

STUDIES ON THE MILKY DISEASE ORGANISMS¹

I. PARASITIC GROWTH AND SPORULATION OF *Bacillus popilliae*

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The biological control of the Japanese beetle, *Popillia japonica* Newman and the European chafer, *Amphimallon majalis* Razoumowsky could be considerably facilitated by the development of a practical method for producing on a large scale spores of *Bacillus popilliae*, the causative agent of type A milky disease, on artificial media. Steinkraus and Tashiro (1955) described a method for producing spores of *B. popilliae* in small quantities in the laboratory.

Suspensions of spores of *B. popilliae* now available commercially are produced by collecting living larvae from infested soil, injecting each with the disease organisms, incubating the larvae until their blood becomes filled with spores, and then grinding and mixing them with an extending material such as talc (Dutky, 1941).

Bacillus popilliae was isolated and described by Dutky (1940). It is a slender nonmotile rod, forming spores that are distinguished from other bacterial spores by the presence of a distinctive, refractile parasporal body accompanying the spore proper within the swollen sporangium. The bacilli and their host appear to be well adapted to each other (Beard, 1945). Generally the bacteria are able to complete their life cycle forming new spores before giving any evidence of damaging or destroying the host.

Before studying the saprophytic growth of cells of *B. popilliae*, it seemed advisable to reinvestigate the parasitic life cycle. Tashiro and White (1954) presented data indicating a considerable variation in the virulence of various strains of milky disease organisms. This paper reports changes in pH and oxidation-reduction potential or larval blood during the course of the disease and describes morphological variations encountered in strains of *B. popilliae* growing parasitically.

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EXPERIMENTAL METHODS

The life cycle of *B. popilliae* was studied in both naturally and artificially infected European chafer larvae. Those infected naturally were collected in the field and were presumably infected by ingestion of spores. Those infected artificially acquired the spores either by ingestion with soil inoculated with the organisms, or by injection directly into the haemocoel. The spore dose in injection studies was one million spores per larva. For the feeding tests, ten billion spores were mixed with one kilogram of soil in which the larvae were introduced and maintained at 25 C. They were examined macroscopically daily for evidence of milkiess. When there was evidence of infection, the larval blood was examined microscopically for changes in numbers and morphology of the multiplying vegetative cells and developing spores. The pH and oxidation-reduction potential were measured to determine whether these characteristics of the blood were altered during the course of the disease. A Beckman Model G meter with glass electrode was used to measure the pH. A platinum electrode was substituted for the glass electrode for the Eh measurement. Blood from three third instar larvae was pooled for each examination.

Microscopic examination was made of the unstained blood in wet mounts. Dried smears were stained by the Gram method or malachite green spore stain and examined for comparative numbers of spores and vegetative cells.

RESULTS AND DISCUSSION

Infected larvae were easily recognized in the advanced stage of the disease (figure 1). The rectal sac which is normally visible through the transparent blood was obscured by the milky white blood containing the high concentration of cells and spores of *Bacillus popilliae* in the diseased larvae. A drop of blood oozing from a snipped leg also revealed the milkiess (figure 1).

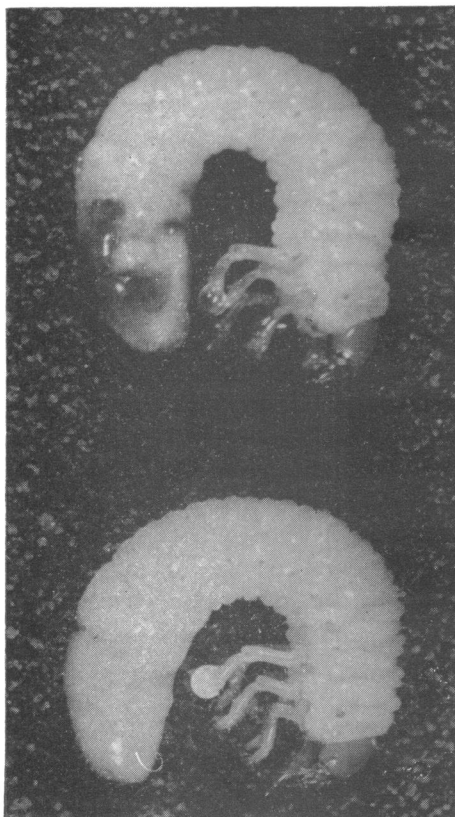


Figure 1. Healthy (top) and milky diseased (bottom) European chafer larvae. $\times 4$. Blood droplets on snipped legs show relative milkiness.

Macroscopic examination was not sufficient to detect milky disease in all infected larvae. Some strains of *B. popilliae* killed the larvae too soon or failed to reach the cell population levels within the blood necessary to cause perceptible turbidity. In critical studies this necessitated microscopic examination or culture of blood of all larvae suspected of having milky disease on artificial media.

It was thought that when the vegetative cells had become sufficiently numerous to cause a perceptible turbidity in the blood, the pH and the oxidation-reduction potential of the blood might be altered. Such a change might induce, or at least accompany the sporulation phase of the organisms. It was found, however, that the pH remained close to neutrality during the entire course of the disease (table 1). The oxidation-reduction potential showed a trend downward as the disease progressed, but no abrupt change occurred when sporulation began.

TABLE 1

Changes in pH and oxidation-reduction potential of blood of larvae of *Amphimallon majalis* following inoculation with spores of *Bacillus popilliae*

Hr after Inoculation	pH	Eh	Bacteria in Blood	
			Vegetative cells	Spores
0*	6.9	<i>mv</i> +454	—	—
24	7.2	399	+	+
48	7.3	444	+	—
72	7.1	434	+	—
96	6.9	414	+	—
120	6.9	414	+	—
144	7.0	414	+	+†
168	6.9	374	+	+
192	6.9	399	+	+
216	7.0	414	+	+
240	7.0	369	+	+
264	7.0	374	+	+
288	7.0	404	+	+
312	6.9	349	+	+

* Uninoculated.

† Atypical.

The oxidation-reduction potential of the blood varied among batches of larvae. Larvae with an Eh as low as +270 mv in the blood when healthy showed a reduction in potential to about +240 mv when diseased. Larvae with an Eh as high as +450 mv when healthy showed a progressive decrease to about +350 mv when diseased (table 1). (Steinkraus, 1957).

Injected directly into the blood or fed to the larvae, the cells of a majority of strains of *B. popilliae* followed a characteristic, predictable, and relatively stable pattern of morphological changes within the blood of living larvae as previously described by Dutky (1940) and Beard (1945).

The vegetative cells (figure 2) multiplied within each larva until there were billions and the blood became perceptibly milky. The vegetative cells were typically slender, nonmotile rods staining predominately gram-negative but containing gram-positive inclusions. Spores were then produced which swelled the sporangia and resembled the spores produced by many other species of bacteria. In most instances, this was followed by the appearance and development of a distinctive parasporal refractile body at one end of the

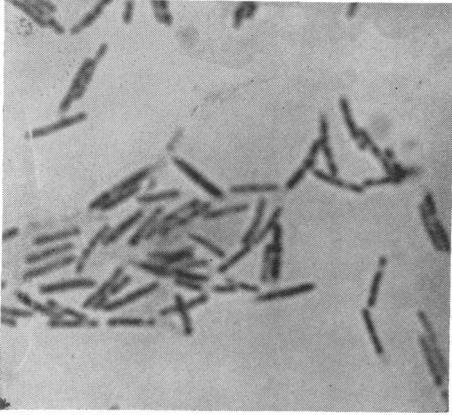


Figure 2. Vegetative cells of *Bacillus popilliae* stained with crystal violet. Bright field. $\times 1600$.



Figure 4. Crystal violet stained spores of *Bacillus popilliae*. Bright field. $\times 2,800$.

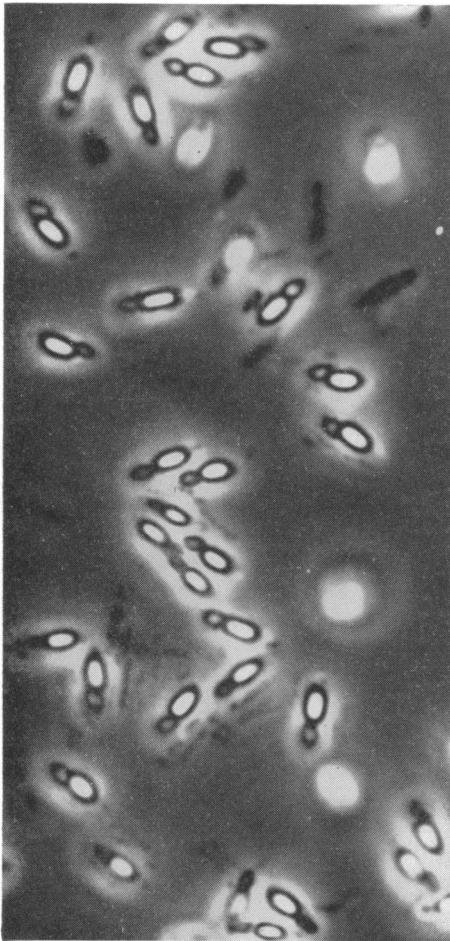


Figure 3. Unstained spores of *Bacillus popilliae* showing spore proper and parasporal body under phase. $\times 2,425$.

sporangium (figures 3 and 4). Approximately 50 to 75 per cent of the vegetative cells formed spores. The remaining vegetative rods appeared to be asporogenic. Spores formed parasitically were never observed free of the sporangium.

Unstained, both the spore and the paraspore were refractile. Staining reactions of the spore proper and the parasporal refractile body were different. The parasitic spore proper, stained with malachite green, did not become the vivid green characteristic of spores of saprophytic species. The paraspore did not take the spore stain. The surface of the spore proper stained deeply with crystal violet. In contrast, the paraspore did not retain the dye under ordinary circumstances and appeared as a clear unstained body (figure 4).

Although nearly all the cells and spores of *B. popilliae* followed the characteristic, predictable pattern of morphological changes under parasitic conditions, a few cells within any strain showed variations of the following types: (1) paraspores at both ends of the sporangium, instead of the single paraspore at one end (figure 4); (2) a tail-like projection at the end of the sporangium opposite the paraspore (figure 4).

Morphological variations were also observed among various species of the milky disease organisms. Although the study was concentrated on *B. popilliae*, cause of type A milky disease,

the closely related species, *Bacillus lentimorbus*, cause of type B milky disease, and a *Bacillus* species, cause of New Zealand milky disease (Dumbleton, 1945) were also given attention. All three species complete similar parasitic life cycles in larvae of the family *Scarabaeidae* but not necessarily in the same genera.

It was found that the three bacterial species were easily distinguished morphologically in unstained wet mounts. The paraspores of *B. popilliae* generally appeared triangular and were never round like the spores of the New Zealand *Bacillus*. *Bacillus lentimorbus* did not have a paraspore visible by either the ordinary light or the phase microscope. The parasporal refractile bodies of *B. popilliae* removed from different lots of larvae ranged in size from those at the limits of resolution of the light microscope to approximately 1.2 μ in maximum length and width. This comparative study of the paraspore morphology enabled the recognition and identification of parasitic type milky disease spores in the later investigation of the growth and sporulation of the organisms on artificial media.

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SUMMARY

A study was made of *Bacillus popilliae* growing parasitically. The object was to develop a founda-

tion for the later study of the growth of this species on artificial media. It was found that most strains of *B. popilliae* derived directly from diseased larvae are capable of parasitizing healthy larvae, producing a typical milky disease. In the course of such activity the bacteria complete a cycle of vegetative growth with formation of spores, morphologically unique because of their parasporal bodies.

Even when the bacteria became exceedingly numerous in the larval blood, they did not cause an appreciable change in pH. Both vegetative cell multiplication and sporulation occurred in a range of Eh from +240 to +450 mv.

Morphological variations among spores of *B. popilliae* within a strain or among related milky disease species were noted.

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