

## Comparative Biochemistry of Entomocidal Parasporal Crystals of Selected *Bacillus thuringiensis* Strains†

DANA J. TYRELL,<sup>1,2</sup> LEE A. BULLA, JR.,<sup>1,2\*</sup> ROBERT E. ANDREWS, JR.,<sup>1,2</sup> KARL J. KRAMER,<sup>2,3</sup>  
LOREN I. DAVIDSON,<sup>2</sup> AND PHILIP NORDIN<sup>3</sup>

*Division of Biology<sup>1</sup> and Department of Biochemistry,<sup>3</sup> Kansas State University, and U.S. Grain Marketing Research Laboratory, Science and Education Administration, U.S. Department of Agriculture,<sup>2</sup> Manhattan, Kansas 66502*

Parasporal crystals of *Bacillus thuringiensis* subsp. *kurstaki*, *tolworthi*, *alesti*, *berliner*, and *israelensis* were compared by electron microscopy, polyacrylamide gel electrophoresis, amino acid analysis, tryptic peptide mapping, immunological analysis, and insecticidal activity. Spore coats also were compared by polyacrylamide gel electrophoresis. *B. thuringiensis* subsp. *israelensis* crystals were lethally toxic to mosquito larvae and nontoxic to tobacco hornworm larvae. Conversely, crystals from the other subspecies killed tobacco hornworm larvae but were ineffective against mosquitoes. Crystalline inclusion bodies of all subspecies contained a protoxic subunit that had an apparent molecular weight of approximately  $1.34 \times 10^6$ . However, polyacrylamide gel electrophoretic patterns of solubilized crystals revealed a small-molecular-weight component (apparent molecular weight, 26,000) in *B. thuringiensis* subsp. *israelensis* that was absent in the other subspecies. Also, differences were noted in amino acid composition and tryptic peptide fingerprints. Crystal proteins were found in spore coats of all subspecies. The results suggest that insecticidal specificity is due to unique polypeptide toxins.

Previously, we characterized the entomocidal parasporal crystal of *Bacillus thuringiensis* subsp. *kurstaki* (5). The crystal contains a single glycoprotein subunit that has an apparent molecular weight of  $1.34 \times 10^6$ . The glycoprotein is a protoxin that, under appropriate conditions, is converted to a toxic molecule whose apparent molecular weight is  $6.8 \times 10^4$  (4).

*B. thuringiensis* subsp. *kurstaki* is an agriculturally important organism because it kills lepidopteran insects (moths). Recently, another potentially useful strain, *B. thuringiensis* subsp. *israelensis*, has been isolated that produces a toxin effective against mosquitoes and black flies (8-12, 30-32). We are interested in comparing the properties of the crystal of *B. thuringiensis* subsp. *israelensis* that is toxic to mosquitoes with those of other strains which synthesize crystals that kill moths to determine whether there are differences in the polypeptide composition of these various strains. Therefore, we have compared some of the biochemical, immunological, and toxicological properties of parasporal crystals isolated from five strains of *B. thuringiensis*. The strains selected include some of the more important subspecies that have been

isolated from different parts of the world. Four of the strains that we investigated produce crystals that are toxic to lepidopterans; the fifth one is *B. thuringiensis* subspecies *israelensis*, whose crystals kill dipteran insects.

### MATERIALS AND METHODS

**Organisms and cultural conditions.** *B. thuringiensis* subsp. *kurstaki* used in this study was isolated from a commercial insecticidal formulation called Dipel (Abbott Laboratories, North Chicago, Ill.); *B. thuringiensis* subsp. *alesti* was a gift from P. C. Fitz-James, University of Western Ontario Medical School, London, Ontario, Canada; *B. thuringiensis* subsp. *berliner* was given to us by M. M. Lecadet, Centre National de la Recherche Scientifique et Universite, Paris, France; *B. thuringiensis tolworthi* was a gift from B. N. Herbert, Imperial College, London, England; and *B. thuringiensis* subsp. *israelensis* was isolated from an experimental preparation provided by Abbott Laboratories. Originally, it was isolated by Goldberg and Margalit (12) from soil samples near mosquito breeding sites, and it is designated strain 60A. All cultures were maintained on MD agar (3) slants. Cells for experimental use were cultured in the manner described previously for *B. thuringiensis* subsp. *kurstaki* (26).

**Determination of growth characteristics.** Cells maintained on MD agar slants were inoculated into 100 ml of liquid MD medium in 300-ml Erlenmeyer flasks and aerated by rotary agitation at 200 rpm

† Contribution no. 81-171-J, Division of Biology and Department of Biochemistry, Kansas Agricultural Experiment Station, Kansas State University, Manhattan, KS 66506.

overnight at 28°C. The cells were phased by transferring a 10% inoculum into 100 ml of modified GYS (22) medium in 300-ml Erlenmeyer flasks and incubating the flasks at 28°C. Mid-exponential cells (6% inoculum) were transferred into 300-ml nephelometer flasks and then shaken at 200 rpm at 28°C for an extended period. Absorbance and pH readings were taken at 1- to 2-h intervals throughout the growth and sporulation cycle of each strain. Morphological changes were monitored by phase-contrast microscopy. Duplicate growth curves were plotted for each *B. thuringiensis* strain.

**Electron microscopy.** Sporulated but unlysed cultures of each *B. thuringiensis* strain were centrifuged at 4°C, and the pellets were suspended in 4% glutaraldehyde in 0.01 M phosphate-buffered saline (pH 7.2) for 5 min at 4°C. The cells were pelleted at 2,000 rpm and suspended in warm (55°C) 2% water-agar. The agar was immediately cooled to 4°C, cut into 1-mm cubes, and placed in fresh cold fixative for 1 h. Samples were washed four times in cold buffer for a total of 80 min. Postfixation, embedding in Epon 812 (20), sectioning, and electron microscopy were done as previously described (2).

**Isolation, purification, and solubilization of parasporal crystals.** Parasporal crystals were separated from spores and cellular debris by buoyant density centrifugation in Renografin gradients (27). Crystals isolated in this manner were washed at least three times in water and lyophilized to constant weight. Purity of preparations was monitored at each step of the separation procedure by phase contrast microscopy. Complete solubilization was accomplished by incubating the crystals in 1% (wt/vol) sodium dodecyl sulfate (SDS); 2% (vol/vol)  $\beta$ -mercaptoethanol (ME); 6 M urea and an equimolar (0.01 M) ratio of  $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  (pH 7.2) for 1 h at 28°C. Soluble toxic material was obtained in the following manner. A suspension of crystals (0.4%, wt/vol) was titrated to pH 12 with 1 N NaOH at 28°C. The crystal suspension was allowed to stay at this pH for 5 h and then dialyzed against 0.02 N phosphate at pH 7.5. Protein was determined by absorbance at 280 nm using an extinction coefficient of 1.1 absorbance units equal to 1 mg of protein per ml. After various times of incubation, samples were subjected to either bioassays or gel filtration as described below.

**Spore coat protein extraction.** Spores purified on Renografin gradients were extracted three times with 6 M guanidinium chloride and 0.1 M  $\beta$ -mercaptoethanol at pH 8.6. The extracts were subsequently dialyzed against 0.1 M Tris-hydrochloride buffer containing 9 M urea (pH 8.6) for 24 h and then against water for 48 h (40 to 44 liters) before they were lyophilized.

**Polyacrylamide gel electrophoresis.** Parasporal crystals solubilized by the methods described above and spore extracts solubilized with SDS, urea, and  $\beta$ -mercaptoethanol as described above were subjected to electrophoresis on SDS polyacrylamide slab gels (1.5 by 140 by 170 cm; Buchler Polyslab polyacrylamide gel electrophoresis system) by using the method of Weber and Osborn (33, 34). Gradient gels containing 3 to 10%, 5 to 15%, 5 to 20%, and 10 to 20% acrylamide were used to characterize spore and crystal polypep-

tides. Electrophoresis was carried out at 40 to 90 V. Gels were stained for 3 to 12 h in 0.25% (wt/vol) Coomassie brilliant blue (Eastman Kodak Co., R250) in methanol-acetic acid-water (25:7.5:62.5, vol/vol/vol), and destained in methanol-acetic acid-water for 16 to 48 h. Molecular weight standards for the 3 to 10% acrylamide gels were myosin (200,000),  $\beta$ -galactosidase (135,000), phosphorylase B (92,000), bovine serum albumin (68,000), and ovalbumin (43,000), which were prepared by Bio-Rad Laboratories. Cross-linked bovine serum albumin (molecular weight, 68,000, 136,000, etc.) from Sigma Chemical Co., bovine albumin (65,000), ovalbumin (45,000), beef pancreas chymotrypsinogen A (25,000), and sperm whale myoglobin (17,000) from Schwarz/Mann, RNase A (13,600) from Pharmacia Fine Chemicals, Inc., and bovine pancreas insulin (2,400) from Sigma were used as molecular weight standards in the other gels. Destained gels were photographed on 35-mm, high-contrast copy film that was developed in Kodak D-19. Gels were dried by using a Bio-Rad model 224 slab gel dryer.

**Reduction and S-alkylation.** Purified crystals were reduced and S-carboxymethylated by the procedure of Crestfield et al. (7) in 6 M guanidinium chloride-0.25 M Tris-hydrochloride-0.003 M EDTA (pH 8.5). S-Carboxymethylation was done by using iodoacetate in 1 N NaOH (14). The material was then dialyzed against distilled water (40 liters) for 48 h.

**Protein determination.** Protein was determined by the procedure of Lowry et al. (19) and by absorbance at 280 nm with an extinction coefficient of 1.1 absorbance unit equal to 1 mg of protein per ml for all crystal preparations.

**Amino acid analysis.** Samples were analyzed on a Beckman 120C analyzer after hydrolysis for 25 h in 6 N HCl. Labile amino acids were quantitated by extrapolation to zero time. Cystine and cysteine were determined as cysteic acid, and methionine was determined as the sulfone after performic acid oxidation (15). Tryptophan was quantitated in samples hydrolyzed in 3 M *p*-toluenesulfonic acid containing a small amount of tryptamine. The hydrolysis was carried out for 24 h at 110°C in vacuo (18).

**Tryptic digestion and peptide mapping.** S-Alkylated crystals were incubated for 24 h at 37°C with diphenylcarbonyl chloride-treated trypsin as previously described (16). Two samples of trypsin (each 2%, wt/wt) were used. One was added at the beginning of incubation, and the other was added after 2 to 4 h of incubation.

Maps of tryptic digests of 300 to 500  $\mu\text{g}$  of S-alkylated crystals were prepared on thin-layer cellulose sheets (20 by 20 cm, Eastman). Material was dissolved in 80% methanol, centrifuged for 15 min at 5,000 rpm, and spotted onto a corner of the sheet, 5  $\mu\text{l}$  at a time. Two-dimensional separation was obtained by electrophoresis at pH 2 (8% [vol/vol] acetic acid, 2% [vol/vol] formic acid at 200 V per plate for 2 h followed by chromatography with butanol-pyridine-acetic acid-water (15:10:3:12, vol/vol/vol/vol) for 3 to 4 h. The peptides were visualized by staining with ninhydrin-cadmium acetate (13). Plates were photographed by using high-contrast copy film which was developed with Kodak D-19.

**Carbohydrate analysis.** Monosaccharides were

determined by high-performance liquid chromatography (21) after hydrolysis with 2 N HCl for 2 h or 4 N HCl for 3 h and evaporation of the acid under vacuum at 0°C. Sugars also were chromatographed on the same column as <sup>3</sup>H-alditols after reduction with NaB<sub>3</sub>H<sub>4</sub> (29). Retention times and elution volumes were compared with those of standard sugars and alditols.

**Preparation of antisera.** Antisera were prepared by inoculating New Zealand white albino rabbits subcutaneously in four sites with a total of 1 mg of solubilized crystal toxin suspended in complete Freund adjuvant. After 4 weeks, the rabbits were reinoculated with another 1-mg dose of solubilized crystal in incomplete Freund adjuvant. The animals were bled at the end of 2 weeks and each subsequent week thereafter.

**Ouchterlony assays.** Ouchterlony gels were prepared in 1% agarose, 2% polyethylene glycol 6000, 0.089 M boric acid, 0.089 M Tris base, and 0.001 M EDTA (pH 8.3). Antigen and antiserum were placed into the gels and allowed to incubate overnight at room temperature. Precipitin bands were visible within 10 to 12 h.

**Gel filtration chromatography.** Sepharose CL-4B (100-200 mesh; Pharmacia) was equilibrated in 0.02 M NaH<sub>2</sub>PO<sub>4</sub> (pH 7.5). The gel slurry was poured to a column bed height of 50 cm in a glass column (1.5-cm inner diameter by 90 cm; Pharmacia). The Sepharose bed was further equilibrated by passing two column volumes of the above buffer through the column. The void and inclusion volumes were determined by using blue dextran 2000 and <sup>3</sup>H-labeled diisopropyl phosphorofluoridate. The elution behavior of alkali-solubilized (see below) parasporal crystals was compared under conditions identical with those of protein molecular weight standards (2 mg/0.4 ml) including aldolase (158,000), egg albumin (45,000), and chymotrypsinogen A (25,000).

**Insect bioassays.** Bioassays were done as previously described against the tobacco hornworm *Manduca sexta* (26) and the mosquitoes *Aedes aegypti*, *Culex pipiens* var. *quinquefasciatus*, and *Anopheles albimanus* (30). Alkali-solubilized crystals were bioassayed by the same techniques after the preparations were dialyzed against 0.02 N NaH<sub>2</sub>PO<sub>4</sub> (pH 7.5). Gel filtration chromatographic fractions also were bioassayed exactly as were the alkali solubilized crystals.

## RESULTS

**Growth characteristics.** All five strains grew at almost the same rate. The doubling times were: *B. thuringiensis* subsp. *kurstaki*, 50 min; *B. thuringiensis* subsp. *berliner*, 70 min; *B. thuringiensis* subsp. *alesti*, 65 min; *B. thuringiensis* subsp. *tolworthi*, 80 min; *B. thuringiensis* subsp. *israelensis*, 65 min. Sporulation was complete (beginning of lysis) at 16 h in *B. thuringiensis* subsp. *berliner* and at 10 h *B. thuringiensis* subsp. *kurstaki*, *alesti*, *tolworthi*, and *israelensis*. Parasporal crystals formed at about stage III of sporulation in all of the strains. The pH of the culture medium during exponential

growth decreased from 7.0 to about 6.6 at the beginning of sporulation and then increased to about 8.0 during the early stages of sporulation. The pH remained constant during the early stages of sporulation. The pH remained constant during the later stages of sporulation. All five subspecies exhibited this pattern, although the pH during the later stages of sporulation varied somewhat among the subspecies.

**Morphology.** Thin sections of *B. thuringiensis* subsp. *kurstaki* and *israelensis* cells are shown in Fig. 1. The four subspecies toxic to lepidopteran insects had bipyramidal crystals and ovoid inclusions in sporulating cells (Fig. 1A). Each cell usually contained only one crystal and one ovoid inclusion. *B. thuringiensis* subsp. *israelensis* cells contained inclusions that varied in size and shape (Fig. 1B). The crystals were either cubic, bipyramidal, ovoid, or amorphous. Also, there were complexes of several inclusion bodies enclosed in netlike structures. Generally, cells of *B. thuringiensis* subsp. *israelensis* contained two or three inclusion bodies (Fig. 1B).

**Insect toxicity.** The effects of parasporal crystals from the four lepidopteran toxic crystals on the tobacco hornworm are presented in Table 1 as lethal concentrations (50% endpoints) along with 95% confidence limits. As can be seen, crystals from all four strains were extremely toxic to the hornworm. *B. thuringiensis* subsp. *berliner* was about 5 times as toxic as the other three strains that had nearly equal 50% lethal concentration values. Approximately 90 to 100% of the larvae were killed at concentrations of 25.1 ng/cm<sup>2</sup> for *B. thuringiensis* subsp. *kurstaki*, 5.5 ng/cm<sup>2</sup> for *B. thuringiensis* subsp. *berliner*, 27.2 ng/cm<sup>2</sup> for *B. thuringiensis* subsp. *alesti*, and 34.1 ng/cm<sup>2</sup> for *B. thuringiensis* subsp. *tolworthi*. *B. thuringiensis* subsp. *israelensis* crystals, however, exhibited no toxicity to *M. sexta* larvae at concentrations as high as 2.0 × 10<sup>4</sup> ng/cm<sup>2</sup>. de Barjac (8) also observed that *B. thuringiensis* subsp. *israelensis* was ineffective against lepidopteran larvae.

The toxicity of *B. thuringiensis* subsp. *israelensis* parasporal crystals on larvae of three species of mosquitoes is shown in Table 2. The crystals were very effective against these particular insects. Parasporal crystals from the other four subspecies affected the mosquito larvae only at very high concentrations. Their toxicity to mosquitoes was 10<sup>3</sup> to 10<sup>6</sup> times less than that of *B. thuringiensis* subsp. *israelensis* crystals.

**Polyacrylamide gel electrophoresis of crystals.** Crystals of all strains are extremely insoluble and require denaturants such as SDS, urea, or guanidine hydrochloride plus a reducing agent such as β-mercaptoethanol to render them

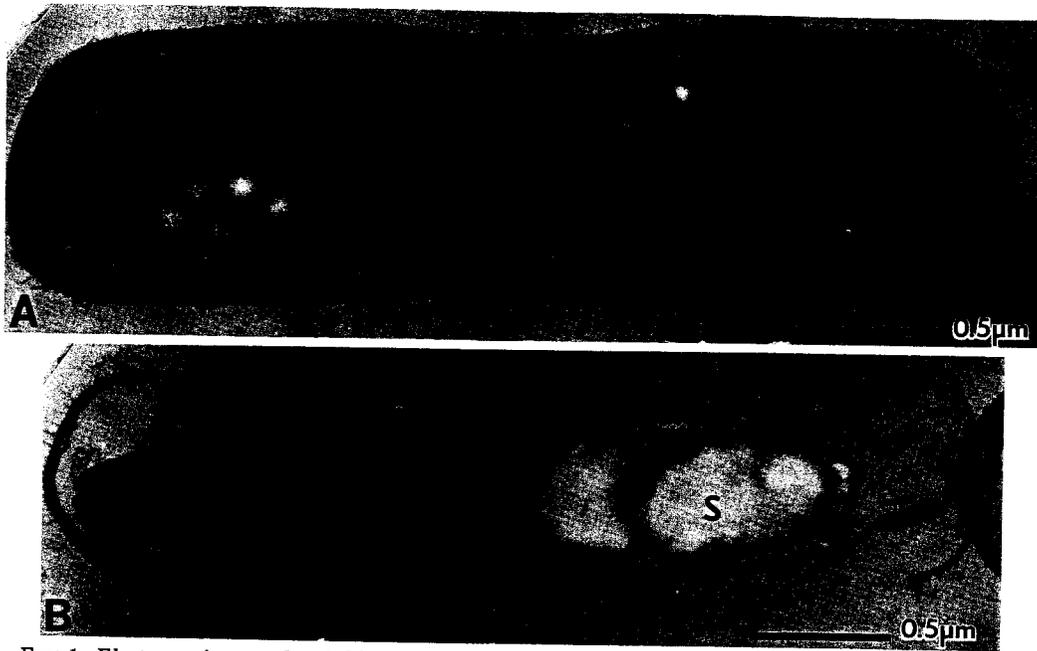


FIG. 1. Electron micrographs of thin-sectioned, sporulated cells of *B. thuringiensis* subsp. *kurstaki* (A,  $\times 44,000$ ) and *B. thuringiensis* subsp. *israelensis* (B,  $\times 37,000$ ). OI, Ovoid inclusion; PC, parasporal crystal; S, spore.

TABLE 1. Lethal concentrations of *B. thuringiensis* parasporal crystals for insects

Subspecies	Lethal concentration, 50% endpoint <sup>a</sup>			
	<i>M. sexta</i> (ng/cm <sup>2</sup> )	<i>A. aegypti</i> (10 <sup>-4</sup> g/ml)	<i>C. pipiens</i> (10 <sup>-4</sup> g/ml)	<i>A. albimanus</i> (10 <sup>-4</sup> g/ml)
<i>B. thuringiensis</i> subsp. <i>kurstaki</i>	6.88 (5.50-8.50)	—	—	—
<i>B. thuringiensis</i> subsp. <i>berliner</i>	1.36 (1.16-2.25)	—	—	—
<i>B. thuringiensis</i> subsp. <i>alesti</i>	6.14 (3.41-13.10)	—	—	—
<i>B. thuringiensis</i> subsp. <i>tolworthi</i>	6.82 (5.53-9.48)	—	—	—
<i>B. thuringiensis</i> subsp. <i>israelensis</i>	>100	1.9 (1.4-2.5)	3.7 (2.0-6.6)	80 (46-140)

<sup>a</sup> Numbers within parentheses are 95% confidence limits.

TABLE 2. Amino acid composition of *B. thuringiensis* crystals

Subspecies	Mol per 100 mol of amino acids <sup>a</sup>																	
	Trp	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	$\frac{1}{2}$ Cys <sup>b</sup>	Gly	Ala	Val	Met <sup>c</sup>	Iso	Leu	Tyr	Phe
<i>B. thuringiensis</i> subsp. <i>kurstaki</i>	0	2.8	1.8	6.7	13.5	6.2	8.3	12.2	4.4	2.0	7.2	5.4	6.2	1.0	4.9	8.2	4.9	5.2
<i>B. thuringiensis</i> subsp. <i>berliner</i>	0.5	2.5	1.7	5.5	15.1	6.9	9.1	12.9	3.1	1.3	9.0	6.5	4.3	0.8	3.0	8.7	4.5	4.6
<i>B. thuringiensis</i> subsp. <i>alesti</i>	0	2.6	1.5	5.3	14.0	6.3	10.2	13.1	4.1	1.8	9.1	6.4	4.6	0.7	3.2	8.6	4.4	4.7
<i>B. thuringiensis</i> subsp. <i>tolworthi</i>	0	2.9	1.7	6.3	13.7	6.6	9.0	12.6	3.2	1.2	8.2	6.4	5.0	0.9	4.3	8.5	4.6	4.9
<i>B. thuringiensis</i> subsp. <i>israelensis</i>	0	4.2	1.3	2.3	14.2	8.0	6.9	10.7	5.0	2.1	5.4	7.3	7.7	1.9	6.1	8.2	3.8	4.8

<sup>a</sup> Determined by hydrolysis in *p*-toluene sulfonic acid (18). Half cystine and methionine were not stable to hydrolysis.

<sup>b</sup> Determined as cysteic acid after performic acid oxidation (15).

<sup>c</sup> Determined as methionine sulfone after performic acid oxidation (15).

soluble (6). Either boiled or reduced and S-carboxymethylated crystal proteins in reducing and denaturing solvents exhibited the same electrophoretic profile on SDS-polyacrylamide gels as proteins dissolved in the reducing and denaturing solvent without alkylation or boiling. Figure 2 is an electrophoretogram of *B. thuringiensis* crystals solubilized in 6 M urea-1% SDS-1%  $\beta$ -mercaptoethanol-0.01 M  $\text{NaH}_2\text{PO}_4$ -0.01 M  $\text{Na}_2\text{HPO}_4$  (pH 7.2). Electrophoresis of the solubilized crystals produced a major band (band 1) in the SDS-polyacrylamide gel with an apparent molecular weight of approximately  $1.35 \times 10^5$  in *B. thuringiensis* subspp. *kurstaki* (track B), *tolworthi* (track E), *alesti* (track F), and a double band with the same apparent molecular weight for *B. thuringiensis* subspp. *berliner* (track G). This protein has been shown to be the protoxin in *B. thuringiensis* subspp. *kurstaki* (Bulla et al., unpublished data) and is a single repeating subunit in the crystal. The smallest protein produced from *B. thuringiensis* subspp. *kurstaki*, *berliner*, *alesti*, and *tolworthi* had an apparent molecular weight of  $6.8 \times 10^4$  (band 2). This

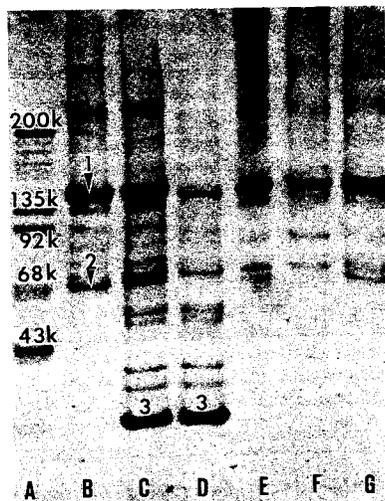


FIG. 2. SDS-polyacrylamide slab gel electrophoresis of *B. thuringiensis* parasporal crystals solubilized in 1% SDS, 1%  $\beta$ -mercaptoethanol, 6 M urea-0.01 N  $\text{NaH}_2\text{PO}_4$ -0.01 N  $\text{Na}_2\text{HPO}_4$  (pH 7.2). This slab contained a continuous gradient of 3 to 10% acrylamide. (A) Molecular weight standards myosin (200,000),  $\beta$ -galactosidase (135,000), phosphorylase B (92,000), bovine serum albumin (68,000), and ovalbumin (43,000); 1  $\mu\text{g}$  of each protein was applied. (B) *B. thuringiensis* subspp. *kurstaki*, 5  $\mu\text{g}$ ; (C) *B. thuringiensis* subspp. *kurstaki* (5  $\mu\text{g}$ ) and *israelensis* (10  $\mu\text{g}$ ) crystals solubilized together; (D) *B. thuringiensis* subspp. *israelensis*, 10  $\mu\text{g}$ ; (E) *B. thuringiensis* subspp. *tolworthi*, 7  $\mu\text{g}$ ; (F) *B. thuringiensis* subspp. *alesti*, 5  $\mu\text{g}$ ; (G) *B. thuringiensis* subspp. *berliner*, 5  $\mu\text{g}$ .

polypeptide is the insecticidal toxin in *B. thuringiensis* subspp. *kurstaki* (4). *B. thuringiensis* subspp. *israelensis* crystals produced only minor amounts of material with apparent molecular weights of approximately  $1.35 \times 10^5$  and  $6.8 \times 10^4$  (bands 1 and 2, track D) as well as several lower-molecular-weight polypeptides. The molecular weights of these bands were determined by electrophoresis on a gel having a 5 to 20% acrylamide gradient (Fig. 3). The major component of *B. thuringiensis* subspp. *israelensis* crystals had an apparent molecular weight of  $2.6 \times 10^4$  (Fig. 3, band 1, tracks B and C; Fig. 2, band 3, tracks C and D).

Crystals from *B. thuringiensis* subspp. *kurstaki* and *israelensis* also were solubilized together, and the electrophoretic profile produced is shown in Fig. 2, track C. The pattern obtained overlapped with the pattern for crystals solubilized separately and electrophoresed (Fig. 2, tracks B and D). Apparently, the unique electrophoretic pattern produced by *B. thuringiensis* subspp. *israelensis* crystals is not due to proteases of different specificity bound to the crystals.

Crystals can be solubilized without reducing and denaturing agents by increasing the pH of the solvent (4). This was accomplished by titration of the crystals with 1 N NaOH to pH 12 and dialysis against 0.02 N phosphate at pH 7.5. These solubilized preparations had 50% lethal concentration values similar to those of whole crystals. Electrophoresis of such preparations (Fig. 4) produced patterns similar to those in Fig. 2, except that there were more "conversion

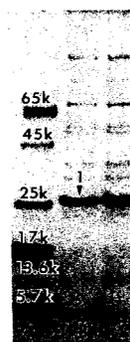


FIG. 3. SDS-polyacrylamide gel electrophoresis of *B. thuringiensis* subspp. *israelensis* crystals solubilized as described in the legend to Fig. 2 on a slab containing a continuous gradient of 5 to 20% acrylamide. (A) Molecular weight standards: bovine albumin (65,000), ovalbumin (45,000), chymotrypsinogen A (25,000), myoglobin (17,000), ribonuclease A (13,600), and bovine insulin (A chain, 2,384); 1 to 2  $\mu\text{g}$  of each protein was applied; (B) *B. thuringiensis* subspp. *israelensis* crystal, 10  $\mu\text{g}$ ; (C) *B. thuringiensis* subspp. *israelensis*, 15  $\mu\text{g}$ .

products" of molecular weights  $6.8 \times 10^4$  to  $1.3 \times 10^5$  produced from the lepidopteran toxic crystals (Fig. 4, tracks C to F) and greater amounts of components ranging in molecular weight from  $6.8 \times 10^4$  to  $2.6 \times 10^4$  from *B. thuringiensis* subsp. *israelensis* (Fig. 4, track G).

**Gel filtration chromatography.** Gel filtration of alkali-solubilized crystals in Sepharose CL-4B and phosphate buffer at pH 7.5 yielded single peaks, suggesting the presence of only one subunit in all of the crystals. Based on elution volumes (Fig. 5), the molecular weight estimation for the lepidopteran crystal subunits was  $1.35 \times 10^6$  whereas that for *B. thuringiensis* subsp. *israelensis* was  $2.5 \times 10^4$ .

**Amino acid analysis.** The amino acid compositions of *B. thuringiensis* crystals are given in Table 2. Glutamic acid and aspartic acid residues were the most abundant in all five strains. *B. thuringiensis* subsp. *kurstaki*, *berliner*, *alesti*, and *tolworthi* had very similar compositions. The amino acid analyses closely resemble those reported previously for crystals of other strains of *B. thuringiensis* (6). Except for the amounts of aspartic acid, half cystine, leucine, and phenylalanine, the amino acid composition of *B. thuringiensis* subsp. *israelensis* was

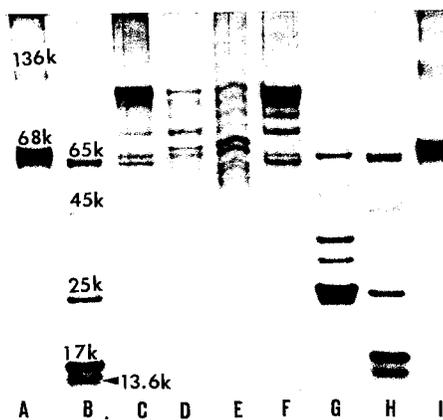


FIG. 4. SDS-polyacrylamide slab gel electrophoresis of the *B. thuringiensis* parasporal crystals solubilized in NaOH and dialyzed overnight against the solubilization buffer described in the legend to Fig. 2. This slab contained a continuous gradient of 5 to 15% acrylamide. (A) Cross-linked bovine serum albumin (68,000, 136,000); (B) molecular weight standards as in Fig. 3, 1.5 to 3  $\mu$ g of protein applied; (C) *B. thuringiensis* subsp. *berliner*; (D) *B. thuringiensis* subsp. *alesti*; (E) *B. thuringiensis* subsp. *tolworthi*; (F) *B. thuringiensis* subsp. *kurstaki*; (G) *B. thuringiensis* subsp. *israelensis*; (H) standards as in track B; (I) cross-linked bovine albumin, same as track A; 10 to 20  $\mu$ g of each crystal protein was applied in tracks C to G.

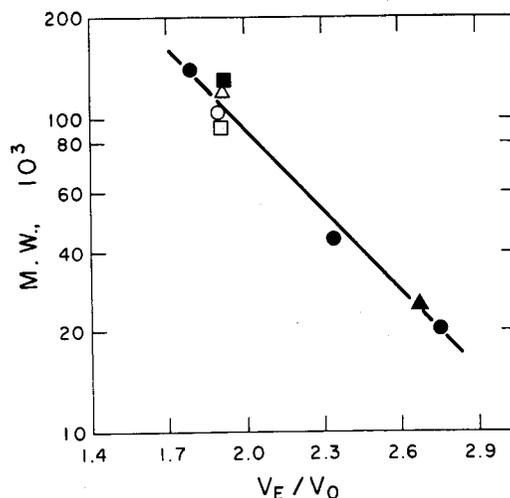


FIG. 5. Determination of molecular weight for major peak from alkali-solubilized crystals. Molecular weight (●) standards (left to right): aldolase (158,000), egg albumin (45,000), chymotrypsinogen A (25,000); (■) *B. thuringiensis* subsp. *kurstaki*; (Δ) *B. thuringiensis* subsp. *alesti*; (○) *B. thuringiensis* subsp. *berliner*; (□) *B. thuringiensis* subsp. *tolworthi*; (▲) *B. thuringiensis* subsp. *israelensis*.

significantly different from those of the other subspecies.

**Carbohydrate content.** The amount of sugar released by 4 N HCl was severalfold greater for *B. thuringiensis* subsp. *israelensis* than for the other subspecies. *B. thuringiensis* subsp. *israelensis* crystals contained glucose, mannose, fucose, rhamnose, xylose, and galactosamine, whereas crystals from the other strains contained only glucose and mannose.

**Immunological characterization.** The antigenicity of alkali-solubilized crystal proteins was examined by means of Ouchterlony double-diffusion procedures (Fig. 6). The top assay in each gel (Fig. 6A to E) shows the reaction of each crystal antigen with its homologous antiserum; the bottom portion of each gel shows reactions of all five crystal antigens with a single antiserum. *B. thuringiensis* subsp. *israelensis* crystal antigen did not react with antisera from the other subspecies. Likewise, crystal antigens from the four lepidopteran toxic strains did not react with *B. thuringiensis* subsp. *israelensis* antiserum; however, the former did cross-react with each other on unstained Ouchterlony gels.

**Tryptic peptide analysis.** Crystals were approximately 90% solubilized by trypsin treatment after reduction and S-alkylation. Polyacrylamide gel electrophoretic patterns of the insoluble residue from *B. thuringiensis* subsp. *kurstaki* and *israelensis* revealed peptides with

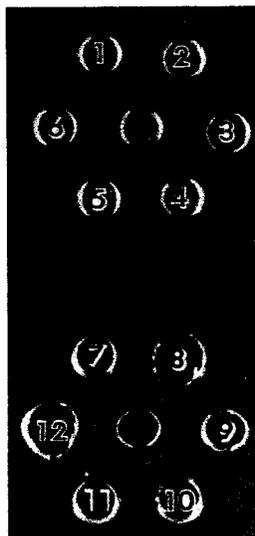
# ERRATUM

## Comparative Biochemistry of Entomocidal Parasporal Crystals of Selected *Bacillus thuringiensis* Strains

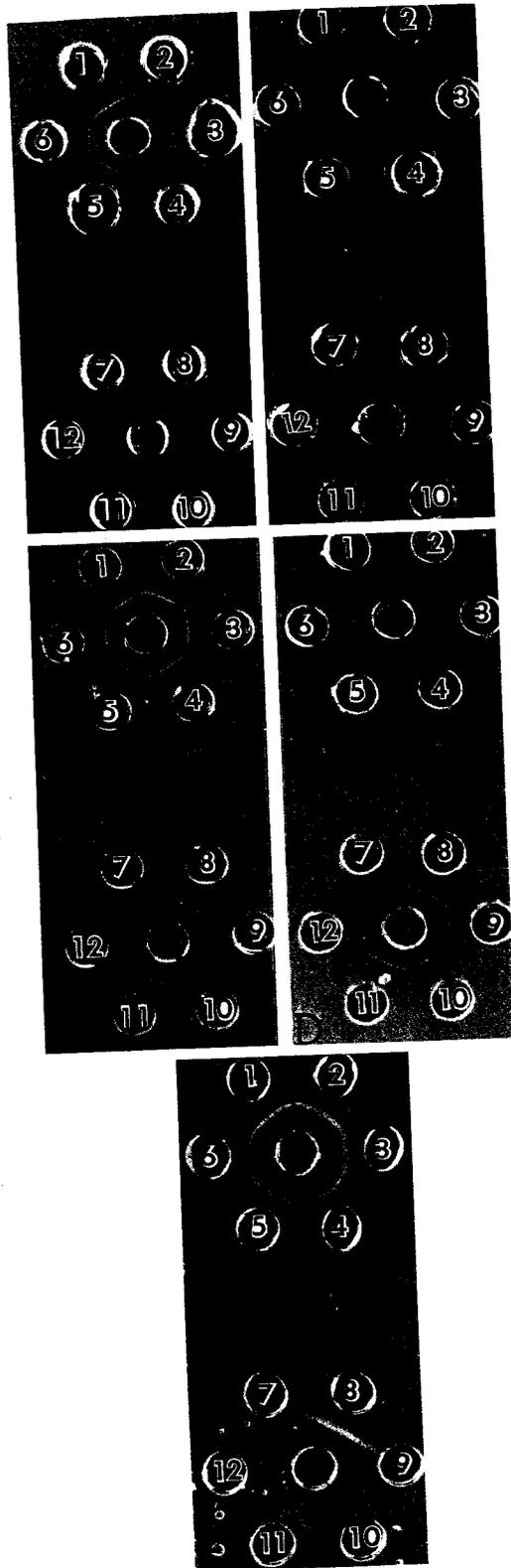
DANA J. TYRELL, LEE A. BULLA, JR., ROBERT E. ANDREWS, JR., KARL J. KRAMER,  
LOREN I. DAVIDSON, AND PHILIP NORDIN

*Division of Biology and Department of Biochemistry, Kansas State University, and U. S. Grain Marketing  
Research Laboratory, Science and Education Administration, U. S. Department of Agriculture,  
Manhattan, Kansas 66502*

Volume 145, no. 2, p. 1058: Fig. 6A is inverted. It should appear as follows:



(Note: The legend to Fig. 6A is correct as printed.)



apparent molecular weights of less than  $2 \times 10^4$ . However, the *B. thuringiensis* subsp. *israelensis* insoluble peptides were slightly larger than those from *B. thuringiensis* subsp. *kurstaki*. The 80% methanol-soluble peptides were separated by two-dimensional chromatography and electrophoresis. Maps of tryptic peptides are shown in Fig. 7. Fingerprints of *B. thuringiensis* subsp. *kurstaki*, *berliner*, *alesti*, and *tolworthi* all were very similar (Fig. 7A to D). All five maps contained the major peptides numbered 1 to 6. *B. thuringiensis* subsp. *israelensis* contained four major peptides (Fig. 7E, numbered 8, 11, 13, and 14, see arrows) either absent or present in extremely low amounts on the other four maps. A comparison of the peptide maps indicates that the *B. thuringiensis* subsp. *israelensis* crystal protein is partially homologous with crystal protein from the other subspecies.

**Spore extracts.** Because spore extracts reportedly contain crystal protein (1, 17, 28; L. A. Bulla, Jr., K. J. Kramer, and L. I. Davidson,

Fig. 6. Ouchterlony double diffusion immunological analysis of parasporal crystals. (A) Center wells contain *B. thuringiensis* subsp. *kurstaki* crystal antibody; wells 1 to 6, *B. thuringiensis* subsp. *kurstaki* crystal antigen; well 7, *B. thuringiensis* subsp. *israelensis* antigen; wells 8 and 11, *B. thuringiensis* subsp. *kurstaki* antigen; well 9, *B. thuringiensis* subsp. *berliner* antigen; well 10, *B. thuringiensis* subsp. *tolworthi* antigen; well 12, *B. thuringiensis* subsp. *alesti* antigen. (B) Center wells contain *B. thuringiensis* subsp. *berliner* antibody; wells 1 to 6, *B. thuringiensis* subsp. *berliner* crystal antigen; well 7, *B. thuringiensis* subsp. *israelensis* antigen; wells 8 and 11, *B. thuringiensis* subsp. *berliner* antigen; well 9, *B. thuringiensis* subsp. *tolworthi* antigen; well 10, *B. thuringiensis* subsp. *kurstaki* antigen; well 12, *B. thuringiensis* subsp. *alesti* antigen. (C) Center wells contain *B. thuringiensis* subsp. *tolworthi* crystal antibody; wells 1 to 6, *B. thuringiensis* subsp. *israelensis* antigen; well 7, *B. thuringiensis* subsp. *tolworthi* antigen; wells 8 and 11, *B. thuringiensis* subsp. *tolworthi* antigen; well 9, *B. thuringiensis* subsp. *berliner* antigen; well 10, *B. thuringiensis* subsp. *kurstaki* antigen; well 12, *B. thuringiensis* subsp. *alesti* antigen. (D) Center wells contain *B. thuringiensis* subsp. *alesti* crystal antibody; wells 1 to 6, *B. thuringiensis* subsp. *alesti* crystal antigen; well 7, *B. thuringiensis* subsp. *israelensis* antigen; wells 8 and 11, *B. thuringiensis* subsp. *kurstaki* antigen; well 9, *B. thuringiensis* subsp. *berliner* antigen; well 10, *B. thuringiensis* subsp. *tolworthi* antigen; well 12, *B. thuringiensis* subsp. *alesti* antigen. (E) Center wells contain *B. thuringiensis* subsp. *israelensis* crystal antibody; wells 1 to 6, *B. thuringiensis* subsp. *israelensis* crystal antigen; well 7, *B. thuringiensis* subsp. *tolworthi* antigen; wells 8 and 11, *B. thuringiensis* subsp. *israelensis* antigen; well 9, *B. thuringiensis* subsp. *kurstaki* antigen; well 10, *B. thuringiensis* subsp. *berliner* antigen; well 12, *B. thuringiensis* subsp. *alesti* antigen.

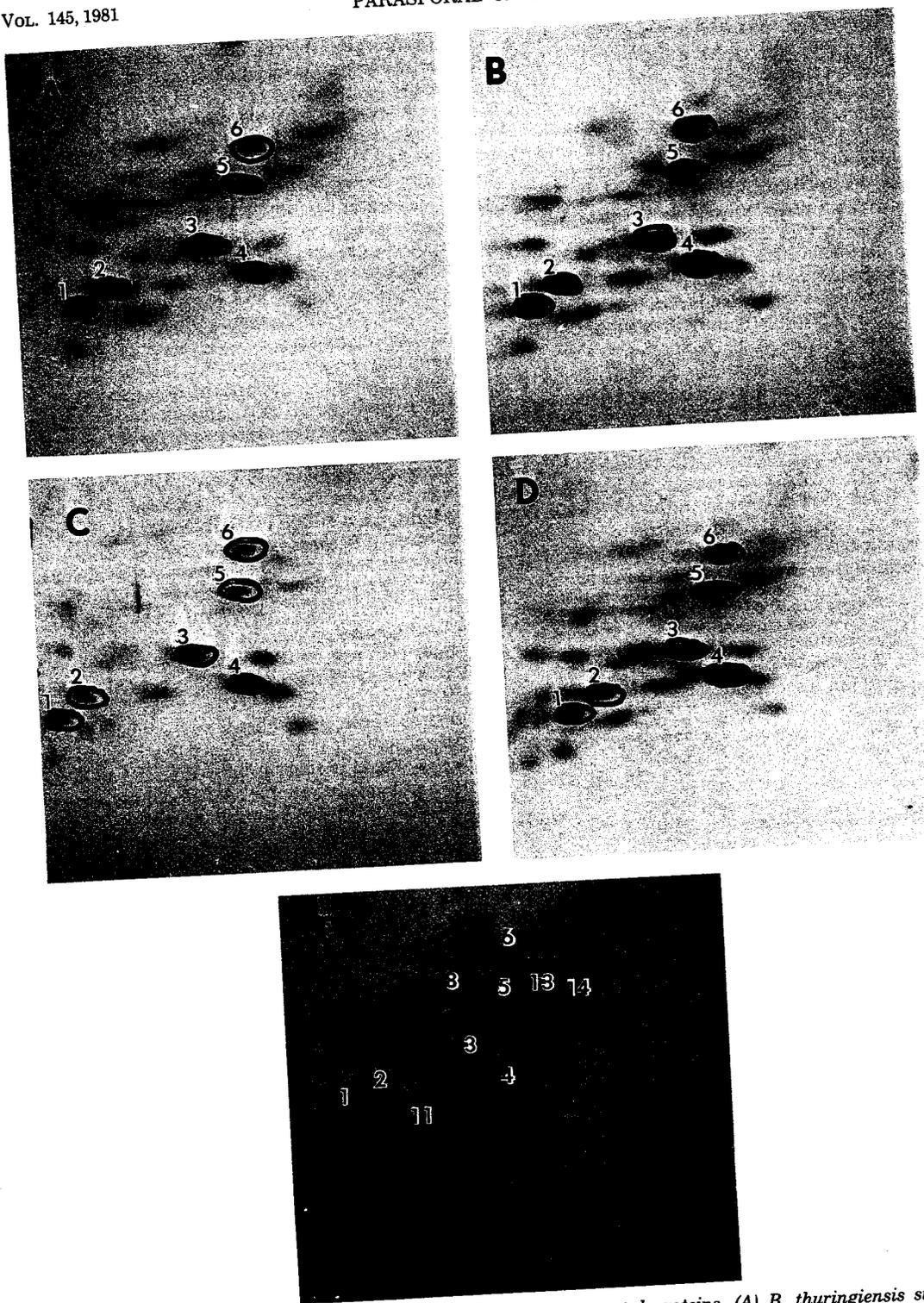


FIG. 7. Fingerprints of tryptic digests of *B. thuringiensis* crystal proteins. (A) *B. thuringiensis* subsp. *kurstaki*; (B) *B. thuringiensis* subsp. *berliner*; (C) *B. thuringiensis* subsp. *alesti*; (D) *B. thuringiensis* subsp. *tolworthi*; (E) *B. thuringiensis* subsp. *israelensis*.

Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I62, p. 165) and we have shown that purified *B. thuringiensis* subsp. *kurstaki* spores are insecticidal (25), we were interested in examining the extractable spore proteins from these bacteria. An electrophoretic profile of guanidine-hydrochloride,  $\beta$ -mercaptoethanol extracts is displayed in Fig. 8, bands 1, 2, and 3). All spores contained their respective crystal proteins as shown in Fig. 2 along with an array of lower-molecular-weight proteins. *B. thuringiensis* subsp. *berliner*, *alesti*, *tolworthi*, and *kurstaki* extracts produced similar profiles (Fig. 8, tracks C to F), differing mainly in the amount of each protein extracted. *B. thuringiensis* subsp. *israelensis* spore extract (Fig. 8, track B) contained many proteins the same size as those in the other subspecies, including a protein (band 2) that is the same size as the toxin of *B. thuringiensis* subsp. *kurstaki* (Fig. 8, track F). Interestingly, a 26,000-dalton polypeptide (see band 3, track B) was present in the *B. thuringiensis* subsp. *israelensis* spore extracts and may be identical to the 26,000-dalton polypeptide predominant in the crystal.

### DISCUSSION

The results of these experiments show that *B. thuringiensis* subsp. *israelensis*, which is toxic to dipteran insects, produced crystals that were

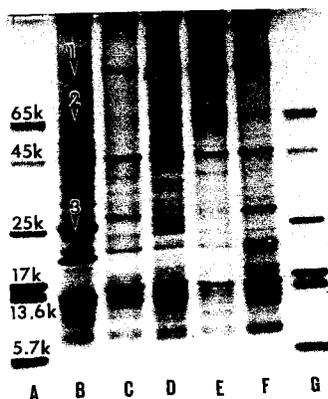


FIG. 8. SDS-polyacrylamide gel electrophoresis of *B. thuringiensis* spore extracts solubilized as described in the legend to Fig. 2. The slab contained a continuous gradient of 5 to 20% acrylamide. (A) Molecular weight standards as in Fig. 3; 1.5 to 3  $\mu$ g of each protein was applied; (B) *B. thuringiensis* subsp. *israelensis* spore extract; (C) *B. thuringiensis* subsp. *berliner* spore extract; (D) *B. thuringiensis* subsp. *alesti* spore extract; (E) *B. thuringiensis* subsp. *tolworthi* spore extract; (F) *B. thuringiensis* subsp. *kurstaki* spore extract; (G) standards as in track A; 60  $\mu$ g of each spore extract was applied.

structurally, biochemically, and immunologically different from lepidopteran-toxic *B. thuringiensis* parasporal crystals. The closely related lepidopteran-toxic *B. thuringiensis* subsp. *kurstaki*, *berliner*, *alesti*, and *tolworthi* produced crystals that are similar structurally, biochemically, immunologically, and functionally.

The four lepidopteran toxic subspecies synthesized bipyramidal crystals, and usually each sporangium contained only one such crystal. *B. thuringiensis* subsp. *israelensis* formed crystals of various shapes, and each sporangium contained two or three crystals. Electrophoretic profiles of all five subspecies revealed proteins with similar molecular weights. In particular, 134,000- and 68,000-dalton polypeptides were present in both lepidopteran and dipteran toxic crystals. The 134,000-dalton protein was identified as the protoxic subunit from *B. thuringiensis* subsp. *kurstaki* (unpublished data). This component was converted to a toxin (apparent molecular weight,  $6.8 \times 10^4$ ) in this subspecies (4). Because crystals of *B. thuringiensis* subsp. *israelensis* are not toxic to lepidopteran insects, we believe that the 68,000-dalton protein of *B. thuringiensis* subsp. *israelensis* is different from that of *B. thuringiensis* subsp. *kurstaki*. At the present time, it is not known which protein in the *B. thuringiensis* subsp. *israelensis* crystal is toxic. Profiles of *B. thuringiensis* subsp. *israelensis* crystals showed a smaller major component (apparent molecular weight, 26,000), which may be a conversion product produced upon solubilization and activation of a 134,000-dalton protoxic molecule.

Crystal protein was a major spore coat component in *B. thuringiensis* subsp. *israelensis* as well as in spores of the other subspecies. However, the differences in the *B. thuringiensis* subsp. *israelensis* extractable coat protein profile compared with those of the other strains were not due entirely to variation in crystal proteins. Differences in small-molecular-weight protein components of the coat also occurred. What significance this aspect has on the biology of *B. thuringiensis* subsp. *israelensis* is not known.

Like the other subspecies, *B. thuringiensis* subsp. *israelensis* is nontoxic to mammals (8). The toxicity spectrum with regard to insects, however, is completely different since purified *B. thuringiensis* subsp. *israelensis* crystals are toxic to dipterans but do not kill lepidopterans, whereas crystals from the other subspecies kill lepidopteran larvae and have only a minimal effect on dipteran larvae. The insecticidal specificity of *B. thuringiensis* may be due to toxins with unique amino acid sequences, different car-

bohydrate content, or both. Recently, Ohba and Aizawa (24) isolated a new subspecies, *B. thuringiensis* subsp. *kyushuensis*, that produces crystals having a toxicity spectrum similar to that of *B. thuringiensis* subsp. *israelensis* (23, 24). Those findings, along with the data reported here, indicate that two groups exist within *B. thuringiensis*: one group that synthesizes lepidopteran toxic crystals, and a new group that produces dipteran toxic crystals. *B. thuringiensis* subsp. *kurstaki*, *berliner*, *alesti*, and *tolworthi* currently are used to control agriculturally important lepidopteran insect pests in various parts of the world (6). *B. thuringiensis* subsp. *israelensis* now is being developed as a control agent for several medically important mosquitoes and black flies (8, 11, 12, 32). Further characterization of the dipteran toxic crystals and elucidation of the genetics of both crystal types is now being conducted in our laboratory.

#### ACKNOWLEDGMENTS

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