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LIST OF CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

DANIEL M. ADAMS, *Department of Food Science, North Carolina State University, Raleigh, North Carolina* (245)

H. BABICH, *Department of Biology, New York University, New York, New York* (55)

L. R. BEUCHAT, *Department of Food Science, University of Georgia Agricultural Experiment Station, Experiment, Georgia* (219)

LEE A. BULLA, JR., *U.S. Grain Marketing Research Center, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, Kansas* (1)

F. F. BUSTA, *Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota* (195)

M. C. CADMUS, *Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois* (19)

RALPH N. COSTILOW, *Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan* (1)

DONALD P. COX, *Chemicals and Plastics Division, Union Carbide Corporation, South Charleston, West Virginia* (173)

R. F. GOMEZ, *Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts* (263)

T. R. GRAUMLICH, *Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan* (203)

YOUN W. HAN, *Agricultural Research Service, U.S. Department of Agriculture, Department of Microbiology, Oregon State University, Corvallis, Oregon* (119)

LLOYD G. HERMAN, *Environmental Safety Branch, National Institutes of Health, Bethesda, Maryland* (155)

S. E. MARTIN, *Department of Food Science, University of Illinois, Urbana, Illinois* (263)

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Biology of *Bacillus popilliae*

LEE A. BULLA, JR.

U.S. Grain Marketing Research Center,
Agricultural Research Service, U.S. Department of Agriculture,
Manhattan, Kansas

RALPH N. COSTILOW

Department of Microbiology and Public Health,
Michigan State University
East Lansing, Michigan

AND

EUGENE S. SHARPE

Northern Regional Research Center,
Agricultural Research Service, U.S. Department of Agriculture,
Peoria, Illinois

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 × 10²			
	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.

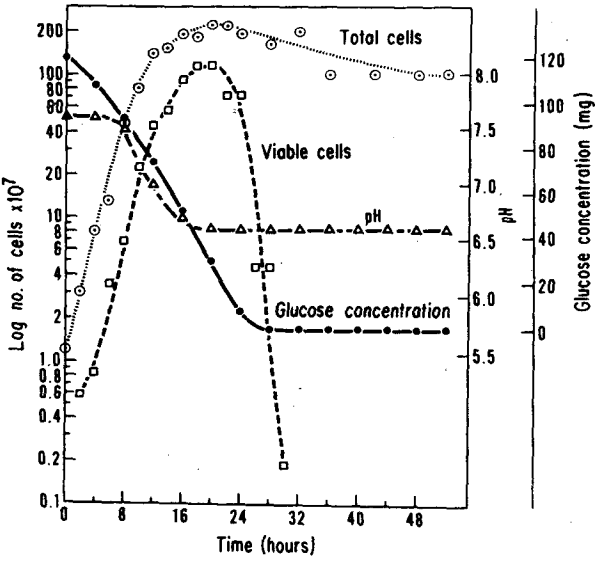

FIG. 1. Characteristic growth pattern of *Bacillus popilliae* in MD medium of 1.5% yeast extract, 0.6% K_2HPO_4, and 0.2% glucose in distilled water. ⊙, total cells; △, pH; □, viable cells; ●, glucose concentration.

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.

V. Metabolism

As mentioned earlier, *B. popilliae* requires sugar for growth and utilizes trehalose found in larval hemolymph more rapidly than it does glucose in artificial culture. The uptake and subsequent cleavage of trehalose is dependent upon the activity of a PEP:sugar phosphotransferase system. Free trehalose is not hydrolyzed by the organism, and the formation of trehalose 6-phosphate appears to be completely dependent on the presence of PEP as a phosphoryl donor (Bhumiratana *et al.*, 1974).

The metabolic pattern with glucose is not as clear. At high substrate concentrations, the oxidation of glucose is inhibited only about 50% by 0.01 *M* sodium fluoride, a potent inhibitor of enolase, whereas trehalose oxidation is inhibited completely (Bhumiratana and Costilow, 1973). However, when glucose concentrations are low ($<10^{-4}$ *M*), the inhibition is complete. Also, uptake studies with the glucose analogue, methyl α-D-glucoside, which is not metabolized by glucose-grown cells, indicate that when these sugars are present at low concentrations, their uptake also depends upon PEP-dependent phosphotransferase (D. C. Taylor and R. N. Costilow, unpublished data).

The maltose oxidation enzymes in this organism are constitutive, but the uptake of maltose appears to be by simple diffusion. There is no evidence of saturation of the uptake system when varied from 10^{-5} to 10^{-3} *M*. Also, the growth rate of *B. popilliae* is limited by maltose concentrations up to about 10^{-2} *M*, and there is no repression of sporulation of the oligosporogenous strain NRRL B-2309M until the maltose concentration exceeds 0.2%. In contrast, glucose strongly represses sporulation of this strain at concentrations in excess of 0.1%. Presumably, the differences observed are due to the uptake systems involved because maltose is oxidized as rapidly as glucose if the maltose concentration is sufficiently high (D. C. Taylor and R. N. Costilow, unpublished data).

Some strains of *B. popilliae* are inducible for the utilization of both methyl α-D-glucoside and methyl α-D-mannoside (Bhumiratana and Costilow, 1973). The latter compound is growth-limiting probably because it is hydrolyzed at such a slow rate. Apparently, the mannoside is cleaved by a glucosidase that has a low affinity for the mannoside. Concentrations of methyl α-D-mannoside as high as 0.5% can be used in the sporulation medium for strain B-2309M without any repression of sporulation.

Bacillus popilliae metabolizes glucose via the Embden–Myerhof–Parnas (EMP) and pentose phosphate (PP) pathways (Pepper and Costilow, 1964; Bulla *et al.*, 1970b). Under anaerobic conditions, *B. popilliae* cells use the EMP system exclusively (Bulla, 1976). Cells produced in laboratory media preferentially use the EMP system, whereas cells harvested from infected

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*.