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Biological Control of Mosquitoes by the Larvicidal Activity of *Bacillus thuringiensis* var. *israelensis* Delta Endotoxin*

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* Dedicated to Prof. W. J. H. Kunicki-Goldfinger on the occasion of his 70th birthday.

Abstract

Bacillus thuringiensis var. *israelensis* (*B.t.i.*) is a promising, safe toxic agent for control of mosquitoes, but the rapid disappearance of its toxicity makes its use in practice economically unattractive. The lack of evidence for *B.t.i.* multiplication in water also makes the natural ecology of *B.t.i.* puzzling. The observation that mosquito larvae readily cannibalize carcasses of *B.t.i.*-killed larvae, and that the carcasses become toxic to scavenging larvae provides a possible solution to this puzzle. Several experimental techniques have been developed to study these phenomena (e.g., quantitative determination of spore numbers despite aggregation, protocols for following toxicity development, etc.). Results suggest a cycle involving larval poisoning and death due to δ-endotoxin of ca. 1,000 spores, germination, vegetative growth and sporulation (with toxin production) of *B.t.i.* after multiplication to several million in the carcass. Implications of these results for the ecology of *B.t.i.*, and for practical applications in mosquito control, are discussed.

Introduction

The bacterium *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) was discovered in Israel (Goldberg & Margalit, 1977) and defined as a new serotype — H14 — of *Bacillus thuringiensis* (*B.t.*) (de Barjac, 1978). It proved to have strong larvicidal activity specific against many groups of mosquitoes and the blackfly (Margalit, Lahkim-Tsror, Pascar-Gluzman, Bobroglo & Barak, 1985), insects that transmit numerous tropical diseases (Schnell, Pfannenstiel & Nickerson, 1984). There is no evidence yet for any mechanism which could lead to larval resistance to its activity.

The toxic activity of *B.t.i.*, like that of other strains of *B.t.*, is

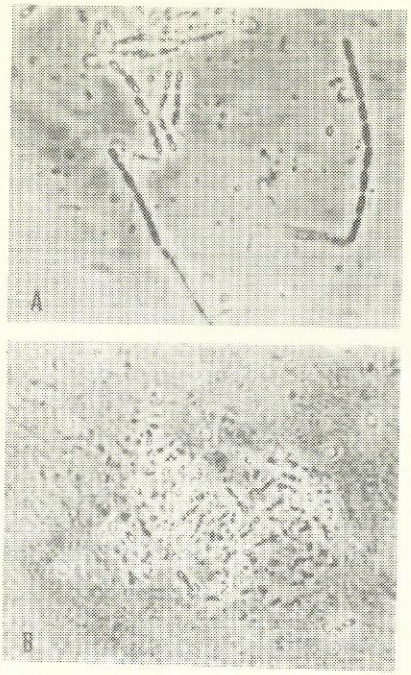

Fig. 1. Aggregated *B.t.i.* in carcasses of *B.t.i.*-killed 3rd instar larvae.
(A) vegetative bacteria in 26 h carcass; (B) spores in 44 h carcass. Final magnification. ×2,000.

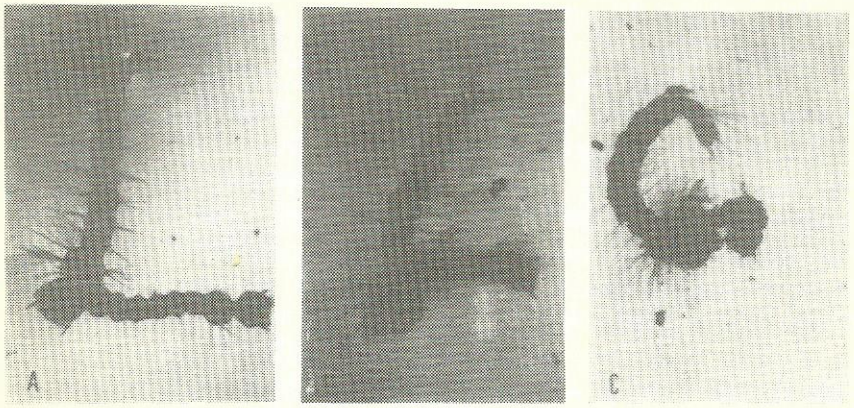

Fig. 2. Third instar larva of *Ae. aegypti* scavenging a 24 h old carcass of *B.t.i.*-killed larva (3rd instar).
The caracass is ingested systematically from tail (A) through abdomen (B) to thorax (C) leaving a severed head intact. Magnification. ×12.

less than one larva per two ml, they develop to adult mosquitoes within one week. (b) *Bacillus thuringiensis* var. *israelensis*. A larvicidal powder (R-153-78, of Roger Bellon Laboratories, U.S.A.), containing *B.t.i.* spores (about 10^7/mg), cell debris and crystals, was used throughout. A concentration of 4 µg/ml (400 times LC_{50}) killed third instar *Ae. aegypti* larvae within half an hour under the experimental conditions previously described (Lahkim-Tsror, *et al.*, 1983).

Bacterial viability was determined as colony-forming ability on LB plates (Miller, 1972), and spores counted similarly after a heat shock (10 min at 70°C). To disperse clumped aggregates of spores, 1% Tween-80 or sonication (4 min at maximum energy, MSE sonifier) were employed.

Observations were performed and pictures photographed under phase contrast microscope for bacteria and a binocular for larvae.

Cannibalism: Carcasses of *B.t.i.*-killed 3rd instar larvae were incubated in 10 ml water for 24 hrs at 27±3°C and then introduced individually into vessels containing fresh larvae, as described (Zaritsky & Khawaled, 1986). Each scavenger larva was transferred at a given time after cannibalism to a beaker with another 3rd instar larva (defined as "secondary scavenger") in 10 ml water, the behaviour of which was continously followed at the same temperature.

Results

Natural Habitat of *B.t.i.* Growth conditions for batch cultures of *B.t.i.* differ among various laboratories, but in all cases rich media are required for multiplication and for toxicity development (Margalit *et al.*, 1983). A decaying carcass of an intoxicated larva was assumed to be a suitable medium for the pathogenic bacteria, as has been shown for *B. sphaericus* (Des Rochers & Garcia, 1984). Preliminary experiments (Ohana, 1985; Khawaled, unpubl. data) revealed that the endotoxin associated with several hundred *B.t.i.* spores apparently was sufficient to kill one *Ae. aegypti* larva (third instar), and that the ingested spores indeed germinated in the carcass so that vegetative cells multiplied to form some 10^5 spores three days later. Similar results were obtained also by Aly, Mulla & Federici (1985).

B.t.i. forms short filaments when cultivated in rich media and in carcasses of intoxicated larvae (Fig. 1A), in a manner similar to the behaviour of *B. subtilis* (Zaritsky, Kihara & Macnab, 1981). Aggregates containing varying numbers of *B.t.i.* spores are usually observed in late stationary phase, in fully-developed spores in water, as well as in >24 h-old carcasses of *B.t.i.*-intoxicated larvae (Fig. 1B). Spore titration and thus quantitation of toxicity is therefore not a trivial matter. Either sonication of the spore suspension or addition of several detergents did not significantly change the situation. However, combining the two procedures (i.e., sonication in the presence of 1% Tween-80) separated the clumped spores (seen under the microscope) and raised the apparent number of colony-forming

units by a factor of 10-20 (data not shown). Similar dispersion of clumped spores was obtained when this procedure was applied to homogenized carcasses of *B.t.i.*-killed larvae (data not shown). The minimal number of spores killing one 3rd instar larva of *Ae. aegypti* is thus about one thousand, and these develop inside the resulting carcass to $3\text{-}4 \cdot 10^6$ at

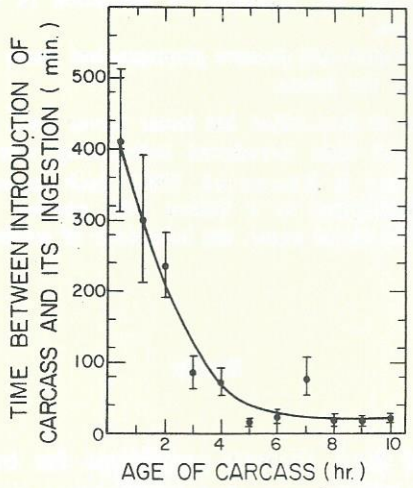

Fig. 3. Time between introducing a caracass and the first effective approach of scavenger, as a function of carcass' age.
Bars represent standard errors of the mean in at least three repetitions.

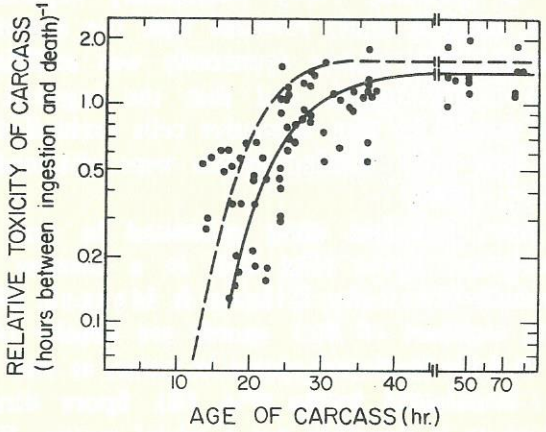

Fig. 4. Relative toxicity (reciprocal of time between its ingestion by and death of "secondary scavenger") of a carcass of 1st instar "primary scavenger" larva, as a function of carcass' age.
"Primary scavenger had consumed a carcass of *B.t.i.*-killed 3rd instar larva 24 hrs earlier.
Dashed line represents similar results obtained with "primary scavenger" being a 3rd instar larva (Fig. 2 in Zaritsky & Khawaled, 1986).

Table I

Toxic Activity of *B.t.i.*-killed Larvae

Number of Larvae Introduced[a]	Mortality[b]	
	Number	%
1	1	100
3	3	100
5	5	100
7	6	86
10	7	70
15	6	40
20	8	40
25	8	32
30	9	30
40	14	35
50	22	44
60	11	18
75	11	15
90	7	8

[a] In each case, one 120 h old carcass of a "primary scavenger" larva was introduced into a beaker containing the indicated number of live 3rd instar larvae in 10 ml sterile water.

[b] Scored after 24 hrs incubation at room temperature (27±3°C). Natural mortality under these conditions was zero, even with the highest larval concentration (9 per ml).

the end of the cycle several days later. At this time, the carcass decays, and the 'encapsulated' spores disperse in the surrounding water, presumably to start a new vegetative cycle after being ingested by other larvae.

Feeding Behaviour of Dipteran Larvae. Larvae of various dipteran species are thought to feed exclusively by filtering particles ranging in size between one and several hundred micrometers (Dadd, 1971), and their mouth parts were accordingly described as evolved to suit such feeding (Harbach, 1977). In the course of our investigations, we discovered that larvae of *A. aegypti*, of *Culex pipiens* and of *Culiseta longiareolata*, which are included among filter-feeder species, can all gnaw and ingest carcasses of larvae of their own and of other dipteran species (Fig. 2), thus being able to develop to adult mosquitoes on these as their only food source (Zaritsky & Khawaled, 1986). The ability of *A. aegypti* larvae to feed on everything that can be torn apart has recently been suggested to us as one of their features, being detritivores (pers. commun, from G. B. Craig, Jr.).

The time taken for a scavenger larva to effectively approach an introduced carcass is inversely related to the carcass' age (i.e., time after its death), as seen in Fig. 3. The minimal time (about 20 min) is reached at a carcass age of about 5 hrs under the conditions employed.

Development of Toxicity in Carcasses of *B.t.i.*-killed Larvae. If the ingested carcass is of a *B.t.i.*-killed *Ae. aegypti* larva, the scavenger dies

soon afterwards, provided the carcass has been incubated for over 15 h at 27°C. If ingested earlier, it does not induce death, implying that the toxic activity does not pass through the carcass to the scavenger, but rather develops inside the carcass. To substantiate this conclusion, the experiments were performed by allowing a "secondary scavenger" to consume a primary scavenger. Toxicity in the caracass of "primary scavenger" (expressed as reciprocal of time between its ingestion by and the death of the "secondary scavenger" develops exponentially and reaches a plateau at about 30 h (Fig. 4). The facts that carcasses of 1st and of 3rd instar larvae develop toxicity at similar rates and reach similar pleteau levels, indicate that this toxicity bioassay (time of death after ingesting intoxicated dead larva) is nor sensitive enough.

A more sensitive and instructive toxicity bioassay, which is also less laborious, is based on scoring the maximum number of larvae killed by a single carcass of *B.t.i.*-killed larva (Table I).

Discussion

Natural Cycling of *B.t.i.*

After destruction of its epithelium by the delta endotoxin, the larval gut provides a medium for germination, vegetative multiplication, and sporulation of *B.t.i.* (Fig. 1; Aly *et al.*, 1985). In nature, the propagation of *B.t.i.* is a more complex problem. If most carcasses of intoxicated larvae eventually disintegrated, one would expect spores to be infective to new larvae for only a day or two (as is found for artificially applied spores). If however, larval carcasses were very rapidly consumed by scavengers, one would again expect a very low propagation rate, since vegetative *B.t.i.* cells do not even seem not to be toxic even to dipteran larvae, but rather to be sensitive to the conditions prevailing in the **intact** larval gut (Ohana, 1985). Natural propagation of *B.t.i.* would then seem to be dependent on an appropriate population density of larvae. A more detailed study is necessary to substantiate such an analysis.

Toxicity developed later and reached a lower plateau level in carcasses of 1st instar larvae than in carcasses of 3rd instar larvae (Fig. 4; Zaritsky & Khawaled, 1986), during *B.t.i.* multiplication (as seen in Fig. 1A) and after full sporulation is reached (as in Fig. 1B), respectively. This difference of 10-20% may have stemmed from slight fluctuations in ambient temperatures during the two experiments. It may however be real, reflecting a true difference in amounts of δ-endvtoxin produced within the two types of carcasses. This is supported by the lower titers of *B.t.i.* (colony-forming units per carcass) observed in carcasses of 1st instar *B.t.i.*-intoxicated larvae than in those of 3rd instar (to be published elsewhere). This evidence is obviously not conclusive, since

a "secondary scavenger" always ingests a whole carcass when cannibalizing a 1st instar larva, while it rarely consumes a whole carcass of a 3rd instar *B.t.i.*-intoxicated larva (as in Fig. 2) before its own death. Further investigations are under way to fully describe *B.t.i.*'s ecology and toxicology.

Natural Encapsulation of Delta Endotoxin

Repeated applications of *B.t.i.* powder are necessary to keep mosquito populations at low levels (Margalit *et al.*, 1983), because it is not toxic to eggs or to adult mosquitoes; larvae that hatch after disappearance of larvicidal activity develop fully and maintain the population.

Much effort is being expended around the world to raise the persistence of *B.t.i.* δ-endotoxin in natural waters (Margalit *et al.*, 1984; Cheung & Hammock, 1985). One approach is encapsulation in semipermeable or degradable polymers. The observations reported here (multiplication of *B.t.i.* in the target organism's carcass and cannibalism among dipteran larvae) together form a relatively simple encapsulation method, which requires no more than raising large numbers of target larvae. After these are killed by *B.t.i.* spores, there occurs a full growth cycle inside the resulting carcass with about a thousand-fold increase in spore numbers (and hence, in toxic activity). It thus remains to collect the carcasses and apply them in mosquito-infested waters. The expenses of raising larvae is probably overcompensated by the saving on *B.t.i.* powder production and toxin encapsulation.

A further saving can perhaps be obtained by simultaneous application of *B.t.i.* powder and carcasses of *B.t.i.*-killed larvae, so that one application be as effective as two: the powder is to act during the first couple of days, the carcasses — during the next. To this end, it is important to better describe the cannibalistic behaviour of dipteran larvae (i.e., to extend the observations of Fig. 3) in waters of varying dimensions (depth, surface, volume) and with competing food sources. Such studies are under way in our laboratories.

Genetic Engineering

One major implication of our observation that dipteran larvae are not simply filter-feeders is that the delta-endotoxin gene might profitably be transferred into larger 'delivery organisms' than into single cell bacteria. A crucial question to be investigated is then which organisms are both consumed by dipteran larvae and readily genetically manipulated.

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