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NOTE

Spores of *Bacillus thuringiensis* serovar *israelensis* as Tracers for Ingestion Rates by *Tetrahymena pyriformis*

A novel system has recently been described (A. Zaritsky, V. Zalkinder, E. Ben-Dov, and Z. Barak, *J. Invertebr. Pathol.* 58, 455–457, 1991) in which the ciliate *Tetrahymena pyriformis* is used to bioencapsulate the larvicidal activity of *Bacillus thuringiensis* serovar *israelensis* and to deliver it to the target organisms, mosquito larvae. Death of larvae preying on *T. pyriformis* loaded with active powder of *B. thuringiensis* serovar *israelensis* clearly demonstrated that the protozoan delivered toxicity to the predator. The powder is composed of spores together with their toxic principle, crystals of δ-endotoxin. Toxin quantity was therefore presumed to be proportional to the number of spores. Here, this assumption is confirmed directly by observing encapsulated *B. thuringiensis* serovar *israelensis* in *T. pyriformis*. In an attempt to quantify the amount of spore-equivalent toxin ingested by *T. pyriformis* and to improve the proposed method for mosquito biocontrol, the ciliates' feeding behavior was investigated, using the spores as tracers. The kinetics of spore clearance was determined, filtration rate was calculated, and the amount of ingested toxin was evaluated.

Ingestion rates were measured by the decrease in spore numbers in the medium during incubation with the ciliate. Figure 1A presents the results of an experiment performed with an exponentially growing culture of *T. pyriformis*. Half of the cells were washed and starved in H_2O 3 hr prior to the start of the experiment. Similar rates of clearance were observed in both populations, with a delay of about 2–3 hr in the nonstarved cells. The starved population may have been adapted to the experimental conditions during the preceding starvation period. Alternatively, endocytosis may be stimulated by spores faster in starved *T. pyriformis* than in nonstarved cells, the vacuoles of which are full (T. R. Ricketts, *Exp. Cell Res.* 66, 49–58, 1971). Clearance of spores almost stopped at 8.5–10 hr, in both cultures.

Filtration rates of both starved and fed cells (Fig. 1B), calculated from the data of Fig. 1A, increased during the 5 hr after commencement of endocytosis and reached similar maximal values (12 μl hr^{-1}/cell for starved cells, and 11.4 μl hr^{-1}/cell for nonstarved) at 5 and 8 hr, respectively. These rates are three orders of magnitude higher than those reported previously for uptake of carbon particles and India ink by *T. pyriformis* (F. E. G. Cox, *Trans. Am. Microsc. Soc.* 86, 261–267, 1967; L. Rasmussen, H. E. Buhse, Jr., and K. Groth, *J. Protozool.* 22, 110–111, 1975), a difference which might be due to different reactions of *T. pyriformis* to organic (spores here) and inorganic (carbon there) particles (T. R. Ricketts, *J. Protozool.* 19, 373–375, 1972). Filtration rates, calculated from bacterial uptake, have earlier been found to be about 20 μl hr^{-1} (J. P. Harding, *J. Exp. Biol.* 14, 422–430, 1937), a similar value to those obtained here. Filtration rates in both cultures decreased later to very low levels, about 1.25 μl hr^{-1}/cell.

Formation of food vacuoles is believed to be membrane limited (L. Skriver and J. R. Nilsson, *J. Protozool.* 21, 421, 1974); it is possible that starved *T. pyriformis* cells have more membrane material available for immediate formation of vacuoles. The number of new food vacuoles formed (incubated with carmine particles) after 1 hr of starvation was indeed higher than that in fed cells (J. R. Nilsson, *C. R. Trav. Lab. Carlsberg* 40, 258–288, 1976). A mucous layer, formed in starved cells, might also play a role in the increased uptake of nutrients under starvation (L. Rasmussen, *Carlsberg Res. Commun.* 41, 143–167, 1976; unpublished results).

Plotting ingestion rates vs spore concentration (Fig. 1A, insert) yields an identical V_{max} (160 spores/cell/hr), but starved cells seem to have a slightly higher affinity to the spores.

The number of spores in individual *T. pyriformis* cells was determined by the colony-forming ability (for living spores) and by direct microscopic counting (for total spores). The number of colony-forming units (CFU), determined at different times, was almost identical in two independent experiments carried out under identical conditions, indicating that the cells were in a steady state of spore ingestion. The average number of spores per cell was 47 in the first experiment and 80 in the second (Table 1). Samples were withdrawn from the first experiment for direct microscopic counting of ingested spores (Fig. 2): Their average number per *T. pyriformis* was similar to that found by colony counting. The variation among different *B. thuringiensis* serovar *israelensis*-loaded cells was large, from 7 to 113 spores per cell.

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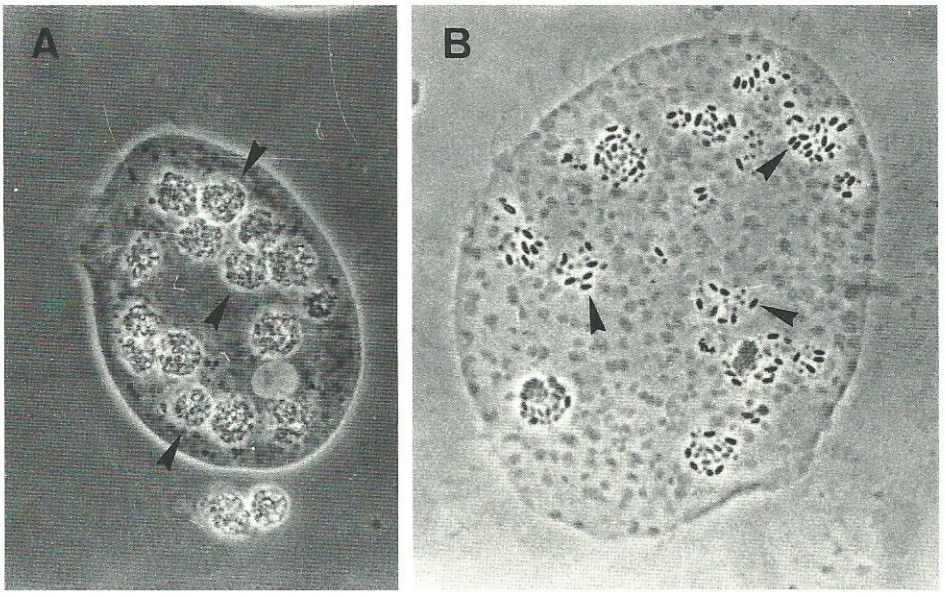

FIG. 2. *B. thuringiensis* serovar *israelensis* spores in food vacuoles of *T. pyriformis* after 105 min of incubation before (A) and after (B) flattening. Conditions of incubation and preparation are as those described in Table 1. Magnification, ×1500. Arrows in (A) point at several food vacuoles filled with *B. thuringiensis* serovar *israelensis* spores and in (B) at individual *B. thuringiensis* serovar *israelensis* spores.

value of this system should, however, be further investigated in the laboratory and tested in nature. Determination of ingestion kinetics of *B. thuringiensis* serovar *israelensis* by *T. pyriformis* and evaluation of the toxin ingested is an important step toward this aim.

KEY WORDS: Spores as tracers; ciliate protozoan; clearance; filtration and ingestion rates; viability of *Bacillus thuringiensis* serovar *israelensis;* mosquito biocontrol.

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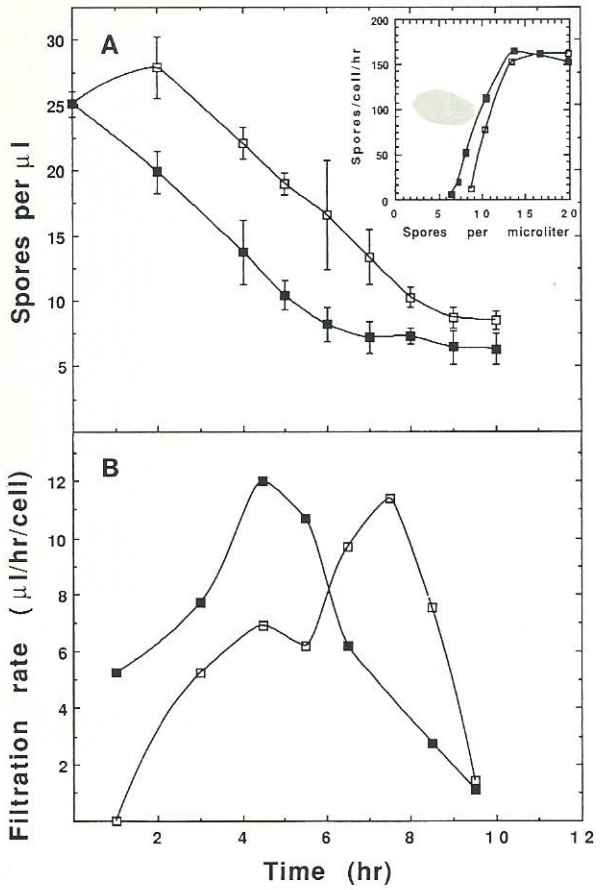

FIG. 1. Clearance (A), filtration rate (B), and ingestion rate (insert) of *B. thuringiensis* serovar *israelensis* spores by *T. pyriformis* cells: Starved (■) and nonstarved (□). Cultures of *T. pyriformis* were maintained and grown in PPY:2% (w/v) Proteose-peptone enriched with 0.2% (w/v) yeast extract and with 10 mM Fe–EDTA complex (L. Jacobson, *Plant Physiol.* **26**, 411–413, 1951). Suspensions containing (per milliliter) 20 cells and 2.5×10^4 spores (from a commercial powder R-153-78; Roger Bellon Laboratories, Belgium) were shaken gently at 28°C. Ten samples (10 µl each), mostly without *T. pyriformis,* were withdrawn periodically into 2.5 ml of melted (50°C) soft (0.6%) LB agar and poured on an LB agar (1.5%) plate for counting colonies (after 20 hr at 30°C). Starved cells were preincubated for 3 hr in sterile distilled water prior to the addition of spores. Filtration rate (B) was calculated for each time by dividing the volume cleared (number of spores cleared/spore concentration in suspension) by the concentration of *T. pyriformis* and by the time difference (A). Bars represent standard deviations about the averages of 10 independent determinations. Dependency of ingestion rate (A) on spore concentration was plotted in the insert.

TABLE 1
Number of Spores in Individual *Tetrahymena pyriformis* Cells

Experiment no.	Time (min)	Spores/cell	
		CFU[a]	Microscope[b]
I	20	—	58.7
	30	42.4	—
	70	40.2	—
	105	—	64.2
	155	59.4	—
II	55	85.1	—
	85	101.8	—
	145	52.9	—

Note. A mixture of *T. pyriformis* cells ($4–5 \times 10^4$ ml^{-1}) and dispersed spores (10^7 ml^{-1}) of *B. thuringiensis* serovar *israelensis* (both washed three times) was shaken gently at 28°C. Aliquots were withdrawn periodically and diluted with PPY medium (Fig. 1) to obtain a mixture with 100 cells ml^{-1}, from which 5-µl drops were screened for cells microscopically under a small objective (4×).

[a] Drops containing *T. pyriformis* were collected to a total of 10 cells per 50 µl and added to 0.95 ml H_2O. The cells were sonicated (MSE sonifier, at 0°C; 4 × 30 sec) to disperse the ingested spores, and 100-µl aliquots were spread over LB agar plates for counting colonies (grown overnight at 30°C). The average number of viable spores per cell was calculated, subtracting background numbers (determined similarly, with drops void of cells).

[b] *T. pyriformis* cells loaded with spores were fixed with 0.5% formaldehyde and then flattened to allow direct counting of spores inside the vacuoles under an immersion objective (×1000) of an Olympus microscope, Model BH. Each number is the average of 10 and 20 cells after 20 and 105 min of incubation, respectively.

Toxicity of a *T. pyriformis* cell loaded with 113 *B. thuringiensis* serovar *israelensis* spores was calculated to be about 0.0068 IU in our experiments (calculated according to H. T. Dulmage, J. A. Correa, and G. Gallegos-Morales, *In* "Bacterial Control of Mosquitoes and Black Flies" (H. de Barjac and D. J. Sutherland, Eds.), pp. 116–117, Rutgers Univ. Press, New Brunswick, 1990). The average number of vacuoles per cell, determined microscopically, was 7.6 at 20 min of incubation and 11.5 at 105 min. The average number of spores per vacuole was thus 7.7 and 5.6 at 20 and 105 min, respectively. The number of spores per cell could reach 200 [the average number of spores per food vacuole (6.65) times the number of vacuoles per cell (about 30; J. R. Nilsson, *C. R. Trav. Lab. Carlsberg* **40**, 258–288, 1976)]. The value observed (60 spores/cell) may be an underestimate, because some spores could have been digested by the protozoan during incubation. As expected, the average number of spores observed in an individual cell was higher than the CFU, albeit only by 33%, indicating that most of the visible spores did not lose their colony-forming ability inside the food vacuoles. (This difference is not statistically significant.)

These findings confirm our previous postulate that larval mortality, caused by their preying on *B. thuringiensis* serovar *israelensis*-loaded *T. pyriformis* cells, resulted from the spores and crystals encapsulated in the ciliate (A. Zaritsky, V. Zalkinder, E. Ben-Dov, and Z. Barak, *J. Invertebr. Pathol.* **58**, 455–457, 1991). The results support our biotechnological approach for controlling mosquitoes. In particular, surface feeder species such as *Anopheles* are affected to a larger extent than bottom feeders (R. Manasherob, Z. Barak, E. Ben-Dov, A. Zaritsky, and J. Margalit, to be published) because they share the natural habitat of *T. pyriformis,* the upper layers of fresh water. The real economic