Fine Structure of *Bacillus megaterium* during Microcycle Sporogenesis¹

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Ultrathin sections were prepared from cultures of *Bacillus megaterium* QM B1551 undergoing microcycle sporogenesis (initial spore to primary cell to second-stage spore without intervening cell division) on a chemically defined medium. The cytoplasmic core of the dormant spore was surrounded by plasma membrane, cell-wall primordium, cortex, outer cortical layer, and spore coats. Early in the cycle, the coat opened at the germinal groove, the cortex swelled, ribosomes and a chromatinic area associated with large mesosomes (which may later be incorporated into the expanding plasma membrane) appeared in the core, and the cell wall became defined at the site of the cell wall primordium. Poly- β -hydroxybutyrate granules began to appear in the primary cell at about 3 hr. By 7 hr, the forespore of the second-stage spore was delineated by typical double membranes. Between 7 and 12 hr, second-stage cell-wall primordium and cortex developed between the separating forespore membranes. The inner membrane became the plasma membrane of the second-stage spore, and the outer membrane eventually disintegrated within the second-stage spore cortex. A densely staining double layer (spore-coat primordium) developed external to the outer forespore membrane. The inner spore coat and the outer cortical layer of the second-stage spore developed from this primordium. The outer part of the spore coat, probably of sporangial origin, was laid down on the external surface of the inner spore coat. By 12 hr, second-stage spores were almost mature. By 20 hr, the mature endospores, with a thickened outer coat, were often still enclosed by degenerate primary cell wall and by the outer cortical layer and spore coat of the initial spore.

The primary cells resulting from germination of spores of *Bacillus cereus* (23) or of *B. megaterium* (11) were capable of elongating and sporulating without intermediate cell division (microcycle sporogenesis). The second-stage spores resulting from microcycle sporogenesis possessed no unusual anatomical properties and were normally refractile under phase-contrast optics. They were not, however, capable of undergoing a second cycle on the minimal medium employed (11).

Heretofore, in spite of separate studies on the fine structure of sporulation (3, 4, 10, 18) and of germination (17, 21), it has been difficult to determine the origins and interrelations of

structures in spores and in vegetative cells. Microcycle sporogenesis now offers the unique opportunity of studying these structures without the complications introduced by cell division.

MATERIALS AND METHODS

Spores of *B. megaterium* QM B1551 were produced on a medium containing 0.5% liver fraction B (Wilson Laboratories, Chicago, Ill.) buffered at pH 6.8 with 0.01 M potassium phosphate, and were harvested, washed, and lyophilized as previously described (15). Aqueous suspensions of the lyophilized spores (30 mg/ml), heated at 60 C for 10 min, were mixed with glucose-ammonia medium (11) to give a final spore concentration of 1.0 mg/ml, and were incubated, 10 ml per 125-ml Erlenmeyer flask, on a New Brunswick rotary shaker at 30 C. Samples of the culture were removed at regular intervals for examination under phase-contrast optics and by electron microscopy of thin sections.

Samples for electron microscopy were prepared by use of a modification of the technique described by Kellenberger, Ryter, and Séchaud (14). Cells were

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fixed with 1% OsO₄ for 18 to 26 hr prior to dehydration in an acetone series. Embedding was accomplished in Durcupan ACM (Fluka AG, Buchs SG, Switzerland) as described for Araldite (9). After polymerization, blocks were sectioned with glass knives on an LKB 4800A Ultratome, and sections were collected on carbon-coated parlodion (Mallinckrodt Chemical Works, New York) films. Sections were stained with aqueous 1% vanadyl sulfate on the grids for up to 30 min at 50 C (2). Grids were examined in a Siemens Elmiskop 1A electron microscope with an accelerating voltage of 80 kv and a 50- μ objective aperture at instrument magnifications of up to 40,000.

We refer to: unincubated spores as initial or zero-time spores; the cell resulting from germination of a spore as a primary cell; occasionally, a primary cell, containing a refractile spore, as a sporangium; and the endospore formed within this sporangium as a second-stage spore. Time designations refer to time elapsed between addition of spores to the glucose-ammonia medium and fixation of the specimen for electron microscopy.

RESULTS

Phase-contrast observations were essentially similar to those of Holmes and Levinson (11). By 0.5 hr, all of the initial spores had germinated and had started to emerge. All of the emerged cells were elongated and typically bacillary in shape by 3 hr, at which time phase-bright granules of poly- β -hydroxybutyrate were evident. By 7 hr, forespores were seen, and, by 10 hr, at least 90% of the primary cells contained refractile second-stage spores. The spore coats of the initial spores were still attached to a majority of the sporangia.

The cytoplasmic core of the initial spore (Fig. 1) was surrounded by a plasma membrane, a cell-wall primordium, cortex, outer cortical layer, and spore coats. The spores of this strain viewed in shadowed preparations (11, 16, 20) appeared to possess characteristic polar knobs which, in section (Fig. 1), were revealed as grooved equatorial ridges of spore coat. Since, during emergence, the spore coats appeared to open along these grooves to release the primary

cell, we have termed them "germinal grooves." The spore coats were separated from the outer cortical layer by a space which was probably not present in the dormant spore. This subcoat space bore no apparent relation to heat activation since it was evident in both heated and in unheated spores. It may be the result of penetration of water through the germinal grooves during specimen preparation. Within this space lay "subcoat material" similar in profile to the inner spore coat. The subcoat material was accumulated in the region of the germinal grooves, where it may function as an annulus or plug sealing the coat of the dormant spore against penetration of water. We cannot entirely discount the possibility that some very early germination-like changes took place during fixation, and that this may account for the enlarged subcoat space and for the small invaginations of the core plasma membrane (Fig. 2). Ribosomes and chromatinic areas were also evident in sections of the zero-time spores, although these were poorly defined. The cortex of the initial spore (Fig. 3) appeared "as an open network of granular material, arranged in concentric bands" (25). The cell-wall primordium may correspond to the structure referred to by Robinow (19) as spore wall and by Warth, Ohye and Murrell (24) as cortical membrane. The outer cortical layer showed a multiple profile, especially evident in the regions subtending the germinal grooves. The spore coat, outer cortical layer, and cell-wall primordium of the initial spore were recognizable throughout the cycle.

By 0.5 hr, a period of rapid membrane formation had occurred in the primary cell and had resulted in large prominent mesosomes (Fig. 4), implying that a functional cell wall was present at the site of the core cell-wall primordium (7). The cortex appeared swollen and had lost its banded appearance. The cell-wall primordium had thickened, giving, by 1 hr (Fig. 5), a profile more characteristic of a cell wall.

By 2 hr (Fig. 6), chromatin was dispersed in the cytoplasm, and the cell wall was more pro-

FIG. 1-3. Thin sections of initial *Bacillus megaterium* spores (zero-time). Markers represent 0.1 μ . The following abbreviations are used in all figures: CE, core envelope (CWP, cell-wall primordium; PM, plasma membrane); CH, chromatin; CW, cell wall; FM, forespore membranes (IFM, inner forespore membrane; OFM, outer forespore membrane); G, germinal groove; M, mesosome; OCL, outer cortical layer; PHB, poly- β -hydroxybutyrate; SC, spore coat (ISC, inner spore coat; OSC, outer spore coat); SCM, subcoat material; and SCP, spore-coat primordium.

FIG. 1. The essential structural features are shown. The fibrous subcoat material, more abundant in the regions subtending the germinal grooves, lies in a space which may be somewhat larger than that of a completely dry, dormant spore.

FIG. 2. Detail showing cell-wall primordium and invaginations of the core plasma membrane (unlabeled arrows).

FIG. 3. Tangential section showing outer cortical layer, bands of cortical material (unlabeled arrows), core envelope, and cytoplasm. Fragments of the fibrous subcoat material are external to the outer cortical layer.

FIGS. 1-3

FIG. 4. Primary cell of *Bacillus megaterium* (0.5 hr of incubation prior to fixation). The spore coat of the initial spore has opened at the germinal groove leaving remnants of subcoat material (bottom of figure). The emerging primary cell shows differentiated chromatin associated with a large mesosome. A cell wall is seen at the site of cell-wall primordium. The outer cortical layer has expanded to surround the primary cell. The cortex has swollen and appears undifferentiated.

FIG. 5 and 6. Primary cells of *Bacillus megaterium*. Markers represent $0.1\ \mu$.

FIG. 5. Emerging cell (1 hr of incubation prior to fixation). Generally similar to Fig. 4, but with thickened cell wall and intimate mesosome-chromatin association. The fibrous subcoat material is more abundant in the region of the unopened germinal groove.

FIG. 6. Emerging cell (2 hr of incubation). Numerous plasma membrane invaginations are seen. Clear spaces in the cytoplasm probably mark the sites of poly- β -hydroxybutyrate granules. Cell wall is more pronounced.

nounced. Poly- β -hydroxybutyrate granules were abundant in the primary-cell cytoplasm. Mesosomes appeared only rarely, but the plasma membrane presented an irregular profile with many small vesicles on the internal surface (Fig. 6-8). Poly- β -hydroxybutyrate granules became more abundant during the 3rd and 4th hr, resulting in large, often polar, accumulations in the primary-cell cytoplasm (Fig. 7), just before development of the forespore septum at 5 to 6 hr (Fig. 10).

Forespore septum formation appeared to follow the process already suggested by Fitz-James (4) and by Ohye and Murrell (18), resulting in an isolated forespore delineated by a double membrane (Fig. 9-11, 24). It is suggested that, at the time of invagination of the forespore membranes, small amounts of cell-wall material (perhaps soluble) lie between the two forespore membranes—a favorable location for later synthesis and assembly of second-stage spore cortex and cell-wall primordium.

By 7 hr (Fig. 11), the forespore membranes had begun to separate and, by 8 hr (Fig. 12 and 13), the cell-wall primordium (and perhaps cortex) of the second-stage spore was evident as a lightly staining layer between them. At about this time, the structural features of the forespore cytoplasm became less evident. Fitz-James (8) has associated a featureless protoplasm with the refractility of the dormant spore. Factors which may contribute to the undifferentiated appearance of the forespore cytoplasm, at about 8 hr, include: loss of function prior to the onset of the dormant state; beginning of calcium and dipicolinic acid accumulation; and poor penetration of fixative through the increasingly complex spore periphery. The separation of the forespore membranes was more evident at 9 hr (Fig. 14 and 15). At this time, the second-stage spore-coat primordium became evident external to the outer forespore membrane. At 10 hr (Fig. 16 and 17), the double-layered spore-coat primordium stained more intensely, and the positions of the germinal grooves were apparent. In section, these often, although not invariably, appeared opposite one another. The second-stage spore cortex increased in size and the remnants of the outer forespore membrane were apparent in the outer layers of the second-stage

spore cortex (Fig. 17). The outer cortical layer of the second-stage spore was first noted in the 11-hr sections (Fig. 18) and appeared to originate at the inner layer of the spore-coat primordium. It had a profile similar to that of the inner spore coat and appeared to be continuous with parts of the subcoat material. The outer layer of the spore-coat primordium later contributed to the inner spore coat of the second-stage spore, and it was upon this inner coat that the outer spore-coat material was progressively laid down (Fig. 18-22).

At 11 hr (Fig. 18), the second-stage spore was still completely enclosed within the intact, and probably functional, primary cell. Between 12 and 20 hr (Fig. 20-23), the outer spore coat gradually thickened. By 20 hr (Fig. 23), the second-stage spores had matured and were in the process of being released from the primary cell by breakdown of the primary cell wall. The second-stage spores, at this time, were enclosed within the intact outer cortical layer of the initial spore. Remnants of the initial spore coat of the primary cell wall and plasma membrane could often be seen (Fig. 23). No exosporium was evident.

DISCUSSION

Membranes. During microcycle sporogenesis, there were at least two periods of rapid membrane formation. During the first 0.5 hr, chromatin, associated with large, newly formed mesosomes, became apparent. Subsequently, during swelling and elongation of the primary cell, the chromatin-associated mesosomes disappeared, and vesicles appeared in the plasma membrane. These vesicles may have arisen, through fusion of mesosomal membrane with plasma membrane, facilitating rapid expansion of the elongating primary cell periphery. A second period of rapid membrane formation occurred with the renewed mesosome activity associated with delineation of the forespore double membrane. Forespore septum formation may be a specialized case of the more general process of vegetative cell septum formation (Fig. 24). Both are initiated by invagination of the plasma membrane (4, 18). In septum formation, membrane invaginations from the periphery of the cell meet and fuse. In forespore delineation, a septum was

FIG. 7 and 8. Primary cells of *Bacillus megaterium*. Markers represent 0.1 μ .

FIG. 7. Cell (3 hr of incubation) showing polar accumulation of poly- β -hydroxybutyrate and numerous membrane invaginations. The persistent outer cortical layer and remnants of the spore coats of the initial spore surround the primary cell.

FIG. 8. Cell (4 hr of incubation), similar to Fig. 7, and showing chromatin, poly- β -hydroxybutyrate, and increasing membrane invaginations. The large "vacuole" is interpreted as an artifact caused by extraction of poly- β -hydroxybutyrate during specimen preparation.

FIGS. 7-8

FIG. 9 and 10. Primary cells of *Bacillus megaterium* (6 hr of incubation). Markers represent $0.1\ \mu$.

FIG. 9. Forespore, rich in chromatinic material, is delineated by double forespore membranes. The site of attachment of the forespore membranes to the plasma membrane is seen at the right.

FIG. 10. A stage in forespore delineation earlier than that shown in Fig. 9. Membranes have not yet coalesced. A mesosome is seen in the forespore region.

FIG. 11. Primary cell of *Bacillus megaterium* (7 hr of incubation). Marker represents 0.1 μ . Completed forespores are bounded by a double membrane. A mesosome is enclosed within the forespore. Ribosomes and a defined central chromatinic area are evident in the forespore. The outer cortical layer of the initial spore surrounds the primary cell.

formed in a similar manner, but this process was followed by further growth of the septum membrane, eventually forming an isolated forespore detached from the sporangial plasma membrane (Fig. 24). It may be emphasized that after stage b (Fig. 24) the plane of the inward folding of the membrane moves toward the pole of the primary cell. Not only is more septum made, but there is membrane movement as well. The similarity between forespore and septum formation was previously noted by Fitz-James (8) and by Ellar and Lundgren (3). The close association of mesosomes with chromatin in the 0.5-hr cells could be interpreted as a phase of primary cell deoxyribonucleic acid replication (12, 13) prior to elongation and second-stage sporulation. A similar association between mesosomes and chromatin at the beginning of forespore formation could account for the separation and eventual isolation of the second-stage spore chromatin from that of the primary cell.

Cell wall and cortex. In both cross-wall and forespore formation, the space between the invaginating membranes corresponds to the external surface of the plasma membrane. This space may include small amounts of cell-wall material (perhaps soluble) derived from the vegetative cell wall. In vegetative septum formation, the new cross wall is laid down in the space between the membranes, and Fitz-James (8) showed that, in sporulation mutants of *B. cereus*, cell-wall material was also laid down between the forespore membranes at an early stage of forespore formation (stage b and c, Fig. 24). Vegetative cell-wall material between the forespore membranes may serve as primer in the assembly of second-stage spore cell-wall primordium and cortex. The new cell-wall primordium, first apparent in the forespore at 8 hr, could be followed through development of the second-stage spore. In the transition from spore to primary cell, the vegetative cell wall developed from this primordium. In certain

FIG. 12 and 13. Primary cells of *Bacillus megaterium* (8 hr of incubation) with delineated forespores. Markers represent $0.1\ \mu$. The large accumulation of poly- β -hydroxybutyrate is concentrated in the cytoplasmic region external to the forespore. Structural differentiation of the forespore cytoplasm is becoming less obvious, although, in Fig. 13, which shows a slightly earlier stage than Fig. 12, ribosomes are still obvious. A differentiated layer (arrow) is apparent between the separating forespore membranes (Fig. 12), but is not yet apparent in Fig. 13.

FIG. 14 and 15. Primary cells of *Bacillus megaterium* (9 hr of incubation) showing early second-stage spores. The numerals 1 and 2 after designations refer to initial and second-stage spores, respectively. Forespore cytoplasm appears more amorphous than in previous sections. The separation of the inner and outer forespore membranes is more pronounced than at 8 hr (Fig. 12). The cell-wall primordium of the second-stage spore and an unstained region, probably representing second-stage spore cortex, are visible between the inner and outer forespore membranes. Early spore coat (spore-coat primordium 2) of the second-stage spore lies external to the outer forespore membrane. Markers represent 0.1 μ .

FIG. 16 and 17. Primary cells of *Bacillus megaterium* (10 hr of incubation) containing second-stage spores. The numerals 1 and 2 after abbreviations refer to initial and second-stage spores, respectively. Markers represent 0.1 μ .

FIG. 16. The spore-coat primordium of the second-stage spore consists of two layers, but becomes multilayered at the site destined to become the germinal groove of the mature spore. Lying beneath the inner layer of spore-coat primordium is a densely staining layer which, together with the unstained zone surrounding the cell-wall primordium, will become the second-stage spore cortex.

FIG. 17. An earlier stage than that in Fig. 16, showing degeneration of the outer forespore membrane in the newly forming second-stage cortex.

FIG. 18 and 19. Primary cells of *Bacillus megaterium* (11 hr of incubation) containing maturing second-stage spores. Numerals 1 and 2 after abbreviations refer to initial and second-stage spores, respectively. Markers represent 0.1 μ .

FIG. 18. The second-stage inner spore coat appears as a densely staining structure with laminations at the site of the germinal grooves. Some outer spore-coat material appears to be assembled (perhaps radially) on the external surface of this structure. The outer cortical layer of the second-stage spore appears as a complete structure, probably originating in the innermost layer of the double-layered spore-coat primordium. Lying immediately internal to the outer cortical layer is a granular layer, part of the second-stage cortex, which probably contains remnants of the outer forespore membrane.

FIG. 19. The degenerating primary cell is surrounded by a complete cell wall and by the outer cortical layer of the initial spore.

FIG. 20. Primary cell of *Bacillus megaterium* (12 hr of incubation) containing maturing second-stage spores. Numerals 1 and 2 after abbreviations refer to the initial and second-stage spores, respectively. The primary cell, emerged from the initial spore coat, is enveloped by the outer cortical layer of the initial spore. The second-stage spore cytoplasm appears homogeneous and undifferentiated. The inner spore coat appears fibrous, especially where it subtends the germinal groove. Some of this inner spore-coat material may eventually become subcoat material. Assembly of the outer spore coat of the second-stage spore on the outer surface of the inner spore coat appears to be the final phase before maturation and release of the second-stage spore. Marker represents $0.1\ \mu$.

FIG. 21. Primary cell of *Bacillus megaterium*. Marker represents $0.1\ \mu$. The numeral 2 after abbreviations refers to second-stage spore. Detail of the germinal groove region of a second-stage spore (12 hr of incubation) showing spore-coat layers and their close relation to the outer cortical layer. The fibrous nature of the inner spore coat and of the subcoat material is evident.

FIG. 22 and 23. Maturation of second-stage spores of *Bacillus megaterium*. Numerals 1 and 2 refer to initial and second-stage spores, respectively.

FIG. 22. At 14 hr, the outer spore coat of the second-stage spore has thickened. Marker represents 0.1 μ .

FIG. 23. At 20 hr, mature second-stage spores are still enveloped by the outer cortical layer of the initial spore. Cell walls of primary cells are seen. One sporangium (on right) shows, in addition, the persistent spore coat of the initial spore. The cell in the upper center of the figure has the typical accumulation of subcoat material in the region of the second-stage spore germinal groove. The undifferentiated cytoplasmic core of this spore and of the spore in the lower part of the electron micrograph appear to be surrounded by new cell wall primordium. Note that marker represents 1.0 μ .

FIG. 24. Diagrammatic representation of forespore formation (references 3, 4, 18). Dark outer line represents cell wall of primary cell. Thinner line represents primary cell plasma membrane. Stages a and b are common to formation of vegetative cell and forespore septa. Stages c, d, and e represent further membrane growth to give a forespore bounded by two membranes. Dots between membranes represent cell-wall material which may serve as the basis for formation of cross wall (b) or of cell-wall primordium and cortex (e).

respects, then, we are in substantial agreement with Robinow's concept (19) of spore formation as a specialized form of cell division or internal budding. The site of cortical and cell-wall synthesis between the forespore membranes further emphasizes the similarity between these structures already noted by Ohye and Murrell (18) and by Warth, Ohye, and Murrell (24).

In almost all sections taken between 6 and 7 hr, a mesosome was included within the forespore cytoplasm. This mesosome may be involved in the biosynthesis of cell-wall primordium (22).

Spore coat. The first anatomical evidence for second-stage spore coat was the appearance, at about 9 hr (Fig. 15), of a double-layered structure (spore-coat primordium) external to the developing cortex and to the outer forespore membrane. The double-layered profile of the spore-coat primordium suggested a membranous structure. Fitz-James (6) reported that, during sporulation of *B. megaterium* KM, there was an accumulation of sporangial ribosomes just external to the developing spore coat. Aronson (1) reported the presence of an exceptionally persistent messenger ribonucleic acid in sporulating

B. cereus. These two observations, together with the membranous profile of the spore-coat primordium and its physical separation from the undifferentiated spore core, suggest that spore-coat synthesis is a function of the sporangium rather than of the forespore, and that the spore-coat primordium provides a suitable site for synthesis and assembly of this structure.

The fibrous nature of the inner spore coat and of the subcoat material evident in Fig. 22 was previously noted by Rode and Williams (20). The smooth membranous integument which they described may correspond to our outer cortical layer. The inner spore coat, the subcoat material, and the outer cortical layer all had similar profiles, and all appeared to originate in the spore-coat primordium. The outer spore coat developed as a thickening on the outer surface of the inner spore coat.

Exosporium. No identifiable exosporium developed during microcycle sporogenesis. The loosely fitting structure surrounding the mature second-stage spore (Fig. 23) and resembling exosporium was the persistent outer cortical layer of the initial spore. This structure, retained during microcycle sporogenesis, would disappear when extensive cell division precedes sporulation.

We believe that germination and sporulation during microcycle sporogenesis can be validly compared to these processes during a more normal growth cycle, when germination and sporulation are separated by vegetative cell divisions. It should, however, be kept in mind that, since second-stage spores cannot go through a second microcycle on the glucose-ammonia medium (11), they may be deficient in some respects. No alteration of spore morphology reflecting this deficiency was detectable in our thin sections.

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