TWO NEW SPORE-FORMING BACTERIA CAUSING MILKY DISEASES OF JAPANESE BEETLE LARVAE

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INTRODUCTION

The existence of diseases among larvae of the Japanese beetle (Popillia japonica Newm.) and the part they play in the reduction of the populations of these larvae has been realized for some time. Probably the most important from the standpoint of the natural control of the insect are the so-called milky diseases. Two distinct milky diseases are recognized, referred to hereafter as type A and type B, whose causal agents are two closely related spore-forming bacteria. The author proposes the name Bacillus popilliae, n. sp., family Bacillaceae, for the species causing the type A disease and Bacillus lentimorbus, n. sp., for the causal agent of the type B disease. A search of the literature relating to insect diseases as well as that on spore-forming bacteria has failed to reveal any forms similar to these two bacilli.

Hawley and White\(^1\) indicated that the diseases of the Japanese beetle could be classified, on the basis of the gross appearance of affected larvae, into three groups, the black group, the white group, and the fungus group. They considered that the majority of the dead larvae found in the field belonged to the black group. They concluded that there was probably only one disease present among larvae of the white group. This disease was characterized by the presence of large numbers of a microorganism in pure or nearly pure culture, which was probably the causal organism.

The writer\(^2\) found that there were several diseases in the white group, of which types A and B milky diseases were the most prevalent and seemed to be responsible for the greater part of the reduction in larval population within the older area of beetle infestation. More recently Hadley\(^3\) has given a brief summary of the status of the disease investigations. The present paper deals with the description of these milky disease organisms.

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THE TYPE A MILKY DISEASE ORGANISM

SYMPTOMS OF THE DISEASE

GROSS APPEARANCE

To the inexperienced eye there appears to be little difference between healthy larvae and those infected with the type A milky disease. However, even in the early stages the diseased grubs show a turbidity of the blood which obscures the dorsal blood vessel readily seen in healthy grubs. As the disease advances, the grubs acquire a milky-white appearance, which an experienced observer can easily distinguish from the fat accumulation in mature larvae (fig. 1).

The activity of the larvae is not affected until within a few days of death, when they become sluggish. At the same time they turn slightly brownish, except in the lower parts of the body, which become chalky white owing to the settling out of spores as the blood circulation slows down.

MICROSCOPIC APPEARANCE

When blood from a diseased larva is observed under the microscope, it is found to be swarming with two types of cells—a highly refractile, spindle-shaped, spore-bearing rod, and a slender, nonmotile rod. These cells are apparently developmental stages of the same organism. Few blood cells are observed, and these few appear little different from those of normal larvae. The milky-white appearance of the blood is due to these highly refractile spores, which may be present in numbers as high as 20 billion in the blood of a single individual (fig. 2). Examination of the fat tissue surrounding the intestine shows a large number of spores. Examination of other tissues by gross dissection does not reveal localization of spores in any number, although some are observed in the layers of cells of the midintestine.

DEMONSTRATION OF THE CAUSAL RELATIONSHIP

The causal relationship between the disease and the organism occurring in the blood was demonstrated as follows: When blood from
a diseased larva was injected into healthy larvae, the typical disease picture appeared. When saline suspensions of blood from a diseased larva were heated to 80°C for 10 minutes and then injected into healthy larvae, the disease developed. The injected larvae showed the presence of the slender rod-shaped cells a few days after inoculation, and later both spores and rods, with the rods present in all transitional stages between the slender and the swollen spindle forms. These inoculation tests have been made with several thousand larvae

![Figure 2](image-url)

**Figure 2.** Photomicrograph of the blood of a larva with type A milky disease, showing spore and rod forms of the disease organism. × 1,200.

and seem to establish the etiology of the disease and to show that the rod forms and spore-bearing spindle cells are only developmental stages of the same organism.

**DESCRIPTION OF THE CAUSAL ORGANISM**

**Morphology and Staining Reactions**

The vegetative form of the organism is a slender, nonmotile rod occurring singly or in pairs. In the living condition the rods measure 0.9 by 5.2 microns. When fixed by Schaudinn's solution and stained by Hucker's crystal violet, the dimensions are about 0.3 by 3.5 microns.
The mode of division appears to be by plate formation rather than constriction, and is evidenced by the squareness of adjoining ends of the paired cells. After separation the ends are somewhat rounded. The cytoplasm in young cells is homogeneous and stains uniformly with Gram stain; in older cells granules are often found, and after fixing and staining, unstained areas are seen which divide the cell into two unequal sections.

The rods become swollen at sporulation. When the cell begins to swell, the spore becomes visible as a slightly refractile vacuole equal in size to the mature spore. As sporulation proceeds, the vacuole becomes more and more refractile until a definite spore is observed. At this time the cell has a pronounced spindle shape, and the spore is located somewhat terminally. One end of the cell broadens, and the cell becomes more pyriform than spindle-shaped. A granule is now observed in the broadened end, which grows until it is about half the size of the spore. With the development of the granule the spore assumes a more nearly central position. The cytoplasm about the spore becomes increasingly refringent.

After the completion of the refractile body and the increase in density of the cytoplasm surrounding the spore, no further morphological changes occur. In the fresh state the spore and granule are homogeneous in internal structure, and they do not take up either stains or iodine. The spore is surrounded by a halo formed by the encircling protoplasm, but it is very definite in outline. Spores free from the sporangium have never been observed. The size of the unstained sporangium is 1.6 by 5.5 microns, and that of the endospore 0.9 by 1.8 microns. When fixed by Schaudinn's solution and stained with Hucker's crystal violet, the refractile body and spore remain unstained, but the latter is obscured by the deeply stained surrounding protoplasmic layer. When fixed and stained, the spore-bearing cells are approximately 1.3 by 3.6 microns in size. When stained by Dorner spore stain, both the refractile body and the spore retain the stain, whereas the cytoplasm is completely decolorized. The membrane of the vegetative rods and both the membrane and the refractile body of the spore-bearing forms are resistant to the action of alkali, remaining intact for at least 2 days in 10-percent sodium hydroxide solution.

Germination of the spores has never been observed in either the blood or the digestive fluids of the insect.

Artificial Culture of the Type A Organism

Repeated attempts to isolate the causal organism from the blood of infected larvae were unsuccessful. No evidence of growth, with either the vegetative or the spore stages, was obtained on nutrient agar by shake, slant, or Petri-dish culture methods. The inverted Petri-dish method of Krumwiede and Pratt, which has frequently been used successfully for the isolation and cultivation of anaerobic bacteria, did not yield positive results. On blood-agar slants a slender, motile rod, forming small discrete colonies, was frequently obtained, but attempts to produce the disease by inoculation into healthy larvae were not successful. In peptone-glucose litmus whole milk

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Heavy inoculations with spore forms yielded a slender, nonmotile rod identical in morphology with the vegetative forms seen in the blood of diseased larvae. No attempts were made to infect healthy larvae with the rods obtained in the milk cultures, since the number of rods produced in the cultures was not much greater than the number of ungerminated spores remaining in the medium.

Recently, when dried-blood films from diseased larvae have been used as the source of spores for inoculation, a large proportion of the attempts to isolate the causal organism have been successful.

Unheated egg-yolk media, used by White for the isolation of Bacillus larvae White, proved to be satisfactory for the isolation of the type A milky disease organism. Heavy inoculations of spores from dried-blood films (700,000 spores per 5-ml. slant) were used, and the inoculated slants were incubated at 33°C. Slants of the basal medium, with and without the addition of sterile unheated egg yolk, were incubated under aerobic conditions at atmospheric pressure and at 700-mm. pressure, with the addition of 10 percent by volume of carbon dioxide. Inoculated slants were also incubated anaerobically under a pressure of 100 mm. of carbon dioxide. Under aerobic conditions growth of the type A organism occurred only on the egg-enriched medium, whereas under anaerobic conditions growth occurred equally with and without egg yolk. The beneficial action of the egg yolk must therefore be due in part to reduction of the oxygen content of the medium. The basal medium was fresh beef-infusion agar adjusted to pH 6.8, containing 0.5 percent each of dextrose and peptone. Sterile unheated egg-yolk suspension was added at the rate of 1 ml. per 5 ml. of the basal medium. Beef-infusion agar without peptone and without dextrose was also satisfactory as the basal medium.

The organisms form small discrete colonies on the slants, and as yet only nonmotile slender rods have been obtained in artificial culture. When the rods obtained in pure culture are inoculated into healthy larvae, the typical disease symptoms are produced and the blood of the inoculated larvae becomes loaded with typical spore forms of the type A organism.

Resistance of the Spores

The spores are heat-resistant, withstanding temperatures of 80°C for 10 minutes, as shown by the production of the disease in larvae by inoculation of heated spore suspensions. The thermal death point of the spores has not been determined. The spores are also resistant to desiccation. Spores in blood films dried for periods as long as 42 months have given consistently high infection when moistened and inoculated into healthy larvae.

Development of the Causal Organism in the Insect's Blood

When healthy larvae are inoculated with spores of the causal organism and held at 30°C, certain developmental changes occur in the bacteria in the blood (fig. 3). For about 12 hours after inoculation no change is detected in either the morphology or the number of spores. Then there is a gradual decrease in the number of spores

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until, after 30 hours, about half the original number remain. At this time vegetative forms are also seen in small numbers; they usually occur in pairs (C, D). After 48 hours about one-third of the original spores remain, and rods are present in extremely large numbers, still largely in pairs. On the third day after inoculation the rods begin to swell and many cells show the presence of an oval central vacuole (E, F). During the next 24 hours there is an increase in refringency of the vacuole with the formation of the mature endospore (G, H). At this time most of the rods are observed in the early stages of sporulation; there is a slight bulging of the rod with the appearance of the vacuole, and a few cells with well-developed endospores, swollen considerably and somewhat pyriform, showing a small granule in the broader pole (I). Periodic examination during the next 24 hours shows an increasing number of cells with initial granule formation and a few cells with the granule enlarged to the well-developed refractile body (J). The cells have the general appearance of those originally injected. By the sixth day the spores are sufficiently numerous so that the turbidity of the blood may be observed as a change in the external appearance of the larva. At this time many of the rods are still present, in the state both of division and of sporulation. The number of spores reaches a maximum about 7 to 10 days later, and at this time the larva is distinctly milky in appearance. The blood is found to be swarming with mature spore-bearing cells, and the rods, which are still present in considerable number, appear granular and many have lost their refringency. These so-called shadow cells are probably for the most part incapable of sporulation. The total num-

![Figure 3](image-url)
The number of spores in the blood at this time is from 2 to 20 billion, averaging about 5 billion, per larva. Subsequent examinations until the larva dies do not show any marked changes in the appearance of the spores, except that the surrounding protoplasmic layers are somewhat thicker and more refringent. Most of the rods appear to be either shadow cells, excessively granular cells, or sporulated cells that are not swollen appreciably. The rods stain only feebly. Examination of droppings from diseased larvae or the contents of the mid- or hind-intestine has not revealed the presence of spores or typical vegetative rods.

EFFECT OF TEMPERATURE ON TIME OF DEVELOPMENT OF DISEASE

To determine the effect of temperature on the time of development of macroscopic symptoms of the disease, larvae were injected with 2 million spores and held at various temperatures. The times of development were as follows: 4 days at 34° C., 6 days at 30°, 9 days at 25°, 11 days at 22°, and 14 days at 17°. Larvae held at 13° were not diseased after 63 days, and those held at 9° were still healthy after 28 days. When the larvae held at 9° for 28 days were placed at 30°, they developed the disease after 5 days.

The foregoing data show a linear relationship between the time of development of the disease and the temperature between 17° and 34° C. This corresponds approximately to the mathematical expression \( T = 24 - 0.6 \theta \) \(^\circ\)C, where \( T \) is the time of development of the disease and \( \theta \) is the temperature of incubation.

Other tests were run with inoculated larvae held at 36°, 37°, and 40° C., for comparison with larvae similarly inoculated and held at 30° as checks. Although the checks showed consistent disease development, in no case did larvae held at the higher temperatures develop the disease. Temperatures as high as 36° to 40° are close to the maximum tolerated by larvae, and in no case did larvae survive after 1 week. In view of the rapid development of the disease at 34°, lack of development after 7 days at higher temperatures seems to indicate that 36° must be approximately the maximum temperature for development of the causal organism.

Larvae held at temperatures of 15° to 16° C. had not developed the disease after 29 days. The blood of larvae held at 15° for 29 days was found to contain a few spores but no vegetative forms. From these observations it is concluded that the most probable temperature range for development of this disease is from 16°-17° to 36°.

THE TYPE B MILKY DISEASE ORGANISM

SYMPTOMS OF THE DISEASE

GROSS APPEARANCE

Larvae infected with type B milky disease found in the late summer and fall cannot be distinguished macroscopically from those infected with the type A disease. In overwintering diseased larvae, however, the general appearance is quite different. Instead of having a milky-white coloration, these larvae are a muddy brown. Overwintering diseased larvae collected in March were milky white with little or no brownish discoloration, but when held at room temperature they
darkened rapidly until at the end of 2 or 3 weeks they had assumed the chocolate color generally found in type B-diseased larvae during April and May. Microscopic examination has shown this darkening of diseased larvae to be due to extensive formation of blood clots which are brown to jet black. Chocolate-brown larvae are still alive and active (fig. 4). The accumulation of these clots in appendages blocks the blood circulation, producing a gangrenous condition which causes the affected parts to blacken. Death of such larvae is probably the result of gangrene.

When healthy larvae injected with blood of the brown diseased larvae develop the disease, they show the milky-white condition rather than the brown coloration of the larvae used as inocula.

**Microscopic Appearance**

The blood of diseased larvae shows the presence of large numbers of spindle-shaped spore-bearing rods and nonmotile vegetative rods (fig. 5). In blood of overwintering larvae the rods are nonrefractile shadow cells, and many of the spore-bearing rods show thickened and darkened membranes, which appear to be encysted by precipitation of blood about the spores. In addition, a large number of irregular opaque bodies are observed, which are blood clots.
DEMONSTRATION OF THE CAUSAL RELATIONSHIP

The disease is readily produced in healthy larvae by the injection of either heated or unheated suspensions of the blood of diseased larvae. Injection of either spores or vegetative rods produces the same sequence of events—namely, the appearance, first, of a large number of vegetative rods, followed by the typical spindle-shaped spore-bearing forms and the milky-white coloration of the larvae.

DESCRIPTION OF THE CAUSAL ORGANISM

MORPHOLOGY AND STAINING REACTIONS

The morphology and staining reactions of the vegetative form of the type B milky-disease organism are similar to those of the type A organism. The spore-bearing forms, however, although having considerable resemblance, are easily differentiated. The refractile body, so prominent in the type A sporangium, is absent in this form, and the sporangium is more decidedly spindle-shaped. The morphological differences apparent in the living spores are just as pronounced in fixed and stained spores. In the type B organism the spore-bearing rods take up crystal violet strongly and evenly, and the stained sporangium has a distinct lemon shape (fig. 6).

The dimensions of the vegetative rod are about 1.0 by 5.0 microns in the living state, and 0.5 by 4.0 microns when fixed by Schaudinn's solution and stained with crystal violet. The dimensions of the endospore are 0.9 by 1.8 microns, and of the sporangium 1.4 by 3.9 microns. When the sporangium is fixed and stained, its apparent dimensions are 1.9 by 2.8 microns.
Both rods and spore-bearing forms are stained by Hucker's modification of the Gram stain. The spores retain a deep red, and the sporangia are decolorized when stained by the Dorner method.

Attempts at artificial culture and isolation of the causal organism have thus far been unsuccessful.

**Figure 6.** Drawings illustrating the major morphological differences between the type A and type B spores: A, Type A, and B, type B spore unstained: 
- a, Refractile body; 
- b, endospore; 
- c, sporangium. Note the absence of a refractile body and greater symmetry in B. 
- C, Type A spore stained with crystal violet: 
  - a, Position of unstained refractile body. Note the lack of uniformity in staining. 
- D, Type B spore stained with crystal violet. Note the uniform staining and lemon shape. × about 10,000.

**Resistance of the Spores**

The spores are resistant to heat, withstanding at least 85° C. for 10 minutes when heated in physiological saline suspensions. They are also resistant to desiccation, producing the disease upon injection into the healthy larvae after drying in thin blood films on glass slides for as long as 42 months.

**Development of the Organism Within the Host**

Larvae were inoculated with 2 million spores by injection with spore suspensions of the causal organism, and held at 30° C. As with the type A disease, the organism is largely a blood parasite, although other tissues may be attacked. Periodic examination of
the blood showed the following changes in the organism: For 2 days after inoculation a gradual reduction in the number of spores was observed. On the third day vegetative rods appeared in considerable numbers, most of which were present in pairs. Adjoining ends of paired cells were truncate, indicating that the division probably occurred by plate formation rather than by constriction. The number of rods increased with time, and swelling of the rods began on the fifth day. At that time the presence of vacuoles was also noted in a few cells. On the sixth, seventh, and eighth days there was a growing preponderance of swollen sporulating rods, until at the ninth day they were present in sufficient numbers to give the first external symptoms of the disease.

At 30° C. development of the causal organism seems to be retarded, and the number of spores per larva seldom exceeds 1 to 2 billion even after 2 weeks at this temperature. At temperatures lower than 30° an increase in the number of spores continues after visible symptoms are observed, and after 2 to 3 weeks the typical milky-white condition of field-collected diseased larvae is reached. At this time between 5 and 10 billion spores per larva are present. Mature third instars, inoculated by injection, frequently had pupated before the disease caused their death.

**EFFECT OF TEMPERATURE ON DEVELOPMENT OF THE DISEASE**

When larvae were inoculated by injection and held at different temperatures, both the time and the extent of development of the disease were different. As mentioned above, although the time of development of the first external symptoms is the same (9 days) at 30° and 25° C., the extent of development is less at the higher temperature. At 22° external symptoms first appeared after 10 days, and at 15.5° after 19 days; at 12° there was no development after 63 days, and spores were in evidence but no rods.

The absolute maximum and minimum temperatures for development have not been determined for the type B organism, but the observations between 12° and 30° C. indicate that 30° is close to the maximum temperature, since the disease develops less than at lower temperatures, and that the minimum temperature is between 12° and 16°, probably closer to the latter. It seems, therefore, that the optimum and maximum temperatures for development of the type B disease are lower than those for the type A disease, and the minimum temperature for development is very nearly the same for both diseases. Type B disease thus has a considerably smaller temperature range than type A disease.

**SUMMARY**

Two new spore-forming bacteria are described. These organisms are the causal agents of types A and B milky disease of the larvae of the Japanese beetle (*Popillia japonica* Newm.).

The type A milky disease organism is a nonmotile Gram-positive rod measuring about 0.9 by 5.2 microns. The rods become swollen at sporulation, assuming first a spindle and then a pyriform shape. The spores are cylindrical and measure about 0.9 by 1.8 microns and are located centrally in the cell. In the broader pole of the cell
is found a refractile body, which is about half the size of the spore and possesses staining reactions similar to those of the spore. The temperature range of development seems to be from 16° to 36° C. The spores are found mainly in the blood of the larvae, reaching numbers as high as 20 billion in a single insect.

The type B organism is similar in appearance to the type A organism in the vegetative stages, but is readily distinguished morphologically after sporulation. The refractile body so prominent in the type A spore is lacking in the type B organism, and the spore-bearing rods are more nearly spindle-shaped. The temperature range of development seems to be somewhat narrower than for the type A organism, although the minimum temperature for development is the same (16° C.).

Both organisms produce a similar disease condition in the larvae of the Japanese beetle, so that upon gross examination the two conditions are usually indistinguishable.

Only the type A organism has thus far been cultured on artificial media.

The author proposes the name *Bacillus popilliae*, n. sp., family Bacillaceae, for the species causing the type A milky disease and *Bacillus lentimorbus*, n. sp., for the species causing the type B disease.