ARTICLES

THE STORY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* (*B.t.i.*)[1]

J. MARGALIT[2] AND D. DEAN[3]

ABSTRACT. The isolation and the characteristics of *Bacillus thuringiensis* var. *israelensis* is reviewed. Included in the review are full descriptions of the nomenclature, mosquito target range of this potent mosquitocidal bacterium, as well as the genetics and biochemistry of the toxins.

INTRODUCTION

The development of residual insecticides over the past 40 years provides a relatively simple and inexpensive tool for the control of vectors of diseases, especially in the vast rural areas of the tropics where diseases such as malaria take a heavy toll in human life and suffering. However, the emergence and spread of insecticide resistance in many species of vectors, the concern with environmental pollution, and the high cost of the new chemical insecticides make it apparent that vector control can no longer be solely dependent on the use of chemicals.

Many important anopheline vector species have developed multiple resistance to organochlorines, organophosphates and carbamates; i.e., *Anopheles albimanus* Wied. in several Latin American countries, *An. sacharovi* Favre in Greece and Turkey, *An. stephensi* Giles in India, Iran, Iraq and Pakistan, and *An. arabiensis* Patton in Africa. More recently, *Simulium soubrense* and *S. sanctipauli* in West Africa have developed resistance to temephos (WHO 1982b).

For all these reasons, increasing attention has been directed toward natural enemies such as predators, parasites and pathogens. Unfortunately, none of the predators and parasites can be mass-produced and stored for a long period. They all must be reared *in vivo*. It became evident that there was an urgent need for a biological agent that would possess the desirable properties of a chemical pesticide, i.e., it must be highly toxic to the target organism, able to be mass-produced on an industrial scale, have a long shelf-life and be transportable.

In 1975–76, Drs. Tahori and Margalit conducted a survey in Israel for biocontrol agents against mosquitoes. During this survey (August 1976) the senior author of this paper came across a small pond in a dried-out river-bed in the northcentral Negev Desert, near Kibbutz Zeelim (Fig. 1). This mosquito breeding site, 15 × 60 m, with a maximum depth of 30 cm, contained brackish water with an approximate salinity of 900 mg Cl/liter and a heavy load of decomposing organic material. A very dense population of exclusively *Culex pipiens* complex dead and dying larvae was found as a "thick carpet" on the surface in an epizootic situation. In addition, pupae and sunk adults attempting to emerge from their pupal cases were floating on the surface.
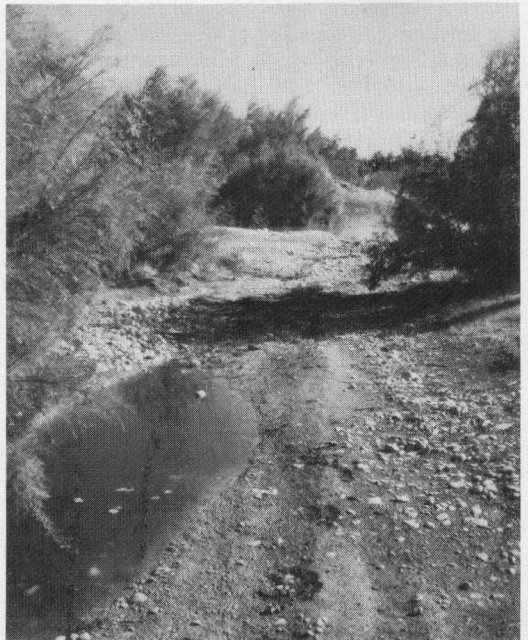

Fig. 1. Temporary pond in the central Negev Desert of Israel, near Kibbutz Zeelim where *Bacillus thuringiensis* var. *israelensis* was first isolated.

[2] Ben Gurion University of the Negev, Center for Biological Control, Department of Biology, P. O. Box 653, Beer Sheva 84 105, Israel.

[3] Department of Microbiology, The Ohio State University, 484 West 12th Avenue, Columbus, OH 43210, U.S.A.

A sample collected from the edge of the pool, containing dead and decomposing larvae, water and silty mud was taken to the laboratory and refrigerated. Bacteria were isolated from this sample in the lab, in association with Mr. L. H. Goldberg, and purified to single colonies. Thus, from a single colony designated ONR 60A, were derived all the known cultures of *B.t.i.* now in use. Sub-samples were taken from the homogenate of the parent sample and cultured on standard media and then processed for larvicidal activity. The larvicidal activity of this strain was tested in 1976 and found to be effective against five species of mosquitoes belonging to the genera *Aedes, Culex, Anopheles* and *Uranotaenia* (Goldberg and Margalit 1977). Clones of this strain were delivered through WHO to Dr. de Barjac, at the reference laboratory of the Pasteur Institute in Paris. The strain was given two separate accession numbers (WHO 1884 and WHO 1897) because it was sent in two separate vials, but both were derived from the single isolate, ONR 60A. Later it was identified by Dr. H. de Barjac as *Bacillus thuringiensis* var. *israelensis* Serotype H–14, and described in detail (de Barjac 1978a).

NOMENCLATURE

The nomenclature of *Bacillus thuringiensis (B.t.)* has recently been reviewed by Burges (1984). The main subdivisions of *B. thuringiensis* are termed varieties. They are defined by the flagellar serotype which is designated by the serotype number (de Barjac 1981). *Bacillus thuringiensis* currently has 27 varieties, 24 H-types, ca 28 crystal serotypes, over 900 isolates and over 50 mutants (Burges 1984).

TARGET ORGANISMS

Since its detection, *B.t.i.* has been tested by scientists all over the world, and was found to be toxic against practically all filter-feeding mosquito and blackfly larvae tested, namely 72 species of mosquitoes: *Anopheles* (21 species), *Aedes* (21), *Culex* (17), *Culiseta* (5), *Limatus* (2), *Uranotaenia* (1), *Psorophora* (1), *Mansonia* (1), *Armigeres* (1), *Trichoprospon* (1) and *Coquillettidia* (1).

Bacillus thuringiensis also proved to be effective against 22 species of *Simulium* blackflies (WHO 1982a): *Simulium* (14 species), *Cnephia* (2), *Prosimulium* (1), *Austrosimulium* (2), *Eusimulium* (1), *Odogmia* (1) and *Stegoptera* (1). Among the many other organisms which have been tested for susceptibility to *B.t.i.* only two filter-feeding chironomid species and one spe-

cies of Dixidae were found to be susceptible, but at a dose two orders of magnitude higher than that required to kill mosquito larvae. All non-target organisms tested, breeding in association with mosquito larvae, were not affected by *B.t.i.* Recently, *B.t.i.* was shown to be effective against adult female *Aedes aegypti* (Linn.). When both purified bacterial crystals and solubilized preparations were administered to adult mosquitoes via an anal route as an enema, the LD_{50} was 0.21 and 0.01 μg/ml of mosquito for intact or solubilized crystal respectively (Klowden, Held and Bulla 1983).

GENETIC PROPERTIES OF *B.T.I.*

The parent strain of *B.t.i.*, ONR 60A has nine plasmids when extracted by the Eckhardt technique (Eckhardt 1978; Gonzalez, Dulmage and Carlton 1981) or the method of Kronstad, Schnepf and Whiteley (1983). The molecular weights of these plasmids are 3.6 Md, 4.2 Md, 4.8 Md., 9.8 Md, 10.2 Md, 65 Md, 72 Md, 105 Md, and 130 Md. Despite confusion about which plasmid encodes the crystal toxin gene of genes (Dean et al. 1982, Faust et al. 1983) it was highly suspected that the toxin gene or genes were borne upon a plasmid or plasmids because of the extreme instability of the crystalliferous phenotype. It has recently been demonstrated that the 72 Md plasmid is the sole plasmid associated with toxicity. This has been shown by curing (Clark and Dean 1983, Kamdar and Jayaraman 1983, Ward and Ellar 1983) and by mating (Gonzalez and Carlton 1984). Unpublished results (Clark, Workman and Dean) indicate that all the proteins of the crystal are encoded by the 72 Md plasmid.

Other isolates of serotype H-14 have been isolated but in each case they have proven to be the same as ONR 60A. A possible exception may be the Roumanian isolate (WHO 2013–9). A shipment of dead mosquito larvae was received by the WHO Collaborative Center for Biological Control (November 28, 1978) from Dr. I. Nosec (Bucuresti, Romania) and sent to Dr. S. Singer for bacterial analysis. The samples of *Culex* larvae were collected from a pond at Novodari on August 16, 1978. Bacterial spores were found in one of the dead *Culex* larvae which were serotyped as H-14 (designated WHO 2013–9) and seemed to have a different larvicidal activity than *B.t.i.* The plasmid profile of this culture was compared with cultures or subcultures of ONR 60A. It was seen that WHO 2013–9 had essentially the same plasmid profile as ONR 60A missing only three plasmids. Of the two cultures of WHO 2013–9 examined each had a slightly different pattern but not

widely different than substrains of ONR 60A which had spontaneously lost a few plasmids (Clark and Dean, unpublished data).

BIOCHEMICAL PROPERTIES OF B.T.I.

Unlike many other varieties of *Bacillus thuringiensis*, the variety *israelensis* does not have a well ordered bipyramidal crystal. The crystal is composed of at least three proteins: a predominant 25–28 kd band (may be separated into two bands of 25 kd and 28 kd on polyacrylamide gels) and two minor proteins 65 kd and 130 kd (Luthy et al. 1982). In electron micrographs the crystal appears to be composed of three sections within a netlike matrix. Sodium hydroxide solubilization dissolves the proteins leaving the net matrix (Huber and Luthy 1981).

To date, solubilization of the crystal greatly reduces or destroys the mosquito larvicidal activity by two to four orders of magnitude. For this reason, it is not yet possible to determine which of these proteins (if not all three) are larvicidal. As mentioned above all three proteins are synthesized only when the 72 Md plasmid is present.

The solubilized crystal has potent toxicity to tissue culture (insect and mammalian), is haemolytic and lethal to suckling mice, when injected but not *per os* (Thomas and Ellar 1983a, 1983b). It has not been clearly demonstrated that the larvicidal and the tissue culture toxicity are the same. Recently, a mutation of ONR 60A has been derived which blocks synthesis of the 65 and 130 kd proteins, without significantly affecting the mosquito toxicity (Yamamoto, Iizuka and Aronson 1983). This has narrowed the suspected larvicidal activity to the 25–28 kd crystal component.

MODE OF ACTION

The principal agent of insecticidal activity of *B.t.i.* is the parasporal body commonly known as the crystal. In Diptera only the crystal is toxic (de Barjac 1978b). Serovar *israelensis* parasporal bodies are irregular in shape and there are usually two or more such bodies associated with the spore (Fast 1982).

In other varieties of *Bacillus thuringiensis* the crystals and their subunits represent protoxins without biological activity. The basic elements are polypeptides with a molecular weight range between 128,000 and 136,000 (Luthy et al. 1982). After the pathogen is ingested by a susceptible insect, gut secretion solubilizes the crystal and gut proteases convert the protoxin into a toxin which has a molecular weight of 65,000. This pattern of solubilization and pro-

tease cleavage is not seen in *B.t.i.* As mentioned above the size of the suspected toxin is smaller (25,000) than that of the other varieties. It also does not appear to require solubilization or proteolytic activation. In fact, solubilization results in loss of toxic activity.

The mode of action of *B. thuringiensis* crystals is not fully understood. It involves the release of a toxin into the insect gut after ingestion of the crystals. The toxic polypeptide has not been isolated yet, however, it appears that the proteins of *B.t.i.* are different from those of other varieties. Crystals of *B.t.i.* kill mosquito larvae within minutes. *Bacillus thuringiensis* causes toxic symptoms in lepidopterous larvae also within minutes. It is therefore assumed that the delta-endotoxins of both varieties have similar modes of action.

The primary target organ of the δ-endotoxins of the *B.t.* serotypes active against lepidopterous larvae is the plasma membrane of gut epithelia and of susceptible cells *in vitro*. For *B.t.i.* it was shown that the gut epithelium also appears to be primary target for its δ-endotoxin (de Barjac 1978b.). Thomas and Ellar (1983a) observed that a soluble preparation of *B.t.i.* toxin caused rapid cytolysis of insect and mammalian cells *in vitro*, but had no effect on bacterial protoplasts. This toxin also showed haemolytic activity. In a later work Thomas and Ellar (1983b) identified certain membrane phospholipids as the primary target for the toxin. The interaction of the *B.t.i.* toxin with specific plasma membrane lipids such as phosphatidyl choline, sphyngomyelin and phosphatidyl ethanolamine, provided the lipids contain unsaturated acyl residues, causes a detergent-like rearrangement of the lipids, leading to disruption of membrane integrity and eventual cytolysis.

PRODUCTION AND FORMULATION

In the Western industrialized countries only three major firms commercially produce *B.t.i.* In China about 10 tons of *B.t.i.* have been produced and research to improve the fermentation technique is proceeding vigorously. Production in Israel takes place by conventional methods in a 500-liter fermentor. A 100-liter fermentor using inexpensive local ingredients has been used in India, and in Nigeria a 20-liter fermentor exploits inexpensive local media. Since *B.t.i.* can be produced in a variety of synthetic media, many developing countries should be able to base a fermentation unit for *B.t.i.* on local by-products, as has already been shown for coconut water and endosperm residues, waste products of the coconut oil industry.

Bacillus thuringiensis var. *israelensis* is produced commercially in submerged or deep-tank fermentation units. It requires a large supply of air, a nitrogen source, glucose or starch and some minerals. The amount of toxin produced during the fermentation process depends on the medium used, the temperature employed and the isolate used. The quantity and insecticidal activity of the toxin may also vary among different production lots and cannot be measured chemically at present. Therefore, potency is now determined by bioassay (standardized by McLaughlin et al. 1984) using insect larvae of an appropriate target species and calculated in international toxic units (ITU) (Dulmage and Cooperators 1981). Enzymes linked immunosorbent assays (ELISA) have been developed for *B. thuringiensis* (Smith and Ulrich 1983) and are useful for the chemical analysis of *B.t.i.* crystals.

Two main types of *B.t.i.* formulations exist. In wettable powders the particles are large aggregates (10 to 30 μ), whereas in suspension concentrates they are suspensions of isolated spores and crystals. The efficacy of the wettable powders increases with increasing size of clumps up to an optimum (40 to 70 μ). For onchocerciasis control the best type of formulations are liquid suspensions of isolated crystals (Guillet 1984). At present, the main formulations available are water dispersible powders and suspension concentrates. In addition, granulates are now being developed, designed to penetrate the vegetation covering breeding habitats but still light enough to float on the water surface where the larvae feed.

Several major differences exist between formulation of a chemical larvicide and formulation of *B.t.i.*

1. In contrast to chemical larvicides the primary products in the formulation of *B.t.i.* may show differences in their composition and physical properties. These variations strongly affect their biological efficacy.

2. Accurate methods have not yet been widely used to assay the content of active ingredients of a formulation. Currently the potency of *B.t.i.* products is evaluated only by biological methods with their inherent range of > 20%. A new method utilizing the haemolytic effect of *B.t.i.* on mammalian red blood cells is being developed at the Hebrew University, Jerusalem by Keynan, Sandler and Margalit. Enzyme-linked immunosorbant assay (ELISA) also can detect nanogram quantities of the toxin in formulations. This method has been published for use with other *B. thuringiensis* varieties (Smith and Ulrich 1983) and has been established for

B.t.i. in several laboratories (unpublished observations).

3. *Bacillus thuringiensis* var. *israelensis* is a natural microbial agent and cannot be patented. Thus, industry tends to keep information on fermentation and formulation secret. Nevertheless, process patents have been issued, and patents may also be issued on genetically engineered variants of *B.t.i.*

4. As a microbial agent, *B.t.i.* may be genetically engineered. Strains may be constructed which can grow at low carbonsource and oxygen concentrations found in mosquito breeding environments, offering long-term vector control with low environmental impact. Strains may also be constructed which grow faster, produce more toxin, or grow on cheaper carbonsources, thus reducing the costs of production and formulations.

SAFETY

Due to its specificity, *B.t.i.* is remarkably safe to non-target organisms, including man. *Bacillus thuringiensis* has not shown a single case of human toxicity after over 23 years of operational use.

Bacillus thuringiensis var. *israelensis* has undergone extensive safety tests for public health use (Shadduck 1980). The Informal Consultation Group on Mammalian Safety of Microbial Control Agents for Vector Control, after reviewing the status of safety testing of *B.t.i.*, concluded in 1980 that the organism has passed the necessary safety tests to warrant its application in large-scale field trials. In subsequent experiments negative results were obtained when *B.t.i.* endotoxin was tested for mutagenicity *in vitro*. Maximum dosages were applied by the conventional oral, parenteral, respiratory and dermal routes, together with allergenicity tests and a mutagenicity screen; all confirmed that *B.t.i.* poses no hazard. It can be concluded that *B.t.i.* is safe without tolerance limits, even for use in drinking water.

It has recently been reported that when the *B.t.i.* toxin in the crystal is dissolved in alkaline buffer, it becomes toxic, in very high concentrations, to suckling mice by intraperitoneal injection, but has no effect orally. Since in practice there is no chance that dissolved crystal sufficient to be harmful will reach the blood stream of man or mammal this presents no tangible hazard.

Over 240 tons of *B.t.i.* were used operationally in West Africa in 1982 without causing any adverse effect. *Bacillus thuringiensis* var. *israelen-*

sis is now considered to be by far the safest mosquito larvicide, chemical or other, developed so far.

FIELD TESTS

Many small-scale field trials all over the world have been carried out with *B.t.i.* These are reviewed in the WHO Mimeographed document 750 (1982a), and by Schaefer (1984). Two lessons can be learned from these field trials: liquid concentrates seem to be more promising than wettable powders and ground application is more economical than aerial spraying.

Large-scale field trials for the control of mosquitoes were carried out in Germany along the Rhine (Becker[4]). *Bacillus thuringiensis* var. *israelensis* powder was applied by helicopter to an area of ca. 1200 ha, and 750 kg of *B.t.i.* powder + 2,550 liters of *B.t.i.* liquid concentrates were applied by knapsack sprayers to an area of ca. 2,200 ha with excellent control results.

In Israel, we conducted field trials under extreme ecological conditions: 1) In untreated sewage where *Cx. pipiens* larvae were almost the only invertebrate organism present, and 2) in a natural well-oxygenated pond with abundant populations of diverse mosquito predators. In both cases, *B.t.i.* initially suppressed larval populations. In the case of sewage, the mosquito population very soon reestablished itself in spite of numerous weekly treatments with *B.t.i.* This was due to the influx of mosquitoes from neighboring breeding habitats. In the natural pond, predators took over and after 4 weekly applications additional *B.t.i.* treatments became unnecessary (Margalit et al. 1985).

PERSISTENCE OF *B.T.I.*

The major economic disadvantage of *B.t.i.* is its low stability after application into the mosquito breeding habitats. The field performance of *B.t.i.* is greatly influenced by the presence of organic matter or of solids in the water. Tests with suspended particles in water have been carried out by a number of researchers (Ramoska et al. 1982, Ignoffo et al. 1981, van Essen and Hembree 1982, Margalit and Bobroglio 1984). They found that the rate of inactivation of *B.t.i.* by organic material, as well as the adsorption of *B.t.i.* on soil particles and on organic matters in the water greatly reduced its persistence and its field efficacy.

PRESENT USE OF *B.T.I.*

Bacillus thuringiensis var. *israelensis* is operational against *Simulium* larvae in the onchocerciasis control program in the Upper Volta region of Africa. A large-scale field trial carried out by boat at high discharge in the Maraboue River in the Ivory Coast resulted in the complete removal of *S. damnosum* larvae for 19 km and in a partial reduction for an additional 15 km (Lacey et al. 1982). Altogether, nearly a million liters of *B.t.i.* were used for aerial and ground applications in West Africa.

Bacillus thuringiensis var. *israelensis* should very soon be tested against mosquitoes in large-scale field trials in tropical developing countries. In Indonesia, limited field evaluation against *An. sundaicus* (Rodenwaldt) has already proven that *B.t.i.* satisfactorily reduces larval densities, and also has an impact on manlanding densities of adult mosquitoes (Schaefer 1984). In Israel, *B.t.i.* is presently used as an exclusive pesticide in natural preserves as well as in all large water treatment plants.

THE FUTURE OF *B.T.I.*

Until now *B.t.i.* has not developed cross-resistance with chemical insecticides, nor have cases of larvae resistant to *B.t.i.* been reported.

Bacillus thuringiensis var. *israelensis* is still relatively expensive. Liquid formulations cost $4.87/liter in 1983. According to Schaefer (1984) the operational use rate was ca $1.87–5.70/ha. However, Becker (personal communication) states that the cost of using *B.t.i.* is only 40% of that of a conventional pesticide. Apparently, this depends on ecological conditions as well as the expertise of the applicator. It is hoped that the price of *B.t.i.* will be reduced in the near future due to improved technology and an expanding market.

The future of *B.t.i.* will very likely involve genetic engineering. Specifically, it will be desirable to transfer the toxin gene(s) into a bacterium which would survive well and produce the toxin under the low organic conditions of most stagnant ponds. A few biological agents such as *B. sphaericus* have been reported to survive well under such conditions but are not as toxic as *B.t.i.* Through the combined efforts of geneticists, microbiologists and insect pathologists, improved control of insect vectors will be forthcoming.

ACKNOWLEDGMENTS

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on the origin of strains. The photograph was kindly taken by Ronald A. Ward during October 1984.

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