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Larvicidal Activity of *Bacillus thuringiensis* subsp. *israelensis* in the Dipteran *Haematobia irritans*

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A strain of *Bacillus thuringiensis* subsp. *israelensis* was found to be larvicidal to horn flies, *Haematobia irritans* (L. [Diptera:Muscidae]). The toxic activity was particulate, appeared during sporulation, and could be prevented by the addition of streptomycin before sporulation. Density gradient centrifugation in Renografin was used to separate endospores, crystals, and low-density particulate matter (fraction 3) from sporulated preparations. Larvicidal activity was restricted to purified crystals and fraction 3, indicating that δ-endotoxin of *B. thuringiensis* subsp. *israelensis* was active against horn fly larvae. Purified crystals produced mortality during larval feeding stages, but not pupal stages. Fraction 3 produced significant mortality during both larval and pupal stages. The mortality data indicated the presence of at least two dipteran-active toxins.

Horn flies, *Haematobia irritans* (L.), are serious pests of beef and dairy cattle in the United States. They are closely related to other dipteran pests of the family Muscidae in which the spread of disease is facilitated by egg deposition on feces or carrion. Commercial insecticides are often toxic or mutagenic to vertebrates, raising serious concern about their widespread use. In addition, the increasing incidence of insect resistance to chemical pesticides has prompted search for safe and effective agents for biological control of these insect pests.

Bacillus thuringiensis subsp. *israelensis* has been shown to be toxic to several families of aquatic dipteran larvae such as mosquitoes (4, 6, 8, 9, 23), black flies (23), and midges (10). *B. thuringiensis* is known to produce two major classes of insecticidal toxins (12). Beta-exotoxin, recently reviewed by Sebesta et al. (17), is effective against several orders of insects, including Diptera (7), but also possesses vertebrate toxicity. The other major toxin, δ-endotoxin or crystal protein (3), is an insoluble proteinaceous crystalline inclusion of *B. thuringiensis* shown to lack vertebrate toxicity in its natural protoxin state (3, 5, 20), making it a good candidate for biological control. δ-Endotoxin has been shown to be responsible for the mosquitocidal properties of *B. thuringiensis* subsp. *israelensis* (9, 22). This paper contains results of studies whose purpose was to determine whether the crystal protein of *B. thuringiensis* subsp. *israelensis* is toxic to nonaquatic dipteran larvae. Purified crystal protein of *B. thuringiensis* subsp. *israelensis* was shown to be insecticidal to horn fly larvae. In addition, the data suggest the presence of another, previously unreported insecticidal toxin.

MATERIALS AND METHODS

Bacterial strains. *B. thuringiensis* subsp. *kurstaki* KBt4 and *B. thuringiensis* subsp. *israelensis* HD522 were supplied by Gail Donaldson, U.S. Department of Agriculture Cotton Insects Research Unit, Brownsville, Tex. Strain KBt4 is a derivative of HD1 which lacks the 51-megadalton plasmid of the parental strain (G. Donaldson, personal communication).

Growth and fractionation of bacterial strains. Cells were grown at 30°C with aeration in 2-liter flasks containing 250 to 500 ml of tryptic soy broth (Difco Laboratories)–0.1% yeast extract (Difco) inoculated from 5-ml starter cultures in L-broth (11). The tryptic soy broth–0.1% yeast extract medium permitted rapid vegetative growth of the bacteria, but strongly inhibited sporulation. At the end of logarithmic growth, bacterial cells were collected by centrifugation and suspended in the minimal salts medium of Davis and Mingioli (2), modified by the omission of citrate and the addition of glucose to 0.5% (wt/vol), L-glutamate to 100 μg/ml, and trace elements (Table 1). This procedure resulted in synchronous and complete sporulation of the bacterial cells during subsequent incubation. Cellular material (endospores, crystals, debris, log-phase cells) was collected by centrifugation. The pellet was dispersed, rinsed two or three times in distilled water, and suspended in 50 mM Tris-HCl (pH8.0) containing 100 mM NaCl and 0.01% (vol/vol) Triton X-100. The suspension was sonicated, and particulate matter was fractionated by centrifugation on Renografin-76 (sodium diatrizoate; E. R. Squibb & Sons) step gradients (13, 19). Renografin concentrations were adjusted to 67, 60, and 50%, allowing sedimentation of endospores, crystals, and low-density particulate matter, respectively. The pellet in each case was suspended and sedimented again in the appropriate concentration of Renografin. Particulate matter that sedimented twice in 50% Renografin was labeled fraction 3. All fractions were washed extensively in distilled water and then lyophilized to dryness. The purity of the particulate fractions was monitored by phase-contrast microscopy and by determination of the number of bacterial CFU by serial dilution onto nutrient agar plates.

Bioassay procedure. Fifty newly hatched horn fly larvae were transferred to petri dishes (10 larvae per dish) containing 15 g of bovine fecal medium. Dishes containing larvae were covered and incubated at 27°C for 6 to 7 days to allow larvae to grow and form pupae. The pH of the fecal medium remained near 7.0 throughout the bioassay period. Pupae were rinsed from the fecal medium, drained, and incubated for an additional 5 to 7 days until all flies had emerged and died. The results were scored by counting the number of pupae and emergent flies.

Bovine feces used in the preparation of fecal medium were collected from animals kept inside screened buildings to prevent access of wild flies. Fecal medium was prepared from fresh or frozen feces as described previously (16) by the addition of water (ca. 40 ml to 60 g of feces) and fractions of

TABLE 1. Composition of mineral salts medium[a]

Compound	Final concn
K_2HPO_4	40 mM
KH_2PO_4	22 mM
$(NH_4)_2SO_4$	7.6 mM
Glucose	28 mM
L-Glutamate	680 μM
Citric acid	52 μM
$MgCl_2 \cdot 6H_2O$	493 μM
$FeCl_2 \cdot 4H_2O$	10 μM
$CaCl_2 \cdot 2H_2O$	14 μM
$MnCl_2 \cdot 4H_2O$	3.5 μM
H_3BO_3	0.81 μM
$Na_2MoO_4 \cdot 2H_2O$	0.043 μM
$CuCl_2 \cdot 2H_2O$	0.29 μM
$CoCl_2 \cdot 6H_2O$	0.21 μM
$ZnCl_2$	0.37 μM

[a] K_2HPO_4, KH_2PO_4, and $(NH_4)_2SO_4$ were made up as a 20-fold concentrate and then diluted and autoclaved. Sterile solutions of the other compounds were added. The final pH was 7.0.

bacterial cultures to be tested for toxicity. The fecal medium was mixed at high speed in a Waring blender, 15 g was added to each petri dish (60 by 15 mm), and the mixture was kneaded with dry cotton gauze to absorb excess moisture before inoculation of larvae. It was important to use urine-free feces because the presence of bovine urine inhibits growth and development of horn fly larvae. Separate controls were run for each batch of fecal medium and usually yielded 0.8 to 0.9 adult flies per larva inoculated.

RESULTS

Culture supernatants and particulate matter of *B. thuringiensis* KBt4 (subsp. *kurstaki*) and HD522 (subsp. *israelensis*) were bioassayed for toxicity to horn fly larvae (Table 2). Particulate matter from sporulated cultures of strain HD522 was toxic to horn fly larvae, whereas HD522 culture supernatants and all KBt4 preparations were essentially nontoxic. β-Exotoxin is produced and released to the medium during logarithmic growth; δ-endotoxin is produced and accumulated as crystalline inclusions during sporulation. Logarithmically growing bacteria were compared with sporulated bacteria for toxicity to horn fly larvae by bioassay to determine the time at which toxic activity appeared. In some experiments, HD522 vegetative cells were mildly toxic (less than 50% mortality at 0.5 mg/g). The inclusion of streptomycin (20 μg/g) in the bioassay medium prevented toxicity of HD522 vegetative cells, but did not affect horn fly larval development or reduce the toxicity of sporulated preparations of HD522. This level of streptomycin (20 μg/ml) completely inhibited growth and sporulation of strain HD522 in liquid culture medium. These results suggest that streptomycin functions to prevent expression of a sporulation-specific gene essential to the development of toxicity.

Larvae killed by sporulated preparations of HD522 were rinsed, crushed, and streaked onto nutrient agar. Although some larvae produced nearly confluent growth of *B. thuringiensis*, most yielded few or no *B. thuringiensis* colonies, indicating that bacteremia was not an obligate step in larval mortality. These results suggested that strain HD522 produced a particulate toxin during sporulation which was independent of bacteremia for the expression of toxicity.

Particulate matter from sporulated cultures of HD522 was sonicated and fractionated by density gradient centrifugation on Renografin to separate endospores, crystals, and light-density particulate matter. Purified particulate fractions were bioassayed, and the results were analyzed to determine relative toxicity and whether mortality occurred during larval growth or during pupation. No toxicity was detected in purified endospores of HD522 even when endospore germination was stimulated by heating at 70°C for 30 min before the bioassay (data not shown). Purified crystals and light-density particulate matter (fraction 3) from HD522 were toxic to horn fly larvae and were not affected by the presence of streptomycin during bioassay. Renografin was not toxic to horn fly larvae at concentrations up to 1 mg/ml. These results confirm that toxicity of *B. thuringiensis* subsp. *israelensis* in horn fly larvae is independent of vegetative cells or endospores and is due to production of toxic substances rather than to bacteremia.

The concentration dependence for toxicity of HD522 crystals (Fig. 1) and HD522 fraction 3 (Fig. 2) is shown as a typical experiment. The slopes of regression lines (Fig. 1 and 2) can be used as a measure of larvicidal activity. A negative slope indicates decreasing larval survival with increasing concentration. A slope of −1 indicates a stoichiometric dose-response relationship. Table 3 lists the slopes of regression lines for mortality during specific stages of larval development. Larval mortality from HD522 crystals was restricted to feeding stages before pupation, whereas HD522 fraction 3 produced high mortality during pupation as well as during preceding stages.

DISCUSSION

This research laboratory has participated for several years in an international cooperative program (H. Dulmage, coor-

TABLE 2. Bioassay of culture fractions of strains KBt4 and HD522

Strain	Fraction[a]	Concn of added fraction[b] (mg/g of fecal medium)	Effect on fly maturation[c]		
			% Pupation	% Emergence	% Mortality
KBt4	TSY	250	81	92	25
	DCE	250	81	105	15
	Veg cells	50	90	94	15
	Sporulated cells	0.5	72	124	11
HD522	TSY	500	77	83	36
	DCE	250	72	110	21
	Veg cells	50	92	106	2
	Sporulated cells	0.5	0		100
		0.1	38	41	84
Control[d]	None		100 (85)	100 (95)	0 (19)
Control	Renografin	1	97	98	5

[a] TSY, Supernatant from vegetative growth in tryptic soy broth–0.1% yeast extract; DCE, supernatant from sporulation in minimal medium; veg cells, wet pellet from tryptic soy broth–0.1% yeast extract culture; sporulated cells, dry pellet from minimal medium culture.

[b] Liquid was concentrated by lyophilization and added as concentrate or as the dry residue remaining after lyophilization of the indicated weight (given for TSY and DCE fractions) of liquid.

[c] Values given are corrected for control values in separate experiments. Pupation is the number of pupae formed per larva; emergence is the number of mature flies formed per pupa; mortality is equal to 1 − (number of mature flies formed per larva).

[d] The average uncorrected control values are given in parentheses.

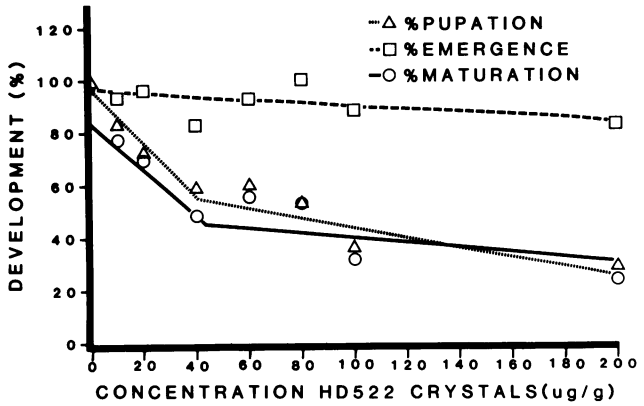

FIG. 1. Purified crystals of HD522 were added to fecal medium at time zero and bioassayed with newly hatched horn fly larvae. The results are presented as percent pupation and percent emergence as in Table 2. The percent maturation is the number of emergent adult flies formed per larva.

dinator, Brownsville, Tex.) screening standardized culture samples of different strains of *B. thuringiensis* for toxicity to horn flies. Horn fly sensitivity to β-exotoxin (1, 16) or to septicemia (R. Gingrich, unpublished results) from a number of *B. thuringiensis* varieties had been previously demonstrated as a result of this program. The present communication has demonstrated that purified crystal protein of *B. thuringiensis* subsp. *israelensis* strain HD522 is toxic to horn fly larvae. This extends previous reports of dipteran toxicity for crystal protein of *B. thuringiensis* subsp. *israelensis* to include nonaquatic larvae of the family Muscidae. The purified crystal protein of strain HD522 was observed to produce rapid death with mortality restricted to prepupal stages of larval development. The nonlinear response (Fig. 1) of horn fly larvae to increasing concentrations of toxin could result either from genetic or physiological heterogeneity of the larval population or from reduced feeding with a resultant decrease in actual ingestion of toxin at the higher concentrations. The latter phenomenon has been previously reported in bioassays against lepidopteran larvae of the European corn borer (14, 15).

Many researchers have reported 50% lethal concentration values for *B. thuringiensis* subsp. *israelensis* crystal protein against mosquitoes to be in the range of 1 to 10 ng/ml (8, 9,

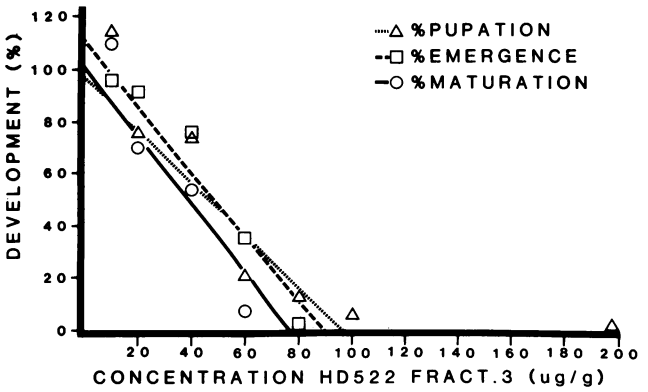

FIG. 2. Fraction 3 of HD522 was bioassayed as described in the legend to Fig. 1.

TABLE 3. Effect of increasing concentration of HD522 particulate fractions on development of horn fly larvae

Fraction[a]	Slope of regression lines		
	% Pupation	% Emergence	% Maturation
Endospores	−0.03	+0.06	+0.02
Crystals	−1.0[b]	−0.06	−0.85[b]
Fraction 3	−1.0	−1.26	−1.28

[a] Particulate matter from HD522 sporulated culture purified by density gradient centrifugation on Renografin.
[b] Maximal values; the effect was discontinuous.

18). Initially, one might assume that the crystal protein of *B. thuringiensis* subsp. *israelensis* is far more toxic to mosquitoes than to horn fly larvae (50% lethal concentration, ca. 50 μg/g of fecal medium); however, this assumption is not justified, as mosquito larvae are filter feeders and greatly concentrate suspended dry matter before ingestion. In addition, carbonate has been shown to reduce toxicity to mosquitoes by up to 100-fold (18), and the presence of certain unsaturated phospholipids may neutralize toxicity (21). It is difficult to assess the relative effects of fecal medium components that may affect toxicity since these factors are not easily controlled and would be expected to vary with animal diet and stress. Therefore, it is possible that the actual effective lethal dose may be similar (relative to body weight) in the two insects when assayed under equivalent conditions.

In addition to purified crystal protein, larvicidal activity was present in the low-density particulate fraction (fraction 3) from strain HD522. Fraction 3 contains assorted cellular debris and small crystalline or amorphous material visible in phase contrast microscopy. The observation that larvicidal activity is not restricted to prepupal larval development, unlike that of the purified crystal protein, suggests a different mechanism for toxic activity of fraction 3. The delayed death observed (during pupation) in bioassays of fraction 3 is reminiscent of β-exotoxin activity, which interferes with RNA synthesis, usually producing death at times of physiological stress or metabolic shifts such as occur during ecdysis and pupation (17). However, β-exotoxin is normally produced and released to the culture medium during logarithmic growth and has not been reported to be produced by *B. thuringiensis* subsp. *israelensis*. The soluble nature of β-exotoxin, together with the low toxicity of culture supernatants, the ability of particulate fractions to withstand extensive dialysis in Renografin density gradients and subsequent washing, and the abolition of toxin formation by streptomycin suggest that the toxic component of fraction 3 was not β-exotoxin, thus suggesting the presence of a previously unreported toxin. Alternate possibilities include formation of β-exotoxin sequestered as an insoluble salt, or formation of poly-β-hydroxybutyrate. The biological and structural properties of the toxic component of fraction 3 remain to be elucidated.

Fly control is a major problem for livestock producers, especially in feedlot locations near residential areas. This report suggests the possible utility of preparations of *B. thuringiensis* subsp. *israelensis* or its toxins as an additive to livestock waste or feedstock for use in fly control. The noninfectious nature of the purified crystal protein, together with the absence of vertebrate toxicity in its native, crystalline state (5, 20), makes the crystal protein of *B. thuringiensis* subsp. *israelensis* an excellent candidate for use in fly

control. Modification of the gene for the crystal protein toxin (δ-endotoxin) and cloning in a suitable host may provide new biological agents with increased toxicity, altered specificity for insect susceptibility, or both, allowing production of environmentally safe, biologically targeted insecticides.

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