Growth Pattern of *Bacillus popilliae* in Japanese Beetle Larvae

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Induction of milky disease in 50% of Japanese beetle (*Popillia japonica*) larvae by feeding requires about 10⁸ spores of *Bacillus popilliae* per gram of soil. The infectious process occurs in four phases: (1) An initial incubation phase of about 2 days during which there is no evidence of infection in the hemolymph. (2) A vegetative phase of proliferation in the hemolymph which lasts until day 5 when prespores occur and a few spores first are observed. (3) An intermediate phase between day 5 and day 10 characterized by concomitant vegetative growth, prespore formation, and sporulation; maximum vegetative populations of about 10⁶ cells per ml hemolymph occur during this phase but the number of spores exceed that of vegetative cells by the end of the phase. (4) Thereafter, a sporulation phase which terminates by day 14 to day 21 with typical milknss and death of larvae; vegetative populations steadily decline and large numbers of spores accumulate during this phase. Milky larvae contain an average of 5 X 10⁹ spores per ml hemolymph. Throughout the process microscopic evidence indicates many vegetative cells die without forming spores; dead cells disappear from the hemolymph by some unknown lytic or phagocytic process. Thus the massive spore populations which characterize milky disease result from accumulation of spores during a prolonged period of simultaneous vegetative growth and sporulation rather than from an extended period of exclusively vegetative growth followed by sporulation of most cells.

**INTRODUCTION**

*Bacillus popilliae* invades the hemolymph of a Japanese beetle (*Popillia japonica*) larva via its digestive tract and produces "milky disease." the name of the disease refers to the milky appearance of the hemolymph after it becomes heavily infected with spores of the bacterium (Dutky, 1963). Spore populations reportedly average about 2 x 10⁸ per larva (2 x 10⁶ spores/ml hemolymph) (Dutky, 1940; Beard, 1945; Tashiro, 1957; Haynes et al., 1961; Pridham et al., 1964; St. Julian and Hall, 1968).

There is considerable interest in propagating *B. popilliae* spores on a large scale to combat the Japanese beetle. However, the bacterium (as compared to other well-known bacilli) grows appreciably only under restricted conditions, has limited viability, and sporulates appreciably only under highly specific conditions in laboratory cultures (Steinkraus and Provvidenti, 1958; Haynes and Rhodes, 1966; Rhodes et al., 1965, 1966, 1967). In addition, other studies have shown that spores of *B. popilliae* are unique among those of related bacilli (St. Julian et al., 1967). *B. popilliae* spores do not respond to common germinating agents, and the germination rate of these spores within sporangia is only about 2%. In gen-
eral, outgrowth of the spores is less than 10%. The percentage of dipicolinic acid is 10–100 times less than that reported for other related bacilli. The special character of the spores and the unique pattern of growth and viability of vegetative cells of this species in artificial culture led us to study the pattern of development of *B. popilliae* in larvae.

This report includes a determination of the number of spores needed to infect larvae in soil and outlines the pattern of development within larvae.

**Materials and Methods**

*Larvae.* Larvae of the Japanese beetle (*Papillia japonica*) were collected by the Plant Pest Control Division, Agricultural Research Service, U.S. Department of Agriculture, from presumably disease-free areas in Midwestern States. Their care before and during infectivity tests has been described by St. Julian et al. (1963).

*B. popilliae* spores. During the spring of 1966, more than 6000 third-instar larvae were each injected with about $1.5 \times 10^6$ spores to induce milky disease. The procedure for infecting larvae has been reported (St. Julian and Hall, 1968).

Infected larvae that exhibited overt milky disease (9–12 days after injection) were washed free of soil in 45–50°C tap water several times and then rinsed for about 30 sec in Clorox$^3$ (5.0% sodium hypochloride). After Clorox treatment, larvae were rinsed 6–8 times in sterile distilled water. Washed larvae were punctured with a sterile dissecting needle and the hemolymph was dripped into a thick-walled 100-ml volume centrifuge bottle until about 30 ml of milky hemolymph was accumulated (ca. 300 larvae). Spores were centrifuged from the hemolymph and subsequently washed three times in water. After the third wash, the packed spores were stored at $-20^\circ$C.

**Preparation of spore-laden soil.** Frozen *B. popilliae* spores were thawed at room temperature and then washed three additional times in sterile water. Direct microscopic counts were made of pooled spore samples with a Petroff-Hauser 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 dry 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 per gram of soil.

An elaborate mixing procedure was followed to ensure even distribution of spores. Finely sieved loam soil was first air-dried at room temperature in a thin layer and then was further dried in an oven at 40°C for 24 hours. The dry soil was weighed into four 2000/g portions. The proper concentration of spores was added to one-fourth of each 2000/g portion and mixed thoroughly by hand. The remaining soil was added in three equal portions and hand-mixed after each addition. After hand mixing, one-fourth portions of each spore-laden soil was mixed in a small PK V-shaped dry blender for 15 min; subsequent one-fourth portions were added with mixing until all the 2000/g spore-laden soil was blended. Each 2000/g spore-laden soil then was mixed for 3 hours.

Spore-laden soil was placed into compartmented trays. These trays were seeded with a mixture of domestic ryegrass, Dutch sweet clover, Kentucky bluegrass, and redtop seeds and were watered with distilled water.

**Pattern of infection.** Preliminary experiments determined the number of *B. popilliae* spores required per gram of soil for maximum infection of larvae. Fifty-four larvae were exposed to each concentration of spores for 4 to 21 days at 28–30°C. After exposure for a given number of days, larvae were removed from spore-laden soil...
and transferred to similar trays of soil free of spores and further incubated for a total of 21 days. Larvae were examined daily for symptoms of milkiness. The soil was seeded and watered when needed. Milky or dead larvae were removed when found.

Test trays of 54 larvae in soil devoid of B. popilliae spores were included in each experiment as controls; no control larvae became diseased.

The growth pattern of B. popilliae during infection was established by exposing larvae to $1 \times 10^9$ spores per gram of soil for 1 to 21 days. After exposure, all living larvae were removed from spore-laden soil, washed as described, and bled into sterile graduated 12-ml centrifuge test tubes. Cells were centrifuged from the hemolymph for 1/2 hour at 1802g and resuspended in 0.1% tryptone to the original volume of hemolymph. Both microscopic count and viable plate counts were made from this 0.1% tryptone suspension according to the procedure of St. Julian et al. (1963).

Photographs were taken with a 35-mm camera attached to a Zeiss WL microscope: phase-contrast optics were used.

**Results and Discussion**

**Infection of larvae.** The number of B. popilliae spores needed to facilitate oral infection is given in Table 1. Of the concentrations used, $1 \times 10^9$ spores per gram of soil resulted in greatest infection. For this reason, trays containing $1 \times 10^9$ spores were used to determine the pattern of development of B. popilliae during infection of larvae.

Both healthy and infected third-instar Japanese beetle larvae are shown in Fig. 1. The contrast is evident between the colorless hemolymph of the healthy larvae (Fig. 1A) and the milky appearance of the hemolymph of a larva grossly infected with B. popilliae (Fig. 1B). The diseased larva was infected with the bacterium for 21 days. Figure 2 is a microscopic view of healthy larval hemolymph containing blood cells. The appearance of “milky” hemolymph after 5 days of infection is seen in Fig. 3; the hemolymph at this stage of infection contains spores and various morphological stages of sporulation in B. popilliae. Hemolymph cells are seldom seen in grossly infected larvae.

**Pattern of infection.** The cellular structures associated with sporogenesis of B. popilliae in larvae have been elucidated by Black (1965). In our work, we have not distinguished between the several prespore stages: swelling of sporangium, delineation of phase-dark spore anlage, formation of spore walls, and development of refractility. Most of these stages are visible in Fig. 3. Rather, we used only three categories to enumerate cells in hemolymph: vegetative cells, all prespore forms, and fully refractile spores.

The quantitative pattern of infection by B. popilliae is given in Table 2. Obviously, sporulation is not a synchronous event in the population. That is, there is no prolonged period of exclusively vegetative growth followed by simultaneous spore formation in most cells to yield suddenly the massive spore populations associated

<table>
<thead>
<tr>
<th>Time larvae exposed to spore-laden soil (days)</th>
<th>Spores/g of soil</th>
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<tbody>
<tr>
<td>$10^2$</td>
<td>$10^3$</td>
</tr>
<tr>
<td>4</td>
<td>1.9</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
</tr>
</tbody>
</table>

*a Larvae incubated for the designated time in spore-laden soil and then removed to untreated soil for balance of time. All larvae incubated a total of 21 days.

$^b$ Percentage of larvae infected $= \frac{\text{Number of larvae infected}}{\text{Total number of larvae fed}} \times 100.$
with terminal disease. Instead, vegetative growth and sporulation occur concomitantly so that spores are continuously formed and ultimately accumulate in large numbers.

There is an initial incubation phase of about 2 days after larvae are put into spore-laden soil during which no bacterial cells are found in the hemolymph. Vegetative growth and some prespore development occurs exclusively for the next 2 to 3 days; a few spores usually are found by day 5. The microscopic appearance of hemolymph in infected larvae during this predominantly vegetative phase is shown in Figs. 4 and 5. About 20% of cells at 3 and 5 days

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**TABLE 2**

<table>
<thead>
<tr>
<th>Time of incubation (days)</th>
<th>Cells/ml hemolymph (count × 10⁵)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Vegetative</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>6.9</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
</tr>
<tr>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>21</td>
<td>2</td>
</tr>
</tbody>
</table>

*a* Larvae incubated in soil containing 10⁶ spores/g for time indicated.

*b* Differential microscopic counts made from pooled larval hemolymph with Petroff-Hauser bacteria counter under phase-contrast optics at 1250×.
are undergoing sporulation (Table 2. Figs. 4 and 5). Spores develop and become refractile within the swollen sporangium before parasporal inclusions are evident.

The intermediate phase of infection occurs between 5 and 10 days after larvae are fed spores. This phase is characterized by a change from predominantly vegetative to extensively sporogenic processes. Maximum vegetative populations of about

![Images of B. popilliae](image_url)

**Fig. 4.** Viable vegetative cells (VC) and granular or nonreproductive cells (GC) of *B. popilliae* in hemolymph of larvae during day 3 of infection.

**Figs. 5, 6, and 7.** Hemolymph containing *B. popilliae* vegetative cells, prespores, and spores. The larvae from which the hemolymph came were infected for 5, 7, and 10 days, respectively.

**Figs. 8 and 9.** *B. popilliae* spores and vegetative cells in larvae infected for 16 and 21 days, respectively.
3 × 10⁶ cells per ml are present, and prespores constitute 20 to 35% of vegetative forms. Spores rapidly become the most numerous cell during this phase (40 to 78% of total population, Table 2). The microscopic appearance of hemolymph during this phase is shown in Figs. 6 and 7.

Subsequent development during the sporulation phase is characterized by decreasing numbers of vegetative cells and prespores while large numbers of spores accumulate (Fig. 8). More than 90% of cells in milky larvae at the terminal stage are spores (Fig. 9). The number of spores that develop during milky disease varies considerably. Individual larvae, which exhibit similar milky appearance after being infected for 14 to 21 days (Fig. 1B), contain between 4 and 20 × 10⁶ spores; the average is about 5 × 10⁶ spores (5 × 10⁶ per ml hemolymph). Because spores accumulate during infection, the final concentration depends upon how long the host survives. Conversely, premature death curtails accumulation of spores. Largest numbers of spores develop in vigorous larvae infected by bacterial strains of low virulence. Dutky (1963) has described the importance of good larval nutrition in allowing maximum yield of spores.

About 50% of the vegetative cells in the hemolymph at early stages of infection contain highly granular cytoplasm (Fig. 4). Granular cells constitute a significant proportion of the population throughout the infectious process. Even cells in process of sporulation become phase-light and apparently incapable of further development. At any period, some vegetative cells proceed to sporulate while others continue to develop vegetatively; at the same time, many cells undergoing either process die. The extent to which these events occur changes as the disease progresses. Dead vegetative cells and prespores probably disappear from the hemolymph by phagocytosis or lytic process. Few spores, if any, disappear. Circulating blood cells decrease during the disease, and it is doubtful that phagocytosis is solely responsible for removing dead cells. B. popilliae is not autolytic in vitro and a lytic system in healthy or diseased hemolymph has not been demonstrated.

Viability of cells in hemolymph. We have made several attempts over the past 5 years to determine by culture techniques the number of viable B. popilliae cells in diseased larvae. The results with larvae used in these experiments are given in Table 3. Lack of growth from unheated 5- and 7-day samples indicates that vegetative cells in diseased hemolymph do not grow when plated on artificial medium. The viable cells detected in diseased hemolymph are from germinated spores. Outgrowth of B. popilliae spores is inconsistent and limited; generally, less than 1% of spores germinate (Splittstoesser and Stein- kraus, 1962; St. Julian et al., 1967). A comparison of data in Tables 2 and 3 indicates that only 0.5% of spores germinated. Somewhat greater numbers are obtained in heated samples because spore germination is stimulated by heat.

<table>
<thead>
<tr>
<th>Time of incubation (days)</th>
<th>Viability of cells in hemolymphb</th>
<th>Heat-treated 50°C, 15 min</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Unheated (%)</td>
<td>(%)</td>
</tr>
<tr>
<td>3–5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>16</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>21</td>
<td>0.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

a Larvae incubated in soil containing 10⁶ spores for time indicated.

b Percent viability of cells in hemolymph = Plate count of viable cells

Total microscopic count of cells × 100.
Apparently vegetative cells from germinated spores become acclimated to conditions in the hemolymph during the initial lag period; these populations are unable to grow promptly when placed on an artificial medium. Vegetative cells of *B. popilliae* die quickly under conditions where they cannot grow, and consequently, their capacity to adapt to new environments is poor. On the other hand, spores survive and outgrowth occurs; this slower process gives rise to viable vegetative cells. However, not all colonies from germinated spores contain vegetative cells which later proliferate equally well in artificial media. Some colonies resulting from spores remain small and the cells do not grow when transferred. Other colonies become larger and provide cultures that grow readily thereafter. It must be concluded that an adaptation by the milky disease organism is required for growth in vitro. Probably cells from artificial culture also must adapt to conditions in hemolymph when they are used to initiate infection.

Acknowledgments

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References


