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Cloning and Characterization of the Crystal Protein-Encoding Gene of *Bacillus thuringiensis* subsp. *yunnanensis*

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Molecular cloning and characterization of a novel *cry* gene, *cry32Aa*, of *Bacillus thuringiensis* subsp. *yunnanensis* was carried out. The Cry32Aa protein was predicted to have a molecular mass of 139.2 kDa and was found to have an unusual 42-amino-acid-long tail at the C terminus. The *cry32Aa* gene was localized on the 103-MDa plasmid of the organism. Bioassays showed no toxicity against several moths and mosquitoes. However, it exhibited weak toxicity against larvae of the diamondback moth, *Plutella xylostella*.

Bacillus thuringiensis is a rod-shaped, ubiquitous, gram-positive bacterium (1, 16). During sporulation, it produces large crystalline parasporal inclusions (Cry proteins), many of which are toxic to certain moths, flies, and beetles. However, ~40% of the crystal forming strains exhibit no known insecticidal activity (16). *Bacillus thuringiensis* subsp. *yunnanensis* belongs to serotype 20ab of the ~60 flagellar serotypes so far identified (M.-M. Lecadet and E. Frachon, XXVIIth Annu. Meet. Soc. Invertebr. Pathol., 1994). In *B. thuringiensis* subsp. *yunnanensis*, spore and crystal formation occurs in separate cells (12). The unique regulation of crystal production in *B. thuringiensis* subsp. *yunnanensis* maintained in each successive generation was found to be mediated by the Cry-encoding 103-MDa plasmid (18). A *B. thuringiensis* strain with similar properties was isolated from certain African soil samples and characterized by Zelazny et al. (24). The presence of the crystal inclusions only in asporogenous cells, the irregular shape of the crystals, and its weak toxicity for the diamondback moth (*Plutella xylostella*) are some of the identifying features of this strain.

In order to gain further molecular and biochemical understanding about this novel Cry protein, the aim of this study was to clone and characterize the *cry* gene of *B. thuringiensis* subsp. *yunnanensis* in *Escherichia coli*.

Construction of a partial genomic library. The total DNA of *B. thuringiensis* subsp. *yunnanensis* was partially digested with *Mbo*I to obtain 9- to 23-kb fragments. The fragments were purified on a sucrose gradient and then cloned between the *Bam*HI-digested λDASHII vector arms (Stratagene Co.). The library amplification and screening of recombinants in the provided XL1-Blue MRA (P2) cells were done according to the manufacturer's instructions.

Cloning of the *cry32Aa* gene. Two heterologous primers, K5UN2 (5′-AGGACCAGGATTTACAGGAGG-3′) and K3UN2 (5′-GCTGTGACAC GAAGGATATAGCCAC-3′), designed on two highly conserved regions of *cry* genes (6) were used to amplify a 2-kb amplicon from *B. thuringiensis* subsp. *yunnanensis* through PCR on a minicycler (MJ Research). The amplicon was cloned into pGEM-T (Stratagene Co.) vector to yield pCRS1. The amplicon was radiolabeled and subsequently used as a probe for screening the library of lambda clones. The positive lambda clones λSBK1 through λSBK6 were digested with various restriction enzymes (*Bgl*II, *Msc*I, *Nco*I, *Pvu*I, *Sal*I, *Sma*I, and *Sph*I), electrophoresed on a 1% agarose gel, and Southern blotted to screen for a suitable insert size. The 5.5-kb *Nco*I fragment from the clone λSBK6 showed up as a single band on Southern hybridization. This was subsequently cloned into pOK12 under the control of the *lacZ* promoter to yield pSDMK1 and transformed into *E. coli* strain DH5α for further characterization of the *cry* gene.

Localization of the gene. The plasmid profile of *B. thuringiensis* subsp. *yunnanensis* was analyzed by vertical slot lysis essentially as described by Gonzalez et al. (5). The agarose gel showed the expected plasmid bands of 103, 91, 61, 52, 45, 4.7, and 3.2 MDa (18). The gel was blotted onto a Hybond N+ nylon membrane, and Southern hybridization was performed by standard procedures described in Bio-Rad's Zeta probe membrane manual. An 862-bp *Sma*I-*Kpn*I internal fragment of the coding region of *cry32Aa* gene from pSDMK1 was used as the probe. The Southern blot revealed that the gene is located on the 103-MDa plasmid of *B. thuringiensis* subsp. *yunnanensis* as predicted earlier by Srinivas et al. (18). The *Bacillus thuringiensis* subsp. *kurstaki* strain HD73 was used as a negative control which did not hybridize with the probe.

Analysis of the *cry32Aa* gene sequence. Sequencing of the 5,589-bp insert in pSDMK1 was performed by Microsynth, Inc., Balgach, Switzerland.

A complete open reading frame composed of 3,711 nucleotides encoding 1,236 amino acid residues was detected within the sequenced region by using the Wisconsin Package (version 9.1; Genetics Computer Group, Madison, Wis.). The protein was predicted to have a molecular mass of 139.2 kDa. A BLAST analysis of the protein revealed close homology to the Cry4 class of proteins and showed nearly 51 and 48% similarity to Cry4Ba and Cry4Aa, respectively. A CLUSTALW comparison (19) of the new Cry protein sequence with several other known Cry protein sequences helped to identify the characteristic eight conserved blocks predicted by Schnepf et al. (16) and

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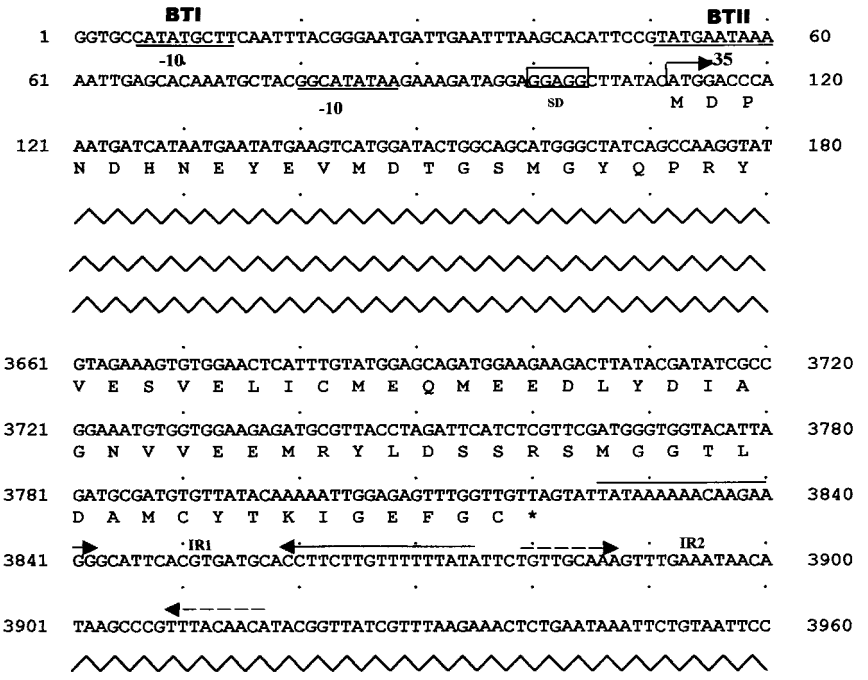

FIG. 1. Positions of the regulatory regions of *cry32Aa*. The promoter region (−10 and −35 sequences) is underlined. The ribosomal binding site is boxed. The deduced amino acid sequence is below the coding region. The terminator region (IR1 and IR2) is indicated with inverted arrows. The initiation codon, ATG (at position 112), and the termination codon, TAG (at position 3822), are indicated with an arrow and an asterisk, respectively.

also an unusual 42-amino-acid tail at the C terminus. Of the two transcriptional start sites (BtI and BtII) defining two overlapping sequentially activated promoters (22) in *cry* genes, we were able to partially characterize a putative BtI region and a complete BtII region in the new *cry* gene represented schematically in Fig. 1. A palindromic sequence (IR1) located downstream of the gene which can form a mRNA hairpin loop with ΔG of −23.04 kcal/mol and a shorter one (IR2) could act as putative factor-independent transcriptional terminators (4, 16, 21). The usual translational termination codon (after the ELICMNE motif that is present at the C terminus of the protoxin in most Cry proteins; ^{1188}ELICMEQ^{1194} in Cry32Aa) was found to be replaced by a methionine residue (^{1195}M), resulting in a 42-amino-acid tail at the carboxy terminus. The fact that this tail was the only significant difference we could find between this protein and the other closely related Cry proteins lends credence to the view that it could somehow be related to the nontoxicity of this protein, which requires further study. The evolutionary divergence of the Cry protein was estimated with phylogenetic tree algorithms as described by Crickmore et al. (3). The protein was identified as a new Cry toxin named Cry32Aa, which was confirmed by the *B. thuringiensis* Pesticidal Crystal Protein Nomenclature Committee (3). The phylogram can be viewed at http://www.biols.susx-.ac.uk/home/Neil_Crickmore/Bt/.

The protein sequence reveals putative proteolytic cleavage sites near the N terminus (R58) and at the C terminus of the toxin (the K613 residue) which are known to be more frequently associated with proteolytic cleavage (2, 11, 16). Hence, the toxin is estimated to have a length of 561 amino acids (position 58 to 618) after proteolytic cleavage, with a predicted molecular mass of 63.5 kDa. A three-dimensional model of the protein was constructed by the unpublished method of Shindyalov and Bourne at the San Diego Supercomputer Center, University of California, San Diego, through the Web-based tool PREDICTPROTEIN. A hydropathic profiling of the protein with the Kyte and Doolittle algorithm (7) did not reveal significant differences from closely related Cry proteins, but analysis of the model with INSIGHTII software (Biosym Technologies, Inc., San Diego, Calif.) revealed that loops 1 and 2 of domain II, which are involved in receptor binding (13, 16), are rich in hydrophilic residues (^{336}DSQDAE^{341} and ^{419}RTDANN^{424}, respectively) instead of the usual hydrophobic residues, which is detrimental to strong receptor binding (14, 16, 17, 23) and may also contribute to the lack of toxicity.

Expression studies on Cry32Aa. The orientation of the 5.5-kb *Nco*I fragment in pSDMK1 was reversed and put under the control of the T7 promoter of pOK12 to yield pPSVS. The transformant *E. coli* strain BL21(DE3) was grown in Luria-Bertani broth (15) at 37°C with shaking and induced with 0.4 mM IPTG (isopropyl-β-D-galactopyranoside) at an optical density at 600 nm of 0.5 to induce Cry32Aa expression. The expression of the Cry32Aa protein in the transformant BL21 (DE3)(pPSVS) was higher than that in DH5α(pSDMK1), where the gene is under the control of the *lacZ* promoter. Total protein extracts from both transformed and untransformed *E. coli* strains were prepared by spinning down the 8-h cultures, suspending the pellet in a suitable quantity of water, and boiling it for 5 min. It was again spun briefly, the supernatant was collected, and the protease inhibitor phenylmeth-

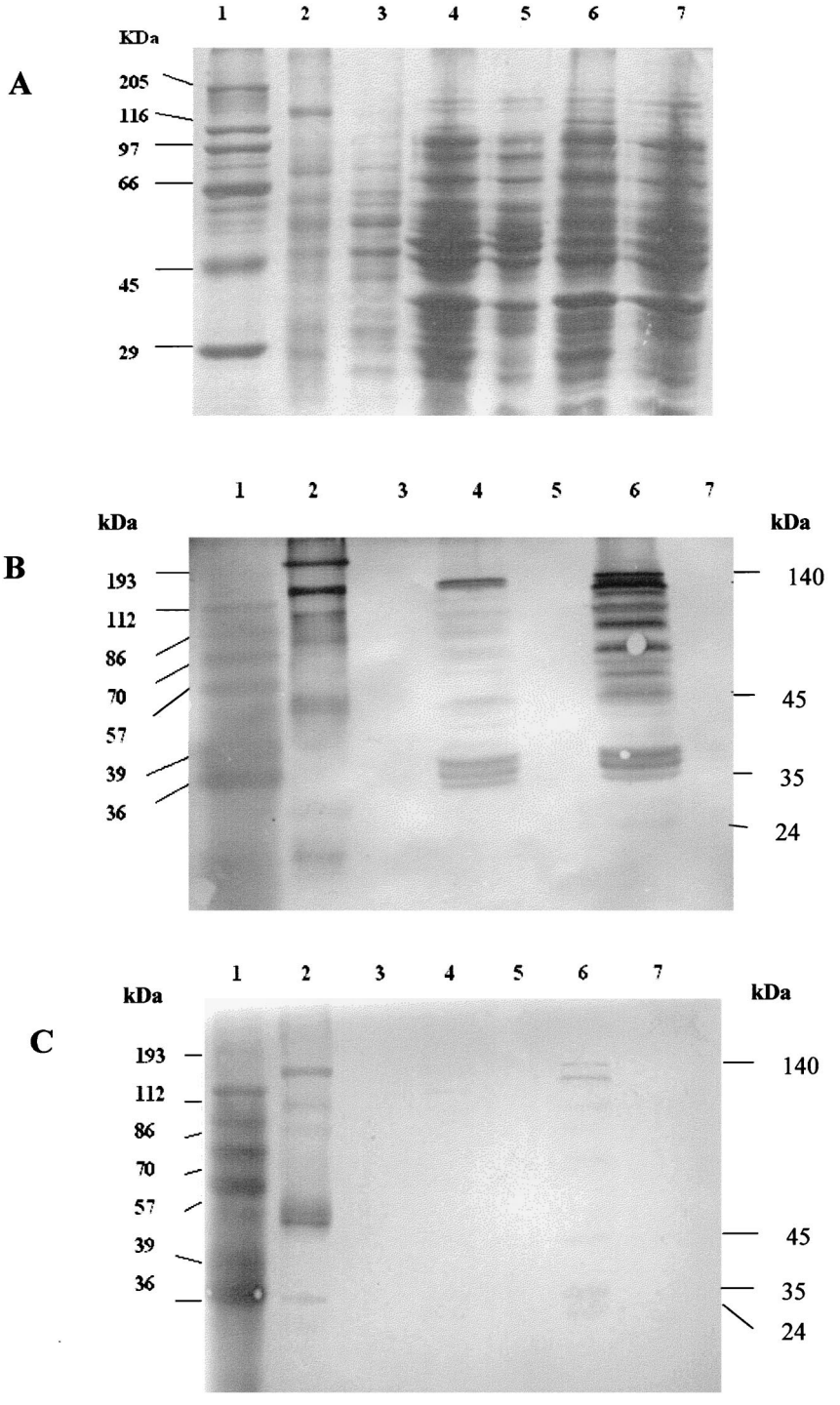

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (A) and Western blot analysis (B and C) of Cry32Aa protein extracts from strains DH5α and BL21(DE3). Immunoblot analysis was performed with the antisera to the 140-kDa (B) and 45-kDa (C) proteins. Lanes: 1, protein marker; 2, solubilized crystal of *B. thuringiensis* subsp. *yunnanensis*; 3, *B. thuringiensis* subsp. *kurstaki* strain HD 73; 4, *E. coli* DH5α(pSDMK1); 5, *E. coli* DH5α; 6, *E. coli* BL21(DE3)(pPSVS) induced with 0.4 mM IPTG; 7, *E. coli* BL21(DE3).

ylsulfonyl fluoride (PMSF; 1 mM) was added to it. Crude protein extracts from *B. thuringiensis* subsp. *yunnanensis* as a positive control and *B. thuringiensis* subsp. *kurstaki* HD73 as a negative control were prepared as described by Murty et al. (10). The crude preparation was solubilized for 3 h at 37°C in 50 mM $NaHCO_3$ supplemented with 10 mM dithiothreitol and 1 mM PMSF for protease inhibition. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8) was performed with the *E. coli* extracts on a 10% polyacrylamide gel (Fig. 2A). The gel was immunoblotted (20) with rabbit antiserum raised against

the 140- and 45-kDa proteins of *B. thuringiensis* subsp. *yunnanensis* to check the expression of the Cry32Aa protein. The immunoblots showed a major 140-kDa reacting band and several smaller cross-reacting bands in the transformant *E. coli* and the positive *B. thuringiensis* subsp. *yunnanensis* lanes for both the antisera used (Fig. 2B and C), even though PMSF was added in all protein preparations. The extensive degradation of Cry32Aa observed in *E. coli* may be due to proteolytic cleavage of the protein in the heterologous host.

Bioassays. Due to the close homology of Cry32Aa to Cry4 and Cry1 classes of proteins, it was thought prudent to check its toxicity against mosquitoes (Diptera) and moths (Lepidoptera). The protein preparation from *B. thuringiensis* subsp. *yunnanensis* was quantified by the method of Lowry et al. (9).

Bioassays against moths (Lepidoptera) were carried out with *B. thuringiensis* subsp. *kurstaki* HD73 as a positive control (1 $\mu g/cm^2$). The solubilization buffer and water were used as negative controls. Bioassays were performed by coating suitable leaves cut to the desired size (young mulberry leaves for silkworm *Bombyx mori* and cotton leaves for the tobacco cutworm *Helicoverpa armigera*) with 50 μg of the preparations per cm^2. Bioassays with *P. xylostella* were done with *B. thuringiensis* subsp. *yunnanensis* toxins as crude, solubilized, and trypsin-digested preparations. For each treatment, five larvae were used and the assays included four replicates. The surfaces of detached mustard leaves were coated with the protein preparations (10 and 25 $\mu g/cm^2$), and 12 such leaves were placed in a petri dish lined with moist filter paper. The mortality of the larvae was scored after 24 and 72 h. The toxin showed weak toxicity only for *P. xylostella* (40 to 55% mortality at 10 $\mu g/cm^2$ and 90% at 25 $\mu g/cm^2$ after 72 h). The positive control strain (HD73) showed very strong toxicity (100% after 24 h) for all moths. Weak toxicity of *B. thuringiensis* B109 for *P. xylostella* larvae has been reported by Zelazny et al. (24).

Toxicity for dipteran insects (mosquitoes like *Culex quinquefasciatus*, *Culex tritaeniorhynchus, Aedes aegypti,* and *Anopheles stephensi*) was assayed by using 50 μg of Cry proteins from *B. thuringiensis* subsp. *yunnanensis* per ml. *B. thuringiensis* subsp. *israelensis* was used as a positive control. The bioassays did not reveal any mortality for any mosquito species.

Nucleotide sequence accession number. The sequence of the 5,589-bp insert in pSDMK1 has been deposited in EMBL/DDBBJ/GenBank with the accession number AY008143.

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