Israel Journal of Entomology

Vol. XXIII(1989) pp.9-15

THE PRESENT STATUS OF *BACILLUS SPHAERICUS*

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ABSTRACT

Certain strains of the sporeforming bacterium, *Bacillus sphaericus,* are highly insecticidal to mosquito larvae. These strains have been found in many soil and water habitats, including both deserts and tropical areas. The insecticidal strains differ from non-insecticidal strains in flagellar antigen serology and susceptibility to lytic phages, and these techniques can be used to group the insecticidal strains into phage- or serotypes which contain strains of approximately the same level ofinsecticidal activity. *Bacillus sphaericus* is highly insecticidal to *Culex* and much less insecticidal to species related to *Aedes aegypti* and is not insecticidal to black fly larvae.

Bacillus sphaericus has been successfully used in small field trials against susceptible species and under conditions of high spore dose and heavy pollution has provided control for several weeks. The bacterium recycles in dead larvae, producing an increase in number of bacterial spores. However, it has not been proven that recycling is an important factor in persistence of this organism at an effective level in the field.

A protein toxin of *ca.* M, 43,000 has been identified as the cause of larval death. This toxin is produced in large quantity at sporulation, and is concentrated in a parasporal inclusion or crystal which accompanies the spore. The crystal is dissolved in the larval gut releasing a toxin of M_r 43,000 which is converted to a cytotoxic Mr 40,000 form by larval digestive enzymes. The toxin also acts on cultured cells of sensitive *C. quinquefasciatus,* but not on cells of nonsensitive species of mosquitoes or other insects. Recently it has been shown that binding of the toxin to these cultured cells preceeds toxic activity, and that specificity of the toxin for glycoprotein receptors on the cells is probably responsible for the restricted host range of the bacterium. The toxin binds to specific regions of the midgut of susceptible larvae, but does not bind to the gut of nonsusceptible larvae.

The gene for the toxin of *Bacillus sphaericus* has been cloned recently by at least four different groups, and the genetic and amino acid sequences of the toxin have been reported. The *B. sphaericus* toxin gene has been inserted into an alga.

This organism exhibits considerable potential a microbial control agent for vector mosquitoes. Recent advances in genetics and understanding of the mode of action of this bacterium should lead to genetic manipulation of the toxin to increase its host range and persistence in the field.

Certain strains of the sporeforming bacterium, *Bacillus sphaericus*, are highly insecticidal to mosquito larvae. These strains have been found in many habitats, including deserts, temperate, and tropical areas (Table 1). Insecticidal *B. sphaericus* has been isolated from dead mosquitoes, black flies, and other insects, and also from soil and water (Brownbridge and Margalit, 1987; Davidson, 1984; Lysenko et al., 1985; Mohsen et al., 1986; Weiser and Prasertphon, 1984). Isolation of *B. sphaericus* is facilitated by three factors; the survival of the spores after pasteurization, the resistance of this organism to the antibiotic streptomycin, and the ability of the bacterium to grow with acetate as the sole carbon source (Hertlein et al., 1979; Massie et al., 1985; Singer, 1980; White and Lotay, 1980; Yousten et al., 1985). A simple method for isolating *B. sphaericus* is given in Appendix 1. Insecticidal strains of this organism are widely distributed (Table 1), and deliberate search for useful strains in local mosquito habitats may prove fruitful.

TABLE 1
Ecological and geographic origins of insecticidal *Bacillus sphaericus* strains

Dead mosquitoes collected in the field: USA (Kellen K); Indonesia (1593): El Salvadore (1691, 1881): Romania (2013); Philippines (1404, 2115, 2117, 2314); India (SSII-1, 2377, 2534); Israel (1894); Egypt (Ghar. 1 × 10, 2 × 20, 3 × 30).

Dead mosquitoes from laboratory cultures: India (2173); Japan (SC 1713).

Dead insects other than mosquitoes: Nigeria, black fly adults (2362); Guyana, lepidoptera (2532-2, 2533-1); Hungary, grasshopper (2601); Czechoslovakia, lepidoptera (2602).

Soil or water from mosquito habitats: Israel (1883, 1885–1893); Sri Lanka (2297); Iraq (Mohsen, unnumbered).

Few of the strains of *B. sphaericus* in general microbiological culture collections (e.g. U.S. ATCC) are insecticidal. The insecticidal strains differ from non-insecticidal strains in flageller antigen serology and susceptibility to lytic phages, and these techniques can be used to group the insecticidal strains into phage- or serotypes which contain strains of approximately the same level of insecticidal activity (deBarjac et al., 1980, 1985; Yousten, 1984). Insecticidal strains may be obtained from the collections of entomogenous bacteria maintained at: the Institut Pasteur, Paris; the Czechoslovak Academy of Sciences, Cèske Budejovice; and the Bacillus Genetic Stock Center, Ohio State University, Columbus. Serotyping of new strains is available at the Institut Pasteur (Addresses in Appendix 2).

Bacillus sphaericus is highly insecticidal to mosquito larvae in the genera *Culex* and *Psorophora* and to some *Anopheles* and *Aedes* larvae. However it is much less insecticidal to mosquito species related to *Aedes aegypti* and is not insecticidal to black fly larvae or other insects (Davidson, 1984; Lacey and Undeen, 1986). The activity of this organism against a large number of mosquito species has been reported in the literature. These data are difficult to compare since a number of different bacterial preparations were used, and bioassay conditions differed from one laboratory to another. If larvae of the highly sensitive *Culex pipiens* (*C. quinquefasciatus*) complex were included in these assays, however, the relative sensitivity of other species may be estimated by comparing the LC_{50} for *C. pipiens* or *C. quinquefasciatus* to the LC_{50} for other species. Spores of strain 1593 or 2362 generally produce LC_{50} values to second- or third-instar *C. pipiens* or *C. quinquefasciatus* larvae of *ca.* 0.003 mg/ml primary, unformulated powder (Bourgouin et al.,1984) or 5×10^2 spores/ml (Davidson, 1983). Table 2 compiles selected published data on suseptibility of various mosquito species as compared to *C. pipiens* complex larvae. The restricted host range of *B. sphaericus* is a very important consideration in its field use, and assessment of the susceptibility of the target mosquito species is essential before field application can be undertaken.

Laboratory bioassay of *B. sphaericus* must take into consideration certain important aspects of the biology of the bacterium and of the mosquito larvae. Containers must be of appropriate size and volume to contain 10 or 20 larvae in 20 to 100 ml of bacterial suspension. Second- or third-instar larvae are generally used. *Anopheles* spp. larvae may require containers of less depth than other species. Most bioassays are held at room temperature (*ca.* 25–28°C). Brief sonication, blending, and/or vortexing of dry powder preparations may be necessary to eliminate clumping of bacteria. Bioassays must be held for 48 hr for full expression of mortality, and the assays should contain a small amount of food such as yeast. Control mortality should not generally exceed 10%, and mortality data should be corrected for control mortality using Abbott's formula (1925) or similar statistical method. LC_{50} may be estimated by log-probability plots or appropriate computer program. At least two and preferably three data points between 0 and 100% mortality are required for valid statistical analysis. An International Standard preparation, RB-80, is available from Institut Pasteur, Paris, for comparison of *B. sphaericus* preparations (Bourgouin et al., 1984).

Bacillus sphaericus is highly insecticidal to mosquito larvae in the genera *Culex, Aedes,*

TABLE 2
Selected laboratory bioassays of *Bacillus sphaericus*

Mosquito species	Relative sensitivity[1]	Bacterial strain	Notes	Reference
Culex				
cinereus	resistant to 2×10^5 spores/ml	2362	beneficial, major competitor	42
Anopheles				
albimanus	10	1593	diluted liquid culture	48
	12.5	2013-4	lyophylized spores	30
stephensi	4	1593	commercial dry powder	3
	50	1593	RB-80 standard	6
quadrimaculatus	35	2013-4	lyophylized spores	30
gambiae	4, 5	1593, 2362	spray dry powder	33
Aedes				46
aegypti	100	1593	liquid culture	48
	8	1593	commercial dry powder	3
triseriatus	63	2013-4	lyophylized spores	30
taeniorynchus	*ca.* $10^5 \times$	1593	by dilution	
stimulans	2	1593	commercial dry powder	54
intrudens	1.3	1593	commercial dry powder	54
vexans	3	1593	commercial dry powder	54
fitchii	8	1593	commercial dry powder	54
Psorophora				
columbiae	3	2013-4	lyophphylized spores	30

[1] LC_{50}/LC_{50} to *C. pipiens* or *C. quinquefasciatus*.

Anopheles, and *Psorophora* spp. mosquito larvae (Davidson et al., 1981; Hougard et al., 1985; Majori et al., 1987; Mulla et al., 1984a,b; Mulligan et al., 1978; Nicolas et al., 1987; Obeta and Okafor, 1983; Ramoska et al., 1978; Vankova, 1984; Wraight et al., 1982). In general, efficacy of the bacterium in the field is predicted by sensitivity of the target species in laboratory bioassay. Under conditions of high spore dose and heavy pollution this bacterium has provided control of *Culex* spp. larvae for several weeks (Hougard et al., 1985; Nicolas et al., 1987). The bacterium recycles in dead larvae, producing an increase in number of bacterial spores (Davidson et al., 1984; Nicolas et al., 1987). However, it has not been proven that recycling is an important factor in persistence of this organism at an effective level in the field.

Nontarget aquatic insects and other invertebrates are in general quite insensitive to *B. sphaericus* (Davidson et al., 1977; Mathavan and Velpandi, 1984; Mulla et al., 1984a,b; Mulligan et al., 1978). Deleterious sublethal effects leading to reduced growth and fecundity of an aquatic hemipteran have been reported (Mathavan et al., 1987).

A protein toxin has been identified as the cause of larval death (Baumann et al., 1985; Davidson, 1983; Narasu and Gopinathan, 1986; Sgarella and Szulmajster, 1987). This toxin is produced in large quantity at sporulation, and is concentrated in a parasporal inclusion or crystal which accompanies the spore (Baumann et al., 1985; Broadwell and Baumann, 1986;, Davidson, 1983; deBarjac and Charles, 1983; Payne and Davidson, 1984; Yousten and Davidson, 1985). The toxin protein can be extracted by alkali or disruption of the spore, and has been described as having molecular weight from 35 to 43 kDa. The crystal is dissolved in the larval gut releasing a 43 kDda toxin which is reduced to a cytotoxic 40 kDa form by larval digestive enzymes. Enzymes from the resistant *A. aegypti* larvae are equally efficient in activating the toxin as enzymes from sensitive *C. quinquefasciatus* (Aly et al., 1986; Broadwell and Baumann, 1986, 1987; Davidson et al., 1987b).

When susceptible larvae ingest a lethal dose of *B. sphaericus* spores and crystals, the first effects are seen on midgut cells. Swelling of the midgut can be seen in as little as 30 minutes, and behavioral changes in 2–3 hr. However several hours are required before histological changes in the gut are observed. Most prominent among these changes is development of large lysosomes in the gut cells of *C. quinquefasciatus* larvae. Further changes include disruption of mitochondrial and endoplasmic reticulum structure and finally necrosis of muscle and nerve tissue (Charles, 1987; Davidson, 1979, 1981; Karch and Coz, 1983; Singh and Gill, 1988).

The toxin acts on cultured cells of sensitive *C. quinquefasciatus*, but is far less active on cells of nonsensitive species of mosquitoes or other insects (Broadwell and Baumann, 1987; Davidson, 1986). The activity of the toxin on cultured cells is very rapid, leading to ultrastructural changes in less than 5 minutes (Davidson and Titus, 1987). Recently it has been shown that binding of the toxin to these cultured cells precedes toxic activity, and that specificity of the toxin for glycoprotein receptors on the cells is probably responsible for the restricted host range of the bacterium (Davidson et al., 1987b). The toxin binds to specific regions of the midgut of susceptible larvae, but does not bind to the gut of nonsusceptible *A. aegypti* larvae (Davidson, 1988).

Cloning of the gene for the *B. sphaericus* toxin was reported in 1987 by at least three different laboratories, and the genetic and amino acid sequences of the toxin have been reported (Baumann et al., 1987; Hindley and Berry, 1987; Tandeau de Marsac et al., 1987). The toxin gene contains some homologies with *Bacillus thuringiensis* delta-endotoxin genes (Hindley and Berry, 1987). Two proteins, the 43 Kda toxin and a 63 Kda nontoxic protein are found in extracts of *B. sphaericus* crystals, and a high molecular weight precursor, or protoxin, was described from these crystals (Broadwell and Baumann, 1986). However the cloned toxin was insecticidal only in the presence of the 63 Kda protein, suggesting a synergistic effect between these two proteins (Baumann et al., 1987). In one study, evidence for a precursor was found (Baumann et al., 1987), while in another study no evidence for such precursor was found (Hindley and Berry, 1987). Genetic and biochemical data reported in the next year or two should clarify the status of these proteins and their toxicity. The *B. sphaericus* toxin gene has been inserted into a cyanobacterium, which reproduces in the upper layers of the water, thereby hopefully retaining toxin activity in the mosquito larval feeding zone (Tandeau de Marsac et al., 1987).

This organism exhibits considerable potential as a microbial control agent for vector mosquitoes. Recent advances in genetics and understanding of the mode of action of this bacterium should lead to genetic manipulation of the toxin to increase its host range and persistence in the field.

APPENDIX 1

SUGGESTED TECHNIQUE FOR ISOLATION OF INSECTICIDAL *BACILLUS SPHAERICUS*

1. Samples are collected using sterile or disposable tools (e.g. plastic spoons), and placed into sterile vials or new plastic bags. One-gram samples are weighed out using sterile tools on new weighing papers.
2. Samples are dispersed by shaking in 1 ml sterile distilled water or saline, and pasteurized (80°C for 1 min).
3. From each pasteurized sample, several flasks of NYSM medium are inoculated using 0.1 ml of the pasteurized sample per flask.
4. Flasks are incubated at 28°C and 150 rpm for 48 hr. At the end of this time the growth in the flasks is inspected for the presence of typical *B. sphaericus* spores (spherical, terminal or subterminal, swelling the sporangium).
5. One-ml samples from each flask are pasteurized as above.
6. These samples are plated on selective agar medium. Plates are incubated at 28–30°C for 24–48 hr. Colonies with characteristic *B. sphaericus* morphology (circular, tan, cratered center) are selected and numbered individually. Bacteria from each colony are observed microscopically

using 100 × objective. Colonies exhibiting typical *B. sphaericus* spore morphology and the presence or absence of parasporal inclusions are noted.

7. Colonies which appear to be *B. sphaericus* are inoculated into fresh NYSM flasks, which are incubated as before.
8. Cultures are bioassayed against *Culex quinquefasciatus* second instar larvae at 1/1000 (10^{-3}). Any culture demonstrating insecticidal activity is streaked for purity, recultured, and assayed along with a viable cell count.

These procedures enrich for *B. sphaericus* at several points, and are designed to eliminate cultures of relatively weak insecticidal activity or which sporulate poorly.

Media:
NYSM broth (Myers, P. and Yousten, A.A. 1978. *Inf. Immun.* 19: 1047–1053)
Nutrient broth as directed by manufacturer
0.05% yeast extract
5×10^{-5}M $MnCl_2$; 7×10^{-4}M $CaCl_2$; 1×10^{-3}M $MgCl_2$

NYSM selective medium
NYSM as above
100 mg/L streptomycin sulfate
2% agar

BATS selective medium (Yousten, A.A., Fretz, S.B. and Jelley, S.A. 1985. *Appl. Environ. Microbiol.* 49: 1532–1533)
per liter:
Na_2HPO_4, 5.57 g; KH_2PO_4, 2.4 g; $MgSO_4 \cdot 7H_2O$, 50 mg; $MnCl_2 \cdot 4H_2O$, 4 mg; $FeSO_4 \cdot 7H_2O$, 2.8 mg; $CaCl_2 \cdot 2H_2O$, 1.5 mg; L-arginine, 5 g; thiamine, 20 mg; biotin, 2 mg; streptomycin sulfate, 100 mg; agar, 20 g.

APPENDIX 2
CULTURE COLLECTIONS OF INSECTICIDAL BACILLI

1. Laboratorie de Lutte Biologique II, Professor H. deBarjac, Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France (serotyping service available).
2. Czechoslovak Academy of Sciences, Institute of Entomology, Department of Insect Pathology, 370 05 Ceske Budejovice, Branisovska 31, Czechoslovakia.
3. Bacillus Genetic Stock Center, Dr. Donald Dean, Department of Biochemistry, Ohio State University, 484 W. 12th Ave., Columbus, Ohio 43210, USA.

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