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Expression of *cryIVA* and *cryIVB* Genes, Independently or in Combination, in a Crystal-Negative Strain of *Bacillus thuringiensis* subsp. *israelensis*

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The *cryIVA* and *cryIVB* genes, encoding the 125- and 135-kDa proteins, respectively, of *Bacillus thuringiensis* subsp. *israelensis*, were cloned either alone or together into a shuttle vector and expressed in a nontoxic strain of *B. thuringiensis* subsp. *israelensis*. The CryIVB protein was produced at a high level during sporulation and accumulated as inclusions; in contrast, the CryIVA polypeptide did not form such structures unless it was cloned on a higher-copy-number plasmid. Transcriptional fusions between the *cryIVA* or *cryIVB* gene promoter and the *lacZ* gene were constructed. The poor synthesis of CryIVA was not due to a poor efficiency of transcription from the *cryIVA* gene promoter. Mosquitocidal assays performed with purified inclusions showed that CryIVA was toxic for larvae of the species *Aedes aegypti*, *Anopheles stephensi*, and *Culex pipiens*, whereas CryIVB displayed activity only toward *Aedes aegypti* and *Anopheles stephensi*. The activity of inclusions containing both polypeptides was higher than that of single-peptide inclusions but was not as high as that of the native crystals, which contain at least four polypeptides.

Bacillus thuringiensis subsp. *israelensis*, a gram-positive bacterium, is highly toxic to dipteran larvae such as mosquitoes and blackflies (15, 29), which are vectors of several tropical diseases. The entomocidal activity of *B. thuringiensis* subsp. *israelensis* is due to crystalline inclusions produced during sporulation.

The inclusions of *B. thuringiensis* subsp. *israelensis* are composed of at least four polypeptides of 125, 135, 68, and 28 kDa, now referred to as CryIVA, CryIVB, CryIVD, and CytA, respectively (17). It was previously shown that the toxin genes were located on a 72-MDa resident plasmid (16, 31). The genes encoding the four crystal polypeptides have subsequently been cloned from this high-molecular-weight plasmid (for a review, see reference 22).

Since a transformation system for *B. thuringiensis* strains has become available (reviewed by Lereclus et al. [22]), several studies on the introduction of cloned genes into acrystalliferous or toxic strains of *B. thuringiensis* have been reported. The *cryIVA*, *cryIVB*, and *cryIVD* genes have been introduced separately into crystal-negative strains of *B. thuringiensis* (2, 9), and the toxicity of inclusions containing individual polypeptides has been determined. However, native *B. thuringiensis* subsp. *israelensis* crystals are composed of several polypeptides, and little information about the activity of inclusions made up of different combinations of toxins is available. Moreover, little is known about the expression of *B. thuringiensis* subsp. *israelensis* genes in crystal-negative strains of the same species. This paper reports (i) the expression of the *cryIVA* and *cryIVB* genes in a *B. thuringiensis* subsp. *israelensis* crystal-negative strain and (ii) the activity of CryIVA and CryIVB proteins, assayed either independently or combined in the same inclusion, toward three mosquito species.

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MATERIALS AND METHODS

Strains. *B. thuringiensis* subsp. *israelensis* 4Q2-72 and 4Q2-81 (kindly provided by D. H. Dean, Ohio State University, Columbus) were used as recipient strains for transformation experiments. Strain 4Q2-81 is a crystal-negative strain cured of all resident plasmids, and strain 4Q2-72 is a crystal-producing strain harboring only the 72-MDa plasmid, which encodes all the *B. thuringiensis* subsp. *israelensis* crystal proteins. *Escherichia coli* JM83 [*ara* Δ(*lac-pro*) *strA* ϕ80 Δ*lacZ* ΔM15] was used for plasmid constructions.

B. thuringiensis subsp. *israelensis* was transformed by electroporation (21), and *E. coli* was transformed as previously described (20). The antibiotic concentrations for bacterial selection were 25 μg of erythromycin per ml and 100 μg of ampicillin per ml.

Plasmids. The shuttle vectors pHT3101 and pHT315 (3, 21) were used for cloning experiments; they contain an erythromycin resistance determinant and differ by their copy number (4 and 15 per cell for pHT3101 and pHT315, respectively). The recombinant plasmid pRX70 (5) was the source of the *cryIVA* gene.

Plasmid pRX80 was constructed as follows. An *Sst*I library of the 72-MDa plasmid was established in *E. coli* JM83 with pUC18 as a cloning vector (5). The library was screened with the nick-translated *Eco*RI-*Xba*I fragment containing the 5′ part of the *cryIVB* gene from pRX8 as the probe (6); one recombinant plasmid, pRX80, was identified as positive, and its restriction map was determined (Fig. 1A).

Plasmid pHT611 was constructed by cloning from pRX80 a 4.1-kb partially restricted *Cla*I fragment, made blunt ended with Klenow fragment, into the *Sma*I site of the pHT3101 vector. This fragment contains the *cryIVB* gene (Fig. 1A).

A 4.3-kb partially restricted *Cla*I fragment containing the *cryIVA* gene was purified from plasmid pRX70 (5) and cloned into the *Sma*I site of pHT3101 to yield plasmid pHT601 (Fig. 1A). *Cla*I sites were made blunt with the Klenow fragment of DNA polymerase I. A 4.3-kb *Sst*I-*Sph*I

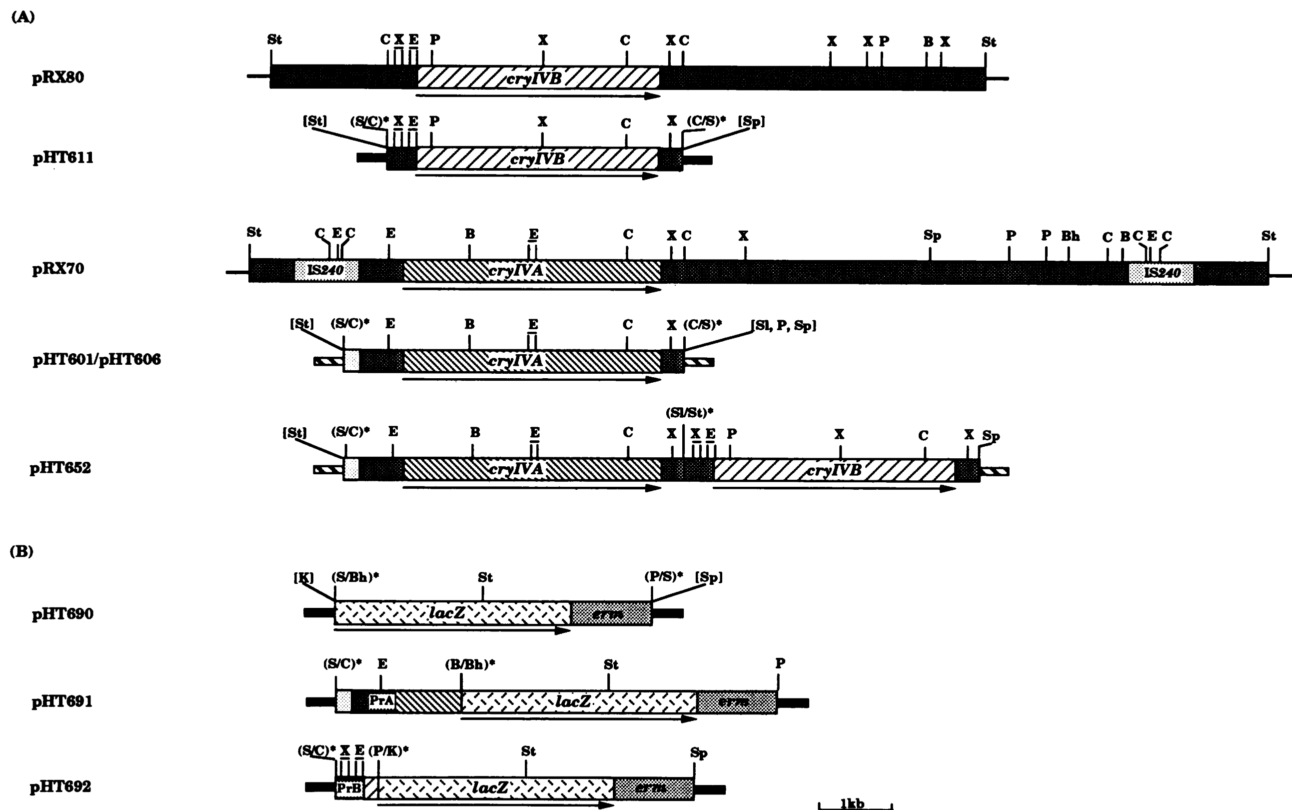

FIG. 1. Simplified restriction maps of the recombinant plasmids containing all or part of the *cryIVA* and *cryIVB* genes. (A) Construction of recombinant plasmids containing the *cryIVA* gene encoding the 125-kDa polypeptide (pRX70, pHT601, and pHT606), the *cryIVB* gene encoding the 135-kDa protein (pRX80, pHT611), or both *cryIVA* and *cryIVB* genes (pHT652). Plasmid pRX70 has been described previously (5). (B) Construction of transcriptional fusions between the *cryIVA* or *cryIVB* gene promoter (represented as PrA and PrB, respectively, on the figure) and the *lacZ* gene of *E. coli*. All plasmids were constructed as described in Materials and Methods. The arrows indicate the positions and directions of transcription of the *cryIVA*, *cryIVB*, and *lacZ* genes. The symbol * indicates that restriction sites have been lost. The vector pUC18 is indicated by a thin line. The vectors pHT315 and pHT3101 are represented by hatched and black lines, respectively; the dotted line represents either pHT315 or pHT3101. Letters in parentheses indicate restriction sites from the vector. Abbreviations: B, *Bgl*II; Bh, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sma*I; Sl, *Sal*I; Sp, *Sph*I; St, *Sst*I; X, *Xba*I.

fragment from plasmid pHT601 was cloned into pHT315 to give plasmid pHT606.

Plasmid pHT652 was obtained by inserting a 4.1-kb *Sst*I-*Sph*I fragment from pHT611 (Fig. 1A) between the *Sal*I and *Sph*I sites of plasmid pHT606. *Sal*I and *Sst*I, which are sites from the vector, were made blunt with Klenow fragment and T4 DNA polymerase, respectively.

Plasmid pHT690 was obtained by cloning a 4.5-kb *Bam*HI-*Pst*I fragment containing the *lacZ* and *erm* genes from pMC11 (12) into the single *Sma*I site of pHT3101 (Fig. 1B). *Bam*HI and *Pst*I sites were made blunt with Klenow fragment and T4 DNA polymerase, respectively.

Plasmid pHT691 was constructed as follows. The 4.5-kb *Bam*HI-*Pst*I fragment of pMC11 was cloned between the *Bgl*II and *Pst*I sites of pHT601, thus eliminating part of the *cryIVA* gene. Plasmid pHT691 contains a transcriptional fusion between the *cryIVA* gene promoter and the *lacZ* gene.

Plasmid pHT692 was constructed by replacing the 3.4-kb *Pst*I-*Sph*I fragment, containing the *cryIVB* gene from plasmid pHT611, with the 4.5-kb *Kpn*I-*Sph*I fragment from plasmid pHT690. *Kpn*I and *Pst*I sites were made blunt with T4 DNA polymerase. Plasmid pHT692 contains a transcriptional fusion between the *cryIVB* gene promoter and the *lacZ* gene.

DNA manipulations. Protocols for restriction enzyme digestions and use of DNA polymerase large fragment (Klenow fragment), T4 DNA polymerase and T4 DNA ligase were carried out as described by Sambrook et al. (25). All enzymes were used as recommended by the manufacturers.

Plasmid DNA was isolated as previously described (4, 18), except that *B. thuringiensis* subsp. *israelensis* cells were incubated in 25 mM Tris-HCl (pH 8)–10 mM EDTA–20% (wt/vol) sucrose–2 mg of lysozyme per ml for 1 h at 37°C and in 0.15 N NaOH–0.7% (wt/vol) sodium dodecyl sulfate (SDS) for 30 min at 50°C.

Protein analysis. *B. thuringiensis* cells were grown in HCT medium (19), supplemented with antibiotics as appropriate, with shaking at 30°C until the cells lysed. Spores and crystals were harvested and treated as described for purification of crystals (14). Crystal protein concentrations were measured by the Bio-Rad assay after solubilization of the extracts (14). SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Thomas and Ellar (28).

β-Galactosidase assays. *B. thuringiensis* cells containing *lacZ* fusions were grown in HCT medium. Cultures were concentrated 10-fold in Z buffer (24) and disrupted by ultrasonic disintegration (for 3 min with a Branson sonifier at 30% duty cycle). Cell debris was eliminated by centrifugation at 8,000 $\times$ g for 15 min, and the β-galactosidase activity in the supernatant was determined (24). The β-galactosidase

specific activity was expressed in Miller units per milligram of protein. The values indicated represent averages from three independent assays. *B. thuringiensis* colonies expressing *lacZ* fusions were detected by plating on HCT medium containing 250 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml.

Mosquitocidal activity assays. Purified crystals were diluted in glass petri dishes containing 10 ml of deionized water and 0.5 mg of yeast extract and tested against larvae of *Aedes aegypti* (fourth instar), *Anopheles stephensi* (third instar), and *Culex pipiens* (fourth instar). Mortality was scored after 24 h of incubation at 27°C. Each sample was independently assayed five times in duplicate, and both the LC_{50}s and LC_{90}s (concentration of crystal protein giving 50% and 90% mortality, respectively) were determined by Probit analysis.

RESULTS

Expression in *B. thuringiensis* subsp. *israelensis* of the *cryIVA* and *cryIVB* genes cloned on a low-copy-number plasmid. The *cryIVA* and *cryIVB* genes were introduced into *B. thuringiensis* subsp. *israelensis* to study the involvement of the encoded polypeptides, the 125- and 135-kDa proteins, in the overall toxicity of the *B. thuringiensis* subsp. *israelensis* crystals. The *cryIVA* and *cryIVB* genes were inserted separately into the low-copy-number vector pHT3101, which is able to replicate in *B. thuringiensis*. The resulting recombinant plasmids pHT601 and pHT611 (Fig. 1A), extracted from *E. coli*, were introduced into the nontoxic, crystal-negative *B. thuringiensis* subsp. *israelensis* 4Q2-81 by electroporation (21). Analysis of upstream and downstream sequences flanking the *cryIVA* and *cryIVB* genes (26) indicates that the *Cla*I fragments cloned into either pHT601 or pHT611 should contain, in addition to the toxin genes, both the putative promoter and terminator of each gene; therefore, these genes should be transcribed from their own promoter in *B. thuringiensis* subsp. *israelensis*.

Recombinant cells 4Q2-81(pHT601) and 4Q2-81(pHT611) sporulated normally, and plasmids pHT601 and pHT611 were maintained. Microscopic observations of recombinant cells containing the *cryIVB* gene revealed the presence of inclusions at the end of sporulation. These inclusions, which were not present in cells containing the vector pHT3101 alone, were smaller than those produced by the toxic strain 4Q2-72. In contrast, no inclusion could be seen in 4Q2-81 (pHT601) cells containing the *cryIVA* gene.

Expression of each toxin gene was further analyzed by SDS-PAGE. Cells of strains 4Q2-81(pHT601), 4Q2-81 (pHT611) and 4Q2-72 were grown with shaking in HCT medium at 30°C until cell lysis. Cells were harvested at the end of sporulation, and any inclusions were purified and subjected to SDS-PAGE followed by staining with Coomassie brilliant blue (Fig. 2). Inclusions were purified from strain 4Q2-81(pHT611); they contained a major polypeptide of approximately 135 kDa (Fig. 2, lane 3), which has the same electrophoretic mobility as the largest polypeptide in the crystals of the toxic strain 4Q2-72 (lane 1). In contrast, no inclusion containing the CryIVA polypeptide could be purified from strain 4Q2-81(pHT601); however, immunodetection after SDS-PAGE of the polypeptides produced by this strain harvested before cell lysis revealed the presence of a polypeptide of 125 kDa (data not shown). These results indicate that (i) at the end of sporulation, sufficient 135-kDa protein is produced from the *cryIVB* gene to form inclusions, and (ii) the *cryIVA* gene cloned into plasmid pHT601 is expressed but the 125-kDa protein does not crystallize, either because the level of production is too low or because the protein is not capable of spontaneously forming crystals. We therefore tested whether expression from the promoter upstream from the *cryIVA* gene was less efficient than expression from the promoter upstream from the *cryIVB* gene.

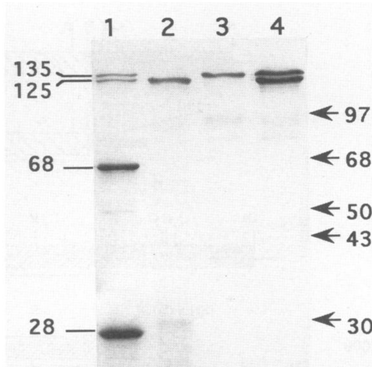

FIG. 2. Protein analysis of inclusions from wild-type and recombinant *B. thuringiensis* subsp. *israelensis* strains. Inclusions corresponding to 10 μg of protein were subjected to electrophoresis on an SDS–10% polyacrylamide gel, which was then stained with Coomassie brilliant blue. Lanes: 1, 4Q2-72; 2, 4Q2-81(pHT606); 3, 4Q2-81(pHT611); 4, 4Q2-81(pHT652). Numbers in the right margin represent molecular masses (in kilodaltons) of standard protein markers.

Efficiency of the *cryIVA* and *cryIVB* gene promoters. Transcriptional fusions with the *lacZ* gene of *E. coli* were constructed in pHT3101 to determine the efficiency of both *cryIVA* and *cryIVB* promoters. The three recombinant plasmids, pHT690, pHT691 and pHT692 (Fig. 1B), were introduced by electroporation into *B. thuringiensis* subsp. *israelensis* 4Q2-81. Cells from strains 4Q2-81(pHT690), 4Q2-81(pHT691), and 4Q2-81(pHT692) were grown in HCT medium at 30°C and harvested at different sporulation stages from t_0 (corresponding to the entry in sporulation) to t_{10} (10 h after t_0). β-Galactosidase produced by recombinant cells was determined by assaying sonic extracts. From t_0 to t_5, there was no β-galactosidase activity in extracts of strain 4Q2-81(pHT692), which contains the *cryIVB* promoter, or of strain 4Q2-81(pHT690), which does not (Fig. 3). In contrast, a significant amount of β-galactosidase was produced by strain 4Q2-81(pHT691). Both strains 4Q2-81(pHT691) and 4Q2-81(pHT692) showed an increase in β-galactosidase activity at t_5, corresponding to midsporulation. This seems to be specific to the crystal gene promoters, since no β-galactosidase activity was detected in extracts from strain 4Q2-81(pHT690).

The level of β-galactosidase activity at t_{10} in strain 4Q2-81(pHT691) was higher than that in strain 4Q2-81(pHT692) (Fig. 3). Therefore, the absence of crystals in strain 4Q2-81(pHT601) is not due to a weak promoter activity but may result from CryIVA instability or a threshold level of the former polypeptide being required for crystal formation; this threshold may not be reached when the gene is expressed on a low-copy-number plasmid (the cloning vector pHT3101 used in this experiment has a copy number of about 4 per equivalent chromosome [3]). To test this hypothesis, the *cryIVA* gene was subcloned onto a higher-copy-number plasmid and introduced into *B. thuringiensis* 4Q2-81.

Use of a high-copy-number plasmid for expression of the

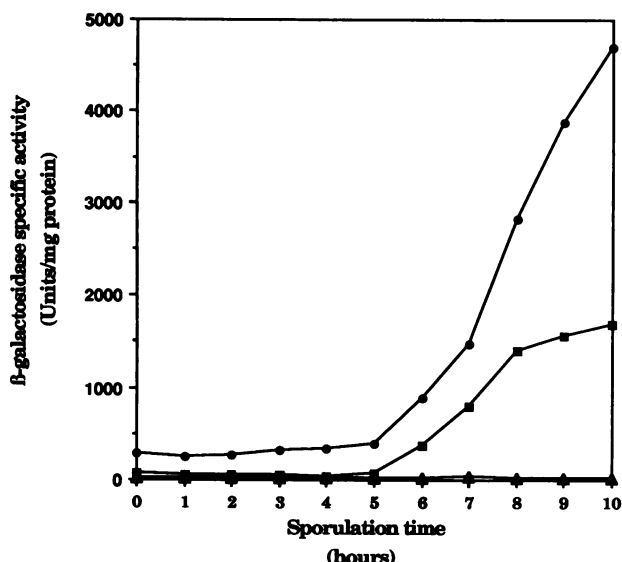

FIG. 3. Time course of *cryIVA* and *cryIVB* expression as measured by *cryIVA'-lacZ* or *cryIVB'-lacZ* transcriptional fusions. β-Galactosidase specific activities in sonic extracts were determined as a function of sporulation time. Symbols: ●, 4Q2-81(pHT691); ■, 4Q2-81(pHT692); ▲, 4Q2-81(pHT690).

***cryIVA* gene alone or in combination.** The *cryIVA* gene was subcloned into plasmid pHT315 to determine whether the vector copy number affects the *cryIVA* gene expression level; this vector is normally present in the cell at a copy number of about 15 per equivalent chromosome (3). The recombinant plasmid thus obtained, pHT606 (Fig. 1A), was introduced by electroporation into *B. thuringiensis* subsp. *israelensis* 4Q2-81.

Microscopic observations of recombinant cells harboring plasmid pHT606 revealed the presence of small inclusions at the end of sporulation. These inclusions were purified, subjected to SDS-PAGE, and stained with Coomassie brilliant blue (Fig. 2). Strain 4Q2-81(pHT606) synthesized a major polypeptide of approximately 125 kDa (Fig. 2, lane 2), which has the same electrophoretic mobility as the lower polypeptide of the 130-kDa protein doublet in the crystals of the wild-type strain 4Q2-72 (lane 1). This result indicates that the increase of vector copy number correlates with a higher-level expression of the *cryIVA* gene and leads to the formation of CryIVA protein-containing inclusions.

To obtain inclusions composed of both CryIVA and CryIVB proteins, which could be tested for their mosquitocidal activity, we subcloned the corresponding genes together into the vector pHT315. The resulting plasmid, pHT652 (Fig. 1A), was introduced by electroporation into *B. thuringiensis* subsp. *israelensis* 4Q2-81, and recombinant cells were tested for their ability to produce inclusions containing both polypeptides. Cells harboring plasmid pHT652 were grown and harvested at the end of sporulation as described for cells containing the *cryIVB* gene. Strain 4Q2-81(pHT652) produced inclusions, and these inclusions were purified and analyzed by SDS-PAGE. Analysis of the Coomassie blue-stained gel showed that these inclusions contained two major polypeptides of 125 and 135 kDa (Fig. 2, lane 4). These polypeptides have the same electrophoretic mobilities as the high-molecular-weight polypeptides in crystals produced by the toxic strain 4Q2-72 (lane 1).

Involvement of the 125- and 135-kDa polypeptides in mosquitocidal toxicity. Purified inclusions from strains 4Q2-72, 4Q2-81(pHT606), 4Q2-81(pHT611), and 4Q2-81(pHT652) were assayed for mosquitocidal activity on larvae of *Aedes aegypti*, *Anopheles stephensi*, and *C. pipiens*.

The CryIVB polypeptide was toxic to *Aedes aegypti* and to a lesser extent to *Anopheles stephensi* larvae (Table 1). It was inactive against *C. pipiens* larvae even at high doses. In contrast, CryIVA was toxic for the three species tested, with the major activity against *C. pipiens*; however, both the LC_{50}s and LC_{90}s determined for *Aedes aegypti* and *Anopheles stephensi* were higher than those of the CryIVB inclusion (Table 1). In all cases, when tested independently, the single-peptide inclusions were not as active as the wild-type crystals.

Inclusions containing both CryIVA and CryIVB were toxic to all three mosquito species. This seems to be the result of a synergistic rather than an additive effect, as discussed below. However, the activity of the crystals composed of both the CryIVA and CryIVB polypeptides was still lower than the toxicity obtained with the native crystals: about 10-fold lower for *Aedes aegypti* and *C. pipiens* larvae, and about 50-fold lower for *Anopheles stephensi*. These results demonstrate that the CryIVA and CryIVB polypeptides, even when present simultaneously, cannot account for the toxicity of the wild-type crystals, at least against the three mosquito species tested.

DISCUSSION

We describe the expression of two toxin genes from *B. thuringiensis* subsp. *israelensis*, namely *cryIVA* and *cryIVB*, in a crystal-negative derivative of the same strain. Both genes were expressed under the control of their own promoters, both alone and in combination. Two plasmids with different copy numbers were used. Inclusions containing either CryIVA or CryIVB or both were obtained and purified from the corresponding *B. thuringiensis* subsp. *israelensis* recombinant strains.

In mosquitocidal assays, crystals containing only the CryIVB protein were active against *Aedes aegypti* and *Anopheles stephensi* larvae but nontoxic for *C. pipiens*. This is consistent with results obtained when testing the cloned gene product from *E. coli* (13). In contrast, the CryIVA protein was active against the three species tested, although the toxicity toward *Aedes aegypti* and *Anopheles stephensi* larvae was lower than that of the CryIVB toxin. Inclusions containing both CryIVA and CryIVB proteins were more toxic to all three species than were the single-peptide inclusions. This higher toxicity appeared to be the result of a synergistic rather than an additive effect. According to Tabashnik (27), the expected LC_{50} of the inclusion containing both CryIVA and CryIVB in the absence of synergism should not be lower than that found for the toxin displaying the highest activity; since inclusions composed of both CryIVA and CryIVB proteins are more toxic than expected on the basis of their individual toxicities, we can conclude that synergistic interaction occurs between the two polypeptides. Despite this synergy between CryIVA and CryIVB, the activity of CryIVA- CryIVB-containing inclusions is still lower than that of the native crystals of *B. thuringiensis* subsp. *israelensis* on all three species of larvae. Thus, CryIVA and CryIVB do not alone account for the toxicity of the wild-type crystals, at least against the three mosquito species tested. As we previously reported, the CytA protein has only a small role in the mosquitocidal activity (14). The

TABLE 1. Mosquitocidal activity of purified crystals from *B. thuringiensis* subsp. *israelensis*

Strain	Crystal composition	Mosquitocidal activity[a] (ng/ml after 24 h) against:					
		A. aegypti		*A. stephensi*		*C. pipiens*	
		LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
4Q2-72	Wild type	11.6 (10.7–12.6)	34.5 (27.3–43.5)	4.9 (4.5–5.4)	16.6 (13.5–20.4)	8.5 (7.8–9.3)	26.4 (21.2–32.8)
4Q2-81(pHT606)	CryIVA	1.6×10^3 [(1.3–2.0) $\times 10^3$]	14.5×10^3 [(8.6–39.7) $\times 10^3$]	7.4×10^3 [(6.6–8.4) $\times 10^3$]	22.3×10^3 [(14.8–33.7) $\times 10^3$]	0.4×10^3 [(0.3–0.5) $\times 10^3$]	4.0×10^3 [(2.4–6.7) $\times 10^3$]
4Q2-81(pHT611)	CryIVB	0.3×10^3 [(0.3–0.4) $\times 10^3$]	1.7×10^3 [(1.2–2.4) $\times 10^3$]	0.55×10^3 [(0.5–0.6) $\times 10^3$]	1.9×10^3 [(1.5–2.5) $\times 10^3$]	>50.0×10^3[b]	ND[c]
4Q2-81(pHT652)	CryIVA + CryIVB	82.6 (73.3–93.0)	0.4×10^3 [(0.3–0.6) $\times 10^3$]	0.3×10^3 [(0.2–0.3) $\times 10^3$]	0.6×10^3 [(0.5–0.8) $\times 10^3$]	63.0 (56.2–70.5)	0.3×10^3 [(0.2–0.3) $\times 10^3$]

[a] Values represent the average of five assays (see Materials and Methods). Numbers in parentheses are 95% confidence limits, as determined by Probit analysis.
[b] At this concentration, only 10% mortality was obtained.
[c] ND, not determined.

presence of the CryIVD protein in the native crystals may therefore be necessary for full activity. The LC_{90}/LC_{50} ratio is characteristic of the kinetics of action of a toxin. For *Aedes aegypti*, the ratios found for CryIVB and native inclusions are similar but differ from that of the CryIVA inclusion; similarly, for *C. pipiens*, CryIVA and wild-type inclusions have different values. In contrast, for *Anopheles stephensi*, ratios obtained with individual or combined toxins are similar. This indicates that the actions of CryIVA and CryIVB could be similar on *Anopheles stephensi* but different on *C. pipiens* and *Aedes aegypti*. Binding experiments performed with CryIVA and CryIVB toxins on the membranes of these mosquito species would be valuable to elucidate their mode of action.

Expression of the *cryIVA* gene cloned on the vector pHT3101, present at about four copies per chromosome, in *B. thuringiensis* did not result in crystal formation. This could have been due to poor promoter activity. To check this hypothesis, we constructed transcriptional fusions between the *cryIVA* or *cryIVB* gene promoters and the *lacZ* gene. Comparison of levels of β-galactosidase produced by strains containing the *cryIVA'-lacZ* or the *cryIVB'-lacZ* fusion gave the following results. (i) Slight expression from the *cryIVA* promoter was detected as early as the beginning of the sporulation phase, probably owing to the presence of the toxin gene promoter, since there was no expression of the *lacZ* gene in the analogous strain without the promoter. (ii) Expression from both *cryIVA* and *cryIVB* promoters increased at the midsporulation stage, indicating that a σ^{28} subunit of RNA polymerase (8) may control the transcription of both *cryIVA* and *cryIVB* genes. Evidence that both promoters are activated at midsporulation is surprising since previous reports showed that crystal genes of *B. thuringiensis* such as *cytA* were transcribed at early sporulation ($t_{1.5}$ to t_4) from a promoter referred to as Bt I (32), which can be recognized by an RNA polymerase associated with a σ^{35} subunit (7). (iii) Expression from the *cryIVA* gene promoter was higher than that from the *cryIVB* promoter. Therefore, the failure of the 125-kDa polypeptide to crystallize is not due to poor transcription of the *cryIVA* gene from its own promoter. Instability and/or a requirement for a threshold of CryIVA protein seems more likely; we suggested that the level of CryIVA obtained when the gene is cloned on a low-copy-number plasmid would not be high enough to allow the formation of inclusions. The finding of CryIVA inclusions in a *B. thuringiensis* strain expressing this gene from a higher-copy-number vector supports this hypothesis. Arantès and Lereclus (3) have described a similar phenomenon: the level of production of CryIIIA toxin from the gene at 15 copies per cell was very much higher than that at four copies per cell.

In native *B. thuringiensis* subsp. *israelensis* 4Q2-72, the *cryIV* genes are located on a 72-MDa plasmid, which is a low-copy-number plasmid. This plasmid contains at least four copies of the 130-kDa-class protein gene, with three copies of the *cryIVB* gene per copy of the *cryIVA* gene (5). Although the *cryIVA* gene is present at a low copy number in this bacterium, the corresponding product is found in the crystals, in contrast to the recombinant strain described above. We can suggest the following. (i) The other polypeptides of wild-type crystals could allow the cocrystallization of the CryIVA protein, even when small amounts of this protein are synthesized. This is supported by the following observation: a recombinant strain containing both *cryIVA* and *cryIVB* genes cloned on the low-copy-number plasmid pHT3101 produced inclusions composed of the two proteins,

suggesting that CryIVB could help crystallization of CryIVA (data not shown). (ii) Regulatory factors encoded by the 72-MDa plasmid could be involved in the expression of the *cryIVA* gene and/or crystallization of the encoded polypeptide. Previous reports (1, 10, 11, 23, 30) have shown that expression of the CytA and CryIIA polypeptides was greatly increased by the presence of proteins that might act as chaperones and protect the toxins from proteolytic degradation before being laid down within the crystalline inclusion. Similar polypeptides may be required for crystallization of the CryIVA toxin.

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