ARTICLES

MICROBIOLOGY, PATHOLOGY AND GENETICS OF *BACILLUS SPHAERICUS:* BIOLOGICAL ASPECTS WHICH ARE IMPORTANT TO FIELD USE[1]

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ABSTRACT. Some strains of *Bacillus sphaericus* have a high level of insecticidal activity toward larvae of many mosquito species. Spores of these strains kill their host by means of a toxin, and recycling can occur in the dead host with net increase in numbers of spores. This toxin affects the larval midgut within 30–60 min, but 48 hr are required for all mortality to be expressed. Although the activity of *B. sphaericus* spores is apparently degraded by sunlight, this effect is not due to the sterilizing ultraviolet component. Antibiotic resistance of *B. sphaericus* has been exploited in the production of selective media useful in recovery of this organism from the aquatic environment. *Bacillus sphaericus* is highly insecticidal to some species of *Culex* and *Anopheles,* but is much less insecticidal to certain *Aedes* species; it is not toxic to black flies. Recent genetic studies of this organism may result in improvement of its host range.

INTRODUCTION

The genus *Bacillus* includes a large number of microbial species which share the common property of production of resistant endospores while growing in the presence of oxygen. Among these bacilli are a few microorganisms with the unique ability to become primary pathogens of insects; these include *Bacillus larvae* White, *B. lentimorbus* Dutky, *B. popilliae* Dutky, *B. cereus* Frankland and Frankland, *B. thuringiensis* Berliner, *B. alvei* Cheshire and Cheyne, *B. moritai* Aizawa and Fujiyoshi and *B. sphaericus* Neide. These organisms may kill their hosts in two different ways; by invasion and multiplication, or by intoxication. Those species which invade host tissues and overgrow the host may be considered "true pathogens," and include *B. larvae, B. popilliae* and *B. lentimorbus.* Those which kill the host by means of toxins produced outside the host, sometimes considered "food poisoning organisms" (Lysenko 1981), include the remainder of the insect-pathogenic bacilli. This latter group includes all the presently-known pathogens of mosquito larvae. Although the toxin-producing bacilli kill the host rather quickly before tissue invasion, some will multiply and invade the host body after death of the host.

MICROBIOLOGY OF *BACILLUS SPHAERICUS*

Bacillus sphaericus is widely distributed in fresh water and soils. Most strains, however, are not pathogenic for insects. All those strains showing pathogenicity for mosquito larvae have been isolated from dead insects (Krych et al. 1980, Yousten et al. 1980, Balaraman 1980, Wickremesinghe and Mendis 1980, Singer 1981, Weiser 1984). There are at present over 30 insecticidal *B. sphaericus* strains originating from 11 different countries (Yousten 1984, Table 1). The insecticidal strains differ among themselves in the level of activity which they produce. Two microbiological techniques, H- or flagellar antigen immunological serotyping, and sensitivity of the bacterium to certain bacterial viruses called phages, have permitted these strains to be classified into at least four major groups. In addition to providing a convenient method of classifying strains, these groupings correspond to observed differences in insecticidal activity. In other words, the sero- or phage type of a newly-discovered strain gives a rather accurate prediction of its insecticidal potential. Two of these groups, serotype H5a5b (phage group 3; strains 1593, 2362, 2013-6, and others), and serotype H25 (phage group 4; strains 2297, 2173, and others) contain the strains currently known to be highly insecticidal (deBarjac et al. 1980, Yousten et al. 1980, Yousten and Hedrick 1982).

The most insecticidal *B. sphaericus* strains develop full toxicity only during sporulation (Myers et al. 1979, Yousten and Davidson 1982); therefore fermentation must be tailored to achieve as complete sporulation as possible. *Bacillus sphaericus* is a highly aerobic organism, requiring considerable oxygen for complete sporulation. In addition to oxygen, a successful fermentation must provide mineral supplementation, pH control, and proper amounts of nitrogen and carbon in the form of amino

[1] The following four papers resulted from a symposium on *Bacillus sphaericus* held at the Annual Meeting of the Society of Vector Ecologists, San Diego, CA, December 14–16, 1983.

Table 1. Origin, bacteriophage type and serotype of mosquito-pathogenic *Bacillus sphaericus* strains.

Strain	Origin	Bacteriophage type	Serotype
Kellen K	Calif. USA	1	H1
Kellen Q	Calif. USA	1	H1
SSII-1	India	2	H2
1404	Philippines	2	H2
1883	Israel	2	H2
1885–1893	Israel	2	H2
1895, 1896	Israel	2	H2
1593	Indonesia	3	H5a5b
1691	El Salvador	3	H5a5b
1881	El Salvador	3	H5a5b
2013-6	Romania	3	*H5a5b
2117-2	Philippines	3	*H5a5b
2362 (=290-8)	Nigeria	3	H5a5b
2297 (=MR4)	Sri Lanka	4	H25
2173 (=ISPC5)	India	4	*H25
2314-2	Thailand	4	H25
2317-3	Thailand	4	H25
2377 (=ISPC6)	India	4	*H25
1894	Israel	5	not done
2115	Philippines	6	not done
2315	Thailand	7	not done

Data from Yousten (1984), deBarjac et al. (1980) and Yousten and Hedrick (1982).
* Inferred from phage type.

acids or proteins as this organism does not utilize carbohydrates (Yousten et al. 1984). Recently, *B. sphaericus* has been successfully fermented at pilot plant scale on soy-based and peptonized milk-based media (H.T. Dulmage and J. Correa, unpublished data; Davidson et al. 1984, B.C. Hertlein, unpublished data) (see Lacey 1984). Failure of a *B. sphaericus* culture to sporulate fully will result in greatly reduced insecticidal activity (Myers et al. 1979, Yousten and Davidson 1982, Kalfon et al. 1983). In cases where highly insecticidal *B. sphaericus* strains are reported to have low activity against susceptible larvae, failure of sporulation of the culture should be suspected (e.g., Bourgouin and deBarjac 1980, Wickremesinghe and Mendis 1981).

Spores of *B. sphaericus,* like those of most *bacilli,* have a remarkable ability to survive long periods of desiccation, cold or temperatures up to 80°C. Sterilizing ultraviolet light rapidly renders *B. sphaericus* 1593 spores unable to grow and multiply on bacteriological medium; however the insecticidal ability of these spores is not affected by exposure to many times the amount of sterilizing ultraviolet normally reaching the earth's surface (Burke et al. 1983). *Bacillus sphaericus* spores in shallow aqueous suspensions are both slowly detoxified and rendered nonviable by exposure to sunlight (Bourgouin and deBarjac 1980, Mulligan et al. 1980). These effects are apparently due to a component of sunlight different from the sterilizing ultraviolet, and are not due to heating of the spores (E. W. Davidson and P. Savastano, unpublished data). Because of the detrimental effects of spore detoxification and sterilization upon field activity and persistence, it is important to determine the precise wavelengths of light responsible. Protectant chemicals may then be added to formulations of these spores to block out these critical wavelengths (Lacey 1984).

Bacillus sphaericus is naturally resistant to high levels of several antibiotics, including streptomycin and chloramphenicol (Burke and McDonald 1983). Antibiotic resistance can be exploited in the design of media which can be used to selectively retrieve *B. sphaericus* from the environment, either during a search for new strains or while monitoring the location of spores following field application (Yousten et al. 1982, Davidson et al. 1984).

Because *Bacillus* spp. spores are more dense than water, they tend to settle rapidly from the larval feeding zone at the water surface. Settling from the upper water and accumulation of spores in mud was observed during two recent field trials (Davidson et al. 1984, Mulla et al. 1984). In both trials, a relationship was noted between control of larvae and the presence of at least 100 spores/ml in the upper water layer; this was also observed by Hornby et al. (1981) and Des Rochers and Garcia (1984).

PATHOLOGY

Bacillus sphaericus spores are ingested by larvae during normal filter-feeding activity. Other suspended particles can interfere with the ingestion of a lethal dose of *B. sphaericus* spores; therefore sites with heavy silt or pollution may require treatment at higher dosages than clean water sites (Ramoska and Pacey 1979, Mulla et al. 1984).

Bacillus sphaericus is among those organisms which kill their hosts by means of toxins. The toxin of *B. sphaericus* accumulates rapidly during sporulation (Myers et al. 1979, Yousten and Davidson 1982, Kalfon et al. 1983), and has recently been shown to be concentrated in the crystal-like parasporal inclusions formed alongside the spore (Payne and Davidson 1984). These inclusions are produced by all the most insecticidal strains, and are rapidly dissolved in the larval gut or by chemical extraction methods (Davidson and Myers 1981, Yousten and Davidson 1982, Davidson 1983, deBarjac and Charles 1983).

When a susceptible mosquito larva ingests a high dose of *B. sphaericus* spores, symptoms of intoxication are detectable both microscopically and grossly within 30–60 min. The midgut swells to lie against the body wall, throwing the peritrophic membrane with its food contents into zigzag folds. Large cytolysosomes appear in midgut cells, and these cells separate from one another at the bases. Death may occur as early as 4 hr after feeding, but at lower dosages up to 48 hr are required for full expression of mortality (Davidson 1979, 1981). *Bacillus sphaericus* spores germinate in the midgut of susceptible larvae, multiply vegetatively and produce fresh spores in the larval cadaver. On the average, 10^5–10^6 spores are formed in each *Culex quinquefasciatus* Say larval cadaver (Davidson et al. 1975, Ramoska and Hopkins 1981, Davidson 1981, Silapanuntakul et al. 1983, Davidson et al. 1984). The ability of *B. sphaericus* to recycle in the larval cadaver has two important implications in the use of this equipment; first, it provides a potential source of spores which may infect reinfesting larvae after field treatment (Des Rochers and Garcia 1984), and second, recycling in bioassay containers may produce erratic results in these assays. For this reason, *B. sphaericus* bioassays should be completed by 2–3 days after initiation of assay.

Perhaps the greatest obstacle to the widespread use of *B. sphaericus* in mosquito control programs is its restricted host range. Most species of *Culex* so far tested are very susceptible to this organism, while most species of *Aedes* are quite insensitive to it. The response of *Anopheles* spp. seems variable; to date only a few *Anopheles* species have been used in assays of this organism. For example, *An. albimanus* Wiedemann and *An. stephensi* Liston are quite sensitive, while *An. quadrimaculatus* Say is less sensitive (Balaraman 1980, Bourgouin and deBarjac 1980, Ramoska and Hopkins 1981, Dagnago and Coz 1982, Lacey and Singer 1982). Black fly larvae are not killed by *B. sphaericus*. The reasons for the variation in host response are not known. What is clear, however, is the importance of assessing the sensitivity of each mosquito species in the laboratory before attempting field evaluation of this agent.

It has been shown that mosquito larvae exhibit decreased susceptibility to *B. sphaericus* with increasing age. First instar larvae of *Culex pipiens* L. are 2–5 fold more susceptible than fourth instar larvae (Wraight et al. 1981). Whether this reflects a simple increase in body size, or is related to changing physiology or behavior is not known. If field populations contain late-instar larvae, application of an increased level of bacterial preparation is indicated.

One benefit of the limited host range of *B. sphaericus* is its safety to nontarget organisms. It has been shown to have little effect on honey bees, nontarget invertebrates found in conjunction with mosquito larvae during field trials, lizards and mammals (Davidson et al. 1977, Cantwell and Lehnert 1979, Mulligan et al. 1980, Shadduck et al. 1980, Hudson 1981; Mulla et al. 1984).

Aside from the possibility of recycling in bioassays, several other problems may arise in laboratory bioassay of this (and some other) microbial control organisms. Although originating from the same colony, batches of mosquito larvae may vary considerably in sensitivity to a microorganism over time (Sweeney 1981). Nonviable *B. sphaericus* spores can contribute to insecticidal activity, and spores can clump, producing misleading counts when plated on bacteriological media. These factors make relation of activity to spore count less than perfect. Finally, variation in methods of calculating LC_{50} can lead to apparently different results among investigators. The best method of producing reliable assays whose data can be compared to the data of others is comparison to an International Standard which is assayed simultaneously with the preparation in question, using the same larvae, methodology and data computation (Burges and Thomson 1971, Dulmage 1973, Dulmage et al. 1976). Such a standard for *B. sphaericus* strain 1593, designated RB-80, has been developed at Institut Pasteur in Paris. This preparation has been assigned an arbitrary potency of 1000 International Units (H. deBarjac, personal communication).

GENETICS

As mentioned above, *B. sphaericus* as a species is composed of a number of strains with no insecticidal activity, and a relatively small number which are toxic to mosquitoes. Some genetic studies have been carried out on the nontoxic strains of *B. sphaericus,* but until recently virtually nothing was known of the genetics of the insecticidal strains. Krych et al. (1980) were the first to investigate the genetic relationships of the insecticidal to the noninsecticidal strains, using a technique which estimates the genetic similarity among DNA sequences. Their data indicated that the insecticidal strains of *B. sphaericus,* although closely related to a few of the noninsecticidal strains, are only distantly related to most of the noninsecticidal strains. Unfortunately, no biochemical test has yet been found which will distinguish the insecticidal strains from other *B. sphaericus* strains (Krych et al. 1980, deBarjac et al. 1980). The serotyping and phage-typing techniques

mentioned above, however, do distinguish nontoxic from toxic strains (deBarjac et al. 1980, Yousten et al. 1980). These techniques detect differences among insecticidal strains, and differences are also seen in onset of toxic activity, level of toxic activity produced, and presence or absence of parasporal crystals (Myers et al. 1979, Davidson and Myers 1981, Yousten 1984), indicating that there must be considerable genetic heterogeneity among the insecticidal strains. The wide variety of *B. sphaericus* strains available provides a rich reservoir of material for future genetic development of this organism.

A recent breakthrough in the study of genetics of insect pathogens has been the discovery that the crystal or delta-endotoxin of most strains of *B. thuringiensis* is produced by genetic information borne on circles of DNA located outside the bacterial chromosome (Schnepf and Whiteley 1981, and others). These circles, called plasmids, have long been known to carry genes for such properties as antibiotic resistance in bacteria. Location of toxin genes on plasmids is significant for two reasons; first this genetic information may be passed naturally from bacterium to bacterium during culture or infection of the host, and, second, these plasmids are amenable to manipulation and deliberate transfer from one strain or species to another, i.e. "genetic engineering."

Bacillus sphaericus strains bear a variety of plasmids (Davidson et al. 1982, Abe et al. 1983, Yoshimura et al. 1983). Many of these are relatively small, and there seems to be no relationship between any of the small plasmids and the activity of these strains toward insects. "Curing" or removal of some of these small plasmids from two insecticidal strains did not lead to loss of activity or crystal production (Kalfon et al. 1983). However the delta-endotoxin of *B. thuringiensis* is generally associated with large plasmids, and these large plasmids are difficult to detect and are often present in low numbers per cell (Gonzalez et al. 1981, and others). Insecticidal *B. sphaericus* strains have also been reported to contain some high molecular weight plasmids (Davidson et al. 1982); these large plasmids were not seen by Abe et al. (1983) or by Kalfon et al. (1983). It is not presently known whether large plasmids are associated with insecticidal activity in *B. sphaericus*.

The gene for toxin production in *B. sphaericus* strain 1593 has been reportedly removed from the organism, isolated, and cloned in the unrelated bacterium *Escherichia coli*. This bacterium was then found to have acquired mosquito larvicidal activity (Ganesan et al. 1983). Recently, genes for antibiotic resistance have been introduced into *B. sphaericus* 1593 using a so-called "vector" plasmid which can be used to transport genetic material from one species to another (McDonald and Burke 1984).

The ultimate goal of investigations of the genetics of this organism is improvement of its activity toward mosquitoes, its ability to be produced commercially, and in particular its host range. If the toxin could be changed to make larvae of *Aedes* spp. more susceptible, for example, the usefulness of *B. sphaericus* would be greatly enhanced. It has been suggested that a "blended" organism might be created, with the wide host range and efficacy of *B. thuringiensis* var. *israelensis* and the potential for persistence and recycling of *B. sphaericus*. Transformation of *B. sphaericus* by genetic material from other bacterial species, as reported by McDonald and Burke (1984), is the first step in this process. Current genetic research on these organisms is opening the way for development of improved microbial control agents.

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