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Biology of *Bacillus popilliae*

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I. Introduction

Bacillus popilliae is a pathogen of various scarabaeid beetles. The bacterium, when ingested by beetle larvae, invades the hemocoel wherein it undergoes vegetative proliferation and subsequent sporulation causing death of the larvae. The mass of spores that accumulates upon approaching death of the insect is ultimately released to the surrounding soil and consequently the pathogen can survive for an extended period. These spores are eaten by newly hatched beetle larvae and, upon germination and outgrowth in the alimentary tract, begin the infectious process again. The name given to this infection is "milky disease" because of the milky appearance of the hemolymph containing spores of *B. popilliae* or *B. lentimorbus*, another closely related organism. Theoretically, *B. popilliae* represents a persistent and perpetual microbial insecticide although soil physical and chemical properties as well as climatic conditions, agricultural and horticultural prac-

tices, and density of the larval population influence its effectiveness in nature.

Several reviews have been written on milky disease (Dutky, 1963; Rhodes, 1965, 1968; St. Julian and Bulla, 1973), and an extensive bibliography has been compiled on milky disease bacteria (Klein *et al.*, 1976). The purpose of this paper is to provide an overview of biological considerations of *B. popilliae* resulting from many years of research related to the agricultural and commercial application of this bacterium as a microbial insecticide. Unfortunately, the biology of *B. popilliae* is not yet well understood. We hope that our review will stimulate new interest for more fundamental research on this potentially powerful microbial control agent.

II. Taxonomy and Classification

Bacillus popilliae (cause of Type A disease) and *B. lentimorbus* (cause of Type B disease) were named and partially described by Dutky (1940). The organisms were isolated from Japanese beetle larvae having milky disease, and both species produced typical disease symptoms when pure cultures were inoculated into healthy larvae. Dutky (1940) differentiated the two species primarily on the basis of (1) the difference in color of diseased larvae and (2) the presence of a refractile parasporal body lying adjacent to the spore in *B. popilliae* which was absent in *B. lentimorbus*. Subsequent investigations indicate that these organisms do represent different species (Gordon *et al.*, 1973). Kaneda (1969) and Bulla *et al.* (1970a) demonstrated that these two organisms differ in lipid composition, and Bulla *et al.* (1969) showed that the endospores have different surface topographies. Hrubant and Rhodes (1968) found no serological reactions between *B. popilliae* and *B. lentimorbus* as determined by agglutination reactions with whole cells, although Krywienczyk and Lüthy (1974) demonstrated a close serological relationship between the two by using disrupted cells as antigens and by determining the presence of common antigens with double-diffusion and immunoelectrophoresis techniques.

Two additional species of bacteria causing milky disease in certain scarabaeid larvae have been described: *B. fribourgensis* (Wille, 1956) and *B. euloomarahae* (Beard, 1956). Also, a number of varieties have been described, the most recent by Milner (1974). A number of comparative studies of the various species and strains have been reported (Krieg, 1961; Steinkraus and Tashiro, 1967; Lüthy, 1968; Wyss, 1971; Lüthy and Krywienczyk, 1972; Gordon *et al.*, 1973; Krywienczyk and Lüthy, 1974). Two basically different classification schemes have been suggested depending primarily on the relative emphasis given to the presence of a parasporal crystal. Wyss (1971) and Krywienczyk and Lüthy (1974) have given more weight to charac-

teristics other than a crystalline inclusion and have suggested that the four species be reduced to *B. popilliae* and *B. euloomarahae*. They proposed three subspecies of *B. popilliae: popilliae, melolonthae* (includes the strains named *B. fribourgensis*), and *lentimorbus*. In contrast, Krieg (1961) and Gordon *et al.* (1973) suggested designating those strains with parasporal inclusions as *B. popilliae*; subspecies would include *fribourgensis* and *new zealand*. *B. lentimorbus* and related strains were retained as a separate species.

Gordon *et al.* (1973) directly compared strains of *B. popilliae* for a variety of characteristics, and the results of this study served as the primary basis for the description of the species included in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974). The vegetative cells are gram-negative rods (0.5–0.8 × 1.3–5.2 μm). However, sporangia and presporal forms are gram-positive. This feature may be the reason why Dutky (1940) originally described the cells as gram-positive. The cells can be cultivated continuously in complex laboratory media but lose viability rapidly after reaching the stationary phase of growth. Viable cells are uniformly phase-dark whereas nonviable cells are "ghostlike" and granular in appearance. Spores are formed when pure cultures of *B. popilliae* are injected into the hemolymph or fed to susceptible insects. The sporangium is swollen and spindle-shaped and contains a typical refractile spore in addition to the smaller refractile parasporal body. The spore has a characteristic fine structure (Black, 1968a,b; Black and Arrendondo, 1966) and surface topography (Bulla *et al.*, 1969). The shape of the inclusion body does not appear to be constant from culture to culture because various shapes have been described by different investigators.

Bacillus popilliae is a catalase-negative facultative anaerobe though growth under anaerobic conditions is quite slow. Gordon *et al.* (1973) found that cultures frequently require 14 days to attain any observable mass anaerobically. R. N. Costilow (unpublished data) has observed growth on agar plates inoculated with heat-shocked (60°C for 10 minutes) spores of *B. popilliae* NRRL B-2309M produced *in vitro* as described by Sharpe *et al.* (1970) and incubated in an anaerobic chamber. Colonies of *Clostridium sporogenes* appear on the surface of agar plates in this chamber within 12–18 hours. However, 4–7 days of incubation were necessary before *B. popilliae* colonies became visible. Colonies were clearly visible on plates incubated aerobically within 2 days. Also, the maximum colony size was much smaller under anaerobic conditions than under aerobic conditions. The responsiveness of *B. popilliae* to oxygen is illustrated in Table I. Significant increases in growth occurred as the aeration rate increased up to an oxygen absorption rate of about 1.0 mmol O_2/liter/minute. This rate appeared to be where maximum growth occurred (R. N. Costilow, unpublished data).

TABLE I

EFFECT OF AERATION AND OF NEUTRALIZATION OF ACIDS PRODUCED ON THE GROWTH OF *B. popilliae* (NRRL B-2043)

	OD at 670 nm × 10^2			
	Unneutralized		Neutralized[b]	
OAR[a]	24 hours	48 hours	24 hours	48 hours
0.26	8	14	3	18
0.87	14	25	14	28
1.12	23	28	24	38
2.91	23	26	23	26

[a] OAR: Oxygen absorption rate (mmoles O_2 absorbed/minute/liter of medium). The medium with an OAR of 0.26 was not shaken, and the others were shaken at different speeds. All were incubated at 30–32°C.

[b] Cultures were neutralized at intervals by addition of sterile 1 *N* NaOH. Samples of duplicate cultures were titrated to determine the amount of base required.

Bacillus popilliae can utilize relatively few sugars. Acid is produced from glucose, fructose, mannose, galactose, maltose, sucrose, and trehalose (Steinkraus, 1957a, b; Steinkraus and Tashiro, 1967). Methyl α-D-glucoside, methyl α-D-mannoside, and salicin can be utilized by some strains for growth after an induction period (Bhumiratana and Costilow, 1973).

It is clear that this organism is properly classified as a *Bacillus* (Buchanan and Gibbons, 1974). It is not a *Clostridium* as Faust and Travers (1975) suggested. The eighth edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974) includes all endospore-forming, rod-shaped, aerobic or facultative anaerobic bacteria in the genus *Bacillus*. Gordon *et al.* (1973) stated that the genus "encompasses the rod-shaped bacteria capable of aerobically forming refractile endospores that are more resistant than vegetative cells to heat, drying, and other destructive agencies." The hemolymph of Japanese beetle larvae in which this organism sporulates is aerobic. Weiner *et al.* (1966, 1969) reported the presence of significant amounts of dissolved oxygen in hemolymph throughout infection and sporulation. There is no rationale for considering reclassification of *B. popilliae* as a *Clostridium*.

III. Nutrition

Bacillus popilliae is a fastidious organism. All the laboratory media that have been devised for its cultivation are complex. Most of them contain yeast extract and digests of casein. Sugar is required in all media as a source of

energy. The organism will grow reasonably well in a vitamin-free hydrolysate of casein medium supplemented with tryptophan, thiamine, glucose, and phosphate (Sylvester and Costilow, 1964). Both tryptophan and thiamine are necessary for growth; biotin, myoinositol, and niacin are stimulatory for growth although they are not required.

Obviously, this organism has become extremely dependent on its natural host for nutrients. Hemolymph of Japanese beetle larvae is rich in amino acids (Shotwell *et al.*, 1963, 1965), and *B. popilliae* requires many of them (Sylvester and Costilow, 1964). Only those amino acids requiring relatively few enzymes for biosynthesis are not required. *Bacillus popilliae* cannot make any of the amino acids in the serine or aromatic amino acid families, nor can it synthesize histidine. Only alanine and glutamic acid of those acids in the pyruvate and glutamate families are not required. The organism can synthesize aspartate, lysine, and thereonine but requires both asparagine and methionine for growth. Still unresolved is the role of barbituric acid which must be added to a synthetic medium to obtain consistent growth (Sylvester and Costilow, 1964). This compound has been shown to stimulate both nucleic acid and protein synthesis in a synthetic medium but there is little or no incorporation of labeled barbiturate into cell material (Coulter and Costilow, 1970). Common purines or pyrimidines in synthetic media do not replace the requirements for barbiturate, nor do they enhance growth.

Evidence for the adaptation of *B. popilliae* to grow in its natural host is provided by the observation that it responds well to the high concentration of trehalose in larval hemolymph (Rhodes, 1968). The enzymes that catalyze the breakdown of this disaccharide are constitutive; both respiration and growth rates are higher with trehalose than with glucose (Bhumiratana *et al.*, 1974). Furthermore, the organism takes up trehalose by the energy-conserving phosphoenolpyruvate (PEP):sugar phosphotransferase system and cleaves the trehalose 6-phosphate formed by a unique phosphotrehalase that has not been found elsewhere.

IV. Growth Characteristics

Bacillus popilliae, as stated earlier, can be cultured in artificial media (Steinkraus, 1957b; Rhodes *et al.*, 1966; Steinkraus and Tashiro, 1955). Liquid and solid media containing yeast extract and glucose support good growth of *B. popilliae*. Figure 1 displays a characteristic growth pattern of the organism under aerobic conditions. The maximum population (1.2×10^9) is achieved at 16–20 hours. Immediately after peak growth occurs, the number of viable cells rapidly declines. However, there is little or no cell lysis. Under anaerobic conditions, similar growth occurs except that the number of viable cells is 5×10^8 per ml. No spores are formed either aerobically or anaerobically.

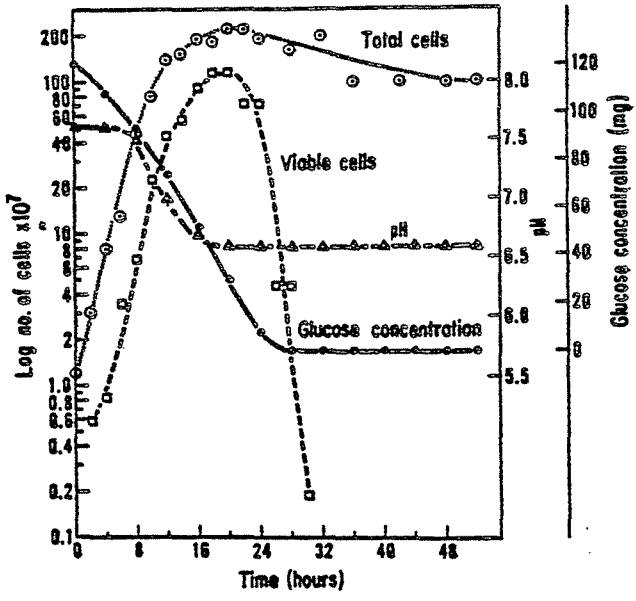

FIG. 1. Characteristic growth pattern of *Bacillus popilliae* in MD medium of 1.5% yeast extract, 0.6% K_2HPO_4, and 0.2% glucose in distilled water. ⊙, total cells; △, pH; □, viable cells; ●, glucose concentration.

What causes death of aerobic *B. popilliae* cells is not well understood, but it could be due, in part, to the lack of catalase (Costilow *et al.*, 1966) or peroxidase activity (Steinkraus, 1957b; Pepper and Costilow, 1965). Without a hydrogen peroxide scavenging system, hydrogen peroxide could accumulate during vegetative proliferation and poison the stationary-phase cells. Cell death also could result from exposure to the superoxide free radical produced when oxygen reacts with reduced flavin mononucleotide and flavin adenine dinucleotide. However, because *B. popilliae* cells contain superoxide dismutase (Costilow and Keele, 1972; Yousten *et al.*, 1973; Yousten and Nelson, 1976), the reactive superoxide free radical can be converted to oxygen and hydrogen peroxide. Consequently, the cells should be free from the lethal effects of the superoxide free radical.

Under anaerobic conditions, *B. popilliae* does not exhibit any detectable superoxide dismutase activity (Yousten and Nelson, 1976). Whether lack of enzyme activity in anaerobically grown cells is a possible cause of cell death is not known. Based on the results of aerobic and anaerobic experiments, there is no apparent correlation between cell death and enzyme activity.

Japanese beetle larvae use the PP pathway with only minimal EMP participation (St. Julian *et al.*, 1975). The mechanism by which this is regulated is not known, although Pepper and Costilow (1964) did demonstrate that the percentage of glucose assimilated by the PP route is greatly enhanced when incubated in an atmosphere of oxygen compared to an atmosphere of air. The hemolymph of healthy and infected larvae have significant levels of oxygen present (Weiner *et al.*, 1966). The larvae may maintain oxygen levels at a higher level than those normally achieved in laboratory cultures or concentrated cell suspensions. Such a phenomenon could lead to higher levels of the enzymes of the PP pathway or lower levels of those of the EMP system or both. Interestingly, there appears to be a very low percentage of glucose oxidized to acetate by cells harvested from diseased larval hemolymph. St. Julian *et al.* (1975) observed that practically all of the CO_2 produced by such cells was derived from carbons one and two of glucose, indicating a considerable amount of pentose cycling. This activity may indeed provide a reasonable explanation for some observations with respect to sporulation. Larval hemolymph contains trehalose at concentrations that repress sporulation of oligosporogenic strains *in vitro*, and the trehalose level remains high in the hemolymph throughout *in vivo* vegetative growth and sporulation (Bennett and Shotwell, 1973). In contrast, when glucose is used as a carbon source, no spore formation occurs in colonies of oligosporogenous strains on agar plates until the sugar is at a very low level (Costilow and Coulter, 1971). So, it is possible that this organism requires a utilizable sugar as an energy source for sporulation. As noted earlier, trehalose is used more readily than glucose in broth cultures. Because the uptake of trehalose is dependent on PEP, the rate of trehalose uptake in the hemolymph may be restricted by the rate of PEP generation. Cycling of carbons via pentoses does not generate PEP, and this fact could explain the relatively slow rate of growth and the failure of trehalose to repress sporulation of *B. popilliae* in hemolymph. Rhodes (1968) has proposed that most of the trehalose in the hemolymph is in an unavailable form. More work is needed to clarify this matter.

The primary products of glucose dissimilation by *B. popilliae* are acetate, lactate, and CO_2 (Pepper and Costilow, 1964). Small amounts of glycerol, ethanol, acetoin, and acetaldehyde have also been found. The rate of acetate to lactate is readily varied by regulating the availability of oxygen. In unlimited oxygen, little or no lactate is formed. Strains of *B. popilliae* vary in their capacity to oxidize acetate. Pepper and Costilow (1964) reported that a variant of strain NRRL B-2309 oxidizes acetate and contains condensing enzyme (citrate synthase) whereas the parent strain does not. McKay *et al.* (1971) found six of the eight strains they examined to oxidize acetate whereas the parent strain (NRRL B-2309) does not. Bulla *et al.* (1970b, 1971) and Yousten *et al.* (1974) failed to find any evidence of acetate oxidation in *B. popilliae*.

However, all of the latter studies (Bulla *et al.*, 1970b, 1971; Yousten *et al.*, 1974) were done with strain B-2309. St. Julian *et al.* (1975) failed to find any acetate oxidation by cells harvested from larvae infected by injection with spores. They incubated the cell suspensions for 60 minutes before addition of the labeled substrate, and this procedure could well have influenced the results obtained. McKay *et al.* (1971) were unable to detect a glyoxalate cycle in strains that oxidize acetate. Therefore, the cells must have a source of necessary four-carbon intermediates of the tricarboxylic acid cycle if the cycle is to function with acetate as the substrate. Incubation without substrate (St. Julian *et al.*, 1975) could deplete these intermediates to a point where no oxidation would occur. Further evidence for acetate oxidation by some strains of *B. popilliae* was obtained in studies of the utilization of methyl α-D-mannoside (Bhumiratana and Costilow, 1973). When the mannoside was present at low concentrations (10 m*M*), resting cells of an acetate oxidizing strain produced 6 μmol of CO_2 per μmol mannoside consumed. Also, significant levels of radioactivity from [1-^{14}C]acetate were found incorporated into citrate, glutamate, succinate, and aspartate by an acetate-oxidizing strain of *B. popilliae* (McKay *et al.*, 1971).

There is no significant accumulation of acetate in larval hemolymph during the infective cycle (Stubblefield *et al.*, 1966). However, Bulla and St. Julian (1972a) have shown that both healthy and infected Japanese beetle larvae readily oxidize pyruvate, acetate, succinate, and glutamate via a tricarboxylic acid cycle. Since there is a constant supply of trehalose present in hemolymph throughout the infective cycle, it would appear unnecessary for the organism to rely on acetate oxidation as an energy source for sporulation. Thus, although all of the oligosporogenous strains tested have been found to oxidize acetate *in vitro* (McKay *et al.*, 1971), this ability may have no bearing on sporulation *in vivo*. In fact, strains which have been found to oxidize acetate most rapidly will not sporulate at all in laboratory media.

As stated earlier, *B. popilliae* is facultatively anaerobic. Cells grown aerobically have both a cytochrome and a direct flavin link for the electrons from nicotinamide adenine dinucleotide, reduced form (NADH), to oxygen (Pepper and Costilow, 1965). The cytochrome-dependent system is present in the particulate fraction of cells and is sensitive to azide, cyanide, and carbon monoxide. However, spectrophotometric data revealed only cytochrome b_1 in particles from cells. The different spectra observed resembled those of anaerobically grown *B. cereus* cells. Unfortunately, no studies of the cytochromes have been conducted with *B. popilliae* grown under anaerobic conditions.

The soluble NADH oxidase of *B. popilliae* is flavin-dependent, and, when isolated from late-exponential or stationary-phase cells, it produces hydrogen peroxide (Pepper and Costilow, 1965). As we mentioned earlier, the

absence of catalase and peroxidase in *B. popilliae* cells (Steinkraus, 1957a; Pepper and Costilow, 1965) has been proposed as a possible explanation for the loss in viability that occurs to stationary cells. Of interest in this respect is the fact that cells which have initiated sporulation produce a heat-stable catalase as one of the earliest events (Costilow and Coulter, 1971). Like all other aero-tolerant organisms tested, this organism contains superoxide dismutase that can protect it from the superoxide free radical but generates H_2O_2 (Costilow and Keele, 1972; Yousten *et al.*, 1973; Yousten and Nelson, 1976).

VI. Strain Variation, Sporogenicity, and Germination

Efforts to develop sporulating strains of *B. popilliae in vitro* have been attempted since its discovery (Smith and Hadley, 1926: Hawley and White, 1935; Hadley, 1938; Dutky, 1940). Many different strains of milky disease bacteria have been isolated from naturally infected scarabaeids and characterized by microscopy and host preference. Variations in virulence of certain strains were noted by several researchers during the early investigations (see Fleming, 1968). For some strains, virulence and host preference could be modified by repeated passage through larval hosts. Steinkraus and Tashiro (1955) induced the first *in vitro* sporulation of *B. popilliae* by transferring vegetative cells grown on a complete agar medium to a solid starvation medium. These spores produced in artificial culture were much less infective perorally than spores formed naturally in larvae. Steinkraus and Provvidenti (1958) found variability among 64 isolates of *B. popilliae* that sporulated in their two-stage procedure. Even the most sporogenic strains, however, were unable to form spores after a few transfers. Rhodes *et al.* (1965) reported a new substrain, NRRL B-2309S, that produced 0.3% spores when acetate was added to a solid medium containing yeast extract, glucose, and phosphate. Sharpe *et al.* (1970) found that selected yeast extracts and filter sterilization were more influential than acetate and developed from B-2309S a second new substrain, NRRL B-2309M, which produced 20% spores in colonies. Trehalose was substituted for glucose and Müeller–Hinton broth solids were added to the medium (MYPT medium). Other investigators (Costilow and Coulter, 1971; Black, 1968b) have confirmed the sporogenicity of B-2309M. In a similar liquid MYPT medium, cells of B-2309M form 1% spores (about 7×10^6 spores/ml) during 12 days of still culture following a 24-hour shaken culture period. Spores of B-2309M appear to be nearly typical to those produced in larvae (Black, 1968b). They are infective by intrahemocoelic injection although sporulation in larvae is incomplete (E. S. Sharpe, unpublished data). The spores are not infective perorally (Schwartz and Sharpe, 1970). Vegetative cells of B-2309 and B-2309S, the parent

strains, are motile whereas B-2309M cells are nonmotile. Motile, sporogenic substrains of NRRL B-2309 have been isolated by Sharpe and St. Julian (1969); they produce 3 to 5% spores in colonies. These *in vitro* spores of substrains B-2309T and B-2309G resemble spores from larvae more than spores of the other substrains and, following intrahemocoelic injection, they produce a normal milky disease. Their peroral infectivity has not been tested. Asporogenic sectors of B-2309M colonies contain vegetative cells that do not sporulate even when injected into larvae. These cells (B-2309N) are completely asporogenic and have not remained viable in lyophil. Lüthy *et al.* (1970) were able to sporulate two strains in tissue culture medium containing hemocytes of *Phyllophage anxia*, a June beetle larva. Cells of NRRL B-2309 grew in the medium but did not form spores.

Some progress has been reported by Haynes and Rhodes (1966, 1969) on the sporulation of *B. popilliae* in liquid medium containing activated carbon. Sufficient spores for peroral infectivity tests with insects were not obtained. Interestingly, when activated carbon or 1% sodium sulfite (Dutky, 1963) was added to MYPT medium, sporulation of B-2309M in colonies was depressed. MYPT agar medium made with yeast extract that was pretreated with activated carbon supported only 3% sporulation of B-2309M strain as compared to 20% in untreated medium. When eluates from the spent carbon were recombined with pretreated yeast extract and used in MYPT medium, 11% sporulation occurred; the same eluates combined with fresh yeast extract depressed sporulation. Yeast extract may contain an inhibitor of sporulation as well as a substance that enhances sporulation. If both are absorbed by carbon, selective elution might explain the results of the recombination experiments.

Bacillus popilliae has been induced to form about 1% spores in continuous culture in a chemostat (E. S. Sharpe, unpublished data). Cultures were maintained continuously for as long as 40 days; spores appeared only at slow growth rates. Variability in the inoculum (B-2309) for continuous cultivation was expressed on solid medium as three morphologically distinct colony types which by reason of their cell content represent slow, intermediate, and rapid growth rates. Rapidly growing cells that produced the largest colony type had been inadvertently isolated in serially transferred batch cultures. Cells of intermediate growth rate were first continuously cultivated cultures and rapidly growing cells were reisolated therefrom. The slow growers have not been purified because of their tendency to progress to faster growth rates. Only the fast-growing cells from large colonies were tested and found not to be infective to Japanese beetle larvae. The variability of *B. popilliae* cultures in laboratory media has been noted by most investigators who have isolated and worked with the different substrains. E. S. Sharpe (unpublished data) found that the three different colony types (Fig. 2a) described above

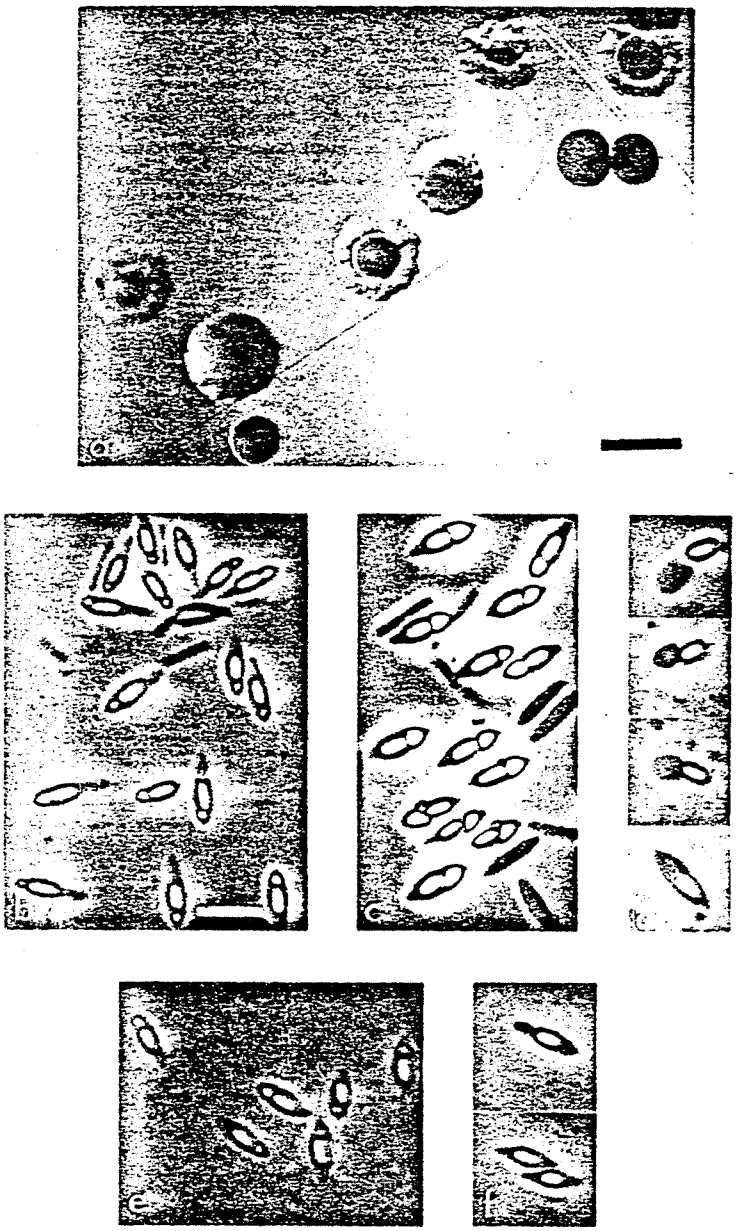

FIG. 2. Variations in *Bacillus popilliae*. (a) Three colony types resulting from outgrowth of lyophilized vegetative cells and from germinating spores of *B. popilliae*. Bar equals 2 mm. (b) Normal Type A *B. popilliae* spores untreated. Bar equals 5 μm. (c) Variant *B. popilliae* spores with large paraspores (from Connecticut). (d) Variant spores after 20 minutes in 0.1% NaOH (note phase-dark swollen paraspores). (e) Type A spores unchanged after 30 minutes in 0.1% NaOH. (f) Type A spores after 60 minutes in 1.0% NaOH (parasporal bodies lose refractility but do not swell). Magnification in c, d, e, and f same as b.

also occur when lyophilized cells return to vegetative growth and also when wild-type spores germinate. Thus, the potential for variability exists in the parent wild-type strains. Perhaps minor variations have occurred in nature without expressing any notable changes in larval disease symptoms.

Spores of *B. popilliae* are unique among related bacilli in that they germinate and outgrow poorly (St. Julian *et al.*, 1967) and outgrowth is essentially asynchronous (Sharpe and Bulla, 1977). Heat does not appreciably affect spore germination; heat causes no immediate loss of spore refractility, and outgrowth is only slightly enhanced. The extent of outgrowth of a population of unheated spores is about 1%; maximum outgrowth of heat-treated (50°C for 20 minutes) spores is only 12%. *B. popilliae* spores are not appreciably affected by germinants, and their thick-walled sporangia are impermeable to lytic agents. Adenosine and L-alanine alone or in combination with glucose increase germination and outgrowth by about 3–10%; sporogangia of the bacteria are not physically altered by lysozyme, trypsin, or crude snail (*Helix pomatia*) enzyme. The addition of pyruvate to complex media has been reported to increase the percentage of spores that germinate (Costilow and Coulter, 1971). Divalent cations may be needed for germination (Splittstoesser and Farkas, 1966), but they are not necessary for outgrowth.

VII. Pathology

Bacillus popilliae is a true pathogen. It meets all criteria of Koch's postulates with regard to milky disease of the Japanese beetle: (1) the organism is always found in diseased larvae but is not present in healthy ones; (2) the organism can be cultivated in pure culture outside the larvae; (3) pure cultures, when inoculated into susceptible insects, initiate the characteristic disease symptoms; and (4) the organism can be reisolated from the experimental larvae and recultured artificially, retaining the characteristics of the originally isolated organism.

The infectious process develops in four phases according to St. Julian *et al.* (1970). There is an initial incubation phase (phase I) of about 2 days in which relatively few bacterial cells are found. From day 3 to day 5 (phase II), vegetative cells rapidly proliferate, and by day 5 prespore forms occur as well as an occasional spore. Phase III is the intermediate phase (days 5–10) in which there is a change from predominantly vegetative growth to prespore and spore development. Phase IV is the sporulation phase that terminates by day 14 to day 21 and involves massive sporulation and ensuing larval death. About 20% of cells at days 3–5 sporulate. During the intermediate phase, prespore forms constitute 20–35% of the total population at days 5 and 6, and they increase to 40–80% by day 10. Further development of prespores during phase IV is characterized by decreasing numbers of vegetative cells and prespores while large numbers of spores accumulate. In this terminal phase,

an individual larva will contain approximately 5×10^9 spores (5×10^{10} per ml of hemolymph) and appears milky. Obviously, sporulation of *B. popilliae* within the Japanese beetle larva is not a synchronous process. There is no exponential phase of exclusive vegetative proliferation followed by a stationary phase and sporulation cycle; instead, vegetative growth and sporulation proceed concomitantly. Sharpe and Rhodes (1973) have shown that sporulation in colonies proceeds in a stepwise manner and proposed that sporulation in larvae becomes more efficient as the disease progresses.

It is not clear how many spores are required to induce milky disease but it is highly unlikely that a single spore can do so. Dutky (1940) observed a graded response by the insect to single larval dosages of 1×10^2 to 2×10^6 administered parenterally. Beard (1945) found that soil containing 4×10^6 fresh spores per gram would cause the death of about 96% of larvae feeding in the soil. Apparently, the age of spores influences their virulence because only 50% of larvae are killed by 4×10^6 aged spores of *B. popilliae* when injected. By feeding, 1×10^9 aged spores per gram of soil are required to kill the same number of larvae (St. Julian and Hall, 1968; St. Julian *et al.*, 1970). In contrast, as few as 140 healthy vegetative cells, when injected into the hemocoel, can disease 63% of a larval population.

Although laboratory-infected larvae generally die during phase IV of milky disease, naturally infected (field-infected) larvae do not (St. Julian *et al.*, 1972). Larvae infected in field plots die during all phases of the disease. The largest percentage of death is during phases II and III. At phase II, 90% of the total cell population in the hemolymph are vegetative cells and at phase III, 65–75% are vegetative cells with 15–30% spores. Most larvae whether dead or alive exhibit the intermediate phase of infection (phase III); less than 30% of larvae reach phase IV of disease. Consequently, buildup of *B. popilliae* spores in experimental field plots does not occur as rapidly as predicted from laboratory studies.

The recent resurgence of Japanese beetles in Connecticut (Dunbar and Beard, 1975b) deserves attention because it appears to be due to the emergence and increasing dominance of a variant *B. popilliae* strain. Dunbar and Beard (1975b) reported that in many areas where Japanese beetles had been controlled by milky disease for 25 years, they are now as numerous as they were originally. The insect has developed resistance to chlordane in Connecticut (Dunbar and Beard, 1975a), in Ohio (Niemczyk and Lawrence, 1973), and in New York (Tashiro and Newhouser, 1973). It is possible that traces of chlordane in the insect hemolymph, where the bacterium must spend its life cycle, have diminished its infective capacity. Also, if chlordane resistance is due, in part, to a thickening of chitinous layers of the integument and the gut, invasion of the hemocoel would be more difficult. The reemergence of heavy infestations are probably due to loss in virulence of *B.*

popilliae in Connecticut because cross-infection studies indicate that larvae from Connecticut are susceptible to *B. popilliae* spores from New York and Ohio whereas spores from Connecticut are not very infective to larvae from elsewhere.

M. G. Klein, Japanese Beetle Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Wooster, Ohio, and E. S. Sharpe (unpublished data) have determined that a new variant strain of *B. popilliae* is present in diseased larvae from Connecticut. The parasporal bodies of this strain are unusually large for *B. popilliae*, and many of the spores are accompanied by two or three such bodies. Infectivity of the variant strain (when injected) is diminished, and diseased larvae contain elongated hyphal-like cells. Sporulation in diseased larvae is only 20% of that expected. The large parasporal bodies of the Connecticut variant are sensitive to 0.05% NaOH; upon treatment, they lose refractility and swell two to three times their original size. Authentic NRRL B-2309 spores produced in larvae are sensitive only to higher concentrations of alkali (0.1% NaOH) and do not swell when they lose refractility (Fig. 2b–f). Hemolymph collected from diseased larvae in Connecticut during 1974 and 1975 seldom showed evidence of the full-blown sporulation that is characteristic of normal milky disease. Hemolymph from other larvae collected in Connecticut as late as 1975 contain normal Type A *B. popilliae* spores, parasporal bodies, and vegetative cells in expected ratios and quantities. Thus, a normal milky disease exists in the area but the dense larval population seems to be resistant to both strains. Evidence that the resurgence of beetles in Connecticut has been caused by a variant strain of *B. popilliae* has raised some questions. First, what has happened to the Type A spore inoculum that was recently present in many of the areas that are now infected with beetles? Second, how can a supposedly less-virulent strain become dominant in the presence of the original virulent Type A strain?

VIII. Summary

Bacillus popilliae represents a unique category among bacteria. It is gram-variable, facultatively anaerobic, catalaseless, insecticidal, and it forms endospores along with parasporal crystals. Whether parasporal crystals are toxic to larvae is not yet fully determined although some data (unpublished) indicate that there is pathogenicity associated with the crystals. Also, characterization of the inclusion bodies is incomplete although we do know that they are predominantly protein and can be separated into three cathodic components when solubilized crystals are subjected to high-voltage electrophoresis. They contain no lipid or nucleic acids but do contain glycoprotein components (Weiner *et al.*, 1974). Of the 17 amino acids present,

glutamic acid and aspartic acid predominate, followed in order of quantity by leucine, valine, glycine, threonine, isoleucine, alanine, and arginine; no unusual amino acids are present (Bulla *et al.*, 1975).

The cause(s) of larval death during the intermediate and terminal stages of milky disease is not explained. A search for toxic compounds, exclusive of the parasporal crystal, elaborated by the bacterium has proven futile. Physiological starvation of larvae during the infectious process (Bulla and St. Julian, 1972b) has not been proved, although there is evidence that the trophocyte cells of the fat body are depleted of lipid globules during the disease (Sharpe, 1976).

What prompts efficient sporulation of *B. popilliae* exclusive of or within its insect host also is not known. Attempts to determine the factor(s) that regulates spore formation *in vivo* and to simulate the sporulation process in liquid artificial cultures have been unsuccessful. Currently, the most efficient means of producing spores of *B. popilliae* for use as a commercial microbial insecticide is by artificially infecting individual larvae. Obviously, such an operation is costly and, therefore, large-scale fermentation production of the spores would considerably reduce the costs. A much clearer understanding of the metabolism of both the insect host and the bacterial pathogen as well as some insight of the regulatory mechanisms that control bacterial sporogenesis during the infectious process are needed to facilitate the development of a successful industrial fermentation for production of *B. popilliae* spores.

References

Beard, R. L. (1945). *Conn. Agric. Exp. Stn., New Haven, Bull.* 491, 505–581.

Beard, R. L. (1956). *Can Entomol.* 88, 640–647.

Bennett, G. A., and Shotwell, O. L. (1973). *Biotechnol. Bioeng.*, 15, 1023–1037.

Bhumiratana, A., and Costilow, R. N. (1973). *Can. J. Microbiol.* 19, 169–176.

Bhumiratana, A., Anderson, R. L., and Costilow, R. N. (1974). *J. Bacteriol.* 119, 484–493.

Black, S. H. (1968a). *J. Invertebr. Pathol.* 12, 148–157.

Black, S. H. (1968b). *J. Invertebr. Pathol.* 12, 158–167.

Black, S. H., and Arredondo, M. J. (1966). *Experientia* 22, 77–78.

Buchanan, R. E., and Gibbons, N. E., eds. (1974). "Bergey's Manual of Determinative Bacteriology," 8th ed. Williams & Wilkins, Baltimore, Maryland.

Bulla, L. A., Jr. (1976). *Spore Research.* Academic Press, London, 1975.

Bulla, L. A., Jr., and St. Julian, G. (1972a). *J. Invertebr. Pathol.* 19, 120–124.

Bulla, L. A., Jr., and St. Julian, G. (1972b). *Spores* 5, 191–196.

Bulla, L. A., Jr., St. Julian, G., Rhodes, R. A., and Hesseltine, C. W. (1969). *Appl. Microbiol.* 18, 490–495.

Bulla, L. A., Jr., Bennett, G. A., and Shotwell, D. L. (1970a). *J. Bacteriol.* 104, 1246–1253.

Bulla, L. A., Jr., St. Julian, G., Rhodes, R. A., and Hesseltine, C. W. (1970b). *Can. J. Microbiol.* 16, 243–248.

Bulla, L. A., Jr., St. Julian, G., and Rhodes, R. A. (1971). *Can. J. Microbiol.* **17**, 1073–1079.
Bulla, L. A., Jr., Rhodes, R. A., and St. Julian, G. (1975). *Annu. Rev. Microbiol.* **29**, 163–190.
Costilow, R. N., and Coulter, W. H. (1971). *Appl. Microbiol.* **22**, 1076–1084.
Costilow, R. N., and Keele, B. B., Jr. (1972). *J. Bacteriol.* **111**, 628–630.
Costilow, R. N., Sylvester, C. J., and Pepper, R. E. (1966). *Appl. Microbiol.* **14**, 161–169.
Coulter, W. H., and Costilow, R. N. (1970). *Can. J. Microbiol.* **16**, 801–807.
Dunbar, D. M., and Beard, R. L. (1975a). *Conn., Agric. Exp. Stn., Bull.* **757**, 1–5.
Dunbar, D. M., and Beard, R. L. (1975b). *J. Econ. Entomol.* **68**, 453–457.
Dutky, S. R. (1940). *J. Agric. Res.* **61**, 57–68.
Dutky, S. R. (1963). *In* "Insect Pathology: An Advanced Treatise" (E. A. Steinhaus, ed.), Vol. 2, pp. 75–115. Academic Press, New York.
Faust, R. M., and Travers, R. S. (1975). *Proc. Soc. Invertebr. Pathol.* p. 23.
Fleming, W. E. (1968). *U.S., Dep. Agric., Tech. Bull.* **1383**, 1–78.
Gordon, R. E., Haynes, W. C., and Pang, C. H. N. (1973). *U.S., Dep. Agric., Agric. Handb.* **427**.
Hadley, C. H. (1938). *J. N.Y. Entomol. Soc.* **46**, 203–216.
Hawley, J. M., and White, G. R. (1935). *J. N.Y. Entomol. Soc.* **43**, 405–412.
Haynes, W. C., and Rhodes, L. J. (1966). *J. Bacteriol.* **91**, 2270–2274.
Haynes, W. C., and Rhodes, L. J. (1969). *J. Invertebr. Pathol.* **13**, 161–166.
Hrubant, G. R., and Rhodes, R. A. (1968). *J. Invertebr. Pathol.* **11**, 371–376.
Kaneda, T. (1969). *J. Bacteriol.* **98**, 143–146.
Klein, M. G., Johnson, C. H., and Ladd, T. L., Jr. (1976). *Bull. Entomol. Soc. Am.* **22**, 305–310.
Kreig, A. (1961). "Grundlagen Der Insektenpathologie." Steinkopff, Darmstadt.
Krywienczyk, J., and Lüthy, P. (1974). *J. Invertebr. Pathol.* **23**, 275–279.
Lüthy, P. (1968). *Zentralbl. Bakteriol., Parasitenkd., Infectionkr. Hyg., Abt. 2* **122**, 671–711.
Lüthy, P., and Krywienczyk, J. (1972). *J. Invertebr. Pathol.* **19**, 163–165.
Lüthy, P., Wyss, C., and Ettlinger, L. (1970). *J. Invertebr. Pathol.* **16**, 325–330.
McKay, L. L., Bhumiratana, A., and Costilow, R. N. (1971). *Appl. Microbiol.* **22**, 1070–1075.
Milner, R. J. (1974). *Aust. J. Biol.* **27**, 235–247.
Niemczyk, H. D., and Lawrence, K. O. (1973). *J. Econ. Entomol.* **66**, 520–521.
Pepper, R. E., and Costilow, R. N. (1964). *J. Bacteriol.* **87**, 303–310.
Pepper, R. E., and Costilow, R. N. (1965). *J. Bacteriol.* **89**, 271–276.
Rhodes, R. A. (1965). *Bacteriol. Rev.* **29**, 373–381.
Rhodes, R. A. (1968). *In* "Proceedings of the Joint U.S.-Japan Seminar on Microbial Control of Insect Pests," pp. 85–92. Kyushu University, Fukuoka, Japan.
Rhodes, R. A., Roth, M. S., and Hrubant, G. R. (1965). *Can. J. Microbiol.* **11**, 779–783.
Rhodes, R. A., Sharpe, E. S., Hall, H. H., and Jackson, R. W. (1966). *Appl. Microbiol.* **14**, 189–195.
St. Julian, G., and Bulla, L. A., Jr. (1973). *Curr. Top. Comp. Pathobiol.* **2**, 57–87.
St. Julian, G., and Hall, H. H. (1968). *J. Invertebr. Pathol.* **10**, 48–53.
St. Julian, G., Pridham, T. G., and Hall, H. H. (1967). *Can. J. Microbiol.* **13**, 279–285.
St. Julian, G., Sharpe, E., and Rhodes, R. A. (1970). *J. Invertebr. Pathol.* **15**, 240–246.
St. Julian, G., Bulla, L. A., Jr., and Adams, G. L. (1972). *J. Invertebr. Pathol.* **20**, 109–113.
St. Julian, G., Bulla, L. A., Jr., and Hanson, R. S. (1975). *Appl. Microbiol.* **30**, 20–25.
Schwartz, P. A., Jr., and Sharpe, E. S. (1970). *J. Invertebr. Pathol.* **15**, 126–128.
Sharpe, E. S. (1976). *Proc. Soc. Invertebr. Pathol.* (in press).
Sharpe, E. S., and Bulla, L. A., Jr. (1977). *J. Invertebr. Pathol.* **30**, 242–248.
Sharpe, E. S., and Rhodes, R. A. (1973). *J. Invertebr. Pathol.* **21**, 9–15.
Sharpe, E. S., and St. Julian, G. (1969). *Bacteriol Proc.* **A111**, 17.

Sharpe, E. S., St. Julian, G., and Crowell, C. (1970). *Appl. Microbiol.* **19**, 681–688.
Shotwell, O. L., Bennett, G. A., Hall, H. H., VanEtten, C. H., and Jackson, R. W. (1963). *J. Insect Physiol.* **9**, 35–42.
Shotwell, O. L., Bennett, G. A., Hall, H. H., Stubblefield, R. D., Peters, J. E., VanEtten, C. H., and Jackson, R. W. (1965). *J. Insect Physiol.* **11**, 671–682.
Smith, L. B., and Hadley, C. H. (1926). *U.S., Dep. Agric., Circ.* 363, 1–67.
Splittstoesser, D. F., and Farkas, D. F. (1966). *J. Bacteriol.* **92**, 995–1001.
Steinkraus, K. H. (1957a). *J. Bacteriol.* **74**, 621–624.
Steinkraus, K. H. (1957b). *J. Bacteriol.* **74**, 625–652.
Steinkraus, K. H., and Provvidenti, M. L. (1958). *J. Bacteriol.* **75**, 38–42.
Steinkraus, K. H., and Tashiro, H. (1955). *Science* **121**, 873–874.
Steinkraus, K. H., and Tashiro, H. (1967). *Appl. Microbiol.* **15**, 325–333.
Stubblefield, R. D., Bennett, G. A., Shotwell, O. L., Hall, H. H., and Jackson, R. W. (1966). *J. Insect Physiol.* **12**, 949–956.
Sylvester, C. J., and Costilow, R. N. (1964). *J. Bacteriol.* **87**, 114–119.
Tashiro, H., and Newhouser, W. (1973). *Search: Agric.* **3**, 1–6.
Weiner, B. A., Kwolek, W. F., St. Julian, G., Hall, H. H., and Jackson, R. W. (1966). *J. Invertebr. Pathol.* **8**, 308–313.
Weiner, B. A., St. Julian, G., and Kwolek, W. F. (1969). *J. Invertebr. Pathol.* **13**, 250–255.
Weiner, B. A., Bulla, L. A., Jr., Rhodes, R. A., and Yousten, A. A. (1974). *Proc. Soc. Invertebr. Pathol.* p. 27.
Wille, H. (1956). *Mitt. Schweiz. Entomol. Ges.* **29**, 271–282.
Wyss, C. (1971). *Zentralbl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt.* **2 126**, 461–492.
Yousten, A. A., and Nelson, K. D. (1976). *J. Gen. Appl. Microbiol.* **22**, 161–164.
Yousten, A. A., Bulla, L. A., Jr., and McCord, J. M. (1973). *J. Bacteriol.* **113**, 524–525.
Yousten, A. A., Hanson, R. S., Bulla, L. A., Jr., and St. Julian, G. (1974). *Can. J. Microbiol.* **20**, 1729–1734.