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Characteristics of the Vegetative Growth of *Bacillus popilliae*

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ABSTRACT

RHODES, R. A. (Northern Regional Research Laboratory, Peoria, Ill.), E. S. SHARPE, H. H. HALL, AND R. W. JACKSON. Characteristics of the vegetative growth of *Bacillus popilliae*. Appl. Microbiol. **14**:189–195. 1966.—Growth characteristics of the insect pathogen, *Bacillus popilliae* Dutky, were studied by propagation in shaken flasks and in 2-liter fermentors. Maximal populations between 5×10^8 and 2×10^9 viable cells per milliliter of culture medium routinely were obtained in incubation periods of 18 to 24 hr at 30 C in a medium composed of 1.5% yeast extract, 0.6% K_2HPO_4, and 0.2% glucose or trehalose. The carbohydrate required for growth in liquid media was fermented with the formation of 2 meq of acid per mmole of carbohydrate utilized; acid products ordinarily were not subsequently metabolized. *B. popilliae* is an aerobe, and the amount of growth obtained varied with aeration to an optimum at oxygen absorption rates of about 0.5. Maximal populations persist in a culture for periods of only 1 to 4 hr; cessation of growth was followed immediately by rapid death of cultures, so that less than 1% of the cells remained viable after 48 hr, and viability often was lost entirely by the end of 72 hr of incubation. No cytological evidence for spore formation was observed under any growth condition. Death was not associated with lysis of the cells, although extensive granulation ultimately occurred. Continuous neutralizaiton, augmented buffering, various techniques of dialysis, or slow feeding of the carbohydrate did not markedly alleviate the characteristic death of the cultures.

Bacillus popilliae Dutky is a causative agent for type A milky disease which afflicts the Japanese beetle (*Popillia japonica* Newman). In nature, billions of cells accumulate in the hemolymph of the insect larva as a consequence of the vegetative proliferation of the organism during the 2- or 3-week course of the disease. Most of the cells sporulate in the hemolymph by the time the insect larva dies. Spores are the means by which the pathogen survives in the soil and transmits the disease. A method developed by Dutky for the artificial production of infectious spores entails injection of large numbers of larvae and subsequent harvest of spores that have accumulated in the hemolymph at the terminal stage of the disease. Data obtained with such spores in field trials clearly indicate the potential of *B. popilliae* as a biological control agent [summarized by Dutky (2)].

However, use of this pathogen for control of the insect required a more effective method of producing large numbers of spores. In turn, the production of infective spores requires first the efficient cultivation of virulent vegetative cells. *B. popilliae* is considered fastidious, and cultivation of it led to complex media (11), prolonged incubation periods (2), or other techniques unsuited to large-scale production. No definitive information is available on the characteristics of the growth of the organism in vitro or on the factors that significantly influence vegetative proliferation. It is the purpose of this paper to describe the vegetative growth pattern of this pathogen.

MATERIALS AND METHODS

Cultures. Results obtained with three strains of *B. popilliae* are reported. Strains NRRL B-2043 and NRRL B-2309 originated as spores from the hemolymph of infected larvae. Pure cultures were isolated by germination of the spores, and the resultant vegetative cultures were maintained as lyophilized preparation (5). A substrain, NRRL B-2309A, was selected because certain characteristics varied from those of the correspondingly numbered parent strain (6). The vegetative growth characteristics of these strains are typical of all strains of *B. popilliae* examined, although the three differ in pathogenicity when tested by inoculation of larvae with vegetative cells.

Activity growing cultures were maintained for experimental use by frequent transfer of vegetative

growth in liquid media. The frequency of transfer necessary for maintenance depended upon the rate and extent of growth. Ordinarily, stock cultures were transferred at biweekly intervals; small inocula and incubation temperatures below optimum were used to retard the rate of growth. Inocula of maximal vigor were obtained from such stock cultures by transfer at 20- to 24-hr intervals for several days before use.

Medium. Unless otherwise noted, the basic medium used for growth of the milky disease organisms was composed of 1.5% yeast extract (Difco), 0.3% K_2HPO_4, and 0.2% glucose; 0.5% tryptone (Difco) was added to the medium for maintenance of stock cultures. Medium components, exclusive of glucose, were dispensed into flasks and sterilized for 15 min at 121 C. Carbohydrates were sterilized separately as 2% solutions and added before inoculation because autoclaving the carbohydrate in the medium retarded growth. The *p*H of the medium after addition of glucose was 7.2 to 7.4. The basal medium solidified with 2% agar was used for plate counts. Agar media were sterilized in 200-ml volumes in screw-cap bottles, and the carbohydrate was added to the warm medium immediately before the plates were poured. Plates were dried at room temperature for 24 to 72 hr before use.

Procedure. The growth characteristics of *B. popilliae* initially were studied in flask cultures (90 ml of medium in 500-ml Erlenmeyer flasks) incubated on a rotary shaker [2.5-inch (6.3-cm) stroke, 200 rev/min]. A 5% inoculum from a 20- to 24-hr culture was used.

Growth of *B. popilliae* also was studied in 2-liter fermentors (Fig. 1). The assembled fermentor containing 2 liters of medium was sterilized by autoclaving for 30 min at 121 C. When *p*H electrodes were to be used, the fermentor was sterilized empty by autoclaving, the *p*H electrodes were inserted into the empty, sterile fermentor, and the whole assembly was sterilized again with ethylene oxide; the medium was sterilized separately by autoclaving and then aseptically pumped into the fully assembled fermentors. Automatic *p*H control was achieved with 1 N NaOH added aseptically by means of a solenoid valve and a Radiometer Titrimeter model TT. Fermentor cultures

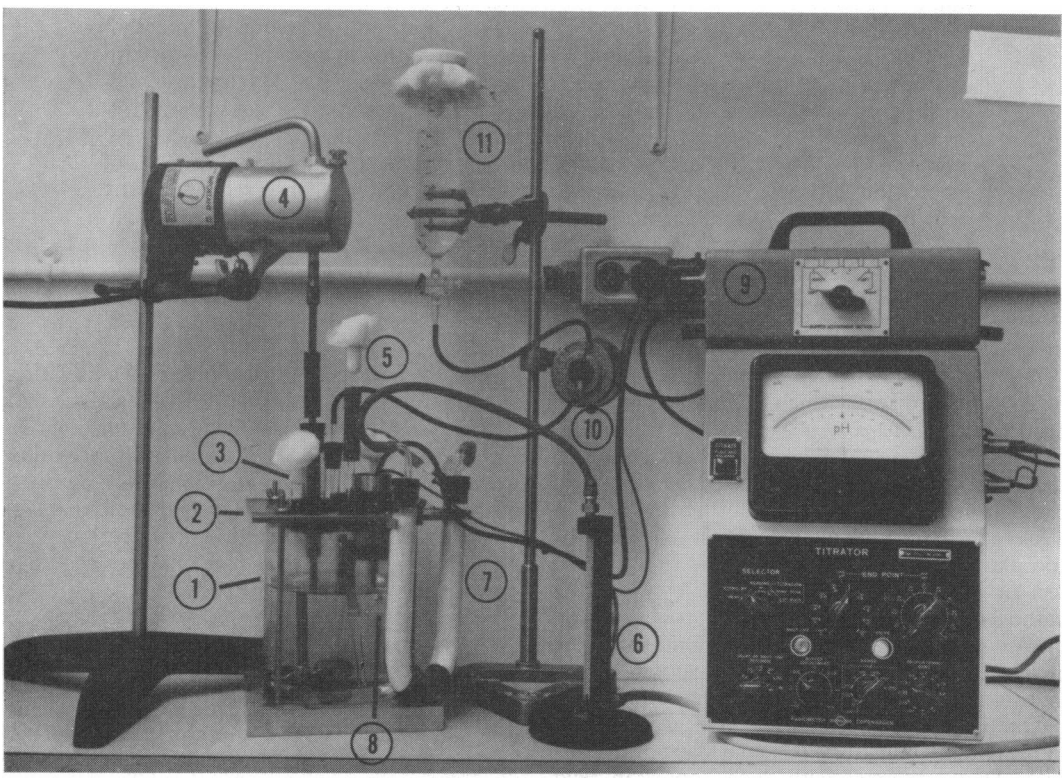

FIG. 1. *Laboratory fermentor used for studies on the growth characteristics of Bacillus popilliae. (1) Pyrex glass vessel, open top flat ground with fire polished edge; (2) ported stainless-steel top and clamping bolts, entry ports tapered to fit suitable rubber stoppers, top sealed to glass vessel with neoprene gasket; (3) exhaust gas port with cotton plug; (4) variable speed stirring motor; (5) port for sampling fermentor liquid; (6) air flow meter; (7) secondary glass-wool air filter, attached and sterilized with fermentor; (8) pH electrodes, sealed in single rubber stopper; (9) automatic titrimeter; (10) solenoid valve controlling alkali flow; (11) alkali reservoir. A second reservoir and solenoid valve assembly actuated by an interval timer was used for slow feed of glucose or other sterile nutrients. Carbon-packed primary air filter and pressure-regulating valves are not shown.*

were agitated with a propeller blade stirrer or, infrequently, only by sparged air flow. A silicone antifoam was added as required; usually foaming occurred only during the initial hours of growth, and a single addition was satisfactory.

All cultures were incubated at 30 C, since preliminary work indicated that maximal populations were most rapidly obtained at this temperature. The results of infectivity tests of the milky disease organisms in the Japanese beetle by Dutky (2) indicated that growth in vivo similarly was maximal at 30 C, although infected larvae survived somewhat longer at slightly lower temperatues. Dutky suggested 27 C as most suitable for work with the infectious process, because the prolonged survival of the host allowed greater numbers of spores to develop.

Viable counts of cultures were made by distributing 0.3 ml of an appropriate dilution in 0.1% tryptone with a glass spreader on the surface of prepared plates. Colonies were counted after incubating the plates at 30 C for 7 days. Optical density measurements were made at 645 mμ. Glucose was determined by reducing power or with glucose oxidase. Oxygen absorption rates were determined by the sulfite-oxidation method (1).

RESULTS

The characteristic pattern of growth of *B. popilliae* is plotted in Fig. 2. Maximal growth as measured by viable-cell count, optical density, or direct microscopic count was achieved in 16 to 20 hr under optimal conditions of culture. A maximum of about 5×10^8 viable cells per milliliter of culture medium was achieved in flask cultures. Characteristically, the point of maximal cell population of this organism was followed immediately by a rapid decline in numbers of viable cells. Usually, fewer than 10^3 viable cells per milliliter remained after 48- to 72-hr incubation; indeed, it was often not possible to detect viable cells in rapidly growing cultures incubated longer than 72 hr. During the period of decline in viable count, no significant decrease occurred in microscopic count or in optical density of cultures; therefore, cells that die do not lyse. By the end of 72-hr incubation periods the vegetative cells developed extensive internal granulation, but microscopically they appeared otherwise intact. No cytological evidence of spore formation was observed.

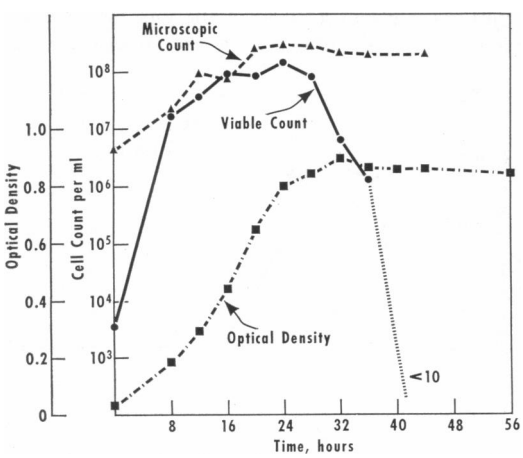

FIG. 2. *Pattern of growth of Bacillus popilliae NRRL B-2043 in shaken flask culture. Flasks inoculated with 5% of a 20-hr shaken-flask culture and incubated on a rotary shaker.*

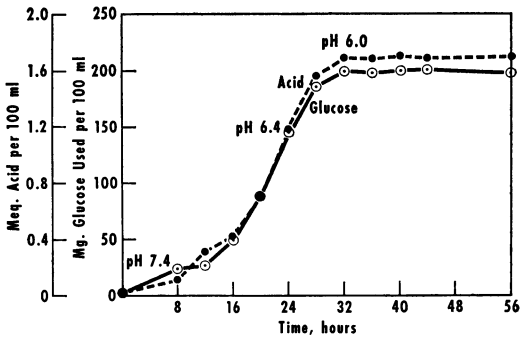

FIG. 3. *Glucose utilization and acid production during growth of Bacillus popilliae NRRL B-2043. Analyses were done on samples taken for growth measurements show in Fig. 2. Glucose was analyzed by copper reduction, acid by titration to pH 7 with a pH meter; the pH of the cultures at representative periods of growth is shown.*

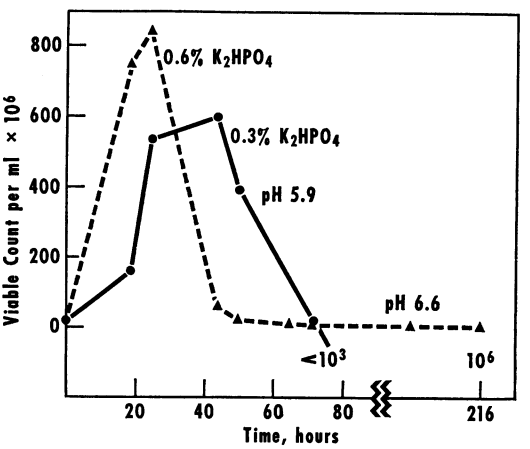

FIG. 4. *Effect of buffer concentration on the growth and survival of Bacillus popilliae NRRL B-2309 propagated in 2-liter fermentors. Final pH values for the fermentations are given. Fermentors inoculated with 5% of a 24-hr shaken-flask culture; 0.5 volume per volume per minute aeration, no pH control.*

Appreciable growth of *B. popilliae* in liquid media occurred only in the presence of ferment-

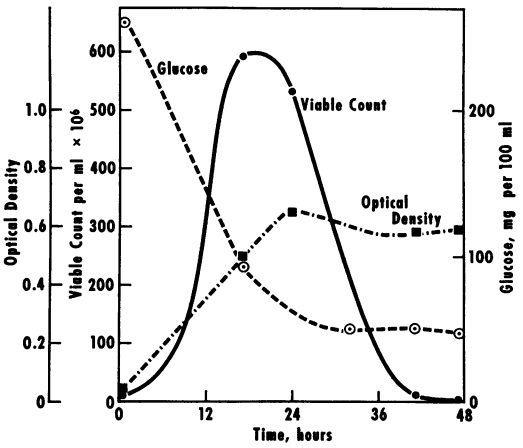

FIG. 5. *Growth of Bacillus popilliae NRRL B-2309 in 2-liter fermentors with pH controlled by automatic addition of NaOH. Fermentors inoculated with 5% of a 24-hr shaken-flask culture; 0.5 volume per volume per minute aeration.*

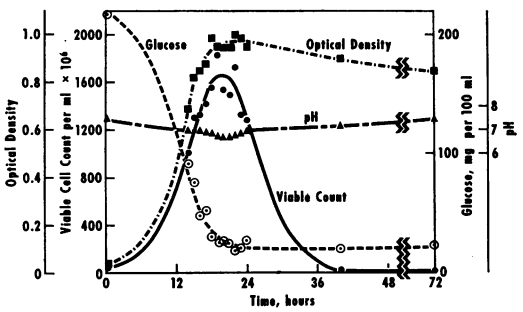

FIG. 6. *Maximal rate of growth and population of Bacillus popilliae obtained in 2-liter fermentors. Strain NRRL B-2309A, fermentors inoculated with 5% inoculum activated by 5% transfer at 18-hr intervals in shaken flasks; 0.5 volume per volume per minute aeration, no pH control, 0.6% K_2HPO_4 buffer. Use of acetate by this strain is illustrated by the pH values.*

able carbohydrate. Metabolism of the carbohydrate resulted in the formation of about 2 meq of acid per mmole of carbohydrate dissimilated (Fig. 3). The acids formed were acetate and lactate; the ratio of the two depended upon the amount of oxygen supplied to the culture (6). The milky disease organisms did not initiate growth below *p*H 6.5 to 6.8. In growing cultures, cell proliferation ceased at about *p*H 6.2 to 6.4, although carbohydrate dissimilation typically continued until accumulation of acid lowered the *p*H to 5.8 to 6.0.

One limitation on growth of the organisms in vitro was the accumulation of acid. The *p*H of larval hemolymph is unchanged during infection (10). Appropriate buffering of the medium delayed the deleterious effect of acid, and somewhat enhanced growth was obtained (Fig. 4). Phosphate buffer appeared to be the most suitable to control *p*H, but concentrations above 0.6% (w/v) retarded growth. Although higher *p*H values were maintained and greater populations were attained in growth media containing more buffer, the characteristic loss of viability was essentially unchanged. In the example cited, only about 10^6 cells per milliliter survived after 44 hr of incubation. The surviving fraction of the population remained for extended periods with slowly decreasing numbers.

Similarly, control of the *p*H in fermentors by automatic or manual addition of alkali did not alter the characteristic pattern of growth and death of the organism (Fig. 5). The *p*H of the medium rose slightly during the death phase of the culture when a neutral *p*H was maintained during the growth period. Whether the increase in *p*H of cultures grown at neutrality resulted from the catabolism of acidic products or from release into the medium of basic metabolic products, such as ammonia, is not known. Strain B-2309 used in this example does not oxidize acetate or lactate. Acetate oxidation following vegetative proliferation is characteristic of other aerobic spore formers and is associated with their sporogenic processes (4). Of the strains of *B. popilliae* studied by Pepper and Costilow (6), only the single variant strain B-2309A oxidized acetate. Oxidation of acetate by this strain occurred only above *p*H 7 in an oxygen atmosphere. Strains which do not oxidize acetate lack the condensing enzyme found in the acetate-oxidizing strain. When strain B-2309A was grown in fermentors with or without *p*H control, cessation of growth and complete utilization of the glucose were followed by a definite rise in *p*H, indicating that

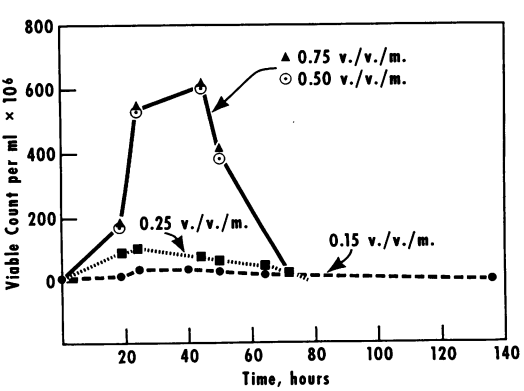

FIG. 7. *Effect of aeration on the growth of Bacillus popilliae NRRL B-2309 in 2-liter fermentors. Fermentors inoculated with 5% of a 24-hr shaken-flask culture; no pH control.*

acetate was being used by the cells (Fig. 6). The apparent metabolism of acetate did not alter the pattern of cell proliferation and death, characteristic of all *B. popilliae* strains.

Dutky has held that the milky disease organisms are facultative anaerobes. In our experience, *B. popilliae* does not grow in the absence of air, and the amount of growth obtained is proportional to the extent of aeration. Pepper and Costilow (6) have established that oxygen is required by these organisms for the metabolism of glucose. Again, the dissolved oxygen content of larval hemolymph containing proliferating *B. popilliae* is one-third that of normal larvae (Weiner and Anderson, Abstr. 142nd Meeting Am. Chem. Soc., 1962). Earlier work had shown that the oxidation-reduction potential of larval hemolymph is lowered as a result of infection (10). The relation of culture growth to aeration rate is seen in Fig. 7. The 0.5 volume per volume per minute aeration rate (oxygen absorption rate, 0.5) results in maximal vegetative growth under the conditions used. Higher aeration rates, such as the 0.75 volume per volume per minute rate shown, afford greater oxygen absorption rates in the 2-liter fermentors but do not increase growth. Aeration at less than 0.5 volume per volume per minute results in correspondingly lower maximal populations. As may be noted from Fig. 6, the rate at which cultures die approximates the rate at which they grow. When less than optimal conditions are established, the rate of cell development is retarded, and correspondingly the rate of death is slowed. For example, with the 0.15 volume per volume per minute limited aeration rate, the relatively slow growing culture of limited population remained alive for extended periods.

Growth of *B. popilliae* in liquid media depends upon the presence of a small amount of fermentable carbohydrate. Ordinarily, greater populations do not result from the inclusion of more than 0.2% carbohydrate (Fig. 8); neither slow feeding nor periodic addition of glucose or trehalose has afforded greater growth or extended longevity. Trehalose is used comparably to glucose during growth of *B. popilliae*. The hemolymph of a third-instar Japanese beetle larva does not contain a significant amount of glucose but does contain a relatively large amount of trehalose (Shotwell, *personal communication*). The amount of trehalose in the hemolymph of diseased larvae is approximately two-thirds the amount in healthy larvae, and may indicate use of trehalose for proliferation of the pathogen in the host.

The loss of viability of vegetative cells in rapidly growing cultures means that cells suitable for inoculum occur only during a limited time in the growth phase. Maximal populations remain in an actively growing culture only for about a 4-hr period. Rapid initiation of growth requires the use of actively metabolizing cells from the late log phase of growth. As illustrated by the data in Fig. 6 (compare with Fig. 5 and 8), a relatively slight change in the schedule of inoculum transfer considerably increases the rate and extent of growth in a fermentor culture. Maximal cell populations of 10^9 to 2×10^9 viable cells per milliliter were achieved in aerated fermentors in 16 to 18 hr by use of modified patterns of inocula preparation. The data represent the maximal rate and extent of growth of *B. popilliae* obtained reproducibly in laboratory cultivation.

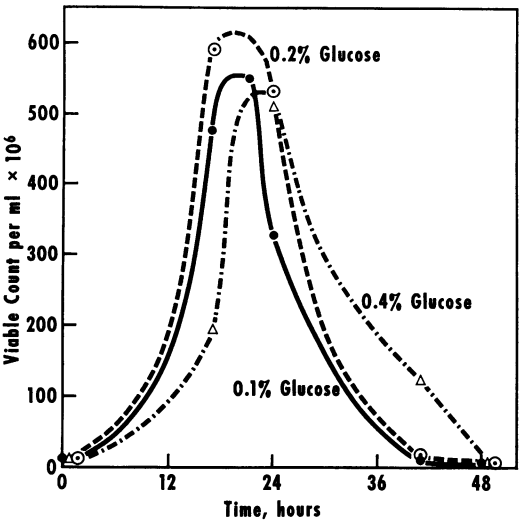

FIG. 8. *Effect of glucose concentration on growth of Bacillus popilliae NRRL B-2309 in 2-liter fermentors. Fermentors inoculated with 5% of 24-hr shaken-flask culture; 0.5 volume per volume per minute aeration rate, pH controlled by automatic addition of NaOH.*

The proliferation of *B. popilliae* in Japanese beetle larvae results in the accumulation of between 2×10^{10} and 3×10^{10} spores per milliliter of larval hemolymph. The hemocele of the larva contains between 0.1 and 0.2 ml of fluid. The density of viable cells obtained in laboratory fermentors therefore is about 10-fold less than that which occurs in insect larvae. Development of massive accumulations of cells during the course of the disease requires about 2 to 3 weeks. During this period of growth, the host larva may contribute to the growth and sporulation of the pathogen by removal of toxic products and by continuous replenishment of nutrients. Consequently, various techniques for dialysis in both flasks and fermentors were tried; these included small-volume dialysis bags suspended in shaken flasks and the incorporation of membrane filter

devices in 2-liter fermentors. The latter designs were based on the work of Gerhardt and Gallup (3). Dialysis techniques in some instances allowed the accumulation of larger populations of viable vegetative cells than those obtained with usual growth techniques. For example, populations of up to 10^{10} cells per milliliter were obtained within the dialysis bag in shaken flasks. Such populations are not far different from those obtained in the hemolymph of the insect larvae. However, in terms of the total nutrient supply contained in the growth vessel (the ratio of medium within the dialysis bag to that without was 1:100), such populations are comparable to those obtained under the usual growth conditions. No evidence of spore formation was obtained in dialysis experiments, although viability of the cultures was somewhat prolonged.

DISCUSSION

Cultures grown under the procedures described are infective when injected as vegetative cells into insect larvae. The extent of virulence is influenced by conditions under which the organisms are grown. For example, cultures grown under conditions of suitable buffering induce milky disease more effectively than do cultures grown in less adequately buffered media (7). Therefore, procedures which we have established for the production of large numbers of vegetative cells do not result in loss of virulence. Furthermore, cultures grown as described sporulate normally when subsequently proliferating in the insect and, consequently, have not become asporogenic during extensive vegetative proliferation in vitro. In addition, the procedure, which results in a small amount of sporulation in vitro, depends on selected strains grown rapidly to large numbers under the conditions described (7*a*).

Analyses of larval hemolymph indicate that the conditions employed for vegetative proliferation are not dissimilar to those that exist in the insect (9; Shotwell et al., Bull. Entomol. Assoc. **9:** 168, 1963; Stubblefield et al., **10:**170, 1964). The nutritive requirements for growth established by Sylvester and Costilow (12) undoubtedly are satisfied by the medium employed. It may be concluded that the conditions established for vegetative growth do not permanently impair either the pathogenicity or sporogenicity of *B. popilliae*.

It is theorized that the characteristic death of *B. popilliae* cells immediately following growth may reflect the commitment of a large proportion of the population to sporulation. Sporulation is an irreversible process. For some reason *B. popilliae* cells are unable to complete the process in vitro and die. The phase of growth following maximal vegetative proliferation in other sporeformers exhibits a similar decrease in viable vegetative cells, which is compensated by the concomitant appearance of refractile spores. The changes accompanying spore formation in these organisms include the induction of an acetate-utilizing system. Acetate is not utilized by most of the strains of the milky disease organisms; however, sporogenesis does not occur in the strain which can use acetate.

Vegetative cells of *B. popilliae* are extremely labile. Rapid death of vegetative cells as measured by plate count accompanies dilution of cells in buffer solution, salt solution, or protein diluents (8). Loss of viability of vegetative cultures is accompanied by the development of granulation and apparent loss of internal structural material from the cells. Future work will involve studies of the compositional and morphological changes of vegetative cells to elucidate the nature of cell lability.

LITERATURE CITED

1. CORMAN, J., H. M. TSUCHIYA, H. J. KOEPSELL, R. G. BENEDICT, S. E. KELLEY, V. H. FEGER, R. G. DWORSCHACK, AND R. W. JACKSON. 1957. Oxygen absorption rates in laboratory and pilot plant equipment. Appl. Microbiol. **5:**313–318.
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. GERHARDT, P., AND D. M. GALLUP. 1963. Dialysis flask for concentrated culture of microorganisms. J. Bacteriol. **86:**919–929.
4. HANSON, R. S., V. R. SRINIVASAN, AND H. O. HALVORSON. 1963. Biochemistry of sporulation. I. Metabolism of acetate by vegetative and sporulating cells. J. Bacteriol. **85:**451–460.
5. 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.
6. PEPPER, R. W., AND R. N. COSTILOW. 1964. Glucose catabolism by *Bacillus popilliae* and *Bacillus lentimorbus*. J. Bacteriol. **87:**303–310.
7. 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.

7*a*. RHODES, R. A., M. S. ROTH, AND G. R. HRUBANT. 1965. Sporulation of Bacillus popilliae on solid media. Can. J. Microbiol. **11:** 779–783.

8. ST. JULIAN, G., 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, C. H. VANETTEN, AND R. W. JACKSON. 1963. Amino acids in the haemolymph of *Popillia japonica* (Newman) larvae. J. Insect Physiol. **9**:35–42.
10. STEINKRAUS, K. H. 1957. Studies on the milky disease organisms. I. Parasitic growth and sporulation of *Bacillus popilliae*. J. Bacteriol. **74**:621–624.
11. STEINKRAUS, K. H. 1957. Studies on the milky disease organisms. II. Saprophytic growth of *Bacillus popilliae*. J. Bacteriol. **74**:625–632.
12. SYLVESTER, C. J., AND R. N. COSTILOW. 1964. Nutritional requirements of *Bacillus popilliae*. J. Bacteriol. **87**:114–119.