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Spore Formation by *Bacillus popilliae* in Liquid Medium Containing Activated Carbon

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ABSTRACT

HAYNES, W. C. (Northern Regional Research Laboratory, Peoria, Ill.), AND LENORA J. RHODES. Spore formation by *Bacillus popilliae* in liquid medium containing activated carbon. J. Bacteriol. **91**:2270–2274. 1966.—Heretofore, it has not been found possible to evoke sporulation of *Bacillus popilliae* in liquid culture. We have discovered that sporulation will occur in tryptone-glucose-yeast extract broth shaken cultures if activated carbon (charcoal) is present during growth. The spores so engendered have survived drying in air and subsequent storage for several months as dry films and also in dry soil, sand, and a mixture of powdered calcium carbonate and talc. Furthermore, the longevity of cultures, even when spores are absent, is extended, in cultures containing activated carbon, to several weeks at a population of millions of cells per milliliter. This extension of life is the result of a marked change from rapid decline in numbers to an almost stationary population.

Although *Bacillus popilliae* Dutky and *B. lentimorbus* Dutky sporulate in the hemocoele of *Popillia japonica* Newman (Japanese beetle) and *Amphimallon majalis* (Razoumowsky; European chafer) larvae, they sporulate sparsely on solid laboratory media (7, 10) and not at all in liquid media. In searching for a means to evoke the formation of spores in liquid media, we became aware of the work of Foster, Hardwick, and Guirard (2). They experienced difficulty in causing *B. larvae* White, another insect pathogen, to sporulate. Sporulation of this species was greatly enhanced in media they treated with activated charcoal. Perhaps it was their success that induced Steinkraus (9) in 1957 to add activated charcoal to the solid medium on which he found *B. popilliae* to produce spores.

It seemed unlikely to us that a practical method for the production of milky disease bacteria spores for use in controlling insect hosts could be developed on the basis of spores formed on solid media. Therefore, we continued to work with liquid media and included the use of activated carbon.

MATERIALS AND METHODS

Microorganism. B. popilliae Dutky NRRL B-2309S was used exclusively. It is a substrain of NRRL B-2309 that was previously selected because of its ability to form spores on solid media (7). Both the parent and the substrain were able to infect Japanese beetle larvae.

Medium. The growth medium described by St. Julian et al. (11) [tryptone, 0.5%;yeast extract, 1.5%; K_2HPO_4, 0.3%; glucose, 0.2% (sterilized separately); distilled water; *p*H 7.3 to 7.5], designated J medium for brevity in this report, was used.

Activated carbon. In the past, powdered carbon has been used to stimulate growth of microorganisms. The nature of milky disease bacteria makes it convenient to work with clear media in which their characteristic birefringent turbidity may be observed. Powdered carbons convert clear media into murky, black ones. Furthermore, phase microscopy is hampered in cultures containing powdered carbon, because microscope fields are cluttered with innumerable refractile particles which make observation difficult, if not impossible. We chose, therefore, to use pelleted and granular carbons. One was obtained from the Union Carbide Corporation, New York, N.Y., under the brand name "Columbia." It was designated grade G, screen size 10–24. Another pelleted carbon marked "Columbia Activated Carbon Grade CX Mesh 8–10" was also used. (The company informs us that they never manufactured any such grade.)

Carbon, usually in 1-g quantities, was sterilized apart from the medium by autoclaving at 121 C for 45 min. The sterile carbon was added to the medium at the time of inoculation. (Sterile medium supplemented with carbon sterilized in this manner remained sterile throughout several weeks of observation.) Preliminary experiments to determine whether carbon must be present during growth of the culture or

whether pretreatment of the medium with carbon is satisfactory suggest that sporulation occurs more readily when carbon is present.

Cultural conditions. Cultures were grown in 100 ml of medium in 300-ml Erlenmeyer flasks on a reciprocal shaker at 25 C. At intervals, samples were removed for determination of *p*H and viable population and for observation by phase microscopy. Inoculation was done with 0.1 ml of a 1- to 3-day-old culture.

Viable population count. Viable counts were performed in the usual manner, except that a spreading technique was used instead of the pour plate method. A 0.1-ml amount of every dilution chosen for plating was spread on each of three plates of J-medium agar. Plating was done from three serial 10-fold dilutions selected on the basis of experience to give 30 to 300 colonies per plate. In keeping with the recommendations of St. Julian et al. (11), 0.1% tryptone was used as dilution fluid. To suppress foam that formed upon shaking the dilution blanks, we added to each liter of 0.1% tryptone 0.5 ml of 10% polyglycol containing a drop of Tween 80.

Preparation and testing of spores. A 2-ml amount of a culture showing refractile *B. popilliae* spores was transferred to each of several sterile 300-ml Erlenmeyer flasks, some empty, others containing 10 g of dry, sterile soil, white sand, or a 1:9 mixture of powdered calcium carbonate and talc. The cultures were 2 to 5 weeks old, and, in each, 5 to 15 refractile spores were seen in the course of examining 100 microscope fields (1,000 times magnification) in wet preparations. The flask preparations were then allowed to dry at 32 C. At intervals thereafter, they were tested for viability and numbers of germinable spores. This determination was accomplished by aseptically pouring 100 ml of sterile medium into each flask selected for testing, shaking it to wet and suspend the spores, and then removing a sample for plating to determine the number of germinable spores.

RESULTS

When *B. popilliae* was grown in shaken J-medium cultures at 25 C, the population rose to a maximum at 48 to 72 hr, depending upon the number and vigor of cells in the inoculum. Having reached its zenith, the population rapidly declined until, at the end of 1 week, the number of viable cells was negligible. A typical growth curve (Control) is shown in Fig. 1. As the population rose to its peak and during the initial period of decline, the *p*H dropped constantly to about 6. Figure 2 shows a typical *p*H curve (dotted line marked "No Carbon").

Initial experiments were performed with Columbia carbon grade G, 10–24 mesh. Inasmuch as the amount of carbon used by others ranged up to 2%, we explored this range. Flasks containing 100 ml each of sterile J medium were charged in duplicate with 0.25, 0.5, 1, and 2 g of carbon at time of inoculation. A fifth pair of flasks received no carbon and served as controls.

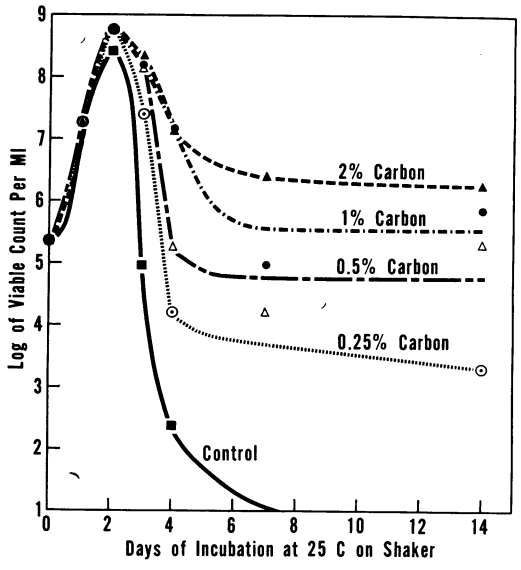

FIG. 1. *Effect of different levels of activated carbon upon the growth curve of Bacillus popilliae NRRL B-2309S.*

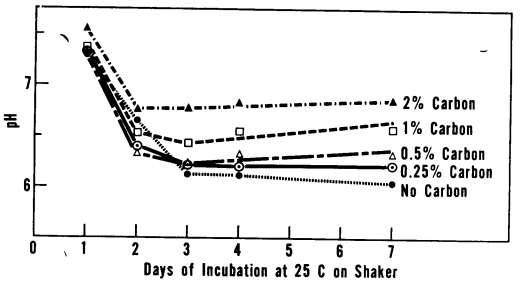

FIG. 2. *Effect of different levels of carbon on the pH in cultures of Bacillus popilliae NRRL B-2309S.*

All flasks were inoculated with 0.1 ml of a 3-day-old J-medium culture of NRRL B-2309S. Typical population curves are shown in Fig. 1, and the course of *p*H is shown in Fig. 2.

To conserve the supply of carbon, we standardized on 1% carbon for subsequent experiments.

Because of reports in the literature that noncarbonaceous surfaces stimulate the growth of microorganisms (1, 5, 12), we tested the effect of glass beads, clay shards, talc, and bentonite. As shown in Fig. 3, glass beads, clay shards, and talc had little or no effect upon the growth curve. Bentonite, on the other hand, was almost as effective as activated carbon in extending the longevity of *B. popilliae*.

Observation by phase microscopy was also

made. In J-medium cultures, without activated carbon, swollen cells were seldom seen. During the logarithmic stage of growth, cells were uniformly rod-shaped and internally appeared homogeneous and phase-dark. After the culture entered the population decline stage, the cells lost their internal homogeneity. They became granular, the granules were phase-dark, and the rest of the cytoplasm appeared gray. In 1 week, most cells were homogeneously gray inside and were dead.

In cultures containing activated carbon, especially the CX 8–10 variety, the picture was similar except that phase-dark forms remained in evidence for many weeks and swollen pleomorphic forms appeared sometimes as early as the 1st day after inoculation. Many of the swollen cells were phase-dark and spindle-, club-, and clostridium-shaped. Some of them rapidly differentiated to show phase-dark concentrations, which usually developed into fully refractile spores and parasporal bodies. A spore was not always accompanied by a parasporal body. A refractile spore might be alone in a sporangium or it might be accompanied by either a refractile or a phase-dark parasporal body. A body resembling a paraspore, either refractile or phase-dark, sometimes occurred alone in a sporangium.

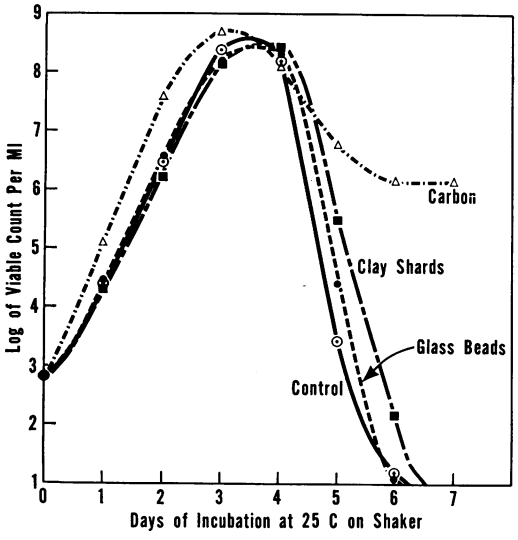

FIG. 3. *Comparison of the effect of activated carbon, clay shards, and glass beads on the growth curve of Bacillus popilliae NRRL B-2309S.*

Bacterial spores have two outstanding properties: they withstand drying for long periods of time, and they survive exposure to temperatures that are fatal to vegetative cells. Of the two, the ability to withstand drying is especially important in this investigation, because spore dusts are marketed in dry form and may be subjected to prolonged storage before use. Furthermore, spores in such preparations must be capable of surviving in soil. We chose to test the authenticity of carbon-engendered spores by comparing their ability to withstand prolonged storage in the dry state. Cultures composed exclusively of vegetative cells, grown either in the presence or absence of activated carbon, repeatedly failed to survive for even a few days when dried. Cultures containing spores of *B. popilliae* withstood drying and subsequent storage at room temperature for long periods of time. Results of some of the tests are shown in Table 1. Among the viable cells added to soil, sand, and $CaCo_3$-talc were an undetermined number of spores. As yet we have not devised a satisfactory method for determining the number of spores in a culture. Direct counts with a Petroff-Hausser bacteria counter are not feasible, because spore populations are such that we seldom can count even one spore. The obvious

TABLE 1. *Longevity of spores of Bacillus popilliae NRRL B-2309S*

Extender	Viable cells added/ml	No. of germinable spores/ml after*				
		4–14 days	30–40 days	60–70 days	90 days	120 days
Soil†	?	1,100	400	1,100	1,700	—‡
	4 million	7,300	13,000	8,100	5,900	—‡
	800 thousand	2,700	2,400	1,500	2,300	—‡
	2 million	1,500	1,700	1,600	1,400	—‡
Sand	3 million		62,000	84,000		73,000
	2 million	4,200	6,000	6,500	6,100	6,200
$CaCO_3$-talc	2 million	3,700	3,200	3,900	3,400	5,000

* Based on 1 ml of inoculum.
† One soil of very low initial spore count still yielded viable cultures after 1 year.
‡ Further tests were not made because samples were expended by earlier tests.

solution to this problem is to count spores in a concentrate. But, when we used this technique, we found the preparations to be so dense with vegetative cells and debris, the latter mostly refractile particles of carbon, that spores were unrecogizable. (In one preparation that was somewhat less impenetrable, we obtained a count of 2 million spores per milliliter.) Thus, for the time being, we are dependent upon the germinable spore counts from soil, sand, dry films, and $CaCo_3$-talc to give an idea of the minimal number of spores produced. Inasmuch as the rate of germination of spores formed in Japanese beetle larvae seldom exceeds 10% (9), it is possible that our cultures contain many spores that are undetected because they do not germinate on J-medium agar.

The cultures that grew up from dried preparations that were reconstituted with J-medium were pure cultures of typical *B. popilliae*.

DISCUSSION

Treatment of culture media with activated carbon (charcoal) to enhance microbial growth has been practiced and sporadically reported during more than three decades. Earliest reports dealt with attempts to improve growth of gonococci and meningococci on nutrient agar (3), and it has been primarily in medical bacteriology that activated carbon has been applied to improve growth of fastidious microorganisms ever since.

The first, and to our knowledge the only, detailed comparison of viable populations in carbon-treated and untreated media was made in 1942 by Roberts and Baldwin (8). They grew *B. subtilis* in treated and untreated peptone water and made viable-cell counts at intervals throughout 4 days. No significant difference was found.

Perhaps no other studies of this nature have been published, because most bacteria have a stationary phase and a logarithmic death phase of such duration that the effects of carbon are not clearly revealed. *B. popilliae*, with its short life cycle, is peculiarly adapted to show the effect of carbon, especially on the terminal stages of growth where the effect is expressed most markedly. We did not anticipate that activated carbon would cause marked changes in the pattern of growth. We were surprised, therefore, when we consistently observed a radical change in the death stages of the culture. The presence of activated carbon did not prevent initiation of the death phase, but, after the rate became rapid and more or less constant, the presence of activated carbon caused an abrupt change to a rate not far from zero. Less than 1% of the population was saved from death by this alteration, but survivors continued viable for several weeks.

An explanation for the more or less abrupt change in the rate of death after the culture has entered the death phase is difficult to provide. The change apparently is not related directly to *p*H or residual glucose, because they reached their nadirs when the population attained its zenith, usually 24 to 48 hr earlier. It seemed to occur at or near the time that pleomorphic swollen forms began to appear, but their small number in relation to the total population at the time seemed to be inadequate to account for the marked change.

Some clues to the manner in which carbon affects the growth and sporulation of *B. popilliae* may be found in the works of Pollock (6), Foster, Hardwick, and Guirard (2), and Hardwick, Guirard, and Foster (4). Pollock (6) suggested that the growth-promoting power of carbon for *Haemophilus pertussis* might be attributable to its ability to combine with toxic fatty acids present in the medium. Foster, Hardwick, and Guirard (2) reported that activated carbon adsorbed factors from complex organic media that inhibited growth and sporulation of *B. larvae*. These factors were shown by Hardwick, Guirard, and Foster (4) to be composed largely of fatty acids. The constituents which accelerate death and inhibit sporulation of *B. popilliae* may be similar to these, but we have not yet attempted to identify them.

One of the main purposes of seeking to evoke spores in liquid culture is that they may be produced in large quantity, and cheaply, for use in controlling insect hosts. Currently, spores are commercially produced for this purpose in limited quantity and rather expensively by procedures developed by Dutky in 1941 and 1942 (U. S. Patents 2,258,319 and 2,293,890). Japanese beetle larvae are collected in heavily invested areas, transported to the laboratory, inoculated, incubated until they become "milky," and then processed to make a spore dust.

The Japanese beetle and the European chafer are most susceptible to milky disease bacteria during the larval stage when they live in soil. To be available to larvae, spores must be able to survive in soil. They are applied to soil in the form of spore dust, in which spores are dispersed in a mixture of calcium carbonate and talc. Months may pass from time of production to time of application. Thus, to be available to infect larvae, spores must be able to survive storage in this mixture. It became necessary, therefore, to determine whether artificially produced spores survive in soil and in calcium carbonate-talc mixture as do those produced in larval hemolymph. Table 1 shows that spores engendered in liquid media containing carbon

remain viable for at least 120 days in soil, $CaCo_3$-talc, and white sand. There is no obvious reason why they should not remain viable much longer.

The ability of spores produced in liquid culture in the presence of activated carbon to infect Japanese beetle larvae remains to be determined.

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