

Mosquito Larvicidal Activity of *Escherichia coli* with Combinations of Genes from *Bacillus thuringiensis* subsp. *israelensis*

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The genes *cryIVA* and *cryIVD*, encoding 134- and 72-kDa proteins, respectively, and the gene for a regulatory 20-kDa polypeptide of *Bacillus thuringiensis* subsp. *israelensis* (serovar H14) were cloned in all seven possible combinations by the *Escherichia coli* expression vectors pT7 and pUHE. The four combinations containing *cryIVA* (*cryIVA* alone, with *cryIVD*, with the 20-kDa-protein gene, and with both) displayed high levels of mosquito larvicidal activity in pUHE. The toxicity of the combination of *cryIVA* and *cryIVD*, with or without the 20-kDa-protein gene, was higher than has ever been achieved with δ -endotoxin genes in recombinant *E. coli*. Fifty percent lethal concentrations against third-instar *Aedes aegypti* larvae for these clones decreased (i.e., toxicity increased) continuously to about 3×10^5 cells ml⁻¹ after 4 h of induction. Larvicidal activities, obtained after 30 min of induction, were lower for clones in pT7 and decreased for an additional 3.5 h. Induction of either *cryIVD* or the 20-kDa-protein gene alone resulted in no larvicidal activity in either pT7 or pUHE20. Cloned together, these genes were slightly toxic in pT7 but not in pUHE20. Five minutes of induction of this combination (*cryIVD* with the 20-kDa-protein gene) in pT7 yielded a maximal mortality of about 40%, which decreased rapidly and disappeared completely after 50 min. *CryIVD* is thus apparently degraded in *E. coli* and partially stabilized by the 20-kDa regulatory protein. Larvicidal activity of the combination of *cryIVA* and *cryIVD* was sevenfold higher than that of *cryIVA* alone, probably because of the cross-stabilization of the polypeptides or the synergism between their activities.

Mosquitoes and blackflies are vectors of many human infectious diseases (37). One of the best biocontrol agents against their larvae is the bacterium *Bacillus thuringiensis* subsp. *israelensis* (serovar H14) (14, 22). Its larvicidal activity is included in polypeptides of a parasporal crystalline body (δ -endotoxin) that is produced during sporulation (8). The use of *B. thuringiensis* subsp. *israelensis* is, however, limited by the low efficacies of current preparations under field conditions (4, 29, 30). The major reasons for the low efficacies of these preparations are (i) sinking to the bottom of the body of water (35), (ii) adsorption onto silt particles and organic matter (27, 32), (iii) consumption by other organisms to which it is nontoxic (5), and (iv) inactivation by sunlight (12, 34). One way to overcome these limitations is by the use of transgenic organisms. Recombinant DNA technology may even enhance the levels of expression of the toxin genes with stronger resident promoters and ribosome-binding sites (RBSs) and appropriately placed enhancers and terminator sequences.

B. thuringiensis subsp. *israelensis* harbors several plasmids ranging in size from 3 to 135 MDa (23). A 75-MDa plasmid is responsible for the mosquitocidal activity; it includes the genes *cryIVA*, *cryIVB*, *cryIVC*, *cryIVD*, and *cytA*, encoding δ -endotoxin proteins 134, 128, 78, 72, and 27 kDa in size, respectively, which are highly expressed during sporulation (15, 17, 33). The gene for a regulatory 20-kDa polypeptide, which is required for efficient production of the structural proteins, is also mapped

on that plasmid (1, 24). The presence of its product (which probably acts as a chaperone) was claimed to raise the levels of CytA, CryIVA, and CryIVD in *Escherichia coli* (1, 41, 46) and in an acrySTALLIFEROUS strain of *B. thuringiensis* (9, 10, 43).

In this study, three of the genes (*cryIVA*, *cryIVD*, and the gene for the 20-kDa protein) were isolated and cloned in all seven possible combinations into two expression vectors with *E. coli* as a host. Some of the combinations displayed high toxicities, which is discussed in this report in light of existing literature. In particular, the synergism between CryIVA and CryIVD, which has never been observed before, is documented.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors. Strain 4Q5 (original code 4Q2-72), a derivative of *B. thuringiensis* subsp. *israelensis* cured of all its plasmids except the 75-MDa plasmid (13), was kindly supplied by D. H. Dean (Bacillus Genetic Stock Center, Columbus, Ohio) and served as the source for this plasmid.

The following *E. coli* strains and plasmids were used: pUC-9 and -19 in strain XL-Blue MRF' (Stratagene, La Jolla, Calif.); pT7-1 and -2 (United States Biochemical, Cleveland, Ohio) in strain BL21(DE3) (38); and pUHE-20 and -24 (University of Heidelberg, Heidelberg, Germany), which are descendants of pDS (19), in strain XL-Blue MRF'. Expression was obtained in the latter two series as follows. (i) pUHE24 contains the T5 RBS (7), and pUHE20 lacks the RBS. Both include two tandem *lac* operators and the early T7 promoter P_{A1}, which utilizes the usual *E. coli* RNA polymerase (19). (ii) pT7-1 and -2 have the strong T7 promoter ϕ 10, which utilizes T7 RNA polymerase and lacks the RBS. On its chromosome, *E. coli* BL21(DE3) contains gene *I* coding for T7 RNA polymerase under the control of the *lacUV5* promoter (38).

Recombinant DNA methods. DNA modification was performed and restriction enzymes were used as recommended by the suppliers (mostly New England BioLabs and United States Biochemical), and cloning was carried out as described by Sambrook et al. (36). Competent cells were prepared and plasmids were isolated by standard procedures. Transformants of *E. coli* XL-Blue MRF' and BL21(DE3) were selected on Luria-Bertani plates containing ampicillin (50 to 100 μ g ml⁻¹). For screening of recombinants in pUC derivatives, X-Gal

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(5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) or MacConkey plates were employed. DNA was analyzed by electrophoresis on horizontal 0.7% agarose slab gels and visualized with ethidium bromide. If necessary, DNA fragments were purified from the gels by electrophoresis onto DEAE-cellulose membranes (36).

(i) **Isolation of large plasmid DNA.** The 75-MDa plasmid DNA was isolated according to the method of Krens and Schilperoord (26) for the Ti plasmids of *Agrobacterium tumefaciens*. *B. thuringiensis* subsp. *israelensis* 4Q5 was grown in Luria-Bertani broth at 30°C. Cells were harvested in the early stationary phase and lysed with sodium dodecyl sulfate (1%) and pronase (0.25 mg ml⁻¹). The pH was raised to 12.2, at which chromosomal DNA but not plasmid DNA denatures, and then was slowly lowered to 8.6. The suspension was brought to 1 M NaCl to precipitate denatured DNA and proteins, and the plasmid in the supernatant was precipitated by polyethylene glycol and purified by centrifugation in a CsCl-ethidium bromide gradient.

(ii) **PCR.** Amplification was carried out with the high-fidelity Vent DNA polymerase (New England BioLabs), which contains 3'-to-5' proofreading exonuclease activity, in a DNA thermal cycler (Hybaid Limited, Teddington, Middlesex, England) for a 17-reaction cycle each. Cycle parameters consisted of 1.2 min at 94°C, 45 s at 47°C, and 4 min at 72°C.

The two primers employed to obtain *cryIVA* were (i) the 37-mer 5'-TCATGCCATGGGGAATCCTTATCAAAAATAAAAATGAA-3', containing a *NcoI* restriction site (boldface nucleotides [nt] 6 to 11), an inserted triplet (GGG) of glycine (nt 11 to 13) for constructing the *NcoI* site after ATG, and an additional 24 nt of the *cryIVA* coding sequence and (ii) the 32-mer 5'-ACTCGGATCCTAATCACTCGTTTCATGCAAATT-3', containing a *BamHI* restriction site (boldface nt 5 to 10) and 22 nt complementary to the coding sequence.

Cloning of *cryIVA*, *cryIVD*, and the 20-kDa-protein gene (Fig. 1). (i) **Cloning into pUHE (Fig. 2; Table 1).** *cryIVA* was removed from pUCE4-A by *NcoI*-*BamHI* of the PCR primers and inserted into the same sites of pUHE24. *cryIVD*, with and without the 20-kDa-protein gene, was removed from the multiple cloning sites (MCS) of pUCE4-DR and pUCE4-D, respectively, by *HindIII*-*Ecl136II* and inserted into *HindIII*-*SmaI*-cut pUHE20. The 20-kDa-protein gene was removed from the MCS of pUCE4-R by *PstI*-*BamHI* and was inserted into the same sites of pUHE20. Three additional gene combinations with *cryIVA* were cloned as follows. The genes in clones pUCE4-DR and pUCE4-D were removed from the MCS by *Ecl136II*-*PstI* and the gene in clone pUCE4-R was removed from the MCS by *BamHI*-*PstI*. Each of these was inserted into pHE4-A downstream *cryIVA* cleaved with *BamHI*-*PstI* (*BamHI* was blunt-ended by the Klenow fragment of DNA polymerase I when ligated with *Ecl136II*, which, unlike *SacI*, produces blunt ends) to yield clones pHE4-ADR, pHE4-AD, and pHE4-AR from *cryIVA*, *cryIVD*, and the 20-kDa-protein gene, *cryIVA* and *cryIVD*, and *cryIVA*, and the 20-kDa-protein gene, respectively.

(ii) **Cloning into pT7.** *cryIVA* was removed from pUCE4-A by *SacI* of the MCS and *BamHI* of the PCR primer and was inserted into the unique *SacI*-*BamHI* sites of pT7-1. The genes in clones pUCE4-D (*cryIVD*) and pUCE4-DR (*cryIVD* with the 20-kDa-protein gene) were similarly removed from the MCS by *SacI*-*HindIII* and inserted into *SacI*-*HindIII*-cut pT7-2 and pT7-1, respectively. The 20-kDa-protein gene was removed from the MCS of pUCE4-R by *HindIII* and inserted into the unique *HindIII* site of pT7-2. Three additional gene combinations with *cryIVA* were cloned as described for pUHE to yield clones pTE4-ADR, pTE4-AD, and pTE4-AR.

Bioassays for mosquito larvicidal activity. The recombinant *E. coli* cells were grown at 37°C in Luria-Bertani broth supplemented with 200 to 300 μ g of ampicillin ml⁻¹ to an optical density of 50 to 70 Klett units (ca. 5×10^8 cells ml⁻¹). Cells were harvested by centrifugation at different times after induction by isopropyl- β -D-thiogalactopyranoside (IPTG) (0.5 mM) and were resuspended in distilled water. Samples were added to 20 third-instar *Aedes aegypti* larvae in disposable cups with 100 ml of sterile tap water, and larvicidal activity was determined after 24 h at 28°C (25). Each bioassay was independently performed at least twice in duplicate. The concentrations of cells which kill 50% of the exposed populations in standard bioassays (LC₅₀s) were determined by probit analyses with duplicate bioassays at each of six doses.

Viability of engineered *E. coli* cells. Expression of cloned genes on pT7 in strain BL21(DE3) can lead to loss of plasmid, accumulation of nonfunctional mutants (38), or loss of CFU (21). Therefore, cultures were sampled just before or during induction and titers were determined for CFU on Luria-Bertani plates with and without ampicillin and IPTG in the top agar.

RESULTS

Cloning of the δ -endotoxin genes into expression vectors. (i)

Isolation of δ -endotoxin genes. *cryIVD* and the gene encoding the 20-kDa regulatory protein were isolated as follows from the 9.7-kb *HindIII* fragment (in pUC9) that also includes *cytA* (1). The fragment (Fig. 1, step 1) was truncated by *BamHI*, and the 6.3-kb remaining fragment (Fig. 1, step 2) was subcloned into *BamHI*-*HindIII*-restricted pUC19. The insert was further truncated by *Clal* to 4.6 kb and subcloned into *BamHI*-*AccI*-

restricted pUC19 (thus forming a *TaqI* site) (Fig. 1, step 3). The unique *HaeII* site (in the middle of *cryIVD*) was cleaved, and two fragments, of 3 and 2.1 kb (Fig. 1, step 4), were isolated. The first (3 kb), which includes about 1.1 kb encoding the N-terminal sequence of *cryIVD*, was reduced by *DraI* to yield a 1.1-kb *DraI*-*HaeII* fragment (Fig. 1, step 5); the second (2.1 kb), including the coding sequence for the rest of *cryIVD* and all of the 20-kDa protein gene, was reduced by *HindIII* to yield a 1.9-kb *HaeII*-*HindIII* fragment (Fig. 1, step 5). The purified fragments were ligated together into the *SmaI*-*HindIII* sites of pUC19 (Fig. 1, step 6). The resultant clone, nicknamed pUCE4-DR, contains the two genes in their original organization with 37 bp upstream only. To obtain each of them separately, pUCE4-DR was cut by *NlaIV* (in the intergenic 281-bp sequence), and the resulting fragments were ligated into the unique *SmaI* site of pUC19: 2 kb with *cryIVD* (yielding clone pUCE4-D [Fig. 1, step 7]) and 1,067 bp with the 20-kDa-protein gene (pUCE-R [Fig. 1, step 8]).

The second toxic gene, *cryIVA*, was amplified by PCR. The 3,568-bp, blunt-ended fragment thus obtained was inserted into the unique *SmaI* site of pUC19 to yield clone pUCE4-A (Fig. 1, item 9).

The final four clones contain the genes for *cryIVD* and the 20-kDa protein, singly or together, and *cryIVA* (Fig. 1, steps 7, 8, 6, and 9, respectively).

(ii) **Cloning for expression.** The three genes and their combinations were cloned in two different expression vectors behind strong promoters, as follows (Fig. 2; Table 1; Materials and Methods).

pUHE24 was exploited to clone *cryIVA* alone and in all three possible combinations with *cryIVD* and the 20-kDa-protein gene (each with its original RBS). In addition, *cryIVD* and the 20-kDa-protein gene, together and separately, were cloned with their original RBSs into pUHE20.

pT7-1 was used to clone *cryIVA* alone and in combinations with *cryIVD* and the 20-kDa-protein gene. *cryIVD* and the 20-kDa-protein gene were cloned on pT7-2, each with its original RBS, and their combination was cloned on pT7-1.

Mosquito larvicidal activity of the recombinant clones. Very high mortality (98%) of *A. aegypti* larvae was obtained in a standard bioassay 45 min after induction of *cryIVA* in pHE4-A, and mortality remained that high during the 4 h of follow-up induction (Fig. 3). The expression of *cryIVA* in pTE4-A yielded a much lower larvicidal activity (75% mortality but with 20-fold-higher cell concentrations). With longer induction times, mortality decreased slightly and seemed to stabilize at about 50%.

The highest mortalities (90 to 95%) were obtained 4 h after induction of pHE4-AD and pHE4-ADR, but with much lower cell concentrations (Fig. 4A). The toxicities of these clones continuously increased during the 4 h and were almost twice as high as those of pHE4-A and pHE4-AR, respectively. The toxicity of pHE4-AR was similar to that of pHE4-A during the 4 h (reaching mortalities of 50 to 55%), demonstrating that the 20-kDa protein does not raise the toxicity of *CryIVA*.

Similar toxicities of the same clones in pT7 (Fig. 4B) required about 40-fold more cells. Mortalities resulting from pTE4-AD and pTE4-ADR were maximal after 30 min of induction (ca. 75%), about 1.5-fold higher than those obtained with pTE4-A and of pTE4-AR (Fig. 4B). They continuously decreased during at least 3.5 h, to reach about 40% for the first two clones and 10% for the latter (percentages calculated after subtracting the mortality for the control).

LC₅₀s decreased (i.e., toxicities increased) about 2.5-fold for the clones in pUHE during the 4-h induction and increased in pT7 about 2.7-fold (Table 2). The high toxicities of pHE4-AD

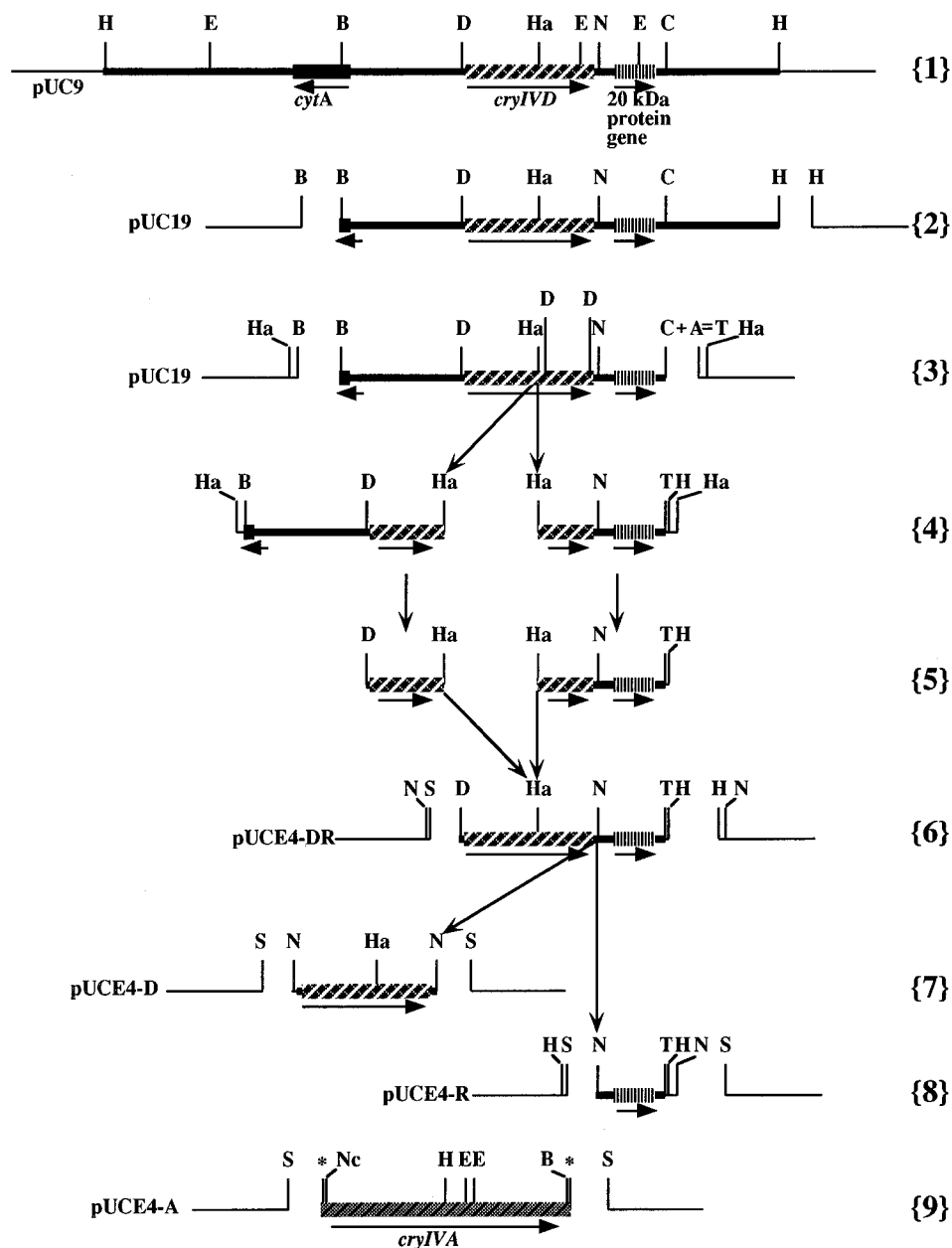


FIG. 1. Cloning the genes for CryIVA, CryIVD, and the 20-kDa protein in pUC vectors. *cryIVD* (step 7) and the 20-kDa-protein gene (step 8) were isolated from a 9.7-kb *Hind*III fragment (step 1) of the 75-MDa plasmid of *B. thuringiensis* subsp. *israelensis*; *cryIVA* (step 9) was amplified by PCR. Restriction enzyme recognition sites: H, *Hind*III; B, *Bam*HI; S, *Sma*I; *, blunt end of PCR product. In addition, for clarity, not all restriction sites are shown for the enzymes designated as follows: E, *Eco*RI; D, *Dra*I; Ha, *Hae*II; N, *Nla*IV; C, *Cl*aI; A, *Acc*I; T, *Taq*I; Nc, *Nco*I.

and pHE4-ADR (LC_{50} s of about 3×10^5 cells ml^{-1} against third-instar *A. aegypti* larvae after 4 h of induction) were about 45-fold higher than those of pTE4-AD and pTE4-ADR (LC_{50} s of about 1.3×10^7 cells ml^{-1} after 30 min of induction) and 5- to 7-fold higher than those of pHE4-A and pHE4-AR (LC_{50} s of about 1.5×10^6 cells ml^{-1} after 4 h of induction), the highest ever reached for engineered bacterial cells with *B. thuringiensis* subsp. *israelensis* toxin genes (6, 9, 44).

Stabilization of CryIVD by the 20-kDa protein. Five minutes of induction of the combination of *cryIVD* with the 20-kDa-protein gene in pT7 (clone pTE4-DR) yielded a maximal mortality of about 40%, which decreased rapidly and disappeared completely after 50 min, while induction of either *cryIVD* or

the 20-kDa-protein gene alone (pTE4-D or pTE4-R) resulted in no larvicidal activity (Fig. 5). CryIVD is thus apparently degraded in *E. coli* and partially stabilized by the 20-kDa regulatory protein. Induction in pUHE20 of each of the three clones (pHE4-DR, pHE4-D, and pHE4-R) yielded no larvicidal activity (Table 1).

The comparison of *cryIVA* and *cryIVD* in the same experiment (Fig. 5) demonstrated that the toxicity of *cryIVA* is two-fold higher than that of *cryIVD* when the genes are expressed from the same vector and that the corresponding protein (CryIVA) is much more stable than CryIVD in recombinant *E. coli*.

Viability of recombinant clones. All clones containing pT7

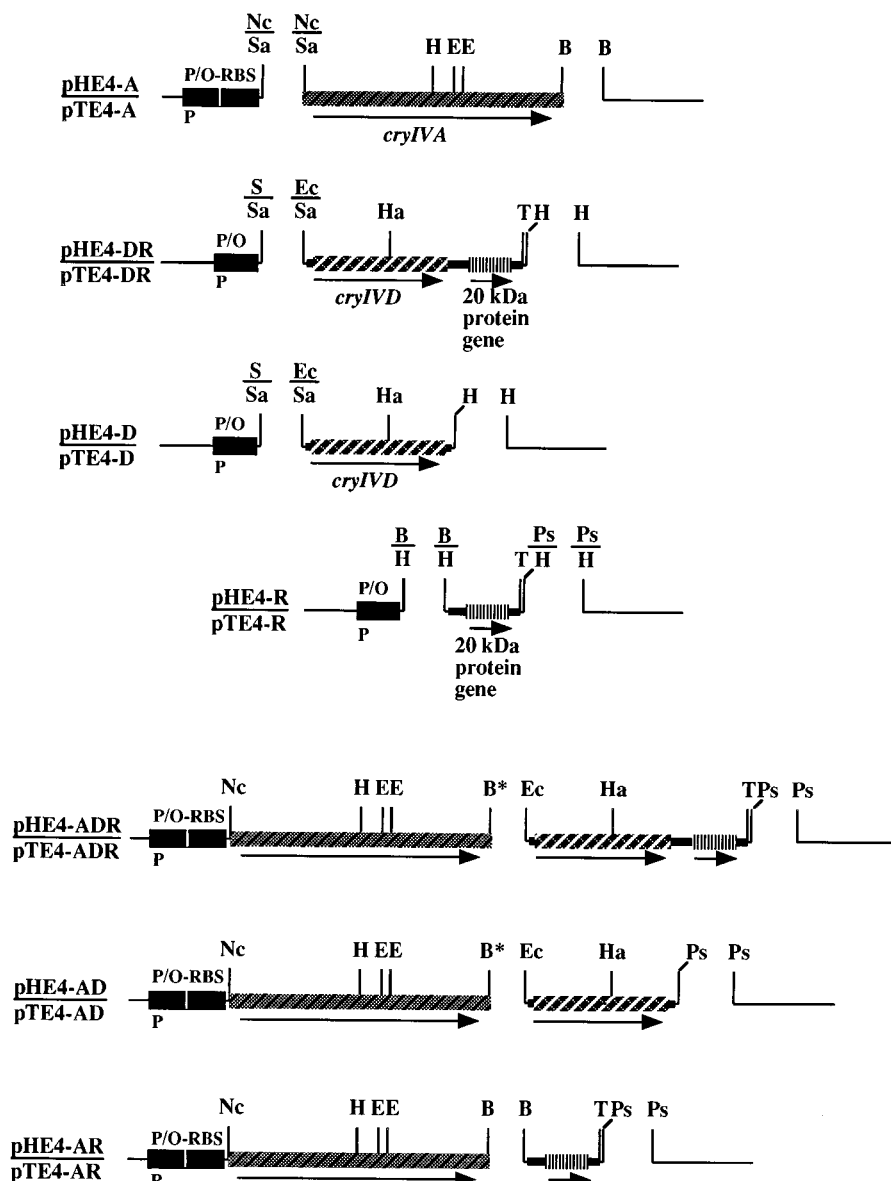


FIG. 2. Cloning of *cryIVA*, *cryIVD*, and the 20-kDa-protein gene into pUHE (pHE4 clones) and pT7 (pTE4 clones). Cloning was performed as described in Materials and Methods. Restriction sites are designated as in Fig. 1, with additional designations as follows: B*, *Bam*HI (blunt ended); Ps, *Pst*I; Sa, *Sac*I; Ec, *Ecl*136II. For clarity, not all *Taq*I, *Eco*RI, and *Nla*IV sites are shown. P, promoter sequence; P/O, promoter-operator sequence.

and its recombinant derivatives caused dramatic drops in the colony-forming ability of their host, *E. coli* BL21(DE3), to about 0.1% after 20 min of induction. The viability of recombinant clones containing pUHE and its derivatives was not changed during at least 3 h of induction (data not shown).

DISCUSSION

The results reported here demonstrate that mosquito larvicidal activity of the δ -endotoxin gene products of *B. thuringiensis* subsp. *israelensis* in transgenic organisms depends not only on the number of genes but also on the expression vector and host cells as well.

The genes *cryIVA* and *cryIVD*, encoding 134- and 72-kDa proteins, respectively, and the gene for the regulatory 20-kDa protein of *B. thuringiensis* subsp. *israelensis* (Fig. 1) were cloned

either alone or together into the expression vectors pT7 and pUHE in *E. coli* (Fig. 2; Table 1), and the toxicity of the resultant clones against *A. aegypti* larvae was quantified (Fig. 3 through 5; Table 2). Neither CryIVD alone nor the 20-kDa protein alone was toxic when expressed from either vector (Fig. 5; Table 1). The clones pTE4-D and pHE4-D lack a 10-nt inverted repeat sequence at the 3' end of *cryIVD* (*Nla*IV cuts between two inverted repeat sequences) that stabilizes its mRNA (20, 42). The clone containing them both (pTE4-DR) was slightly and temporarily toxic in pT7 only (Fig. 5); the lack of toxicity in pHE4-DR may be due to its weaker promoter (P_{A1}), taken from an early T7 gene, which utilizes the usual *E. coli* RNA polymerase (19). The strong T7 promoter ($\phi 10$) in pTE4-DR is transcribed efficiently by T7 RNA polymerase, the gene of which had been recombined into the chromosome of strain BL21(DE3) (38, 40). The observed toxicity was low,

TABLE 1. Recombinant clones and their toxicities^a

Gene(s)	Recombinant clone in pT7-1 or pT7-2	Toxicity ^b	Recombinant clone in pUHE-24 or pUHE-20	Toxicity ^b
<i>cryIVA</i>	pTE4-A	+	pHE4-A	++
<i>cryIVD</i>	pTE4-D	-	pHE4-D	-
20-kDa-protein gene	pTE4-R	-	pHE4-R	-
<i>cryIVD</i> and 20-kDa-protein gene	pTE4-DR	±	pHE4-DR	-
<i>cryIVA</i> and 20-kDa-protein gene	pTE4-AR	+	pHE4-AR	++
<i>cryIVA</i> and <i>cryIVD</i>	pTE4-AD	++	pHE4-AD	++++
<i>cryIVA</i> , <i>cryIVD</i> , and 20-kDa-protein gene	pTE4-ADR	++	pHE4-ADR	++++

^a The genes *cryIVA* and *cryIVD*, encoding 134- and 72-kDa proteins, respectively, and the gene for the regulatory 20-kDa polypeptide of *B. thuringiensis* subsp. *israelensis* were cloned either alone or together by the expression vectors pT7 and pUHE in *E. coli* (Fig. 2).

^b Symbols designate toxicities of recombinant clones against *A. aegypti* larvae in standard bioassays. -, no toxicity; ±, +, ++, +++, and +++++, levels of toxicity from lowest to highest.

however, and disappeared a short time after induction (Fig. 5); the 20-kDa protein thus seems to partially stabilize CryIVD, as has been suggested before (10, 41).

Expression in *E. coli* JM83 of *cryIVD* and the gene for the 20-kDa protein from their original promoters had previously been found to be nontoxic to *A. aegypti* larvae (28); two smaller peptides (50 and 30 kDa) which were the proteolytic cleavage products of the 72-kDa CryIVD appeared (41). Cells carrying the 20-kDa-protein gene produced substantially more CryIVD than did those without it. The amounts of the smaller peptides also increased in the presence of the 20-kDa protein (41). In addition, the toxicity of CryIVD to *E. coli* (like that of CytA [21, 43]) must be considered, because expression of some genes from the T7 RNA promoter by T7 RNA polymerase indeed kills the cells (38, 40).

CryIVD alone was produced, and it formed parasporal inclusions in other *B. thuringiensis* subspecies (9, 10), but higher levels of these inclusions were observed in the presence of the 20-kDa protein (10). Expression of *cytA* in the presence of the 20-kDa protein in acrySTALLIFEROUS strains of *B. thuringiensis* yielded bipyramidal crystals of CytA that were larger than those of wild-type *B. thuringiensis* (43). These results suggest that the 20-kDa protein promotes crystal formation, perhaps

by chaperoning CytA molecules during synthesis and crystallization, concomitantly preventing lethal interaction with the host (21, 43). The combination of CryIVD and the 20-kDa protein (20) was larvicidal in *Bacillus megaterium* but not in *E.*

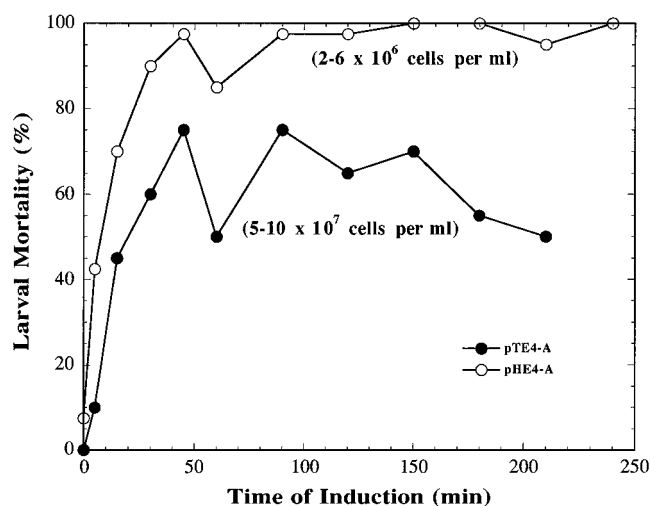


FIG. 3. Toxicities of CryIVA expressed by clones pHE4-A and pTE4-A (Fig. 2; Table 1). Each exponentially growing culture was induced with 0.5 mM IPTG when its density reached about 6×10^8 cells per ml during the indicated time. The cells were centrifuged, and the pellets were suspended in distilled, sterile water and introduced at the indicated concentrations into 20 third-instar *A. aegypti* larvae in 100 ml. Mortality was recorded after 24 h at 28°C.

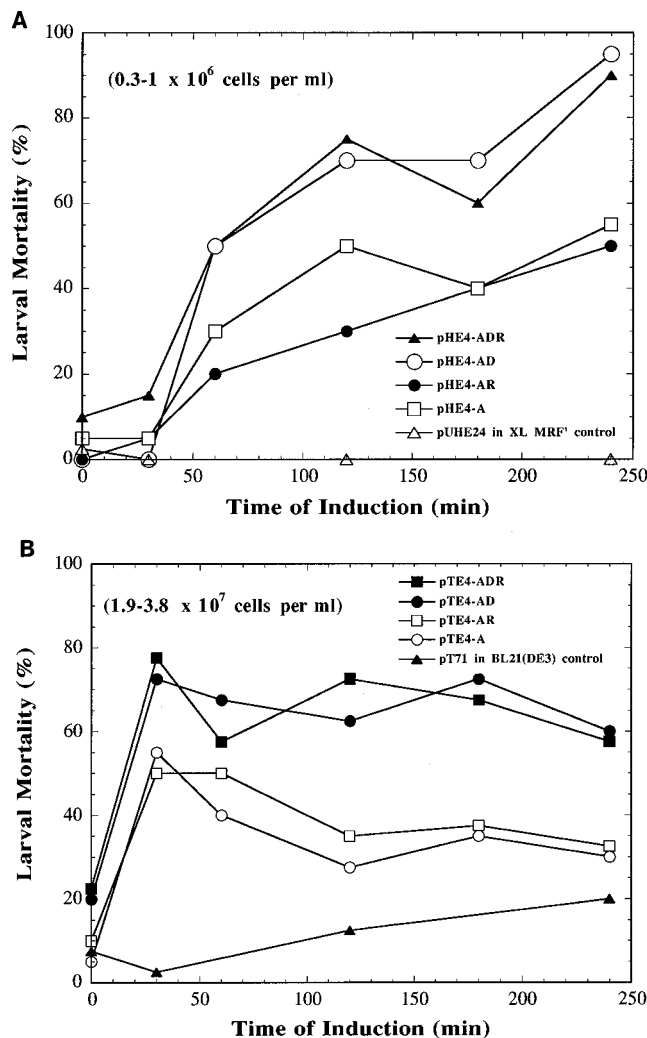


FIG. 4. Toxicities of pHE4-A, pHE4-AR, pHE4-AD, and pHE4-ADR (A) and pTE4-A, pTE4-AR, pTE4-AD, and pTE4-ADR (B) (Fig. 2; Table 1). Each culture was induced and the cells were treated as described in the legend to Fig. 3, but at the indicated concentrations.

TABLE 2. Toxicities of *E. coli* recombinant clones containing combinations of *B. thuringiensis* subsp. *israelensis* genes against third-instar *A. aegypti* larvae

Strain	LC ₅₀ (cells ml ⁻¹) after induction for ^a :		
	0.5 h	2 h	4 h
pHE4-A	2.8 × 10 ⁶	2.2 × 10 ⁶	1.6 × 10 ⁶
pHE4-AR	3.7 × 10 ⁶	2.8 × 10 ⁶	1.5 × 10 ⁶
pHE4-AD	7.3 × 10 ⁵	3.9 × 10 ⁵	2.3 × 10 ⁵
pHE4-ADR	8.0 × 10 ⁵	4.7 × 10 ⁵	3.3 × 10 ⁵
pTE4-A	2.4 × 10 ⁷	4.5 × 10 ⁷	7.0 × 10 ⁷
pTE4-AR	3.2 × 10 ⁷	4.0 × 10 ⁷	6.8 × 10 ⁷
pTE4-AD	1.4 × 10 ⁷	2.3 × 10 ⁷	2.9 × 10 ⁷
pTE4-ADR	1.3 × 10 ⁷	2.4 × 10 ⁷	5.3 × 10 ⁷

^a Values are averages of three bioassays performed as described in Materials and Methods.

coli. When *cryIVD* from *Bacillus thuringiensis* subsp. *morrisoni* PG-14 was inserted into the shuttle vector pHT3101 together with the 20-kDa-protein gene for expression in an acrystalliferous mutant of *Bacillus thuringiensis* subsp. *kurstaki*, parasporal inclusions of CryIVD which were comparable in size, shape, and toxicity to those produced by the parent strain were observed. In contrast, expression from the same vector of *cryIVD* in *E. coli* was very low (demonstrating no toxicity to *Culex quinquefasciatus*) and detectable by immunoblot analysis only as 34- to 40-kDa proteolytic fragments (9). Differences in expression of the δ -endotoxin genes, naturally expressed during sporulation, between *E. coli* and *Bacillus* strains would depend on a sporulation-specific transcription factor(s), such as σ^{35} , other than those used during vegetative growth (24, 45).

In our study, the clone expressing *cryIVD* with the 20-kDa-protein gene (pTE4-DR) was toxic in recombinant *E. coli*, apparently because the genes were expressed from T7 rather than from their original promoters. This combination had no toxicity in pHE4-DR, as mentioned above. The toxicity of CryIVA, on the other hand, was higher (causing 98% mortality of *A. aegypti* larvae after 45 min of induction) in pHE4-A than in pTE4-A (Fig. 3). Higher translation efficiency is expected in pUHE24 than in pT7 because the former contains an RBS (7). In addition, induction in strain BL21(DE3) carrying pTE4

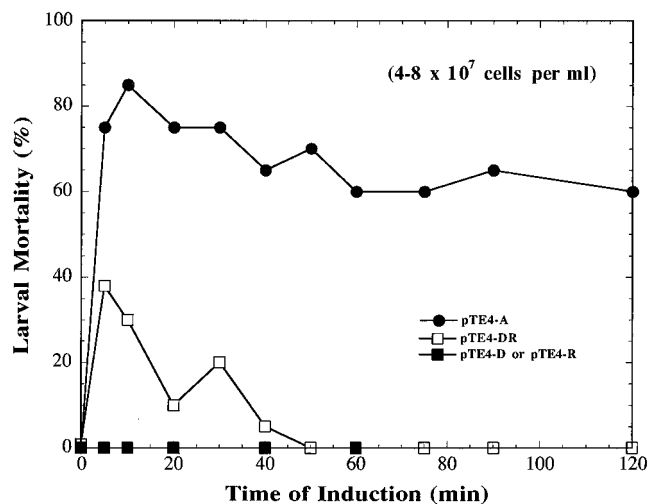


FIG. 5. Toxicities of pTE4-A, pTE4-DR, pTE4-D, and pTE4-R (Fig. 2 and Table 1). Each culture was induced and the cells were treated as described in the legend to Fig. 3, but at the indicated concentrations.

clones caused loss of colony-forming ability but induction in XL-Blue MRF' (pHE4-A) did not (data not shown). Apparent toxicity would thus seem lower because of growth inhibition of the former and the appearance of nonfunctional mutants (38). Transcription terminators from gram-positive bacteria enhance expression in *E. coli* (2, 44), but for T7 RNA polymerase [in strain BL21(DE3)] only a natural T7 terminator (T ϕ) will terminate most transcripts (38). CryIVA appears to be more stable than CryIVD in the same strain (Fig. 5); purified CryIVD was claimed to be rather more toxic to *A. aegypti* than purified CryIVA (11). Recombinant *E. coli* cells harboring *cryIVA* with its promoter demonstrated toxicity against certain *Aedes*, *Culex*, and *Anopheles* spp. (6), but toxicity was very low compared with that of the strain carrying pHE4-A used in our study.

Recombinant *E. coli* harboring a chimera of *cryIVA* with $\Delta lacZ$ (in frame) expressed under the control of *plac* was nontoxic (42). However, when this fusion protein was expressed with the 20-kDa-protein gene, in *trans* from another (compatible) low-copy-number pACYC-type plasmid, an increase in production of the fused CryIVA (on a high-copy-number pUC-type plasmid) was detected (46). The combination we have now constructed, with both genes in *cis* and in both expression vectors, did not raise the toxicity of *cryIVA* (Fig. 4; Table 2). Very low levels of expression of the 20-kDa-protein gene on plasmid pACYC184 were sufficient to stimulate production of CytA from pUC9 (1). (This expression was more prominent than that obtained when both genes were carried on pUC9.) The balance of intracellular concentrations of CytA and the 20-kDa protein could thus be important. We cloned *cryIVA* and the 20-kDa-protein gene in tandem into each of the high-copy-number plasmids pUHE24 and pT7-1 so that *cryIVA* was expressed under a strong promoter and the 20-kDa-protein gene was expressed with its own promoter as well. It is conceivable that production of the major crystal components such as CytA and CryIVD is stimulated by the 20-kDa-protein to a larger extent than is the production of the minor components such as CryIVA (46).

Inclusion bodies were not formed when *cryIVA* was cloned on a low-copy-number plasmid in a crystal-negative strain (4Q7) of *B. thuringiensis* subsp. *israelensis* (18). This recombinant strain, containing *cryIVB* as well, produced inclusions composed of the two proteins, suggesting that CryIVB could promote crystallization of CryIVA (18). Our clones with *cryIVA* and *cryIVD* in *cis* on the two high-copy-number plasmids (pT7 and pUHE) displayed two- to sevenfold-higher toxicities than their equivalents with *cryIVA* alone (Table 2), while those with *cryIVD* alone were nontoxic (Table 1). These results suggest that CryIVA promotes CryIVD crystal formation, and vice versa. The two proteins are expected to synergize as a result of different modes of action (33), but a contradictory conclusion has recently been reached (39): weak antagonism rather than synergism between purified 130- and 65-kDa proteins was observed (11). It is likely that synergism is operating at the level of synthesis and not at the level of activity, but one should bear in mind that the "purified" 130-kDa protein is a mixture of CryIVA and CryIVB. A recombinant clone producing all three CryIV proteins (A, B, and D) would resolve this apparent paradox.

We plan to construct an operon with a combination of all the δ -endotoxin genes from *B. thuringiensis* subsp. *israelensis*, anticipating that the stabilization of the polypeptides and the synergism between their activities (10, 15, 16, 39) will significantly improve the toxicity of the bacterium while reducing the chances that mosquitoes exposed to the toxin will gain resistance to it (3). Our final goal is to clone the complex construct

into indigenous cyanobacterial strains (31, 33), which are ingested by mosquito larvae, for reintroduction into their natural habitats, where the adaptive values and competition potentials of such strains are high.

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