OBSERVATIONS ON THE NUCLEAR CYTOLOGY OF SPORE GERMINATION IN BACILLUS MEGATERIUM

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Spore germination has generally been recognized to consist of a swelling of the spore followed by a splitting of the spore coat in a characteristic manner. Less often an absorption of the spore coat has been described. This process is accompanied by a decrease in refringence and an increase in stainability. Considerable difference of opinion is apparent, however, concerning the existence, form, and location of a spore nucleus and its activity during germination. The following brief historical review is concerned only with those investigations which deal with the internal structure of the resting and germinating spore. The reader is referred to the original papers for more detail and to the review of Knaysi (1948) for other aspects of spore germination.

Meyer (1897, 1899) was unable to observe a nucleus in the resting spore. However, in swollen spores in the process of preparing for germination, a distinct peripherally located nucleus was observed. In the young bacillus, following germination, one to two nuclei were seen.

The spore was regarded by Preisz (1919) as consisting of a strongly refractile "shiny body" made up of reserve material, surrounded by a thin layer of protoplasm and a spore coat. Apparently no nucleus was visible until germination commenced. At this time a round or elongated nucleus appeared between the equatorial side of the shiny body and the spore coat. During the course of germination the nucleus often swelled at the periphery of the spore. The "shiny body" became more stainable. Eventually the spore took on a homogeneous appearance, and the nucleus and the "shiny body" could no longer be distinguished. The young bacillus, as it emerged from the spore coat, contained one or two nuclei.

Badian (1933, 1935) was able to stain spores of *Bacillus mycoides*, *Bacillus subtilis*, and *Bacillus megaterium* only after germination had commenced, at which time a central chromatinic rod could be observed. This rod took on a transverse position and divided longitudinally into two daughter "chromosomes".

Two types of spores were recognized by Allen, Appleby, and Wolf (1939). The first type was considered to be "haploid" and showed no internal structure either in the resting state or during germination. The second type was considered to be "diploid" and showed, according to these authors, nuclear figures suggesting meiosis during germination.

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Feulgen-positive bodies located at the periphery of the spore have been described for various members of the genus *Bacillus* by the following investigators: Pietschmann and Rippel (1932), Milovidov (1935), Stille (1937), Schaede (1939), Piekarski (1940), Stapp (1942), Robinow (1945), and Delaporte (1948). Stille claimed that in old spores (from 120-day-old cultures) the nucleus took on a central position. Stille, Piekarski, and Stapp each studied spore germination and reported similar results. The appearance of two Feulgen-positive bodies in the swollen spore or the young rod was interpreted as evidence for division of the single spore nucleus during germination.

Robinow (1945), studying chiefly *Bacillus mycoides* and *Bacillus mesentericus*, employed N HCl as a means of rendering the spore coat permeable to Giemsa stain. He described the spore as consisting of three concentric layers, a refractile and stainable core, a layer of less refractile and less stainable cytoplasm, and the stainable, nonrefractile spore membrane. The nucleus, the most refracting element, "is attached to the outer surface of the cytoplasm." It has the shape of a biconcave disk. Germination is described as follows: the nucleus "actively enters or is engulfed by the cytoplasm" where it is not easily distinguished. The affinity of the cytoplasm for the dye greatly increases, obscuring the nucleus. A distinct chromatinic structure in the form of a distorted C, S, V, 8 figure, or elongated ring soon reappears. This gradually contracts, appearing first as a knotted string of chromatin and later as a deeply stained polygonal body. Thereafter, this body is found in various stages of division, giving rise to the dumbbell-like chromatinic bodies observed in the vegetative cell by Robinow and others.

Delaporte (1948) recognizes and describes two kinds of spores in *B. mycoides*. The first type, representing the majority of spores, is strongly refractile and shows, after hydrolysis, a nucleus in the form of an ovoid or elongated central granule. The second type is nonrefractile, possesses a thick membrane, and contains a nuclear element which is stainable with vital stains. This nucleus, which may assume the shape of a granular ring, is located at the periphery and appears to lie outside the spore. A second nuclear granule is sometimes seen inside the spore. When germination commences with a number of sporeformers, the nuclear element swells, assuming the shape of a granular ring or possibly a disk with a thin center. It then stretches through the entire length of the cell, forming a continuous axial thread. The nuclear structure may increase in size.

Knaysi (1946) made a study of sporulating cells of *Bacillus cereus* with the Feulgen procedure. Bodies containing Feulgen-positive central rods or granules within a colorless zone were interpreted as mature spores. More recently, Knaysi, Baker, and Hillier (1947), and Knaysi and Baker (1947), investigated *B. mycoides* with the electron microscope. They concluded that the "forespore" contains two to six nuclei. The electron microscope failed to reveal any internal structure in resting or germinating spores.

The purpose of the present paper is to record preliminary observations on the nuclear phenomena associated with spore germination as observed by means of the newly developed procedures cited. It is felt that these methods afford a

greatly increased opportunity to observe nuclear behavior in undistorted cells. Present observations also constitute a departure from those of past workers, a fact which again appears to depend mainly upon methodology.

MATERIALS AND METHODS

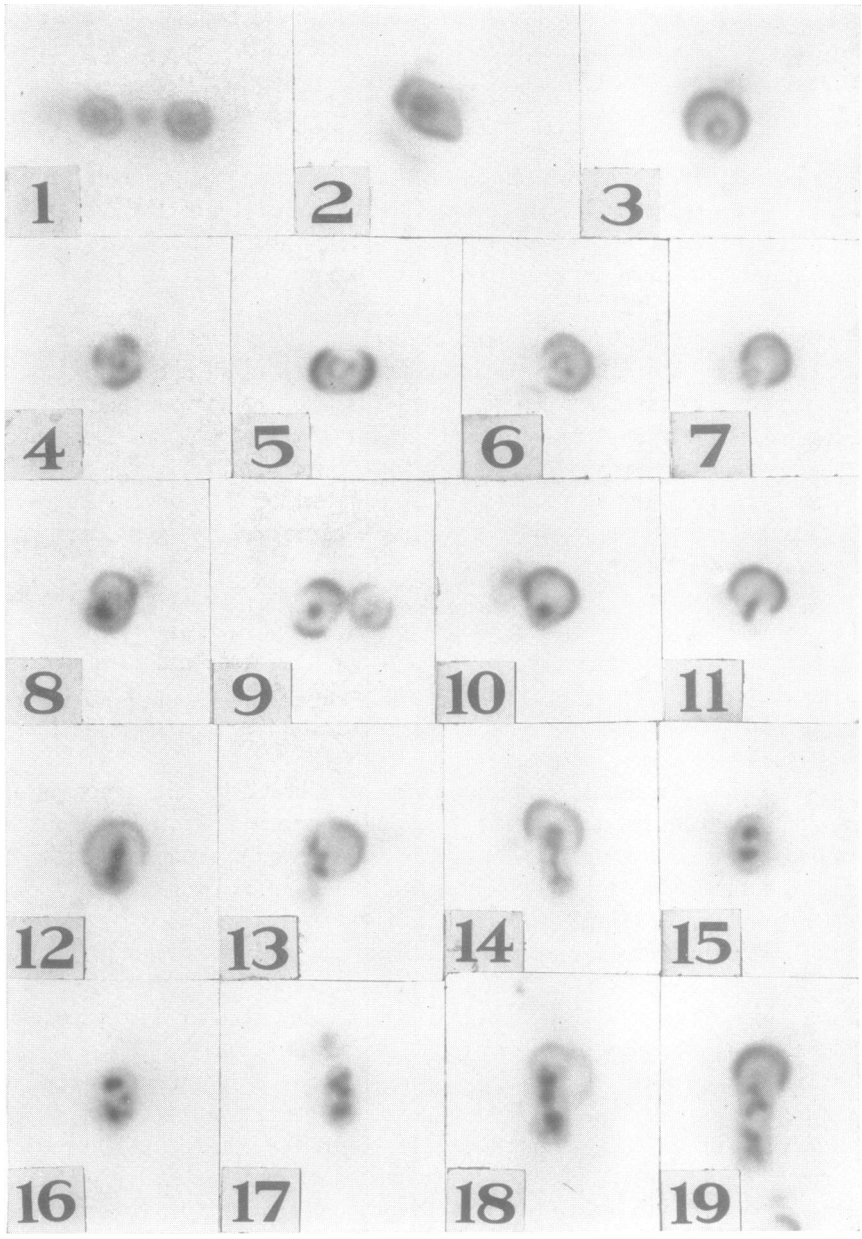
The organism used was the same strain of *B. megaterium* used in previous studies (DeLamater, 1951*b*; DeLamater and Mudd, 1951; DeLamater and Hunter, 1951–1952). Spore suspensions were prepared in the following manner: sporulation was induced, as described in the preceding paper (DeLamater and Hunter, 1952), by cultivation on agar containing 0.1 per cent casamino acids (Difco) and 0.5 per cent NaCl, a modification of a medium described by Tarr (1932). A 72-hr plate culture was flooded with 5 ml of sterile distilled water, and the growth agitated with a wire loop. The suspension was then retrieved with a pipette and concentrated to about half its volume by centrifugation. The vegetative cells were destroyed by heating to 60 C for 10 minutes. The customary 80 C was found to be unnecessarily high. The suspension was stored in the refrigerator. Fresh spores were prepared each week because an increase in germination time with aging of the spore was noted.

The suspension, as prepared, consisted almost entirely of spores. A certain percentage of inviable spores was always present. Some of these may represent spores that had commenced germination on the old medium and were destroyed with the vegetative cells by exposure to heat and distilled water. They could be recognized in stained preparations by their lack of the characteristic heavily stained spore wall.

In early work, germination of the spores was allowed to take place on agar containing 3 per cent casamino acids (Difco). Later work indicated that nutrient agar enriched with 0.5 per cent yeast extract (Difco) was superior in two respects: germination proceeded more rapidly, and the nuclear elements were larger and, therefore, more clearly delineated. Plates were inoculated by spreading 0.05 ml of the spore suspension over an area of about 2 square inches. The first new bacilli appeared after about 3 hours' incubation at 37 C on the yeast extract medium, and in 4 hours on the casein hydrolysate agar. Four- and five-hr cultures were ideal for observing spores in all stages of germination.

Preparations were made according to the freezing-dehydration method described by DeLamater (1951*a*). The details of the procedure have been set forth in the preceding paper (DeLamater and Hunter, 1952) and will not be repeated here. Acid hydrolysis of spores in early stages of germination proved to be unnecessary because of the low ribosenucleic acid content of the cytoplasm which, however, rises very rapidly. Hydrolysis was always conducted because of the presence of spores in various stages of germination in the same preparation.

Photographs were taken with a Gamma photomicrographic camera and a Bausch & Lomb research microscope having the following lens system: a 97× achromatic objective, a 12.5× widefield ocular, and a 4 lens 1.40 NA research condenser. All lenses were balcoated. A Bausch & Lomb ribbon filament lamp was used for illumination. Images were intensified on Panatomic X film with



Figures 1–19. Bacillus megaterium. Spore germination on 3.0 per cent casein hydrolysate agar

Figure 1. Photograph no. 1408—×4,450—Resting spores; nuclei somewhat obscure.

Figure 2. Photograph no. 1387—×4,450—Six hours' germination; enlarged spore with ring-like nucleus.

Figures 3, 4, 5. Photograph nos. 1492, 1496, 1408—×4,450—Five and one-half hours' germination; spore coat split; nucleus in "ring" form with one chromosomal granule visible.

Figures 6, 7. Photograph nos. 1406, 1427—×4,450—Five and one-half hours' germination; spore coat split; three chromosomal granules visible in nucleus.

light green and amber Corning glass filters. Exposure times ranged between 12 and 20 seconds. Primary magnification of 1,112.5× was used, with a final magnification of 4,450× in the finished prints.

RESULTS

The following series of observations is in direct continuity with that of the preceding paper, which defines sporulation as a vegetative process, and considers the activities of the nuclei of the sporulating cell. The following observations deal with the process of spore germination and, as will be seen, tend to emphasize the vegetative aspects of the process.

Figures 1 through 19 depict germinating spores as they appear on casein hydrolysate agar. In figure 1 unswollen spores are observed in which both the nuclei and the spore coat are clearly defined. As spore germination proceeds, the spore swells, as observed in figures 2, 3, etc., and the spore coat ruptures. As described by Knaysi and Hillier (1949), the rupture may occur at the equator or between the equator and the pole, as a slit at one side, or may split circularly completely around the spore so that the expanding cell has two caps of spore coat at each end, as seen in figures 5 and 9. During this early phase of spore germination, the basophilic substance surrounding the nucleus of the spore tends to disappear so that the hydrolysis period required to visualize the spore nucleus is reduced almost to zero. The spore nucleus is observed to be central in the cell, and at no time has it been observed to be peripheral to the spore cytoplasm. Following rupture of the spore coat, a protoplasmic projection or germination tube is extruded through the aperture of the spore coat, as seen in figures 7, 10, and 14. The nucleus becomes less dense and tends to take on a ring-like configuration, often simulating a signet ring. One granule at the periphery of the ring is frequently very obvious. Not infrequently three distinct granules are very clearly definable (figures 6 and 7). It is felt that these represent the three condensed and contracted vegetative chromosomes. Subsequent to this the chromatinic material of the spore may again become dense as though the cell were becoming meta-

Figure 8. Photograph no. 1398—×4,450—Six hours' germination; nucleus increasing in density; chromatin massed on one side of nucleus; individual chromosomes not distinguishable.

Figures 9, 10. Photograph nos. 1434, 1379—×4,450—Five and one-half hours' germination; nuclear chromatin in compact mass.

Figures 11, 12, 13. Photograph nos. 1428, 1382, 1390—×4,450—Five and one-half hours' germination; nuclei in division; chromosomes too small to be individually distinguished.

Figure 14. Photograph no. 1430—×4,450—Five and one half hours' germination; telophase divisional stage of nucleus; germinating cell elongating.

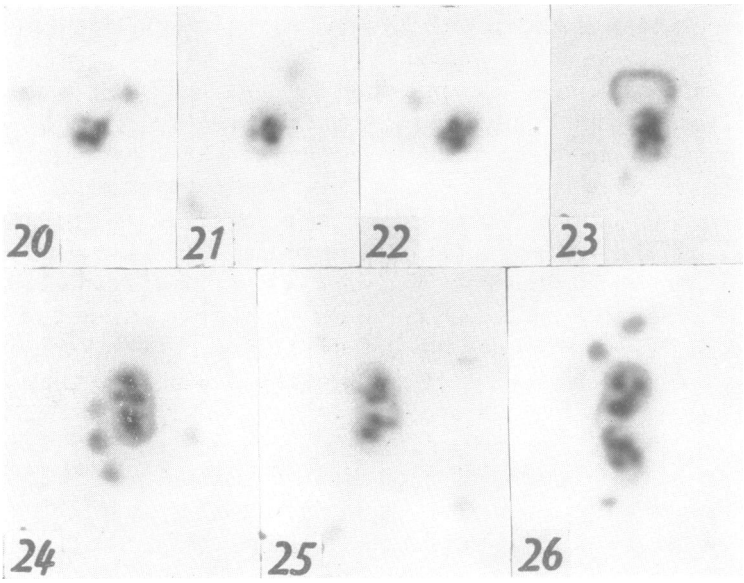
Figure 15. Photograph no. 1394—×4,450—Five and one-half hours' germination; separation of chromosomes complete; spore coat lost.

Figures 16, 17. Photographs nos. 1402, 1494—×4,450—Five and one-half hours' germination; binucleate stages in which one nucleus is commencing second mitosis; spore coat lost.

Figure 18. Photograph no. 1498—×4,450—Five and one-half hours' germination; binucleate stage; note telophase spindle in lower half of cell.

Figure 19. Photograph no. 1432—×4,450—Five and one-half hours' germination; binucleate stage; chromosomes elongating in late telophase or early interphase; one polar body still faintly visible at top of cell.

bolically active and synthesizing desoxyribose nucleic acid and laying this down in the germinating spore nucleus. Shortly after this increase in density occurs, the spore nucleus undergoes its first division, as seen in figures 11 to 14. The nucleus at this stage on this medium is too small to define the process with clarity, since the structures being observed approach the resolving power of the microscope. They appear, however, to parallel and simulate the mitotic processes described in vegetative cells, and very soon take on the aspects of the mitotic process as the



Figures 20–26. Bacillus megaterium. Spore germination on 0.5 per cent yeast extract agar

Figure 20. Photograph no. 1978—×4,450—Four hours' germination; prophase with three chromosomes and a single centriole visible.

Figures 21, 22. Photographs nos. 1992, 1972—×4,450—Four hours' germination; metaphase stages.

Figure 23. Photograph no. 1959—×4,450—Five hours' germination; ana-telophase stage.

Figure 24. Photograph no. 1980—×4,450—Four hours' germination; telophase.

Figure 25. Photograph no. 1990—×4,450—Four hours' germination; telophase; three chromosomes can be counted in upper half of spindle.

Figure 26. Photograph no. 1981—×4,450—Four hours' germination; binucleate cell in interphase; chromosomes in long, beaded threads.

cell enlarges. More conclusive pictorial evidence for mitosis is given in figures 20 through 26 and will presently be described. Figure 13 is construed as a late telophase stage in which the two sister nuclei, daughter nuclei of the spore nucleus, tend to rotate during separation so that each lunar structure observed represents a cluster of chromosomes. Figure 14 is considered to show the pulling apart of the chromatinic material with the central band of chromatin still connecting the daughter nuclei in a manner suggestive and reminiscent of that observed in yeast by DeLamater (1950). Figures 15 and 16 demonstrate the two

condensed and separate daughter nuclei in a young vegetative cell which has shed its spore coat. Figure 17 shows the early second division of the two nuclei in the young vegetative cell. This is construed as an ana-telophase stage in which the polar bodies are not in focus. Figure 18 shows a similar stage with the spore coat still attached to one end of the vegetative cell. In figure 19 a somewhat later stage in the mitotic division of the two daughter nuclei is to be observed. Again, the spore coat can be seen remaining attached to the upper pole of the young vegetative cell.

Figures 20 through 26 represent later work, in which germination was induced on nutrient agar containing yeast extract. The greater size of the nuclear elements on this medium makes possible the clarification of the stages in which the chromosome groups appear as unresolved, condensed masses in figures 8 through 16, and removes doubt as to the mitotic nature of the first germination division. Figure 20 shows a germinating spore with the nucleus in prophase. The three chromosomes and a single centriole are clearly defined. Metaphase stages are illustrated in figures 21 and 22. The condensed chromatinic masses of figures 8, 9, and 10 probably correspond to prophase or metaphase stages. Figure 23 is interpreted as an ana-telophase stage, with the two chromosome groups still partially attached. Later telophase stages are shown in figures 24 and 25. Rotation of the two halves of the spindle has occurred. The three chromosomes can again be separately distinguished in figure 25. In figure 26 the binucleate stage has been reached. The chromosomes have elongated into the fine, beaded threads of interphase, and the first mitotic cycle has been completed. Loss of the spore coat appears to occur earlier on this medium than on the casein hydrolysate medium, as indicated in figures 20 to 26 by the absence of the coat except in figure 23.

DISCUSSION

It is felt that the first germination division has been clearly demonstrated to be a mitotic one, and that this feature emphasizes the vegetative nature of the sporulation process in bacteria. The nucleus at all times is observed to be central in the cell, and at no time has been seen to be eccentric. It seems likely that Robinow and others have induced the eccentric position of the spore nucleus by the drastic nature of the procedures which they have used. It may be emphasized in this connection that the methods herein utilized, in which a rapid freezing-dehydration process is used, probably constitute a much more gentle procedure.

It seems to the authors that the laying down of pari-nuclear substance during the process of sporulation tends to obscure the details of the nucleus, and may also aid in accounting for the difficulties of past workers in visualizing the process.

Since three distinct granules of chromatin have been visualized during the process of spore germination, it seems likely that these three chromatin granules represent the three haploid chromosomes and that the process of sporulation and spore germination probably represents a strictly vegetative one similar to that which occurs in fungi. Upon germination the nucleus appears to reorganize and go directly into mitotic vegetative reproduction. No evidence for sexuality or

autogamy has been observed in either sporulation or spore germination. The processes described tend to emphasize the similarity of bacteria, as a general group, to other microorganisms in their essential processes, and tend, with the demonstration of a typical mitotic process, to establish this large group of microorganisms morphologically into the general biological economy where they belong.

SUMMARY

The nuclear activities which occur during spore germination of *Bacillus megaterium* have been described. The observations indicate that the process is a vegetative one, and that as reorganization from the condensed spore nucleus proceeds, it immediately begins a typical mitotic process which is carried on in the vegetative cell.

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