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Characterization of the Parasporal Inclusion of *Bacillus thuringiensis* subsp. *kyushuensis*

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Electron microscopy of *Bacillus thuringiensis* subsp. *kyushuensis* revealed that the parasporal inclusions are composed of a homogeneous center surrounded by a thick, electron-dense coating. Antibodies directed against the 135- and 65-kilodalton *B. thuringiensis* subsp. *israelensis* peptides cross-reacted with the 70- and 26-kilodalton peptides, respectively, of *B. thuringiensis* subsp. *kyushuensis*.

Proteinaceous parasporal crystals, toxic predominantly to lepidopterous larvae, are produced by *Bacillus thuringiensis* during sporulation (1). However, a few subspecies, such as *B. thuringiensis* subsp. *israelensis*, produce crystals that are highly toxic to the larvae of mosquitoes and blackflies (1). The effectiveness of such subspecies against medically important insect pests has led to the search for other strains with similar host ranges. *B. thuringiensis* subsp. *kyushuensis* (10, 11) is a mosquitocidal isolate. The crystals of this strain, like those of *B. thuringiensis* subsp. *israelensis*, appear irregularly shaped when examined by phase-contrast microscopy (1, 13). A cloned gene coding for the 130-kilodalton (kDa) mosquitocidal protein of *B. thuringiensis* subsp. *israelensis* cross-hybridizes weakly with the 60-MDa plasmid of *B. thuringiensis* subsp. *kyushuensis*, indicating that peptides of the two strains may be related (12). The current study was undertaken to determine which, if any, of the crystal proteins of the two subspecies are related.

B. thuringiensis subsp. *kyushuensis* 4U1 was obtained from the *Bacillus* Genetic Stock Center. *B. thuringiensis* subsp. *israelensis* was obtained from Lee A. Bulla, Jr.

B. thuringiensis subsp. *israelensis* crystals were purified as previously described (3). *B. thuringiensis* subsp. *kyushuensis* crystals were purified by adjusting the spore-crystal mixture to 40% (wt/wt) sodium bromide and centrifuging it at 15,000 × *g* for 20 min. Under these conditions, spores and debris float to the surface, and most crystals pellet. Crystal purity was monitored by phase-contrast microscopy. Both types of crystals were alkali solubilized for bioassay as previously described (3).

B. thuringiensis subsp. *israelensis* crystals were solubilized for polyacrylamide gel electrophoresis (7) in sample buffer (0.05 M Tris hydrochloride [pH 6.8], 2% 2-mercaptoethanol, 2% sodium dodecyl sulfate) by boiling for 2 min. *B. thuringiensis* subsp. *kyushuensis* was incubated in a solution of KOH (pH 12.0) for 2 min prior to being boiled in sample buffer.

Mosquito larvae (*Aedes aegypti*) were bioassayed as previously described (3). *Manduca sexta* was bioassayed by placing 10 neonate larvae per treatment individually in 4-oz (ca. 115 ml) cups containing diet on which was adsorbed a known quantity of crystal. Mortality was determined after 48 h and again at 7 days. After 7 days, survivors were weighed and the treatment results were compared by single-classification analysis of variance of log-transformed weights and Tukey's honest significant differences (9). Analysis was performed with the assistance of the program PC-MULTI (version 1.1; Gerard E. Dallal, U.S. Department of Agriculture Human Nutrition Research Center, Tufts University, Boston, Mass.). Monoclonal antibodies (3) and immunoblots were prepared as previously described (6).

Examination of sporulated cells of *B. thuringiensis* subsp. *kyushuensis* by electron microscopy (Fig. 1) revealed that the crystals were homogeneous, irregularly shaped bodies with thick, closely adhering, electron-dense coatings. In contrast, *B. thuringiensis* subsp. *israelensis* crystals were typically composed of several distinct and often agglomerated bodies enclosed in a loose covering (13).

The crystals of *B. thuringiensis* subsp. *kyushuensis* were composed primarily of peptides of four sizes (Fig. 2A), from about 15 kDa (not shown) to 150 kDa.

Two of the *B. thuringiensis* subsp. *kyushuensis* peptides cross-reacted with monoclonal antibodies formed against *B. thuringiensis* subsp. *israelensis* crystal peptides (Fig. 2). An anti-68-kDa-peptide monoclonal antibody cross-reacted strongly with the 26-kDa peptide of *B. thuringiensis* subsp. *kyushuensis* (Fig. 2B). The 75- and 80-kDa peptides of *B. thuringiensis* subsp. *kyushuensis* (Fig. 2E) cross-reacted with a monoclonal antibody directed against the 135-kDa peptide of *B. thuringiensis* subsp. *israelensis*. The other two peptides visible in this size range (Fig. 2A) did not cross-react with any of the monoclonal antibodies or the polyclonal antibody. The two peptides that cross-reacted may represent proteolytic degradation products of a single peptide. The presence of the non-cross-reactive bands suggests that these bands represent at least one other distinct peptide. The strong cross-reaction between the *B. thuringiensis* subspp. *israelensis* and *kyushuensis* peptides suggests that the crystal proteins of these two subspecies are related. Both of the cross-reactive *B. thuringiensis* subsp. *kyushuensis* peptides were significantly smaller than the corresponding homologous *B. thuringiensis* subsp. *israelensis* antigens.

Although *B. thuringiensis* subsp. *kyushuensis* crystals were only partially solubilized when boiled in sample buffer, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed polypeptides of the same sizes as those obtained from crystals incubated with KOH for 2 min prior to boiling (data not shown). Therefore, the major *B. thuringiensis* subsp. *kyushuensis* crystal polypeptides observed appear to be unaffected by the alkali pretreatment.

Monoclonal antibody to the hemolytic and mammalian toxic *B. thuringiensis* subsp. *israelensis* 28-kDa crystal polypeptide (2–5, 8) failed to cross-react with any of the *B. thuringiensis* subsp. *kyushuensis* peptides (Fig. 2B). Consis-

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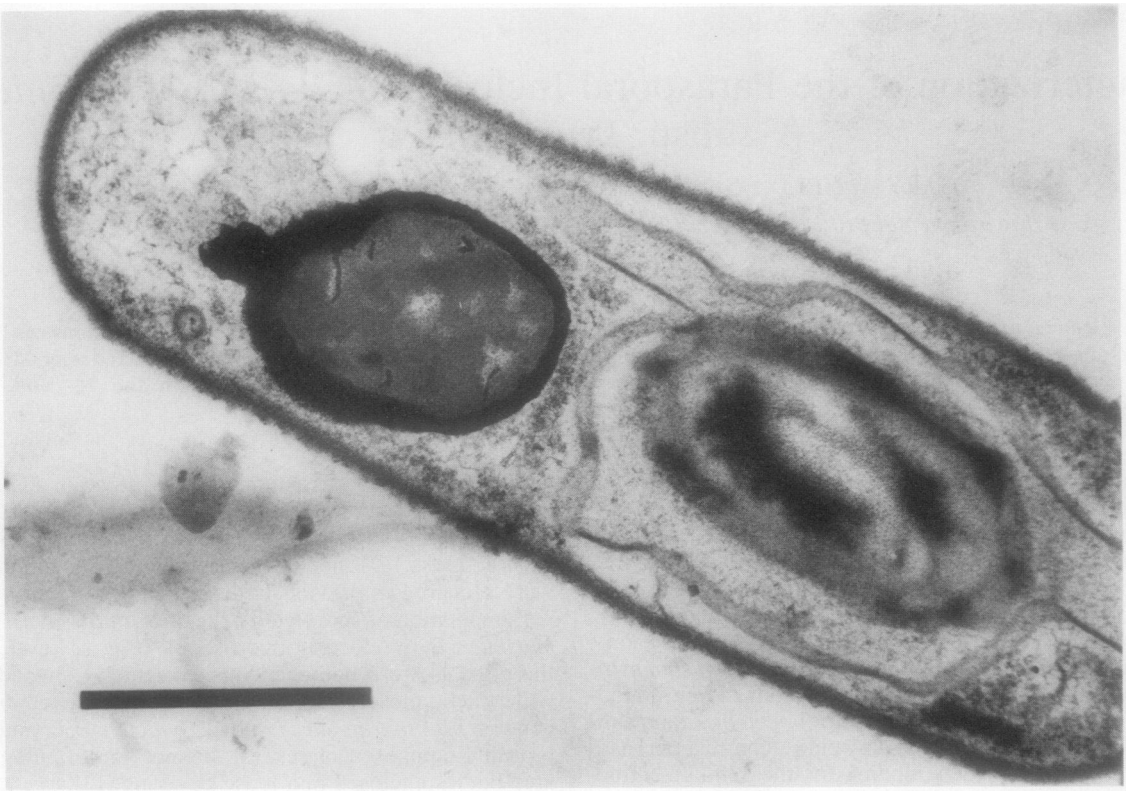

FIG. 1. Electron micrograph of a sporulated cell of *B. thuringiensis* subsp. *kyushuensis* containing a crystal. Late-stationary-phase cells were grown in GYS medium (3) and then harvested and washed by centrifugation. Cells were fixed in Karnovsky fixative and 1% OsO_4, stained with 1% uranyl acetate en bloc, and embedded in Spurr epoxy resin. Bar = 0.5 μm.

tent with these results, solubilized *B. thuringiensis* subsp. *kyushuensis* crystal proteins were not hemolytic.

For crystals of *B. thuringiensis* subsp. *kyushuensis*, the 50% lethal concentration against third-instar larvae of *A. aegypti* was 12 μg/ml. In comparison, for *B. thuringiensis* subsp. *israelensis* crystals, the 50% lethal concentration was 0.032 μg/ml. The reduction in toxicity of alkali-solubilized proteins relative to whole crystals was significantly greater with *B. thuringiensis* subsp. *kyushuensis* than with *B. thuringiensis* subsp. *israelensis*. Preliminary experiments suggested that poor dissolution of *B. thuringiensis* subsp. *kyushuensis* crystals in the mosquito gut resulted in lower toxicity. However, solubilization prior to bioassay resulted in an even greater difference in toxicity between the two subspecies (50% lethal concentration of *B. thuringiensis* subsp. *israelensis*, 0.88 μg/ml; 640 μg of *B. thuringiensis* subsp. *kyushuensis* per ml resulted in only 10% mortality). The toxicity of *B. thuringiensis* subsp. *kyushuensis* crystals to neonate *M. sexta* larvae was also tested (Table 1). Only 3 of 10 larvae died at the highest concentration (25 μg/cm^2) of crystals. However, the average weight of the larvae that survived for 7 days was only 5.7 mg, compared with 22.7 mg for the control larvae. This suggests that *B. thuringiensis*

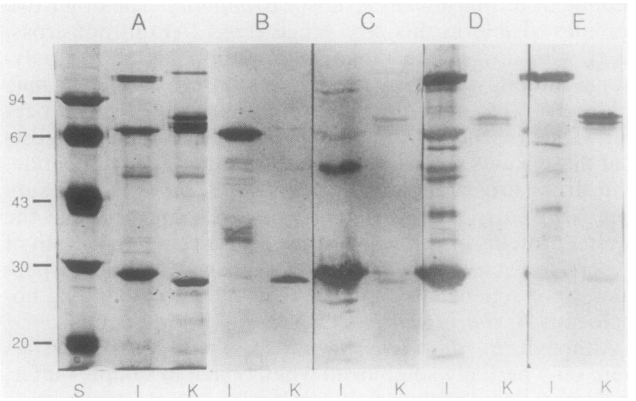

FIG. 2. Immunoblot of *B. thuringiensis* subspp. *israelensis* and *kyushuensis* crystals. (A) 10% Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis; (B) immunoblot with anti-68-kDa monoclonal antibody; (C) immunoblot with anti-28-kDa monoclonal antibody; (D) immunoblot with anti-*B. thuringiensis* subsp. *israelensis* crystal peptide polyclonal antibody; (E) immunoblot with anti-135-kDa monoclonal antibody. Lane S, Molecular weight standards (molecular weights in kilodaltons indicated at left); lanes I, *B. thuringiensis* subsp. *israelensis* crystal peptides; lanes K, *B. thuringiensis* subsp. *kyushuensis* crystal peptides.

TABLE 1. Toxicity of *B. thuringiensis* subsp. *kyushuensis* to *M. sexta* neonate

Amt of toxin (μg/cm^2)	No. of deaths/initial no. of larvae	Wt (mg/larva) of surviving larvae (range)[a]
0.0	1/10	22.7 (15.4–33.5)
0.25	3/10	28.1 (24.8–31.8)
2.5	3/11	15.3 (8.90–26.2)
25.0	3/10	5.7 (2.8–11.3)

[a]Weighed after 7 days. *B. thuringiensis* subsp. *kurstaki* crystals (10 ng/cm^2) killed 100% of larvae within 48 h. Confidence intervals were all 95%.

subsp. *kyushuensis* crystals either had an extremely low level of toxicity to the hornworm larvae or inhibited feeding at very high concentrations. In contrast, a 10-$\mu g/cm^2$ concentration of crystals isolated from *B. thuringiensis* subsp. *kurstaki*, which is known to be very toxic to *M. sexta*, killed 100% of the neonate larvae within 48 h.

The lack of hemolytic activity of *B. thuringiensis* subsp. *kyushuensis* crystal protein preparations is probably not the cause of its low mosquitocidal activity, since the addition of the purified hemolytic *B. thuringiensis* subsp. *israelensis* 28-kDa peptide did not result in an increase in toxicity (unpublished data).

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