Stored Bacillus popilliae Spores and Their Infectivity against Popillia japonica Larvae

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Bacillus popilliae spores were stored for about 7 years under three separate conditions: frozen in sterile distilled water, smeared on glass microscope slides, and stored in loam soil at room temperature. In separate experiments, each of the 7-year-old preparations was fed to Popillia japonica larvae at concentrations of 10⁶, 10⁷, 10⁸, and 10⁹ spores/g of soil. A significant decrease in the percentage of larvae infected occurred in all of the aged spore tests. B. popilliae spores stored in soil, for the extended period, produced 3% larval infection only at the 10⁷ spores/concentration; similar results were obtained from frozen spores. When P. japonica larvae were fed spores stored dried on slides, about 20% of the larvae developed milky disease. When aged frozen spores were artificially injected into larvae, 12% became infected at concentrations of 1 x 10⁶ spores/larvae; dried spores at the same concentration infected about 38% of the insect larvae. We conclude from these data that aged B. popilliae spores are significantly less infective against P. japonica larvae than young spores.

Key Words: Bacillus popilliae; Popillia japonica; milky disease; bacterial spores; spores; longevity of; Coleoptera; Scarabaeidae.

INTRODUCTION

Bacillus popilliae belongs to the family Bacillaceae that comprises spore-forming rod-shaped bacteria. The microorganism is well known for its pathogenicity to Popillia japonica (the Japanese beetle) larvae. A brief history, including the discovery of the host parasite, by E. L. Dickerson and H. B. Weiss, observations on the larvae susceptibility to bacterial infection, isolation, description, and naming the bacterium (B. popilliae) by S. R. Dutky, and the notable field studies of the milky disease by R. L. Beard and others can be found in a review chapter by St. Julian and Bulla (1973). A bibliography of the bacteria associated with Popillia japonica and closely related

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Scarabaeidae refers to most pertinent publications from 1937 to 1975 (Klein et al., 1976). P. japonica is in the order Coleoptera, family Scarabaeidae (scarab beetles). Previous studies indicate that the number of bacterial spores needed to cause a disease in the beetle larvae varies with the condition of the experiment and the strain of the microorganism (Beard, 1944, 1945; Dutky, 1941; St. Julian and Hall, 1968; St. Julian et al., 1970, 1972; Tashiro and White, 1957). Research on B. popilliae spores at this Center has dealt with mostly one strain. NRRL B-2309. Bacillus popilliae strain NRRL B-2309 is the parent strain originally isolated by S. R. Dutky, and the predominant strain used in commercial preparations.

B. popilliae enters a P. japonica larva via its digestive tract, then invades the hemolymph and there proliferates vegetatively and eventually sporulates. Recent publications of outstanding merit detail the invasion process of B. popilliae in the European chafer. Amphimallon majalis (Kawanishi et al., 1978; Splittstoesser et al., 1978). The
INFECTIVITY OF *B. popilliae*

bacterium is an excellent insecticide; however, it has unique characteristics, specifically regarding growth and viability of the vegetative cells, and germination and outgrowth of the spore (Steinkraus, 1957; Splittstoesser and Steinkraus, 1962; Splittstoesser and Farkas, 1966; Steinkraus and Tashiro, 1967; St. Julian et al., 1967, 1970; St. Julian and Bulla, 1973; Splittstoesser et al., 1975; Sharpe and Bulla, 1976).

Spores of *B. popilliae* are used currently to control various scarab beetle populations; therefore, knowledge of their longevity is critical to the effective and efficient use of the spores as an insecticide. Previous studies on the viability of *B. popilliae* spores indicate that the spores remain viable for several years in air-dried soil or stored in dried blood film (Beard, 1945; White and Dutky, 1940); our data indicate spores lose viability after prolonged storage. This report includes the determination of viability, dose requirement, and infectivity of aged *B. popilliae* spores in *P. japonica* larvae. The tested spores were stored for about 7 years either in frozen sterile distilled water, smeared on glass microscope slides, or in loam soil. Spores from most stored conditions were both fed and artificially injected into the larvae.

**MATERIALS AND METHODS**

*Larvae.* Third-instar larvae of *P. japonica* were collected by the Animal Plant Health Inspection Service, U.S. Department of Agriculture, from presumably disease-free areas in Midwestern States. The larvae care and handling have been described (St. Julian et al., 1963). Data presented here summarize four separate experiments involving about 5000 larvae. The larvae for the original feeding experiments were collected in the fall of 1968. Feeding tests on stored spores were performed with larvae collected in the spring and fall of 1975.

*Preparation of spores.* *B. popilliae* were collected from artificially infected larvae (St. Julian and Hall, 1968). Washed larvae (St. Julian et al., 1970) were punctured with a sterile dissecting needle, and their hemolymph was dripped into sterile distilled water contained in a glass thick-walled 100-ml volume centrifuged bottle partially submerged in an ice-water bath. Nearly 10,000 diseased larvae were bled in this manner. Collected spores then were centrifuged from the hemolymph and subsequently washed in water at least 10 times. After the final wash, the spores were suspended in sterile distilled water at a concentration of about $40 \times 10^9$ ml. The spore suspension then was divided into three portions, one of which was stored at about $-10^\circ C$. The second portion of suspended spores was pipetted and smeared onto several hundred glass microscope slides (0.5 ml/slide) and air-dried for several hours. The microscope slides containing dried spores then were placed in (100-slide capacity) microscope boxes and stored at room temperature. Direct microscope counts were made of the remaining spore portion with a Petroff-Hausser$^3$ counting chamber. Samples containing $2 \times 10^{12}$, $2 \times 10^{10}$, $2 \times 10^8$, and $2 \times 10^6$ spores were prepared to provide four 2000-g portions of finely sifted dry loam soil with a final concentration each of $1 \times 10^9$, $1 \times 10^7$, $1 \times 10^5$, or $1 \times 10^3$ spores/g of soil. These spore–soil mixtures were to be used to test the infectivity of the freshly harvested spores for later comparison with their aged relatives. An elaborate mixing procedure was followed to ensure even distribution of spores in the soil (St. Julian et al., 1970).

*Infectivity test of fresh spores (1968).* Each spore-laden soil mixture was seeded with domestic rye grass, Dutch sweet clover, Kentucky bluegrass, and red top seeds and placed into compartmented metal trays. Finally, each tray was watered with distilled water and one larva added per compartment. Fifty-four larvae were exposed to each concentration of spores for 4 to 21 days at

$^3$ The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.
28–30°C. Larvae were examined daily for symptoms of milky disease. The spore-laden test soils were reseeded and watered when needed. Milky or dead larvae were removed when found. Several test trays of 54 larvae in soil devoid of *B. popilliae* spores were included in each experiment as controls.

**Infectivity test of stored spores.** In the spring of 1975, *B. popilliae* spores (previously washed and stored in sterile distilled water) were removed from freezer temperature and thawed at room temperature for several hours. The thawed spores were centrifuged for 10 min at 1800g and resuspended into about 20 ml of sterile distilled water, after which direct microscopical counts were made of the bacterium. Proper dilutions were made, and the spores were added to soil and mixed as mentioned above to give final concentrations of $1 \times 10^9$, $1 \times 10^8$, $1 \times 10^5$, or $1 \times 10^3$ spores/g of soil. The respective soil–spore mixtures were seeded and placed into glass culture tubes, and each tube was covered with a cork stopper.

*B. popilliae* spores stored for about 7 years on glass microscope slides were removed from the slides in the following manner: About 50 slides each were placed into several 400-ml glass beakers filled with sterile water, and the beakers were covered securely with aluminum foil and put in a refrigerator overnight. The next day, each of the slides was scraped with a microspatula to remove the remaining spores; the resulting spore suspension was centrifuged and resuspended into water at a heavy concentration. The spores were counted, and appropriate numbers were added to dry soil in the same manner and concentrations previously described.

For feeding tests, no more than three healthy larvae were added to each test tube; 200 larvae were fed each spore concentration, a total of 800 larvae per each spore storage condition. The test larvae were examined every 3 days for disease symptoms. All dead and diseased larvae were removed when discovered.  

**Artificial infection.** In these experiments, we wanted to compare a new collection of fresh spores with original young spores of 1968. Due to time required and an insufficient supply of insects, it was impossible to obtain the large quantity of fresh spores needed for an additional feeding test. Therefore, for an additional fresh spore test, we resorted to artificially infecting the larvae by injecting spores directly into the hemolymph via a hypodermic needle. A Dutky–Fest microinjector was used for the injections (Dutky and Fest, 1942). Fresh 1975 spores of NRRL B-2309 were obtained from artificially infected larvae, washed immediately after harvesting, stored at refrigerated temperature, and injected into test larvae within 1 week after harvest. Previously, aged and frozen, and dried spores also were injected into healthy larvae. The spores (from each condition) were injected into 50 larvae, each at numbers of $1 \times 10^4$, $1 \times 10^5$, and $1 \times 10^6$ per larva. After injection, the larvae were incubated in soil at 28–30°C and examined as before. Control larvae were injected with sterile water.

**Determination of spore viability.** The procedures for determining outgrowth of *B. popilliae* spores were those previously reported (St. Julian and Hall, 1968). All spores tested for viability were heat-shocked at 50°C for about 15 min prior to plating them onto the yeast extract–phosphate–agar medium. Plated spores were allowed to incubate for 5 to 14 days at 28–30°C.

**RESULTS**

**Aged *B. popilliae* Infectivity**

The pathogenicity of young and aged *B. popilliae* spores against *P. japonica* larvae is shown in Table 1. When young, fresh spores are fed to the larvae at high concentrations, about 50% of the larvae become diseased and exhibit the classical milky disease symptoms. When the spores are stored for 7 years and then fed to the larvae, a significant decrease in the number of diseased insects occurs. The spores, previously
TABLE 1
AGED Bacillus popilliae Spores Infectivity against Popillia japonica Larvae

<table>
<thead>
<tr>
<th>Storage condition of spores</th>
<th>Time spores stored (years)</th>
<th>No. of spores fed per gram of soil</th>
<th>Larvae diseased average (%)</th>
<th>Larvae diseased range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loam soil</td>
<td>(&lt;2 months)</td>
<td>1 x 10^8</td>
<td>50</td>
<td>(40–60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^7</td>
<td>15</td>
<td>(10–18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^6</td>
<td>0</td>
<td>(0–2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^5</td>
<td>2</td>
<td>(0–5)</td>
</tr>
<tr>
<td>Loam soil</td>
<td>7</td>
<td>1 x 10^6</td>
<td>3</td>
<td>(2–5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^5</td>
<td>0.5</td>
<td>(0–1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^4</td>
<td>0</td>
<td>(0–2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^3</td>
<td>0</td>
<td>(0–4)</td>
</tr>
<tr>
<td>Frozen</td>
<td>7</td>
<td>1 x 10^6</td>
<td>1.5</td>
<td>(0–4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^5</td>
<td>0</td>
<td>(0–2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^4</td>
<td>0</td>
<td>(0–3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^3</td>
<td>0</td>
<td>(0–5)</td>
</tr>
<tr>
<td>Dried</td>
<td>7</td>
<td>1 x 10^6</td>
<td>20</td>
<td>(10–30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^5</td>
<td>2</td>
<td>(0–5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^4</td>
<td>0</td>
<td>(0–2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^3</td>
<td>0</td>
<td>(0–3)</td>
</tr>
</tbody>
</table>

* Percentage of larvae diseased: (number of larvae infected/total number of larvae fed) x 100.

b Original spore feeding test (1968) prior to long-term storage.

d Washed and stored on microscope slides as dried smears, infected 20% of the larvae fed. The bacterial spores, stored in dry loam soil or in frozen water, infected 3 or 2% of the larvae, respectively. None of the control larvae became diseased.

The aged B. popilliae spores also were injected directly into the hemolymph of larvae to evaluate more precisely their infectivity (Table 2). The data in Table 2 indicate that 7-year-old B. popilliae spores do not maintain a high degree of infectivity. Apparently, dried spores survive the aging process better than those frozen in water. When 1 x 10^6 dried spores are injected per larva, about 38% of the larvae develop milky disease, whereas only 12% of the larvae become infected with the same concentration of frozen spores injected. Young spores, at the highest spore concentration used, infect 90% of the larvae routinely. None of the control larvae became diseased.

One of the apparent reasons for the low infectivity of aged B. popilliae spores against P. japonica larvae is their relative inability to outgrow and reproduce. Table 3 presents data that compares the viability of young and 7-year-old spores. Aged spores outgrowth rate is about 7% compared to a frozen spore rate of 1%; about 16% of the young spores grow out to form colonies.

DISCUSSION

For more than 30 years, B. popilliae spores were presumed to be similar to many other spore-forming bacteria in their ability to survive for many years. Indeed, the authors previously extrapolated from literature data that indicated a large percentage of B. popilliae spores could survive in soil for an indefinite period of time and then still cause disease in Popillia japonica larvae. In 1965 (unpub.), we prepared a small-scale experiment to feed 12 larvae about 1 x 10^8 fresh young spores/g of soil and observed that 25% of the larvae became infected. The following year, using the same spore-soil mixture, some 20% of the larvae tested were infected. But when the experiment was performed again in 1967 (3-year-old spore-soil mixture), none of that year’s larvae supply became diseased; these preliminary results
prompted us to reinvestigate the infection rate of aged *B. popilliae* spores.

There are two noteworthy points to be discussed that are indirectly related to the data presented here but directly relevant and crucial to the validity of our conclusion: first, larvae resistance to specific strains or substrains of the bacterium *B. popilliae*; and second, variation in disease susceptibility of larvae collected at different times and from different field locations.

A recent field survey in Connecticut indicates that *B. popilliae* spores may be less infective to *P. japonica* now than in the past (Dunbar and Beard, 1975). Dunbar and Beard's data suggest that a change either in the bacterium or in the susceptibility of larvae to the bacterium has occurred in the past 30 years. We have experimented with *P. japonica* larvae (only in laboratory studies) for about 20 years; the larvae were collected from several locations in Ohio, Indiana, Illinois, Michigan, and Pennsylvania. Most of the larvae we used came from Ohio. Our laboratory experiments lead us to conclude that low infectivity rate of *B. popilliae* against *P. japonica* is primarily due to the inability of the bacterium to reproduce itself within the larvae in sufficient numbers for the chance invasion into the hemolymph. For example, we have found no larvae resistant to *B. popilliae* NRRL B-2309 (spores or vegetative cells), although different tests require different numbers of the bacterium to cause infection. "Resistance" is defined here as immunological or physiological response (to the bacterium) sufficient to eliminate the potential for bacterial invasion. We observed no larvae resistant to any of 14 different strains via artificial injection (spores) (unpublished data); again, different numbers of the bacterial spores in any given injection experiment were needed to disease the larvae. Of the larvae, 40 to 80% could be diseased by injection with these different strains. However, when attempts were made to feed larvae spores of the 14 strains, only 10 to 25% of the larvae became diseased.

### TABLE 3

**Viability of Aged *Bacillus popilliae* Spores**

<table>
<thead>
<tr>
<th>Storage condition of spores</th>
<th>Time spores stored (years)</th>
<th>Spore outgrowth range (%)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refrigerated</td>
<td>(1 week)</td>
<td>7–24</td>
<td>16</td>
</tr>
<tr>
<td>Frozen</td>
<td>7</td>
<td>0.4–2</td>
<td>1</td>
</tr>
<tr>
<td>Dried</td>
<td>7</td>
<td>4–10</td>
<td>7</td>
</tr>
</tbody>
</table>

* Average number calculated from vegetative colony counts after spores were plated onto yeast extract high-phosphate growth medium.

* Fresh young spores washed and then stored at refrigerated temperature until plated.

Vegetative growth studies (unpubl.) of the *B. popilliae* strains referred to above indicate that the first five generations of vegetative progeny have a high percentage of lytic cells. After several transfers of the surviving cells to artificial media, the fragile cells are reduced in number, and the most vigorous cells then dominate the bacterial population. The first to fifth generation of vegetative cells do not disease larvae when injected, presumably because the fragile cells lyse within the larvae hemolymph. However, when young healthy vegetative cells (at least 10 generations removed from their parent spore) are injected into larvae, the larvae become infected; again, this suggests that the state of the bacterium is the critical factor for milky disease development. We now believe that most *B. popilliae* spores in a population germinate within the larvae to produce fragile vegetative cells that can easily be disposed of by the various defense mechanisms available to the larvae, as shown in the European chafer (Splittstoesser et al., 1978; Kawanishi et al., 1978). Regarding larvae variation from season to season, we have noted 3 to 60% variation in the survival rate of healthy larvae from one supply to another if the larvae are only fed and left in their original field soil. However, we routinely use only about 10% of all larvae collected for experiments of the type reported here. Prior to use, the test larvae are...
screened for color, size, feeding, vigor, body parasites, and body wounds, and then incubated in nursery loam soil for 1 week, after which they are rescreened for healthiness and then used in experiments.

Only about 3 to 10% of the screened control larvae die of natural causes during the experiments, compared to about 50% death rate for unscreened larvae. Our larvae screening method is intended to choose only the hardiest and most resistant insects for the experiments. A 5% variation in larvae disease susceptibility occurs from one season to another when screened larvae are fed or injected with ideal spores or vegetative cells of the type elucidated above. Therefore, we believe that the inherent differences in the larvae we use are apparently insignificant to our results. We conclude from these data that aged stored *B. popilliae* spores are significantly less infective against *P. japonica* larvae than are young spores of the bacterium.

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**REFERENCES**


