A BACTERIAL SPORE DEMONSTRATING RAPID LARVICIDAL ACTIVITY AGAINST *ANOPHELES SERGENTII, URANOTAENIA UNGUICULATA, CULEX UNIVITATTUS, AEDES AEGYPTI* AND *CULEX PIPIENS*

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ABSTRACT. A spore-forming bacillus demonstrating a high level of larvicidal activity in nominally 12 hr. following challenge has been isolated from the screening of roughly 1,000 clones from some 10 soil samples taken at known mosquito larval breeding sites. Roughly 1 in 100 such clones demonstrated larvicidal activity of the same order of magnitude as *Bacillus sphaericus* var. *fusiformis* (SSII-1) but only 1 isolate (60A) demonstrated rapid activity against species in 4 genera as evidenced by the 5 test species used, i.e., *Anopheles sergentii* (Theobald), *Uranotaenia unguiculata* Edwards, *Culex univitattus* Theobald, *Aedes aegypti* (Linnaeus), and *Cx. pipiens* Linnaeus. The activity of this isolate (60A) is some 30–100 times greater than that observed using *B. sphaericus* (SSII-1), when assayed using *Cx. pipiens* test larvae. Successful screening was dependent upon the use of a proprietary nutrient N2X which was made available by Nutrilite. This media is a standard production media for *Bacillus thuringiensis* (HD-1). With only one exception (60A), all other toxic isolates did not demonstrate larvicidal activity when grown on nutrient agar (Difco). The isolate, 60A, demonstrated no significant loss in larvicidal activity after being heat-shocked for 20 min. at 60°C, lyophilized or exposed to ultra violet (2537 Å), sufficient to reduce the viable spore count to less than 0.1% of its initial count. The mode of action can be attributed to an ultra violet and heat-stable endotoxin.

INTRODUCTION. The successful widespread use of *Bacillus thuringiensis* against Lepidoptera larvae has suggested the possibility of a bacillus existing which would demonstrate a broad-spectrum of mosquito larvicidal activity. *Bacillus thuringiensis* (HD-1), in addition to several other strains of *thuringiensis*, demonstrated larvicidal activity against *Culex tarsalis* (est. ED_{95} -10^6 cells/ml) (Goldberg unpublished). Using a "sewage flora" screening procedure which provides a stable but "worst test condition," the authors screened bacterial clones isolated from soil samples obtained from known mosquito larval breeding sites for possible larvicidal activity against *Cx. pipiens* (complex).

MATERIALS AND METHODS. N2X, a proprietary media used in the commercial fermentation of *B. thuringiensis* (HD-1) was selected as the standard agar screening media. Incubation for 24 hr. at a temperature of 30°C was selected based on previous data using B.T. Single passage "sewage flora" (obtained from the raw sewage pond of Kibbutz Hulda, Israel) was selected for screening use since it demonstrated both the "worst test condition" and the most consistent test results. Field-collected water at larval breeding sites was also used to provide the microbial flora for selected laboratory tests. The larval test challenge fluid consisted of filtered "alfalfa tea" made by autoclaving 16 gr. of fresh frozen alfalfa in 0.5 liter of tap water for 1 hr. After the alfalfa tea was cooled to 27°C, 0.5 liter of rough filtered (to remove solid debris) field water or sewage water was added to provide a simulation of normal microbial flora and plant nutrients. This liquid was incubated at 27°C for 18–24 hr. prior to use in larval test challenges. One or two larvae per test well (Bruen et al. 1976), initially containing 4 ml of inoculated and incubated alfalfa tea, were used for subsequent test challenge. The challenge liquid volume was limited to 1–3% of the well volume to minimize

disturbances due to challenge. The microbial flora within the alfalfa infusion provided adequate nutrition for the test larvae for 24 hr. Additional daily feeding was provided (20 mg. Tetramin/well/day) until pupation occurred. The *Cx. pipiens* larvae were originally obtained from a local sewage pond but subsequently maintained as a laboratory colony. Test larvae of *Anopheles sergentii, Uranotaenia unguiculata* and *Cx. univitattus* were field collected. *Aedes aegypti* larvae were obtained from a local experimental test colony.

RESULTS AND DISCUSSION. Initial comparative larvicidal tests using *Cx. pipiens* larvae, with selected microbial test floras, in combination with a standard challenge of *Bacillus sphaericus* var. *fusiformis* (SSII-1) (Goldberg unpublished) clearly demonstrated that the use of a single passage sewage flora (obtained from a raw sewage pond of Kibbutz Hulda) demonstrated both the "worst test condition" and the most consistent test results. Although some 10 new bacterial isolates were obtained, using *Cx. pipiens* larval screening that demonstrated a larvicidal activity comparable in magnitude to SSII-1, only 60A will be reported in detail (see Fig. 1). Following the isolation of 60A which demonstrated a 30–100 fold improvement in toxic activity over SSII-1 measured against *Cx. pipiens* larvae, screening was broadened in scope to include field-collected *An. sergentii, Ur. unguiculata* and *Cx. univitattus*. Larvae of laboratory-reared *Ae. aegypti* were also added.

A nominal 100-fold range in larvicidal activity is observed between *An. sergentii* and *Cx. pipiens*. In all cases, however, primary activity occurs in less than 12 hr. with very little difference between 1st to 2nd and late 3rd to 4th instar challenge (Fig. 2). In addition, the data suggest a "3-hit" model of dose response (Fig. 2). Following lyophilization, heat shock for 20 min. or ultra violet (2537 Å) exposure (sufficient to reduce spore viability to less than 0.1%), no significant loss in larvicidal activity was noted. (Fig. 3, 4, 5). Toxicity decreased approximately fourfold when the incubation temperature was raised from 30 C to

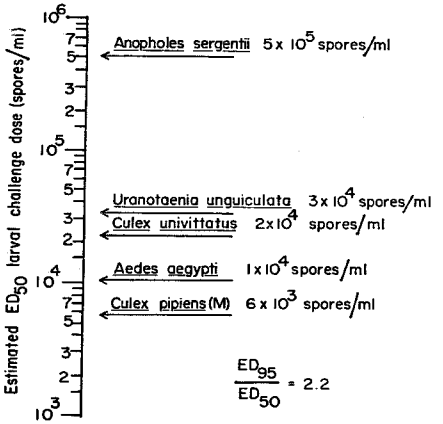

Fig. 1. Estimated ED_{50} (spores/ml) for 1st–2nd instar larvae challenged with a 48 hour, 30 C nutrient agar (Difco) test culture from clone 60A.

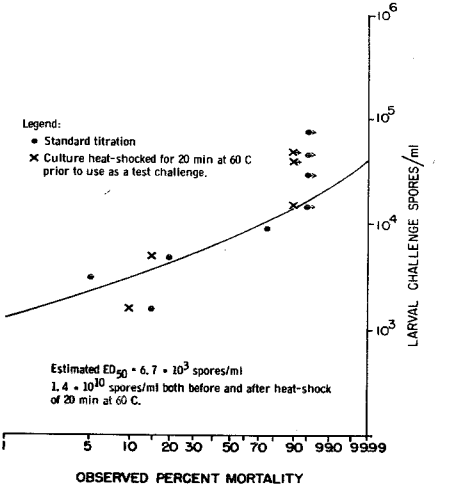

Fig. 2. Suggested 3 hit dose response of 1st—2nd instar larvae and of late 3rd—4th instar larvae of *Culex pipiens* (complex) to challenge with a 30 C nutrient agar (Difco) test culture of isolate 60A.

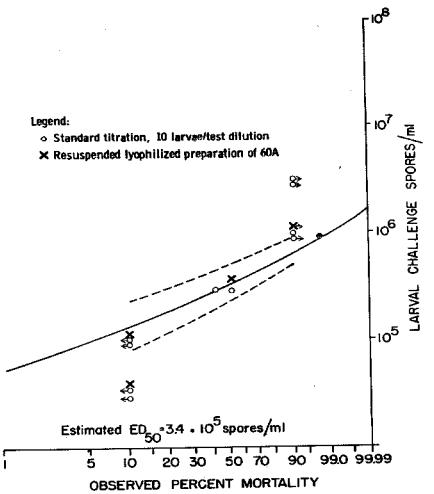

Fig. 3. Comparative dose response of field-collected 1st—2nd instar larvae of *Anopheles sergentii* to challenge with a 48 hr, 30 C nutrient agar (Difco) test culture of isolate 60A and to challenge with a rehydrated lyophilized test culture.

37 C. A careful check using parallel tests with fresh nutrient agar plates vs. 1-week (or older) plates demonstrated that an optimal toxic and cell yield occurred on older (or drier) agar surfaces (Fig. 4, 5). Although 60A toxicity was equally as good using N2X or nutrient agar (Difco) when incubated for 24–48 hr. at 30° C, nutrient agar was preferred since incubation for 48 hr. at 30° C provided a more stable cell suspension in alfalfa infusion.

Since *Anopheles* mosquito larvae are predominantly surface feeders, it would be highly advantagous to have a formulation which would result in the cells of 60A concentrating just under the liquid surface but remaining as a distinct colloidal suspension. Such a microencapsulated formulation has been achieved without loss in toxicity and will be reported in a separate publication.

Lyophilized cells provide approximately $2–10^{11}$ spores per gram. Using such a preparation, projected experimen-

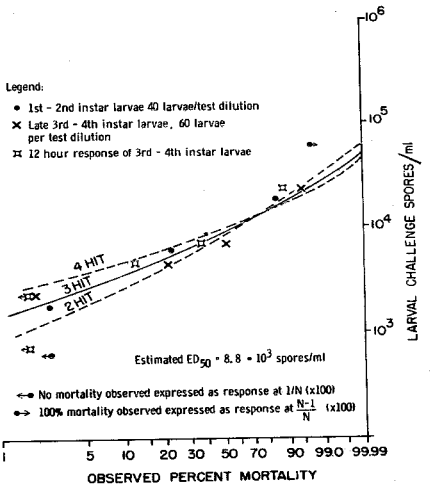

Fig. 4. Comparative dose response of 1st instar *Culex pipiens* (complex) larvae to challenge with a 52 hr, 30 C nutrient agar (Difco) test culture of isolate 60A; before and after heat-shock for 20 min. at 60 C.

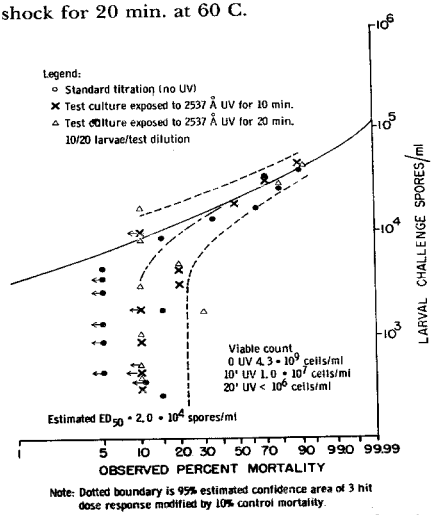

Fig. 5. Comparative dose response of 1st instar *Culex pipiens* (complex) larvae to challenge with a 48 hr, 30 C nutrient agar (Difco) test culture of isolate 60A, exposed to 0, 10 min and 20 min (2537 Å) UV.

tal application rates should provide for 0.1 to 1.0 μg/ml against other than *Anopheles* larvae. Levels of 5.0 μg/ml are suggested for *Anopheles* larvel control, but with a microencapsulated formulation to achieve a toxic concentration just under the liquid surface, this figure in field application may be reduced by a factor of 10 or even more, due to the potential benefit derived from immediate subsurface concentration over that required for general dilution.

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THE CONCEPT OF INTEGRATED CONTROL OF VECTOR-BORNE DISEASE

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Historically, vector-borne disease control programmes have concentrated upon the practical application of measures offered by a single discipline. Where other disciplines have been involved this has often been upon an incidental basis. For example, since the development of residual insecticides effective against anopheline mosquitoes, their application has been emphasized in the attack phase of malaria control programmes. Other measures, such as the use of anti-malarial drugs were recommended as supplementary procedures and often were used only in difficult circumstances after problems had arisen or in later phases of the control programme. This recommended procedure has been effective in many parts of the world, has succeeded in eradicating diseases such as malaria in several countries and has achieved a significant degree of control in many others. However, vector-borne infectious diseases remain a major hindrance to economic progress in many parts of the tropical and sub-tropical areas of the world. Apart from the morbidity and mortality directly attributable to these diseases, especially malaria, there is a chronic impairment of the health of the population giving rise to increased mortality from other causes and to a reduction of efficiency in physical and mental activity. A growing awareness of the importance of these diseases, and the problems associated with their control, has prompted a re-appraisal of the measures currently available to us and of the range of diseases against which these measures might be effective.

An appreciation of the problems involved suggests that the integration of the various measures available to us may have considerable benefits. Improvement in effective liaison between the various disciplines involved has made this possible. Thus, the objective of integrated control