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Protozoan-Enhanced Toxicity of *Bacillus thuringiensis* var. *israelensis* δ-Endotoxin against *Aedes aegypti* Larvae

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The toxicity of *Bacillus thuringiensis* var. *israelensis* (*Bti*) in mosquito larvae was enhanced by encapsulation in the protozoan *Tetrahymena pyriformis*. *Aedes aegypti* larvae which fed on *T. pyriformis* loaded with *Bti* died about three times faster than when fed on the same concentrations of *Bti* alone due to ingestion of higher toxin concentrations, reflected by shorter death times of exposed populations. The best larvicidal activities were achieved at ratios of cell/spore numbers in the range of 1:200 to 1:500. This enhancement of mortality by preincubation with *T. pyriformis* was higher at low *Bti* concentrations or in late third-instar larvae. Ninety minutes of preincubation yielded the best enhancement effect. Toxicity enhancement is very likely a consequence of concentrating large quantities of *Bti* spores and crystals (containing δ-endotoxin) by *T. pyriformis* cells and delivering them to the larvae. Shortening larval mortality time by encapsulation in *T. pyriformis* should reduce the exposure time of *Bti* to unfavorable field conditions that inactivate its larvicidal activity. Whether this method will indeed improve *Bti* efficacy is still to be determined.

KEY WORDS: ***Bacillus thuringiensis* var. *israelensis*; bioencapsulation; Δ-endotoxin; larvicidal mortality; mosquito biocontrol; *Tetrahymena pyriformis*.**

INTRODUCTION

Mosquitoes and blackflies are vectors of many human infectious diseases (e.g., Mattingly, 1969; Arata *et al.*, 1978). One of the best biocontrol agents against mosquitoes and blackfly larvae is the bacterium *Bacillus thuringiensis* var. *israelensis* (*Bti;* Goldberg and Margalit, 1977; de Barjac, 1978; for reviews, see Part 1 of de Barjac and Sutherland, 1990). Its larvicidal activity is included in polypeptides of a parasporal crystalline body (δ-endotoxin) that is produced during sporulation (Bulla *et al.*, 1980). The use of *Bti* is, however, limited by the low efficacy of current preparations under field conditions, under which it does not reproduce (Mulligan *et al.*, 1980; Mulla, 1985; Becker *et al.*, 1992). The major reasons for the low efficacy are: (1) sinking to the bottom of the water body (Rashed and Mulla, 1989), (2) adsorption onto silt particles and organic matter (Ohana *et al.*, 1987), (3) consumption by other organisms to which it is nontoxic (Blaustein and Margalit, 1991), and (4) inactivation by sunlight (Cohen, 1991).

To increase the persistence of *Bti* larvicidal activity, a commercial powder was incubated with *Tetrahymena pyriformis*, a protozoan in which δ-endotoxin is not inactivated (Zaritsky *et al.*, 1991). Each cell has been shown to concentrate between 180 and 240 *Bti* spores and their toxins in its food vacuoles and to keep the spores at the water surface (Ben-Dov *et al.* 1994, and unpublished data). The motile protozoan cells would be ingested by mosquito larvae, thus delivering the toxin to the larvae. This active process of concentrating and delivering intact δ-endotoxin to the target organisms was termed (Zaritsky *et al.* 1991) "bioencapsulation."

Here, we use this bioencapsulation system to demonstrate in the laboratory that larvae of *Aedes aegypti* fed on *Bti*-loaded *T. pyriformis* quickly ingest large, lethal quantities of toxin, resulting in a fast death rate.

MATERIALS AND METHODS

Mosquito larvae. Dry strips of paper bearing eggs of *Ae. aegypti* larvae were kindly provided by Professor J. Margalit. Eggs were submerged, and larvae were grown in 1 liter of sterile tap water supplemented with 1.5 g of Pharmamedia (Traders Protein, U.S.A.) at 30°C as described before (Khawaled *et al.* 1988).

B. thuringiensis var. israelensis. The commercial powder (Roger Bellon Laboratories, Belgium, R-153-78) used contained *Bti* spores, crystals of δ-endotoxin (about 1000 International Units/mg (Dulmage *et al.*, 1990)), diatom algae, and debris of unknown origin. The powder was suspended in sterile distilled water (1 mg/ml including approximately 10^8 colony-forming units) and pretreated by heat shock (10 min, 70°C) and sonication (MSE Sonifier, four times for 30 sec each with 30-sec intervals, 0°C) before the experiments to disperse spore aggregates and to eliminate contamination.

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T. pyriformis. The protozoan was maintained and grown axenically in PPY medium: 2% (w/v) proteose peptone enriched with 0.2% (w/v) yeast extract and with 10 mM Fe–EDTA complex (Jacobson, 1951). Stock cultures (10 ml) were kept in tightly closed test tubes at 10°C. Experiments were performed with cells derived from exponentially growing cultures shaken in a water bath (28°C, 60 strokes/min) after reaching 1–3 × 10^5 cells/ml (microscopically counted in a Sedgwick chamber after fixation in 1% formaldehyde). The cells were washed twice with sterile distilled water by centrifugation (90 sec at 3000 rpm).

Bioencapsulation. Unless otherwise stated, washed *T. pyriformis* cells (20,000/ml) were preincubated for 2 hr (28°C, 60 strokes/min) with *Bti* spores (10^7/ml) in 5-ml total volume of sterile distilled water in a 20-ml vial.

Bioassay. Twenty third-instar larvae of *Ae. aegypti* were incubated (at 28°C) with 100 ml of appropriate dilutions of *Bti,* alone or encapsulated (at 100-fold higher concentrations) in *T. pyriformis,* in sterile tap water in 150-ml disposable plastic cups. Larval mortality was determined every 10 min. Bioassays were carried out in duplicate. Mortality in the absence of *Bti* was negligible in all experiments (see also Zaritsky *et al.,* 1992).

Short-exposure experiments. Twenty larvae (third-instar) of *Ae. aegypti* were used to bioassay *Bti* (1 μg/ml), either bioencapsulated in *T. pyriformis* (200 cell/ml) or alone. The larvae were washed twice with sterile tap water at the indicated times and transferred to cups with fresh sterile tap water in order to remove the spores and crystals, and mortality was determined every 20 min.

RESULTS

T. pyriformis cells can ingest *Bti* spores and deliver their toxicity to mosquito larvae (Zaritsky *et al.* 1991). This bioencapsulation at concentrations of 25,000 spores (0.25 μg) in 200 cells per milliliter improved the larvicidal activity against *Ae. aegypti* (Fig. 1): Larvae died three times faster when treated with *Bti*-loaded *T. pyriformis* than when treated with *Bti* alone at the same concentration. The increased larval mortality was reflected in an earlier starting death time (lag) and in shorter times of death of 50 and 90% of the exposed populations (LT_{50} and LT_{90}, respectively).

A series of similar experiments with variable *T. pyriformis* concentrations (0–1680 cells/ml) and a constant concentration of *Bti* spores (10^5/ml) was carried out; the results are summarized and plotted in Fig. 2. The highest larvicidal activities (reflected in all three time parameters—lag, LT_{50}, and LT_{90}) were obtained at 200 and 500 cells/ml. At concentrations lower than 200 *T. pyriformis* cells/ml as well as higher than 500 cells/ml, larvicidal activity declined. It was higher at

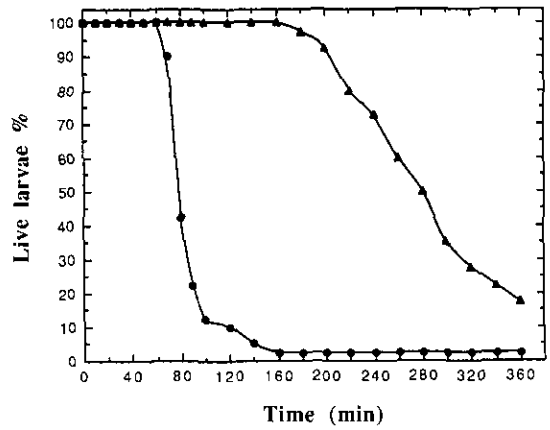

FIG. 1. Raising mortality rate of *Ae. aegypti* larvae by encapsulating *Bti* in *T. pyriformis.* Third-instar larvae were exposed to 0.25 μg/ml of a *Bti* commercial powder, either alone (▲) or encapsulated in 200 *T. pyriformis* cells/ml (●).

all tested concentrations (even with 10 cells/ml) than with the same concentration of *Bti* alone.

The ratio (for any time parameter) between larval death time caused by *Bti* to that caused by the same concentration of *Bti* when encapsulated in *T. pyriformis* was defined as the "toxicity amplification factor." This factor increased as the concentration of *Bti* was reduced (from 1.0 to 0.2 μg/ml) with a constant *T. pyriformis* concentration of 500 cells/ml and when times needed to kill higher fractions of the exposed larvae were considered (Fig. 3). The toxicity amplification factor was also higher with old than with young third-instar larvae (LT_{90} of 3.0 and 1.16, respectively).

The dependence of toxicity on the loading time of *T. pyriformis* cells with *Bti* spores (preincubation) was studied at constant concentrations (200 cells and 1 μg powder per milliliter, respectively). Bioassays were conducted immediately after preincubation with late third-instar larvae. Ninety minutes of preincubation was optimal for mortality (Fig. 4).

The improved delivery of *Bti* through *T. pyriformis*

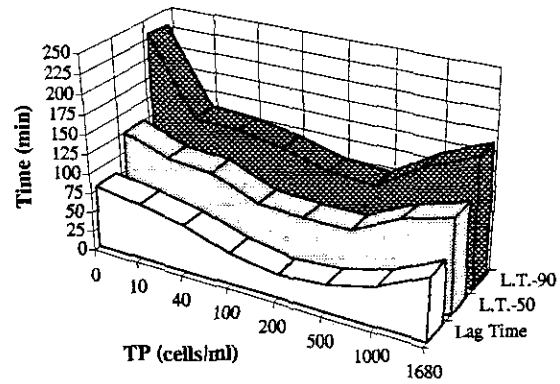

FIG. 2. The effect of *T. pyriformis* concentrations on time parameters of *Ae. aegypti* larval mortality. Concentration of *Bti* powder (1 μg/ml) was held constant.

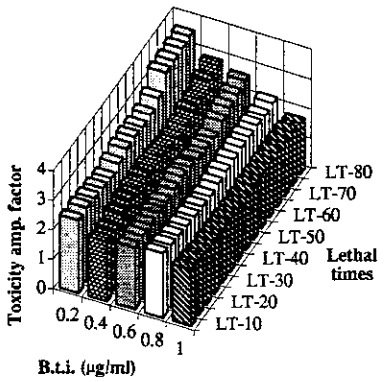

FIG. 3. The effect of *Bti* concentrations on the toxicity amplification factor at a constant concentration (500 cells/ml) of *T. pyriformis*.

was confirmed when mortalities caused by brief exposures of larvae to *Bti*-loaded *T. pyriformis* were compared to those caused by *Bti* alone (Fig. 5). The LT_{50} obtained after 4 min exposure to *Bti*-loaded *T. pyriformis* (200 cells/ml) was the same as that obtained after 35 min exposure to the same concentration (10^5/ml) of *Bti* spores alone. Short exposure times (16, 12, and 7 min), which were sufficient to yield LT_{90} values of 215, 225, and 265 min, respectively, resulted in death of only 30, 10, and 10% larvae after 24 hr when exposed to the same concentrations of *Bti* alone.

DISCUSSION

The toxicity of *Bti,* as judged by the rate of *Ae. aegypti* larval mortality, is improved by loading the spores with δ-endotoxin in the protozoan *T. pyriformis* (Fig. 1). Enhancement of larvicidal activity of the commercial powder (1 µg/ml, with 10^5 spores/ml) was best achieved at 200–500 *T. pyriformis* cells/ml, namely, at 500–200 spores per cell (Fig. 2).

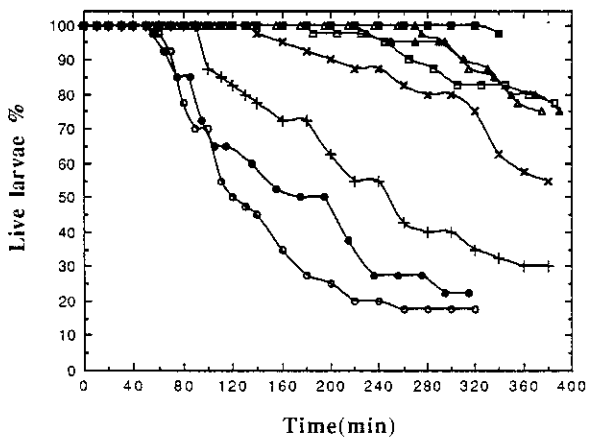

FIG. 4. The effect of loading time of *T. pyriformis* with *Bti* spores on larval mortality. Third-instar larvae were exposed to 1 µg/ml *Bti* powder encapsulated in 200 cells/ml. Preincubation times were 1 (△), 5 (▲), 10 (□), 30 (×), 75 (+), 90 (○), and 120 min (●). Control with *Bti* alone (■).

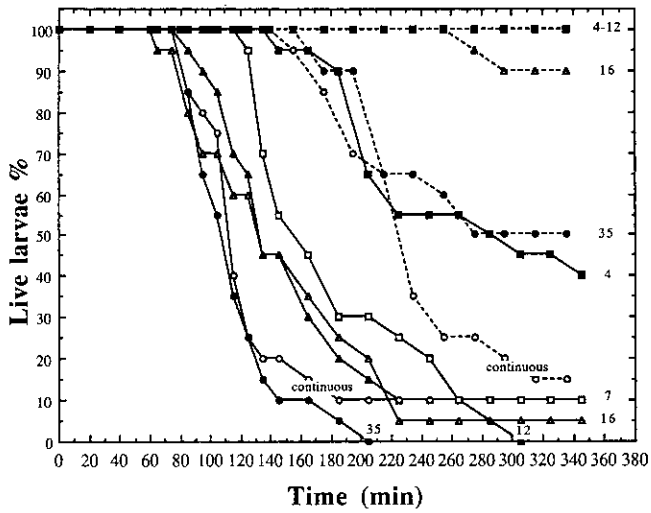

FIG. 5. Short time exposures of older third-instar *Ae. aegypti* larvae to *Bti* alone (- - -) and encapsulated in 200 *T. pyriformis* cells/ml (—). Exposure times are displayed numerically and symbolically as follows: 4 (■), 7 (□), 12 (▲), 16 (△), and 35 min (●) and continuous exposure (○).

An exponentially growing *T. pyriformis* cell can form up to about 30 food vacuoles (Chapman-Andresen and Nilsson, 1968). We find that each food vacuole contains 6–8 spores of *Bti* with their toxins (Ben-Dov *et al.*, 1994, and unpublished data). Thus, each cell can concentrate about 180–240 spores and will be fully loaded at ratios above 240:1 between *Bti* and *T. pyriformis*. At lower ratios *Bti* becomes a limiting factor and cells do not reach their maximal loading potential. This is one of the reasons that the larvicidal activity declined (Fig. 2) at concentrations of *T. pyriformis* higher than 500 cells/ml with a constant (10^5/ml) *Bti* spore concentration. In addition, excess *T. pyriformis* cells (being a food source; Zaritsky *et al.*, 1992) improves the larval resistance to *Bti,* as found for other food sources (Ignoffo *et al.*, 1981). On the other hand, below 200 cells/ml each cell is fully loaded with *Bti,* but their total number is too low to express the full larvicidal capacity of bioencapsulated spore suspension at this concentration.

An average of 3 vacuoles are formed during 10 min of growth at 28°C in each cell (Chapman-Andresen and Nilsson, 1968; Hoffmann et al., 1974; Nilsson, 1972). This frequency is in good agreement with our finding (Fig. 4) that 90 min of preincubation with *Bti* was optimal for larval mortality (3/10 = 27/90): All potential food vacuoles were formed during the 90 min of preincubation and were apparently filled with spores and crystals. At different preincubation times *T. pyriformis* cells were not fully loaded and larval mortality declined: At times shorter than 90 min the number of food vacuoles formed was smaller; at longer times, some of the *Bti*-loaded vacuoles were excreted, and the cells required at least 60 additional minutes to recover

their potential for a new cycle of vacuole formation (Nilsson, 1972). In addition, the concentration of free spores declined during loading, thus reducing the rate of formation of new food vacuoles and their filling with *Bti*.

Delivery of *Bti* toxicity to *Ae. aegypti* larvae by *T. pyriformis* shortened the ingestion time needed to achieve a lethal dose (Fig. 5). Higher toxicity amplification factors were therefore found under conditions in which larval ingestion rates were low, i.e., when old larvae (Results) or lower concentrations of *Bti* (Fig. 3) were employed (Khawaled *et al.*, 1988).

The increased mortality rate of *Ae. aegypti* larvae by encapsulating δ-endotoxin in *T. pyriformis* cells is very likely the consequence of concentrating large quantities of spores and crystals and their efficient delivery to the target organism. We predict that this phenomenon will shorten the time *Bti* is exposed to harmful field conditions that inactivate its larvicidal activity and will thus improve its efficacy. This prediction must still be tested in nature.

Utilization of *Bti* encapsulated in *T. pyriformis* as a biological control agent of mosquitoes would be advantageous over treatments with commercial formulations of *Bti* because the protozoan cells: (1) prevent fast sinking of spores and toxic crystals and their adsorption to soil; (2) concentrate large quantities of spores and toxins that cause rapid larval mortality and increase the spectrum of the affected population, such as late fourth-instar larvae; and (3) retain the toxic principle in the feeding zone of surface-feeding species such as *Anopheles* due to the motile behavior and attraction to oxygen of the protozoan (to be published elsewhere). An additional advantage can be achieved by exploiting a new phenomenon we have recently discovered, recycling of *Bti* spores in the excreted food vacuoles (to be published elsewhere).

Ciliated protozoa such as *Tetrahymena* exhibit a substantial biotechnological potential (Munro, 1985), but fermentation strategies yielding biomass comparable to those of bacteria and yeast cultures have been lacking for these organisms. Kiy and Tiedtke (1992) recently described an efficient method of growing ciliated protozoa in a 2-liter perfused bioreactor, which routinely achieved cell concentrations and dry weights 30- to 40-fold higher than those achieved in standard batch fermentations, but this method has not yet been exploited for biotechnological purposes. The tactics of raising large amounts of *Tetrahymena* and loading them with *Bti* for efficient delivery in nature are the subjects of future studies.

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