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Cloning and Expression of Two Homologous Genes of *Bacillus thuringiensis* subsp. *israelensis* Which Encode 130-Kilodalton Mosquitocidal Proteins

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Two homologous genes encoding 130-kilodalton (kDa) mosquitocidal proteins of *Bacillus thuringiensis* subsp. *israelensis* have been cloned and expressed in *Escherichia coli* or *Bacillus subtilis* or both. One of these genes, pPC130, was expressed as a *lacZ* transcriptional fusion in *E. coli* at a level sufficient to produce phase-bright inclusions, which were purified and shown to be toxic to *Aedes aegypti* larvae. The second gene, pCH130, was expressed at a low level in recombinant *E. coli* cells and was therefore cloned in *B. subtilis* as a transcriptional fusion of the promoter sequences corresponding to a *B. thuringiensis* subsp. *israelensis* 27-kDa δ-endotoxin (E. S. Ward, A. R. Ridley, D. J. Ellar, and J. A. Todd, J. Mol. Biol. 191:13–22, 1986) and the structural gene. Recombinant *B. subtilis* cells produced phase-bright inclusions during late sporulation; these were partially purified and shown to be toxic to *A. aegypti* larvae at an LC_{50} (concentration required to cause 50% mortality of larvae after 24 h of assay) which is significantly lower than that of the pPC130 protein. Neither 130-kDa protein was hemolytic under the assay conditions. Comparison of the nucleotide sequences of these two genes indicates that they share a high degree of homology in the C-terminal portions, but relatively little similarity in the N termini. In addition, significant homologies were found between the pCH130 gene and the HD-1 Dipel gene of *B. thuringiensis* subsp. *kurstaki* (H. E. Schnepf, H. C. Wong, and H. R. Whiteley, J. Biol. Chem. 260:6264–6272, 1985).

Bacillus thuringiensis subsp. *israelensis* (17) is a gram-positive, sporeforming organism which produces a parasporal δ-endotoxin crystal during sporulation (6, 42). The δ-endotoxin is extremely toxic to the larvae of mosquitoes and blackflies (13, 44). Purified *B. thuringiensis* subsp. *israelensis* crystalline inclusions consist of several major polypeptide species (of 130 kilodaltons (kDa) [doublet], 65 kDa, and 27 kDa), together with other minor proteins (25, 44, 55). The relative contribution of these polypeptides to the overall toxicity of the *B. thuringiensis* subsp. *israelensis* crystal is of considerable interest. Recent data suggest that the crystal proteins may interact synergistically to enhance toxicity (55; C. N. Chilcott and D. J. Ellar, submitted for publication).

Recently there have been two approaches to the elucidation of the activities of the individual polypeptide components which make up the *B. thuringiensis* subsp. *israelensis* crystal. Several groups have reported the separation by biochemical methods of *B. thuringiensis* subsp. *israelensis* proteins from crystal-spore mixtures (2, 11, 12, 26, 27, 31, 43, 47, 55; W. E. Thomas, Ph.D. thesis, University of Cambridge, Cambridge, United Kingdom, 1984). By using this approach, the 130-kDa doublet and the 65- and 27-kDa polypeptides have been studied in the absence of other polypeptide species. Alternatively, separation by genetic means has been reported in several cases (1, 5, 37, 41, 45, 48, 52, 53). The structural gene encoding the larvicidal 27-kDa protein has been cloned and expressed in *Escherichia coli* (48, 52) and in two strains of *B. subtilis* (53). In addition, the nucleotide sequence of this gene has been published (48, 50). A structural gene encoding a mosquitocidal protein of 72 kDa has been cloned and sequenced (45) and shown to share homology with the 5.3-kilobase-pair (kb) gene isolated from *B. thuringiensis* subsp. *kurstaki* HD-1 Dipel (39). In addition, a *B. thuringiensis* subsp. *israelensis* gene encoding a mosquitocidal protein has been isolated by using *B. megaterium* (41) and *E. coli* (1, 5) as cloning hosts. The nucleotide sequence of all or part of this gene has recently been reported (1; T. Yamamoto, personal communication), and it appears to code for one of the *B. thuringiensis* subsp. *israelensis* 130-kDa doublet proteins. Recombinant *B. megaterium* and *E. coli* cells harboring this gene synthesize a polypeptide of 130 kDa which reacts immunologically with antisera raised against *B. thuringiensis* subsp. *israelensis* crystal protein (1, 5, 40).

In the present study, two genes encoding *B. thuringiensis* subsp. *israelensis* 130-kDa proteins have been isolated and expressed by using *E. coli* and *B. subtilis* as cloning hosts. One of these has been shown to be the same as that reported by Sekar and Carlton (41), Bourgouin et al. (5), and Angsuthanasombat et al. (1). The second gene has been sequenced (51), and the amino acid sequence shares a high degree of homology in the C-terminal portion with the sequence (Yamamoto, personal communication) of the cloned *B. thuringiensis* subsp. *israelensis* gene. The N-terminal regions of the two genes show little similarity, however. This gene also shares significant similarities with the *B. thuringiensis* subsp. *israelensis* gene described by Thorne et al. (45) and a cloned *B. thuringiensis* subsp. *kurstaki* HD-1 Dipel gene (39) encoding a lepidoptera-specific δ-endotoxin. In an attempt to understand more of the contribution of these 130-kDa proteins to the toxicity of the *B. thuringiensis* subsp. *israelensis* crystal, we have investigated the mosquitocidal and hemolytic properties of these proteins.

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MATERIALS AND METHODS

Strains and plasmids. *E. coli* TG1=[K-12 Δ(*lac-pro*) *supE thi hsdD5*/F' *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*] (Gibson, Ph.D. thesis) (a generous gift of T. Gibson) and pUC18 (56) were used as cloning host and vector, respectively. *B. subtilis* MB24 (*metC3 trpC2 rif-2*) (a generous gift of P. J. Piggot, Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pa.) was also used as a cloning host, with pC194 (23) as described previously (53) as a *B. subtilis* replicon. For DNA sequencing, M13tg130 (Amersham Corp.) was used. *E. coli* BMH mutL71-17 was used as a cloning host during mutagenesis experiments (7) and was a generous gift of G. Winter, Medical Research Council Laboratory of Molecular Biology, Cambridge, England.

Enzymes and reagents. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., and were used in the buffers recommended by Maniatis et al. (36). T4 DNA ligase and the Klenow fragment of DNA polymerase were provided by R. T. Hunt, University of Cambridge. Calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim Biochemicals) was used to dephosphorylate vector DNA in the medium salt buffer of Maniatis et al. (36). L-[^{35}S]methionine, [γ-^{32}P]ATP, [α-^{32}P]dATP, and [α-^{35}S]dATP were purchased from Amersham. End repairing was carried out as described previously (3), except that the incubation was performed with 5 U of Klenow polymerase at 30°C for 15 min.

Cloning of the *B. thuringiensis* subsp. *israelensis* δ-endotoxin genes. Purified 72-MDa plasmid was isolated as described previously (52) and used as a source of *B. thuringiensis* subsp. *israelensis* DNA during the cloning experiments. *Pst*I-restricted 72-MDa plasmid was ligated into PstI-restricted pUC18 treated with CIP, and the resulting ligation mix was used to transform *E. coli* TG1 to ampicillin resistance by the $CaCl_2$ method (36). DNA isolated from recombinants by a Triton lysis (52) or sodium dodecyl sulfate (SDS)-NaOH (4) method was used to prime protein synthesis in an *E. coli* in vitro transcription-translation system (10) (a generous gift of K.-F. Chak, University of Cambridge). Products from the in vitro system were analyzed as described previously (8, 24) by using antisera raised against purified *B. thuringiensis* subsp. *israelensis* 130-kDa proteins. By using this procedure, clones directing the synthesis of an immunoprecipitable polypeptide were isolated and shown to harbor an 8-kb *Pst*I insert. Restriction fragments derived from this *Pst*I insert were gel purified (15) and radiolabeled to a specific activity of 10^8 to 10^9 cpm/μg by the method of Feinberg and Vogelstein (16). Recombinants obtained by transforming *E. coli* TG1 with *Cla*I-restricted 72-MDa plasmid ligated into *Cla*I-restricted pUC18 treated with CIP were screened for the presence of homologous genes by using the radiolabeled probe as described previously (19).

DNA sequencing. The dideoxynucleotide chain termination method (3, 38) was used. Random fragments derived from the cloned *B. thuringiensis* subsp. *israelensis* DNA were generated by using a cup horn sonicator (model W-375; Heat Systems Ultrasonics, Inc.). The sonicated DNA was end repaired, and the repaired fraction was size fractionated and purified by low-gelling agarose (FMC Colloids Ltd.) gel electrophoresis. End-repaired, size-fractionated DNA was then ligated into *Sma*I-restricted M13tg130 treated with CIP.

Protein sequence comparisons. Amino acid sequences corresponding to several *B. thuringiensis* proteins were compared by using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package, ver. 5.0 (14). Diagon printouts were obtained by using Dotplot with a 4014 terminal emulator program on a Macintosh SE computer (Apple Computers).

Polyacrylamide gel electrophoresis and immunoblotting. SDS-polyacrylamide gel electrophoresis and immunoblotting were carried out as described previously (50) by published methods (22, 30, 46). Proteins were transferred from the SDS-gels onto nitrocellulose by using an LKB Novablot apparatus.

Preparation of antiserum. Antiserum was raised by injecting New Zealand White rabbits with *B. thuringiensis* subsp. *israelensis* 130-kDa polypeptides isolated by gel purification from SDS-polyacrylamide gels (49) and was a generous gift of T. Sawyer. The purified protein was emulsified with Freund complete (first injection) or incomplete (subsequent injection) adjuvant prior to subcutaneous injection. Antiserum was analyzed for specificity and titer by immunoblotting.

Site-directed mutagenesis. To generate a *Bam*HI restriction site 30 nucleotides upstream of the initiation codon of pCH130, we used the synthetic oligonucleotide 5'-CTCA ATTTGGATCCACTCTTTT-3' to direct two base changes at positions 29 (T to G) and 25 (T to C) nucleotides upstream of the initiation AUG. Oligonucleotide-directed mutagenesis was carried out as described previously (7).

Transformation of *B. subtilis*. The method of Chang and Cohen (9) was used to transform *B. subtilis*, except that chloramphenicol was used at 4 μg/ml in regeneration medium.

Growth of recombinants. Recombinant *E. coli* cells were grown in 2xTY (3) containing ampicillin at 100 μg/ml. During induction experiments, cells were grown to an optical density of 1.0 (A_{600}), at which time the *lacZ* inducer isopropyl-β-D-thiogalactopyranoside (IPTG) was added at 0.5 mM. The cells were harvested after 24 to 48 h of induction. Recombinant *B. subtilis* cells were grown as described previously (53).

Purification of inclusions from recombinant cells. Recombinant *E. coli* cells were grown under inducing conditions until phase-bright inclusions could be seen by phase-contrast microscopy in ca. 90% of the cells. Cells were harvested by centrifugation, the resulting cell pellet was suspended in 10 mM Tris hydrochloride (pH 7.4)–10 mM NaCl–10 mM disodium EDTA, and then cells were lysed by using a sonic probe as described previously (52). Inclusions were purified from the resulting cell lysate by sucrose density gradient centrifugation (44). Phase-bright inclusions were isolated from recombinant *B. subtilis* cells as described previously (53).

Toxicity assays. Inclusions isolated from recombinant *E. coli* or *B. subtilis* were assayed for in vivo toxicity by using third-instar larvae and following published procedures (52) (*Aedes aegypti* larvae were kindly provided by D. Funnel, Shell Research Ltd.).

Hemolysis assays. Inclusions were solubilized as described previously (44), in both the presence and absence of gut extract from *A. aegypti* larvae (21). Gut extracts were generous gifts of C. N. Chilcott and J. Horsnell. Solubilized protein was assayed for hemolytic activity by using human or rabbit erythrocytes (44).

RESULTS

Isolation of two 130-kDa δ-endotoxin genes. Plasmid DNA was extracted from groups of recombinant clones harboring

*Pst*I-restricted *B. thuringiensis* subsp. *israelensis* 72-MDa plasmid DNA ligated into pUC18 and used to prime protein synthesis in an in vitro *E. coli* system.

Immunoprecipitation of the products synthesized in vitro by using antisera raised against the *B. thuringiensis* subsp. *israelensis* 130-kDa proteins identified several groups synthesizing immunoprecipitable polypeptides. Further analysis of one of these groups identified two recombinants (p130/P1-1 and p130/P1-2) harboring a chimera of a *Pst*I 8-kb fragment and the cloning vector pUC18 which directed the synthesis of approximately 100-kDa cross-reacting polypeptides and truncated derivatives in the in vitro system (not shown). Restriction analysis of p130/P1-1 (Fig. 1) indicated that it contains the 3.34-kb *Pst*I-*Xba*I fragment previously described (1, 5, 41) and recently shown by sequence analysis to encode most of a 130-kDa *B. thuringiensis* subsp. *israelensis* polypeptide (Yamamoto, personal communication). In an attempt to isolate the promoter region and N terminus of this gene, a gene bank was constructed by ligating *Cla*I-restricted *B. thuringiensis* subsp. *israelensis* 72-MDa plasmid into pUC18. The resulting recombinants were screened for the presence of homologous DNA by using radiolabeled 1.54-kb *Pst*I-*Xba*I and 1.8-kb *Xba*I fragments (Fig. 1).

Recombinants harboring a 3.6-kb *Cla*I insert gave strong hybridization signals with these fragments as probes; further analysis indicated that these inserts overlapped with the 8-kb *Pst*I fragment of p130/P1-1. A construct containing the complete 130-kDa δ-endotoxin gene was made as shown in Fig. 1 and is designated pPC130.

Interestingly, during the hybridization studies of the *Cla*I gene bank, hybridizing colonies were identified which produced weaker signals than colonies which contained the 3.6-kb insert. These colonies were found to harbor chimeras of a 3.9-kb *Cla*I fragment and pUC18 (designated pCC130); analysis of DNA extracted from one of these recombinants in the *E. coli* in vitro system indicated that it encoded a polypeptide of approximately 100 kDa, in addition to truncated derivatives, which were immunoprecipitated by anti-130-kDa-protein antisera (not shown). The restriction map of pCC130 is shown in Fig. 2. Nucleotide sequencing of this 3.9-kb insert identified an extended open reading frame (ORF) of 3.05 kb, truncated at the 3′ end. To isolate DNA fragments encoding the carboxy-terminus of the truncated gene carried by pCC130, a 1.8-kb *Hin*dIII-*Cla*I fragment (Fig. 2) was gel purified, radiolabeled, and used as a gene-specific probe. Hybridization studies of a gene bank constructed by ligating *Hin*dIII-restricted *B. thuringiensis* subsp. *israelensis* plasmid DNA into pUC18 resulted in the

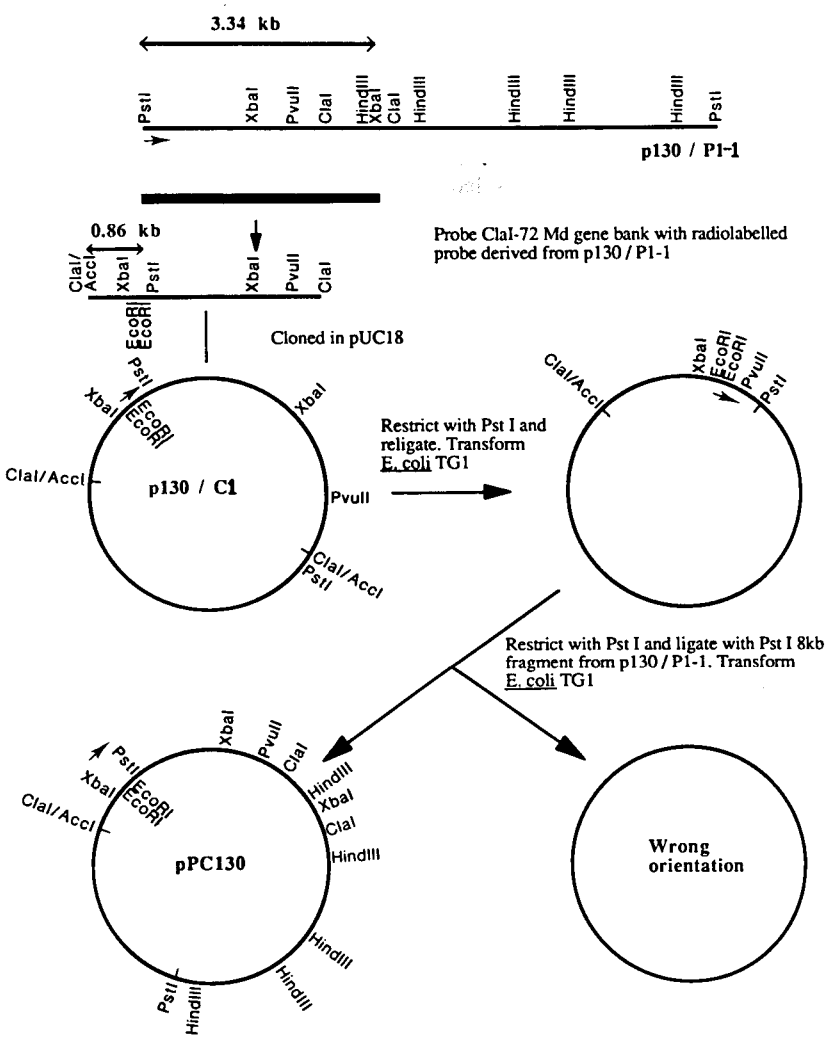

FIG. 1. Construction of the complete 130-kDa δ-endotoxin gene (pPC130). The direction of transcription of the gene is indicated by arrowheads. For clarity, not all restriction sites are shown.

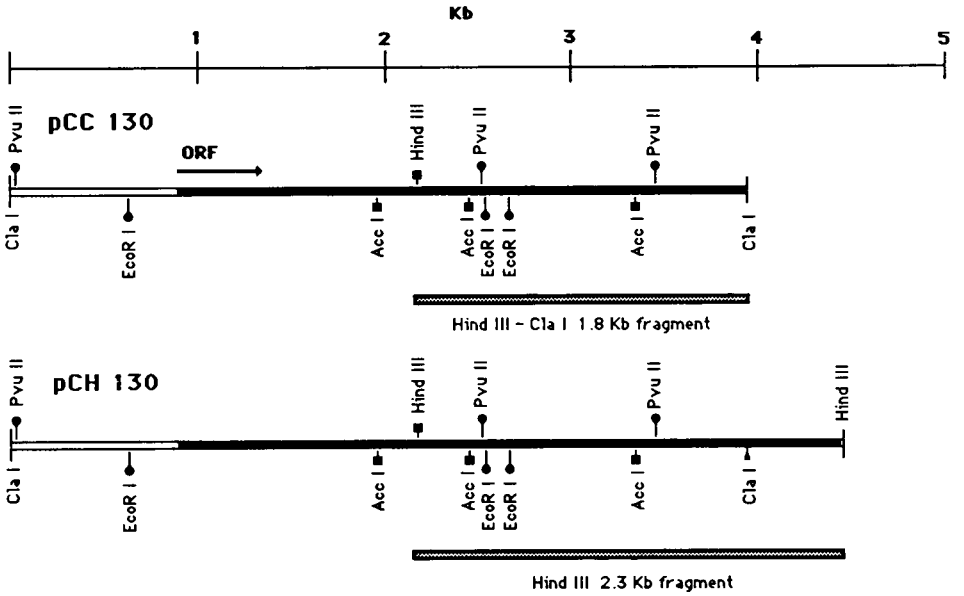

FIG. 2. Restriction map of pCC130 and pCH130. The 1.9-kb radiolabeled *Hin*dIII-*Cla*I fragment and the *Hin*dIII 2.3-kb fragment isolated with this probe are indicated by hatched boxes. The ORF is represented by the solid line. The direction of transcription is indicated by an arrow.

isolation of a 2.3-kb *Hin*dIII fragment which extends 520 base pairs to the 3′ side of the *Cla*I site (Fig. 2). This 2.3-kb *Hin*dIII fragment was used to construct plasmid pCH130, which contains the translational stop codon corresponding to the ORF of pCC130. Since both the structural genes encoded by pPC130 and pCH130 have been isolated, by using overlapping probes, from gene banks made by ligating restricted *B. thuringiensis* subsp. *israelensis* 72-MDa plasmid into pUC18, this suggests that these genes are present in vivo in *B. thuringiensis* subsp. *israelensis*.

Amino acid sequence comparisons. The nucleotide sequence of the complete ORF encoded by pCH130 has recently been reported (51). Comparison of the structural gene sequence of this ORF with the amino acid sequences of *B. thuringiensis* δ-endotoxin genes previously reported (39, 45, 48, 50; Yamamoto, personal communication) has revealed that they share some interesting similarities (Fig. 3). First, the structural gene for the pCH130 gene has been compared with the amino acid sequence of the *B. thuringiensis* subsp. *kurstaki* P1 5.3-kb δ-endotoxin gene (39); significant similarities can be found (Fig. 3a), particularly in the corresponding C-terminal regions. Second, amino acids 713 to 1180 of the pCH130 gene show near identity with the C-terminal residues of the *B. thuringiensis* subsp. *israelensis* pPC130 gene (Yamamoto, personal communication) (Fig. 3b). The N-terminal regions, in contrast, show a limited degree of similarity. Comparison of the amino acid sequence of the *B. thuringiensis* subsp. *israelensis* 27-kDa gene (48, 50), however, with that of pCH130 indicates that they show no significant similarities (Fig. 3c). Interestingly, the pCH130 gene also shares similarities throughout the coding sequence with the structural gene encoding a 72-kDa polypeptide previously described (45) (Fig. 3d). In addition, Höfte (Proceedings of the Third European Workshop on Bacterial Protein Toxins, Uberlingen, Federal Republic of Germany, 1987) have recently reported that many δ-endotoxin genes have a conserved sequence of 10 amino acids located between residues 590 and 700 of the protein. This sequence is shown for the pCH130 gene, together with that of the pPC130 and *B. thuringiensis* subsp. *kurstaki* P1 genes, in Fig. 4. Further work is necessary to elucidate the function of this common structural feature of δ-endotoxin genes.

Expression of the 130-kDa δ-endotoxin genes. In an attempt to study the properties of the proteins encoded by the *B. thuringiensis* subsp. *israelensis* DNA in pPC130 and pCH130, we grew cultures of *E. coli* as described in Materials and Methods. After 24 to 48 h of induction, inclusions could be visualized in the majority of pPC130 cells. Similar inclusions have been previously observed for recombinant cells harboring δ-endotoxin genes (20). Analysis of purified inclusions by immunoblotting (Fig. 5) indicate that these inclusions contain polypeptides which cross-react with 130-kDa antisera, and a major cross-reacting protein comigrates with the protein of lower electrophoretic mobility in the 130-kDa doublet of the *B. thuringiensis* subsp. *israelensis* crystal (44). Purified inclusions isolated from pPC130 cells have been assayed for toxicity against *A. aegypti* larvae and erythrocytes. The estimated LC_{50} (concentration required to cause 50% mortality of larvae after 24 h of assay) of this protein against *A. aegypti* larvae is 5 to 10 μg/ml. At 10 μg/ml the solubilized δ-endotoxin does not appear to have hemolytic activity against human or rabbit erythrocytes. Treatment of the solubilized protein with *A. aegypti* gut extract did not affect the hemolytic activity.

E. coli cells harboring pCH130, however, produce relatively low quantities of a 130-kDa polypeptide which cross-reacts with the anti-130-kDa antisera, and inclusions cannot be visualized in these cells even after prolonged periods of growth (not shown). To facilitate construction of chimeric plasmids with the pCH130 structural gene and promoters which function efficiently in *E. coli* or *B. subtilis*, a *Bam*HI restriction site was created 30 nucleotides upstream of the initiation ATG codon by using oligonucleotide-directed mutagenesis. In addition, since 3′ sequences have been reported (54) to enhance the expression of other cloned δ-endotoxin genes, a 3′ inverted repeat sequence derived from pPC130 was inserted at the 3′ end of the pCH130 gene (Fig. 6a). The presence of this inverted repeat was found to

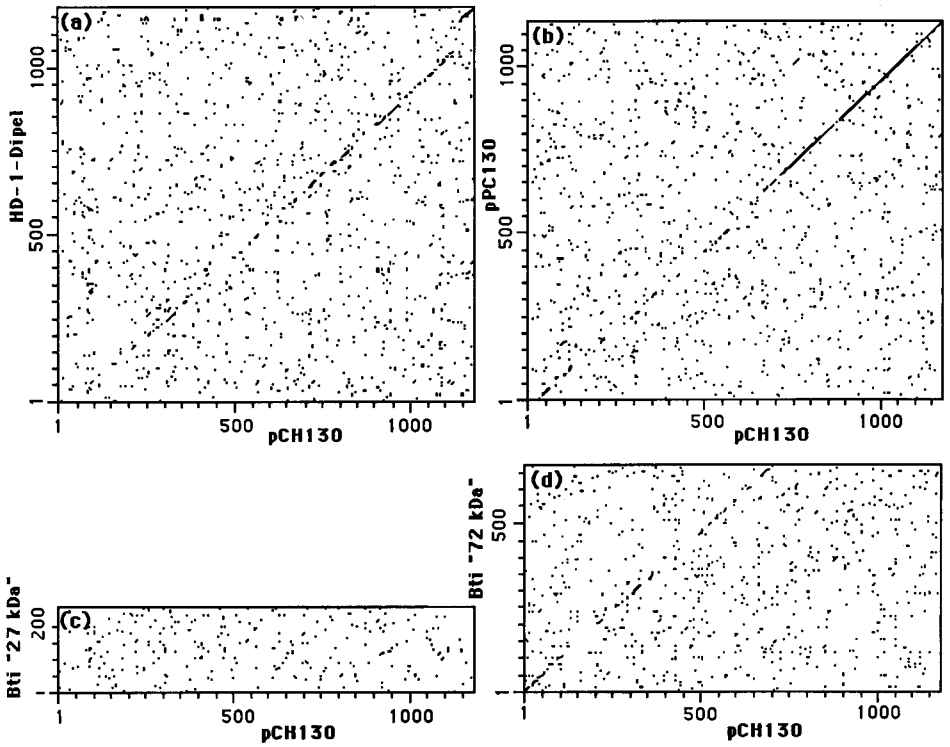

FIG. 3. Use of matrix analyses to compare the amino acid sequence of a *B. thuringiensis* subsp. *israelensis* 130-kDa δ-endotoxin gene (pCH130) with the sequences of several δ-endotoxin structural genes. These genes are as follows: (a) *B. thuringiensis* subsp. *kurstaki* δ-endotoxin gene encoding a lepidoptera-specific toxin (39); (b) pPC130, encoding a 130-kDa *B. thuringiensis* subsp. *israelensis* toxin (1, 5; Yamamoto, personal communication); (c) a gene encoding a *B. thuringiensis* subsp. *israelensis* 27-kDa δ-endotoxin previously described (48, 50); and (d) a *B. thuringiensis* subsp. *israelensis* gene encoding a 72-kDa polypeptide previously described (45). Amino acid numbers are shown for each gene on the corresponding margins. The program used was "Compare/pair = 1.5" with conditions of window = 5 and stringency = 5.0.

enhance expression (not shown). Recombinant *E. coli* cells harboring a chimera of pBC130 in transcriptional frame with the *lacZ* promoter of pUC18 were, however, found to produce insufficient levels of 130-kDa antigen for toxicity assays to be carried out. In an attempt to improve the level of expression, we used *B. subtilis* as a cloning host. Since previous experiments had indicated that expression of the pCH130 gene from its native promoter in *B. subtilis* cells is low (results not shown), a construct was made in which the promoter sequences of the *B. thuringiensis* subsp. *israelensis* 27-kDa gene (50, 53) were fused to the structural gene of pBC130 (Fig. 6b). A *B. subtilis* replicon, pC194 (23), was also ligated, as an end-repaired *Hin*dIII fragment, into the *Sma*I site of the pUC18 polylinker (Fig. 6b). p27BC130 was

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var israelensis (pCH130) GTACTTATTGATAAAATTGAATTTCTGCCA
 (codons 667-676)         V  L  I  D  K  I  E  F  L  P

var israelensis (pPC130) GTGATTATTGACAGAATCGAAATTATTCCA
 (codons 623-632)         V  I  I  D  R  I  E  I  I  P

var kurstaki "5.3"       GTTTATATAGATCGAATTGAATTTGTTCCG
 (codons 596-605)         V  Y  I  D  R  I  E  F  V  P
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FIG. 4. Homologous regions of several 130-kDa δ-endotoxin genes. The *B. thuringiensis* subsp. *israelensis*(pCH130) sequence (51) and the *B. thuringiensis* subsp. *kurstaki* 5.3-kb gene sequence (39) were reported previously. The *B. thuringiensis* subsp. *israelensis*(pPC130) sequence was obtained from T. Yamamoto (personal communication). The amino acid sequence is shown below each corresponding nucleotide sequence; amino acids identical for the three genes are in italics.

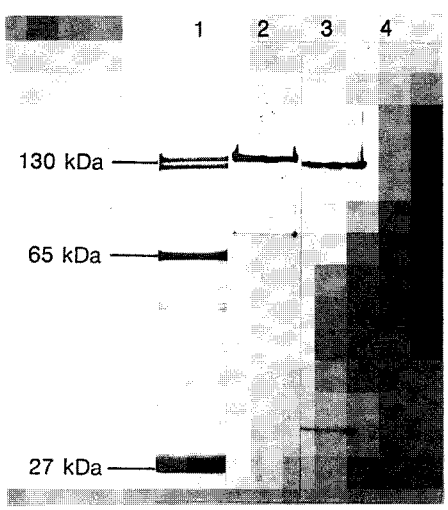

FIG. 5. Immunoblot analysis of the protein inclusions harvested from recombinant *E. coli*(pPC130) or *B. subtilis*(p27BC130) cells. For lanes 2 to 4, proteins were transferred onto nitrocellulose after electrophoresis on SDS–10% polyacrylamide gels; they were then incubated with anti-130-kDa antiserum as described in Materials and Methods. Lanes: 1, 80 μg of *B. thuringiensis* subsp. *israelensis* crystal protein, stained with Coomassie brilliant blue; 2, isolated pPC130 inclusions; 3, isolated p27BC130 inclusions; 4, 100 μg of cell lysate derived from *E. coli* cells harboring pUC18. The molecular masses corresponding to the major polypeptides of the *B. thuringiensis* subsp. *israelensis* crystal are shown on the left margin.

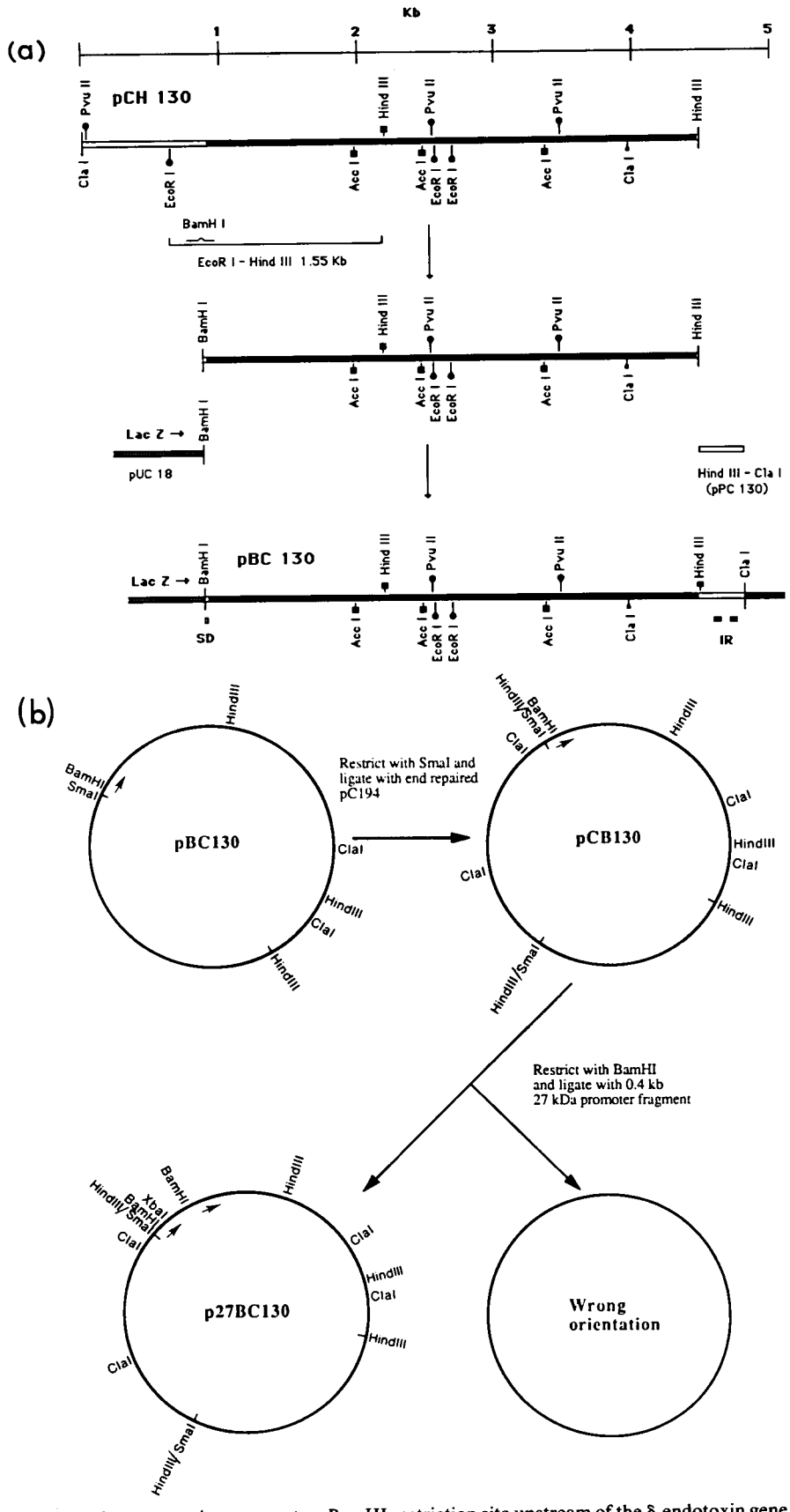

FIG. 6. (a) Use of site-directed mutagenesis to generate a *Bam*HI restriction site upstream of the δ-endotoxin gene (pCH130), and insertion of a 3′ inverted repeat sequence (IR) to generate pBC130. Symbols: ⁓, Mutagenic oligonucleotide; ▬, ORF. SD, Shine-Dalgarno sequence. (b) Strategy used for the construction of p27BC130. Arrowheads indicate the direction of transcription of the δ-endotoxin gene. *Hin*dIII/*Sma*I indicates the junction, following ligation, of an end-repaired *Hin*dIII fragment and a blunt-ended *Sma*I site.

then used to transform sporogenic *B. subtilis* cells. Analysis of 130-kDa gene expression in these cells indicates that at the end of sporulation, sufficient 130-kDa δ-endotoxin is synthesized to be deposited as phase-bright inclusions in approximately 20% of the recombinant cells. These inclusions are smaller than those observed for the 27-kDa δ-endotoxin (53), and as yet only partially purified preparations have been obtained. Immunoblotting experiments with these inclusions indicate that the protein encoded by p27BC130 comigrates with the 130-kDa polypeptide of higher electrophoretic mobility in the *B. thuringiensis* subsp. *israelensis* doublet (Fig. 5). Toxicity assays have been carried out, and the estimated LC_{50} to *A. aegypti* larvae is 0.5 to 1 μg/ml. This protein does not appear to show hemolytic activity when solubilized, digested with *A. aegypti* gut extract, and assayed at 5 μg/ml against rabbit erythrocytes.

DISCUSSION

The results describe the cloning of two δ-endotoxin genes which share considerable homology and encode polypeptides which cross-react with antisera raised against the *B. thuringiensis* subsp. *israelensis* 130-kDa proteins. The pPC130 gene has been described previously (1, 5, 41; Yamamoto, personal communication), and we have used an in vitro *E. coli* system to isolate a *Pst*I fragment, derived from the *B. thuringiensis* subsp. *israelensis* 72-MDa plasmid, which encodes almost the entire structural gene for this 130-kDa protein. Hybridization probes derived from this clone have been used to isolate the complete gene (pPC130), in addition to a 3.9-kb *Cla*I fragment encoding a major part of the coding region of a second homologous gene, pCC130. The remaining portion of this gene has been cloned and used to generate pCH130, which contains a complete ORF encoding an 1,180-amino-acid polypeptide of M_r 134,545. The sequence of this gene has been reported elsewhere (51), and in this paper the corresponding amino acid sequence has been compared with the sequences previously published (39, 45, 48, 50; Yamamoto, personal communication) for several *B. thuringiensis* δ-endotoxin genes. Interesting similarities have been found, both with other *B. thuringiensis* subsp. *israelensis* genes and with a lepidoptera-specific toxin from another serotype. In particular, the homology in the C-terminal regions of the two *B. thuringiensis* subsp. *israelensis* 130-kDa proteins raises questions concerning the evolution of this *B. thuringiensis* strain. Several groups (28, 29, 32–35) have identified transposonlike elements juxtaposed to δ-endotoxin genes, and the ubiquitous nature of these elements suggests a role in mobilizing toxin genes and generating novel toxins. In addition to this, genetic transfer by a conjugationlike mechanism (18) has been observed to occur under laboratory conditions for several strains of *B. thuringiensis*, and this, combined with the fact that many strains harbor multiple copies of δ-endotoxin genes, suggests a mechanism by which *B. thuringiensis* species might enhance their efficacy.

The expression of the cloned genes (pPC130 and pCH130) in recombinant cells has been studied. The pPC130 gene is expressed in *E. coli* recombinants at a level sufficient to produce phase-bright inclusions, and these have been purified and shown to be toxic to *A. aegypti* larvae, with an estimated LC_{50} of 5 to 10 μg/ml. This protein does not have any hemolytic activity under the assay conditions. For the gene encoded by pCH130, however, expression is low in recombinant *E. coli*, when an efficient *E. coli* promoter is ligated adjacent to the structural gene. The reasons for the low level of expression of this gene are not clear. The data suggest that translation of the gene-specific mRNA is limiting, since strong *E. coli* promoters fused to the pCH130 gene produce a relatively low level of expression of this δ-endotoxin.

In an attempt to increase the expression of the pCH130 gene in recombinant cells, we used *B. subtilis* as a cloning host. In addition to a 3′ inverted repeat sequence (retroregulator), the promoter sequences of the *B. thuringiensis* subsp. *israelensis* 27-kDa gene (50, 53) were ligated in transcriptional frame with the structural gene in the chimera used to transform *B. subtilis*. This promoter has been previously shown to function efficiently in *B. subtilis* (53). Expression of the gene in recombinant sporulating *B. subtilis* cells is sufficient to produce inclusions in 20% of the cells; the remaining 80% presumably produce the δ-endotoxin at a level insufficient to accumulate as inclusions. This protein has been partially purified and shown to be toxic to *A. aegypti* larvae, with an LC_{50} of approximately 0.5 to 1 μg/ml. In addition, this protein has been shown to be nonhemolytic under the assay conditions. This polypeptide thus appears to have a higher in vivo toxicity against *A. aegypti* larvae than the protein encoded by pPC130 does. The recent observation (Höfte, Proceedings of the Third European Workshop on Bacterial Protein Toxins) that the N-terminal 634 amino acids of the polypeptide encoded by pPC130 are sufficient for toxicity is consistent with the in vivo toxicity data in this report; the proteins encoded by pPC130 and pCH130 have homologous C-terminal regions, but are significantly different in their N-terminal portions. It will be of interest to determine the function of the homologous regions of these proteins. It is also significant that the isolated *B. thuringiensis* subsp. *israelensis* 130-kDa doublet has been reported to have a higher in vivo activity (47; Chilcott and Ellar, submitted) than either of the two proteins individually, suggesting that these two polypeptides may interact synergistically. Current experiments are directed toward using the two cloned genes described in this report to investigate synergism.

In vitro and in vivo studies of the toxicity of 130-kDa proteins from a wide variety of *B. thuringiensis* strains reveal that even within the same strain, 130-kDa proteins with substantial homology display quite different toxicity spectra (20; B. H. Knowles and D. J. Ellar, *in* F. Fehrenbach, ed., *Bacterial Protein Toxins II*, in press; P. E. Granum, S. M. Pinnavaia, and D. J. Ellar, Eur. J. Biochem., in press). Further analyses of toxicity against a broad range of insect cell lines and dipteran larvae is now in progress to determine whether this is the case for the two toxins in *B. thuringiensis* subsp. *israelensis*.

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