STUDIES ON THE MILKY DISEASE ORGANISMS[1]

II. Saprophytic Growth of *Bacillus popilliae*

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Dutky (1940, 1947) cultivated vegetative cells of *Bacillus popilliae* on artificial media aerobically and anaerobically. Aerobic cultivation required the use of highly poised, strongly reducing media. The media used were not adequate for sporulation.

Since the development of a method for producing large quantities of spores of *B. popilliae* on artificial media would have considerable value in extending the biological control of insects susceptible to milky disease, the problem of saprophytic culture of this species was reinvestigated. The primary objective was to develop artificial media and environmental conditions under which *B. popilliae* would duplicate its parasitic life cycle including sporulation. Results of the above study are reported.

EXPERIMENTAL METHODS

Sources of inocula. Dr. S. R. Dutky kindly supplied two suspensions of vegetative cells and two dried spore inocula of *B. popilliae*. These were used for comparison with the strains isolated in our laboratory.

At the start of the investigation, inocula were stored as dried blood smears from infected larvae. These, however, always contained a small percentage of contaminants which interfered with the isolation of new cultures. Thus, for the majority of the studies organisms for inocula were removed aseptically directly from the infected living larvae.

Larvae were surface sterilized by three successive 15-min immersions in fresh 0.5 per cent sodium hypochlorite solutions. The larvae were then placed individually in sterile petri plates containing 5 ml of sterile distilled water. The hemocoele of each larva was punctured through the dorsum with a sterile needle, carefully avoiding the gastro-intestinal tract. Blood containing the spores and vegetative cells of the bacilli was allowed to flow into the water. By the use of sterile equipment, the suspended materials were pipetted and transferred to test tubes. Unless a vegetative cell inoculum was desired, all suspensions were heated to 70 C for 15 min to destroy these cells. Each inoculum was tested for purity by streaking it on a suitable medium in petri plates, incubating at 32 C for 24 to 96 hr, and examining the colonies that developed. The vegetative cells derived from pure suspensions were, in turn, used as inocula in further studies.

Determination of virulence. The typical parasitic spores of *B. popilliae* could be recognized with reasonable certainty, even in the presence of contaminants. The vegetative cells, however, were indistinguishable from those of many other species. Positive identification was possible only by proving their ability to cause milky disease. Whenever practicable, therefore, virulence of isolated strains was determined by feeding tests (Steinkraus, 1957) or by injecting the cells and/or spores into the living larvae, incubating in soil at 25 C and examining periodically for development of milky disease.

Development of artificial media and conditions for growth. Since the primary objective of this study was the development of artificial media and conditions for growth and sporulation of *B. popilliae*, this topic is covered under results. A wide variety of media were used. Brevity permits inclusion of only those which were relatively successful with a general classification of those which were not satisfactory. To be satisfactory, a medium had to have the ability not only to permit growth of *B. popilliae* but also to maintain the organisms in their typical parasitic morphology.

Use of larval macerates and extracts for media. Consideration was given to the possibility that larval macerates sterilized by heating or larval extracts sterilized by filtration through a Seitz

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filter might either separately or when added to other nutrients prove a satisfactory medium for both growth and sporulation.

Healthy third instar European chafer, *Amphimallon majalis* Razoumowsky larvae were ground in a mortar and the macerates were sterilized in test tubes at 121 C for 15 min with or without the addition of other nutrients. Extracts of healthy and of milky diseased larvae were prepared by mixing larvae and solvent in the ratio of 5 ml of solvent per larva (average weight 355 mg) in a Waring Blendor jar for 3 min at room temperature. Solvents used were distilled water, physiological saline, 70 per cent ethyl alcohol, and 95 per cent ethyl alcohol. In order to preserve soluble heat labile components the extracts were filtered through sterile Seitz filters. Excess ethanol was removed under vacuum prior to addition to media.

The extracts were added to various media in petri plates at a temperature of 45 C. Among these media were 1.5 per cent agar buffered with 0.3 per cent K_2HPO_4, in which the extracts themselves were the only sources of nutrient, and tryptone-glucose-yeast extract medium buffered with 0.3 per cent K_2HPO_4 and containing 1 per cent soluble starch. The latter medium permitted growth of *B. popilliae* in the vegetative cell state without added larval extracts. The extracts were added singly and in combination to the basal media in concentrations ranging from 0.1 ml to 2.0 ml per 10 ml of medium. They were added in combinations as follows: aqueous + saline, aqueous + ethanol, saline + ethanol, aqueous + saline + ethanol.

Heat activation of spore germination. In order to determine whether a preliminary heat treatment would stimulate germination of the spores and their subsequent recovery in the form of visible colonies, a series of heat treatments was applied to selected spore inocula.

Direct microscopic spore counts were made of each spore suspension in a Petroff-Hausser blood counting chamber. The aqueous spore suspensions then were heated at temperatures of 50, 60, 70, 80, and 90 C. At intervals of 0, 5, 10, 20, 40, and 80 min, samples of each spore suspension were removed, diluted serially, and inoculated into petri plates. The growth medium was added to the plates which were incubated at 32 C for 4 to 6 days. The colonies developing on the plates were counted, and the percentage recoveries of total spores in the form of visible colonies were calculated.

RESULTS

Use of larval macerates or extracts for media. The living larvae were the only completely satisfactory medium for growth and sporulation of *B. popilliae*. Healthy European chafer larvae macerated and heat sterilized still supported growth of the organisms in the vegetative state. The life cycle, however, was interrupted and no spores[2] formed.

Similarly, aqueous extracts of larvae added alone or with saline and ethanol extracts to 1.5 per cent agar base in the higher concentrations used, supported some vegetative growth of *B. popilliae*. Addition of the larval extracts to tryptone-glucose-yeast extract medium enhanced the growth of vegetative cells as measured by colony size and profuseness of growth. No typical spores formed. Although the extracts were sterilized by filtration to prevent destruction of heat-labile factors, it was subsequently found that sterilizing the extracts by heat at 120 C for 15 min did not interfere with their ability to stimulate vegetative growth. An effect similar to that obtained by addition of larval extracts was achieved by doubling the amount of glucose added to the medium.

A number of test tube experiments were performed in which vegetative cells of *B. popilliae* were concentrated by centrifuging and mixed with sterile larval extracts singly and in combination. Sporulation failed to occur. Results were similar using extracts prepared from healthy or milky larvae. Spores also failed to form when vegetative cells were inoculated into whole larval blood removed aseptically from healthy larvae.

Thus, some factor or condition present in the living larval blood permitting sporulation was not duplicated when the dead larval macerates or extracts were used as media.

Development of artificial media and conditions for growth. At the beginning of the study, considerable difficulty was encountered in growing *B. popilliae* on artificial media. Those tried were

[2] As the term spore is used in this paper, it refers to the morphological type formed parasitically (Steinkraus, 1957) and does not include those spores of atypical morphology which will be described in a later paper.

reducing types such as brain-heart infusion, brain-liver infusion, and fluid thioglycolate (Difco). Growth was relatively sparse; large inocula (1,000 to 2,000 spores/visible colony) were required. The use of liquid media was abandoned due to the difficulty of controlling variants which grew faster than the typical parasitic vegetative cells of *B. popilliae*. Growth of the organisms on the surface of solid media in petri dishes resulted in high concentrations of cells even when the colonies remained small. Growth was consistently heavier in colonies directly exposed to the air than it was in submerged colonies. Since the blood of both healthy and diseased larvae had relatively high oxidation-reduction potentials (Steinkraus, 1957) and typical sporulation was never observed to occur in cells grown on the highly reducing media, these media were discarded except for comparison purposes.

The search for new media was directed to the tryptone-glucose-yeast extract type which appeared to have oxidation-reduction potentials in the general range of those found within the larval blood. Although the organisms actually grew at a pH as high as 7.8 and growth was not completely inhibited until the pH was lowered to 6.1, the media were buffered in the range 7.0 to 7.2, close to that of larval blood, which was satisfactory for both growth and sporulation of the organisms.

The first medium of the tryptone-glucose-yeast extract type developed and found satisfactory for growing *B. popilliae* contained the following: tryptone, 5 g; yeast extract, 3 g; K_2HPO_4, 3 g; soluble starch, 10 g; glucose, 1 g; maltose, 1 g; agar, 15 g; distilled water, 1,000 ml. This medium permitted good growth of the vegetative cells from spores inoculated on its surface and appeared to maintain the cells in a relatively stable, typical parasitic morphology. The colonies were tiny and nearly colorless (figure 1), consisting of typical rods. However, no typical spores formed in the undisturbed vegetative cells which grew on it.

The addition of 0.01 to 0.1 per cent soluble starch or activated charcoal (10 to 20 per cent of total solids) to the tryptone medium was essential for good germination and appreciable growth of the organisms outside their living host. Although the starch was not fermented, it probably neutralized inhibitors, as suggested by Olsen and Scott (1946), and Foster and Wynne (1948), and Wynne and Foster (1948). The activated charcoal presumably absorbed inhibitors (Foster *et al.*, 1950).

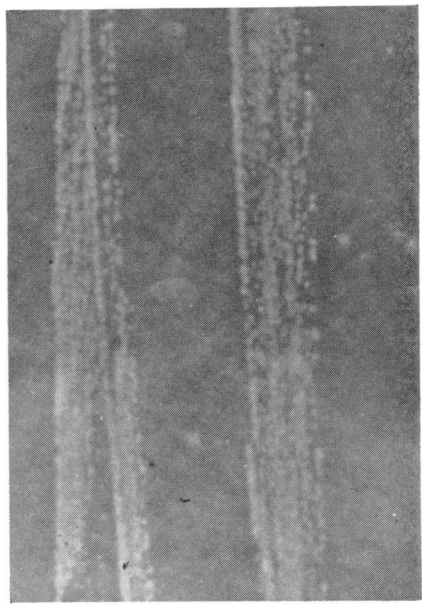

Figure 1. Colonies of *Bacillus popilliae*. × 1.

The strains of *B. popilliae* studied fermented the following carbohydrates as evidenced by a distinct lowering of the pH of the medium (tables 1 and 2): glucose, mannose, galactose, maltose, and salicin. Fructose was fermented by all strains examined except strain 1 (table 1). Inocula consisting of spore suspensions freshly removed from diseased larvae (strains 4–6, table 2) were not generally as active in utilization of sucrose, salicin, and occasionally other sugars as were the aqueous spore suspensions stored at 1 C for a period of months (strains 7–10, table 2). Sucrose supported little, if any, growth of vegetative cell cultures (strains 1 and 2, table 1; strains 1–3, table 2). It was fermented and markedly stimulated germination and growth when aqueous spore suspensions stored for 13 months at 1 C were used as inocula (strains 7–10, table 2). However, subcultures of the vegetative cells failed to utilize sucrose. Thus, the sucrose fermentation must be connected in some way with the germination process.

On the basis of the above study, glucose, fructose, mannose, sucrose, maltose, and salicin were added to the growth medium.

Of a number of sources of organic nitrogen,

TABLE 1

pH Changes in liquid basal medium containing added carbohydrates*

Carbohydrate Added†	pH Measurements (Incubation at 32 C)								
	Uninoculated			Inoculated with strains‡					
				1		2		3	
	0 days	*6 days*	*9 days*	*6 days*	*9 days*	*6 days*	*9 days*	*6 days*	*9 days*
None	7.2	7.1	7.1	7.2	7.1	7.2	7.1	7.2	7.0
Arabinose	7.1	7.1	7.1	7.0	6.8	6.9	6.8	7.0	6.9
Xylose	7.1	7.1	7.0	7.0	7.0	7.0	6.9	6.9	6.8
Rhamnose	7.1	7.1	7.1	7.1	6.8	7.0	5.9	7.1	7.0
Glucose	7.1	7.1	7.0	5.8	5.9	5.7	5.8	5.8	5.9
Fructose	7.1	7.2	7.1	7.1	7.0	7.0	6.8	6.7	6.6
Galactose	7.0	7.1	7.1	6.5	6.4	6.3	6.3	6.4	6.4
Mannose	7.1	7.1	7.0	6.3	5.8	5.9	5.8	6.5	6.4
Lactose	7.1	7.2	7.1	7.0	7.0	7.1	7.1	7.1	7.0
Maltose	7.1	7.1	7.1	6.8	6.5	6.7	6.5	6.7	6.4
Sucrose	7.1	7.0	—	7.1	7.0	7.1	7.1	7.0	6.4
Raffinose	7.1	7.1	7.1	7.1	7.1	7.2	7.1	7.1	7.0
Glycogen	7.1	7.2	7.1	7.2	7.1	7.0	7.1	7.1	7.0

* Basal medium: Tryptone, 10 g; yeast extract, 6 g; K_2PO_4, 1 g; soluble starch, 10 g; distilled water, 1,000 ml.

† 1 per cent soluble starch in basal medium + 1 per cent added carbohydrate. Carbohydrates with exception of starch sterilized by filtration of aqueous solution.

‡ Strains: *1*, A-1 vegetative cells transferred 95 times on artificial media; *2*, A-3 vegetative cells transferred 95 times on artificial media; *3*, Spore suspension prepared from milky diseased European chafer larvae.

TABLE 2

pH Changes in liquid basal medium containing added sucrose or salicin*

Carbohydrate Added	pH Measurements (Incubated 6 Days at 32 C)									
	Uninoculated (control)	Inoculated with strains†								
		1	2	3	4	5	6	7	8	9	10
Salicin (1%)	7.1	6.4	6.5	6.4	6.4	7.0	7.1	6.4	6.3	6.4	6.4
Sucrose (1%)	7.2	7.2	7.1	7.0	7.2	7.0	7.2	6.8	6.8	5.7	6.8

* Basal medium tryptone, 5 g; yeast extract, 3 g; K_2HPO_4, 1 g; soluble starch, 4 g; distilled water, 1,000 ml.

† Strains: 1–3, vegetative cells; 4–6, aqueous spore suspensions newly prepared from diseased larvae; 7–10, aqueous spore suspensions stored 13 months at 1 C.

including peptone, proteose-peptone, casitone, casamino acids, tryptone (all from Difco), and trypticase (BBL), tryptone was found to be the most stimulatory to germination and to growth of the organisms. All strains tested were indole negative and did not reduce nitrates.

After studies of numerous variations of media, the one found most suitable for the germination and growth of milky disease organisms contains the following: tryptone, 10 g; yeast extract, 6 g; K_2HPO_4, 3 g; soluble starch, 10 g; activated carbon, 6 g; glucose, fructose, mannose, maltose, sucrose, salicin, 1 g each; agar, 15 g; distilled water, 1,000 ml. The medium is sterilized by heating at 120 C for 20 min and is hereafter referred to as the "growth medium." No typical spores formed in the undisturbed vegetative cells which grew on this medium. However, some strains did form spores when the vegetative cells were handled by the method of Steinkraus and Tashiro (1955).

Although *B. popilliae* grew slowly at 18 C

and a few strains grew at temperatures as high as 37 C, the optimum for culturing of the organisms was found to be 32 C. At this temperature, initial germination and growth from spore inocula generally required 4 days on the growth medium. Subsequent subcultures of vegetative cells required 2 days for colony formation.

The organisms showed a heavier, more rapid, and more typical growth under aerobic than under strictly anaerobic conditions. However, the vegetative cells were found to be low or completely deficient in catalase as revealed by their failure to release oxygen bubbles when they were mixed with 3 per cent hydrogen peroxide. Addition of catalase (previously sterilized by filtration through a sterile Seitz filter) to the medium failed to stimulate either growth or sporulation.

Poising the oxidation-reduction potentials at lower levels by the addition of cysteine, ascorbic acid, or sodium thioglycolate to the medium did not stimulate growth or sporulation. Growth of the organisms aerobically followed by anaerobic cultivation also failed to stimulate sporulation. It was concluded that the *B. popilliae* is preferably aerobic.

Parasitically, the vegetative cells observed were comparatively uniform and stable morphologically. On artificial media, the vegetative cells formed from the germination of typical spores removed directly from diseased larvae varied in morphology. Some strains developed vegetative cells morphologically unlike the slender parasitic rods as soon as the spores were germinated on artificial media. These variants will be described in detail later.

Motility of vegetative cells. Within the living diseased larvae, the vegetative cells appeared nonmotile. Dutky (1940) reported that it was the nonmotile rods isolated on artificial media which produced milky disease when injected into healthy larvae. He also reported frequent isolation of a slender, motile rod forming small discrete colonies on blood agar slants. Attempts to produce the disease by inoculation of these motile rods into healthy larvae were not successful.

The vegetative cells first derived from spore inocula on artificial media in this study were generally nonmotile. However, motile strains of vegetative cells were derived from carefully handled spore suspensions of *B. popilliae*. When these motile vegetative cells were injected into healthy larvae, they killed the larvae within 24 to 48 hr. Death of the larvae occurred before typical milky disease with formation of spores developed. These typically motile strains, therefore, were apparently too pathogenic to parasitize the larvae and cause typical milky disease.

A prolonged study was made of two typical nonmotile strains of vegetative cells of *B.*

TABLE 3

Variation in motility of two typical strains of vegetative cells of Bacillus popilliae transferred at two day intervals on a standard medium† incubated at 32 C*

Strain	Number of Transfer											
	1	2	3	4	5	6	7	8	9	10	11	12
A-1	NM	NM	NM	M	M	NM	M	NM	M	M	NM	NM
A-3	NM	NM	NM	NM	M	NM	NM	M	M	M	NM	NM
	13	14	15	16	17	18	19	20	21	22	23	24
A-1	M	M	M	M	M	M	M	M	NM	M	NM	NM
A-3	M	M	M	M	M	M	M	M	NM	NM	NM	NM
	25	26	27	28	29	30	31	32	33	34	35	36
A-1	NM	NM	NM	M	NM	NM	M	M	NM	NM	M	M
A-3	NM	NM	NM	M	NM	NM	M	M	NM	NM	M	M

* Streaked on surface of medium in petri plates.

† Tryptone, 5 g; yeast extract, 3 g; K_2HPO_4, 3 g; soluble starch, 10 g; glucose, 1 g; maltose, 1 g; agar, 15 g; distilled water, 1,000 ml.

NM = Nonmotile.

M = Motility of a characteristic type; vegetative cells twirl end over end as though rotating on an axis.

popilliae transferred on the same basic medium regularly at two day intervals and incubated always at 32 C. The medium contained the following: tryptone, 5 g; yeast extract, 3 g; K_2HPO_4, 3 g; glucose, 1 g; maltose, 1 g; soluble starch, 10 g; agar, 15 g; distilled water, 1,000 ml. All transfers were made as streaks on the surface of the medium in petri plates. Initial germination and growth from the spore inocula required 4 days. Microscopic examination was made of the vegetative cells in wet mounts every 48 hr at time of transfer. The medium used maintained the cultures in the form of typically small, colorless colonies for the duration of the study. It was found (table 3) that both strains were typically nonmotile, slender rods for the first three transfers. Starting with the fourth transfer, however, a peculiar type of motility was observed in a portion of the cells in many of the transfers. The motile rods rotated end-over-end as though on axes at their centers. The twirling cells were not observed in every transfer and the proportion of twirling to nonmotile cells varied from transfer to transfer. There were always, with one exception, some nontwirling cells present in every transfer.

In order to insure that these vegetative cells were still virulent cultures of *B. popilliae*, they were injected into healthy third instar European chafer larvae at the time of the following transfers on artificial media: 12, 23, 27, 29, and 35. Six larvae were injected with each strain at the

TABLE 4

Per cent recovery of inoculated spores in the form of visible colonies in pour plate cultures after various heat treatments of the spores

Heat Treatment		Percent Recovery				
		Old suspensions (stored 13 months)		New suspensions		
Temp	Time	1	2	1	2	3
C	*min*					
None*				3.8	4.8	0.5
50	20			6.3	4.2	0.3
50	40			5.4	5.3	0.5
50	80			7.3	4.8	0.4
60	20*	28.7	47.6	9.6	5.8	0.6
60	40			12.3	2.8	0.7
60	80			7.8	16.7	0.4
70	10		20.7	6.3	4.0	0.2
70	20	33.0	66.7	7.2	2.8	0.7
70	40	31.3	78.3	6.5	2.8	0.1
70	80	34.6				
80	10	29.5	34.5	11.5	4.1	0.2
80	20	33.2	30.7	4.6	3.4	0.2
80	40	23.6	23.8	15.0	3.0	0.2
80	80	19.0		6.0	8.9	0.1
90	5	32.0		1.8	3.3	0.2
90	10	22.1		11.6	4.0	0.2
90	20	19.2		6.0	3.4	0.4
Direct count (millions/ml)		105	60	40	45	
Ratio of spores inoculated to visible colonies produced		3–5:1	1.2–5:1	7–26:1	6–40:1	140–1,000:1

* Control.

given transfers. Approximately 10 million vegetative cells were injected per larva. The injected larvae were held at 32 C. Within 24 hr, the larvae injected with 12th transfer cells were becoming grossly milky. Microscopic examination of a drop of blood removed from representative larvae revealed only vegetative cells in large numbers. At 50 hr, some sporulation was noted. Vegetative cells injected into larvae at the 23rd transfer, produced spores within 48 to 72 hr. At the 27th transfer, the vegetative cells grew rapidly within the injected larvae, but sporulation was delayed for 5 to 9 days. This was true also of the cells injected after 35 transfers on artificial media.

The above experiment was continued through 120 transfers on artificial media. The vegetative cells were tested for virulence periodically. It was found that the twirling cells did not interfere with the virulence. The twirling cells continued to twirl within the larval blood. Spores were produced after 5 to 9 days within the larvae. One culture which appeared to be entirely made up of twirling cells was an exception. Injected into healthy larvae, this culture killed the larvae in 24 to 48 hr before typical milky disease and sporulation were apparent. It was evident that strains transferred on artificial media for 27 or more times required a longer period of adjustment in order to complete their life cycles with sporulation within the larvae under the conditions of this experiment. Sporulation was not only delayed but it was diminished in the later transfers.

Heat activation of spore germination. It was observed that recovery of spores of *B. popilliae* inoculated onto media, as indicated by the production of visible colonies, was often poor. Heat treatment of spores has been reported to result in activation of spore germination (Evans and Curran, 1943; Curran and Evans, 1945) There was no evidence (table 4) that heat treatment stimulated germination of spores of *B. popilliae*. Aqueous spore suspensions stored for a period of thirteen months at 1 C yielded a higher percentage of visible colonies than did fresh spore suspensions.

It has been indicated that the source of inoculum influenced the results. In general, it was necessary to consider every spore suspension removed from individual diseased larvae as a different strain. Dutky (1940) reported that he had little success in growing cultures of *B. popilliae* on artificial media until he started using dried blood smears containing the spores as his source of inocula. The dried spores remained viable for at least 42 months.

The above mentioned was used at the start of these studies. However, contaminants generally present in the dried blood spore smears grew more rapidly and had to be separated from *B. popilliae*. This difficulty was encountered much less frequently in spore suspensions removed aseptically from living milky diseased larvae.

As revealed by the sugar fermentation reactions and the per cent germination in the heat activation experiments, spores freshly removed from diseased larvae and suspended in water were not as active as the same suspensions stored for a period of months at 1 C. The aged aqueous suspensions and dried spores gave comparable results. This suggests the presence of inhibitors in or on the spores freshly removed from larvae.

DISCUSSION

Although *B. popilliae* could be cultivated in the vegetative state on artificial media, the typically parasitic strains did not grow as rapidly or as profusely as ordinary saprophytic bacterial species. The concentration of vegetative cells in the colonies on artificial media, of course, exceeded those within the living larvae, but the life cycle, particularly typical sporulation, remained incompleted.

Characteristically, vegetative cell growth within the living larva was followed by sporulation. Vegetative cells growing undisturbed on artificial media did not sporulate. However, the media and conditions of vegetative growth developed in this study led to methods whereby limited sporulation could be achieved on artificial media. Sporulation of *B. popilliae* on artificial media will be discussed in detail in a later paper in this series.

While the morphology of the vegetative cells growing parasitically appeared to be relatively stable and uniform, that growing on artificial media exhibited considerable variation and a sensitivity to changes in nutrient or environmental conditions.

Thus, one or more factors essential to the maintenance and completion of the life cycle of these parasitic bacteria remained beyond control in artificial media.

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SUMMARY

While living larvae of European chafer, *Amphimallon majalis* Razoumowsky, permitted multiplication of vegetative cells and sporulation of *Bacillus popilliae* within their blood, dead macerated larvae sterilized by heat or extracts sterilized by filtration supported growth only in the vegetative cell stages. No typical spores formed.

As a foundation for duplicating the parasitic life cycle of *B. popilliae* on artificial media, a study was made of the growth requirements of various strains of this species.

New media and conditions for growth of *B. popilliae* were devised. These maintained vegetative cells of many but not all strains of the species in their typical parasitic morphology. No typical spores formed, however, in the undisturbed cells.

The surface of solid media was found to be superior to liquid media for culturing organisms. The organisms appeared preferably aerobic.

Glucose, fructose, mannose, maltose, galactose, and salicin were utilized. Sucrose, although not fermented by vegetative cells isolated on artificial media, stimulated germination and growth from spore inocula.

Tryptone and yeast extract satisfied at least the minimal vegetative growth requirements of the organisms for complex organic molecules and growth factors.

A characteristic type of twirling motility was noted in vegetative cells of *B. popilliae* after several transfers on artificial media.

Germination of the spores was not activated by heat.

Aqueous suspensions of spores stored for one year at 1 C germinated more completely than spores freshly removed from diseased larvae.

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