Israel Journal of Entomology

Vol. XXIII(1989) pp. 109-113

COMPATABILITY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AND *LAGENIDIUM GIGANTEUM* FOR MOSQUITO CONTROL. I. EFFECT OF TIME OF APPLICATION AND TOXIN CONCENTRATION

SERGIO ORDUZ[1] AND RICHARD C. AXTELL[2],
[1]*Mosquito Control Program Bucaramanga Health Secretary*
Apartado Aereo 40187, Bucaramanga, Colombia
[2]*Department of Entomology, North Carolina State University,*
P.O. Box 7613, Raleigh, N.C. 27695, USA

ABSTRACT

In laboratory experiments, 3-day-old larvae of the mosquito *Culex quinquefasciatus* Say exposed to *Bacillus thuringiensis* subsp. *israeliensis (B.t.i.)* and to the pathogenic fungus *Lagenidium giganteum* showed no difference in susceptibility when *B.t.i.* was applied before or after the larvae were exposed to *Lagenidium* zoospores. However, the number of dead larvae infected with the fungus and the extent of mycelial growth within the larvae were less when *B.t.i.* was applied first and this diminishing pattern was also shown in relation to increasing *B.t.i.* concentration.

Zoosporogenesis from larval cadavers was accclerated when *B.t.i.* was used in combination with the fungus. *B.t.i.* delayed but did not prevented the production and maintenance of epizootics by *Lagenidium* under the conditions tested.

INTRODUCTION

Integrated control of vector species has been supported to reduce the present dependence on chemicals and because of the success achieved in the control of agricultural pests. Although in 1982 the WHO Expert Committee on Vector Biology and Control suggested "to consider the combined effects of various biological control agents in the reduction of vector populations," the main effort has been directed at testing *Bacillus thuringiensis* subsp. *israeliensis (B.t.i.)* with chemicals (Merriam and Axtell, 1983 b; Laird et al., 1985) and the effect of chemicals and some microbes on *Lagenidium giganteum* (Merriam and Axtell, 1983 a; Lord and Roberts, 1985), and little is known of the interaction of two biological control microorganisms in vector control.

Since 1977, when Goldberg and Margalit reported the discovery *of B.t.i.,* considerable attention has been directed to this pathogenic bacterium, as well as other species as promising biological control agents for vector of diseases; and extensive research has been done on *B.t.i.* and *Lagenidium giganteum*, as summarized by Davidson (1981), Federici (1981), Lacey (1985), and Lacey and Undeen (1986).

The main incentives for research and development of *B.t.i.* as a biological control agent are its efficacy, specificity, biodegradable nature (Lacey, 1985), and the fact that B.t.i. does not produce a broad spectrum heat-stable exotoxin, which is toxic to vertebrates (de Barjac, 1978).

B.t.i. delta-endotoxin is synthesized as a protoxin, and is active only when ingested by the mosquito larvae with subsequent activation in the alkaline gut environment (Lacey, 1985). These conditions are found in the gastric coeca and mid-to posterior part of the larval midgut (Dadd, 1975). The pathogenicity of this bacterium on the larval gut has been studied by Charles and de Barjac

microorganisms for pest control purposes. India, for example, requires that only indigenous species of *B.t.* be used for insect control. The situation can be alleviated, as in India, where *B.t.* strains are isolated from local material.

Bacillus sphaericus (Neide) is also an excellent entomopathogenic species for mosquito control. Unfortunately its development was overshadowed by the discovery of *B.t.i.* However, with the isolation of strains showing increased levels of pathogenicity (Lacey and Singer, 1982; Weiser, 1984) interest was renewed in this bacterium. It is now a valuable candidate organism for large scale mosquito control programmes. It is therefore, also desirable that additional strains of *B. sphaericus* possessing high mosquito larvae toxicity and other advantageous traits become available for possible field use.

MATERIALS AND METHODS

1. Isolation of mosquito-active *Bacillus* spp. in Israel

During 1984 and 1985, a survey was undertaken to isolate bacteria, pathogenic to mosquitoes, from soil samples collected and processed in Israel. The protocol of isolation adopted was simple and relatively straightforward (Brownbridge and Margalit, 1986). Briefly, the method used was as follows:

Mud or soil samples were suspended in tubes containing sterile buffered saline solution and vigorously agitated using a vortex to release bacteria adsorbed onto the soil particles. The tubes were heated at 78°C for 12 minutes to reduce the unwanted micro-organisms, i.e. non-spore-formers and vegetative cells. A sample of the heat treated suspension was then spread evenly over the surface of solid medium. The plates were incubated at 30°C and examined for bacterial colonies after 48 and 72 hours.

The isolated growth of the colonies facilitated their removal from the plates by means of an inoculation loop when colonies were selected and subcultured for evaluation in a screening assay.

Mosquito pathogenic strains were selected by assay of 48 hour liquid cultures against *Culex pipiens* and *Aedes aegypti* larvae. This method was employed in preference to the use of selective media as it enabled both *B.t.* and *B. sphaericus*, as well as other potentially mosquito pathogenic strains of bacteria, to be recovered using only one procedure.

Isolates showing pathogenicity to the mosquito larvae were cultured in a peptone-glucose-salts medium and assayed over a range of dilutions. From these tests, the most toxic isolates were chosen.

2. Isolation of *B.t.* strains in Kenya

Work is currently underway to isolate *B.t.* strains from soil samples taken from different ecological and geographical zones in Kenya. The protocol adopted thus incorporates the use of defined media to reduce other bacteria from the processed material. The methodology is based on that of Travers et al. (1987), and involves the use of a liquid medium containing 0.25m Na acetate, which selects against other *Bacillus* spp. in the sample material.

Initially, a sample of the soil is placed in 100 ml. flasks containing PBS and a number of micro elements. The flasks are incubated at 30°C, 300 rpm for 24 hours. The treatment is designed to release bacteria from the soil and promote spore formation in the *Bacillus* spp.

A 1 ml. sample of the suspension is then transferred to 100 ml. flasks containing 10ml of L broth buffered with 0.25m Na. acetate. The flasks are incubated at 30°C, 300 rpm for 4 hours. Use of Na acetate at this concentration supresses the germination of any *B.t.* spores contained in the sample, but facilitates the germination of other soil bacilli. Heat treatment at 80°C for 4 minutes kills the vegetative cells and non-spore-forming bacteria. The heat-treated suspension is then plated on enriched nutrient agar which allows *B.t.* spores to germinate, with the majority of the unwanted background bacteria removed. The isolates thus obtained are subcultured and examined microscopi-

cally for the presence of crystals. The crystals formers are then screened for activity against Lepidopteran pest species, e.g. *Chilo parellus*, and mosquito larvae.

RESULTS

1. Israel isolates

Over 130 soil samples from 80 diverse mosquito breeding habitats were processed and several hundred sporeforming bacteria were isolated and assayed. Nine *B.t.* strains, toxic to mosquito larvae, were recovered. These were identified, using the serum agglutination test devised by de Barjac and Bonnefoi (1962), as *B.t. israelensis/aizawi*, *B.t. israelensis*, and *B.t. entomocidus* (Brownbridge and Margalit, 1987).

Three isolates, s–2–6, s–2–13 and 37. K 30, identified as *B.t.* subsp. *israelensis/aizawi*, were highly toxic to *A. aegypti* larvae. Isolate s–2–6, with a calculated toxicity of 17 800 ITU/mg, was the most toxic strain recovered (Table 1) (Brownbridge and Margalit, 1987).

TABLE 1
Mosquito pathogenic strains of *B.t.* isolated from soil and mud samples collected in Israel

Isolate code	*B.t.* serovar	ITU/mg vs. *A. aegypti* L4 larvae[1]
IPS–82[2]	israelensis	15000
1884[3]	israelensis	7000
S–2–6	israelensis/aizawai	17800
37.k 30	israelensis/aizawai	15600
S–2–13	israelensis/aizawai	13800
36.k 2297	israelensis	7100
33.14	israelensis	6100
35.28	israelensis	4700
35.k 36	israelensis	3500
S–2–4	entomocidus	250
S–2–5	entomocidus	140

[1]ITU values calculated by comparison with the IPS–82 powder. All assays performed on lyophilized powder preparations against early fourth stage *A. aegypti* larvae.
[2]International Standard Reference Preparation of *B.t.i.* obtained from the Institut Pasteur, Paris, with an arbitrarily assigned value of 15000 ITU/mg vs L4 *A. aegypti* larvae.
[3]Reference strain of *B.t.i.* obtained from the Institut Pasteur cultured and preserved in our laboratory under identical conditions of media and regime as other *B.t.* types isolated in this survey.

Bacteria of typical *B. sphaericus* appearance were the most common types isolated from material collected in the survey (Brownbridge and Margalit, 1987). Nineteen isolates were sufficiently toxic to be retained for bioassay against *C. pipiens* larvae. The isolates were classified according to their reaction to a series of lytic bacteriophages (Yousten, personal communication).

The phage testing enabled the *B. sphaericus* strains to be placed in either Phage Group 3 or Group 4 (Yousten, 1984). The most toxic strains recovered were all in Phage 3. Two strains, coded 2615

TABLE 2
Bacillus sphaericus strains isolated from soil/mud samples collected in Israel (only the most active isolates listed)

WHO/CCBC accession number	Phage group	ITU/mg US L4 *C. pipiens larvae*[1]
RB–80[2]	3	1000
2362[3]	3	910
2613	3	1100
2615	3	1500
2619	3	1300
2631	3	1450
2626	4	130
2630	4	350

[1]ITU values calculated by comparison with the RB–80 Standard. Assays performed on acetone powders vs early fourth stage *C. pipiens* larvae.
[2]International Standard Reference Preparation produced from *B. sphaericus* strain 1593–4 and obtained from the Institut Pasteur. Assigned on arbitrary value of 1000 ITU/mg vs. *C. pipiens* larvae.
[3]*B. sphaericus* reference strain obtained from Dr. A.A. Yousten and produced in our laboratory under the same regime as all other isolates tested.

and 2631, with approximately 1500 ITU/mg were the most potent isolates recovered (Table 2). They were approximately 50% more active than the RB–80 *B. sphaericus* reference preparation.

There was also an interesting geographical distribution of the material from which the different phage groups were isolated. Bacteria belonging to Phase Group 3 were recovered from material collected in the Central Negev region of Israel. Material taken from sources close to the Dead Sea produced isolates belonging to Group 4, indicating that the environment may exert an influence upon the occurrence of different entomopathogenic strains (Brownbridge and Margalit, 1987).

The protocol adopted in Kenya has so far produced eight crystal forming isolates pathogenic to one or more of the pest species used in the preliminary screening assays (Table 3). These and others

TABLE 3
B.t. isolates from soils collected in Kenya

Isolate Code	Pest species	
	C. partellus	*A. aegypti*
MF–1–1	+	–
MF–1–3	+	–
MF–1–4	+	–
MF–2B–3	+	+/–
MF–3A–1	+	–
MF–4B–1	+	–
MF–4B–2	+	–
MF–9–3	+	–

+ = pathogenic
– = non-pathogenic
+/– = slightly pathogenic

that will become available, are now the subject of further quantitive evaluation. These experiments are designed to elucidate the most toxic strains for use in future field experiments.

DISCUSSION

Entomopathogenic *Bacillus* strains were recovered in both surveys from soil samples collected in a variety of ecological and environmental locations. In this respect, this study differs from the usual isolation of entomopathogenic bacteria, where the micro-organisims are obtained from diseased or dead insects.

This study serves to illustrate the value of soils as a source of insect pathogenic bacteria, where *B.t.* and *B. sphaericus* spores are known to survive for long periods (Hertlein et al., 1979; Petras and Casida, 1985). Soils thus appear to have excellent potential for further exploitation, and if such isolation schemes are adopted in several countries, then it seems likely that indigenous strains of entomopathogenic bacteria would become available for development and use against a variety of pest species.

REFERENCES

Brownbridge, M., and Margalit, J. 1986. New *Bacillus thuringiensis* strains isolated in Israel are highly toxic to mosquito larvae. *J. Invertebr. Pathol.* 48:216–222.

Brownbridge, M., and Margalit, J. 1987. Mosquito active strains of *Bacillus sphaericus* isolated from soil and mud samples collected in Israel. *J. Invertebr. Pathol.* 50:106–112.

Brownbridge, M., and Margalit, J. 1987. Identification of *Bacillus thuringiensis* strains, toxic to mosquitoes, recently isolated in Israel. *J. Invertebr. Pathol.* 50:322–323.

De Barjac, H., and Bonnefoi, A. 1962. Essai de classification biochimique et serologique de 24 souches de *Bacillus* du type *B. thuringiensis*. *Entomophage*, 7:5–31.

Goldberg, L.J., and Margalit J. 1977 . A bacterial spore demonstrating rapid larvicidal acticity against *Anopheles sergentii, uranotaenia unguiculate, Culex univittatus, Aedes aegypti* and *Culex pipiens*. *Mosq. News* 37:355–358.

Hertlein, B.C., Levy, R., and Miller, T.W., Jr. 1979. Re-cycling potential and selective retrieval of *Bacillus sphaericus* from soil in a mosquito habitat. *J. Invertebr. Pathol.* 33:217–221.

Krieg, A., Huger, A.M., Langenbruch, G.A., and Schnetter, W. 1983. *Bacillus thuringiensis* var *tenebrionis*; ein neuer gegenuber Larven von Coleopteran wirksamer Pathotyp. *Z. angew Ent.* 96:500–508.

Lacey, L.A., and Singer, S. 1982. Larvicidal activity of new isolates of *Bacillus sphaericus* and *Bacillus thuringiensis* (H–14) against anopheline and culicine mosquitoes. *Mosq. News* 42:537–543.

Padua, L.E., Ohba, M., and Alzawa, K. 1984. Isolation of a *Bacillus thuringiensis* strain (serotype 8a:8b) highly and selectively toxic against mosquito larvae. *J. Invertebr. Pathol.* 44:12–17.

Petras, S.F. and Casida, L.E., Jr. 1985. Survival of *Bacillus thuringiensis* spores in soil. *Appl. Environ. Microbiol.* 50:1496–1501.

Travers, R.S., Martin, P.A.W., and Reichelderfer, C.F. 1987. Selective process for efficient isolation of soil *Bacillus* spp. *Appl. Environ. Microbiol.* 53:1263–1266.

Yousten, A.A. 1984. Bacteriophage typing of mosquito pathogenic strains of *Bacillus sphaericus*. *J. Invertebr. Pathol.* 43:124–125.