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Digestion of *Bacillus thuringiensis* var. *israelensis* Spores by Larvae of *Aedes aegypti*

KAMAL KHAWALED, TSIONA COHEN, AND ARIEH ZARITSKY[1]

Department of Life Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Be'er-Sheva, Israel, 84105

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The larvicidal activity of *Bacillus thuringiensis* var. *israelensis* against mosquitoes and the blackfly is included in parasporal crystalline bodies which are produced during sporulation. Following ingestion, the crystals are solubilized in the larval midgut and induce death within a short time; the spores germinate in the dead larvae and complete a growth cycle. The fate of the spores in surviving live larvae was elucidated by using a nonlarvicidal *B. thuringiensis* var. *israelensis* mutant. When introduced as the only food source, spores of this mutant support development to the adult stage of newly hatched *Aedes aegypti* larvae at a rate directly related to spore concentration. The conclusion that spores of *B. thuringiensis* var. *israelensis* are digested in the larval gut was substantiated by following the incorporation of [^{35}S]methionine-labeled spores into larval tissues.

KEY WORDS: ***Aedes aegypti* larvae; *Bacillus thuringiensis* var. *israelensis;* spores; ingestion; digestion.**

INTRODUCTION

Mosquitoes are known as vectors of several human diseases (Mattingly, 1969). Development of resistance to chemical pesticides, as well as environmental concerns, limit their use for mosquito control (Brattsten *et al.*, 1986). An alternative control means is the bacterium *Bacillus thuringiensis* var. *israelensis* (serotype H14) (Goldberg and Margalit, 1977). The larvicidal activity of *B. thuringiensis* var. *israelensis* is included in parasporal crystalline bodies (δ-endotoxin) which are produced during sporulation (Tyrell *et al.*, 1981). Upon ingestion of *B. thuringiensis* var. *israelensis* spores, these crystals are solubilized in the alkaline environment of the larval midgut (Warren *et al.*, 1984), the epithelial cells of which swell, lyse, and slough off into the gut lumen (Lahkim-Tsror *et al.*, 1983). The disruption of the epithelial cells leads to paralysis and death within a short time (Singh *et al.*, 1986). The ingested spores germinate in the carcasses of the dead larvae and complete a growth cycle (spore germination, vegetative growth, and sporulation; Khawaled *et al.*, 1988). The fate of *B. thuringiensis* var. *israelensis* spores administered at sublethal doses in the surviving, *live* larvae has, however, not been elucidated. Here, we use spores of a nonlarvicidal *B. thuringiensis* var. *israelensis* mutant to demonstrate that they are digested by *Aedes aegypti* larvae.

MATERIALS AND METHODS

Mosquito larvae. Dry strips of paper bearing eggs of *Ae. aegypti* larvae, kindly provided by Professor J. Margalit, were submerged for 1 day in 1 liter of sterile tap water supplemented with 1 g of Pharmamedia (Traders Protein, USA) at 30°C, as described before (Khawaled *et al.*, 1988).

Algae. Chlorella emersonii var. *emersonii* (CCAP-211/11n), a unicellular green alga, kindly provided by Dr. E. Cohen, was grown at 24 ± 1°C under sterile conditions in 700 ml conical tubes as previously described (Arad *et al.*, 1985). Cells were counted with a hemocytometer.

Bacterial strain and growth conditions. A strain of *B. thuringiensis* var. *israelensis* (AO50), which was derived from strain 4Q2-72 (Bourgouin *et al.*, 1986; kindly supplied by the *Bacillus* Genetic Stock Center, Ohio State University, USA) by curing of its single plasmid using 50 μg ml^{-1} acridine orange (H. G. Nandadasa, and A. Zaritsky, unpublished results) and is thus not larvicidal, was used throughout. As a control for toxicity assays, an active powder was used, as described before (Khawaled *et al.*, 1988; Lahkim-Tsror *et al.*, 1983; Ohana *et al.*, 1987). Growth was monitored by measuring the change of optical density with time. Isolation of bacterial colonies on LB plates (Miller, 1972) (containing 1% tryptone, 1% NaCl, and 0.5% yeast extract, plus 1.5% agar) and phase contrast microscopy served to assess culture purity. Logarithmic cells, grown on LB medium, were harvested at a turbidity of 70 Klett Units and used for inoculation at 10,000-fold

[1] Currently on sabbatical leave at Department of Biological Sciences, Florida Institute of Technology, 150 West University Boulevard, Melbourne, FL 32901–6988.

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dilution after washing twice (to avoid carry over of nutrients from the rich medium) at room temperature (8000 rpm, 10 min). Diluted cells were cultivated in an aqueous sulfate-free salt solution (K-S, to be published elsewhere) supplemented with an autoclaved mixture of the 18 non-sulfur-containing amino acids (100 μg ml^{-1} each) adjusted with NaOH to pH 7.4 and methionine (at the indicated concentration), and with glucose (5 mg ml^{-1}). (K-S solution contained NH_4Cl, 1.6 g; K_2HPO_4, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 80 mg; $MnCl_2 \cdot 4H_2O$, 60 mg; $ZnCl_2$, 3 mg; $CuCl_2 \cdot 2H_2O$, 4 mg and $FeCl_2 \cdot 4H_2O$, 0.4 mg per 1 liter of distilled water.)

Radiolabeling of spores. Spores were radiolabeled by growth in K-S medium with 1 μCi in 10 μg ml^{-1} of [^{35}S]methionine. Aliquots were incubated with shaking (250 rpm) at 30°C for 120 hr. Incorporated radioactivity was measured by Packard liquid scintillation counter (Model 300).

Incorporation of radiolabel into larval tissues. Second instar larvae were incubated with ca. 10^8 radiolabeled spores per milliliter (1 cpm $spore^{-1}$). Single larvae were washed at the indicated times, and their gut content was purged by further incubation for 20 min with a suspension of *Ch. emersonii* (10^8 cells ml^{-1}). The larvae were then washed, homogenized, and the incorporated radioactivity was measured.

Bioassay. The larvicidal activity of a culture was determined as described by Lahkim-Tsror *et al.* (1983). The concentration that killed 50% of the larvae (LC_{50}) was determined by probit analysis (Finney, 1971).

RESULTS AND DISCUSSION

The lack of larvicidal activity of strain AO50 against different developmental stages of *Ae. aegypti* larvae was firstly confirmed (Fig. 1). The fate of the spores in live larvae was then investigated by two methods in parallel: (A) Indirectly, by demonstrating that larvae can develop on spores as the only food source; (B) Di-

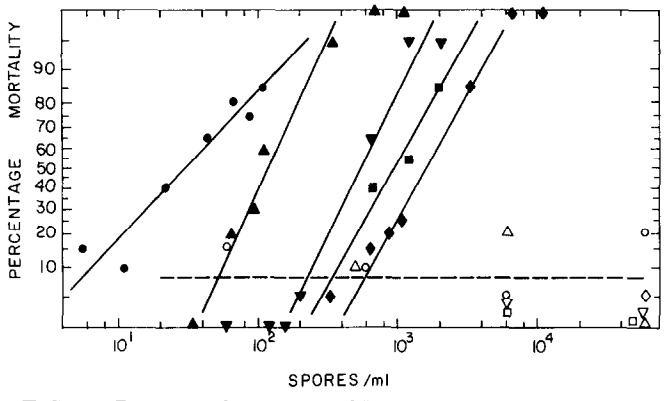

FIG. 1. Bioassays for toxicity of *B. thuringiensis* var *israelensis* spores, from the wild-type strain (solid symbols) and the cured mutant (open symbols). Larvae of *Ae. aegypti* at different instars (○, ●, early I; △, ▲, Late I; ▽, ▼, II; □, ■, III; ◇, ◆, IV) were incubated at 30°C with different spore concentrations. Dashed line represents average mortality caused by the mutant at all concentrations to all tested instars. Mortality was determined after 24 hr.

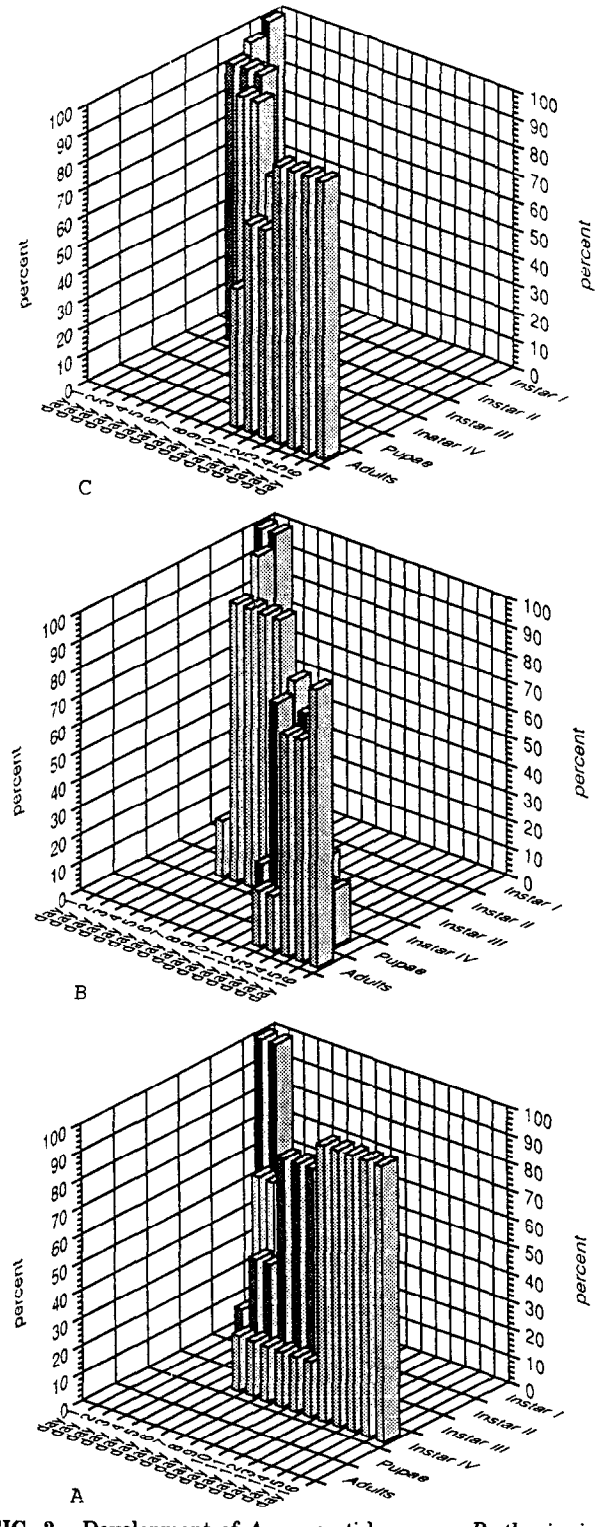

FIG. 2. Development of *Ae. aegypti* larvae on *B. thuringiensis* var. *israelensis* spores as the only food source. Newly hatched larvae were incubated at 30°C with a daily supply of spores of strain AO50 (10^6 ml^{-1}, A; 10^7 ml^{-1}, B; $2 \cdot 10^7$ ml^{-1}, C). Developmental stages were recorded daily.

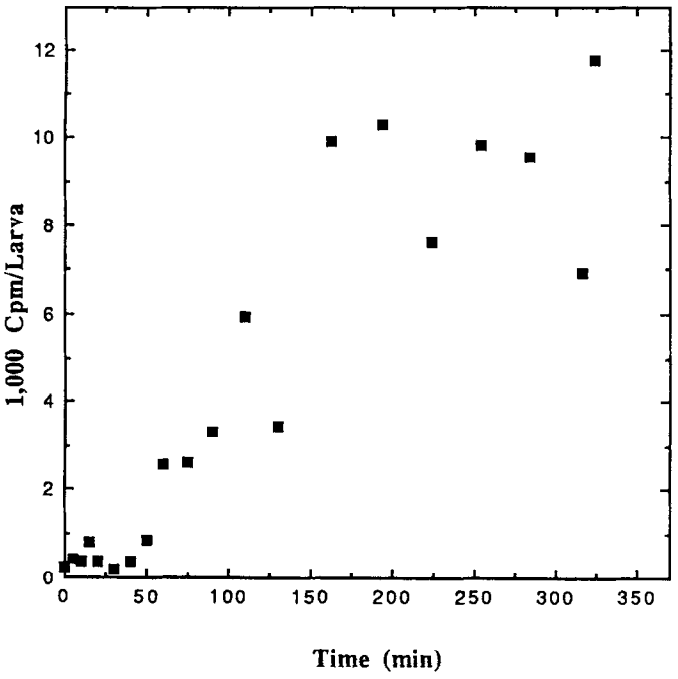

FIG. 3. Ingestion by *Ae. aegypti* larvae (second instar) of *B. thuringiensis* var. *israelensis* (strain AO50) spores. Larvae were incubated with about 10^8 radiolabeled spores per milliliter (1 cpm spore^{-1}). One larva at each of the indicated times was washed and homogenized, and the ingested radioactivity was measured.

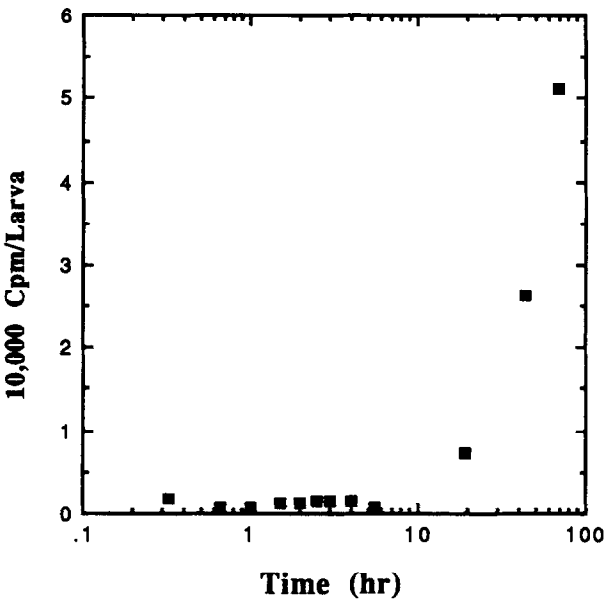

FIG. 4. Incorporation of [^{35}S]methionine into tissues of second instar larvae of *Ae. aegypti*. The larvae were incubated with about 10^8 radiolabeled spores per milliliter, and their gut content was removed at the indicated times by further incubation (20 min) with a suspension of *Ch. emersonii* (10^8 cells ml^{-1}). The larvae were then washed and homogenized, and the incorporated radioactivity was measured.

rectly, by measuring the incorporation of radiolabeled spores into larval tissues.

Introducing spores of strain AO50 to newly hatched larvae as the only food source led to development at a rate directly related to spore concentration (Figs. 2A–2C). Similar results were obtained in a control experiment with spores of the wild-type strain, purified by lysozyme (1%) and SDS (1%) (data not shown) to separate them from cell debris, indicating that larval development resulted from feeding on the spores themselves. (Carcasses of any larvae which died during the experiment were removed before being scavenged (Zaritsky and Khawaled, 1986).) These results indicate that spores of *B. thuringiensis* var. *israelensis* are digested in the larval gut.

More convincing evidence that *Ae. aegypti* larvae do digest *B. thuringiensis* var. *israelensis* spores was obtained by following incorporation into larval tissues of [^{35}S]methionine-labeled spores. Second instar larvae were incubated for different times with radiolabeled spores (1 cpm spore^{-1}), and the ingested radioactivity was determined after washing with sterilized distilled water. Results of a typical experiment are displayed in Fig. 3. The maximum ingested radioactivity was reached 3 hr after introduction, indicating the time needed for filling of the larval gut with spores. In the following series of experiments, the larvae were incubated with radiolabeled spores for longer periods. They were then washed off and their gut content removed by further incubation with the unicellular green alga *Ch. emersonii* (at a concentration of 10^8 cells ml^{-1}). The purging of the larval gut with algae was highly efficient: it removed almost 90% of the ingested radioactivity in 20 min (data not shown). Significant amounts of radioactivity were incorporated into larval tissues (Fig. 4), demonstrating unequivocally that larvae of *Ae. aegypti* digest *B. thuringiensis* var. *israelensis* spores and incorporate digestion products into their tissues. A long period of time (about 10 hr) before incorporation was evident (Fig. 4) and may mean that spore digestion requires an adaptive process such as induction of specific enzymes not usually present in the larval digestion system.

The low persistence of *B. thuringiensis* var. *israelensis* in natural conditions has been attributed to several factors such as adsorbtion to silt particles (Ohana *et al.*, 1987). Digestion of the spores at sublethal concentrations by mosquito larvae could also contribute to the low persistence of the spores in nature. Larval adaptability to digest biological material as tough as spores of *B. thuringiensis* var. *israelensis* (Fig. 4) raises the likelihood that effective cloning of the δ-endotoxin genes for improved biocontrol is not restricted to a narrow range of carriers such as algae and vegetative bacteria (Ward *et al.*, 1984, 1986; Angsuthanasombat and Panyim, 1989).

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