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Molecular cloning and characterization of the insecticidal crystal protein gene of *Bacillus thuringiensis* var. *tenebrionis*

(crystal toxin/coleopteran pathogen/biological insect control)

VAITHILINGAM SEKAR*, DAVID V. THOMPSON, MICHAEL J. MARONEY, ROGER G. BOOKLAND, AND MICHAEL J. ADANG

Agrigenetics Advanced Science Company, 5649 East Buckeye Road, Madison, WI 53716

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ABSTRACT **The insecticidal crystal protein gene of the coleopteran-toxic *Bacillus thuringiensis* var. *tenebrionis* has been isolated, and the nucleotide sequence has been determined. A total DNA library from var. *tenebrionis* was made in the plasmid vector pUC12. By using a synthetic 27-base oligonucleotide corresponding to a stretch of nine N-terminal amino acids of a tryptic fragment of purified crystal protein of var. *tenebrionis* as a probe, recombinant colonies were screened by *in situ* hybridization for the presence of the crystal protein gene. Positive clones obtained from this screening were further tested for toxicity. One recombinant, NSBP544 (which contained a 5.9-kilobase *Bam*HI insert), was toxic to larvae of Colorado potato beetle. Immunoblot analysis revealed that this clone produces two crystal-specific antigens of 65 and 73 kDa as do sporulating var. *tenebrionis* cells. However, purified crystal inclusions from var. *tenebrionis* contain a primary peptide component of 65 kDa. A 1932-base-pair open reading frame with a coding capacity of 73,119 Da has been identified by nucleotide sequencing analysis of the cloned crystal protein. In addition, mung bean nuclease mapping indicates that transcription of the crystal protein of var. *tenebrionis* initiates 130 base pairs upstream from the translational start site. Southern blot analysis using an internal 0.7-kilobase *Eco*RI fragment of pNSBP544 as a probe revealed that the crystal protein gene is located on a 90-MDa plasmid.**

Bacillus thuringiensis is unique in its ability to produce proteinaceous, crystalline inclusions (δ endotoxins) during the process of sporulation, which are found to be highly toxic to larvae of several lepidopteran insect pests of agricultural importance (1). Numerous isolates of *B. thuringiensis* strains belonging to over two dozen distinct flagellar serotypes have been isolated and classified to date (2). The crystal proteins of several of these varieties have a rather narrow host range and hence are used commercially as very selective biological insecticides. Strains of *B. thuringiensis* that are toxic to larvae of dipteran insects have also been isolated recently (3, 4).

The δ-endotoxin genes of various *B. thuringiensis* varieties have been cloned by several research groups (5–10). Kronstad *et al.* (11) have shown that these genes are generally located on one or more large plasmids. These authors have also demonstrated that while several lepidopteran-active genes share extensive nucleotide sequence similarity, no homology exists between the lepidopteran and dipteran toxin genes.

Since several agronomically important insect pests belong to the order Coleoptera (e.g., Colorado potato beetle, boll weevil, and corn root worm), an extensive search for coleopteran-toxic *B. thuringiensis* strains has been undertaken by several laboratories. Such efforts have resulted in the recent isolation of two strains of *B. thuringiensis*, namely var. *tenebrionis* (12) and var. *san diego* (13), that are pathogenic to coleopteran insects. Both strains produce flat, rectangular crystal inclusions and have a major crystal component of 64–68 kDa (13, 14). We report here on the cloning and expression in *Escherichia coli* and extensive molecular characterization of the crystal toxin gene of the coleopteran-active *B. thuringiensis* var. *tenebrionis*.†

MATERIALS AND METHODS

Strains. *B. thuringiensis* var. *tenebrionis* was provided by Boehringer Mannheim. Other *B. thuringiensis* strains were supplied by H. T. Dulmage (U.S. Department of Agriculture, Brownsville, TX). *E. coli* strain JM83 was used as the host organism, and plasmid pUC12 was used as the cloning vehicle (15). Subcloning was carried out in *E. coli* strain HB101 using the plasmid vector p1C20H (16).

Peptide Analysis and Oligonucleotide Synthesis. Crystal inclusions were purified from a crude spore/crystal mixture of *B. thuringiensis* var. *tenebrionis* by passing the mixture twice through linear sucrose gradients (55–88%) as described (13). Crystal proteins were solubilized in 100 mM sodium bicarbonate (pH 10.2) containing 10 mM dithiothreitol and were lyophilized, and the protein content was estimated (17). Urea-denatured crystal protein (4.0 μg) was digested with trypsin. The tryptic fragments were separated on a Varian 5000 HPLC system using a Vydac C_8 reverse-phase column with a linear gradient from 100% solvent A (0.1% trifluoroacetic acid in water) to 40% solvent B (0.1% trifluoroacetic acid in acetonitrile). Purified fragments obtained from highly resolved peaks were subjected to N-terminal protein sequencing on a Microsequenator (Applied Biosystems, Foster City, CA). A 27-base oligonucleotide (TATAAACAATATC-CATTTGTTATGATG) corresponding to the sequence of nine N-terminal amino acids of a tryptic fragment was synthesized. The preferred codon usage in the crystal protein genes of *B. thuringiensis* var. *kurstaki* (9, 18, 19) and var. *israelensis* (10) was used as a guideline in generating a consensus codon bias for the synthesis of the oligonucleotide.

Molecular Cloning. A library of *Bam*HI fragments of total *B. thuringiensis* var. *tenebrionis* DNA was made in *E. coli* JM83 using pUC12 as the cloning vector.

Nitrocellulose filters containing the lysates of putative recombinant clones were prepared (20) and were probed with the ^{32}P-labeled oligonucleotide probe according to Torczynski *et al.* (21). Hybridization was carried out at room

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Abbreviation: ORF, open reading frame.

*To whom reprint requests should be addressed.

†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02978).

temperature for 18 hr using 10^6 cpm per 10 ml for each filter, and the filters were repeatedly washed at room temperature prior to a final wash at 37°C for 5 min as described by Torczynski *et al.* (21).

Bioassay. The biological activity of recombinant clones that showed specific hybridization to the oligomer probe was tested by treating neonate larvae of Colorado potato beetle (*Leptinotarsa decemlineata*) with the total protein extracts, prepared as described (13), obtained from these clones. Excised leaves dipped in *E. coli* extracts were fed to larvae, and bioassays were conducted as presented in Table 1. The amount of protein bound to the leaves was measured by rinsing the leaves with water and estimating the protein contents of the rinse according to Bradford (17).

Immunoblot Analysis. *E. coli* clones were grown overnight in 100 ml of L broth. Cell extracts were prepared as described (13). $NaDodSO_4$/PAGE electrophoresis was carried out according to Laemmli (22), and antigenic peptides were detected as described by Towbin *et al.* (23). The primary mouse antiserum was prepared against the 65-kDa crystal protein of var. *tenebrionis*, the secondary antiserum was rabbit anti-mouse (Kierkegaard and Perry), and ^{125}I-labeled protein A was used for detection.

Southern Blot Hybridization. Plasmid DNA of various *B. thuringiensis* strains was separated in a 0.6% vertical agarose gel by the modified (24) slot-lysis method of Eckhardt (25) and was transferred to a nitrocellulose membrane filter. Using a purified, nick-translated 0.7-kilobase (kb) internal *Eco*RI fragment of pNSBP544 as a probe, DNA–DNA hybridization experiments were performed as described (26).

DNA Sequencing and Mung Bean Nuclease Mapping. DNA sequencing was performed according to Maxam and Gilbert (27). The 5′ end of the crystal toxin RNA was determined using mung bean nuclease transcript mapping (28). A 737-base-pair (bp) *Pst* I/*Hin*dIII fragment (end-labeled at the *Pst* I terminus) was used as the probe. Total RNA (30 μg of each) isolated from early stationary (2 hr after the onset of stationary phase, stage II of sporulation) and midstationary (7 hr into stationary phase, stage III-IV) *B. thuringiensis* var. *tenebrionis* cells (29) were used in the mapping experiments.

RESULTS

Cloning of the Crystal Protein Gene. *Bam*HI fragments of var. *tenebrionis* total DNA were ligated into pUC12 and transformed into *E. coli* JM83. When over 600 possible recombinants were probed with a ^{32}P-labeled oligonucleotide specific to the crystal toxin gene of var. *tenebrionis*, 14 colonies hybridized. Insect bioassays on extracts of these 14 clones indicated that one clone (*E. coli* NSBP544) exhibited toxicity to Colorado potato beetle larvae at a level comparable to that of the insecticidal protein of var. *tenebrionis* (Table 1).

Plasmid pNSBP544 contained a 5.9-kb *Bam*HI insert fragment (Fig. 1*A*). Subclones were constructed to partially define the coding region of the crystal protein. *E. coli* 544Bam(+)SC and pNSBP544 contain the *Bam*HI fragment (in pIC20H and pUC12, respectively) in the same orientation with respect to the *E. coli lacZ* gene. *E. coli* 544Bam(−)SC harbors the insert in the opposite orientation. *E. coli* 544HindSC, shown in Fig. 1*B*, was constructed by cloning the 3.0-kb *Hin*dIII fragment of pNSBP544 into pIC20H.

DNA Sequencing and 5′ End Mapping. The nucleotide sequence for positions 601 to 3000 is shown in Fig. 2. An open reading frame (ORF) extends from nucleotide (nt) 817 to 2775. However, the methionine codon at position 841 is most likely the initiation methionine because of a potential ribosome binding site (with a calculated free energy of base pairing to the 16S rRNA of *Bacillus subtilis* of −21.3 kcal) prior to the ATG at nt 823–832. This ORF encodes a peptide of 644 amino acids corresponding to a molecular mass of 73,119 daltons. Comparison (not shown) of the deduced amino acid composition of the cloned crystal protein shows good agreement with the reported composition of var. *tenebrionis* (14).

Table 1. Effect of insecticidal protein on *L. decemlineata* larvae

Treatment	Dosage: μg of protein per cm²	Dosage: ng of IP per cm²	% mortality
Buffer		0	5
E. coli pIC20H	36	0	0
	3.6	0	0
E. coli pIC20H + IP*	36	1800	80
	3.6	180	47
	0.36	18	23
	0.036	1.8	3
E. coli pNSBP544	36	1800	78
E. coli 544Bam(+)SC	36	1800	76
	3.6	180	43
	0.36	18	13
E. coli 544Bam(−)SC	36	180	42
	3.6	18	3
	0.036	1.8	7
E. coli 544HindSC	36	1800	79

Leaves treated with 3 mg of protein per ml contained an estimated 36 μg of protein per cm². The amount of insecticidal protein (IP) was estimated by ELISA analysis. Mortality was scored 2 days after applying *L. decemlineata* larvae. The results are the average of at least three trials with a minimum of 10 larvae per trial.
**E. coli* pIC20H + IP was obtained by adding solubilized IP to crude *E. coli* extract.

The 5′ end of the crystal protein RNA transcript in early sporulating (stage II) and midsporulating (stage III-IV) *B. thuringiensis* var. *tenebrionis* was mapped (Fig. 3). A single transcriptional start site located 130 bp prior to the initiation ATG was found to be utilized in both sporulation stages. However, two bands are seen at this position, indicating some heterogeneity in initiation.

At the 3′ end of the coding sequence, an inverted repeat is located (Fig. 2). An RNA transcript corresponding to this region will presumably fold into a stem-loop structure with a predicted $\Delta G = -28.4$ kcal. Wong and Chang (30) found that a similar sequence distal to a var. *kurstaki* crystal protein gene terminates transcription and enhances expression of the upstream gene.

Immunoblot Analysis. Immunoblot analysis of purified crystal inclusions of *B. thuringiensis* var. *tenebrionis* revealed the presence of a major crystal antigen of 65 kDa (Fig. 4, lane 2). When a higher concentration of the crystal protein sample was subjected to electrophoresis, the presence of additional minor peptide components of 73 and 56 kDa became evident (Fig. 4, lane 1). Sporulating cells of var. *tenebrionis* (stage III-IV), on the other hand, were found to produce a major crystal antigen of 73 kDa and a minor component of 65 kDa (Fig. 4, lane 3).

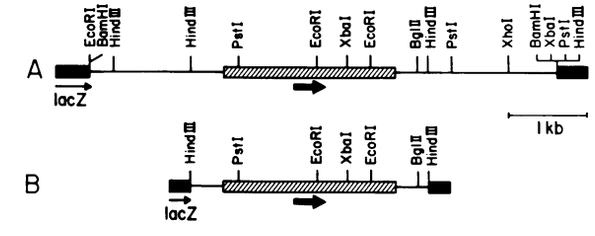

FIG. 1. Partial restriction maps of pNSBP544 (*A*) and p544-HindSC (*B*). Bold arrows show the direction of the crystal protein transcription. Hatched bars indicate crystal protein coding DNA. Solid bars indicate vector.

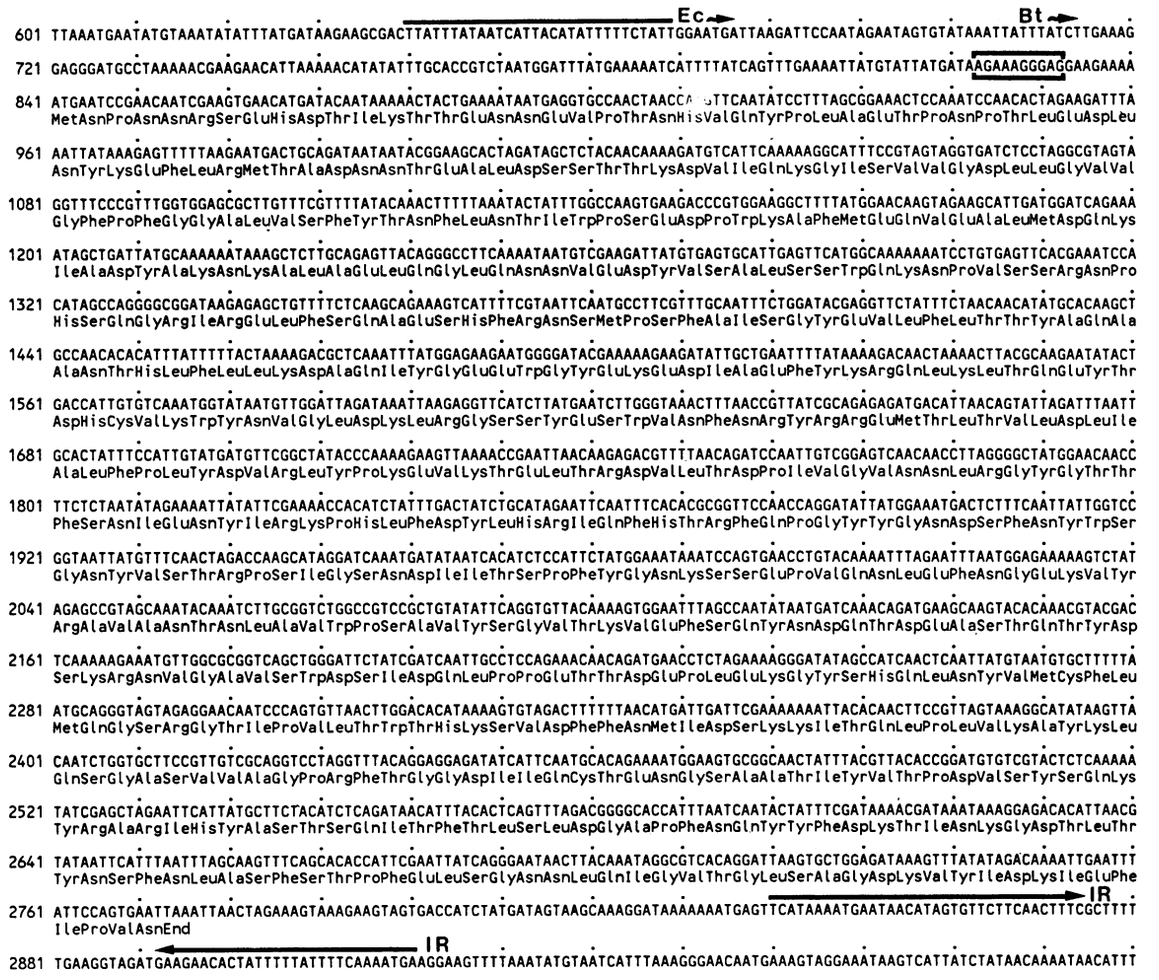

FIG. 2. Nucleotide sequence of 2400 bp of var. *tenebrionis* DNA encoding the 73-kDa crystal protein. The deduced amino acid sequence for the ORF is presented underneath the nucleotide sequence. The A+T-rich region upstream from the site of RNA initiation is overlined. The putative Shine–Dalgarno sequence is boxed. Wavy arrows show the position of transcription initiation in *B. thuringiensis* (Bt) and *E. coli* (Ec) as determined by mung bean nuclease mapping. An inverted repeat (IR) beyond the 3′ end of the gene is shown by arrows.

Analysis of the total soluble proteins of *E. coli* NSBP544 showed that this clone produced 65- and 73-kDa crystal antigens (Fig. 4, lane 5). Although both peptides were present irrespective of the orientation of the 5.9-kb *Bam*HI insert with respect to the *lacZ* gene of the vector plasmid, the concentration of both antigenic peptides was greatly reduced in the *E. coli* 544Bam(−)SC extract (i.e., in which the orientation of the insert was opposite to the *lacZ* promoter of the vector; Fig. 4, lane 6). We have estimated by ELISA experiments that the concentration of the crystal antigens in

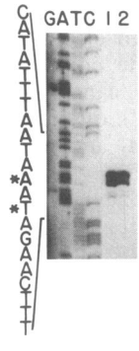

FIG. 3. Mung bean nuclease mapping of the transcriptional start point used in *B. thuringiensis* var. *tenebrionis* for the crystal protein gene. A 737-bp *Hin*dIII–*Pst* I fragment (5′ end-labeled at the *Pst* I terminus) was used as a probe. Base-specific chemical cleavages are shown in the lanes G, A, T, and C. Lanes 1 and 2, *B. thuringiensis* var. *tenebrionis* RNA samples isolated at early and midsporulation stages, respectively. The sequence complementary to positions 692–725 of Fig. 2 is shown at left. *, Transcription start site.

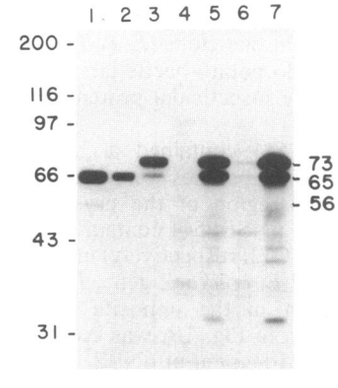

FIG. 4. Immunoblot analysis. Various *E. coli* transformants were tested by immunoblot analysis. Lanes: 1, 250 ng of var. *tenebrionis* crystal protein; 2, 50 ng of crystal protein; 3, 25 μg of protein from sporulating (stage III-IV) cells of var. *tenebrionis*. Total protein (50 μg of each) from each of the following samples were analyzed in lanes 4–7. Lanes: 4, *E. coli* pIC20H; 5, *E. coli* pNSBP544; 6, *E. coli* 544Bam(−)SC; 7, *E. coli* 544HindSC. Protein standards (in kDa) are shown at left. Major antigenic polypeptides and their apparent sizes are indicated at right.

E. coli NSBP544 is ≈5% and that in the Bam(−) subclone is 0.5% of the total protein. In addition, the subclone *E. coli* 544-HindSC, which contains the crystal protein gene flanked by 588 nt at the 5′ end and 480 nt at the 3′ end, expresses the 73- and 65-kDa peptides at a level equivalent to the parental *E. coli* pNSBP544 strain (Fig. 4, lane 7).

Southern Blot Analysis. Slot-lysis electrophoretic analysis of *B. thuringiensis* var. *tenebrionis* revealed the presence of at least five different plasmids (Fig. 5*A*, lane 5). By comparison to the marker *B. thuringiensis* HD2 strain (31), the sizes of the plasmids of var. *tenebrionis* were determined to be approximately 105, 90, 58, 56, and 9.5 MDa. Using the internal 0.7-kb *Eco*RI fragment of pNSBP544 as a probe, hybridization was observed only to var. *tenebrionis* DNA, primarily to the 90-MDa plasmid (Fig. 5*B*, lane 5). No hybridization was seen to any plasmid or chromosomal DNA of vars. *thuringiensis*, *kurstaki*, and *israelensis* (Fig. 5*B*, lanes 1–4). Using pNSBP544 as a probe, however, hybridization was seen to HD1 and HD73 plasmids but not to HD2 or HD567 plasmids (data not shown). Purified total and plasmid DNA preparations from var. *tenebrionis* that were digested with *Bam*HI and *Hin*dIII were hybridized with the 0.7-kb *Eco*RI fragment. An identical hybridization pattern was observed for both total and plasmid DNA samples. In addition, hybridization to only one fragment of the predicted size was found in each digest (Fig. 5*C*).

DISCUSSION

The insecticidal crystal protein gene of the coleopteran-toxic *B. thuringiensis* var. *tenebrionis* has been isolated, and the nucleotide sequence has been determined. This crystal protein gene is contained on a 5.9-kb *Bam*HI fragment. A subclone containing the 3-kb *Hin*dIII fragment from pNSBP544 was constructed. This *Hin*dIII fragment contains an ORF that encodes a 644-amino acid polypeptide of 73,119 Da. Extracts of both subclones exhibited toxicity to larvae of Colorado potato beetle at a level comparable to that of the insecticidal protein of var. *tenebrionis* (Table 1). They produced 73- and 65-kDa peptides that cross-react with an antiserum against the crystal protein of var. *tenebrionis* (Fig. 4). Sporulating var. *tenebrionis* cells contain an immunoreactive 73-kDa peptide that corresponds to the expected product from the ORF of pNSBP544 (Fig. 4). However, isolated crystals primarily contain a 65-kDa component. Several explanations for this difference are possible. The 65-kDa peptide may result from proteolytic cleavage of the 73-kDa peptide in sporulating cells prior to crystal formation. Alternatively, proteases may be degrading the subunits after the crystal is released from the mother cell. This could occur either in the culture medium or during subsequent crystal purification. Bernhard (14) showed that var. *tenebrionis* crystals harbor proteases that are released during solubilization. Further experiments will be required to determine the events that generate the 65-kDa component from the 73-kDa translation product.

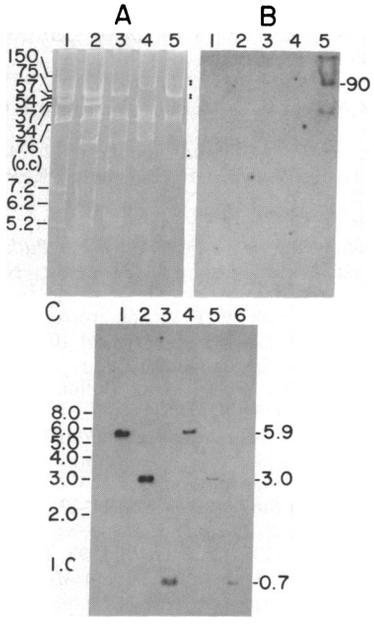

FIG. 5. Southern blot analysis. (*A*) Plasmid DNA of several *B. thuringiensis* strains was separated on a 0.6% agarose gel. (*B*) A filter containing the plasmid DNA samples was probed with ^{32}P-labeled 0.7-kb internal *Eco*RI fragment. Lanes 1–5 contain the following *B. thuringiensis* strains. Lanes: 1, *thuringiensis* HD2 (the molecular masses, in MDa, of the plasmids of this strain are shown at left); 2, *kurstaki* HD1; 3, *kurstaki* HD73; 4, *israelensis* HD567; 5, *tenebrionis*. Individual components of the plasmid array of var. *tenebrionis* are indicated by dots, and the size (in MDa) of the presumptive crystal coding plasmid is shown at right. (*C*) Purified total DNA (lanes 1–3) and plasmid DNA (lanes 4–6) preparations obtained from var. *tenebrionis* were digested with *Bam*HI (lanes 1 and 4), *Hin*dIII (lanes 2 and 5) or *Eco*RI (lanes 3 and 6) and probed with the same probe as in *B*. The relative mobilities and the sizes (in kb) of the linear size markers are shown at left. The sizes (in kb) of the fragments exhibiting specific hybridization are shown at right.

Mung bean nuclease mapping was used to locate the transcriptional start point in var. *tenebrionis*. We observe a single start site, located 130 bp upstream from the translational initiator ATG codon, in var. *tenebrionis* during both early and midsporulation stages. This is similar to the results of Waalwijk *et al.* (10) and Ward and Ellar (32) for the 28-kDa gene of *B. thuringiensis* var. *israelensis*, but it contrasts with the results of Wong *et al.* (29), who have found two different initiation sites in *B. thuringiensis* var. *kurstaki* during early and midsporulation stages but only a single site in logarithmic and stationary *E. coli* cells. A search of the region proximal to the RNA start site showed no significant homologies to *B. subtilis* consensus −10 and −35 promoter regions (33). Some homology, 5 bp out of 8 for the −35 region, **GAATGATT**, and 4 out of 8 for the −10 region, **GTATAAAT** (bases in boldface type indicate identity), is present to the PB1 promoter of var. *israelensis* (32) and the BT1 promoter of var. *kurstaki* (29). The var. *tenebrionis* gene has an A+T-rich region (Fig. 2) upstream from the site of RNA initiation. Similar A+T-rich regions are located prior to the *israelensis* (32) and *kurstaki* (29) genes. A preliminary analysis of crystal protein gene transcripts in *E. coli* pNSBP544 using mung bean nuclease mapping indicates that transcription starts approximately 170 bp upstream from the initiation ATG (data not shown). A consensus *E. coli* −10 promoter region (TATAAT) is located near this start.

Using an internal 0.7-kb *Eco*RI fragment of pNSBP544 as a probe, we have concluded that the crystal toxin gene of var. *tenebrionis* is located on a 90-MDa plasmid. Hybridization signals seen at the top as well as at the center of the gel are probably due to open circular and degraded forms, respectively, of the 90-MDa plasmid. The plasmid-borne nature of the crystal toxin gene was confirmed by hybridizing purified total and plasmid DNA preparations digested with *Bam*HI and *Hin*dIII. In these digests, only fragments of the predicted sizes were found to exhibit specific hybridization in both the total as well as the plasmid DNA samples. This result also indicates that, unlike var. *kurstaki* strain HD1, which contains three related but slightly different crystal protein genes (11), var. *tenebrionis* contains only a single crystal protein gene.

The coleopteran gene has little DNA homology with the lepidopteran-active *B. thuringiensis* var. *kurstaki* HD73 or dipteran-active *B. thuringiensis* var. *israelensis* genes by the criteria of Southern hybridization. However, computer anal-

ysis indicates there is 50.2% DNA homology between the var. *tenebrionis* gene and the 5′ 1827 nt of the 3537 nt of var. *kurstaki* HD73 gene. The hybridization of pNSBP544 probe (but not the internal 0.7-kb *Eco*RI fragment probe) to var. *kurstaki* strains may be due to this homology at the 5′ region. When the amino acid sequences of these two proteins are aligned, all matches occur between the complete var. *tenebrionis* protein and the N-terminal 609 amino acids of the var. *kurstaki* HD73 protein. It is this region of the var. *kurstaki* HD73 protein that contains the active 60-kDa toxin (9). Fig. 6 shows the alignment of the var. *tenebrionis* gene product against the first 609 amino acids of the var. *kurstaki* HD73 gene. Overall, there is 22.7% identity in this region. From amino acid 106 to 382 (33.0% homology) and from amino acid 451 to 644 (22.4% homology), the var. *tenebrionis* protein shows similarity to the corresponding residues in the toxin region of the var. *kurstaki* HD73 gene. As shown in Fig. 6, after a 33-amino acid gap is inserted at the start of the var. *kurstaki* HD73 sequence, residues 67–94 of the var. *tenebrionis* protein align with residues 35–70 of the var. *kurstaki* HD73 protein. These regions have a similar predicted hydropathy value. Possible functional roles have been predicted for this region of a lepidopteran-active var. *kurstaki* HD1 protein (18, 35).

Since var. *tenebrionis* and var. *kurstaki* strains like HD73 have distinct host ranges, some of the predicted specific structural features of their crystal protein genes may indeed contribute toward their insecticidal biological function. Further careful comparisons of these genes should lead to a strategy that can direct the modification of the activity of these insecticidal proteins.

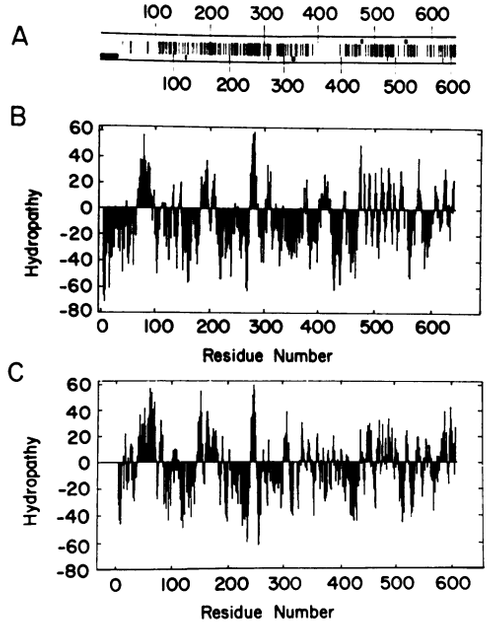

FIG. 6. Alignment and hydropathic profiles of *B. thuringiensis* var. *tenebrionis* and var. *kurstaki* HD73 crystal protein genes. (*A*) Graphic alignment of var. *tenebrionis* (*Upper*) with var. *kurstaki* HD73 (*Lower*) amino acid sequence. Numbering is from the N-terminal methionine. Conserved residues are indicated by vertical bars. Inserted gaps are represented by horizontal bars. Hydropathic profiles of the var. *tenebrionis* (*B*) and var. *kurstaki* HD73 (*C*) proteins were calculated according to Kyte and Doolittle (34) using a window of 7 amino acids. For optimal alignment, hydropathic profiles include the gaps shown in *A*.

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