Structural Similarity between the Lepidoptera- and Diptera-Specific Insecticidal Endotoxin Genes of *Bacillus thuringiensis* subsp. “*kurstaki*” and “*israelensis*”

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A gene from *Bacillus thuringiensis* subsp. “*israelensis*” was cloned from the large plasmids of this subspecies and was shown to code for a mosquitocidal polypeptide. The gene could be expressed in either *Escherichia coli*, *Bacillus subtilis*, or *B. thuringiensis* subsp. “*israelensis*” to produce the larvicidal activity. Similarly, a Lepidoptera-specific toxin gene from *B. thuringiensis* subsp. “*kurstaki*” was also cloned and expressed in *E. coli* and *B. subtilis*. Both cloned genes were sequenced and subjected to computer analysis. A long open translational reading frame coded for the *B. thuringiensis* subsp. “*kurstaki*” gene product. However, the *B. thuringiensis* subsp. “*israelensis*” clone was composed of two adjacent open reading frames oriented as if they were in a transcriptional operon. The products of the cloned genes retained their specificity for either Lepidoptera or Diptera. The control regions immediately preceding the toxin genes of both *B. thuringiensis* subspecies showed considerable DNA homology, most likely because both toxins are expressed only during sporulation. In addition, the deduced amino acid sequences from the two contiguous *B. thuringiensis* subsp. “*israelensis*” genes bore a striking resemblance to the deduced amino acid sequence from the single larger *B. thuringiensis* subsp. “*kurstaki*” gene, as if these two arrangements were evolutionarily related.

Insecticidal toxins are synthesized during sporulation of cells of *Bacillus thuringiensis* subsp. “*israelensis*” and subsp. “*kurstaki*” (for recent reviews, see references 8 and 24). Variants of *B. thuringiensis* produce toxins with different spectrums of activity. Upon ingestion, the toxin made by *B. thuringiensis* subsp. “*kurstaki*” kills the larvae of several species of Lepidoptera which are major agricultural pests (6). The *B. thuringiensis* subsp. “*israelensis*” insecticides are toxic to the larvae of mosquitoes (5, 10) and black flies (33, 34). From both a health and economic standpoint, control of these Dipteran species is important, because they are vectors for human diseases such as malaria and onchocerciasis (16).

For both subspecies, the toxins accumulate after log-phase growth ceases in the form of parasporal crystalline inclusions that can account for up to about one-third of the total cellular protein. The parasporal crystals have been purified by passage through gradients of renograffin (28) or sodium bromide (1). For *B. thuringiensis* subsp. “*israelensis*,” the purified crystals contain polypeptide species with relative molecular sizes of about 120, 68, 38, and 26 kilodaltons (kDa) (19, 29, 32, 41). Other minor species are also present. It is not known whether these proteins cooperate to confer the larvicidal activity or whether they act independently. We do know that the intact crystal structure of this subspecies is not absolutely required for toxicity, because alkali-solubilized crystal proteins can be absorbed onto latex beads and can still retain toxicity, although of a much lower potency (22). Just as for intact crystals, the larvae ingest the bead-bound toxins by filter feeding. The solubilized crystal proteins of *B. thuringiensis* subsp. “*israelensis*” are also cytotoxic to cultured animal cells and

hemolytic to human erythrocytes (29). For *B. thuringiensis* subsp. “*kurstaki*,” the crystal is composed of at least two large proteins of about 130 to 135 kDa (4). There is also a minor crystal component of 65 kDa, which surprisingly has some mosquitocidal activity in addition to being toxic to Lepidoptera (11). However, we know of no report describing the isolation from mosquitos of a pathogenic subspecies like *B. thuringiensis* subsp. “*kurstaki*.”

Isolation of DNA clones that code for the protein components of the crystals of *B. thuringiensis* will make it possible not only to direct the synthesis of each protein separately but also to generate specific mutations in *B. thuringiensis* subsp. “*israelensis*.” This will facilitate studies of the function of each gene product alone and in mixtures. Three different fragments of DNA derived from a 72- to 75-megadalton (MDa) plasmid of this subspecies have recently been cloned by three different groups and have been shown to code for mosquitocidal products (27, 36; A. M. Walfield, F. Garduno, L. Thorne, M. Zounes, D. J. Decker, M. A. Wild, and T. J. Pollock, in A. T. Ganesan and J. A. Hoch, ed., *Genetics and Biotechnology of Bacilli*, in press). One of the clones codes for a polypeptide of about 26 kDa that is not only mosquitocidal but also cytotoxic to cultured mosquito cells and hemolytic to erythrocytes (36). The protein product of the second gene has not been reported yet (27). One of the *B. thuringiensis* subsp. “*israelensis*” clones that we initially isolated (Walfield et al., in press) codes for a product of about 58 kDa in either *Escherichia coli*, *Bacillus subtilis*, or a *B. thuringiensis* subsp. “*israelensis*” strain that was first cured of the naturally occurring large plasmid associated with toxicity. The product of this gene is mosquitocidal but lacks the cytotoxic or hemolytic activities.

Schnepf and Whiteley (23) reported the first cloning of a *B. thuringiensis* subsp. “*kurstaki*” toxin gene taken from a

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sample of the commercial pesticide Dipel. Subsequent analysis of similar toxin genes cloned from *B. thuringiensis* subsp. "*kurstaki*" strains shows the existence of different toxin genes located on different plasmid species in a single subspecies strain (12, 38). The *B. thuringiensis* subsp. "*kurstaki*" clone that we describe here is most like the "5.3" gene described by Whiteley et al. (38), because it is derived from the 43-MDa plasmid of *B. thuringiensis* subsp. "*kurstaki*" HD-1 and codes for about 131 kDa of protein. The designation 5.3 refers to the size in kilobase pairs of the restriction fragment, generated by digestion of total cellular DNA with the endonuclease *Hind*III, which hybridizes to an intergenic probe prepared from the initial cloned gene, the 4.5 gene. A third related gene in this subspecies is designated 6.6. In this paper we compare our 5.3 gene to the *B. thuringiensis* subsp. "*israelensis*" gene we previously cloned (Walfield et al., in press). Similarities at the DNA level for the regulatory regions flanking the genes are described. In addition we found homology between the deduced amino acid sequences of the toxin proteins of both *B. thuringiensis* subspecies. The possible implications of the homologies in the regulatory regions and in the structural genes are discussed.

MATERIALS AND METHODS

Bacterial and insect strains. *B. thuringiensis* subsp. "*kurstaki*" HD-1 and *Trichopulsia ni* (cabbage loopers) were obtained from H. T. Dulmage (U.S. Department of Agriculture, Brownsville, Tex.). *B. thuringiensis* subsp. "*israelensis*" ONR60A and *B. subtilis* I168 were provided by J. Hoch (Scripps Clinic and Research Foundation, La Jolla, Calif.). *Aedes aegypti* (mosquitoes) came from A. R. Barr (University of California at Los Angeles, Los Angeles).

Cloning the *B. thuringiensis* subsp. "*kurstaki*" toxin gene. Large plasmids were isolated from stationary-phase cultures (3) and were purified by sucrose gradient velocity sedimentation from *B. thuringiensis* subsp. "*kurstaki*" HD-1. A collection of fragments of the plasmid DNA was generated by partial digestion with *Sau*3A endonuclease, and fragments of about 10 kilobase pairs (kbp) in length were separated by velocity sedimentation in sucrose gradients and purified. Fragments of about 10 kbp were ligated to the *Bam*HI site of the *E. coli* plasmid pBR322, and ampicillin-resistant, tetracycline-sensitive transformants of strain HB101 were isolated to constitute a gene library. The indirect radioimmunoassay (RIA) devised by Erlich et al. (9) was used to identify the clones producing antigens. First, parasporal crystals were purified from sporulated cultures of strain HD-1 by centrifugation through renograffin gradients (1). More than 95% of the crystal protein was composed of a toxin polypeptide of about 130 kDa. A mixture of whole crystals and crystal protein solubilized in alkali and mercaptoethanol was injected into rabbits to raise antiserum. Cell samples to be screened by the RIA were lysed by freeze-thawing in 10 mM Tris hydrochloride (pH 8)–1 mM EDTA–egg white lysozyme (1 mg/ml). To reduce proteolysis and viscosity, we added phenylmethylsulfonyl fluoride to 4 mM, $MgCl_2$ to 10 mM, and pancreatic DNase to 10 μ g/ml. Samples of cells (0.5×10^9 to 1×10^9) were included in each microtiter well. Sample wells were cut out and counted by liquid scintillation to quantitate bound ^{125}I -protein A.

Measurement of Lepidoptera-specific and mosquitocidal activities. Larvae of *T. ni* were fed an artificial diet containing lysates of bacterial cultures prepared as described above for the RIA, but with the phenylmethylsulfonyl fluoride omitted.

The assay protocol and insect propagation methods were adapted from Dulmage et al. (7). Generally we used about 10 larvae in a covered cup containing about 20 ml of the diet, and the assays were scored after 4 days for killing and weight gain (or loss) as a function of dose. Samples containing mosquitocidal activity were added to stagnant tap water containing third- and fourth-instar larvae of *A. aegypti*. Mortality was scored at 24 or 48 h.

Protein analysis. For direct measurement of toxin polypeptides by polyacrylamide gel electrophoresis, samples of bacterial cultures were collected by centrifugation and suspended in a mixture of 1% (wt/vol) sodium dodecyl sulfate, 0.1% (vol/vol) mercaptoethanol, 10% (vol/vol) glycerol, and bromophenol blue. They were heated in a boiling water bath for 4 min immediately before electrophoresis (14). The proteins were stained with Coomassie blue dye or analyzed by RIA after their transfer to nitrocellulose sheets (Walfield et al., in press).

Partial purification of the cloned *B. thuringiensis* subsp. "*israelensis*" toxin. The protein product from the cloned gene of *B. thuringiensis* subsp. "*israelensis*" was partially purified from *B. subtilis* containing recombinant plasmid pSY408 (Walfield et al., in press). A culture of cells in stationary phase was concentrated by centrifugation and lysed with a French press, and bacterial debris was removed by centrifugation in the cold. Proteins were precipitated from the clarified supernatant by slowly adding ammonium sulfate to 10% of saturation at room temperature. The proteins were recovered by centrifugation in the cold, suspended in 10 mM Tris hydrochloride (pH 7.4), and dialyzed against the same buffer.

DNA restriction mapping, sequencing, and computer analysis. Restriction endonucleases were used according to instructions of the manufacturers (International Biotechnology Inc.; New England BioLabs, Inc.; and Bethesda Research Laboratories, Inc.). Restriction mapping, subcloning, and separation of fragments by electrophoresis through horizontal agarose gels were by standard techniques (15). DNA sequences were determined by a chemical method (17) and the chain termination procedure (21). We used a reagent kit purchased from New England Nuclear Corp. for the chemical method. Both universal and sequence-specific synthetic oligomer primers (made on an Applied Biosystems 380A DNA synthesizer) were used for the dideoxy method. The sequencing strategy included overlapping endpoints and reading from more than one gel autoradiograph, with about one-third of the sequence determined for both strands. Analysis of sequence homology included the use of software from International Biotechnologies (20; Forward Homology program) and Intelligenetics. Protein hydropathy was computed by the algorithm of Kyte and Doolittle (13).

RESULTS

Isolation and characterization of the *B. thuringiensis* subsp. "*kurstaki*" toxin clone. A library of recombinant clones with DNA inserts originating from the large plasmids of *B. thuringiensis* subsp. "*kurstaki*" was prepared as described in Materials and Methods. Our preparation of large plasmids included species from about 20 to 50 MDa, but not the very large plasmids of about 150 MDa seen by others (12). The point of insertion in vector pBR322 was the *Bam*HI site within the tetracycline resistance gene. A recombinant plasmid, pSY112, was found that coded for the synthesis in *E. coli* of material that was seroreactive by an indirect RIA with antisera raised against purified crystal protein. Cultures of *E. coli* containing pSY112 were grown for insect toxicity assays

and for identification of the seroreactive antigen by polyacrylamide gel electrophoresis. *E. coli* cells carrying this plasmid accumulated a protein antigen of the size expected for the *B. thuringiensis* subsp. "*kurstaki*" crystal toxin (about 130 to 135 kDa) and were toxic to *T. ni* in diet incorporation assays with either cell extracts or whole cells. The toxin protein was faintly visible by simply staining a gel with Coomassie blue dye.

The cloned toxin gene normally resides on a plasmid of *B. thuringiensis* subsp. "*kurstaki*" of about 43 MDa. A recombinant plasmid carrying the cloned insert was radioactively labeled by nick translation and was hybridized to plasmids of this subspecies after transfer to a nitrocellulose filter. Only the 43-MDa species was homologous (data not shown). The absence of hybridization to the related toxin genes described by others (12) could reflect the combined effects of the annealing stringency and the low amount of the 150-MDa plasmid DNA in our DNA preparations. Because the fragments to be cloned from the partial *Sau3A* digest were initially selected for sizes of about 10 kbp, the 10-kbp insert in pSY112 must have represented contiguous sequences on the 43-MDa plasmid of *B. thuringiensis* subsp. "*kurstaki*."

A partial restriction map of pSY112, including the 10-kbp insert, is shown in Fig. 1. Also shown is a more detailed map of the internal *HincII* fragment. For subclone pSY177, the *HincII* fragment of 4 kbp was inserted into the *HincII* site within the tetracycline resistance gene of pBR322. The insert orientation with respect to the gene for tetracycline resistance was the same for pSY112 and pSY177, but in pSY176 the toxin gene was oriented in the opposite direction. The entire structural gene for the *B. thuringiensis* subsp. "*kurstaki*" toxin was carried on each of these two subclones. They coded for an antigen of about 130 kDa in *E. coli*.

To measure the amount of the 130-kDa protein in cell extracts, we separated proteins by electrophoresis through polyacrylamide gels. By comparison with Coomassie-stained standards, we judged that at least 5% of the cellular protein of *E. coli* accumulated as the *B. thuringiensis* subsp. "*kurstaki*" toxin for the pSY177 subclone and about 3%

FIG. 1. Restriction endonuclease sites of plasmid pSY112 and the internal *HincII* fragment. In the top diagram, the flanking boxes indicate vector pBR322 sequences. In the bottom diagram, the box indicates the structural gene for the toxin protein and the numbers refer to nucleotide positions. The restriction sites are abbreviated: B, *BclI*; C, *Clal*; HC, *HincII*; HD, *HindIII*; K, *KpnI*; NR, *NruI*; ND, *NdeI*; PS, *PstI*; PV, *PvuII*; R, *EcoRI*; SA, *Sall*; SC, *ScaI*. Other abbreviations: ORI, origin of replication; AMP, ampicillin resistance gene; NH, amino terminus; COOH, carboxy terminus.

FIG. 2. Separation of proteins by polyacrylamide gel electrophoresis from *E. coli* cells carrying the cloned *B. thuringiensis* subsp. "*kurstaki*" toxin gene. Lanes: A, purified crystal protein; B, pBR322 vector; C, pSY176; D, pSY177; E, pSY112. The contents of equal numbers of cells were loaded into lanes B through E, and the gel was stained with Coomassie blue.

accumulated for pSY176. This is much more than was seen for the original clone pSY112. The gel is shown in Fig. 2. Both pSY177 and pSY176 also produced 20 to 50 times more insecticidal activity per cell than did the original construction pSY112. The results of the bioassays with *T. ni* larvae are presented in Table 1.

A series of specific deletions were generated to locate the region of the structural gene that is essential for toxin activity. The deletions were constructed by using the restriction sites shown in Fig. 1 with and without the exonuclease BAL 31. The deletion derivatives were examined for synthesis of polypeptides that reacted with antiserum raised against *B. thuringiensis* subsp. "*kurstaki*" crystals and for their toxicity to larvae of the cabbage looper. The results (not shown) supported the findings of others (25, 37, 39) for the 4.5 toxin gene of this subspecies. First, the DNA sequences immediately distal to the coding regions of both the 4.5 and 5.3 genes were required for maximal gene expression. Second, internal deletions in the C-terminal half of each gene, but not the N-terminal half, retained larvicidal activity.

Isolation and characterization of the *B. thuringiensis* subsp. "*israelensis*" toxin clone. A summary of the cloning manipulations (Walfield et al., in press) is provided below. The large plasmids of *B. thuringiensis* subsp. "*israelensis*" strain ONR60A were purified and digested partially with *Sau3A* restriction enzyme. Fragments of about 10 kbp in length were purified and inserted into the bacteriophage vector λ L47.1. Plaques were screened in situ for the expression of crystal protein antigens with rabbit antiserum raised against purified crystal proteins. The antigenic proteins made by the seroreactive recombinant bacteriophage were cataloged by using an *E. coli* maxicell system: UV-irradiated cells were infected with recombinant bacteriophage, and the proteins were labeled with [35 S]methionine and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The clone of the toxin gene described here was referred to as

TABLE 1. Toxicity of plasmid-bearing *E. coli* HB101 to *T. ni* larvae

Relative	Wt (g) ^a of 10 larvae fed with cells bearing plasmid:			
	pBR322	pSY112	pSY177	pSY176
1	1.3	1.0	0.45	0.5
3	1.1	1.0	0.3	0.5
10	1.3	0.9	0.1	0.1
30	0.9	0.7	0.05	0.05
100	1.0	0.35	0.03	0.03
300	0.7	0.1	0.03	0.03

^a Determined after 4 days.^b A relative amount of 1 was equal to 10⁷ cells bearing plasmid pBR322, pSY112, pSY177, or pSY176 added to 20 ml of the diet.

F1 and carried an insert of about 11 kbp from the 70- to 75-MDa plasmid of *B. thuringiensis* subsp. "*israelensis*" as shown by DNA hybridization experiments. Extracts of bacteria infected with bacteriophage F1 were toxic to *A. aegypti*. We do not know whether the toxin has a wider spectrum of activity among the Diptera; however, it was not toxic to *T. ni* (Lepidoptera) even in large doses.

A restriction cleavage map for the F1 insert and a subclone, pSY368, that retains mosquitocidal activity is shown in Fig. 3. Also indicated are the extents of several deletion mutations that helped to define the region essential for mosquitocidal toxicity in relation to the two open translational reading frames (ORF1 and ORF2) identified in the DNA sequence. Plasmid isolates pSY408 and pSY626 were both toxin producers, and this suggests that ORF1 was necessary for toxicity. Because of the position of the 626 deletion endpoint, however, we cannot rule out the involvement of the N-terminal portion of ORF2 in toxicity.

Lyophilized *B. subtilis* cells harboring plasmid subclone pSY408 (a composite plasmid made up of the *Staphylococcus aureus* plasmid pUB110 and the *B. thuringiensis* subsp. "*israelensis*" insert) were considerably more toxic to mosquito larvae than were cells of *E. coli* bearing the subclone pSY368 (a composite of pUC12 and *B. thuringiensis* subsp. "*israelensis*" DNA). The toxicity of the *B. subtilis* subclone is indicated in Table 2. The 50% lethal concentration for *B. subtilis* (pSY408) was about 0.1 mg of lyophilized cells per ml of assay volume. The 50% lethal concentration for *E. coli* (pSY368) was about 2 mg/ml.

We partially purified the cloned gene product from *B. subtilis* containing the recombinant plasmid pSY408 as described in Materials and Methods. Protein samples from the purification were electrophoresed through sodium dodecyl sulfate-polyacrylamide gels. A Coomassie-stained gel is shown in Fig. 4. Lane 2 shows the unfractionated cell extract, and lane 4 shows the partially purified toxin from the cloned gene. Both the bacterial debris after cell lysis and the dialyzed ammonium sulfate precipitate were toxic to mosquito larvae. However, when compared with the 20 or more Coomassie blue-stained protein species seen after electrophoresis of debris samples, the precipitate showed only 3 prominent and equally stained proteins. The three proteins had relative molecular sizes of about 150, 70, and 58 kDa. Rabbit antisera raised against total *B. thuringiensis* subsp. "*israelensis*" crystal protein or denatured gel-purified 26-kDa crystal protein of this subspecies (Walfield et al., in press) reacted only with the 58-kDa protein on Western blots (see lanes 6 and 7). The crystals from strain ONR60A that were used to raise antisera were first purified by sedimentation through sodium bromide density gradients (1). The

proteins present in these crystal preparations are shown in lane 5.

We compared the hemolytic activity of the product of the clone that accumulated in *B. subtilis* with alkali-solubilized crystal proteins. The 58-kDa product represented about 25% of the total protein in extracts precipitated with ammonium sulfate, as judged by inspecting gel bands stained with Coomassie blue dye (Fig. 4). Protein samples at 10 mg/ml were solubilized in 50 mM NaOH–10 mM EDTA at pH 11.8 and then brought to pH 7.8 with Tris buffer and HCl. Samples of 0.05 ml each were mixed with an equal volume of human erythrocytes in microtiter wells to detect hemolysis. Red halos of differing intensity surrounding the sedimented unlysed cells in the bottom of the concave dish indicated hemolysis. Solubilized crystal proteins of *B. thuringiensis* subsp. "*israelensis*" caused increasing hemolysis as the added protein increased from 2 to 50 µg; the lysis was barely detectable for the 1-µg sample. However neither buffer nor proteins from *B. subtilis* carrying plasmid vector pUB110 or recombinant pSY408 caused detectable hemolysis at these same protein concentrations.

Previously we had isolated derivatives of *B. thuringiensis* subsp. "*israelensis*" ONR60A that were cured of the large plasmid, that failed to make the major crystal antigens, and that lacked toxicity to mosquitos (Walfield et al., in press). We reintroduced our toxin clone into the cured *B. thuringiensis* subsp. "*israelensis*" cells by transformation with pSY408, selecting for resistance to kanamycin. The size of the accumulated antigen was 58 kDa, just as in *E. coli* and *B. subtilis*. The toxicity expressed per cell was about 10-fold lower than that of the wild-type strain ONR60A.

FIG. 3. Restriction endonuclease sites and deletion map for the toxin gene from *B. thuringiensis* subsp. "*israelensis*." The top diagram is the original insert in the bacteriophage vector λL47.1. The middle diagram shows an expansion of the region subcloned in plasmid pSY368. At the bottom are boxes that represent deleted regions in plasmid derivatives. +, Toxicity to mosquito larvae; –, no toxicity. The region of DNA that was sequenced is also shown along with the location of ORF1 and ORF2.

DNA sequences for toxin genes. The *B. thuringiensis* subsp. "kurstaki" *HincII* fragment of 4,074 base pairs was sequenced. The complete sequence is given in Fig. 5a. The sequence includes 154 nucleotides upstream from the start of the structural gene, 3,468 bases coding for a toxin protein of 1,156 amino acids, and an untranslated region downstream of 452 bases. We compared our DNA sequence of the 5.3 gene with that of the 4.5 gene published previously (26). The regulatory region and structural gene sequences showed extensive homology except in two regions. (i) There was divergence between the small internal *EcoRI-EcoRI* fragment of 580 base pairs from the 4.5 gene and the corresponding 586-base-pair fragment of the 5.3 gene. (ii) The 5.3 gene was missing 26 contiguous codons that begin at nucleotide 2903 of the 4.5 gene. Both of these differences were initially noticed when we compared the hydropathy profile of the 4.5 gene product (38) with a similar plot from our deduced amino acid sequence. The apparent deletion removed about 2,800 daltons of protein and by itself could have accounted for the smaller size of the 5.3 gene product (ca. 131,000 compared with 133,500 daltons). In fact, a deletion may have arisen by recombination between two direct repeats of AAAGTGTG that are found in the 4.5 gene sequence.

The sequence of the *B. thuringiensis* subsp. "israelensis" gene that codes for the mosquitocidal toxin is given in Fig. 5b. We sequenced 3,753 nucleotides of the cloned DNA and found two large open reading frames for translation, ORF1 and ORF2, which were oriented in the same direction. As described above, we think that ORF1 coded for the toxin. ORF1 and ORF2 could have coded for proteins of about 72 kDa and at least 28 kDa, respectively. ORF2 extended beyond the boundary of our sequence determination. Three clustered potential start points for translation of ORF1 were located at bases 911 (GTG), 926 (ATG), and 941 (ATG), as well as two downstream sites at bases 1088 (GTG) and 1187 (ATG). The ATG codon at base 941 was preceded by the sequence GGAGG centered at base 931, which is consistent with a consensus ribosome-binding site. The other potential starts showed less recognizable homology and deviant spacing arrangements when compared with the consensus ribosome-binding sequence. The last base of ORF1 was at position 2965 and was followed by a TAA codon. A potential start for ORF2 was located at position 3035 and was preceded by the possible ribosome-binding-site sequence AAAGGTCGTGAAC. Therefore, as few as 64 bases might have separated ORF1 and ORF2. We were unable to compare the DNA sequence of the clone with the N-terminal amino acid sequences of the *B. thuringiensis* subsp. "israelensis" crystal proteins or with the cloned gene prod-

FIG. 4. Partial purification of the cloned *B. thuringiensis* subsp. "israelensis" toxin from *B. subtilis*. The fractionation of cell extracts is described in Materials and Methods. The sodium dodecyl sulfate-polyacrylamide gel in lanes 1 through 5 was stained with Coomassie blue. Lanes: 1, Molecular weight standards of Bio-Rad Laboratories; 2, total lysate of *B. subtilis* I168 carrying recombinant plasmid pSY408; 3, bacterial debris from cell lysate collected by centrifugation; 4, proteins precipitated by adding ammonium sulfate to 10% of saturation; 5, *B. thuringiensis* subsp. "israelensis" crystal proteins; 6, crystal proteins as in lane 5; 7, ammonium sulfate precipitate as in lane 4. Lanes 6 and 7 were electrophoresed on a separate gel; the proteins were transferred to nitrocellulose and immunoblotted with anti-*B. thuringiensis* subsp. "israelensis" toxin antiserum. ▲, Partially purified 58-kDa antigen.

uct accumulated in *B. subtilis*, because the polypeptides did not yield to Edman degradation.

Comparison of toxin gene sequences. We compared the *B. thuringiensis* subsp. "kurstaki" DNA sequence to the sequences for the two contiguous *B. thuringiensis* subsp. "israelensis" genes. Two regions of strong homology were identified by the Forward Homology computer analysis and are shown in Fig. 6. The DNA homologies were not located in the structural genes. For the *B. thuringiensis* subsp. "kurstaki" gene, the homology lay about 70 to 100 bases upstream from the start point for translation. For this gene, the start point for translation was known from sequencing the amino terminus of the crystal protein (data not shown). The position of the homology in the putative *B. thuringiensis* subsp. "israelensis" control region was about 80 base pairs upstream from the most likely start point of translation.

Although there was limited homology between the structural gene sequences of the two subspecies at the DNA level, there were striking similarities in the deduced amino acid sequences in three regions. We show hydropathicity plots for the two genes in Fig. 7. Both proteins began with a hydrophilic region followed by a hydrophobic segment of about 32 amino acids. Approximately 200 ± 10 amino acids downstream there was a sharp transition from hydrophobic to hydrophilic residues. This structural similarity caused us to directly compare the amino acid sequences, again by the Forward Homology analysis. As we varied the stringency and the window of the homology search, regions of similarity emerged on the dot matrices. A representation of the Forward Homology printout is given in Fig. 8. The vertical axis is the deduced amino acid sequence for the two contiguous genes from *B. thuringiensis* subsp. "israelensis" with the DNA sequence from the intergenic region (bases 3011 through 3034) deleted. The horizontal axis is the protein sequence for the 5.3 *B. thuringiensis* subsp. "kurstaki" gene.

TABLE 2. Toxicity of plasmid-bearing *B. subtilis* I168 to larvae of *A. aegypti*

Concn of lyophilized cells ($\mu\text{g/ml}$) ^a	% Larvae mortality ^b from:	
	pUB110	pSY408
30	0	20
60	0	30
125	0	70
250	0	80
500	0	100
1,000	0	100

^a Whole cells were harvested by centrifugation and were lyophilized, and weighed samples were added to water containing larvae.

^b Determined at 24 h with 10 larvae per sample.

a	10 20 30 40 50	60 70 80 90 100	110 120 130 140 150	160 170 180 190 200
	GTAAACACCE TGGGTCAAAA ATTGATATTT AGTAAATTA GTTGCATTT	GTGCTTTTT CATAAGATGA BTGATATGTT TTAATTTGCA GTAATGAAA	ACAGTATTAT ATCATAATGA ATTGATATCT TAATAAAGA GATGGAGTA ACT	
195	ATGGATACACCTCCACATCAATGAATTCCTTATATTTGTTAGTAACTTGAAGTAGAA	MetAspAsnAsnProAsnIleAsnGluCysIleProIleAsnCysLeuSerAsnProGluValGlu	220	
221	GTATTAGTGGAGAAAGATAGAACTGTTACACCAATCAGATATTCCTTGTCTCAACGCAA	ValLeuGluGlyGluArgIleGluThrGlyTyrThrProIleAspIleSerLeuSerLeuThrGln	286	
287	TTCTTTTGTAGTAAATTTGCTCCGCTGATTTGTGTAGGACTAGTGATATAATATGGGGA	PhoLeuLeuSerGluPheValProGlyAlaGlyPheValLeuGlyLeuValAspIleIleTrpGly	352	
353	ATTTTGTGCTCTCAATGGAGACATCTTGTACAAATTAACAGATTAATTAACCAAGATA	IlePheGlyProSerGlnTrpAsnAlaLeuValGlnIleGluGlnLeuIleAsnGlnArgIle	418	
419	BAAGATTCCTTGAACCACTGATTCAGTATTAAGGACTAAGCAATCTTATCAAAATTAAC	GluGluPheAlaArgAsnGlnAlaIleSerArgLeuGluGlyLeuSerAsnLeuTyrGlnIleTyr	484	
485	GCAGATCTTTTGAAGATGGAGACATCTCTAATCCAGCATTAGAGAGAGATGCGTATT	AlaGluSerPheArgGlyTrpGluAlaAspProThrAsnProAlaLeuArgGluGluMetArgIle	550	
551	CAATTCAATGACATGAACATGCTTACACCCCTTCTCTTTTTCAGTCAAAATATTACAA	GlnPheAsnAspMetAsnSerAlaLeuThrThrAlaIleProLeuPheAlaValGlnAsnTyrGln	616	
617	GTCTCTCTTTTATCAGTATATGTTCAACATGCAATTTACATTTATCAGTTTGAAGATGTTCA	ValProLeuLeuSerValThrValGlnAlaAlaAsnLeuHisLeuSerValLeuArgAspValSer	682	
683	GTGTTTGAACAGGTGGGATTTGATGCTGCTATTAAGTATGATTAATGATTAAGTATGAG	ValPheGlyGlnArgTrpGlyPheAspAlaAlaThrIleAsnSerArgTyrAsnAspLeuThrArg	748	
749	CTATTGCAACTATACAGATCATGTTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	LeuIleGlyAsnTyrThrAsnHisArgValArgTrpTyrAsnThrGlyLeuGluArgValTrpGly	814	
815	CCGATCTAGAGATTTGATGATGATTAATCAATTTAGAGAGAAATTAACATTAATGAT	ProAspSerArgAspTrpIleArgTyrAsnGlnPheArgGluGluLeuThrLeuValLeuAsp	880	
881	ATGCTGCTCTATTTCCAGCATATGATGATGATGATGATGATGATGATGATGATGATGATGAT	IleValSerLeuPheProAsnTyrAspSerProHisLeuMetAspIleLeuAsnSerIleThrIleTyrThrAsp	946	
947	AGAGAAATTTATACAAACCAATATTAAAGAAATTTGATGATGATTTTCGAGCTGCTGCTGCTGCT	ArgGluIleTyrThrAsnProValLeuGluAsnPheAspGlySerPheArgGlySerAlaGlnGly	1012	
1013	ATAGAGAAATTTATAGAGATCCACATTTGATGATGATGATGATGATGATGATGATGATGATGAT	IleGluGlySerIleArgSerProHisLeuMetAspIleLeuAsnSerIleThrIleTyrThrAsp	1078	
1079	GCTCATAGAGAGAAATTTATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	AlaHisArgGlyGlyTyrTyrTrpSerGlyHisGlnIleMetAlaSerProValGlyPheSerGly	1144	
1145	CCAGAAATTTCACTTTTCCGCTATATGAACTATGAGAAATGCACTCCACACACGATTTGCT	ProLeuPheThrPheProLeuTyrGlyThrMetGlyAsnAlaAlaProGlnGlnArgIleValAla	1210	
1211	CAACTAGGTCAGGCTGATATGAACTATTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	GlnLeuGlyGlnGlyValTyrArgThrLeuSerSerThrLeuTyrArgArgProPheAsnIleGly	1276	
1277	ATAAATATCAGCACTATCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	IleAsnAsnGlnGlnLeuSerGlyPheAspGlyGlyArgIleCysLeuTrpAsnLeuLeuLysPhe	1342	
1343	GGCAACCCGCTATACAGAAAGCGGAGCTGATGATGATGATGATGATGATGATGATGATGATGAT	GlyGlnProProTyrThrGluLysAlaGluProValAspSerProAspGluIleProProGlnAsn	1408	
1409	AACACCTGCACTATGCAAGGATTTAGTATCATGATGATGATGATGATGATGATGATGATGATGAT	AsnAsnValProProArgGlnGlyPheSerHisArgCysLeuAlaTyrValSerMetPheTyrSer	1474	
1475	GGCTTTATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	GlyPheSerAsnSerSerValSerValIleArgAlaProAsnAspSerSerTrpThrTyrCysSer	1540	
1541	GCTGAATTTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	AlaGluPheGlyAspValIleProSerSerGlnIleThrGlnIleProLeuThrLysLeuGlnSer	1606	
1607	TGGCTGCACTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	TrpLeuTrpAsnSerValValLysGlyLeuGlyPheThrGlyGlyAspIleLeuGluGluLeuThr	1672	
1673	GGCAGATTTCACTTTAAGATTAATATTATGCACTATACCAAGATATCGGATGAAGAT	GlyGlnIleSerThrLeuArgValAsnIleThrAlaProLeuSerGlnArgTyrArgValArgIle	1738	
1739	CGCTACGCTTACCAAAATTTACAAATTTACATCATTAATGACGAAACCTATTAAACGGGG	ArgTyrAlaSerThrThrAsnLeuGlnPheHisThrSerIleAspGlyArgProIleAsnGlnGly	1804	
1805	AAATTTTCACTATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	AsnPheSerAlaThrMetSerSerGlySerAsnLeuGlnSerGlySerPheArgThrValGlyPhe	1870	
1871	ACTACTGCTTTAACTTTTCAATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	ThrThrProPheAsnPheSerAsnGlySerSerValPheThrLeuSerAlaHisValPheAsnSer	1936	
1937	GGCAATGATTTATATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	GlyAsnGluValTyrIleAspArgIleGluPheValProAlaGluValThrPheGluAlaGlyTyr	2002	
2003	GATTTAGAGAGACAGAAAGCGGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	AspLeuGluArgAlaGlnLysAlaValAsnGluLeuPheThrSerSerAsnGlnIleGlyLeuLys	2068	
2069	ACAGAT	ThrAspValThrAspTyrHisIleAspGlnValSerAsnLeuValGluCysLeuSerAspGluPhe	2134	
2135	TGCTGGAT	CysLeuAspGluLysLysGluLeuSerGluLysValLysHisAlaLysArgLeuSerAspGluArg	2200	
2201	AAATTTACTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	AsnLeuGluGlnAspProAsnPheArgGlyIleAsnArgGlnLeuAspArgGlyTrpArgGlyAsn	2266	
2267	ACGATATTTACATCCAGGAGGCTATGATGATGATGATGATGATGATGATGATGATGATGATGAT	ThrAspIleThrIleGlnGlyGlyHisAspValPheLysGluAsnTyrValThrLeuLeuGlyThr	2332	
2333	TTTATGAT	PheAspGluCysTyrProThrTyrLeuTyrGlnLysIleAspGluSerLysAlaTyrThr	2398	
2399	CGTTACCAATTAAGAGGCTATATCAGATGATGATGATGATGATGATGATGATGATGATGATGAT	ArgTyrGlnLeuArgGlyTyrIleGluAspSerGlnAspLeuGluIleTyrLeuIleArgTyrAsn	2464	
2465	GCCAAACACCAACAGTAAATGTCAGGATGATGATGATGATGATGATGATGATGATGATGATGAT	AlaLysHisGluThrValAsnValProGlyThrGlySerLeuTrpProLeuSerAlaProSerPro	2530	
2531	ATCGAAATGTCGCTATCTCCATCATTTCTCTTGGACATGATGATGATGATGATGATGATGATGAT	IleGlyLysCysAlaHisHisSerHisHisPheSerLeuAspIleAspValGlyCysThrAspLeu	2596	
2597	AAATGAGCTTATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	AsnGluAspLeuGlyValTrpValIlePheLysIleLysThrGlnIleAspGluAlaLysValLys	2662	
2663	AACTAGAAATTTCTCAAGAGAACCATTAAGAGAGACATGATGATGATGATGATGATGATGATGAT	AsnLeuGluPheLeuGluGluLysProLeuValGlyGluAlaLeuAlaLeuValLysArgAlaGlu	2728	
2729	AAATGAGGAGAGACAACTGTCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	LysLysTrpArgAspLysArgGluLysLeuGluGluTrpGluThrAsnIleValTyrGluAlaLys	2794	
2795	GAATCTGAT	GluSerValAspAlaLeuPheValAsnSerGlnTyrAspArgLeuGlnAlaLysValThrAsnIleAla	2860	
2861	ATGATCTATCGGAGAGATTAACGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	MetIleHisAlaAlaAspLysArgValHisSerIleArgGluAlaTyrLeuProGluLeuSerVal	2926	
2927	ATTCGAGGTCGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	IleProGlyValAsnAlaAlaIlePheGluGluLysGluGlyArgIlePheThrAlaPheSerLeu	2992	
2993	TATGATCGGAGAGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	TyrAspAlaArgAsnValIleLysAsnGlyAspPheAsnAsnGlyLysSerCysTrpAsnValLys	3058	
3059	GGCATGAT	GlyHisValAspValGluGluGluGlnAsnHisArgSerValLeuValProIleTrpGluAla	3124	
3125	GAATGTCACAGAGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	GluValSerGlnGluValArgValCysProGlyArgGlyTyrIleLeuArgValThrAlaTyrLys	3190	
3191	GAGGATATGAGAGAGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	GluGlyTyrGlyGlyCysValThrIleHisGluIleGluAsnAsnThrAspGluLeuLysPhe	3256	
3257	AGCAATCTGATGAGAGAGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	SerAsnCysValGluGluValTyrProAsnAsnThrValThrCysAsnAspTyrThrAlaThr	3322	
3323	CAAGAGAAATATGAGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	GlnGluGluLysGlyGlyTyrThrTyrSerArgAsnArgGlyTyrAspGlyAlaTyrGlnSerAsn	3388	
3389	TCTCTGAT	SerSerValProAlaAspTyrAlaSerAlaTyrGluGluLysAlaTyrThrAspGlyArgArgAsp	3454	
3455	AATCTTGTGAT	AsnProCysGluSerAsnArgGlyTyrGlyAspTyrThrProLeuProAlaGlyTyrValThrLys	3520	
3521	GAATGAT	GluLeuGluTyrPheProGluThrAspLysValTrpIleGluIleGlyGluGluGluGluGluGluGluGlu	3586	
3587	ATCGTGGACAGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	IleValAspSerValGluLeuLeuLeuMetGluGlu	3652	
		3630 3640 3650		
		TATAT GCTTTAAAT GTAGGTGTG		
	3660 3670 3680 3690 3700			
	CAATAAAGA ATGATTACTG ACTGTGATG ACAGATAAT AAGGAATTT			
	3710 3720 3730 3740 3750			
	TTATATGAT AAAAAGCGG CATCATCTT AAAAGATGA GTGCCCTTT			
	3760 3770 3780 3790 3800			
	TTGTATGAT TAACGATGA TATTTAATG TTTTTTCG AGGCTTTAC			
	3810 3820 3830 3840 3850			
	TTACGCGGT ACCGCCCAT GCCCATCA TTAAGATT BCATACCC			
	3860 3870 3880 3890 3900			
	CAAGTGTCA AAACGTTAT TCTTTCTAA AGCTAGCTA GAAGGATGA			
	3910 3920 3930 3940 3950			
	CATTTTAT GAATCTTCA ATTCAGATG AATTACACT ATTTCTGAA			
	3960 3970 3980 3990 4000			
	GAGCTGATC GTCATTTAC CCCCCTCT TGGAGAAC TCCTAAGA			
	4010 4020 4030 4040 4050			
	ATTAGTTTT GTAAAGAA AACGGAAG TTCAGAAAT GAATTAGCTA			
	4060 4070			
	CCATATGTAT CTGGCTGAT CGAC			

At least three regions of protein homology were apparent from the Forward Homology analysis, and the corresponding amino acid sequences are indicated in Fig. 9. The numbers refer to the respective amino acid positions. The numbers with asterisks for *B. thuringiensis* subsp. "*israelensis*" were assigned as if the two nonsense codons of the

intergenic region were deleted from the DNA sequence. This then causes the ORF1, intergenic region, and ORF2 to be considered as a single polypeptide chain. Between the sequences the triple dots represent exact homology, and the single dots indicate similar amino acids. The steep transition between the hydrophobic region of 15 amino acids and the

FIG. 5. DNA sequence and deduced amino acids for the toxin genes of *B. thuringiensis* subsp. “*kurstaki*” (a) and “*israelensis*” (b). Both sequences are shown in the 5'-to-3' direction. The nucleotide coordinates are the same as in the restriction maps in Fig. 1 and 3.

adjacent hydrophilic region of 16 residues that is seen on the hydropathicity plot in Fig. 7 is centered at about amino acid 298 for *B. thuringiensis* subsp. “*israelensis*” in the uppermost set.

DISCUSSION

We cloned the 5.3 toxin gene, as designated by Whiteley et al. (38), from *B. thuringiensis* subsp. “*kurstaki*” HD-1. By

DNA hybridization analysis, the toxin gene was shown to reside on a 43-mDa plasmid in this subspecies. The DNA sequence of the gene indicated that the protein has a molecular weight of about 131,000. The other *B. thuringiensis* subsp. “*kurstaki*” toxin gene studied in considerable detail was the 4.5 gene (23, 25, 26). A comparison of the two genes showed extensive conservation of DNA and protein

sequences. We must note, however, that the most divergent region between these related genes was found within the essential toxin domain, the N-terminal half of the structural gene. The apparently nonessential C-terminal half was highly conserved.

Certain properties of the two *B. thuringiensis* subsp. "kurstaki" toxin products and their mode of expression were shared as well. (i) Both genes were expressed in *E. coli*. (ii) Expression of the 4.5 gene in *E. coli* was enhanced either by insertion of Tn5 between the *B. thuringiensis* subsp. "kurstaki" promoter sequence and an AT-rich region 50 to 175 bases upstream from the transcriptional start points or simply by deletion of the AT-rich sequences (H. R. Whiteley, H. E. Schnepf, and H. C. Wong, in A. T. Ganesan and J. A. Hoch, ed., *Genetics and Biotechnology of Bacilli*, in press). We observed a similar effect on expression for the 5.3 gene. In the process of subcloning the toxin gene from the original isolate (pSY112), we generated constructions (pSY177 and pSY176) that accumulated 20 to 50 times more toxic activity and toxin in *E. coli*. The subclones were missing the DNA sequences more than 70 base pairs upstream from the promoter region. (iii) There were sequences distal to the C terminus that enhanced expression of the 4.5 gene by stabilizing the mRNA (39). Our deletion analysis for the 5.3 toxin gene suggests a similar phenomenon (data not shown). (iv) The 5.3 and the 4.5 toxin genes shared amino acid sequences with a toxin isolated from *B. thuringiensis* subsp. "dendrolimus" T84A1 within the N-terminal quarter of the protein. When the latter toxin is treated with trypsin, a fragment of about 55 to 60 kDa is produced that has full activity against larvae of *Bombyx mori* and has the N-terminal sequence Ile-Glu-X-Gly-Tyr-Thr (18). We treated the crystal protein purified from *B. thuringiensis* subsp. "kurstaki" HD-1 with trypsin and generated a set of three similar trypsin-resistant fragments that each had an N-terminal sequence of Ile-Glu-Thr-Gly-Tyr (G. P. Textor, C. E. Richardson, and T. J. Pollock, unpublished data). The three fragments of about 55 to 60 kDa each were indistinguishable by polyacrylamide gel electrophoresis under denaturing conditions but did separate by high-performance liquid chromatography. Presumably these three derived from the three toxin genes found in HD-1. Contrary to the results with *B. thuringiensis* subsp. "dendrolimus," however, we found that the trypsin treatment raised the specific activity of the toxin by four- to fivefold in assays against *T. ni*. This is similar to the results of Tojo and Aizawa (31), who were the first to show activation of the *B. thuringiensis* subsp. "kurstaki" HD-1 crystal proteins with gut juice protease from *B. mori*. Furthermore, the region of the HD-1 toxin removed by the protease closely matches the region that can be deleted without eliminating all of the insecticidal activity (35, 37).

The polypeptides associated with the parasporal crystals made by *B. thuringiensis* subsp. "israelensis" include major

FIG. 6. DNA sequence homologies between the control regions of the genes of *B. thuringiensis* subsp. "kurstaki" (BTK) and "israelensis" (BTI). —, Homologous sequences; ==, ribosome binding sites; ===, initiation codons.

FIG. 7. Analysis of hydropathy of the deduced amino acid sequences of the toxin genes of *B. thuringiensis* subsp. "israelensis" (BTI) and "kurstaki" (BTK). The hydropathic index was calculated and plotted by the method of Kyte and Doolittle (13), by averaging values over a span setting of seven amino acids. Positive values, hydrophobic; negative values, hydrophilic. The N terminus is at the left. The intergenic region between *B. thuringiensis* subsp. "israelensis" ORF1 and ORF2 occurs at position 675.

species with molecular sizes of about 120, 68, 38, and 26 kDa. The functional and structural relationships among these proteins are not well known. Of these, the smallest polypeptide (26 kDa) has received the most attention. From an analysis of the crystal proteins made by various strains derived by mutagenesis of *B. thuringiensis* subsp. "israelensis" ONR60A, Yamamoto et al. (41) have concluded that a partially purified 25- to 28-kDa protein is toxic to mosquito larvae, cytotoxic to cultured animal cells, and hemolytic to erythrocytes. An extensively purified form of this protein that was generated by treating solubilized crystal proteins with trypsin and proteinase K has similar activities (2). These findings are consistent with earlier observations made for mixtures of crystal proteins (29). Recently, the gene that codes for this protein was cloned from the 72- to 75-MDa plasmid of *B. thuringiensis* subsp. "israelensis," and extracts of *E. coli* cells carrying the cloned DNA were toxic to mosquito larvae, cytotoxic to cultured insect cells, and hemolytic to erythrocytes (36).

We cloned a separate gene from the large plasmids of *B. thuringiensis* subsp. "israelensis" and expressed the gene in *E. coli* and *B. subtilis*. The gene product of the clone accumulated in *B. subtilis* as a protein with a relative molecular size of about 58 kDa and was toxic to mosquito larvae but had no hemolytic activity. We believe that the accumulated species might have been the result of protein processing, because the cloned insert coded for polypeptides as large as about 72 kDa when gene expression was carried out in vitro with an *E. coli* transcription and translation system (data not shown). We found no homology between

FIG. 8. Diagram of a matrix showing the homologous amino acids present in the toxin proteins of *B. thuringiensis* subsp. "kurstaki" (BTK) and "israelensis" (BTI). The protein of the former is displayed on the horizontal axis, and ORF1 and ORF2 from the latter are shown on the vertical axis. The sequence of the latter is plotted as if the intergenic region of 64 nucleotides were deleted, resulting in a single polypeptide chain. Absence of datum points indicates homology of less than 40% over a window of 21 amino acids.

the deduced amino acid sequence of our cloned toxin gene and the partial sequence determined previously from the protease-resistant form of the 26-kDa toxin (2). Furthermore, the detailed restriction map of our cloned DNA does not resemble the maps of either of the previously isolated toxin genes (27, 36).

The computer-assisted comparisons of the DNA and amino acid sequences of our cloned genes of *B. thuringiensis* subsp. "israelensis" and "kurstaki" indicate two interesting homologies. (i) We found DNA sequence homology between the region immediately upstream from the open reading frame of the cloned *B. thuringiensis* subsp. "israelensis" gene and the promoter recognition sequences for the *B. thuringiensis* subsp. "kurstaki" toxin gene. The homology was consistent with the common pattern of regulation of the two toxin genes. Both toxins are accumulated only in sporulating bacteria and are not synthesized during vegetative growth. The *B. thuringiensis* subsp. "israelensis" sequence was homologous to two adjacent regions in the *B. thuringiensis* subsp. "kurstaki" DNA. These two regions of the latter were probably responsible for controlling the appearance of two temporally regulated transcription products observed during sporulation of *B. thuringiensis* subsp. "kurstaki" cells (40). It will be interesting to determine whether this particular *B. thuringiensis* subsp. "israelensis" gene codes for a single RNA species in sporulating *B. thuringiensis* subsp. "israelensis" cells and whether the sequence will prove to be a common feature of the other toxin genes in this subspecies and of the temporally regulated insecticidal genes of different subspecies of *B. thuringiensis*.

(ii) Despite our inability to show any antigenic cross-reactivity for antisera raised against crystal proteins of either subspecies, we found surprising similarities between the toxin proteins of *B. thuringiensis* subsp. "israelensis" and

"kurstaki" in the corresponding regions of the deduced amino acid sequences. These similarities are seen by alignment of the plots of hydropathic indices and by direct comparison of the amino acid sequences. The overall length and structural features of the ORF1 component of the *B. thuringiensis* subsp. "israelensis" clone were similar to those of the region within the *B. thuringiensis* subsp. "kurstaki" gene that coded for a protease-resistant core that had elevated insecticidal specific activity. Thus at least one of the mosquitocidal gene products of the former subspecies might have mimicked the proteolytic breakdown product of the major crystal protein of the latter. The synthesis of a shortened toxin by *B. thuringiensis* subsp. "israelensis" might eliminate the need for a proteolytic activation in the gut of the mosquito larvae. This might in turn extend the spectrum of activity of this strain to more species of mosquitos. In a similar vein, why would these two subspecies each maintain multiple toxin genes when each of the genes alone has insecticidal activity? We propose that the existence of related but different toxin genes in a single isolate affords the cell the luxury of a more rapid mutation frequency, while not risking the elimination of all the toxin activity in one step. Again, the result could be a potential broadening of the spectrum of activity for a given strain and could lead to an increased likelihood for propagation in susceptible insects. The interesting question remains as to whether the longer *B. thuringiensis* subsp. "kurstaki" toxin gene is an evolutionary progenitor of the two-gene arrangement of our *B. thuringiensis* subsp. "israelensis" clone or vice versa.

The protein sequence similarities between the toxin genes of these two subspecies may reflect a common molecular mechanism underlying their toxicities. However, the protein features that lead to the different insecticidal spectrums for these two toxins remain unknown. One approach that we are currently taking to unravel the contributions made by the different toxin genes in a single strain makes use of the individual cloned genes as a means to specifically mutate the bacteria. This is accomplished by replacing the normal gene with a mutant gene carrying an internal selectable marker. In

FIG. 9. Direct comparison of three regions of amino acid homology between proteins of *B. thuringiensis* subsp. "israelensis" (BTI) and "kurstaki" (BTK). ●●●, Exact homology; ●, similar amino acids. The numbers refer to amino acid positions relative to the N terminus at position 1. For *B. thuringiensis* subsp. "israelensis" the numbers of asterisks refer to positions from ORF2. To assign these numbers, we proceeded as if the two nonsense codons separating ORF and ORF2 were deleted from the DNA sequence.

addition, we are focusing our attention on the regions of amino acid sequence homology to determine whether one or a combination may confer the toxic activity to the proteins of both subspecies. However, we recognize that simple notions of separate protein domains governing crystal formation, binding to cell receptors, or cytotoxicity may prove inadequate in understanding toxin function. By analogy to other toxin proteins that work from outside the cell, we think that a hydrophobic chain of amino acids may be involved either in the initial interactions between the toxin and the epithelial gut lining of the insect or in a subsequent step in disruption of the membrane. In fact, evidence exists that the major toxin in a mixture of *B. thuringiensis* subsp. "*israelensis*" crystal proteins is inactivated by membrane phospholipids (8, 30). We are interested in whether the N-terminal hydrophobic region or the abrupt hydrophobic-hydrophilic transition region constitutes a membrane-binding or membrane-crossing domain.

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LITERATURE CITED

- Ang, B. J., and K. W. Nickerson. 1978. Purification of the protein crystal from *Bacillus thuringiensis* by zonal gradient centrifugation. *Appl. Environ. Microbiol.* **36**:625-626.
- Armstrong, J. L., G. F. Rohrmann, and G. S. Beaudreau. 1985. Delta endotoxin of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.* **161**:39-46.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513-1523.
- Calabrese, D. M., K. W. Nickerson, and L. C. Lane. 1980. A comparison of protein crystal subunit sizes in *Bacillus thuringiensis*. *Can. J. Microbiol.* **26**:1006-1010.
- DeBarjac, H. 1978. Une nouvelle variété de *Bacillus thuringiensis* très toxique pour les moustiques: *B. thuringiensis* var. *israelensis* serotype 14. *C.R. Acad. Sci. Ser. D* **286**:797-800.
- Dulmage, H. T. 1981. Insecticidal activity of isolates of *Bacillus thuringiensis* and their potential for pest control, p. 193-222. In H. D. Burgess (ed.), *Microbial control of pests and plant diseases 1970-1980*. Academic Press, Inc. (London), Ltd., London.
- Dulmage, H. T., O. P. Boening, C. S. Rehnberg, and G. D. Hansen. 1971. A proposed standardized bioassay for formulations of *Bacillus thuringiensis* based on the international unit. *J. Invertebr. Pathol.* **18**:240-245.
- Ellar, D. J., W. E. Thomas, B. H. Knowles, S. Ward, J. Todd, F. Drobniewski, J. Lewis, T. Sawyer, D. Last, and C. Nichols. 1985. Biochemistry, genetics, and mode of action of *Bacillus thuringiensis* δ -endotoxins, p. 230-240. In J. A. Hoch and P. Setlow (ed.), *Molecular biology of microbial differentiation*. American Society for Microbiology, Washington, D.C.
- Erlich, H. A., S. N. Cohen, and H. O. McDevitt. 1980. A sensitive radioimmunoassay for detecting products translated from cloned DNA fragments. *Cell* **13**:681-689.
- Goldberg, L. J., and J. Margalit. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranatena unguiculata*, *Culex univittatus*, *Aedes aegypti* and *Culex pipiens*. *Mosq. News* **37**:355-358.
- Iizuka, T., and T. Yamamoto. 1983. Possible location of the mosquitocidal protein in the crystal preparation of *Bacillus thuringiensis* subsp. *israelensis*. *FEMS Microbiol. Lett.* **19**:187-192.
- Kronstad, J. W., H. E. Schnepf, and H. R. Whiteley. 1983. Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *J. Bacteriol.* **154**:419-428.
- Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* **157**:105-132.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning, a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Margalit, J., and D. Dean. 1985. The story of *Bacillus thuringiensis* var. *israelensis* (*B. t. i.*). *J. Am. Mosq. Control Assoc.* **1**:1-7.
- Maxam, A., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific cleavage. *Methods Enzymol.* **65**:499-560.
- Nagamatsu, Y., Y. Itai, C. Hatanaka, G. Funatsu, and K. Hayashi. 1984. A toxic fragment from the entomocidal crystal protein of *Bacillus thuringiensis*. *Agric. Biol. Chem.* **48**:611-619.
- Pfannenstiel, M. A., E. J. Ross, V. C. Kramer, and K. W. Nickerson. 1984. Toxicity and composition of protease-inhibited *Bacillus thuringiensis* var. *israelensis* crystals. *FEMS Microbiol. Lett.* **21**:39-42.
- Pustell, J., and F. Kafatos. 1984. A convenient and adaptable package of computer programs for DNA and protein sequence management, analysis and homology determination. *Nucleic Acids Res.* **12**:643-655.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
- Schnell, D. J., M. A. Pfannenstiel, and K. W. Nickerson. 1984. Bioassay of solubilized *Bacillus thuringiensis* var. *israelensis* crystals by attachment to latex beads. *Science* **223**:1191-1193.
- Schnepf, H. E., and H. R. Whiteley. 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **78**:2893-2897.
- Schnepf, H. E., and H. R. Whiteley. 1985. Protein toxins of *Bacillus* spp., p. 209-216. In J. A. Hoch and P. Setlow (ed.), *Molecular biology of microbial differentiation*. American Society for Microbiology, Washington, D.C.
- Schnepf, H. E., and H. R. Whiteley. 1985. Delineation of a toxin-encoding segment of a *Bacillus thuringiensis* crystal protein gene. *J. Biol. Chem.* **260**:6273-6280.
- Schnepf, H. E., H. C. Wong, and H. R. Whiteley. 1985. The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *J. Biol. Chem.* **260**:6264-6272.
- Sekar, V., and B. C. Carlton. 1985. Molecular cloning of the delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. *Gene* **33**:151-158.
- Sharpe, E. S., K. W. Nickerson, L. A. Bulla, Jr., and J. N. Aronson. 1975. Separation of spores and parasporal crystals of *Bacillus thuringiensis* in gradients of certain X-ray contrasting agents. *Appl. Microbiol.* **30**:1052-1053.
- Thomas, W. E., and D. J. Ellar. 1983. *Bacillus thuringiensis* var. *israelensis* crystal delta endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. *J. Cell Sci.* **60**:181-197.
- Thomas, W. E., and D. J. Ellar. 1983. Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal delta-endotoxin. *FEBS Lett.* **154**:362-368.
- Tojo, A., and K. Aizawa. 1983. Dissolution and degradation of *Bacillus thuringiensis* δ -endotoxin by gut juice protease of the silkworm *Bombyx mori*. *Appl. Environ. Microbiol.* **45**:576-580.
- Tyrell, D. J., L. A. Bulla, Jr., R. E. Andrews, Jr., K. J. Kramer, L. I. Davidson, and P. Nordin. 1981. Comparative biochemistry of entomocidal parasporal crystals of selected *Bacillus thuringiensis* strains. *J. Bacteriol.* **145**:1052-1062.
- Undeen, A. H., and D. Berl. 1979. Laboratory studies on the effectiveness of *Bacillus thuringiensis* var. *israelensis* de Barjac against *Simulium damnosum* (Diptera: Simuliidae) larvae. *Mosq. News* **39**:743-745.
- Undeen, A. H., and W. L. Nagel. 1978. The effect of *Bacillus*

- thuringiensis* ONR60A strain (Goldberg) on *Simulium* larvae in the laboratory. Mosq. News **38**:524–527.
35. Wabiko, H., G. A. Held, and L. A. Bulla, Jr. 1985. Only part of the protoxin gene of *Bacillus thuringiensis* subsp. *berliner* 1715 is necessary for insecticidal activity. Appl. Environ. Microbiol. **49**:706–708.
 36. Ward, E. S., D. J. Ellar, and J. A. Todd. 1984. Cloning and expression in *Escherichia coli* of the insecticidal delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. FEBS Lett. **175**:377–382.
 37. Whiteley, H. R., J. W. Kronstad, and H. E. Schnepf. 1985. Structure and expression of cloned *Bacillus thuringiensis* toxin genes, p. 225–229. In J. A. Hoch and P. Setlow (ed.), Molecular biology of microbial differentiation. American Society of Microbiology, Washington, D.C.
 38. Whiteley, H. R., H. E. Schnepf, J. W. Kronstad, and H. C. Wong. 1984. Structural and regulatory analysis of a cloned *Bacillus thuringiensis* crystal protein gene, p. 375–386. In A. T. Ganesan and J. A. Hoch (ed.), Genetics and biotechnology of bacilli. Academic Press, Inc., Orlando, Fla.
 39. Wong, H. C., and S. Chang. 1985. Identification of a positive retroregulator that functions in *Escherichia coli* and *Bacillus subtilis*, p. 104–109. In J. A. Hoch and P. Setlow (ed.), Molecular biology of microbial differentiation. American Society for Microbiology, Washington, D.C.
 40. Wong, H. C., H. E. Schnepf, and H. R. Whiteley. 1983. Transcriptional and translational start sites for the *Bacillus thuringiensis* crystal protein gene. J. Biol. Chem. **258**:1960–1967.
 41. Yamamoto, T., T. Iizuka, and J. N. Aronson. 1983. Mosquitocidal protein of *Bacillus thuringiensis* subsp. *israelensis*: identification and partial isolation of the protein. Curr. Microbiol. **9**:279–284.