THE NUCLEAR CYTOLOGY OF SPORULATION IN BACILLUS MEGATERIUM

EDWARD D. DELAMATER[1] AND MARY ELIZABETH HUNTER

Department of Dermatology and Syphilology and the Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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A complete review of the literature on the extensive subject of sporulation in bacteria will not be undertaken here. The reader is referred to the exhaustive review of Knaysi (1948).

The literature dealing with spore formation in bacteria is generally divided into three different categories, corresponding to three major interpretations of the process. The first of these interpretations, originated by Cohn (1876) for *Bacillus subtilis*, and by Koch (1876) for *Bacillus anthracis*, places the origin of the spore in a single granule. This granule makes its appearance as spore formation commences and rapidly increases in size and refringence.

The second interpretation originated with Zopf (1883), who explained spore formation in *Bacillus tumescens* as an aggregation of several refractile granules. A process which must be placed in this category was described by Schaudinn in 1902 for the disporic organism, *Bacillus bütschlii*, and is of considerable importance because of the biologic significance attached to it by the author. The disappearance of a cross wall and the rearrangement of chromatinic granules into an axial spiral preceding their aggregation were interpreted as a primitive copulation of incompletely separated daughter cells. Dobell (1908) described a process in *Bacillus flexilis* identical to that proposed by Schaudinn. Later, presenting an altered concept (1909, 1911), Dobell challenged Schaudinn's interpretation of sexuality on the basis that a similar situation, with the formation of two spores, can arise in the monosporic organism, *Bacillus spyrogyra*, and merely represents an abortive cell division, not a sexual process.

The third interpretation supposes the formation of spores by progressive condensation of the protoplasm. The gradual accumulation of "volume" of the "prespore" area suggested by the first two concepts differs from that suggested here in that spore formation is heralded by the sudden appearance of an evenly opaque spore primordium. This is denser than the cytoplasm and slightly larger than the finished spore. The contents of this area gradually increase in density and refringence, and a spore wall forms. Preisz (1904), who described this process in great detail for *Bacillus anthracis* and *Clostridium tetani*, observed a round or elongated nucleus within the spore primordium which disappears during maturation of the spore. Advocates of sporulation by condensation of the protoplasm are many, and their names need not be mentioned here.

[1] Research Professor of Dermatology and Syphilology, and Research Professor of Microbiology.

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The following more recent works do not fit precisely into the three interpretations outlined, nor do they take particular issue with them. The excellent motion photomicrographs of Bayne-Jones and Petrilli (1933), taken of unstained, living cells of *B. megaterium*, give impetus to the concept that sporulation cannot be entirely accounted for as a growth or aggregation of granules, as described by earlier workers. According to these authors, sporulation consists of "a condensation of the protoplasm in the ends of the cells and a rapid contraction of material in this region." Granules believed to have been displaced from the presporal areas were observed to strike against the edge of the cleared primordium, but failed to penetrate into it. The work of Bayne-Jones and Petrilli on *B. megaterium* is of particular interest because their pictures are very suggestive of the nuclear arrangements that the present authors are about to describe in the same organism.

Also worthy of note in this connection are the excellent ultraviolet photomicrographs of *B. subtilis* by Wyckoff and Ter Louw (1931). Because cells containing mature spores are equally as opaque to ultraviolet rays as other cells, these authors doubt whether a mere condensation of the protoplasm is an explanation.

Lewis (1934) described a process of spore formation in *Bacillus mycoides* which corresponds in general outline to that described by Preisz (1904) and Bayne-Jones and Petrilli (1933). Lewis, however, attributes the greater density of the spore primordium not to a condensation, but to "a greater capacity to produce new substance," since the primordium continues to increase in density after the membrane forms.

Knaysi and Baker (1947), by allowing spores to germinate on a nitrogen-free medium, were able to study, with the electron microscope, sporulating cells of *B. mycoides*, made transparent to the electron beam by starvation. In his review on the endospore Knaysi (1948) writes:

> "The nuclei of the cell which is ready to sporulate, 2 to 6 in number, gather into two groups of 1 to 3 nuclei each. The distance between the distal ends of the groups is equal to the length of a spore. One then observes an area of semielliptical outline, and denser than the cytoplasm of the mother cell, grow from one group toward the other; the two areas finally merge into one, and, together with the nuclei now occupying polar positions, form the forespore. The forespore gradually increases in density and some of the nuclei may now occupy other positions in the forespore. The details of the transition from forespore to mature spore were not observed."

The interpretation of these bodies as nuclei is based on their behavior during cell division and sporulation and the constancy of their presence. Knaysi's concepts have been most recently presented in his book on the cytology of bacteria (Knaysi, 1951).

The contention that sporulation is preceded by autogamy has received support from several investigators in recent years following the work of Badian (1933, 1935). The process of autogamy described by this author differs essentially from that proposed earlier by Schaudinn (1902). It is Badian's belief that the vegetative cell of *B. mycoides*, *B. subtilis*, and *B. megaterium*, which he considers to be haploid, contains a single transverse rod-like "chromosome". This, immedi-

ately before sporulation, divides longitudinally into two, with no accompanying division of the cytoplasm. The daughter "chromosomes" then fuse end to end to form a single thread, aligned parallel to the long axis of the cell. This thread shortens and thickens until it resembles the original "chromosome". It then becomes oriented perpendicular to the long axis of the cell and splits longitudinally into four rod-like bodies. One of these becomes enclosed in the spore, while the other three are excluded. The preceding sequence of events was interpreted by Badian as the formation of a diploid nucleus followed by reduction division. The interpretations of other investigators, including Klieneberger-Nobel (1945), Flewett (1948), and Bisset (1950*a*,*b*), differ from those of Badian in the number of "chromosomes" in the vegetative cell and the manner of division of the "fusion nucleus". The division is described as transverse by these investigators. Bisset contends that nuclear fusion in bacilli of rough morphology is sexual rather than autogamous, because of the multicellular nature of these bacilli.

Recently Delaporte (1950) recapitulated her views on sporulation. These can be placed in either of the following categories: (1) sporulation by growth of a granule, or (2) sporulation by condensation of the protoplasm. The nuclear substance of *Bacillus cereus* is described as consisting of chromatinic granules located between lipid globules, or less frequently as an axial thread. During spore formation one of the nuclear granules at one end of the cell appears a little larger. The cytoplasm around it increases in density, and the whole area grows until it reaches the size of a spore. Robinow (1945) has presented evidence for the extra-cytoplasmic position of the nucleus in this spore.

It is readily apparent that information so far accumulated concerning the process of sporulation in bacteria is confused and far from definitive. Following the development of new methods by DeLamater (1951*a*), which permitted the demonstration of mitosis in *B. megaterium* (DeLamater, 1951*b*; DeLamater and Mudd, 1951; DeLamater and Hunter, 1951), study of the processes of sporulation and spore germination was undertaken.

The purpose of this communication is to present the observations made. Since the present work constitutes a departure from the observations of others, no critical evaluation of past work in terms of that presented here will be undertaken.

MATERIALS AND METHODS

The organism was the same as that used in previous studies (DeLamater, 1951*a*,*b*; DeLamater and Hunter, 1951; DeLamater and Mudd, 1951). Sporulation was induced 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). That investigator demonstrated that dilution of the nitrogenous components of the medium is conducive to early sporulation and high spore yields. In cultures incubated at 37 C nuclear changes leading to sporulation were observed as early as 7 hours after inoculation, and mature spores appeared in about 16 hours.

Cytological preparations were made according to the freezing-dehydration

technique for microorganisms described by DeLamater (1951*a*). Cells in various stages of sporulation were fixed on the agar in the vapors of 2 per cent osmium tetraoxide. Smears were made on coverslips. Hydrolysis was conducted for from 4 to 8 minutes in N HCl maintained at 60 C in a waterbath. The time required for removing the cytoplasmic ribosenucleic acid varied with the age of the culture and had to be adjusted in each case. The coverslips were then placed for one hour or longer in 10 ml of 0.25 per cent thionin to which one drop of thionyl chloride had been added. Staining was followed by dehydration overnight in absolute alcohol previously chilled to and maintained at a temperature of about −50 C by packing in solid CO_2. The coverslips were then passed through absolute alcohol at room temperature and two changes of xylene. They were mounted in "permount".

RESULTS

From the foregoing history it is obvious that there has been no clear-cut delineation of nuclear activity during the process of sporulation. The process as described herein constitutes a completely new departure from all past concepts and will be presented as such. An effort will be made to avoid specific issues with previous work. Where similarities in observations are to be noted, these will be pointed out.

As vegetative growth proceeds, the process of sporulation appears to be a direct and continuous sequence of events so far as the nucleus is concerned. It also appears to be a vegetative process, as will be emphasized. During active vegetative growth the mitotic process as described by DeLamater (1951*a*,*b*; DeLamater and Mudd, 1951; DeLamater and Hunter, 1951) proceeds uninterruptedly until the cells are ready to undergo sporulation. The last nuclear division prior to sporulation appears to be a typically mitotic one (figure 1). Following this division, the rods segment so that each cell becomes binucleated (figures 3, 4, 11). Following this last mitotic division, one nucleus of the pair of sister nuclei formed by this division remains condensed, while the other undergoes a progressive expansion to be described. In this strain of *B. megaterium* it is usually the terminal nucleus in the rod which remains condensed and becomes the spore nucleus. This condensed or unexpanded condition appears to constitute a resting state.

The sister nucleus expands progressively, as shown in figures 2 to 11, and develops into a typical interphase nucleus comparable to that observed in active vegetative cells during the mitotic process. In this process of expansion to an interphase nucleus, the nuclear membrane reforms following the division, and the chromosomes lie within a discrete nuclear vesicle. The chromosomes undergo a progressive elongation into distinct threads and are arranged in a tangled, irregular manner within the nuclear vesicle, but tend to be in apposition to the internal surface of the nuclear membrane, i.e., they tend to lie at the periphery of the nucleus (figures 6, 9 to 13). While this process of expansion of one nucleus into an active vegetative interphase nucleus occurs, the condensed sister nucleus which forms the spore primordium remains very dense and compact (figures 2 to 12). About this dense nucleus, basophilic substance, probably mainly ribose-

nucleic acid, is laid down. This accounts for the area of increasing density which develops around this condensed nucleus as the process of sporulation proceeds. The basophilic substance in the cytoplasm may become so dense as partially or completely to obscure the condensed nucleus which lies *centrally* within it. In hydrolyzed preparations outlines of the condensed nucleus become hazy, as demonstrated in figures 4, 6 to 11.

A delicate wall is then laid down about the condensed nucleus and its surrounding basophilic substance, as shown in figure 11. As the wall becomes thicker, the spore nucleus becomes more indistinct and difficult to stain and visualize (figure 14). At about this time the sister vegetative nucleus coagulates into a dense mass of chromatin and appears to die and undergo dissolution (figures 14, 15). At last the chromatinic material constituting it appears to undergo autolysis or absorption into the surrounding medium or the spore. At any rate, it disappears (figure 15). The mother cell wall (called sporangium-wall by Knaysi and others) tends to collapse around the enclosed spore (figures 15, 16).

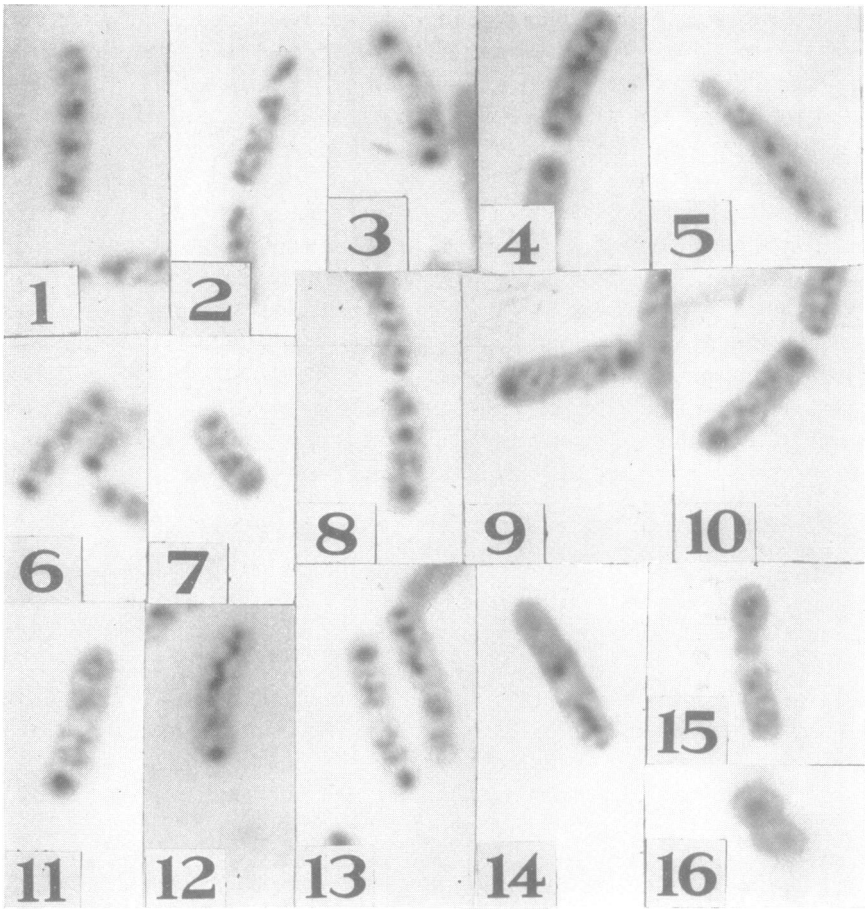
DISCUSSION

The process as described indicates that sporulation in this organism is a strictly vegetative process, and that there is a direct continuity between the mitotic process of actively dividing vegetative nuclei and spore formation. The last division prior to spore formation appears to be a typically mitotic one. One nucleus becomes the focus about which the spore is laid down within the mother cell. The second nucleus expands into a vegetative interphase nucleus, which, by comparison with vegetative cells and nuclei of other organisms, probably represents an active vegetative metabolic type of nucleus. It appears to us that this vegetative interphase nucleus contributes to the support of the cell during the process of sporulation, and to the metabolic activities of the cell necessary to the sporulation process. Once this result has been achieved, this nucleus dies.

No evidence has been observed which suggests that a sexual process, similar to that described by Schaudinn (1902), Badian (1933, 1935), Bisset (1949, 1950*a*,*b*), and others, has been observed.

The similarity of the configurations of the stained chromatin of the cells observed in this work and the granules and prespore areas described by Bayne-Jones and Petrilli (1933) is striking. The similarity of the pictures presented with those of Bisset (1950*b*, chapter 6, figure 28, no. 5) in which he describes sporulation of *B. mycoides* is also striking. It seems likely that Bisset has observed the elements of the process as described, but that he has not followed the details. At no time has evidence for a reduction of chromosomes during the process of sporulation been observed.

DeLamater and his associates have described three chromosomes in the vegetative mitotic process. It is felt that in figures 2, 5, 6, and 7 of this work the three chromosomal granules are also apparent here. At no time has there been evidence observed for the occurrence of a rod-like nucleus during the process of sporulation. Neither has there been evidence observed for the occurrence of the spore nucleus at the periphery of the spore.



Figures 1–16. Bacillus megaterium—Sporulation induced on 0.1 per cent casein hydrolysate agar

Figure 1. Photograph no. 1322—×4,450—7 hours' sporulation; upper terminal nucleus beginning to expand into interphase; adjoining prespore nucleus in early condensation stage.

Figure 2. Photograph no. 1338—×4,450—8 hours' sporulation; upper terminal nucleus in early condensation stage; upper central nucleus still in telophase prior to expansion; lower central nucleus with elongated chromosomes in early interphase; lower terminal nucleus in most highly condensed form.

Figure 3. Photograph no. 1342—×4,450—11 hours' sporulation; two dense prespore nuclei at poles; two central nuclei just beginning to expand.

Figure 4. Photograph no. 1327—×4,450—9 hours' sporulation; lower terminal nucleus condensed; adjoining nucleus beginning to expand.

Figure 5. Photograph no. 1331—×4,450—10 hours' sporulation; outlines of prespore nucleus at upper end of cell becoming less distinct due to basophilic substance being laid down about it; adjoining nucleus in early interphase; other four nuclei coagulated in death.

Figures 6, 7. Photograph nos. 1342, 1366—×4,450—11 and 16 hours' sporulation; terminal dense prespore nuclei with adjoining early interphase nuclei; lowest nucleus of figure 7 in early "ring" form.

The process of sporulation as described here suggests that the process is equivalent to that observed in fungi which produce vegetative haploid spores as a means of propagation of the vegetative phase. Sexuality in such organisms is known to be a distinctive and quite different process. It seems likely that the construing of a sexual process in sporulation by Schaudinn, Bisset, Badian, and others has been due to the effect of the methodology used upon the cell structure.

Tatum (1946) cites Burkholder and Giles as producing genetic evidence which suggests that the vegetative spore of *B. subtilis* is a haploid spore. Genetic work in progress in this laboratory strongly favors the same view. It should be pointed out in this connection that should a process of autogamy occur, such as that described by Bisset and others, it would be impossible to subject it to experimental analysis. It appears likely (DeLamater, to be published) that *B. megaterium* has a sexual process distinctive and separate from sporulation, which involves the fusion of cellular elements from different sources. This further suggests that sporulation in this organism is a vegetative process.

SUMMARY

The nuclear activities occurring during the process of sporulation from *Bacillus megaterium* have been described. These processes include a final mitotic division prior to the establishment of a vegetative supportive nucleus and the spore primordial nucleus. The sister nucleus which forms the vegetative nucleus develops into a typical and recognizable interphase nucleus, while the spore nucleus remains condensed and becomes surrounded, first, probably by ribose-nucleic acid and other basophilic materials, and subsequently by a discrete spore wall. The process appears to the authors to be a distinctly vegetative one and does not appear to involve either nuclear fusion or reduction divisions. The vegetative supportive nucleus, following completion of the development of the spore, undergoes coagulation and subsequently dissolution, and disappears, the

Figure 8. Photograph no. 1342—×4,450—11 hours' sporulation; lower terminal nucleus and upper central nucleus of lower cell condensed; adjoining nuclei in early interphase.

Figures 9, 10. Photograph no. 1327—×4,450—9 hours' sporulation; terminal prespore nuclei with supportive, interphase nuclei.

Figure 11. Photograph no. 1346—×4,450—16 hours' sporulation; upper terminal prespore nucleus in "ring" form, two fully expanded interphase nuclei.

Figure 12. Photograph no. 1322—×4,450—7 hours' sporulation; lower terminal dense prespore nucleus with adjoining supportive interphase nucleus; elongated telophase divisional stage in upper half of cell.

Figure 13. Photograph no. 1342—×4,450—11 hours' sporulation; spore membrane forming around upper nucleus of left cell; dense terminal nucleus and two supportive interphase nuclei.

Figure 14. Photograph no 1350—×4,450—16 hours' sporulation; spore formation complete; adjoining interphase nucleus coagulated into dense mass.

Figure 15. Photograph no. 1366—×4,450—16 hours' sporulation; spore formation complete; constriction in cell; interphase nucleus dissolved or absorbed.

Figure 16. Photograph no. 1387—×4,450—Mature ungerminated spores after 6 hours on 3 per cent casein hydrolysate agar.

spore remaining enclosed in the membrane of the vegetative cell within which it was formed.

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