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Cloning and Expression of a Novel Toxin Gene from *Bacillus thuringiensis* subsp. *jegathesan* Encoding a Highly Mosquitocidal Protein

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A gene, designated *cry11B*, encoding a 81,293-Da crystal protein of *Bacillus thuringiensis* subsp. *jegathesan* was cloned by using a gene-specific oligonucleotide probe. The sequence of the Cry11B protein, as deduced from the sequence of the *cry11B* gene, contains large regions of similarity with the Cry11A toxin (previously CryIVD) from *B. thuringiensis* subsp. *israelensis*. The Cry11B protein was immunologically related to both Cry11A and Cry4A proteins. The *cry11B* gene was expressed in a nontoxic strain of *B. thuringiensis*, in which Cry11B was produced in large amounts during sporulation and accumulated as inclusions. Purified Cry11B inclusions were highly toxic for mosquito larvae of the species *Aedes aegypti*, *Culex pipiens*, and *Anopheles stephensi*. The activity of Cry11B toxin was higher than that of Cry11A and similar to that of the native crystals from *B. thuringiensis* subsp. *jegathesan*, which contain at least seven polypeptides.

Most biological control of mosquito larvae relies on the use of two entomopathogenic bacteria, *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*. Both bacteria during sporulation synthesize proteins that assemble into crystals which are toxic for the larvae upon ingestion (for a review, see reference 31). *B. thuringiensis* subsp. *israelensis* crystals are composed of four major polypeptides with molecular masses of 125, 135, 68, and 28 kDa, now referred to as Cry4A, Cry4B, Cry11A, and Cyt1A, respectively (9). Crystals from *B. sphaericus* are composed of only two polypeptides of 51 and 42 kDa. The contribution of each of these proteins to the overall toxicity of the bacteria has been extensively studied: they have different specificities, and most of them interact synergistically (2, 8, 15, 26, 29, 30, 39). The mode of action of these toxins is being investigated.

Identification of novel mosquitocidal toxins that differ in structure and mode of action from those produced by *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* may be of great value. In particular, this could tend to improve in the entomopathogenic properties of these bacteria. New pesticides could be developed, combining in the same organism several genes encoding toxins with different specificities and/or levels of activity. Combining various toxins in one recombinant cell may prevent or delay the onset of resistance. Indeed, there is a report which suggests that the appearance of mosquito resistance to *B. thuringiensis* subsp. *israelensis* toxins is inversely correlated to the number of toxins used for selection, at least in the laboratory (19). Therefore, several programs have been established to isolate and characterize mosquitocidal active *B. thuringiensis* strains.

There have been several reports of other *B. thuringiensis* strains of various serotypes with mosquitocidal activity. These strains can be classed into four groups according to their larvicidal activity, crystal protein composition, and presence of *B. thuringiensis* subsp. *israelensis*-related genes (12, 32): (i) group 1 contains the six *B. thuringiensis* strains, namely, *B. thuringiensis* subsp. *morrisoni* PG14 (H8a8b), *B. thuringiensis* subsp. *canadensis* 11S2-1 (H5a5c), *B. thuringiensis* subsp. *thompsoni* B175 (H12), *B. thuringiensis* subsp. *malaysiensis* IMR81.1 (H36), K6 (autoagglutinating), and B51 (autoagglutinating) with larvicidal activities and crystal polypeptides similar to those of *B. thuringiensis* subsp. *israelensis*; (ii) group 2 includes the two strains *B. thuringiensis* subsp. *medellin* 163-131 (H30) and *B. thuringiensis* subsp. *jegathesan* 367 (H28a28c), which are nearly as toxic as *B. thuringiensis* subsp. *israelensis* but produce different polypeptides; (iii) group 3 comprises the strain *B. thuringiensis* subsp. *darmstadiensis* 73E10-2 (H10a10b), which synthesizes polypeptides different from those found in *B. thuringiensis* subsp. *israelensis* crystals but is active only on one mosquito species; and (iv) group 4 includes the two strains *B. thuringiensis* subsp. *fukuokaensis* fukuokaensis (H3a3d3e) and *B. thuringiensis* subsp. *kyushuensis* 74 F6-18 (H11a11c), which are only weakly active. Because of the poor toxicity of the group 3 and 4 strains, they have not been extensively studied. Only one gene, encoding a cytolysin now designated Cyt2A, has been cloned from *B. thuringiensis* subsp. *kyushuensis*. The Cyt2A protein has limited similarities to Cyt1A (21).

Group 2 strains seem to be the most interesting, and we have focused on *B. thuringiensis* subsp. *jegathesan* 367 isolated from Malaysia (35). It is as toxic as *B. thuringiensis* subsp. *israelensis* on *Anopheles stephensi* but slightly less active on *Aedes aegypti* and *Culex pipiens*. Crystals from *B. thuringiensis* subsp. *jegathesan* contain seven major polypeptides with molecular masses of 80, 70 to 72, 65, 37, 26, and 16 kDa (32). The 37-kDa protein is immunologically related to a *B. thuringiensis* subsp. *israelensis* crystal component, whereas the other proteins give only weak and variable cross-reaction. No *B. thuringiensis* subsp. *israelensis*-related gene has been detected in this strain, indicating that the crystal proteins could be encoded by new classes of toxin genes (32).

We report the isolation and sequence of the gene hereafter referred to as *cry11B* encoding the *B. thuringiensis* subsp. *jegathesan* 80-kDa protein. The corresponding amino acid sequence (Cry11B) is similar to Cry11A (previously CryIVD) from *B. thuringiensis* subsp. *israelensis*. To understand better the contribution of Cry11B to the toxicity of *B. thuringiensis* subsp. *jegathesan* crystals, the larvicidal activity of the Cry11B

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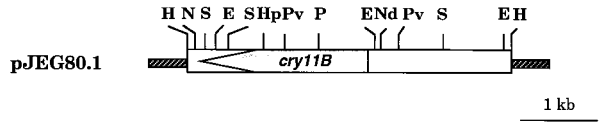

FIG. 1. Restriction map of the recombinant plasmid pJEG80.1 containing the *cry11B* gene. The position and direction of transcription of the *cry11B* gene are indicated. Hatched bar, vector pHT315. Abbreviations: E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nsi*I; Nd, *Nde*I; P, *Pst*I; Pv, *Pvu*II; S, *Sty*I.

protein was determined after expression of the corresponding gene in a crystal-negative *B. thuringiensis* host.

MATERIALS AND METHODS

Bacterial strains and plasmid. *Escherichia coli* TG1 [K-12, Δ(*lac-proAB*) *supE thi hdsD* F′(*traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15)] and pHT315 (3) were used as cloning host and vector, respectively. *B. thuringiensis* subsp. *jegathesan* 367 (from the IEBC Collection of the Unité des Bactéries Entomopathogènes) was used for purifying wild-type crystals and DNA for cloning experiments. *B. thuringiensis* subsp. *thuringiensis* SPL407 (23) was used as a recipient for transformation experiments. The recombinant *B. thuringiensis* subsp. *israelensis* 4Q2-81 (pHT640) was the source of Cry11A inclusions (29).

B. thuringiensis SPL407 was transformed by electroporation (23), and *E. coli* was transformed as previously described (22). The antibiotic concentrations for bacterial selection were 25 μg of erythromycin per ml and 100 μg of ampicillin per ml.

DNA manipulations. Restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were used as described by Sambrook et al. (33) and as recommended by the manufacturers.

Total DNA was isolated from *B. thuringiensis* subsp. *jegathesan* cells as previously described (14). Plasmid DNA was extracted from *E. coli* by a standard alkaline lysis procedure (6) and further purified by using the Qiagen kit. The DNA fragments were purified from an agarose gel with the Prep A Gene DNA purification matrix kit (Bio-Rad, Hercules, Calif.).

Hybridization experiments were performed on Hybond N$^+$ filters (Amersham; Buckinghamshire, United Kingdom). The oligonucleotides were labeled with fluorescein by using the ECL 3′ Oligolabeling system (Amersham).

DNA sequences were determined from alkali-denatured plasmids by the dideoxy chain termination method (34) with a Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio) and α-^{35}S-dATP (>37 TBq/mmol; Amersham). A series of synthetic oligonucleotides (Eurogentec) was used to determine the sequences of both strands.

The Genetics Computer Group sequence analysis software package programs were used (University of Wisconsin, Madison).

Cloning of the *B. thuringiensis* subsp. *jegathesan* toxin gene. The NH_2-terminal sequence of the 80-kDa protein from *B. thuringiensis* subsp. *jegathesan* was determined (Laboratoire de microséquençage des protéines, Institut Pasteur) by using an automatic sequencer model 473 (Applied Biosystems), after transfer of the protein to Problot membranes (Applied Biosystems). The sequence of the 20 first amino acids (MQNNNFNTTEINNMINFPMY) was used to design a degenerate 26-mer oligonucleotide probe, AATAATATGATIAATTTTCCIATG TA. This probe corresponds to the sequence between N (position 12) and Y (position 20); deoxyinosine was used as a neutral base for all threefold or fourfold degenerate positions. To determine the sizes of restriction fragments containing at least the NH_2-terminal region of the gene for the 80-kDa protein, the 26-mer probe was labeled with fluorescein and used in DNA hybridization experiments with restriction enzyme-digested total DNA from *B. thuringiensis* subsp. *jegathesan* 367. The probe specifically hybridized at 42°C to a single *Hin*dIII restriction fragment of approximately 4 kb and to a single *Eco*RI fragment of approximately 2 kb.

Size-selected (3- to 5-kb) *Hin*dIII fragments of *B. thuringiensis* subsp. *jegathesan* 367 DNA were inserted into the *Hin*dIII site of the shuttle vector pHT315 (treated with alkaline phosphatase). Transformed *E. coli* TGI recombinant colonies were tested for hybridization with the labeled probe. The probe hybridized strongly to one colony which contained a plasmid designated pJEG80.1 (Fig. 1).

Protein analysis. *B. thuringiensis* cells were grown in UG medium (11) supplemented with erythromycin when necessary, with shaking at 30°C until cell lysis. Spores and crystals were harvested, washed once in 1 M NaCl and twice in cold double-distilled water. Crystals were further purified on discontinuous sucrose gradients as previously described (36). Crystal protein concentrations were measured by the Bradford assay (Bio-Rad) after alkaline solubilization of the extracts (14).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Thomas and Ellar (36). Proteins were transferred to Hybond-C super membranes (Amersham) and detected immunologically by using the ECL Western Blotting System kit (Amersham), as recommended by the manufacturer.

Mosquitocidal activity assay. Mosquitoes were from colonies of *C. pipiens pipiens* (strain Montpellier), *A. aegypti* (strain Bora-Bora), and *A. stephensi* (strain ST15) reared in the laboratory at 26°C and 80% relative humidity with a 14-10-h day-night photoperiod. Larvae were reared in dechlorinated water and fed with commercial cat biscuits. Purified inclusions were diluted in plastic cups containing 150 ml of deionized water and tested in duplicate against 25 fourth-instar larvae of *A. aegypti* and *C. pipiens* and third-instar larvae of *A. stephensi*. Each bioassay was repeated at least five times. Larval mortality was recorded after 48 h, and 50% lethal concentrations were determined by Probit analysis.

Nucleotide sequence accession number. The nucleotide sequence data in Fig. 2 are available in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number X86902.

RESULTS

Cloning and sequence analysis. The gene encoding the 80-kDa protein from *B. thuringiensis* subsp. *jegathesan* 367 was isolated as follows. The NH_2-terminal sequence of the 80-kDa polypeptide was determined, and an appropriate oligonucleotide was synthesized. A DNA library was constructed in *E. coli* TGI (see Materials and Methods), and positive clones were selected by colony hybridization with the oligonucleotide. The plasmid from one clone (pJEG80.1) was selected and used for further experiments. pJEG80.1 contains a 4.3-kb *Hin*dIII DNA insert, and its restriction map was determined (Fig. 1). The position of the gene on the cloned fragment as well as its direction of transcription were determined by PCR experiments.

The sequence of pJEG80.1 in the region containing the gene encoding the 80-kDa protein (designated *cry11B*) was determined on both strands (Fig. 2). There was one open reading frame encoding a polypeptide of 724 residues with a predicted molecular mass of 81,293 Da. The sequence was searched for any regions that ressembled *B. thuringiensis* promoter structures. No likely promoter sequences were found within approximately 500 nucleotides upstream from the start codon. Downstream from the stop codon (position 2249 to 2282; Fig. 2) inverted repeated sequences that could form a stem-loop structure with a ΔG of −76.9 kJ/mol, calculated according to the rules of Tinoco et al. (37), were identified. This structure may act as a transcriptional terminator. Twelve nucleotides upstream of the start codon, a sequence (AAAGAAGAGGG) that probably acts as a ribosome binding site was found (Fig. 2).

The amino acid sequence of the gene isolated from *B. thuringiensis* subsp. *jegathesan* was compared with those of other known *B. thuringiensis* toxins. It was most similar to *cry11A* from *B. thuringiensis* subsp. *israelensis*: 58% identity between the deduced amino acid sequences (the Cry11A sequence was reported by Donovan et al. [17] and Frutos et al. [18]; Fig. 3). It also shows a low but significant homology (around 15%) with the Cry2 proteins (data not shown).

Five blocks are conserved in all Cry1, Cry3, and Cry4 toxins (20). However, only block 1 was found in Cry11B (Fig. 3), with an alternating arginine region reminiscent of, but not significantly similar to, conserved block 4 (Fig. 3). Cry11B presents a stretch of 82 amino acids containing five cysteine residues at its COOH-terminal part, absent from Cry11A. The protease-sensitive region of Cry11A (10), located in the middle of the protein (amino acids 348 to 357; Fig. 3), is not conserved in Cry11B.

Expression of *cry11B* in a crystal-negative strain of *B. thuringiensis*. Plasmid pJEG80.1 was introduced into the crystal-negative strain *B. thuringiensis* SPL407 to study the toxicity of the Cry11B protein. Recombinant cells 407(pJEG80.1) sporulated normally, and inclusions were visible under a microscope at the end of sporulation. No such inclusions were present in cells containing the vector pHT315 alone.

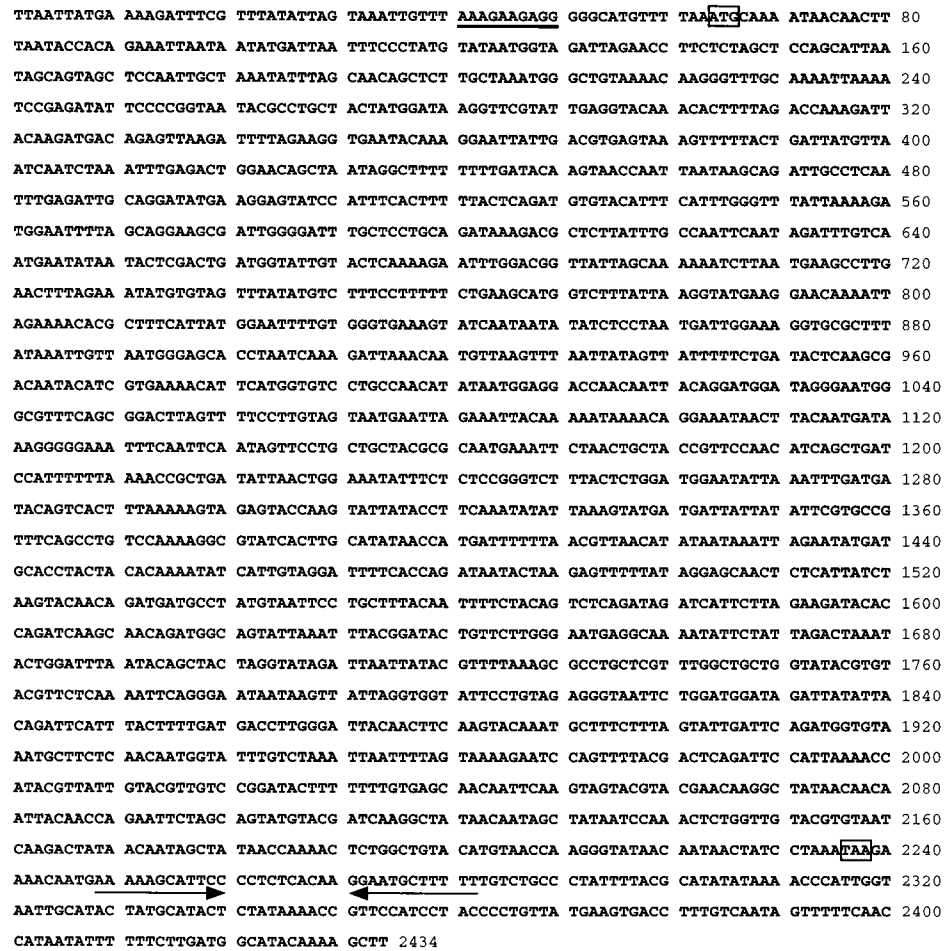

FIG. 2. Nucleotide sequence of the *cry11B* gene. The potential ribosome binding site is underlined. The start and stop codons are boxed. Arrows, inverted repeat sequences outside the gene.

Expression of *cry11B* was further analyzed by SDS-PAGE. Cells of recombinant strain 407(pJEG80.1) and wild-type strain *B. thuringiensis* subsp. *jegathesan* 367 were grown with shaking in UG medium at 30°C until cell lysis. Cells were harvested at the end of sporulation, and inclusions were purified and subjected to SDS-PAGE followed by staining with Coomassie brilliant blue (Fig. 4A). The major polypeptide in inclusions purified from recombinant strain 407(pJEG80.1) was approximately 80 kDa (Fig. 4A, lane 2), the same as the largest polypeptide in the crystals of the toxic wild-type strain 367 (Fig. 4A, lane 1).

The immunological relationships between Cry11B and other mosquitocidal toxins were studied by Western blotting (immunoblotting). Cry11B reacted strongly with a serum directed against total crystal proteins from *B. thuringiensis* subsp. *jegathesan*, as expected (Fig. 4B, lane a2). It also reacted with antisera directed against total crystal proteins from *B. thuringiensis* subsp. *israelensis* (Fig. 4B, lane d2). Interestingly, there was also cross-reaction with proteins from *B. thuringiensis* subsp. *medellin* (Fig. 4B, lane b2), a highly mosquitocidal strain, but not with proteins from *B. thuringiensis* subsp. *darmstadiensis* (Fig. 4B, lane c2), a poorly active strain. Inclusions from strain 407(pJEG80.1) were tested for cross-reaction with antisera raised against Cry4A, Cry4B, Cry11A, and Cyt1A. Antisera directed against Cry4B and Cyt1A gave no signal (Fig. 4B, lanes f2 and h2). In contrast, Cry11B reacted strongly with both anti-Cry4A and anti-Cry11A (Fig. 4B, lanes e2 and g2). Extra bands ranging around 65 to 68 kDa were detected when Cry11B was tested with antisera directed against total proteins from either *B. thuringiensis* subsp. *jegathesan* or *B. thuringiensis* subsp. *medellin* (Fig. 4B, lanes a2 and b2). Same bands were found with anti-Cry11A antibodies (Fig. 4B, lane g2). These bands may correspond to degradation products of Cry11B, still recognized by these antibodies.

Larvicidal activity of Cry11B toxin. Purified inclusions from strain 407(pJEG80.1) were assayed for mosquitocidal activity on larvae of *A. aegypti*, *A. stephensi*, and *C. pipiens*. Crystals purified from the wild-type strain *B. thuringiensis* subsp. *jegathesan* 367 and the recombinant strain 4Q2-81(pHT640), producing only the Cry11A protein, were also tested.

The Cry11B polypeptide was toxic to all three species tested, with greatest activity against *C. pipiens* (Table 1). It was more toxic than the wild-type strain to *A. aegypti* and equally toxic to both *C. pipiens* and *A. stephensi*.

Interestingly, although Cry11B has similarities with Cry11A, it is more toxic: about 7 times more for *A. aegypti* and *A. stephensi* and 37 times more for *C. pipiens.*

DISCUSSION

We report the cloning and characterization of a novel *B. thuringiensis* gene, the *cry11B* gene, from *B. thuringiensis* subsp.

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Cry11B MQNNNFNTTEINNMINFPMYNGRLEPSLAPALIAVAPIAKYLATALAKWAVKQGFAKLKS 60
Cry11A MEDSSLDTLSIVNETDFPLYNNYTEPTIAPALIAVAPIAQYLATAIGKWAAKAAFSKVLS 60

Cry11B EIFPGNTPATMDKVRIEVQTLLDQRLQDDRVKILEGEYKGIIDVSKVFTDYVNQSKFETG 120
Cry11A LIFPGSQPATMEKVRTEVETLINQKLSQDRVNILNAEYRGIIEVSDVFDAYIKQPGFTPA 120

Cry11B TANRLFFDTSNQLISRLPQFEIAGYEGVSISLFTQMCTFHLGLLKDGILAGSDWGFAPAD 180
Cry11A TAKGYFLNLSGAIIQRLPQFEVQTYEGVSIALFTQMCTLHLTLLKDGILAGSAWGFTQAD 180
                                  <-------Block 1------->

Cry11B KDALICQFNRFVNEYNTRLMVLYSKEFGRLLAKNLNEALNFRNMCSLYVFPFSEAWSLLR 240
Cry11A VDSFIKLFNQKVLDYRTRLMRMYTEEFGRLCKVSLKDGLTFRNMCNLYVFPFAEAWSLMR 240

Cry11B YEGTKLENTLSLWNFVGESINNISPNDWKGALYKLLMGAPNQRLNNVKFNYSYFSDTQAT 300
Cry11A YEGLKLQSSLSLWDYVGVSI-PVNYNEWGGLVYKLLMGEVNQRLTTVKFNYSFTNEPADI 299

Cry11B IHRENIHGVLPTYNGGPTITGWIGNGRFSGLSFPCSNELEITKIKQEITYNDKGGNFNSI 360
Cry11A PARENIRGVHPIYDPSSGLTGWIGNGRTNNFNFADNNGNEIMEVRTQTFYQNPNN--EPI 357
                                                  ^^           ^

Cry11B VPAATRNEILTATVPTSADPFFKTADINWKY---FSPGLYSGWNIKFDDTVTLKSRVPSI 417
Cry11A APRDIINQILTA--PAPADLFFKNADINVKFTQWFQSTLY-GWNIKLGTQTVLSSR-TGT 413

Cry11B IPSNILKYDDYYIRAVSACPKGVSLAYNHDFLTLTYNKLEYDAPTTQNIIVGFSPDNTKS 477
Cry11A IPPNYLAYDGYYIRAISACPRGVSLAYNHDLTTLTYNRIEYDSPTTENIIVGFAPDNTKD 473

Cry11B FYRSNSHYLSTTDDAYVIPALQFSTVSDRSFLEDTPDQATDGSIKFTDTVLGNEAKYSIR 537
Cry11A FYSKKSHYLSETNDSYVIPALQFAEVSDRSFLEDTPDQATDGSIKFARTFISNEAKYSIR 533

Cry11B LNTGFNTATRYRLIIRFKAPARLAAGIRVRSQNSGNNKLLGGIPVEGNSGWIDYITDSFT 597
Cry11A LNTGFNTATRYKLIIRVRVPYRLPAGIRVQSQNSGNNRMLGSFTANANPEWVDFVTDAFT 593
                  <-Block 4 ?->

Cry11B FDDLGITTSSTNAFFSIDSDGVNASQQWYLSKLILVKESSFTTQIPLKPYVIVRCPDTFF 657
Cry11A FNDLGITTSSTNALFSISSDSLNSGEEWYLSQLFLVKESAFTTQINPLLK 643

Cry11B VSNNSSSTYEQGYNNNYNQNSSSMYDQGYNNSYNPNSGCTCNQDYNNSYNQNSGCTCNQG 717

Cry11B YNNNYPK 724
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FIG. 3. Comparison of Cry11B and Cry11A protein sequences. Identical amino acids are shaded. Residues which are functionally similar are indicated by colons (accepted conservative-replacement groupings are I, L, V, and M; D and E; Q and N; K and R; T and S; G and A; and F and Y). The regions similar to blocks 1 and 4 present in all Cry1, Cry3, and Cry4 proteins are indicated. Vertical arrows, protease cleavage sites in solubilized Cry11A toxin (10). The last residue on each line is numbered.

jegathesan 367. The *cry11B* gene encodes a protein of 81,293 Da.

The Cry11B protein is similar to the mosquitocidal toxin Cry11A from *B. thuringiensis* subsp. *israelensis*. Indeed it is the only known protein significantly similar to Cry11A, suggesting that these two proteins evolved from a common ancestor. The Cry11B protein also has weak similarity with Cry2 proteins, comparable to that between Cry11A and Cry2 proteins. However, there are substantial differences between Cry11B and Cry11A, particularly in the carboxy-terminal part of the protein. Cry11B harbors a stretch of 82 amino acids including five cysteine residues, which is absent from Cry11A. Bietlot et al. (5) described the importance of such residues in the stability of several δ-endotoxins produced by various *B. thuringiensis* strains. This structure may be essential for crystal formation and insecticidal activity. Mutagenesis may help identify the role of the unique carboxy-terminal part of Cry11B. There are also differences between the regions flanking the *cry11A* and *cry11B* genes. The *cry11A* gene is the second gene of an operon containing two other genes, *p19* and *p20* (1, 16). Although the corresponding polypeptides, P19 and P20, are not essential for expression of *cry11A*, they may act as chaperone proteins to stabilize some of the *B. thuringiensis* subsp. *israelensis* crystal components (8, 16, 38). No such environment was found for the *cry11B* gene: no p20 homolog was found downstream from *cry11B*. However, the DNA fragment cloned in plasmid pJEG80.1 may be too small to contain the start point of another gene. Similarly, no *p19*-related gene was found within the 1 kb upstream from *cry11B*. Instead, 487 bp upstream from *cry11B*, we identified an open reading frame oriented in the opposite direction (data not shown). Comparison of the deduced amino acid sequence (partially determined) with others in the Swiss-Prot data bank revealed similarities with the putative transposase of the insertion sequence IS*240* from *B. thuringiensis* subsp. *israelensis* (13). Two copies of IS*240* flank the *cry4A* gene in subsp. *israelensis* (7), but none was found surrounding *cry11A*, although an IS*231* variant was found downstream from the *p20* gene (1). These insertion elements may account for the dispersion of toxin genes among various *B. thuringiensis* strains. Determination of the total sequence of the element in *B. thuringiensis* subsp. *jegathesan* will help assess whether it is a variant of IS*240* or a novel member of the insertion element family including IS*240*, IS*431* (4) IS*26* (25), IS*51* (28), and IS*6100* (24).

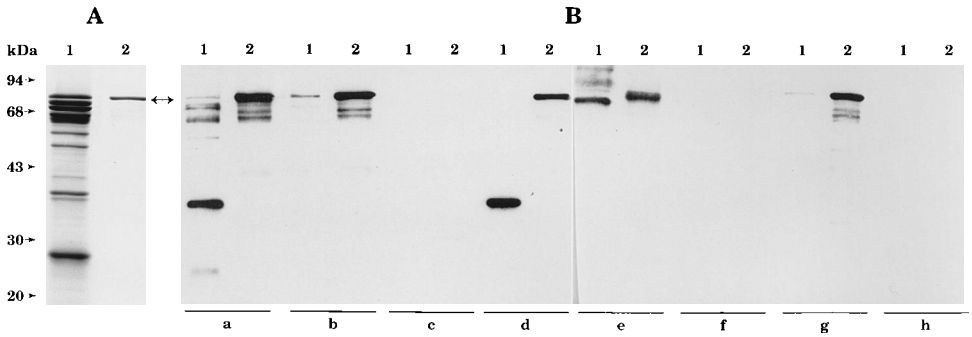

FIG. 4. Protein analysis of Cry11B-containing inclusions. (A) Purified inclusions corresponding to 10 μg of protein were subjected to electrophoresis in SDS–10% polyacrylamide gels and stained with Coomassie brilliant blue. (B) Purified inclusions corresponding to 1 μg of protein were subjected to electrophoresis (as above) and then transferred onto a nitrocellulose filter. The filter was incubated with antiserum (diluted 5,000-fold) raised against either total solubilized crystals from *B. thuringiensis* subsp. *jegathesan* (a), *B. thuringiensis* subsp. *medellin* (b), *B. thuringiensis* subsp. *darmstadiensis* (c), or *B. thuringiensis* subsp. *israelensis* (d) or individual solubilized inclusions composed of Cry4A (e), Cry4B (f), Cry11A (g), or Cyt1A (h). Immunoreactive polypeptides were revealed with a peroxidase-conjugated second antibody (diluted 20,000-fold). Lanes: 1, wild-type *B. thuringiensis* subsp. *jegathesan* 367; lane 2, recombinant 407(pJEG80.1). Arrow between panels A and B, Cry11B. The molecular masses of standard protein markers are indicated on the left.

TABLE 1. Mosquitocidal activity of purified inclusions from *B. thuringiensis* strains

Strain	Inclusion composition	LC_{50} against[a]:		
		A. aegypti	*A. stephensi*	*C. pipiens*
B. thuringiensis subsp. *jegathesan* 367	Wild type	47.4 (41.5–54.2)	54.5 (45.1–99.9)	9.6 (8.6–10.7)
407(pJEG80.1)	Cry11B	18.8 (15.0–23.2)	42.7 (36.0–50.6)	10.1 (7.7–13.1)
4Q2-81(pHT640)	Cry11A	121.5 (96.0–154.0)	326.0 (265.7–393.3)	372.4 (301.5–464.1)

[a] LC_{50}, 50% lethal concentration in nanograms per milliliter after 48 h. The data are averages of five assays (see Materials and Methods) with confidence limits, as determined by Probit analysis, in parentheses.

No data about the regulation of *cry11B* gene expression are currently available. The *cry11A* gene is transcribed from two promoters, recognized by the RNA polymerase associated with the σ^{35} or the σ^{28} factor of *B. thuringiensis* (16). Sequence analysis of the region upstream from the *cry11B* gene did not reveal the corresponding *B. thuringiensis* promoter consensus. Possibly, *cry11B* is transcribed from a promoter recognized by σ factors different from σ^{35} and σ^{28}, or the promoter is located far upstream from the *cry11B* gene, i.e., upstream from the IS*240*-related sequence. Experiments are in progress to determine the transcriptional start site(s).

Cry11B cross-reacted with antibodies directed against Cry11A and Cry4A. Although the *cry11B* and *cry4A* genes do not present any extensive similarity, the corresponding proteins must share similar domain(s). Cry11B also reacted with a serum raised against total proteins from *B. thuringiensis* subsp. *medellin*. Presently, we do not know which polypeptide from *B. thuringiensis* subsp. *medellin* is immunologically related to Cry11B or whether it is also related to Cry11A; previous experiments did not reveal a Cry11A-like polypeptide in this strain (27, 32).

Inclusions composed of the Cry11B protein alone were as toxic as the wild-type inclusions from *B. thuringiensis* subsp. *jegathesan* against *C. pipiens* and *A. stephensi* larvae and were more toxic than the wild type when tested on *A. aegypti* larvae. This is the first report of a single mosquitocidal protein displaying an activity similar to that of a mixture of several polypeptides. In the case of *B. thuringiensis* subsp. *israelensis*, individual polypeptides and even combinations of two or three crystal components are less toxic than the wild-type crystals (2, 15, 30, 39). The high activity of *B. thuringiensis* subsp. *israelensis* is due to synergistic interactions between several crystal polypeptides. For *B. thuringiensis* subsp. *jegathesan*, we cannot exclude such interactions. Indeed, Cry11B is not the major component of the *B. thuringiensis* subsp. *jegathesan* crystals (Fig. 4A). If it were the only active component of wild-type inclusions, one would expect the Cry11B inclusions to be much more toxic than wild-type inclusions. However, the Cry11B inclusions are only one to four times as toxic as wild-type inclusions. Therefore, there must be other polypeptides in the native crystals, presumably either the 65-kDa protein or the 37-kDa protein, or both, responsible for the remaining activity, especially against *A. stephensi* and *C. pipiens.*

Cry11B is much more toxic (7 to 37 times more toxic, depending on the mosquito species tested) than Cry11A, despite the high level of similarity. Further experiments are required to determine the reasons for this higher activity. It may be due to either a differential activation by the midgut proteases or a differential stability involving the additional carboxy-terminal part of Cry11B.

This difference of activity between the two toxins may reflect different modes of action. Therefore, the characterization of putative receptors would be of great value in understanding the mechanism of action of the two toxins. It may be of value in the development of insecticides either in replacing the *cry11A* gene by *cry11B* in a toxic *B. thuringiensis* subsp. *israelensis* strain or in combining both genes in the same strain. Similarly, the *cry11B* gene may also be introduced into a toxic *B. sphaericus* strain.

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