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II. Milky Disease of the Japanese Beetle[1]

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INTRODUCTION

Milky disease of the Japanese beetle, *Popillia japonica* Newman, is caused by either of two related bacteria, *Bacillus popilliae* Dutky or *B. lentimorbus* Dutky. The characteristics of these pathogens are those of an effective biological control agent. They grow in the hemolymph of the insect larva slowly enough to permit the ultimate accumulation of billions of cells before the host dies. Most of these cells form spores during the course of the disease; the spores permit the pathogen to survive inert for long periods in the soil and are the means of disease transmission. These facts were established nearly 30 years ago by Dutky, who first characterized the disease and described the milky disease bacteria. On the basis of this knowledge, he developed a means for artificially propagating the pathogen as a parasite in larvae that remains the only method of producing the spores for use as a control agent [summarized by Dutky (2)]. However, such a production process, which in effect mimics the disease, is not an economic method of obtaining the spores. A crucial attribute of any biological control agent is that the organism must be amenable to large-scale production in a virulent form.

The inability to grow the milky disease organisms efficiently and to cause them to sporulate in vitro has prevented their use for control of the Japanese beetle except under limited circumstances. The Northern Regional Research Laboratory undertook research on this problem several years ago. The work has involved techniques for the isolation and the maintenance of the organisms in pure culture, investigation of their characteristics of growth and of methods for growing comparatively large numbers of cells, studies on the means for evaluating infectivity of artificially propagated cells, a determination of the chemistry of the normal and infected larval hemolymph to define the natural growth and sporulation environment of the pathogen, and development of techniques for the production of large quantities of virulent spores in a manner suitable for commercial practice. The results represent the efforts of many researchers: G. A. Bennett, H. H. Hall, W. C. Haynes, G. R. Hrubant, R. W. Jackson, T. G. Pridham, M. S. Roth, G. St. Julian, E. S. Sharpe, O. L. Shotwell, R. D. Stubblefield, and B. A. Weiner. The material has been selected from the point of view of what has been learned of the characteristics of the disease process in the host and how these characteristics relate to the properties of the pathogens studied in vitro as a basis for the development of an effective method for production of virulent spores.

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NUTRITIONAL ASPECTS

Traditionally, *B. popilliae* and *B. lentimorbus* have been maintained as spores in dried hemolymph on glass slides. Such preparations are entirely suitable to induce the disease in the selective environment of the host insect. Patently, such sources cannot be considered pure cultures in the microbiological sense, and they are not suitable as stock inocula for cultural studies. It was necessary first to obtain pure cultures of these organisms and to determine whether or not they could be maintained as stock cultures in a more useful state. Many strains of both the milky disease pathogens have been isolated from spores and grown in pure culture in artificial media; the resultant vegetative cultures have been preserved

as lyophilized preparations. Such cultures are infective when injected into the appropriate host and cause the typical symptoms of milky disease (3). For various reasons, including pathogenicity under field conditions (2) and vigor in laboratory manipulation, the study of *B. popilliae* has been emphasized, although corresponding work has been done with *B. lentimorbus*.

Either species may be grown on a variety of complex media. A medium composed of 1.5% yeast extract, 0.3% K_2HPO_4, and 0.2% glucose in distilled water is used routinely; this medium sometimes is supplemented with 0.5% tryptone for maximal culture viability, although extent of growth is not markedly affected. The glucose necessary for the vegetative growth of either organism should be sterilized separately for maximal cell growth. The yeast extract can be replaced in considerable part by certain other organic nitrogen sources, including corn steep liquor. However, the relatively large amount of yeast extract required indicates an extensive nutritional demand by these organisms, and yeast extract appears to be necessary to maintain viability through repetitive transfer (7).

The precise nutritional requirements of *B. popilliae* have been defined by Sylvester and Costilow (14), who developed both semisynthetic media based on casein hydrolysate and a synthetic medium formulated of individual amino acids. Requirements for biotin, thiamine, and 11 amino acids were established. Three additional amino acids were stimulatory but not absolutely required for vegetative growth. The inclusion of barbituric acid was necessary for reproducible growth of this organism in the synthetic medium; its function is not known, and it cannot be replaced by purine or pyrimidine bases. Compared with the amount required and utilized during growth in the yeast extract medium, about five times the amount of glucose is necessary for growth in either the synthetic medium or any of the semisynthetic media.

The hemolymph of the larvae of the Japanese beetle contains relatively large amounts of both soluble and insoluble protein (10). During the course of the disease, the ratio of these two forms of nitrogen is not altered, nor are the absolute amounts. However, the concentrations of certain free amino acids in the hemolymph of diseased larvae do differ from those in healthy larvae (Table 1). Tyrosine, histidine, and glycine, all of which are required for vegetative growth, decrease markedly during infection. Conversely, among the seven amino acids that increase as a result of infection, three are required for growth of the pathogen—aspartic acid (asparagine), phenylalanine, and serine. Any significance attached to the decrease during infection of amino acids required for growth is uncertain because of the concomitant increase in the hemolymph of the other amino acids also required for growth. The magnitude of change of the required amino acids that decrease during infection is large, however, compared with the required amino acids that increase. Nonetheless, such changes do indicate a participation of the pathogen in the amino acid economy of the host.

TABLE 1. *Changes in the amino acid content of hemolymph during infection with Bacillus popilliae**

Composition of larval hemolymph		Relation to growth in vitro of *B. popilliae*
Amino acid†	Change during infection	
Glutamic acid	Marked increase	Not required
β-Alanine	Marked increase	Not required
Aspartic acid	Increase	Required (asparagine)
Glycine	Marked decrease	Required
Histidine	Decrease	Required
Lysine	Increase	Not required
Phenylalanine	Increase	Required
Serine	Increase	Required
Threonine	Increase	Not required
Tyrosine	Decrease	Required

* Information concerning the composition of larval hemolymph is from the data of Shotwell et al. (9, 10); growth requirements of *B. popilliae* are summarized from the work of Sylvester and Costilow (14).

† No significant change detected in amino acids other than those listed.

In any comparative examination of diseased and healthy larvae, it must be realized that usually there is no way of distinguishing between changes in the host tissues directly wrought by the metabolic activities of the pathogen and the alternative possibility that such changes represent modifications in the physiology of the host as a consequence of infection. In reality, both processes may well occur simultaneously. The most marked changes in the amino acid composition of hemolymph involve glutamic acid and β-alanine. Addition of either of these amino acids to media does not influence growth or sporulation of *B. popilliae* in our experience, although glutamate might be expected to offer an alternative mechanism for carbon to enter the oxidative metabolism of the organism.

GROWTH CHARACTERISTICS

As a consequence of the diseased state, billions of vegetative cells develop in the hemolymph of

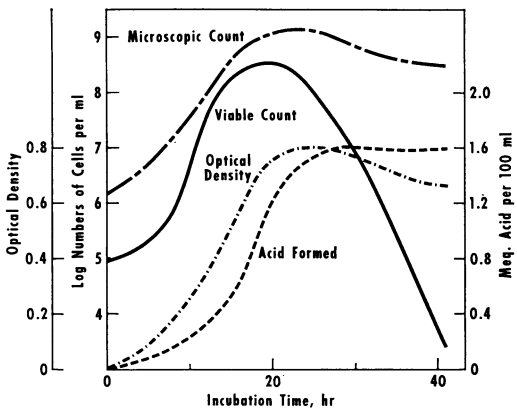


FIG. 1. *Characteristic pattern of growth of milky disease organisms in aerated fermentation vessels.*

diseased larvae, and ultimately at least 80% of them sporulate. In the course of a normal infection, this process requires between 14 and 21 days, during which a steady increase in the numbers of vegetative cells and spores occurs until usually 2×10^{10} to 3×10^{10} spores per milliliter are present in the hemolymph at the terminal stage of the disease. Although information is not definitive, sporulation appears to occur concomitantly with continued vegetative growth after vegetative populations reach 5×10^8 or more cells per milliliter of hemolymph. The volume of hemolymph in third instar larvae is 0.1 to 0.2 ml. The growth characteristics of *B. popilliae* in vitro are illustrated in Fig. 1. The growth pattern shown is typical of all strains of the milky disease organisms examined (7). Maximal populations of 10^9 to 2×10^9 viable cells are obtained in aerated media in 18 to 24 hr. The usual logarithmic growth phase is followed by a brief phase during which the numbers of viable cells remain relatively constant. This point of maximal vegetative population characteristically is followed immediately by a rapid and complete death of cells, as measured by plate count. Conversely, growth measured by optical density or by microscopic count reaches a corresponding plateau that continues indefinitely with only a slow decline. Therefore, the death of cells, as shown by the abrupt decrease in viable count, does not represent dissolutive lysis of the cells. The metabolism typical of vegetative growth, e.g., glucose or pyruvate oxidation, also diminishes during the decline phase at a rate comparable to the loss of viability.

A small amount of fermentable carbohydrate (glucose, trehalose, or fructose) is required for appreciable vegetative growth of these organisms in liquid media. The metabolism of the glucose always results in the formation of about 2 meq of acid per mmole of carbohydrate dissimilated, and the *p*H of the medium decreases correspondingly from an initial *p*H of 7.2 (2, 4, 7). The declining *p*H is only slightly related to the rapid death observed during growth of these organisms in vitro. When laboratory media are appropriately buffered to maintain the *p*H more effectively, the amount of growth obtained is somewhat greater, and survival of the population is slightly enhanced. Growth media may be advantageously buffered up to 0.6% K_2HPO_4; under these conditions, the *p*H decreases to about 6.2 to 6.4, compared with a *p*H less than 6.0 at lower buffer concentrations. Higher concentrations of phosphate buffer are toxic. Similarly, continuous neutralization with alkali provides slightly more vigorous growth and somewhat prolonged viability of the culture.

It is evident that a host may influence the growth of a pathogen by modifying the environment through replenishment of nutrients or by selectively removing or inactivating metabolic products. For example, although acid is an invariable product of growth of the milky disease bacteria in vitro, the *p*H of larval hemolymph does not change significantly as a consequence of infection. So far as is known, growth of the organisms in vivo is restricted to the hemolymph contained in the hemocoele chamber (1, 2). Such an environment can be considered analogous to a dialysis bag containing both dialyzable and nondialyzable nutrient and waste materials, and also, possibly, enzymes indigenous to the larva. Various methods of dialysis in fermentors and flasks, as well as diphasic growth systems in shaken flasks, to a degree retard the death of cells but in our experience do not increase the numbers of cells obtained per volume of total nutrients. No treatment yet devised effectively alters the typical pattern of growth and death in rapidly growing cultures.

The deleterious effect of accumulated acid on the milky disease organisms is demonstrated also when vegetative cells are injected into larvae and the extent of disease formation is measured (Table 2). The maximal extent of disease induced by comparable numbers of cells grown in the more highly buffered medium was nearly twice that caused by cells grown in low phosphate medium. One strain, which was not virulent when grown in low phosphate medium, exhibited a slight degree of pathogenicity when propagated in the buffered medium. Other data indicate that fewer numbers of viable vegetative cells are required to produce disease when they are propagated under conditions of optimal growth. Evidently, there is a relationship between the

TABLE 2. *Effect of growth medium on virulence of vegetative cells of milky disease organisms**

Milky disease organism	Growth medium	
	Low buffer	High buffer
Bacillus popilliae		
NRRL B-2309	35†	63
NRRL B-2043	0	0
NRRL B-2519	0	6
B. lentimorbus		
NRRL B-2522	61	96

* Data from Pridham et al. (5).

† Maximal per cent milkiness calculated as (number of milky larvae/number of larvae injected) × 100.

growth of cells in a medium and their subsequent action as a pathogenic agent when introduced into the insect.

METABOLIC PRODUCTS

The major products of glucose catabolism by *B. popilliae* have been exactly defined in the work of Pepper and Costilow (4). Their results show that essentially all fermented glucose is converted into lactic and acetic acids and CO_2. Small amounts of ethyl alcohol and glycerol, and trace amounts of acetoin and acetaldehyde, also are formed. The organism uses both the Embden-Meyerhof and the hexose monophosphate pathways for glucose dissimilation. The participation of these alternative pathways depends upon the availability of oxygen; up to 40% of the catabolism may occur via the hexose monophosphate mechanism in pure oxygen atmosperes and correspondingly less in lower oxygen concentrations. Although the total amount of acid produced from the fermentation of glucose by these organisms remains constant, the proportion attributable to either of the two acids varies with the amount of oxygen supplied to the organism. The amount of acetate formed increases as more oxygen is available to the organisms, and, conversely, the amount of lactate decreases proportionally. Since glucose is not dissimilated anaerobically, lactate never constitutes more than about 80% of the total acid.

Both lactic and acetic acids, the major products of glucose metabolism by the milky disease organisms, are present in the larval hemolymph to a considerable extent. However, the amounts of these acids in the hemolymph are not changed as a consequence of infection and the associated massive proliferation of the organisms (11, 13). Pyruvate is not a detectable product of *B. popilliae* in vitro, and the organism readily dissimilates pyruvate to lactate, acetate, and CO_2 (4). The amount of pyruvate in the hemolymph doubles as a result of infection (150 μg/ml normal) and may reflect a significant alteration in the metabolism of the bacterium in the host environment or, alternatively, modification of the physiology of the host as a consequence of disease. Because the amounts of lactate and acetate in the hemolymph do not change during infection, the former concept becomes an intriguing possibility. However, addition of pyruvate to cultures in vitro does not significantly alter the pattern of growth or of sporulation.

Essentially, no free glucose exists in the hemolymph of either healthy or diseased larvae. Rather, as in many other insects, there is a relatively large amount of trehalose in the hemolymph of the Japanese beetle, and the amount of trehalose declines about one-third as a result of the proliferation of the pathogen. Trehalose is utilized equivalently to glucose by *B. popilliae* during growth in vitro.

OXYGEN RELATIONSHIP

Dutky has held that the milky disease bacteria are anaerobic (2). In our experience, *B. popilliae* is an aerobic organism which dissimilates glucose and grows only aerobically. The growth response of *B. popilliae* to varied aeration rates in 2-liter fermentors is shown in Fig. 2. Only meager growth occurs at the 0.15 volume per volume per minute aeration rate, and the amount of growth correspondingly increases as aeration is increased to about 0.5 volume per volume per minute. Higher aeration rates, capable of providing more dissolved oxygen to the medium as measured by bisulfite oxidation, do not cause increased growth. Apparently, the 0.5 volume per volume per minute rate is adequate, and has been used routinely in our laboratory. Comparable results have been obtained in shaken-flask cultures agitated so as to provide differing dissolved oxygen concentrations. It should be noted from Fig. 2 that viability is greatly prolonged under conditions of restricted aeration and limited proliferation. From our experience, it is axiomatic that the death rate of vegetative cultures of the milky disease organisms is proportional to the rate of growth. Cultures remain alive longest when propagated under conditions of minimal proliferation.

The use of dissolved oxygen in the fermentor was measured polarographically with a miniature electrode developed in our laboratory (15). The cathode consists of a one-mill platinum wire housed in a 27-gauge hypodermic needle; the

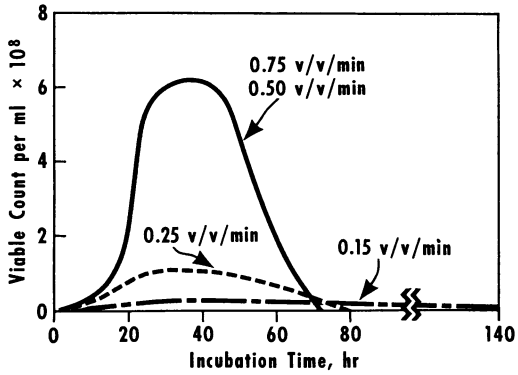


FIG. 2. *Effect of rate of aeration on the extent of growth of Bacillus popilliae in 2-liter fermentors. Aeration rates for the growth curves are shown as volume of air per volume of culture medium per minute (v/v/min).*

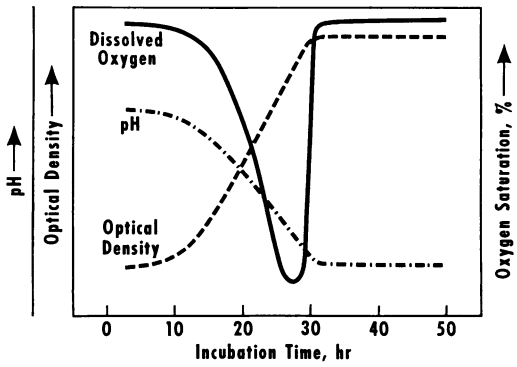


FIG. 3. *Use of dissolved oxygen during growth of Bacillus popilliae in aerated fermentors. The dissolved oxygen content of the culture medium was measured directly in the fermentor by a polarographic technique. Data courtesy of B. A. Weiner.*

electrical circuit is completed by a salt bridge to an external calomel anode. The results are shown in Fig. 3. The metabolism of the carbohydrate required for growth results in the formation of acid, and the *p*H of the medium decreases proportionally to the increase in optical density. As the *p*H decreases to a certain point, growth ceases. During growth of the organism, the dissolved oxygen content of the medium decreases simultaneously with the metabolism of the sugar and with the increase in numbers of cells. When growth and acid production cease, the use of oxygen stops abruptly, and the amount of dissolved oxygen in the medium immediately is restored to the level originally present. There would seem to be no question that the milky disease bacteria utilize dissolved oxygen during vegetative growth in vitro under conditions in which the cells are virulent when tested by injection into a susceptible host.

The small size of the oxygen electrode permits it to be used also for measurement of the oxygen content of hemolymph in living *P. japonica* larvae. By use of a cradle device, the needle cathode is positioned from the lateral aspect into the hemocoele of the larva. The calomel anode contacts a potassium chloride-saturated filter paper under the larva; larva and electrode are shielded from an aluminum base by Teflon. Temperature is controlled by circulating water through the metal base, and selected gas mixtures in the atmosphere can be established by means of a ported Plexiglas housing which fits the assembly.

So far as is known, oxygen exists in the hemolymph of the Japanese beetle larva only in the dissolved form; no oxygen carrier system is known to exist in the hemolymph itself. The mean oxygen content of normal third instar larvae is about one-third saturation (0.079 mmole of O_2 per liter of 0.25 M KCl at 25 C, which approximates the ionic molarity of hemolymph). Typically, the hemolymph of larvae, which microscopically show evidence of infection by the presence of vegetative cells of *B. popilliae*, contains only about one-half the amount of dissolved oxygen contained in healthy larvae. As milky disease progresses in larvae, the bacterial population changes from vegetative cells to spores. During the time sporulation is occurring extensively in the hemolymph, the amount of dissolved oxygen in the hemolymph increases to the value found in normal larvae. Hence, it appears that the proliferation of vegetative cells in the hemolymph utilizes the dissolved oxygen, as deso growth in vitro, and that sporulation occurs when the dissolved oxygen content is relatively low (about one-tenth saturation). It may be that sporulation does not require so much oxygen as does vegetative growth. Oxygen dissolves normally into the hemolymph during the disease, as indicated by the resumption of normal dissolved oxygen concentrations as vegetative growth diminishes. Thus, simple asphyxiation by a permanent impairment of oxygen transfer does not kill the insect. The data show, however, an evident relationship between the physiology of the bacterium as measured in vitro and the corresponding parameter of the environment in the hemolymph of the insect.

SPORULATION

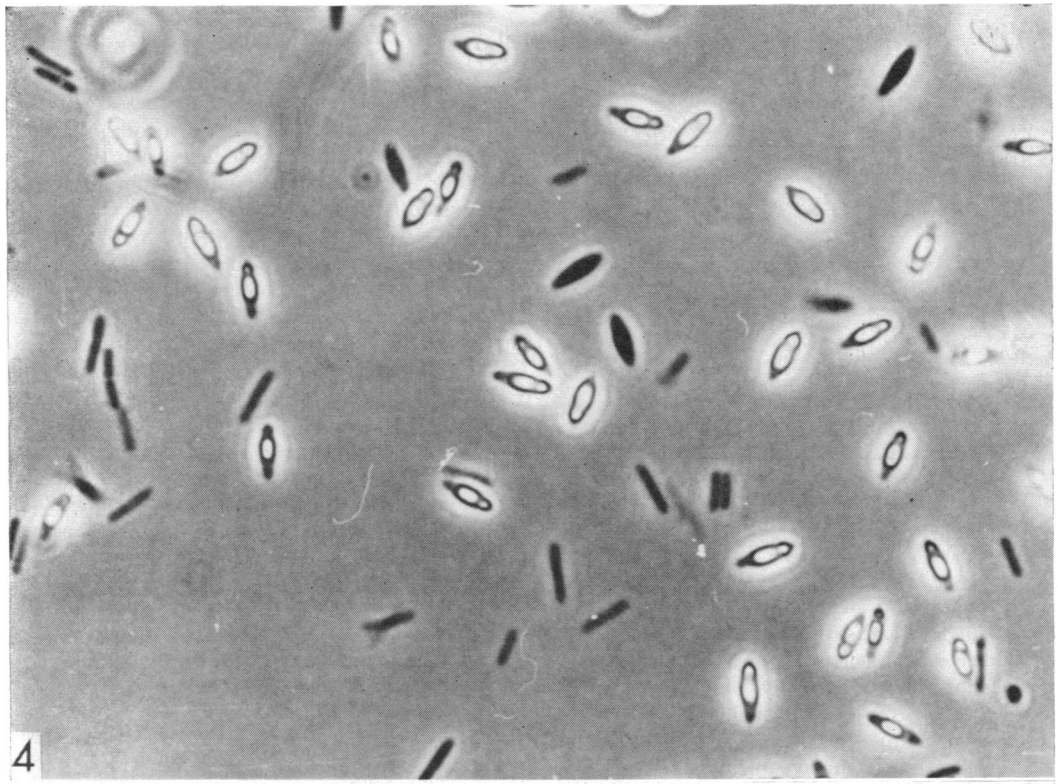
The terminal stages of milky disease in the insect are characterized by the presence in the

hemolymph of massive quantities of spores. In contrast, as has been seen, the organisms do not sporulate during laboratory cultivation. A phase-contrast photomicrograph of infected hemolymph at the time of simultaneous vegetative growth and sporulation is shown in Fig. 4. The unique shape and structure of the sporangium with included spores and parasporal body can be seen and compared with the vegetative cells and prespore structures. The vegetative cells and prespore stages, which involve swelling and enlargement of the vegetative cell, are uniformly phase-dark and do not exhibit, for the most part, the internal granulation characteristic of any cells that remain after sporulation is nearly complete. The refractile spore in the sporangium is formed in vivo before development of the fully refractile paraspore. The spores are not known to exist outside of the enveloping sporangial structure in nature, and in our experience it has not been possible to remove the sporangium effectively in the laboratory by either chemical, enzymatic, or physical means. Spores of *B. popilliae* germinate in vitro only to a limited extent (12). By comparison with vegetative cells, large numbers of spores are necessary to cause infection by injection into larvae (1, 2), which may mean that the spores germinate poorly even in the host. The spores are not particularly heat-resistant (55 to 60 C for 15 to 20 min), but do withstand drying for extended periods and apparently germinate somewhat more readily after aging. Since the organism presumably can sporulate in nature only in the host, and since vegetative populations have fastidious growth and survival requirements (8), it would seem essential that the spores not germinate prematurely. Heat resistance probably is of no particular benefit to the organism in nature, whereas resistance to desiccation is a prime attribute for survival in soil.

A degree of sporulation in vitro has been obtained with a selected strain of *B. popilliae* under highly specific conditions. Cells from 16- to 20-hr shaken-flask cultures of *B. popilliae* NRRL B-2309S are diluted in 0.1% tryptone and spread on the surface of a sporulation medium composed of 1.5% yeast extract, 0.3% K_2HPO_4 0.07 to 0.14% sodium acetate, and 2.0% agar; the *p*H of the medium is 7.4. Dilutions are selected to give fewer than 30 colonies per plate; plates are incubated for 4 weeks at 28 to 32 C. Although the procedure has since been modified somewhat, basically it remains the only method we have discovered by which sporulation in vitro can be reproducibly obtained. Several conditions must be established to obtain any spore formation. First, spores are formed only during colony formation by a selected strain originally isolated as a colonial variant on the acetate medium. Second, sporulation occurs only during colony development after prolonged incubation and when fewer than 30 colonies are present on a plate. Third, a selected amount of acetate is necessary for sporulation under these conditions. Although fermentable carbohydrate is required for growth of the milky disease organisms in liquid media, it is not required when the same medium is solidified with an agar, as illustrated by the comparable number of colonies which develop when a given dilution is plated on the agar medium formulated with and without glucose. Acetate is not inhibitory to growth under the conditions employed. Sporulation occurs only above *p*H 7 and optimally at about *p*H 7.4. Acetate is not appreciably utilized by most strains of these organisms; when demonstrated, acetate utilization occurs only in the presence of oxygen atmospheres and above *p*H 7.4 (4). Strains which do not oxidize acetate lack condensing enzyme (4). Fourth, glucose markedly inhibits sporulation.

The repressive effect of glucose on the sporulation process is shown in Table 3. It is evident that the presence of even minute amounts of glucose severely restricts sporulation and that the repression is only slightly reversed by added acetate. Some sporulation will occur in the absence of glucose on the described medium without added acetate, probably because of the acetate in yeast extract. Larval hemolymph is devoid of glucose but contains significant amounts of trehalose. Trehalose also inhibits sporulation in vitro but is considerably less privative than glucose. The trehalose in hemolymph may not be present in an available form, since the analytical method used does not distinguish free and bound forms of the carbohydrate.

The sporulation process of these organisms is being studied in an effort to learn what must be done to achieve reasonable control of spore formation in the laboratory. Something of the extent and cytology of sporulation in vitro can be seen in Fig. 5. It can be seen that there is more internal differentiation in prespore structures formed in vitro compared with those observed in hemolymph, and the parasporal body develops refractility before the spore does. Significantly, throughout the sporulation process extensive internal granulation may occur in cells or in prespore structures; this phenomenon is interpreted to mean that the sporulation process is abortive in that particular cell. In contrast, the vegetative cells and structures associated with sporogenesis in the hemolymph generally are uniformly phase-dark. Similar granulation is characteristic of the vegetative cells during and after the period of



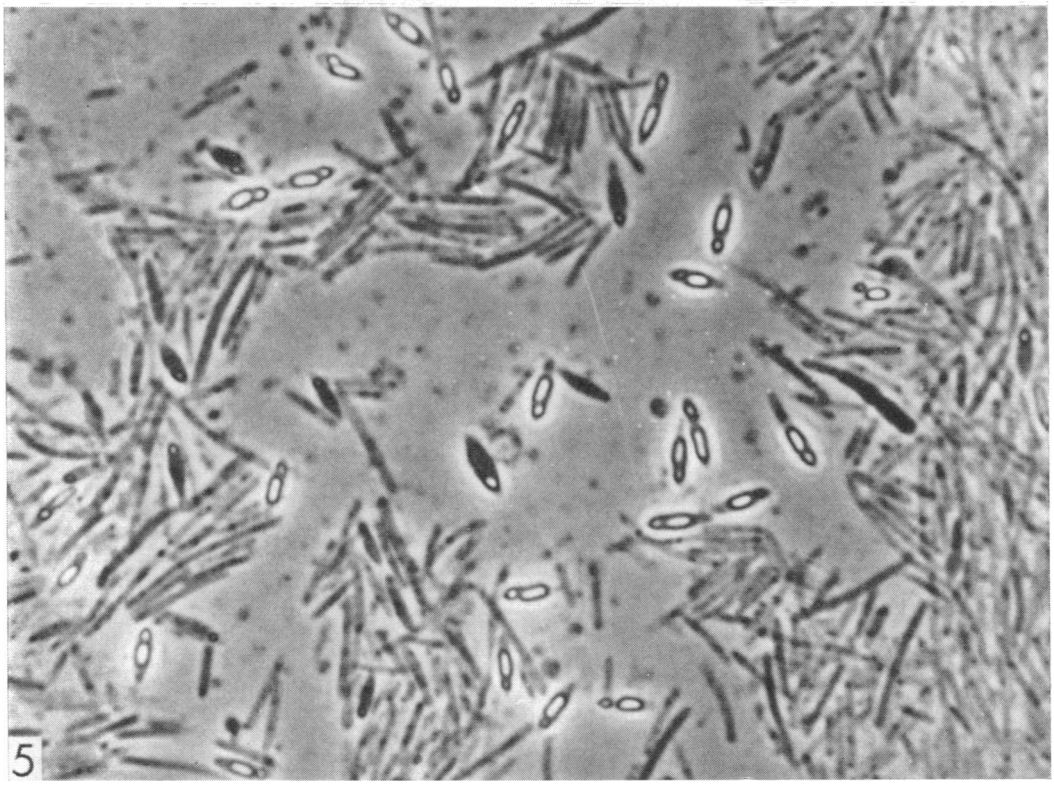


FIG. 4. *Spores of Bacillus popilliae NRRL B-2309 from diseased larval hemolymph (dark-contrast phase, 2,250 ×).*

FIG. 5. *Spores of Bacillus popilliae NRRL B2309S formed in colonies on acetate agar (dark-contrast phase, 2,250 ×). The granular and filamentous nature of the vegetative cells may be noted; such cells are not capable of proliferation or of spore formation.*

TABLE 3. *Effect of glucose on sporulation of Bacillus popilliae NRRL B-2309S*

Glucose concn	Basal medium		Acetate medium	
	Colonies per plate*	Sporulation†	Colonies per plate*	Sporulation†
mg/ml				
0	9	+	9	+++
0.1	9	+	13	++
0.25	11	0	11	+
0.5	9	0	13	±
1.0	11	0	10	0
2.0	10	0	11	0

* Number of colonies which appeared when a 10^7 dilution was plated onto the indicated medium.

† Extent of sporulation indicated by number of plus signs.

rapid population decline in cultures grown in liquid media. Under optimal conditions, about 3% of the cells in colonies complete the process of sporulation, and, maximally, only 20% ever are committed to spore formation as evidenced by cytological changes. Fully refractile spores such as those shown will survive drying and heat-shock treatments. Vegetative cultures obtained from heat-shocked material are infective when injected into the Japanese beetle larva. Therefore, pathogenicity is conveyed through spores produced in vitro. However, pathogenicity of the intact spore has not been incontrovertibly established.

SUMMARY

A technique has been established for permanently maintaining pure cultures of the milky disease organisms in a state from which they can be readily propagated in a virulent form. The pathogens now can be grown to reasonable numbers rapidly and under conditions suitable for commercial practice. Vegetative cells produced under these conditions are infective, and something is known of how virulence may be influenced by the in vitro growth conditions. In addition, there is some assurance from study of the environment within the larva that the conditions established for growth of large quantities of the vegetative cells of these organisms in vitro are not diametric to those existing in the insect. However, it is not known why the organisms do not sporulate in liquid culture or, for that matter, exactly why they do sporulate to some degree on selected solid media. Regardless, for the first time, a means is available for obtaining a reproducible degree of sporulation under conditions which allow study of the process of sporogenesis in these organisms.

Finally, it is suggested that these organisms have the capacity to sporulate readily and completely. Indeed, there is likelihood that the abrupt death of cells is a consequence of commitment to sporogenesis on the part of most of the population. Once initiated, spore formation is an irreversible process. It is theorized that the milky disease organisms become committed to spore formation as they reach a point of vegetative proliferation. For some reason, they are unable to complete the process of sporulation and die. Therefore, research at the Northern Regional Research Laboratory is being concentrated on the sporulation process, and upon malfunctions of the cells which may occur at the time of sporulation, as a key to final development of a means for producing infective spores on a large scale.

LITERATURE CITED

1. BEARD, R. L. 1945. Studies on the milky disease of Japanese beetle larvae. Conn. Agr. Expt. Sta. Bull. **491**:505–581.
2. DUTKY, S. R. 1963. The milky diseases, p. 75–115. *In* E. A. Steinhaus [ed.], Insect pathology, vol. 2. Academic Press, Inc., New York.
3. HAYNES, W. C., G. ST. JULIAN, JR., M. C. SHEKLETON, H. H. HALL, AND H. TASHIRO. 1961. Preservation of infectious milky disease bacteria by lyophilization. J. Insect Pathol. **3**:55–61.
4. PEPPER, R. E., AND R. N. COSTILOW. 1964. Glucose catabolism by *Bacillus popilliae* and *Bacillum lentimorbus*. J. Bacteriol. **87**: 303–310.
5. PRIDHAM, T. G., G. ST. JULIAN, JR., G. L. ADAMS, H. H. HALL, AND R. W. JACKSON. 1964. Infection of *Popillia japonica* (Newman) larvae with vegetative cells of *Bacillus popilliae* Dutky and *Bacillus lentimorbus* Dutky. J. Insect Pathol. **6**:204–213.
6. RHODES, R. A., M. SHEKLETON, G. R. HRUBANT, H. H. HALL, AND R. W. JACKSON. 1963. Studies on sporulation of *Bacillus popilliae*. Bacteriol. Proc., p. 10.
7. RHODES, R. A., M. C. SHEKLETON, E. S. SHARPE, H. H. HALL, AND R. W. JACKSON. 1962. Characteristics of *in vitro* growth of the milky disease pathogen *Bacillus popilliae*. Bull. Entomol. Soc. Am. **8**:158.
8. ST. JULIAN, G., JR., T. G. PRIDHAM, AND H. H. HALL. 1963. Effect of diluents on viability of *Popillia japonica* (Newman) larvae, *Bacillus popilliae* Dutky, and *Bacillus lentimorbus* Dutky. J. Insect Pathol. **5**:440–450.
9. SHOTWELL, O. L., G. A. BENNETT, H. H. HALL, R. D. STUBBLEFIELD, J. E. PETERS, C. H. VANETTEN, AND R. W. JACKSON. 1963. Amino acids in the hemolymph of diseased

Popillia japonica (Newman) larvae. Bull. Entomol. Soc. Am. **9**:168.

10. SHOTWELL, O. L., G. A. BENNETT, H. H. HALL, C. H. VANETTEN, AND R. W. JACKSON. 1963. Amino acids in the haemolymph of *Popillia japonica* (Newman) larvae. J. Insect Physiol. **9**:35–42.
11. SHOTWELL, O. L., G. A. BENNETT, R. D. STUBBLEFIELD, H. H. HALL, AND R. W. JACKSON. 1963. Nonvolatile organic acids in hemolymph of healthy and diseased *Popillia japonica* (Newman) larvae. Bull. Entomol. Soc. Am. **9**:168.
12. SPLITTSTOESSER, D. F., AND K. H. STEINKRAUS. 1962. Factors influencing germination and outgrowth of *Bacillus popilliae* spores. I. Effect of potassium ions. J. Bacteriol. **84**:278–282.
13. STUBBLEFIELD, R. D., G. A. BENNETT, O. L. SHOTWELL, H. H. HALL, AND R. W. JACKSON. 1964. Volatile and keto acids in hemolymph of healthy and diseased *Popillia japonica* (Newman) larvae. Bull. Entomol. Soc. Am. **10**:170.
14. SYLVESTER, C. J., AND R. N. COSTILOW. 1964. Nutritional requirements of *Bacillus popilliae*. J. Bacteriol. **87**:114–119.
15. WEINER, B. A. 1965. A microelectrode to measure dissolved oxygen in insect larvae. J. Insect Physiol., *in press*.