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Identification of a δ-Endotoxin Gene Product Specifically Active against *Spodoptera littoralis* Bdv. among Proteolysed Fractions of the Insecticidal Crystals of *Bacillus thuringiensis* subsp. *aizawai* 7.29

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At least three different insecticidal crystal protein genes were shown to be expressed in *Bacillus thuringiensis* subsp. *aizawai* 7.29, a strain that is potentially active against the cotton leafworm *Spodoptera littoralis* Bdv. Among crude K-60 fractions (60- to 70-kilodalton [kDa] molecules) that were products of proteolysed crystals containing the active domains of the protoxin molecules, we were able to distinguish several distinct components on the basis of their antigenic relationship and their larvicidal properties. A purified fraction designated SF2 was a 61-kDa component specifically active against *Pieris brassicae* L. and homologous to the *B. thuringiensis* subsp. *berliner* 1715 plasmid-encoded crystal protein. A second fraction designated SF1 was composed of 63- and 65-kDa polypeptides and was specifically active against *S. littoralis*. The SF1 fraction and particularly the 65-kDa component were not antigenically related to the 61-kDa component. The purified fractions were compared with the products of three different crystal protein genes we previously cloned from total DNA of *B. thuringiensis* subsp. *aizawai*, among them a new type of crystal protein gene encoding a protein that is specifically active against *S. littoralis* and other insects of the Noctuidae family. This approach led us to consider the 65-kDa component as a minimum active part of a δ-endotoxin that is encoded by this new gene. Products of the two other cloned genes can be correlated with the 61- and 63-kDa components, respectively. Thus, in *B. thuringiensis* subsp. *aizawai* 7.29, multiple δ-endotoxin genes of different structural types direct the synthesis of several δ-endotoxins with different host specificities which were identified as components of the insecticidal crystals.

A recent feature in the field of the insecticidal *Bacillus thuringiensis* toxins is the idea that multiple δ-endotoxin genes encoding a variety of distinct crystal proteins (6, 13) could be responsible for the extreme variability of the insect host range of *B. thuringiensis* strains (8, 12).

Based on the size of the *Hin*dIII restriction fragments containing the 5′ end of the δ-endotoxin gene, several classes of homologous genes have been defined by Kronstad and Whiteley (19) and designated as 4.5-, 5.3-, and 6.6-kilobase (kb) class genes. These researchers have shown that genes belonging to the three classes of crystal protein genes can be identified in *B. thuringiensis* subsp. *kurstaki* HD1. In *B. thuringiensis* subsp. *berliner* 1715, two distinct crystal genes with different localizations have been cloned (16). The two genes corresponded to two different structural types defined on the basis of the size of the *Pvu*II fragments (2 or 8 kb) including the major part of the toxin gene (17, 22). Also, the deduced amino acid sequences of seven or more crystal protein genes, as reviewed by Whiteley and Schnepf (31), Aronson et al. (4), and Andrews et al. (3), indicate that highly variable subdomains generally located in the N-terminal half of the gene could account for the functional diversity among δ-endotoxin families.

An interesting example of agronomical importance among lepidopteran species concerns insects belonging to the Noctuidae family that are poorly controlled with the commercial isolates of *B. thuringiensis* presently used as pesticides. Such is the case for the cotton leaf worm *Spodoptera littoralis* Bdv. It is now well established that *B. thuringiensis* strains among serotypes H7 (subsp. *aizawai*) and H6 (subsp. *entomocidus*) display significant levels of activity toward *S. littoralis* (14, 26, 32).

In a previous study (21), we have shown that *B. thuringiensis* subsp. *aizawai* 7.29 and subsp. *entomocidus* 601 are specifically toxic against *S. littoralis*, although they also display significant activity toward other insect species such as *Pieris brassicae* L. Furthermore, the crystals dissolve efficiently in a suspension of host gut proteases, forming several active products with molecular masses ranging from 60 to 70 kilodaltons (kDa) (designated K-60) that are the true toxin molecules. In a detailed study including several isolates of *B. thuringiensis* subsp. *aizawai*, Jarrett (13) demonstrated that insecticidal crystals are composed of at least two distinct polypeptides of 130 and 138 kDa.

Cloning of δ-endotoxin genes from plasmid DNA of three different *B. thuringiensis* subsp. *aizawai* strains was reported by Klier et al. (16), Oeda et al. (24), and Chak and Ellar (7). In all these cases, the cloned genes seemed to correspond unambiguously to the same type of crystal protein gene, the 5.3-kb class gene according to the Kronstad and Whiteley classification (19) or the 8-kb *Pvu*II class gene according to the classification of Klier and co-workers (17, 22). More recently, we (27) have reported cloning and characterization of multiple δ-endotoxin genes from *B. thuringiensis* subsp. *aizawai* 7.29. In addition to genes belonging to the known classes of crystal protein genes, two new types of genes were identified; one of these, obtained as a truncated gene, was shown to direct the synthesis of a 90-kDa protein that is specifically toxic against *S. littoralis* and against several other species of the Noctuidae family.

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The expression products of the cloned genes in *Escherichia coli* differ in their antigenic properties as well as in the specificity of their larvicidal activity. At the same time, Visser et al. (30) isolated five crystal protein genes from DNA of *B. thuringiensis* subsp. *entomocidus* 605, and one of these was shown to code for a product with specificity toward *Spodoptera* species.

One of the questions that remains to be answered is how the regulation of the expression of distinct δ-endotoxin genes and the independent or synergistic action of the products can determine the overall toxicity of a given strain.

Taking advantage of our preliminary study of the protein fractions derived from crystals of *B. thuringiensis* subsp. *aizawai* 7.29 (21), we undertook further characterization of the protease-activated polypeptides (K-60 fraction). A complementary approach, the individual analysis of each of the expression products of the *B. thuringiensis* subsp. *aizawai* 7.29 cloned genes, led us to compare such products synthesized in *E. coli* with the original crystal protein derivatives and to identify more precisely some of them.

Results obtained from such experiments are reported here. Most of these results concern *B. thuringiensis* subsp. *aizawai* 7.29, although comparison with δ-endotoxin components of other *S. littoralis*-active strains, particularly *B. thuringiensis* subsp. *aizawai* 7.21 and *entomocidus* 601, are also considered.

MATERIALS AND METHODS

Chemicals. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); dithiothreitol and phenylmethylsulfonyl fluoride were from Sigma Chemical Co. (St. Louis, Mo.); DEAE-cellulose (DE52) was from Whatman, Inc. (Clifton, N.J.); Sephadex G-75 was from Pharmacia, Inc. (Piscataway, N.J.); and Ultrogel AcA34 was from LKB (Bromma, Sweden). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins were from Bio-Rad Laboratories (Richmond, Calif.). Protein markers for gel calibration were from Pharmacia.

Buffers. Buffer A contained 50 mM sodium carbonate-bicarbonate (pH 9.5). Buffer B contained 25 mM Tris hydrochloride (pH 8.25). Buffer C contained 50 mM sodium carbonate-bicarbonate (pH 9.5) and 0.25 M NaCl. Buffer D contained 25 mM Tris hydrochloride (pH 8.4) and 0.5 M NaCl. Buffer E contained 25 mM Tris hydrochloride (pH 8.4) and 0.15 M NaCl. Tris-saline contained 50 mM Tris hydrochloride (pH 7.5) and 0.15 M NaCl.

Bacterial strains. *B. thuringiensis* subspecies *aizawai* 7.29, *aizawai* 7.21, *entomocidus* 601, and *berliner* 1715 used in this study were obtained from the WHO Collaborating Center for Entomopathogen Bacillus (Institut Pasteur); we are grateful to H. de Barjac for providing these strains.

E. coli JM83 recombinant strains containing one of the different crystal protein genes isolated from *B. thuringiensis* subsp. *aizawai* 7.29 and designated JM83(pHTA2), JM83(pHTA4), JM83(pHT671), or JM83(pHT71) were obtained as described previously (27).

B. thuringiensis cultures were grown at 30°C in HCT medium as described previously (20) until complete liberation of spores and crystals occurred. *E. coli* recombinant strains were grown at 37°C in LB medium supplemented with ampicillin (100 μg/ml) that was re-added twice during growth and at the beginning of the stationary phase. For inducing the *lac* promoter, IPTG was added (5×10^{-5} M) twice after the culture had reached an optical density (600 nm) of 1 unit. For preparing extracts, cells were harvested at either 16 or 24 h after the inoculation of the culture.

Isolation and proteolytic activation of parasporal crystals. *B. thuringiensis* spore-crystal mixtures harvested after the end of sporulation were washed and treated as already described (21), using the water-tetrabromoethane biphasic procedure for isolating crystals. Gut proteases recovered from fifth instar larvae of *S. littoralis* and partially purified by gel filtration (21) can be directly used for dissolving crystals under mild alkaline conditions by the procedure already described (21).

Alternatively, a two-step procedure was used for obtaining protease-activated toxins. Crystals (7 mg/ml in 0.1 M glycine buffer [pH 9.5]) were preincubated at 30°C for 30 min and then supplemented with 25 mM dithiothreitol; dissolution proceeded at 30°C for 30 min. After residual spores or undissolved crystals were eliminated, the soluble supernatant was dialyzed at 4°C against large volumes of the same glycine buffer for 1.5 to 2 h. Then the protease solution was added to give an enzyme/substrate ratio of about 1/100 (with regard to protein concentration). After incubation for 2 h at 32°C, the proteolysis was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride and the preparation was immediately used for the first purification step.

Purification of proteolysed fractions. (i) Gel filtration on Sephadex G-75. A 4-ml sample of the proteolysed δ-endotoxin solution was filtered through a column (1.7 by 45 cm) loaded with Sephadex G-75 previously equilibrated with buffer A. Fractions containing activated toxin molecules with a molecular mass of >50 kDa were eluted in the main peak, whereas proteases and smaller digestion products were retained on the gel. The active fractions of peak 1 were collected and designated as the K-60 fraction.

(ii) DEAE-cellulose chromatography. About 15 ml of this K-60 fraction was dialyzed for 16 h at 4°C against two 1-liter batches of buffer B. The protein solution was then loaded on a DEAE-cellulose (DE52) column (1.4 by 23 cm) freshly equilibrated with the same buffer. After the column was washed with 50 ml of this buffer, a linear gradient (200 ml) from 0 to 0.3 M KCl was started. The toxic fractions eluted with the gradient were pooled and kept frozen at −25°C for further characterizations. In some cases, a third purification step was performed, using a part of the DE52 fraction.

(iii) Chromatography on Ultrogel AcA34. About 10 to 15 ml of the main DE52 active fraction was treated with ammonium sulfate at 40% satuartion; the resulting precipitate was solubilized in 1 to 1.5 ml of 0.1 M sodium carbonate buffer (pH 9.5). A part of this preparation (0.8 ml) was applied to a Bio-Gel column (Ultrogel AcA34) (0.8 by 42 cm) previously equilibrated with buffer C, thus allowing the recovery of the 60- to 70-kDa toxin molecules in the main peak. The active fractions were pooled and stored frozen at −25°C.

Preparation of antiserum. Rabbit antibodies directed against δ-endotoxins from different strains were obtained after two subcutaneous injections (3-week intervals) of 0.5-ml protein solutions mixed with 0.5 ml of Freund adjuvant, followed by a third subcutaneous injection of the same solution without adjuvant. Rabbits were bled 7 to 8 days after the last injection.

The different antigen solutions consisted of dithiothreitol-dissolved crystals prepared as described in reference 21 from *B. thuringiensis* subsp. *aizawai* 7.29, *berliner* 1715, or *entomocidus* 601. Antisera against two different K-60 fractions obtained from the DE52 chromatography were also prepared.

Electrophoresis and immunoblotting. SDS-acrylamide gel electrophoresis was conducted by the method of O'Farrell (25), using either a 7.5 to 15% acrylamide gradient gel or 10% single-concentration gels with an acrylamide/*N*,*N*-methylene bisacrylamide ratio of 100:1 (9).

Electrotransfer on nitrocellulose membranes followed by immunodetection was done as described by Towbin et al. (29) with goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase as the second antibody.

Preparation of cell extracts from *E. coli*. Soluble protein extracts from *E. coli* recombinant clones bearing the different types of crystal genes from *B. thuringiensis* subsp. *aizawai* 7.29 were prepared as follows. Washed *E. coli* cells (300 ml) concentrated to 6 ml in a lysing solution (10 mM Tris hydrochloride [pH 8.4], 10 mM EDTA, 100 μg of lysozyme per ml) were incubated at 30°C for 15 min and then mechanically disrupted in the presence of 2 mM phenylmethylsulfonyl fluoride with an ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London, England). Then 6 ml of 0.1 M sodium carbonate buffer (pH 9.5) containing sodium thioglycolate at a final concentration of 0.2 M was added, and the mixture was incubated at 35°C for 30 min. After a centrifugation at 17,000 × *g* for 30 min, the resulting supernatant was dialyzed for 2 h at 4°C against 500 ml of buffer D and then for 4 h against 1 liter of buffer E. Such extracts were stored frozen at −25°C.

Immunodetection of insecticidal crystal proteins in cell extracts. To 25 μl of a given antiserum, increasing amounts (not exceeding 100 μl) of *E. coli* extracts were added and made up to 0.5 ml with Tris-saline. The mixture was incubated for 1 h at room temperature and then overnight at 4°C. Precipitates that were collected by centrifugation for 4 min in an Eppendorf centrifuge were then washed in 0.5 ml of Tris-saline, kept overnight at 4°C, and centrifuged again; the pellets were dissolved in 0.25 ml of 0.1 N NaOH. After the mixture rested for 1 to 2 h, the amount of protein as solubilized antigen-antibody complexes was estimated in 100-μl aliquots by the Lowry procedure (22a) performed with a final volume of 1.1 ml including reagents and then by measuring the A_{750}. The amount of proteins contained in the 100-μl aliquots of the dissolved precipitates ranged precisely within the range of sensitivity of the Lowry method (5 to 25 μg of proteins in the assay). When necessary, smaller aliquots were used.

A calibration curve corresponding to known amounts (usually 3 to 30 μg) of a crystal protein solution incubated with antiserum under the same conditions was used as a reference to calculate the amount of specific proteins detected in the extracts. The possible effect of cross-reactions between the immune serum and *E. coli* endogenous proteins was eliminated by preincubating the extracts with preimmune serum (1 h at room temperature) before starting the reaction with the specific antiserum.

In some cases, the relative concentration of crystal proteins in the extracts was increased by precipitating them by the addition of 2 M acetic acid (50 μl for 1 ml of extract); the resulting precipitate can be readily dissolved in 0.5 volume of sodium carbonate buffer (pH 9.5).

This concentrated solution was used either directly for immunoprecipitation or for a further purification step performed by gel filtration on an Ultrogel AcA34 column (0.8 by 42 cm) equilibrated with buffer C. Elution was carried out with the same buffer.

Bioassays of insecticidal activity. For estimating toxicity of crystals and their soluble derivatives, including K-60 purified fractions, and of the soluble *E. coli* extracts, biological assays with fifth instar larvae of the two insect species *S. littoralis* and *P. brassicae* were conducted as previously described (21) by the forced feeding technique.

Activities against the two insect species were compared in terms of 50% lethal concentration (LC_{50}) (micrograms of protein) ratios (LC_{50} for *S. littoralis*/LC_{50} for *P. brassicae*), designated as indexes of specificity.

Concerning *E. coli* extracts, LC_{50} in terms of total proteins was first determined by using dilutions of the extracts for bioassays; the true LC_{50} in terms of specific proteins was then estimated on the basis of the different expression rates deduced from immunoprecipitation experiments.

The 50% lethal dose (LD_{50}) in terms of micrograms per gram of larvae was determined on the basis of an average individual weight of 41 or 113 mg for *S. littoralis* or *P. brassicae*, respectively.

RESULTS

Purification of proteolysed K-60 fractions. The 130- to 140-kDa protoxins of native *B. thuringiensis* subsp. *aizawai* 7.29 crystals or crystals dissolved with dithiothreitol at pH 9.5 were cleaved by *S. littoralis* gut proteases, resulting in smaller molecules, polypeptides of 60 to 70 kDa. These 60- to 70-kDa polypeptides are the true toxins (21) and are components of the K-60 fraction obtained after the first purification step (gel filtration, see Materials and Methods). This fraction represents about 45% of the protoxin material.

Results of DE52 chromatography are shown in Fig. 1A. As is usually the case with the δ-endotoxin or derivatives (2, 5, 15), a major peak of proteins was eluted at 0.2 M KCl, preceded in this experiment by a minor peak eluted at 0.18 M KCl. This minor peak did not always appear so clearly and was more usually seen as a shoulder of the main peak. However, it could be easily detected whatever procedure or batch of crystals was used, and it was still observed when crystals of *B. thuringiensis* subsp. *aizawai* 7.21 were the substrate.

SDS-gel electrophoretic analysis indicated that the crude K-60 fraction was composed of five components ranging from 70 to 60 kDa (Fig. 1B, lane 4), which were numbered from 1 to 5 by decreasing order of molecular mass. Occasionally, depending on the preparation and on the storage conditions, lower-molecular-weight products were present.

The minor fraction eluted at 0.18 M KCl was designated subfraction SF1 and contained only two components (Fig. 1B, lane 1) represented by bands 3 and 4 (65 and 63 kDa, respectively) of the crude fraction. The main fraction was designated MF and contained three major components of 68, 65, and 61 kDa (Fig. 1B, lanes 2 and 3) corresponding to the bands 2, 3, and 5, respectively, of the crude extract; in addition, traces of component 4 are still present and a fifth component represented by the upper band (band 1) can be seen more easily after ammonium sulfate precipitation (data not shown). This 70-kDa component tended to disappear progressively after prolonged storage or after additional proteolytic digestion (see Fig. 7B, lane 5) and thus could correspond to intermediate proteolysis products. Similar observations were reported by Kim et al. (15) for products of other strains.

It is important to note that very similar patterns were observed with different K-60 preparations of *B. thuringiensis* subsp. *aizawai* 7.29, as well as fractions from crystals of *B. thuringiensis* subsp. *aizawai* 7.21 (see Fig. 3A, lanes 5 and 6).

When step 3 of purification (chromatography on Ultrogel)

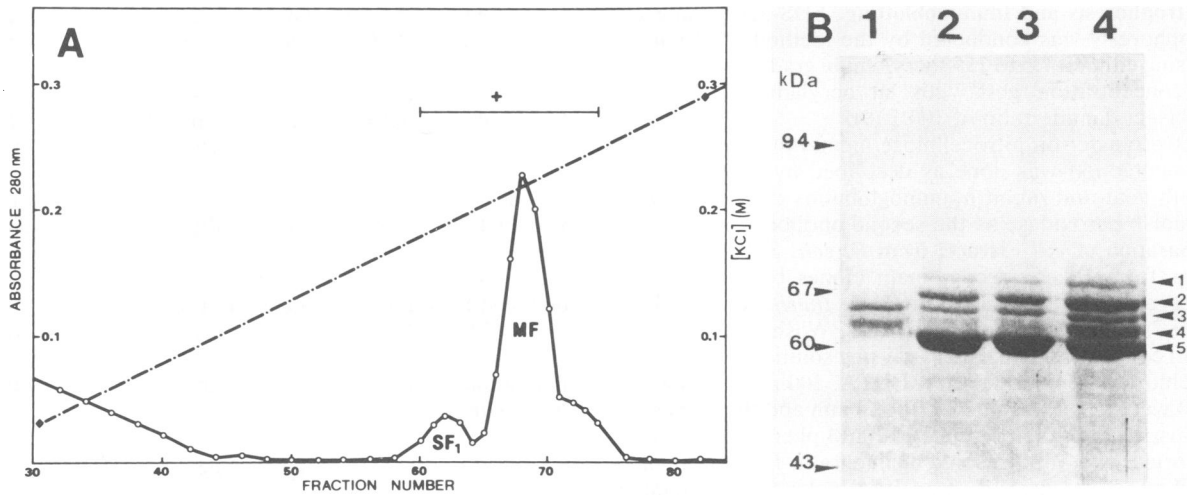

FIG. 1. (A) DEAE-cellulose chromatography of the K-60 fraction (step 2). The crude K-60 fraction (15 ml; 8 to 10 mg of proteins) was adsorbed on a DE52 column (1.4 by 23 cm) and eluted with a linear gradient (200 ml) of KCl at a flow rate of 30 ml/h at 4°C. Fractions of 3 ml were collected and then analyzed for protein concentration (optical density at 280 nm). —, A_{280}; —.—.—, KCl concentration. Pooled fractions 60 to 63 became the SF1 subfraction; pooled fractions 66 to 71 became the MF fraction; +, fractions with larvicidal activity. (B) SDS-PAGE of the different fractions in a 7.5% acrylamide gel. Lanes: 1, SF1; 2 and 3, MF; 4, crude K-60 fraction eluted from the Sephadex column (step 1). Based on these results and on the average values from several different experiments, the molecular size values were estimated to be 70, 68, 65, 63, and 61 kDa for components 1 to 5, respectively.

was applied to the MF fraction, a major peak (Fig. 2A) whose composition was quite similar to that of the MF fraction (Fig. 2B, lane 2) was eluted in the region expected for the 60- to 70-kDa proteins. Interestingly, the decreasing part of the peak, appearing as a shoulder (subfraction SF2) contained essentially the 61-kDa component of the K-60 fraction that is represented by band 5 (Fig. 2B, lane 1). It is not very easy to state precisely the nature of the process leading to the elimination of the other components, but it was a very reproducible experiment. Therefore, the different steps of purification allowed us to distinguish several fractions differing in their composition. We decided to focus our interest on the three fractions that are presented Fig. 2B in lanes 1, 2, and 3 and designated, respectively, SF2, MF, and SF1.

Toxicity of different fractions compared with that of crystals. Table 1 presents the results of toxicity assays, in terms of LC_{50} toward the two insect species, *S. littoralis* and *P. brassicae*. If we consider the activity against *S. littoralis*, it is clear that the activated K-60 fraction and the two derivatives SF1 and MF display LC_{50} values on the same order as that of the native crystals of *B. thuringiensis* subsp. *aizawai* 7.29 from which they are derived. Similar values were also found for crystals from *B. thuringiensis* subsp. *aizawai* 7.21. In contrast, the activity of subfraction SF2 was much lower, as seen from the LC_{50}, which is 10-fold higher.

On the other hand, considering activity against *P. brassicae*, the same SF2 fraction manifested the highest activity, whereas the SF1 fraction appeared much less active than SF2 and native crystals. When the results are compared in terms of LC_{50} ratios toward the two insect species (indexes of specificity), fraction SF1 is characterized by the lowest value of this ratio (lower than for crystals or the MF fraction), suggesting that this fraction is the most specific toward *S. littoralis* larvae.

Another striking feature which is revealed in comparing the values of these ratios concerns the SF2 subfraction. This fraction appeared to be very specific toward *P. brassicae*, as is also a preparation of native crystals of *B. thuringiensis* subsp. *berliner* 1715, a strain considered as a reference and which is known for its specificity against *P. brassicae* and other insects of the same family.

It can be concluded that the three purified fractions differ in their specificity and that at least one component of the SF1 subfraction could correspond to the active moiety of a δ-endotoxin specifically toxic against *S. littoralis*. The MF fraction in which component 4 (63 kDa) is not present in significant amounts is still fully active toward this insect species; consequently, this component is not essential for toxicity toward *S. littoralis*. Similarly, another component isolated in subfraction SF2, more specific toward *P. brassicae*, could be responsible for the relative toxicity of the native *B. thuringiensis* subsp. *aizawai* crystals against this insect species.

These two types of activity were also clearly observed for *B. thuringiensis* subsp. *entomocidus* 601, for which the value of the index of specificity of the native crystals reaches 2 (Table 1). In this case, the LC_{50} toward *S. littoralis* is of the same order as values obtained with crystals of *B. thuringiensis* subsp. *aizawai* strains (21), whereas the value toward *P. brassicae* is much lower.

Immunological relationships between components of the fractions. Another approach to identify the components of the K-60 fraction consisted of examining their reactions with antisera directed against δ-endotoxins of different specificities.

Results of immunoblotting experiments are shown in Fig. 3. Polyclonal antibodies directed against solubilized *B. thuringiensis* subsp. *aizawai* 7.29 crystals reveal all the components of the K-60 fraction as seen with subfractions SF1, MF, and SF2 (Fig. 3A, lanes 2, 3, and 4, respectively), as well as components of the homologous *B. thuringiensis* subsp. *aizawai* 7.21 crystals (lanes 5 and 6) and of the crude K-60 fraction from *B. thuringiensis* subsp. *entomocidus* 601 crystals (lane 1). Antibodies directed against the *B. thuringiensis* subsp. *berliner* 1715 δ-endotoxin (Fig. 3B) revealed uniquely the 61-kDa component (band 5) of the K-60 crude fraction or of the MF fraction (lane 8) and the unique

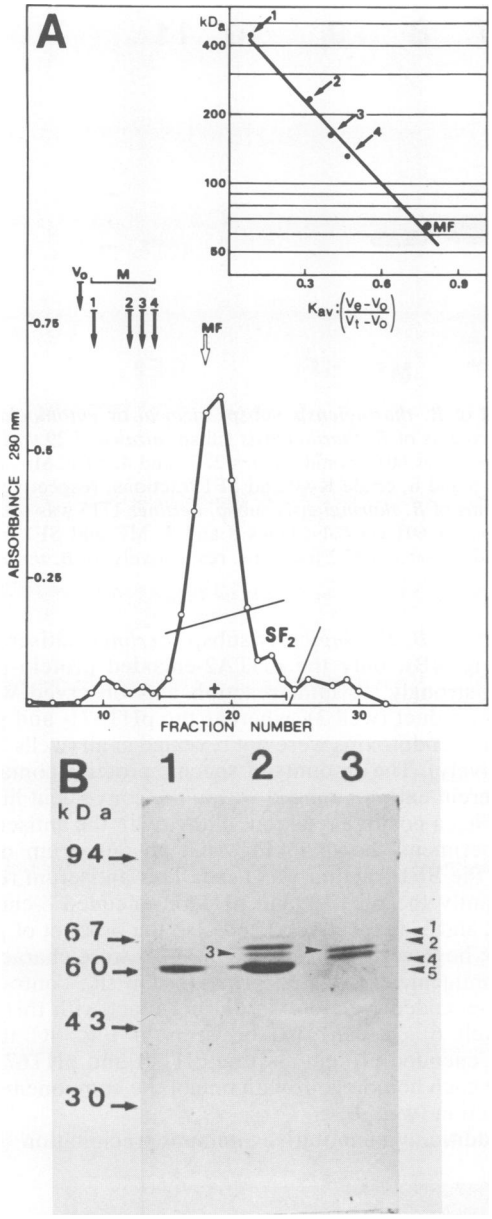

FIG. 2. (A) Gel filtration of the MF fraction on an Ultrogel AcA-34 column (0.8 by 43 cm) (step 3). A 20-ml sample of the MF fraction was precipitated with ammonium sulfate as described in Materials and Methods. The dissolved precipitate was loaded on the column and then eluted with buffer C. Fractions of 1 ml were collected. Pooled fractions 16 to 21 became the MF fraction; pooled fractions 22 to 25 became the SF2 subfraction. A calibration of the column was then performed with markers of known molecular mass (indicated with the arrows): 1, ferritin, 440 kDa; 2, catalase, 232 kDa; 3, aldolase, 158 kDa; 4, bovine serum albumin dimer, 134 kDa. V_e, elution volume; V_o, exclusion volume; V_t, total bed volume of the column. (B) SDS-PAGE of the different fractions on a 7.5 to 15% acrylamide gradient gel. Lanes 1, 2, and 3 represent fractions SF2, MF, and SF1, respectively. The arrowheads on the right of the gel indicate components 1, 2, 4, and 5. The arrowheads within the gel indicate component 3 (65 kDa).

component of the SF2 subfraction; a weak reaction was generally observed with the 63-kDa component of the SF1 subfraction (lane 9), whereas no reaction at all was observed with the 65-kDa component of SF1 and with the other components (bands 1, 2, and 3) of the MF fraction (lane 8). The *B. thuringiensis* subsp. *berliner* antibodies also reacted with the 61- and 63-kDa components of the crude K-60 fraction of the *B. thuringiensis* subsp. *aizawai* 7.21 crystals (lane 10), with the 61-kDa component of the *B. thuringiensis* subsp. *aizawai* 7.21 SF2 fraction (lane 11), and with at least two components of the *B. thuringiensis* subsp. *entomocidus* K-60 fraction (lane 7), the main one of which is still a 61-kDa component.

TABLE 1. Toxicity of the K-60 fraction against fifth instar larvae of two insect species

Prepn	LC_{50} for[a]:		Index of specificity[b] (SI/Pb)
	S. littoralis	*P. brassicae*	
B. thuringiensis subsp. *aizawai* 7.29			
Native crystals	0.021	0.040	0.5
Crude K-60 fraction	0.018	ND[c]	ND
Purified fraction			
MF	0.013	0.025	0.5
SF1	0.027	0.100	0.27
SF2	0.240	0.011	22
Native crystals			
B. thuringiensis subsp. *aizawai* 7.21	0.015	0.038	0.4
B. thuringiensis subsp. *entomocidus* 601	0.028	0.012	2.3
B. thuringiensis subsp. *berliner* 1715 (as a reference)	0.110	0.007	16

[a] LC_{50} is the protein concentration (micrograms per microliter) at which 50% of larvae were killed (72 h). For each dilution of the different preparations, 20 larvae at the fifth instar were inoculated (forced ingestion) with 5 μl of the preparation.

[b] Values of the index of specificity are estimated in terms of LC_{50} for the two insect species as described in Materials and Methods. SI, *S. littoralis*; Pb, *P. brassicae*.

[c] ND, Not determined.

Interestingly, antibodies directed against the SF1 subfraction from *B. thuringiensis* subsp. *aizawai* 7.29 reacted with components of the homologous SF1 subfraction and with components 1, 2, 3, and 4 of the MF and crude K-60 fraction of the *B. thuringiensis* subsp. *aizawai* 7.21 strain. This antiserum reacted also with two of the five components of the *B. thuringiensis* subsp. *entomocidus* K-60 fraction that are homologous in size with SF1 (data not shown). It should be mentioned that purification of the crude K-60 extract of this *B. thuringiensis* subsp. *entomocidus* strain has not yet been performed; consequently, it is not possible to define the fractions more precisely.

We conclude that significant differences among the components of the K-60 fraction were revealed by the use of the three antisera. It is also clear that important homologies can be detected between K-60 fractions originating from strains that have the same specificity, particularly those active against *S. littoralis*, and particularly with regard to components of the SF1 subfractions.

Analysis of expression products of the cloned genes in *E. coli*. In a previous study (27), we demonstrated the existence of at least five δ-endotoxin genes in *B. thuringiensis* subsp. *aizawai* 7.29 and reported the cloning of four genes from total DNA of this strain. Among these genes, two were shown to belong to the 5.3-kb class of crystal protein gene, as for the *B. thuringiensis* subsp. *berliner* plasmid gene, and the two other genes were identified as new classes of crystal

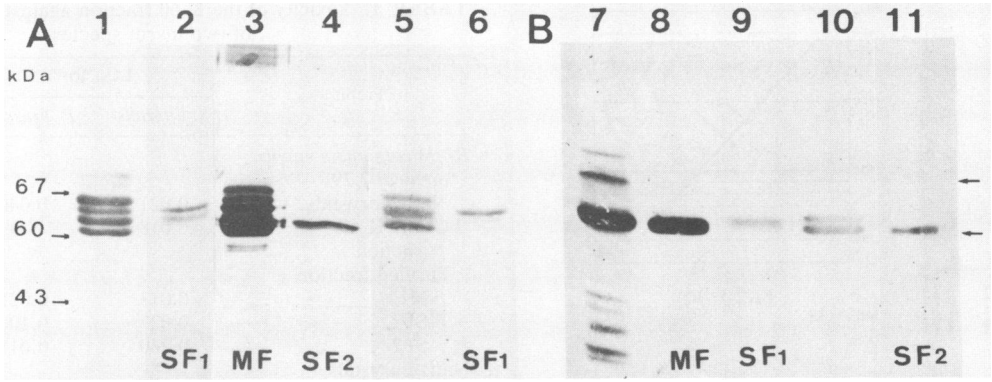

FIG. 3. Immunodetection of the K-60 components originating from crystals of *B. thuringiensis* subsp. *aizawai* or *entomocidus*. The eletrophoresis was run in a 10% acrylamide gel. (A) Antiserum against crystal proteins of *B. thuringiensis* subsp. *aizawai* 7.29 was used as the first antibody. Lane 1, Crude K-60 fraction of *B. thuringiensis* subsp. *entomocidus* 601 crystals; lanes 2, 3, and 4, SF1, MF, and SF2 fractions, respectively, from *B. thuringiensis* subsp. *aizawai* 7.29 crystals; lanes 5 and 6, crude K-60 and SF1 fractions, respectively, from *B. thuringiensis* subsp. *aizawai* 7.21 crystals. (B) Antiserum against crystal proteins of *B. thuringiensis* subsp. *berliner* 1715 was used as the first antibody. Lane 7, Crude K-60 fraction of *B. thuringiensis* subsp. *entomocidus* 601 crystals; lanes 8 and 9, MF and SF1 fractions, respectively, of *B. thuringiensis* subsp. *aizawai* 7.29 crystals; lanes 10 and 11, crude K-60 and SF2 fractions, respectively, of *B. thuringiensis* subsp. *aizawai* 7.21 crystals.

protein genes. One of these, borne by the recombinant plasmids pHT71 and pHT671, was isolated as a truncated gene encoding a 90-kDa protein which was specifically active against *S. littoralis*. In a preliminary study, the products of the cloned genes expressed in *E. coli* were compared by immunodiffusion experiments. Differences between these products were shown (27).

Taking advantage of the data obtained with the K-60 fractions reported above, we developed experiments using several different antisera directed against *B. thuringiensis* subsp. *aizawai* or *berliner* crystals or against the MF and SF1 fractions. Results of immunodiffusion experiments are shown in Fig. 4. An antiserum specific to the MF fraction that presumably contains antibodies against the various δ-endotoxins of the strain revealed the products of the three cloned genes (Fig. 4A). It is clear that the products of the crystal protein genes isolated in plasmids pHT71 and pHT671 (wells 2 and 5, respectively) differ from the pHTA4-encoded crystal protein (well 3) and that both differ from the pHTA2 product (well 4).

When the *B. thuringiensis* subsp. *berliner* antiserum was used (Fig. 4B), only the pHTA2-encoded protein (well 4) reacted strongly. A faint reaction was observed with the pHTA4 product (well 3), whereas the pHT671- and pHT71-encoded δ-endotoxins were not revealed at all (wells 2 and 5, respectively). The amounts of specific proteins contained in the different extracts ranged within the convenient limits for developing a positive reaction, if any, with the antiserum. In the experiment shown in Fig. 4C, an antiserum directed against the SF1 fraction was used. This antiserum revealed significantly the pHT71- and pHT671-encoded δ-endotoxin (wells 2 and 5, respectively) and also the product of pHTA4 (well 3); however, the patterns obtained were characteristic of nonantigenically related proteins. On the contrary, the pHTA2-encoded δ-endotoxin did not react with this antiserum (well 6). It can also be seen in Fig. 4C that the pHTA4-encoded protein and the pHT71 and pHT671 products are each homologous with one of the components of the SF1 fraction (well 4).

In addition, quantitative immunoprecipitation experi-

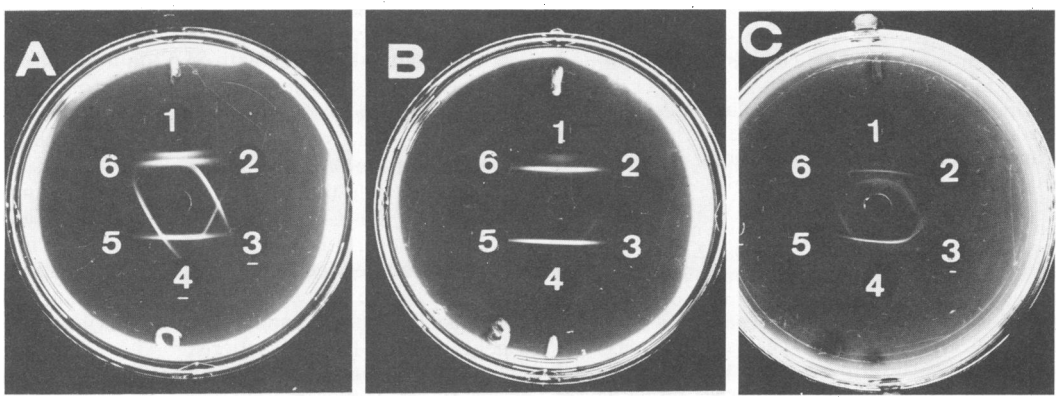

FIG. 4. Reslts of immunodiffusion experiments comparing the crystal protein antigens in the recombinant *E. coli* JM83 clones. (A) Center well, Antiserum against the MF fraction of *B. thuringiensis* subsp. *aizawai* 7.29 crystals. Wells: 1, crystal proteins of *B. thuringiensis* subsp. *aizawai* 7.29; 2, JM83(pHT671) extract; 3, JM83(pHTA4) extract; 4, JM83(pHTA2) extract; 5, JM83(pHT71) extract; 6, JM83(pUC18) extract. (B) Center well, Antiserum against *B. thuringiensis* subsp. *berliner* 1715 crystal proteins. Wells 1, 2, 3, 4, 5, and 6 are as described in panel A. (C) Center well, Antiserum against the SF1 subfraction. Wells: 1, crude K-60 fraction from *B. thuringiensis* subsp. *aizawai* 7.29 crystals; 2, JM83(pHT671) extract; 3, JM83(pHTA4) extract; 4, SF1 subfraction from *B. thuringiensis* subsp. *aizawai* 7.29; 5, JM83(pHT71) extract; 6, JM83(pHTA2) extract.

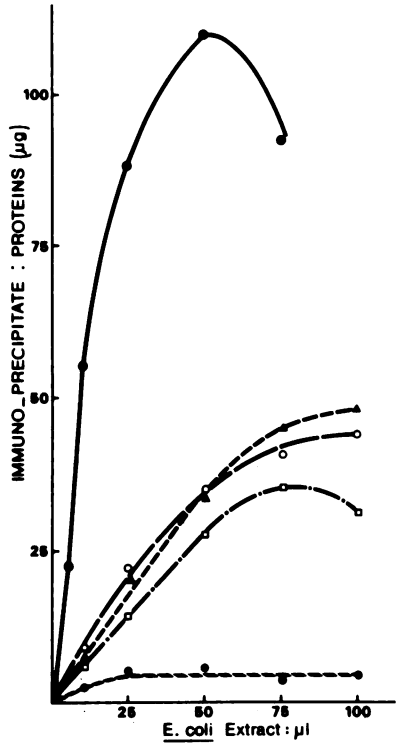

FIG. 5. Immunoprecipitation of the crystal protein antigens from the *E. coli* extracts of the different recombinant clones. Precipitation with antiserum against the *B. thuringiensis* subsp. *aizawai* 7.29 crystal proteins was performed as described in Materials and Methods. Symbols: ●---●, JM83(pUC18) extract; □, JM83(pHT71) extract; ○, JM83(pHT671) extract; ▲, JM83(pHTA4) extract; ●——●, JM83(pHTA2) extract.

ments performed with the recombinant *E. coli* extracts as described in Materials and Methods led to similar observations. Thus, it is clear that the three antisera used are tools which enabled us to differentiate the products of the different cloned genes and to compare them with components of the K-60 fraction.

Specific toxicities of *E. coli* extracts compared with those of crystal proteins. When estimating the toxicity of *E. coli* extracts from recombinant clones, it is difficult to quantify the results in terms of specific crystal proteins. To overcome this problem, we took advantage of immunoprecipitation experiments to determine as precisely as possible the levels of expression of the different cloned genes. Figure 5 shows examples of such experiments done with *E. coli* extracts, using antibody against the solubilized *B. thuringiensis* subsp. *aizawai* 7.29 crystals. Based on these results and on calibration curves as described in the Materials and Methods, the levels of expression of the cloned genes in *E. coli* were estimated (Table 2). The LC_{50}s in terms of specific proteins were determined at least two or three times, and these values were within the limits of the confidence intervals. Furthermore, depending on the clones, variations in the level of expression of the cloned δ-endotoxin genes could be observed according to growth conditions; this was particularly true for genes that were very poorly expressed in *E. coli*. For example, the expression of the pHTA4 clone was increased by two- or threefold in cultures to which ampicillin and IPTG (see Materials and Methods) were repeatedly added. Similar observations were reported by Oeda et al. (24). For these reasons, bioassays and immunoprecipitations to determine the expression rates were rigorously performed on the same batches of extract.

On these bases and owing to the fact that bioassays were performed by the forced feeding technique, it was feasible to determine the toxicities of the *E. coli* protein extracts in terms of LD_{50} (micrograms of toxin per gram of larvae) and to compare them with the toxicities of *B. thuringiensis* crystal proteins and their derivatives. The results presented in Table 2 suggest the following conclusions. (i) The products of the cloned crystal protein genes differed markedly in their specificity; (ii) the pHT71- and pHT671-encoded proteins appeared to be very specific toward *S. littoralis*, and the LD_{50}s, although appreciably higher, were of the same order of magnitude as those observed with *B. thuringiensis* subsp. *aizawai* native crystals, with the K-60 fraction, or with the SF1 and MF subfractions; (iii) the pHTA2-encoded crystal protein displayed a specific activity against *P. brassicae* larvae, with values of the same order of those for *B. thuringiensis* subsp. *berliner* native crystals or the SF2 subfraction; (iv) the pHTA4-encoded protein did not manifest any significant toxicity toward the two insect species.

Finally, it was also observed that *S. littoralis* second instar larvae are very susceptible to pHT71- and pHT671-encoded proteins. This was demonstrated by using the free ingestion technique for bioassays.

TABLE 2. Specific toxicities of the recombinant *E. coli* extracts and comparison with native *B. thuringiensis* crystals[a]

E. coli soluble extracts	% Expression in *E. coli*	*S. littoralis*		*P. brassicae*	
		LC_{50} (µg/µl)[b]	LD_{50} (µg/g)[c]	LC_{50} (µg/µl)[b]	LD_{50} (µg/g)[c]
JM83(pUC18)		NT	NT	NT	NT
JM83(pHTA2)	7.5	1.10 (0.61–2.03)	136	0.0054 (0.0042–0.0069)	0.24
JM83(pHTA4)	1	NT	NT	NT	NT
JM83(pHT671)	2	0.035 (0.018–0.066)	4.3	0.626 (0.486–0.798)	27.6
JM83(pHT71)	1.5	0.030 (0.023–0.039)	3.6	0.266 (0.142–0.499)	11.8
Native crystals					
B. thuringiensis subsp. *aizawai* 7.29		0.021 (0.016–0.028)	2.4	0.049 (0.035–0.069)	2.15
B. thuringiensis subsp. *berliner* 1715		0.110 (0.060–0.160)	12.5	0.007 (0.003–0.016)	0.31

[a] Fifth instar larvae were inoculated orally with 5 µl of the different preparations. NT, No toxicity at all with undiluted extracts.

[b] LC_{50}s with their confidence intervals (in parentheses) are given in terms of specific proteins. For *E. coli* extracts, such values were estimated on the basis of the levels of expression reported in the table, which are those corresponding, respectively, to the extracts used for bioassays.

[c] LD_{50}s, deduced from the LC_{50}s, were estimated in terms of micrograms of toxin per gram of larvae on the basis of an average individual weight of 41 or 113 mg for *S. littoralis* and *P. brassicae*, respectively.

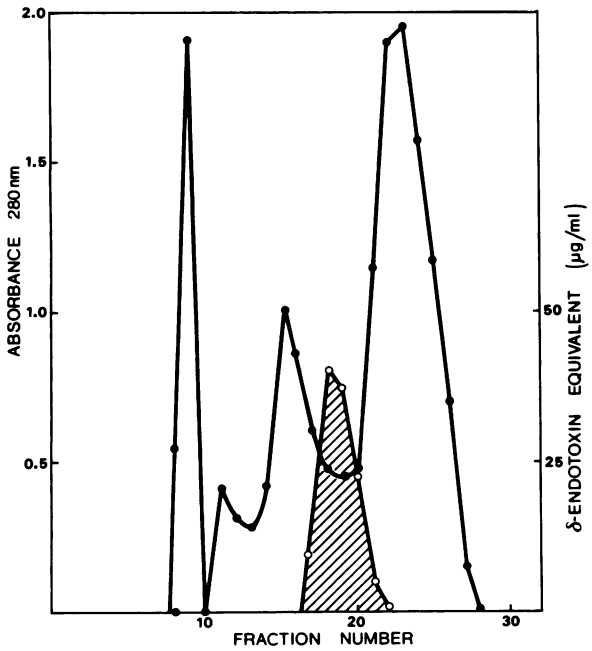

FIG. 6. Gel filtration of *E. coli* JM83(pHT671) extract. A 0.5-ml of sample of the *E. coli* extract previously enriched in crystal protein antigen as described in Materials and Methods was loaded on an Ultrogel AcA34 column (0.8 by 43 cm). Fractions of 1 ml eluted with buffer C were analyzed for A_{280} (●) and crystal protein antigen detection (○). The hatched peak represents the amount of δ-endotoxin equivalent as deduced from immunoprecipitation experiments.

Identification of *S. littoralis*-specific δ-endotoxin in *E. coli* extracts. The results reported above showed that a quantitative detection of δ-endotoxin molecules in *E. coli* extracts is possible. To aid the detection and identification of δ-endotoxins expressed in *E. coli*, we developed a procedure to obtain enriched extracts. Precipitation of the δ-endotoxin by decreasing the pH to 4 to 5 followed by gel filtration on an Ultrogel column of convenient pore size increased by a factor of 5- to 10-fold the proportion of the desired protein. Results of such an experiment conducted with an extract of JM83(pHT671) are presented in Fig. 6. Fractions containing the δ-endotoxin were detected in a defined region with an antiserum directed against solubilized *B. thuringiensis* subsp. *aizawai* crystal; the same fractions also reacted with antisera directed against the K-60 fractions MF and SF1 (data not shown). In the purified extract enriched in specific proteins, the δ-endotoxin material represents about 10% of total protein.

To compare this enriched fraction with the *E. coli* crude extracts and with the K-60 δ-endotoxin components, we performed, immunoblotting experiments with the *B. thuringiensis* subsp. *aizawai* antiserum (Fig. 7A). With crude extracts of *E. coli*(pHT671) or *E. coli*(pHT71) (lanes 4 and 5, respectively), specific proteins were revealed in a part of the gel corresponding to 90-kDa molecules, with derivatives in the 65- to 70-kDa region, as already reported (27). With Ultrogel fraction 18 (lane 3), the main component was detected in the 70-kDa region at the level of the upper band (band 1) of the crude K-60 fraction or of the MF fraction (lane 1). It is easy to observe that the 90-kDa component (very faint band) disappeared spontaneously during the purification and storage periods to give a 70-kDa component.

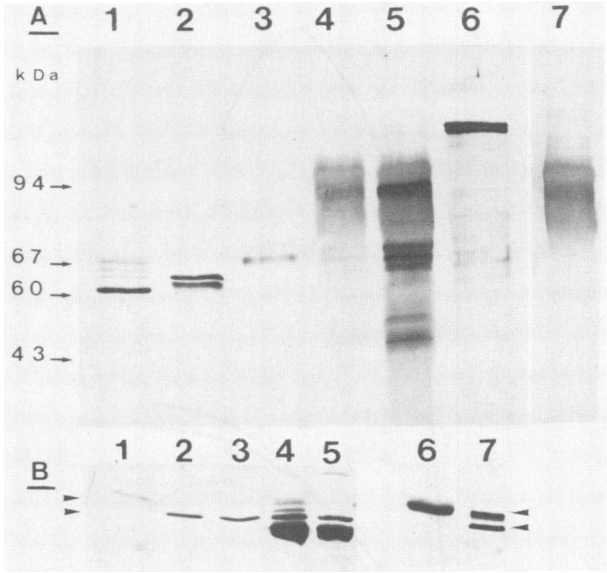

FIG. 7. Comparison of a crystal protein antigen in the *E. coli* JM83(pHT671) extract with the K-60 fraction. SDS-PAGE was run on a 10% acrylamide gel. Immunoblotting was done with antiserum against the *B. thuringiensis* subsp. *aizawai* 7.29 crystal proteins as the first antibody. (A) Lanes 1 and 2, MF and SF1 fractions, respectively, from *B. thuringiensis* subsp. *aizawai* 7.29 crystals; lane 3, crystal protein antigen in fraction 18 eluted from the Ultrogel column (Fig. 6); lane 4, JM83(pHT71) extract; lane 5, JM83(pHT671) extract; lane 6, purified *B. thuringiensis* subsp. *aizawai* crystals; lane 7, JM83(pUC18) extract. (B) Lanes: 1, crystal protein antigen in fraction 18 of the Ultrogel column (fig. 6); 2 and 3, the same fraction treated with trypsin (0.5 or 1 μg of enzyme, respectively, for 40 μl of preparation incubated at 30°C for at least 30 min); 4, MF fraction; 5, MF fraction treated with trypsin as described for lane 3; 6, fraction 18 as described for lane 1; 7, fraction 18 treated with trypsin as described for lane 2, but in two independent experiments. The two arrowheads on the left and on the right indicate the 70- and 65-kDa components, respectively.

This component did not react at all with the antiserum specific to the *B. thuringiensis* subsp. *berliner* δ-endotoxins, but it reacted positively with antibodies raised against the SF1 fraction. Furthermore, the results of a mild proteolytic digestion of enriched fraction 18 by trypsin indicated that the 70-kDa component disappears to give a major final product migrating at the level of the 65-kDa component of the SF1 fraction that is antigenically related to the 70-kDa protein (Fig. 7B, 1, 2, 3, 6, and 7); in the experiment presented in lanes 6 and 7, the transformation into a 65-kDa component was not totally achieved (lane 7). This is also consistent with the fact that the 70-kDa component of the K-60 fraction was shown to progressively disappear as a result of further proteolysis. In Fig. 7B (lanes 4 and 5) are shown the results of a trypsin treatment of the MF fraction leading to the disappearance of the 70-kDA component and also the 68-kDa component (lane 5).

We assumed that the product of the δ-endotoxin truncated gene cloned in pHT671 and pHT71 which is specifically active against *S. littoralis* can be easily detected in *E. coli* protein extracts; furthermore, it is apparently related to the 65-kDa component of the SF1 fraction purified from the K-60 mixtures.

In the same way, we were able to detect the product of the pHTA4 cloned gene that is very probably the 63-kDa component of the SF1 fraction.

DISCUSSION

In an attempt to investigate factors determining the potency of certain *B. thuringiensis* strains against the cotton leafworm *S. littoralis*, we demonstrated unambiguously that several different δ-endotoxin molecules are present in the insecticidal crystals. From *B. thuringiensis* subsp. *aizawai* 7.29, we have cloned (27) and identified multiple δ-endotoxin genes with diverse localizations. Owing to their potency against insects of the Noctuidae family and of other families among Lepidoptera, strains of the *B. thuringiensis* subsp. *aizawai* serotype (H-7) are the subject of many studies. Indeed, besides a specific activity toward *Spodoptera* species, these strains can display a large host spectrum. Recently, Haider et al. (10) and Haider and Ellar (9) reported the toxicity of a new *B. thuringiensis* subsp. *aizawai* strain (IC1) against both lepidopteran and dipteran species. This toxicity was due to a unique polypeptide and apparently depended on differential processing of the protoxin molecule. At the same time, Knowles and Ellar (18) characterized two types of cytolytic activity directed against two different lepidopteran cell lines in vitro and resulting from different crystal polypeptides in *B. thuringiensis* subsp. *aizawai* HD249.

A simple SDS-gel electrophoresis study of crystals from *B. thuringiensis* subsp. *aizawai* 7.29 indicated the presence of at least two distinct polypeptides, seen as a doublet band at 130 to 135 kDa (as for many strains). Furthermore, from experiments involving a *Bacillus cereus* transconjugant that received a 45-MDa *B. thuringiensis* subsp. *aizawai* plasmid bearing a copy of the crystal protein gene (homologous to a 5.3-kb class gene) (22), we have evidence that such a gene encodes a 130-kDa protein which corresponds to the lower band of the doublet (data not shown).

Under a variety of conditions believed to be similar to those prevailing in vivo within the insect gut, proteolysis of crystals led to multiple components which we were able to separate into distinct fractions. Among these components, there are polypeptides which must correspond to the minimum toxic domain, as delineated by Schnepf and Whiteley (28), Höfte et al. (11), Nagamastsu et al. (23), or other researchers (1). One of these components is a 61-kDa polypeptide specifically active against *P. brassicae*, whereas another (a 65-kDa polypeptide included in SF1) looks like a component specifically active against *S. littoralis*. We have also observed that the 68- and 70-kDa components, which are also components of the *S. littoralis*-active MF fraction, appear to be antigenically related to the 65-kDa component (data not shown). These components might correspond to intermediate proteolysed products, particularly to N-terminal domains in which the peptide bond between Arg-27 and Ile-28 (23, 28) or other peptide bonds at secondary sites were not cleaved.

It is important to note that isoleucine has been found to be the main N-terminal amino acid in the crude K-60 fraction; methionine, in lower amounts, was also detected. Furthermore, the values for the molecular weight we proposed are in good agreement with information revealed by the sequence of amino acids deduced from nucleotide sequencing data (unpublished data). It seems, therefore, that the 65-kDa component corresponds to the minimum toxic domain in which the 28 first amino acids were eliminated through proteolysis, whereas the 70- or 68-kDa component could represent the actual complete N-terminal active moieties of a δ-endotoxin molecule.

Whether the different components, apparently distinct, act independently or synergistically to give the global activity estimated in K-60 mixtures or in native crystals is a question that remains unresolved. The SF1 fraction has a slightly lower toxicity than crude K-60 or MF fractions, and this may be due either to the absence of the 61-kDa component or to the presence of the 63-kDa component (band 4) which is unrelated to *S. littoralis*-active polypeptides. To elucidate this point, it would be necessary to test various mixtures of unique components.

A second approach that would help to clarify this question would be to obtain purified polypeptides encoded by each of the cloned genes to examine their nature and to determine how they act together in mixtures. Because we used *E. coli* extracts with high protein concentrations, we were able to identify and to estimate with relative precision the distinct specific proteins that are the product of the different genes cloned in the recombinant plasmids pHTA2, pHTA4, pHT671, and pHT71. Then, such proteins were correlated with polypeptides of the K-60 fraction that are normal components of the insecticidal crystals, particularly regarding the products of pHT671 and pHT71, which are specific toward *S. littoralis*. Interestingly, these products reacted positively with monoclonal antibodies (provided by H. Höfte from Plant Genetic Systems, Ghent, Belgium) directed against a 135-kDa polypeptide common to a number of strains active against *S. littoralis*; a postive reaction was also observed with the 65-kDa polypeptide we identified in the SF1 fraction and with the 68- and 70-kDa components, whereas the 61-kDa polypeptide (SF2) and the 63-kDa component of the SF1 fraction did not react at all with these antibodies. Furthermore, we also know that this last component does not appear to be necessary for a full activity against *S. littoralis*.

Taking together the different results, we can reasonably assume that at least three distinct δ-endotoxin genes are expressed in *B. thuringiensis* subsp. *aizawai* 7.29. The presence of products of other genes homologous to known classes of crystal protein genes, but as yet unidentified, cannot be ruled out.

Also, it should be noted that a decisive point in the identification of the gene products was the estimation of the actual LD_{50}, only possible when known amounts of crystal protein are given to each larva. In this way, specificities that radically differ were clearly determined; in the case of the expression products of the δ-endotoxin genes isolated in plasmids pHT671 and pHT71, the LD_{50}s are equivalent to those of native *B. thuringiensis* subsp. *aizawai* crystals. However, there are no decisive results regarding the pHTA4 product, for which we still have not detected a target insect.

It must be kept in mind that comparing LD_{50}s of these expression products with those of crystals is not entirely correct, because the crystals are themselves the product of several toxin genes. For this reason, referring to the different purified K-60 subfractions was a positive aspect.

In conclusion, in *B. thuringiensis* subsp. *aizawai* 7.29, multiple δ-endotoxin genes (of different structural types) direct the synthesis of several δ-endotoxins with different specificities, which were identified as components of the insecticidal crystals. One of these proteins was characterized as responsible for the larvicidal activity against *S. littoralis* and other insects of the Noctuidae family and was also shown to be present in crystals of other strains displaying the same specificity.

In this way, our present results are consistent with the suggestion (13) that various combinations of the δ-endotoxin families could explain the extreme diversity and complexity

of the host range manifested by the lepidopteran-active strains of *B. thuringiensis*.

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