each respective plasmid; EG4961, strain EG4961. Numbers indicate the molecular masses, in kilodaltons, of protein standards (stnd) and of EG4961 crystal proteins.

The wild-type strain EG4961 produced both the CryIIIB2 protein and the 31-kDa crystal protein (Fig. 4). To test for insecticidal synergism between the CryIIIB2 and 31-kDa proteins, a sporulated culture that contained only the CryIIIB2 protein [HD73-26(pEG269)] was bioassayed with and without the addition of a sporulated culture that contained only the 31-kDa protein [HD73-26(pEG286)]. The mixture of HD73-26(pEG269) and HD73-26(pEG286), which contained a 3:1 (wt/wt) ratio of CryIIIB2 and 31-kDa proteins, had a toxicity to CPB larvae similar to that of the HD73-26(pEG269) culture alone (Table 1). Furthermore, the mixture of HD73-26(pEG269) and HD73-26(pEG286) had a toxicity to SCR larvae similar to that of the HD73-26(pEG269) culture alone (Table 1). Thus, the 31-kDa protein did not significantly enhance the insecticidal activity of the CryIIIB2 protein.

TABLE 1. Toxicity of the CryIIIB, CryIIIB2, and 32- and 31-kDa crystal proteins to CPB and SCR larvae[a]

Protein	LC_{50} for:	
	CPB larvae	SCR larvae
CryIIIB	1.35 (1.08–1.72)	>4,570
CryIIIB-32 kDa (2:1, wt/wt)	2.21 (1.29–3.64)	Not tested
CryIIIB2	0.41 (0.33–0.52)	107 (78–138)
CryIIIB2-31 kDa (3:1, wt/wt)	0.41 (0.34–0.52)	72 (9–153)
32 kDa	>19	>371
31 kDa	>28	>371

[a] Results are 50% lethal doses (LC_{50}) (with 95% confidence intervals) and are expressed as nanograms of protein per square millimeter of diet surface. Values were determined from two replications of eight-dose serial bioassays. For the mixtures of the CryIIIB and 32-kDa proteins and the CryIIIB2 and 31-kDa proteins, the LC_{50} represents only the concentration of the CryIIIB and the CryIIIB2 protein, respectively.

DISCUSSION

In this study, we have characterized the *cryIIIB* and *cryIIIB2* genes, which are part of a family of *B. thuringiensis* genes toxic to coleopterans. The *cryIIIB* and *cryIIIB2* genes shared 96% sequence identity and were 75% identical to a third member of the family of genes toxic to coleopterans, the *cryIIIA* gene. Recently, the sequence of *cryIIID*, a gene toxin to coleopterans, was reported (20), and the *cryIIIB* and *cryIIIB2* genes are each 70% identical to *cryIIID*. Interestingly, the *cryIII* genes exist in four different subspecies of *B. thuringiensis*: *cryIIIB* in *B. thuringiensis* subsp. *tolworthi* (28, 31), *cryIIIB2* in *B. thuringiensis* subsp. *kumamotoensis* (28), *cryIIIA* in *B. thuringiensis* subsp. *morrisoni* (5, 19, 30), and *cryIIID* in *B. thuringiensis* subsp. *kurstaki* (20). The *cryIIIA*, *cryIIIB*, and *cryIIIB2* genes are carried on large plasmids of 88, 100, and 95 MDa, respectively (9, 28). We have shown that the 88-MDa *cryIIIA* plasmid is transferred by a conjugation-like process from *B. thuringiensis* EG2158 to a recipient *B. thuringiensis* strain in broth culture (9). In addition, Jarrett and Stephenson (17) found a high rate of plasmid transfer between strains of *B. thuringiensis* toxic to lepidopterans growing in infected caterpillar larvae. Thus, the presence of related *cryIII* genes in different subspecies of *B. thuringiensis* may be the result of the conjugal transfer of an ancestral *cryIII*-containing plasmid from one subspecies of *B. thuringiensis* to another followed by evolutionary divergence of the genes.

As a first step in investigating *cryIII* gene regulation, we determined the transcription start sites of the *cryIIIB* and *cryIIIB2* genes. Our findings, summarized in Fig. 5, demonstrated that transcription was initiated from adjacent cytosine and thymine nucleotides located at positions −128 and −129 in the *cryIIIB2* gene and at positions −120 and −121 in the *cryIIIB* gene. Similarly, transcription of the *cryIIIA* gene was initiated from adjacent cytosine and thymine nucleotides located at positions −128 and −129 in the *cryIIIA* gene (Fig. 5). In contrast to our findings, Sekar et al.

FIG. 5. Comparison of the promoter sequences of the *cryIIIA*, *cryIIIB*, and *cryIIIB2* genes. A 5-bp conserved sequence (underlined) including the RNA initiation sites (arrows) and the −10 and −35 regions (boxed) are indicated for each of the *cryIII* genes.

(30) reported that transcription of the *cryIIIA* gene was initiated at adenine and thymine nucleotides located at positions −130 and −132 in the *cryIIIA* gene. The reason for this discrepancy is not known, but it may be due to the use of S1 nuclease mapping and the Maxam and Gilbert sequencing protocol employed by Sekar et al. (30) to determine the transcription start site of the *cryIIIA* gene. Our results clearly showed that transcription of all three genes was initiated from the fourth and fifth nucleotide positions within the conserved sequence (ttaTC) and that the three genes had similar promoter regions, with a consensus −10 sequence of (T/C)ATA(T/A)A and a consensus −35 sequence of ATGATT (Fig. 5). The putative −10 and −35 regions of the *cryIII* genes do not appear to be related to the −10 and −35 regions reported for other crystal protein genes (2, 35, 36). Similarities exist between the −10 region [(T/C)ATA(T/A)A] and the −35 region (ATGATT) of the *cryIII* genes (Fig. 5) and the consensus −10 region (TATAAT) and the −35 region (TTGACA) of vegetatively expressed genes of *Bacillus subtilis* (25, 26). Significantly, the *cryIIIA* gene is expressed during vegetative growth (30), unlike other crystal protein genes (1, 36, 37).

The *cryIIIB* gene encoded a protein (CryIIIB) of 651 residues that shared 94% sequence identity with the CryIIIB2 protein (652 residues) encoded by the *cryIIIB2* gene. Despite their high sequence similarity, the two proteins displayed distinct insecticidal activities: the CryIIIB2 protein was approximately threefold more toxic to CPB larvae than the CryIIIB protein, and only the CryIIIB2 protein displayed measurable toxicity to SCR larvae. There are no obvious clusters of nonhomologous amino acids between the CryIIIB and CryIIIB2 proteins, and thus it is difficult to predict which amino acid sequences within CryIIIB2 contribute to its specificity for SCR larvae. Nevertheless, the knowledge of the sequences of the CryIIIB and CryIIIB2 proteins reported here will be invaluable in answering the question of why the CryIIIB2 protein is toxic to SCR larvae whereas the CryIIIB protein is not.

The *31kD* and *32kD* genes, encoding two unusually small crystal proteins of approximately 31 and 32 kDa, were located immediately downstream of the *cryIIIB2* and *cryIIIB* genes, respectively. The small proteins lacked toxicity to CPB larvae, and they failed to enhance the toxicity of the CryIIIB and CryIIIB2 proteins to coleopterans. It is interesting to compare the organization and insecticidal specificities of the *cryIII*, *31kD*, and *32kD* genes with those of the *cryIVD* and *cytA* genes of *B. thuringiensis* subsp. *israelensis*. The *cryIVD* gene encodes a 72-kDa crystal protein (CryIVD) that is toxic to mosquito larvae (7), and the *cytA* gene encodes a protein (CytA) of 28 kDa (34). The insecticidal activity of the 28-kDa CytA protein is controversial: the 28-kDa protein has been reported to enhance the toxicity of the CryIVD protein (15, 16, 38), while other investigators have reported that the CytA protein does not contribute significantly to the mosquitocidal activity of *B. thuringiensis* subsp. *israelensis* (6, 21). Interestingly, the *cytA* gene is immediately adjacent to the *cryIVD* gene (7), which is somewhat analogous to the organization of the *31kD* and *32kD* genes, which are adjacent to their respective *cryIII* genes. The significance of this gene organization, in which genes for highly insecticidal crystal proteins (i.e., *cryIVD*, *cryIIIB*, and *cryIIIB2*) are located adjacent to genes for small, apparently noninsecticidal crystal proteins (i.e., *cytA*, *31kD*, and *32kD*), is presently unknown. If a primary function of the crystal proteins of *B. thuringiensis* is insecticidal activity, then the apparent lack of activity of the 31- and 32-kDa proteins may be explained by assuming that the target insect(s) for these proteins has not been found. Alternatively, these proteins may perform some function not involved with insecticidal activity. The 31- and 32-kDa proteins do not appear to be essential for the formation of CryIII crystals: recombinant *B. thuringiensis* strains that harbored the *cryIIIB* and *cryIIIB2* genes but not the *31kD* and *32kD* genes produced large amounts of the CryIIIB and CryIIIB2 crystal proteins.

ACKNOWLEDGMENTS

We thank C. Jany for bioassays with lepidopteran insects and J. Cristea for assistance with coleopteran bioassays.

REFERENCES

1. **Brizzard, B. L., H. E. Schnepf, and J. W. Kronstad.** 1991. Expression of the *cryIB* crystal protein gene of *Bacillus thuringiensis*. Mol. Gen. Genet. **231**:59–64.
2. **Brown, K. L., and H. R. Whiteley.** 1990. Isolation of the second *Bacillus thuringiensis* RNA polymerase that transcribes from a crystal protein gene promoter. J. Bacteriol. **172**:6682–6688.
3. **Cidaria, D., A. Cappai, A. Vallesi, V. Caprioli, and G. Pirali.** 1991. A novel strain of *Bacillus-thuringiensis* (NCIMB 40152) active against coleopteran insects. FEMS Microbiol. Lett. **81**: 129–134.
4. **Dankocsik, C., W. P. Donovan, and C. S. Jany.** 1990. Activation of a cryptic crystal protein gene of *Bacillus thuringiensis* subspecies *kurstaki* by gene fusion and determination of the crystal protein insecticidal specificity. Mol. Microbiol. **4**:2087–2094.
5. **de Barjac, H., and E. Frachon.** 1990. Classification of *Bacillus thuringiensis* strains. Entomophaga **35**:233–240.
6. **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.
7. **Donovan, W. P., C. Dankocsik, and M. P. Gilbert.** 1988. Molecular characterization of a gene encoding a 72-kilodalton

mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **170**:4732–4738.

8. **Donovan, W. P., C. C. Dankocsik, M. P. Gilbert, M. C. Gawron-Burke, R. G. Groat, and B. C. Carlton.** 1988. Amino acid sequence and entomocidal activity of the P2 crystal protein. J. Biol. Chem. **263**:561–567. (Erratum, **264**:4740, 1989.)
9. **Donovan, W. P., J. M. Gonzalez, Jr., M. P. Gilbert, and C. Dankocsik.** 1988. Isolation and characterization of EG2158, a new strain of *Bacillus thuringiensis* toxic to coleopteran larvae, and nucleotide sequence of the toxin gene. Mol. Gen. Genet. **214**:365–372.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
11. **Gonzalez, J. M., Jr.** 1984. Genetic analysis of the toxin plasmids of *Bacillus thuringiensis*. Ph.D. thesis. University of Georgia, Athens.
12. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72**:3961–3965.
13. **Herrnstadt, C., T. E. Gilroy, D. A. Sobieski, B. D. Bennett, and F. H. Gaertner.** 1987. Nucleotide sequence and deduced amino acid sequence of a coleopteran-active delta-endotoxin gene from *Bacillus thuringiensis* subsp. *san diego*. Gene **57**:37–46.
14. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53**:242–255.
15. **Hurley, J. M., L. A. Bulla, Jr., and R. E. Andrews, Jr.** 1987. Purification of the mosquitocidal and cytolytic proteins of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **53**:1316–1321.
16. **Ibarra, J. E., and B. A. Federici.** 1986. Isolation of a relatively nontoxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **165**:527–533.
17. **Jarrett, P., and M. Stephenson.** 1990. Plasmid transfer between strains of *Bacillus thuringiensis* infecting *Galleria mellonella* and *Spodoptera littoralis*. Appl. Environ. Microbiol. **56**:1608–1614.
18. **Korn, L. J., and C. Queen.** 1984. Analysis of biological sequences on small computers. DNA **3**:421–436.
19. **Krieg, A., A. M. Huger, G. A. Langenbruch, and W. Schnetter.** 1983. *Bacillus thuringiensis* var. *tenebrionis*, a new pathotype effective against larvae of Coleoptera. Z. Angew. Entomol. **96**:500–508.
20. **Lambert, B., W. Theunis, R. Aguda, K. Van Audenhove, C. Decock, S. Jansens, J. Seurinck, and M. Peferoen.** 1992. Nucleotide sequence of gene *cryIIID* encoding a novel coleopteran-active crystal protein from strain BTI109P of *Bacillus thuringiensis* subsp. *kurstaki*. Gene **110**:131–132.
21. **Lee, S. G., W. Eckblad, and L. A. Bulla, Jr.** 1985. Diversity of protein inclusion bodies and identification of mosquitocidal protein in *Bacillus thuringiensis* subsp. *israelensis*. Biochem. Biophys. Res. Commun. **126**:953–960.
22. **Macaluso, A., and A.-M. Mettus.** 1991. Efficient transformation of *Bacillus thuringiensis* requires nonmethylated plasmid DNA. J. Bacteriol. **173**:1353–1356.
23. **Marinus, M. G., M. Carraway, A. Z. Frey, L. Brown, and J. A. Arraj.** 1983. Insertion mutations in the *dam* gene of *Escherichia coli* K-12. Mol. Gen. Genet. **192**:288–289.
24. **Marrone, P. G., F. D. Ferri, T. R. Mosley, and L. J. Meinke.** 1985. Improvements in laboratory rearing of the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber (Coleoptera: Chrysomelidae), on an artificial diet and corn. J. Econ. Entomol. **78**:290–293.
25. **Moran, C. P., Jr.** 1989. Sigma factors and the regulation of transcription, p. 167–184. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development: structural and functional analysis of bacterial sporulation and germination. American Society for Microbiology, Washington, D.C.
26. **Moran, C. P., Jr., N. Lang, S. F. J. LeGrice, G. Lee, M. Stephens, A. L. Sonenshein, J. Pero, and R. Losick.** 1982. Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. **186**:339–346.
27. **Norton, N. B., K. A. Orzech, and W. F. Burke, Jr.** 1985. Construction and characterization of plasmid vectors for cloning in the entomocidal organism *Bacillus sphaericus* 1593. Plasmid **13**:211–214.
28. **Rupar, M. J., W. P. Donovan, R. G. Groat, A. C. Slaney, J. W. Mattison, T. B. Johnson, J.-F. Charles, V. C. Dumanoir, and H. de Barjac.** 1991. Two novel strains of *Bacillus thuringiensis* toxic to coleopterans. Appl. Environ. Microbiol. **57**:3337–3344.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
30. **Sekar, V., D. V. Thompson, M. J. Maroney, R. G. Bookland, and M. J. Adang.** 1987. Molecular cloning and characterization of the insecticidal crystal protein gene of *Bacillus thuringiensis* var. *tenebrionis*. Proc. Natl. Acad. Sci. USA **84**:7036–7040.
31. **Sick, A., F. Gaertner, and A. Wong.** 1990. Nucleotide sequence of a coleopteran-active toxin gene from a new isolate of *Bacillus thuringiensis* subsp. *tolworthi*. Nucleic Acids Res. **18**:1305.
32. **Slaney, A. C., H. L. Robbins, and L. English.** 1992. Mode of action of *Bacillus thuringiensis* toxin CryIIIA: an analysis of toxicity in *Leptinotarsa decemlineata* (Say) and *Diabrotica undecimpunctata howardi* Barber. Insect Biochem. Mol. Biol. **22**:9–18.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
34. **Waalwijk, C., A. M. Dullemans, M. E. S. van Workum, and B. Visser.** 1985. Molecular cloning and the nucleotide sequence of the M_r 28,000 crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. Nucleic Acids Res. **22**:8207–8217.
35. **Ward, E. S., A. R. Ridley, D. J. Ellar, and J. A. Todd.** 1986. *Bacillus thuringiensis* var. *israelensis* δ-endotoxin: cloning and expression of the toxin in sporogenic and asporogenic strains of *Bacillus subtilis*. J. Mol. Biol. **191**:13–22.
36. **Widner, W. R., and H. R. Whiteley.** 1989. Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. J. Bacteriol. **171**:965–974.
37. **Wong, H. C., E. E. Schnepf, and H. R. Whiteley.** 1983. Transcriptional and translational start sites for the *Bacillus thuringiensis* crystal protein gene. J. Biol. Chem. **258**:1960–1967.
38. **Wu, D., and F. N. Chang.** 1985. Synergism in mosquitocidal activity of 26 and 65 kDa proteins from *Bacillus thuringiensis* subsp. *israelensis* crystal. FEBS Lett. **190**:232–236.
39. **Zuber, P., J. M. Healy, and R. Losick.** 1987. Effects of plasmid propagation of a sporulation promoter on promoter utilization and sporulation in *Bacillus subtilis*. J. Bacteriol. **169**:461–469.