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Synchronous Growth and Sporulation of *Bacillus megaterium*

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Filtration of late log-phase cultures of *Bacillus megaterium* ATCC 19213 grown on defined sucrose salts medium (SS) or SS plus glutamate medium (SSG) through nine layers of Whatman no. 40 filter paper in a fritted-glass disc Büchner funnel resulted in filtrates containing cells which showed synchronous growth and proceeded to sporulation. SS cells completed one synchronous division after filtration; sporulation ensued after the cessation of growth. SSG cells completed two synchronous divisions and sporulation occurred during the second division. A high degree of synchrony of vegetative growth of SSG cells was evident by the stepwise pattern of growth, by the doubling of cell numbers at each division, the high division index, and by the rapid formation of sporulation cell types and homogeneity of cell types in the filtered cultures when compared with asynchronous cultures. Because the described system gives both good growth and sporulation synchrony, the method should be useful in delineating early events in sporulation and their regulation.

It is beyond the realm of conventional culture techniques to study the metabolic activities of a single bacterial cell. Therefore, the manipulation of a culture when, ideally, all the cells are engaged in the same metabolic activities has been a useful tool in physiological studies.

Several well-defined events take place during the process of sporulation (12, 13, 22). These events include the production of acids, the turnover of protein, the division and separation of nuclear material, and the formation of spore components and structure. These steps have been characterized in cells grown asynchronously or with the use of the active culture technique (11). The latter gives good sporulation synchrony and has been useful in defining many late stages of sporulation.

Synchronous culture techniques have achieved sufficient development to be applied to a complex physiological system such as sporulation (7, 30). Preliminary experiments (J. R. Gillis et al., Bacteriol. Proc., p. 37, 1965) indicated that a population of *Bacillus megaterium* could be made to proceed synchronously from division to division and subsequently to sporulation. A study of these cells would enable one to postulate the sequence of events occurring in a single cell during the process of sporulation.

This report describes an easily performed "selective synchrony" method which gives both growth and sporulation synchrony and which, because of good division synchrony, should be useful in studying early events of sporulation and their regulation. A comparison is made of cells grown asynchronously and synchronously in a defined sucrose salts medium without (SS) and with glutamate (SSG). In the latter medium, the results suggest that terminal cell division and sporulation can proceed together.

MATERIALS AND METHODS

Organism and culture media. B. megaterium ATCC 19213, originally obtained from the Department of Microbiology at the University of Texas, was grown on SS medium prepared according to Slepecky and Foster (27). In some experiments, the SS medium was supplemented with additional sucrose (to 0.5%), Casamino Acids (0.01 to 0.1%; Difco), Trypticase (0.01 to 0.1%; BBL), mixtures of amino acids (0.01% each; Nutritional Biochemicals Corp., Cleveland, Ohio), or individual amino acids, particularly glutamic acid (SSG).

Culture technique. A stock spore suspension was prepared by inoculating 50 ml of half strength Nutrient Broth (Difco) in a 250-ml Erlenmeyer flask with cells from a 24-hr Nutrient Agar slant. After incubation at 30 C for 24 hr on a New Brunswick rotary shaker (187 rev/min), 5 ml of the culture was inocu-

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lated into a 3-liter Fernbach flask containing 250 ml of SS medium which was incubated at 30 C on a reciprocating shaker [219 oscillations per min, stroke 1.5 inches (3.8 cm)]. Spores formed by 60 hr were harvested by centrifugation in a Sorvall Superspeed Refrigerated Centrifuge at 2,000 × *g* for 15 min at 6 C. The spores were washed once with distilled water and suspended in saline to a turbidity of 350 Klett units, measured in a Klett-Summerson photoelectric colorimeter with a no. 54 filter. The spore suspension was stored at −20 C. Prior to use, the frozen suspension was thawed at 6 C for 6 hr. Samples (16 ml) were heat-shocked at 60 C for 1 hr, with Vortex mixing every 10 min, and then inoculated into 200 ml of the designated medium in a 3–1 Fernbach flask; the culture was incubated at 30 C on the reciprocating shaker. When the culture reached late log phase (approximately 140 Klett units when cells were grown in SS medium), it was filtered. For asynchronous cultures, this filtration step was omitted.

Filtration procedure. The final filtration technique adopted was a modification of the procedure of Maruyama and Yanagita (20). Each of two 600-ml Pyrex Buchner-type funnels with a coarse fritted-glass disc, 9.0 cm in diameter, was packed with nine layers of Whatman no. 40 filter paper. The filter-paper pad was then moistened with distilled water and packed down on the fritted-glass disc with a blunt glass rod. Distilled water, 500 ml, was then slowly passed through the pad under suction from a water aspirator. Care was taken so that the pad remained fitted to the funnel. The water in the collection flask was discarded, and the entire assembly was sterilized by autoclaving for 20 min at 121 C. After sterilization and immediately before culture filtration, 50 ml of the sterile medium under study was passed through the filters with suction and was discarded. The culture, ready for filtration, was divided into two equal samples, and each sample was filtered through a separate funnel in about 1 min by suction from a water aspirator. The filtrates containing those cells passing through the filters were then aseptically pooled into a sterile 3–1 Fernbach culture flask. The culture was returned to the shaker machine and incubated at 30 C. The entire filtration process took less than 5 min and was performed at room temperature.

Turbidity measurements. At the times indicated in the results, a 5-ml sample was withdrawn from the flask for turbidity estimations with a Klett-Summerson photoelectric colorimeter with a no. 54 filter; a portion of each sample was used for total counts and estimations of the percentage of cell types and staining were as described below.

Microscopic counts. The number and types of cells were counted periodically in a Petroff-Hausser counting chamber with a Zeiss dark-contrast phase microscope. Cells of *B. megaterium* grown in the media described form well-defined chains containing two to eight cells. A cell is defined as the smallest single unit of a chain, and a chain is defined as cells linked to form a unit greater than one. A cell count is a total count of the cells present in the chains and as single cells. Nonrefractile rod-shaped cells were recorded as vegetative cells; rod-shaped cells containing phase-dense bodies destined to become sporangia were considered forespores; rod-shaped cells having within them distinct refractile bodies were regarded as sporangia, and oval or spherical highly refractile bodies were counted as free spores. Measurement of the size of the cells and chains before and after filtration was done under the oil-immersion objective by use of a previously calibrated ocular micrometer.

Staining of cells. At various times, smears of cultures were made, air-dried, heat-fixed, stained with a 2% alcoholic solution of crystal violet (National Aniline Div., Allied Chemical Corp., New York, N.Y.) for 1 min, and examined in the phase-contrast microscope with the oil-immersion objective.

Division index. The division index, defined as the percentage of cells showing a definite transverse septum at any given time, was determined from crystal violet-stained preparations viewed in the phase-contrast microscope.

Criterion for growth. The term growth as used in this paper refers to an increase in cell numbers.

RESULTS AND DISCUSSION

The typical growth and sporulation pattern of an asynchronous culture of *B. megaterium* grown in SS medium is given in Fig. 1. Growth of the cells as determined by turbidity measurements and total counts reached a maximum at about 14 hr. After this time, cells no longer divided, but at about 18 hr forespores and sporangia could be detected. Forespore production continued until 22 hr when about 60% of the cells were in the forespore stage. After 22 hr, the number of fore-

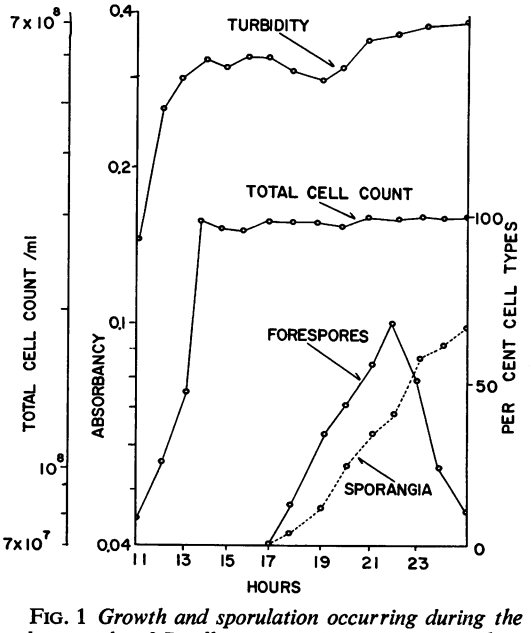

FIG. 1 *Growth and sporulation occurring during the culture cycle of Bacillus megaterium grown asynchronously in SS medium.*

spores in the culture declined with time. The number of sporangia in the culture continued to rise from 18 to 25 hr when 70% of the cells were sporangia. Continued cultivation of the cells resulted in the completion of the spore cycle, and at about 48 hr the culture contained 90 to 95% free spores. The rise in turbidity after 19 hr is due to the increased light scattering caused by the rise in the number of refractile sporangia.

Such a growth pattern, usually obtained with batch cultures of aerobic sporeformers grown on on a variety of sporulation media, is marked by the onset of sporulation after the cessation of growth (12, 13, 22, 29) and by a heterogeneous population of cell types during vegetative growth and sporulation. For example, with the asynchronous culture of *B. megaterium*, noted previously (Fig. 1), during the early hours the culture contained a mixture of vegetative cell types, and from 18 to 21 hr contained a mixture of vegetative cells, forespores, and sporangia. Between 21 and 25 hr, the culture contained mainly forespores and sporangia.

When the SS medium was supplemented with mixtures of amino acids, Casamino Acids, Trypticase, or individual amino acids, the cell yield was more than doubled but sporulation still occurred after the cessation of growth. One such case is illustrated in Fig. 2 in which the growth and sporulation pattern is shown for *B. megaterium* grown in SS medium supplemented with 0.01% L-glutamic acid (SSG medium). When compared with SS-grown cells, the growth rate of the SSG cells is increased in that the cell yield is greater in approximately the same time of cultivation, and the cells go through more divisions. Only slight variation was found in the time of onset of sporulation and in the pattern of formation of the various cell types. There was, however, heterogeneity of cell types at all hours.

Various attempts were made to obtain a homogeneous culture in which cells undergo synchronous cell division. The filtration technique of Maruyama and Yanagita (20) was finally selected. Numerous filters and filter piles were tried before obtaining synchrony of the cells. Büchner-type funnels with fritted-glass filtering areas gave more uniform filtration than plain Büchner or Millipore funnels. After many trials with various combinations of filter papers of different characteristics, the best reproducible synchrony was obtained with nine layers of Whatman no. 40 filter paper. When a typical asynchronous culture growing in SS medium (Fig. 1) was filtered 12.5 to 13.5 hr after inoculation, the pattern of growth and sporulation of the cells in the filtrate was as shown in Fig. 3. There was a reduction in filtrate turbidity to an optical density of 0.11. The turbidity rose to an optical density of about 0.22 in the 1st hr and remained stationary until 6 hr, when it began to increase; it reached an optical density of 0.28 at 10 hr. This rise in turbidity was

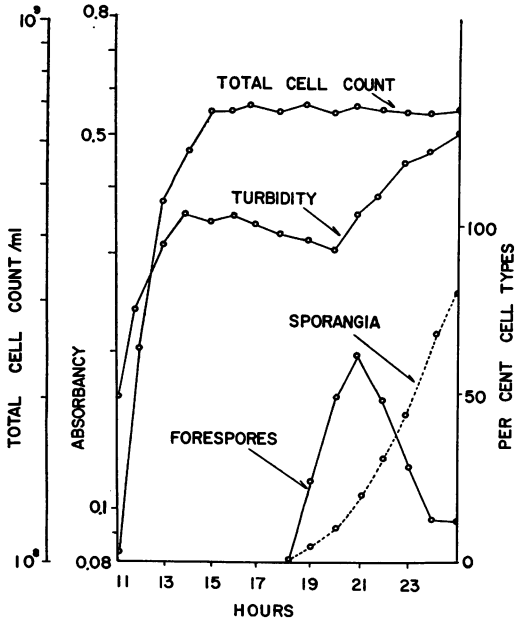

FIG. 2 *Growth and sporulation occurring during the culture cycle of Bacillus megaterium grown asynchronously in SSG medium.*

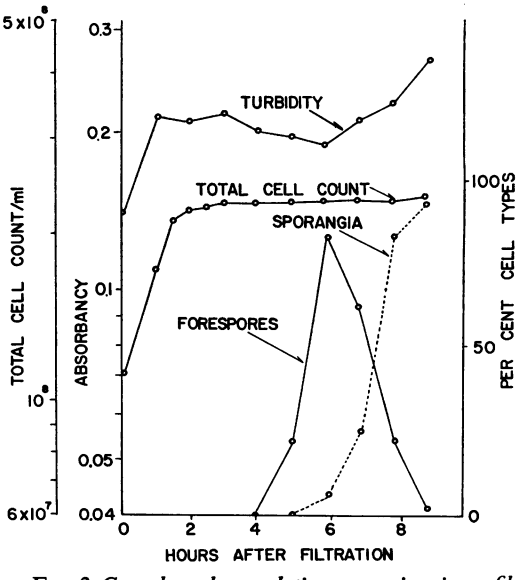

FIG. 3 *Growth and sporulation occurring in a filtered culture of Bacillus megaterium grown in SS medium.*

coincident with the formation of sporangia. Total cell count after filtration was 12×10^7 per ml, and in 3 hr the count increased to 25×10^7 per ml. This almost exact doubling of cells, as well as the 3-hr generation time, was consistently obtained under these growth conditions. At 4 hr (1 hr after the cessation of growth), the cells formed forespores, followed by the formation of sporangia. After 6 hr, the cells were more out of phase than in the previous hours; however, the degree of sporulation synchrony was still much greater than that found in the asynchronous system. Filtration of cells at times earlier or later than those times indicated resulted in asynchrony.

Evidence suggesting that the filtration selects for particular size chains of cells is given in Fig. 4. These frequency histograms show the distribution of chains and cells before and after filtration in a typical experiment. The data indicate that after filtration the chains were mainly composed of two cells, each 4 μ long. Counts of chains and number of cells per chain at various times during subsequent growth in the filtrate did not show a synchronous pattern but indicated that chains of two and four prevailed. This suggested that the doublet cells divided to give chains of four and that the division was followed by some subsequent splitting of the chains during growth.

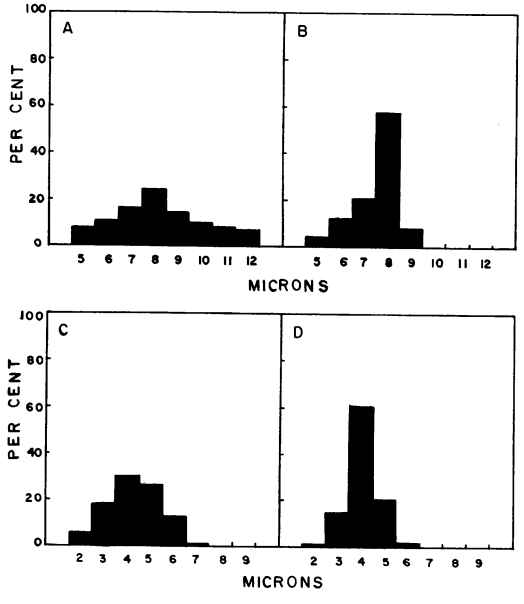

FIG. 4 *Frequency histograms of the distribution before and after filtration of cell and chain sizes in microns of Bacillus megaterium grown in SS medium. (A) Chains before filtration; (B) chains after filtration; (C) cells before filtration; and (D) cells after filtration.*

The synchrony obtained may have been achieved by the selection of a certain size chain; the originators (20) of the filtration technique made this interpretation. However, recent findings (9, 14, 15) with anion-exchange cellulose filters which bind bacteria indicate that only newly formed daughter cells are eluted and then grow synchronously. The finding that doublet cells predominate in the filtrate by use of the present technique suggests a similar phenomenon. Perhaps filtration in the cold or chemical inhibition of possible division during filtration could resolve this point; however, the possibility of introducing physiological abnormalities by such treatment exists. This fact has led to criticism of methods of induced synchrony (1, 18).

Our rapid filtration method, involving a minimum of handling and physiological changes, for attaining a synchronous growth division followed by fairly well-phased sporulation stages differs from the "sporulation synchrony" methods used by others (8, 11, 29). "Sporulation synchrony" obtained by the active culture technique is based on sporulation ensuing after the last of a series of transfers of cells. The rapid appearance of sporangia was the only criterion for synchrony since no synchrony of cell division prior to sporulation was reported.

In some preliminary experiments we obtained a second growth division after filtration. Therefore, a search was made for conditions which would allow more than one division of the cells after filtration. In one series of experiments, various amounts of additional sucrose (up to 0.5%), Casamino Acids (0.01 to 0.1%), and Trypticase (0.01 to 0.1%) were added separately to the SS medium. When the cultures were in approximately the same growth phase which gave synchrony in the SS controls, as estimated by turbidity measurements, the cultures were filtered and the filtrates were treated as previously described. Synchronous cultures showing more than one division were obtained only with the filtrates of cells which had been grown on SS medium supplemented with Trypticase (to 0.06%). Similar results were obtained when the supplements were added immediately after filtration. Subsequent experiments indicated that more than one division occurred in SS medium supplemented with 0.01% L-glutamic acid or 0.01% L-aspartic acid. Figure 5 shows the results of a typical SSG asynchronous culture, similar to the one whose growth and sporulation pattern was illustrated in Fig. 2, filtered when the culture was in the late log phase. The turbidity pattern was similar to that found with filtered SS cultures (Fig. 3). Immediately after filtration, the culture contained 11×10^7 cells per ml, and in 2 hr there was a doubling to

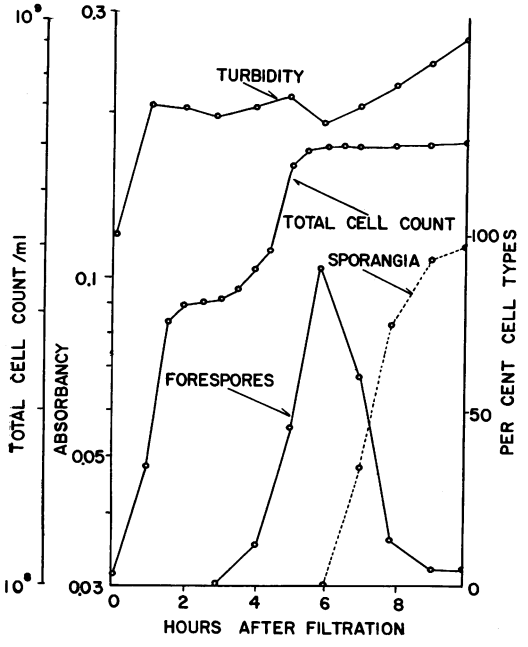

FIG. 5 *Growth and sporulation occurring in a filtered culture of Bacillus megaterium grown in SSG medium.*

about 22 × 10^7 cells per ml. Thus, the generation time of the cells during this first division was 1 hr, which is shorter than that of the cells from the SS medium-filtered cultures. A second division ensued from 3 to 5.5 hr, when the cells again doubled to 42 × 10^7 cells per ml. This second division occurred during the same times that forespores were detected. The pattern of formation of forespores and sporangia was similar to that found in filtered SS cultures, but sporulation began slightly earlier. Furthermore, as shown by a comparison of the unfiltered with filtered SS culture (Fig. 1 versus Fig. 3), a comparison of the unfiltered SSG culture with the filtered culture of the same medium (Fig. 2 versus Fig. 5) shows that the degree of sporulation synchrony is greater in the filtered culture. Forespores appeared during the second division, and the maximal number of forespores was reached at 6 hr after filtration. At that time, very few sporangia were detected, but they shortly appeared in greater number and were produced through 10 hr, when the maximal sporangial population was found. Strikingly different from what happened with the SS-filtered culture was the formation of forespores before the end of the last cell division.

It appeared to make little difference whether the cells had been grown in glutamate before filtration or whether the glutamate was added to SS-grown cells immediately after filtration. In both cases, two divisions were obtained after filtration. No immediate explanation can be given for this glutamate effect. It is known that glutamate plays an important role in sporulation (3, 5, 19, 21, 26); however, in the present system, if glutamate is required for sporulation, the endogenous glutamate would probably be sufficient since sporulation occurred in the SS medium alone. It would appear that glutamate plays a role mainly in the additional synchronous division only upon its addition to the medium (Fig. 5). Glutamate has been shown to bring about a marked shift in growth rate (Fig. 1 versus Fig. 2); subsequently, filtration of the SSG-grown cells results in different division behavior (Fig. 5). Kjeldgaard, Maaløe, and Schaechter (16) have shown an increase in number of nuclei per cell followed by a shift in the division rate when *Salmonella typhimurium* was transferred from a minimal to a rich medium. Work is in progress to determine whether this is a possibility in the described *B. megaterium* system.

In all the experiments, microscope cell counts, particularly of those cells in chains, were based on the ability to discern individual cells in the phase-contrast microscope. Upon this basis, two divisions have been recorded (Fig. 5); however, this assessment indicated that forespore formation accompanied cell division. After staining the cells with crystal violet at various times after filtration, definite transverse septations could be noted at least 1 hr before they could be seen in the unstained preparations (Fig. 6). This means that, based on staining, cells had divided prior

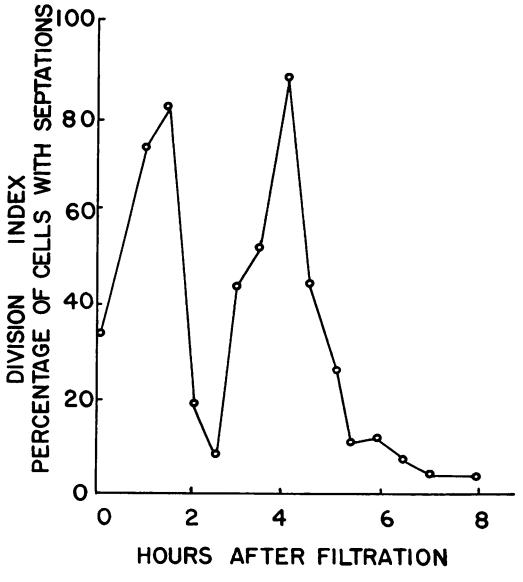

FIG. 6 *Division index (percentage of cells with septations) of a filtered culture of Bacillus megaterium grown in SSG medium.*

to the appearance of forespores. However, it is still highly likely that sporulation events are in process even during the last division, since it is known (5) that thin sections observed in the electron microscope show the first morphological stages of sporulation 1 to 2 hr before they are seen under phase optics. That growth and sporulation are not incompatible is evident from the work of others (24, 28) who have demonstrated sporulation during the logarithmic phase of growth.

Furthermore, the high division index (Fig. 6) substantiates the degree of synchrony obtained in this system. The division index is analogous to the mitotic index in synchronous eucaryotic organisms (4, 23, 31) and is considered to be a good indication of overall synchrony (7, 30). The division index is constant at about 40% in a SSG culture growing asynchronously.

The stepwise pattern of growth and the doubling of cell numbers at each division (Fig. 2 and Fig. 5) fit the criteria for synchronous growth as described in other systems (2, 4, 6, 10, 17, 25; H. Halvorson et al., Federation Proc. **23**:1002, 1964). In addition to the stepwise doubling of cell numbers, further evidence for synchrony has been given by the high division index, by the rapid formation of sporulation cell types, and by the increase in the homogeneity of the cell types when compared with the asynchronous culture.

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