JOURNAL OF INVERTEBRATE PATHOLOGY 56, 312–316 (1990)

The Fate of *Bacillus thuringiensis* var. *israelensis* in *B. thuringiensis* var. *israelensis*-Killed Pupae of *Aedes aegypti*

KAMAL KHAWALED, EITAN BEN-DOV, ARIEH ZARITSKY, AND ZE'EV BARAK

Department of Biology, Ben Gurion University of the Negev, P.O. Box 653, Beer Sheva, Israel, 84105

Received September 29, 1989; accepted February 26, 1990

Carcasses of mosquito larvae killed by *Bacillus thuringiensis* var. *israelensis* allow its complete growth cycle (germination, vegetative growth, and sporulation), thus becoming toxic themselves to scavenging larvae. In this study, we demonstrate that the bacterium is capable of inducing death of *Aedes aegypti* pupae and of recycling in the resulting carcasses. *B. thuringiensis* var. *israelensis*-killed pupae were obtained by treating 40-hr-old synchronized fourth instar larvae with a low dose of spores (8000/ml). The fraction of dead pupae was reduced by higher or lower spore concentrations as well as by treating younger or older larval populations (both fourth instar): Increased proportions of *dead* larvae were obtained at higher concentration or by earlier treatment, whereas lower concentrations or later treatment resulted in more *living* pupae. Multiplication of *B. thuringiensis* var. *israelensis* is shown to occur in the carcasses of dead pupae. The number of spores in each pupal carcass followed a similar kinetic as in larval carcasses, but the final yield was about 10-fold higher, apparently reflecting the difference in dry weight between the two mosquito developmental stages (426 μg vs 83 μg, respectively). The specific larvicidal activity in a homogenized dead pupa was similar to that of *B. thuringiensis* var. *israelensis* powder, LC_{50} of about 600 spores/ml.

KEY WORDS: *Aedes aegypti* larvae; δ-endotoxin; pupicidal activity; spore recycling; *Bacillus thuringiensis* var. *israelenis*.

INTRODUCTION

Mosquitoes are known as vectors of severe human diseases (Mattingly, 1969). Chemical insecticides are conventionally used to control these vectors, but development of resistance as well as environmental concerns restrict their use as efficient control agents (Kirschbaum, 1985). Much effort is thus being expended around the world to develop alternative means. A widely used biological control agent is *Bacillus thuringiensis* var. *israelensis*, a Gram-positive bacterium which produces parasporal crystalline bodies (δ-endotoxin) during sporulation (Lee et al., 1985). The toxic crystals are highly specific to larvae of mosquitoes and blackflies (Lacey and Undeen, 1986; Mulla et al., 1982). Toxicity to adults has also been reported (Klowden et al., 1983, 1984, 1985), but not to other developmental stages (i.e., eggs and pupae). Since *B. thuringiensis* var. *israelensis* was discovered, it has not been found to reproduce in natural bodies of water and the larvicidal activity was repeatedly reported to disappear within short periods (24–48 hr) following application (Ramoska, 1982; van Essen and Hembree, 1982; Margalit et al., 1983; Mulla, 1985; Silapanuntakul et al., 1983)

Recycling in dead larvae of several dipteran species has recently been described (Aly et al., 1985; Ohana, 1985; Zaritsky and Khawaled, 1986; Barak et al., 1987). The *B. thuringiensis* var. *israelensis*-killed larvae were shown to develop high larvicidal activities during a complete bacterial growth cycle (spore germination, vegetative growth, and sporulation) in the carcass.

In this study, we describe conditions for obtaining *B. thuringiensis* var. *israelensis*-killed pupae of *Aedes aegypti*, as well as toxicity development in the resulting carcasses due to recycling of *B. thuringiensis* var. *israelensis*.

MATERIALS AND METHODS

Rearing mosquito larvae. Dry strips of

0022-2011/90 $1.50

paper bearing eggs of *A. aegypti* (Linnaeus) (provided by Dr. J. Margalit) were submerged in 1 liter of sterile tap water supplemented with 1 g of Pharmamedia (Traders Protein, U.S.A.) for 1 day and then removed. The hatching larvae were further incubated in an open-air water bath at 28 ± 2°C.

Larval synchronization. For each larva, the molting time to the fourth instar was recorded. Groups of 10 larvae molting within 30 min were then transferred to a fresh suspension of Pharmamedia (1 mg/ml).

Bioassays. Two procedures were employed, one (a) for carcasses of *B. thuringiensis* var. *israelensis*-infected pupae and the other (b) for *B. thuringiensis* var. *israelensis* powder (Roger Bellon Laboratories, R-153-78), as follows:

(a) Forty-hour-old fourth instar larvae were incubated in a suspension of *B. thuringiensis* var. *israelensis* powder (8000 spores/ml or as stated) at 28 ± 2°C. The pupal carcasses resulting during the incubation were homogenized in 2 ml of sterile tap water at different times following their death, and their toxicity was determined. The larvicidal activity was measured after 24 hr of incubation of 20 third instar larvae in disposable cups containing 100 ml of appropriate dilutions (in sterile tap water) of the pupal homogenate. In control experiments, uninfected pupae were homogenized at different times following piercing their head capsules with a needle.

(b) Synchronized fourth instar larvae were treated with varying concentrations of *B. thuringiensis* var. *israelensis* powder at various times following molting to the fourth instar. The percentage mortality of fourth instar larvae and of pupae developed during the bioassay was determined after 24 hr.

Spore counts. B. thuringiensis var. *israelensis*-killed pupae were washed with sterile tap water at intervals following their death, homogenized in 2 ml of 1% Tween 80, sonicated for 4 min, 4°C at maximum energy (MSE sonifier), and heat shocked for 10 min at 70°C. The number of *B. thuringiensis* var. *israelensis* colonies formed after 24 hr of incubation at 30°C was determined as described before (Khawaled et al., 1988).

Dry weight determinations. Groups of 10 third instar larvae and of 10 pupae of *A. aegypti* were washed with sterile distilled water and dried for 16 hr at 60°C on dry polycarbonate membranes (Uni-Pore). The net dry weight of each individual was calculated.

RESULTS

Pupicidal activity of B. thuringiensis *var.* israelensis. Dead pupae were obtained by treating synchronized fourth instar larvae with a low dose of *B. thuringiensis* var. *israelensis* powder. The proportion of dead pupae after 24 hr changed with larval age and with the concentration of spores in the powder (Fig. 1). The highest fraction of dead pupae (40%) was obtained upon treating the larvae at 40 hr with 8000 spores/ml. Treating younger or older ages resulted in decreased fractions. The latter increased the percentage of *live* pupae and the former led to an increase in the proportion of *dead larvae* (Fig. 1A). Changing spore concentrations reduced the pupicidal activity; an

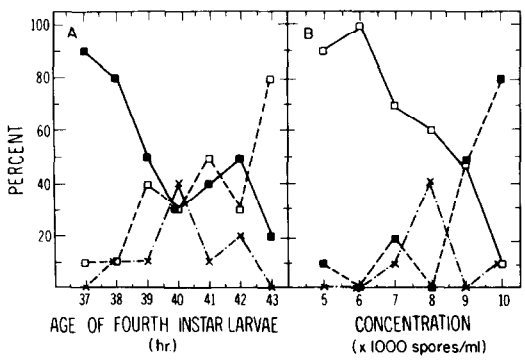

FIG. 1. Larvicidal and pupicidal activities of *Bacillus thuringiensis* var. *israelensis*. Synchronized fourth instar larvae of *Aedes aegypti* were treated with *B. thuringiensis* var. *israelensis* powder (8000 spores/ml) at different times (A) and with various concentrations at 40 hr following molting (B). Percentage of dead larvae ■ and pupae (x), as well as live pupae (□), was determined after 24 hr.

increase in the proportion of dead larvae was obtained at higher concentrations whereas lower concentrations resulted in more living pupae (Fig. 1B). Incubation of pupae with high concentrations of *B. thuringiensis* var. *israelensis* spores (10^7 spores/ml) did not induce any mortality for at least 24 hr and adults emerged within 48 hr.

Recycling in dead pupae. The number of spores in each dead pupa was determined at intervals following pupal death. It is noteworthy that the initial number of spores found in the pupae up to 10 hr following their death (500/pupa) was almost identical regardless of their death time (either immediately or several hours after pupation). This number increased afterward (Fig. 2) with similar kinetics as in *B. thuringiensis* var. *israelensis*-killed third instar larvae (Aly et al., 1985; Khawaled et al., 1988). The highest value (7×10^6 spores/pupa), which was reached after 60 hr, was, however, about 10-fold higher than in larval carcasses. This difference could stem from differences between the composition of larvae and pupae. The dry weight of a single *A. aegypti* pupa was 5-fold higher than that of third instar larva (426 ± 7 μg compared to 83 ± 2.3 μg, respectively). Pupae which were homogenized later showed a decrease in spore content (Fig. 2).

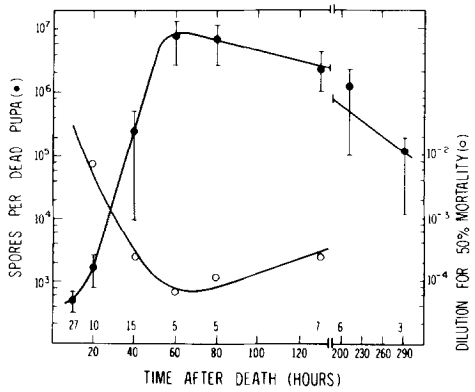

FIG. 2. Sporulation and toxicity development in *Bacillus thuringiensis* var. *israelensis*-killed *Aedes aegypti* pupae. Dead pupae, obtained by treating 40-hr-old fourth instar larvae with 8000 spores/ml, were homogenized, sonicated, and heat shocked for spore counting. Each value is an average of the spore numbers found in all the pupae which died during the preceeding time interval. The number of pupae used at each time is presented above the abscissa. Toxicity to third instar larvae is expressed by the homogenate dilution which is sufficient to kill 50%. Bars represent standard errors.

Live pupae did not allow multiplication of *B. thuringiensis* var. *israelensis*. The number of spores (1000/pupa) remained constant for at least 30 hr postpupation. Adults emerged within 40 hr.

Development of larvicidal activity. Carcasses of *B. thuringiensis* var. *israelensis*-killed pupae, homogenized 10 hr following death or before, did not induce mortality among third instar larvae. The homogenate dilution sufficient to kill 50% of the larvae, expressing pupal carcass' toxicity, increased during the following 50 hr and slightly decreased later (Fig. 2). In control experiments, 130-hr-old or younger decapitated pupae did not induce any mortality for at least 24 hr of incubation with third instar larvae.

DISCUSSION

Treatment with *B. thuringiensis* var. *israelensis* powder of fourth instar *A. aegypti* larvae can lead to death of the developing pupae by manipulating the dosage (Fig. 1B) and their age (Fig. 1A). The most effective conditions employed in this study (40-hr-old fourth instar larvae treated with 8000 spores/ml) killed 40% of the treated population. Consumption by younger fourth instar larvae or of higher doses of spores resulted in high larval mortality while treatment of older larvae or with lower doses did not interfere with the pupation process. Incubating the pupae with high concentrations of *B. thuringiensis* var. *israelensis* did not kill them, probably because pupae were unable to ingest particles (Jones, 1978). Pupal death is thus likely to be the result of δ-endotoxin ingested at the late larval stage. An elusive competition between the processes leading to pupation and the toxic activity of *B. thuringiensis* var. *israelensis* is evident.

Following pupal death, the ingested spores apparently germinated, and vegetative growth followed by sporulation and toxin production were observed microscopically in the decaying carcasses (data not shown). The low number of spores found in a fresh *B. thuringiensis* var. *israelensis*-killed pupa is lower than the lethal dose for the larval stage (Khawaled et al., 1988). It would therefore appear that ingested spores have lost their heat resistance because they germinated or were digested. Determination of the total number of colony-forming *B. thuringiensis* var. *israelensis* would allow discrimination between germination and being digested, but other bacteria proliferating in the decaying carcass interfere. Selection against the latter can be achieved by using antibiotic-resistant mutants (Barak et al., 1987).

The number of spores in each pupal carcass (Fig. 2) followed a similar kinetic as in larval carcasses (Aly et al., 1985; Khawaled et al., 1988). Sporulation begins at about 20 hr following death and reaches a maximum at about 60 hr. The higher final yield of spores here could be explained partially by the difference in the amount of the nutrient contents between third instar larvae and pupae; the dry weight of the latter is fivefold higher (426 ± 7 μg compared to 83 ± 2.3 μg for a third instar larva). The twofold higher specific yield of an average pupal carcass (16.5 spores/ng dry weight) than of an average larval carcass (8.4 spores/ng) may be caused by qualitative differences in the overall environmental conditions (e.g., oxygen tension, carcass' composition, *p*H). The apparent decrease in spore content after 80 hr could be a consequence of aggregation or heat sensitization.

The LC_{50} of homogenized old pupal carcasses (calculated from the data of Fig. 2) was about 600 spores/ml, similar to that of *B. thuringiensis* var. *israelensis* powder (1000 spores/ml, Khawaled et al., 1988). The apparent high larvicidal activity (low LC_{50}) of fresh (less than 40 hr old) *B. thuringiensis* var. *israelensis*-killed pupae (10–80 spores/ml) was due to early δ-endotoxin synthesis during the sporulation process but before heat resistance was gained.

Our results show that multiplication of *B. thuringiensis* var. *israelensis* is not restricted to dead larvae but also occurs in dead pupae of *A. aegypti*. The question of whether other organic sources, including carcasses of other organisms cohabiting with mosquito larvae, can support its multiplication in natural habitats in certain conditions has yet to be looked at.

ACKNOWLEDGMENTS

Thanks are due to Dr. J. Margalit for a free supply of *A. aegypti* eggs. This investigation received a financial support from the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases and from the U.S. Agency foe International Development/CDR.

REFERENCES

ALY, C., MULLA, M. S., AND FEDERICI, B. A. 1985. Sporulation and toxin production by *Bacillus thuringiensis* var. *israelensis* in cadavers of mosquito larvae (Diptera: Culicidae). *J. Invertebr. Pathol.*, **46**, 251–258.

BARAK, Z., OHANA, B., ALLON, Y., AND MARGALIT, J. 1987. A mutant of *Bacillus thuringiensis* var. *israelensis* (BTI) resistant to antibiotics. *Appl. Microbiol. Biotechnol.*, **27**, 88–93.

JONES, J. C. 1978. The feeding behavior of mosquitoes. *Sci. Amer.*, **328(6)**, 112–120.

KHAWALED, K., ZARITSKY, A., AND BARAK, Z. 1988. Feeding behavior of *Aedes aegypti* larvae and toxicity of dispersed and of naturally encapsulated *Bacillus thuringiensis* var. *israelensis*. *J. Invertebr. Pathol.*, **52**, 419–426.

KIRSCHBAUM, J. B. 1985. Potential implication of genetic engineering and other biotechnologies to insect control. *Annu. Rev. Entomol.*, **30**, 51–70.

KLOWDEN, M. J., HELD, G. A., AND BULLA, L. A., JR. 1983. Toxicity of *Bacillus thuringiensis* subsp. *israelensis* to adult *Aedes aegypti* mosquitoes. *Appl. Environ. Microbiol.*, **46**, 312–315.

KLOWDEN, M. J., AND BULLA, L. A., JR. 1984. Oral toxicity of *Bacillus thuringiensis* subsp. *israelensis* to adult mosquitoes. *Appl. Environ. Microbiol.*, **48**, 665–667.

KLOWDEN, M. J., BULLA, L. A., JR., AND STOLTZ, R. L. 1985. Susceptibility of larval and adult *Simulium vittatum* (Diptera: Simuliidae) to the solubilized parasporal crystal of *Bacillus thuringiensis*. *J. Med. Entomol.*, **22**, 466–467.

LACEY, L. A., AND UNDEEN, A. H. 1986. Microbial

control of blackflies and mosquitoes. *Annu. Rev. Entomol.*, **31**, 265–296.

LEE, S. G., ECKBLAD, W., AND BULLA, L. A., JR. 1985. Diversity of protein inclusion bodies and identification of mosquitocidal protein in *Bacillus thuringiensis* var. *israelensis*. *Biochem. Biophys. Res. Commun.*, **126**, 953–960.

MARGALIT, J., ZOMER, E., EREL, Z., AND BARAK, Z. 1983. Development and application of *Bacillus thuringiensis* var. *israelensis* H14 as an effective biological control agent against mosquitoes in Israel. *Biotechnology*, **1**, 74–75.

MATTINGLY, P. F. 1969. The biology of mosquito-borne diseases. *In* "The Science of Biology, Series 1" (J. D. Carthy and J. D. Sutcliffe, Eds.), pp. 13–183, London.

MULLA, M. S. 1985. Field evaluation and efficacy of bacterial formulations against mosquito larvae. *In "Integrated Mosquito Control Methodologies"* (M. Laird and J. W. Miles, Eds.), Vol. 2, pp. 227–250. Academic Press, San Diego.

MULLA, M. S., FEDERICI, B. A., AND DARWAZEH, H. A. 1982. Larvicidal efficacy of *Bacillus thuringiensis* serotype H14 against stagnant-water mosquitoes and its effects on nontarget organisms. *Environ. Entomol.*, **11**, 788–795.

OHANA, B. 1985. "The Fate of *Bacillus thuringiensis* var. *israelensis*, Its Larvicidal Activity in Simulated Stagnant Water and Its Behavior in the Gut of Mosquito Larvae." M.Sc. Thesis, supervised by Dr. Z. Barak, Ben Gurion University of the Negev, Beer Sheva.

RAMOSKA, W. A. 1982. An examination of the long term epizootic potential of various artificially introduced mosquito larval pathogens. *Mosq. News*, **42**, 603–607.

SILAPANUNTAKUL, S., PANTUWATANA, S., BHUMIRATANA, A., AND CHAROENSIRI, K. 1983. The comparative persistence of toxicity of *Bacillus sphaericus* strain 1593 and *Bacillus thuringiensis* serotype H-14 against mosquito larvae in different kinds of environments. *J. Invertebr. Pathol.*, **42**, 387–392.

VAN ESSEN, F. W., AND HEMBREE, S. C. 1982. Simulated field studies with four formulations of *Bacillus thuringiensis* var. *israelensis* against mosquitoes: Residual activity and effect of soil constituents. *Mosq. News*, **42**, 66–72.

ZARITSKY, A., AND KHAWALED, K. 1986. Toxicity in carcasses of *Bacillus thuringiensis* var. *israelensis*-killed *Aedes aegypti* larvae against scavenging larvae: Implications to bioassay. *J. Amer. Mosq. Control Assoc.*, **2**, 555–559.