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Ultrastructural Analysis During Germination and Outgrowth of *Bacillus subtilis* Spores

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Electron microscopy of thin sections of dormant and germinating spores of *Bacillus subtilis* 168 revealed a progressive change in the structure of the cortex, outer spore coat, and inner spore coat. The initial changes were observed in the cortex region, which showed a loose fibrous network within 10 min of germination, and in the outer spore coat, which began to be sloughed off. The permeability of the complex outer spore layers was modified within 10 min, since, at this time, the internal structures of the spore coat were readily stainable. A nicking degradation action of the laminated inner spore coat began at 20 min, and this progressed for the next 20 min leading to the loosening of the inner spore coat. By 30 min, the outer spore coat showed signs of disintegration, and at 40 min, both the outer and inner spore coats were degraded extensively. At 30 to 40 min, a period just preceding net deoxyribonucleic acid synthesis, mesosomes became very prominent in the inner spore core and the cell wall began to thicken around the spore core. At 50 min, an emerging cell was observed, and by 60 min, there was clear evidence for elongation of the emerging cell and the presence of two nuclear bodies. At 90 min, elongation had been followed by the first cell division. There was evidence for spore coat fragments at the opposite poles of the dividing cell.

The dormant bacterial spore has many unique biological properties which have been documented (13, 24). The development of the spore is the result of a sequence of regulated morphological and biochemical events (4, 6, 10, 16, 21, 26). Recent studies, on the effects of ribonucleic acid polymerase mutations on the sporulation process in *Bacillus subtilis*, have reported that the normal cytological events of sporulation could be affected dramatically when ribonucleic acid polymerase activity was abnormal (2, 9, 19, 20, 22).

The dormant state is particularly suitable for survival of the cell under adverse nutritional conditions, and spores may remain dormant for many years (24). However, suitable environmental conditions can cause rapid and relatively synchronous activation, germination, and outgrowth of a spore population (3, 5, 23). The germination stage consists of degradative processes and is accompanied by the release of spore-specific components and an irreversible change in dormant spore properties. This is followed by a period of outgrowth, in which synthetic processes prepare the cell for emergence and the first cell division. Within a few minutes after germination, ribonucleic acid and protein synthesis begins (45), and in *B. subtilis*, after about 45 min, net deoxyribonucleic acid (DNA) synthesis begins followed by cell division at about 90 min (14). These facts are well documented for the *Bacillaceae* as are some of the morphological changes (7, 25) which occur during the germination and outgrowth periods.

One problem encountered in our studies on bacterial sporulation and germination was a lack of knowledge of a complete cytological sequence of germinating spores of *B. subtilis*. To obtain a more detailed picture of the cytological changes which occur during germination, particularly in the complex layers around the inner spore core, an ultrastructural study was undertaken to examine the core and the multiple layers at various times during germination and outgrowth of spores of *B. subtilis*. The results show a sequential alteration in the structure of the cortex, the outer spore coat, and the inner spore coat. Furthermore, the presence of prominent mesosomes was observed within the spore core about 30 min after germination, just prior to DNA synthesis (14), and during the periods of DNA synthesis and cell elongation (45 to 90 min).

MATERIALS AND METHODS

Bacterial strain and growth medium. *B. subtilis* 168 spores were obtained by growth and sporulation of the cells in a modified Schaeffer medium (11). The strain was obtained from N. Sueoka and was used in synchronous DNA replication studies (14). The spores were cleaned by use of the Y-system (18). For germination and outgrowth studies, the spores were inoculated without heat shock treatment into a 1.22% Penassay medium (14), to give a starting Klett unit of 100, and were shaken at 37 C in a New Brunswick gyrotory shaker. Approximately 50% of the spores germinated and proceeded through outgrowth under these conditions. Samples were removed at 10-min intervals for the first hour and then at 15-min intervals, until the first cell division.

For electron microscopy, 15-ml samples were taken, prefixed with 0.5 ml of 1% OsO_4 (8), and centrifuged in a clinical centrifuge at 2,000 rpm. The pellet was suspended in 1 ml of OsO_4 and 0.1 ml of tryptone medium (Difco) by vortexing and was fixed overnight at room temperature. After washing in the Kellenberger buffer (8), the cell pellet was suspended in melted agar and drawn up into a Pasteur pipette; after solidification, the agar was forced out of the pipette and sliced into 2-mm sections, fixed in uranyl acetate, and dehydrated through an ethanol series. Samples were embedded in Epon 812 (12), sectioned on a Porter Blum MT-1 ultramicrotome, and stained with uranyl acetate and Reynold lead citrate (15). Micrographs were taken on an AEI 6B electron microscope with an accelerating voltage of 50 to 60 kv.

RESULTS

The purpose of this study was to obtain a complete cytological sequence during germination and outgrowth of *B. subtilis* spores and to relate it to biochemical changes which had been reported previously (3, 14). The same batch of spores was used for all studies. The spores were germinated under the exact conditions reported earlier (14). The overall pattern of germination was followed by loss of optical density of the spore suspension (Fig. 1). A rapid and highly reproducible pattern of germination was obtained. Electron micrographs representative of thin sections of spores taken at 10, 20, 30, 40, 50, 60, and 90 min after germination, a period which covers outgrowth and the first cell division, are shown in Fig. 2.

The dormant spore, as many previous studies have shown (25, 26), is relatively resistant to staining; it is characterized by a thick outer spore coat, a laminated inner spore coat, a thick non-staining cortex, and the weakly stained inner spore core with its cytoplasmic membrane and germ cell wall (Fig. 2a). These spores were refractile, as observed by phase microscopy. Ten minutes after incubation (Fig. 2b), one could see a partial sloughing off of the outer spore coat, a diminution in size and a fibrous appearance of the cortex, and a prominent well-stained spore core with a definite envelope. Although this particular section does not show them, DNA fibrils were prominently stained at 10 min. The laminated inner spore coat appeared reasonably intact at this time, and phase microscopy showed a darkening of the spore periphery.

At 20 min, nicks or breaks first appeared in the inner spore coat (Fig. 2c), and a thickening of the cell wall around the spore core became apparent. The cortex area had a very fibrillar appearance and had shrunk in size. Some mesosomes were present, and phase microscopy showed completely dark spores. The nicks in the inner spore coat became very prominent by 30 min (Fig. 2d). The outer spore coat thinned out considerably, and the core showed prominent mesosomal activity. Swelling was observed by phase microscopy.

By 40 min, the cortex area had been filled by the swelling core, and the inner and outer spore coats were almost completely disintegrated (Fig. 2e). Mesosome activity became very prominent and coincided with the time that net DNA synthesis first occurs during germination (14). A thick cell wall surrounded the protoplast. At 50 min, the new cell started to emerge from the disintegrating spore coat (Fig. 2f). Spectacular mesosomal activity was observed in most sections of these cells. The cell walls looked like vegetative cell walls.

After emergence (at 60 min), the cell elongated (Fig. 2g), and the spore coats being shed

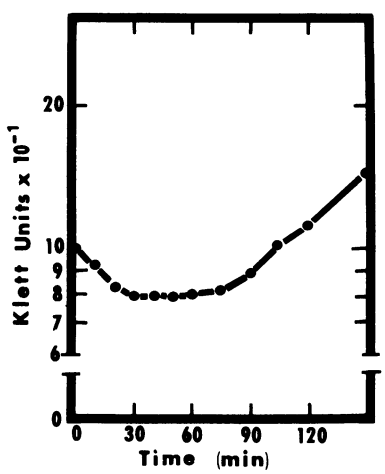

FIG. 1. *Germination of B. subtilis spores. The spores were germinated under the conditions described. The optical density was followed with a Klett-Summerson colorimeter (red filter, 660 nm).*

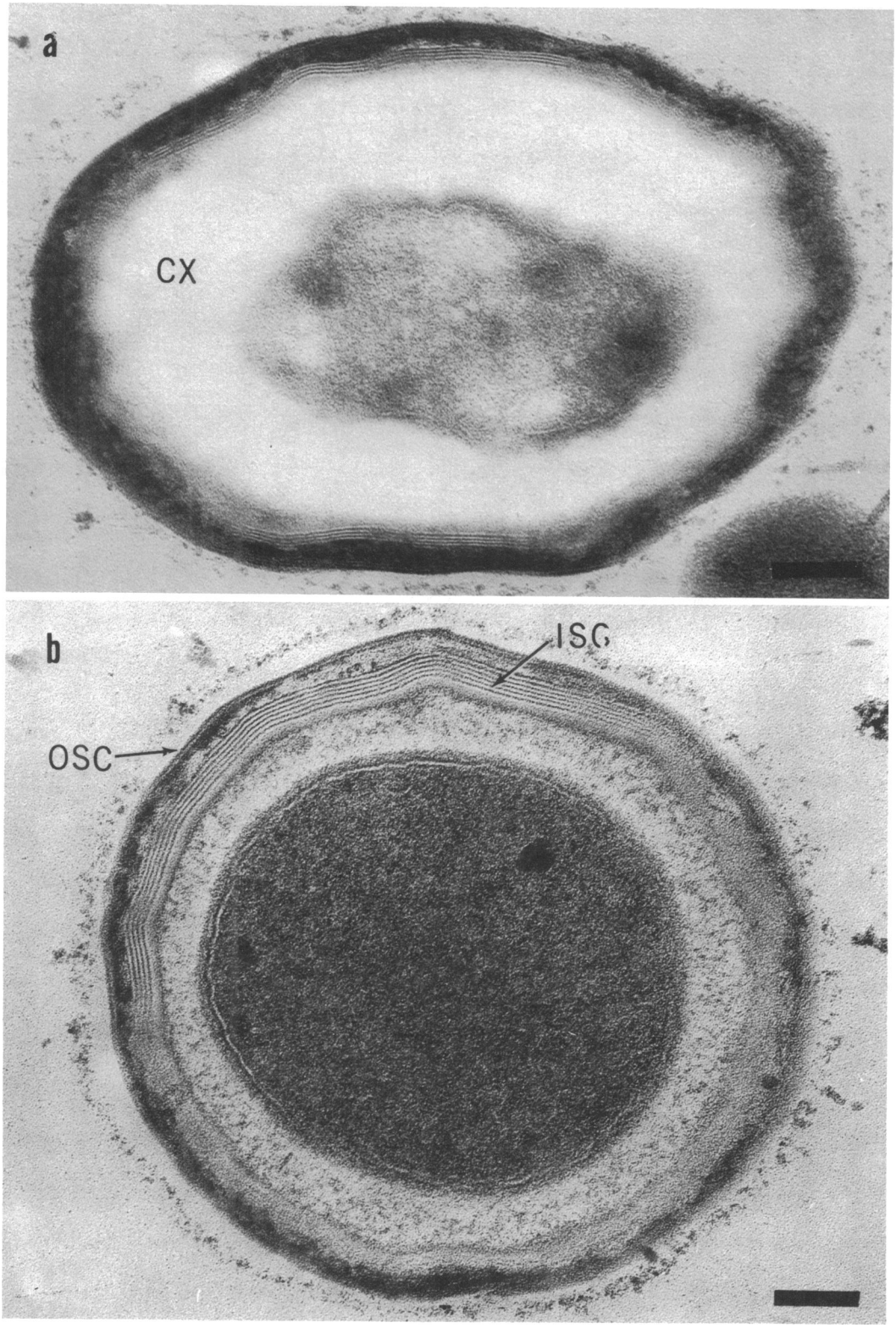

FIG. 2. *a and b*

FIG. 2. *Electron micrographs of thin sections of dormant, germinating, and outgrowing spores. a, Dormant spore; b, 10 min; c, 20 min, arrows indicate nicks; d, 30 min, arrows indicate nicks; e, 40 min; f, 50 min; g, 60 min; h, 90 min. The bars represent 0.1 μm in figures a through g. In figure h, it represents 1.0 μm. CX, Cortex; OSC, outer spore coat; ISC, inner spore coat; M, mesosome.*

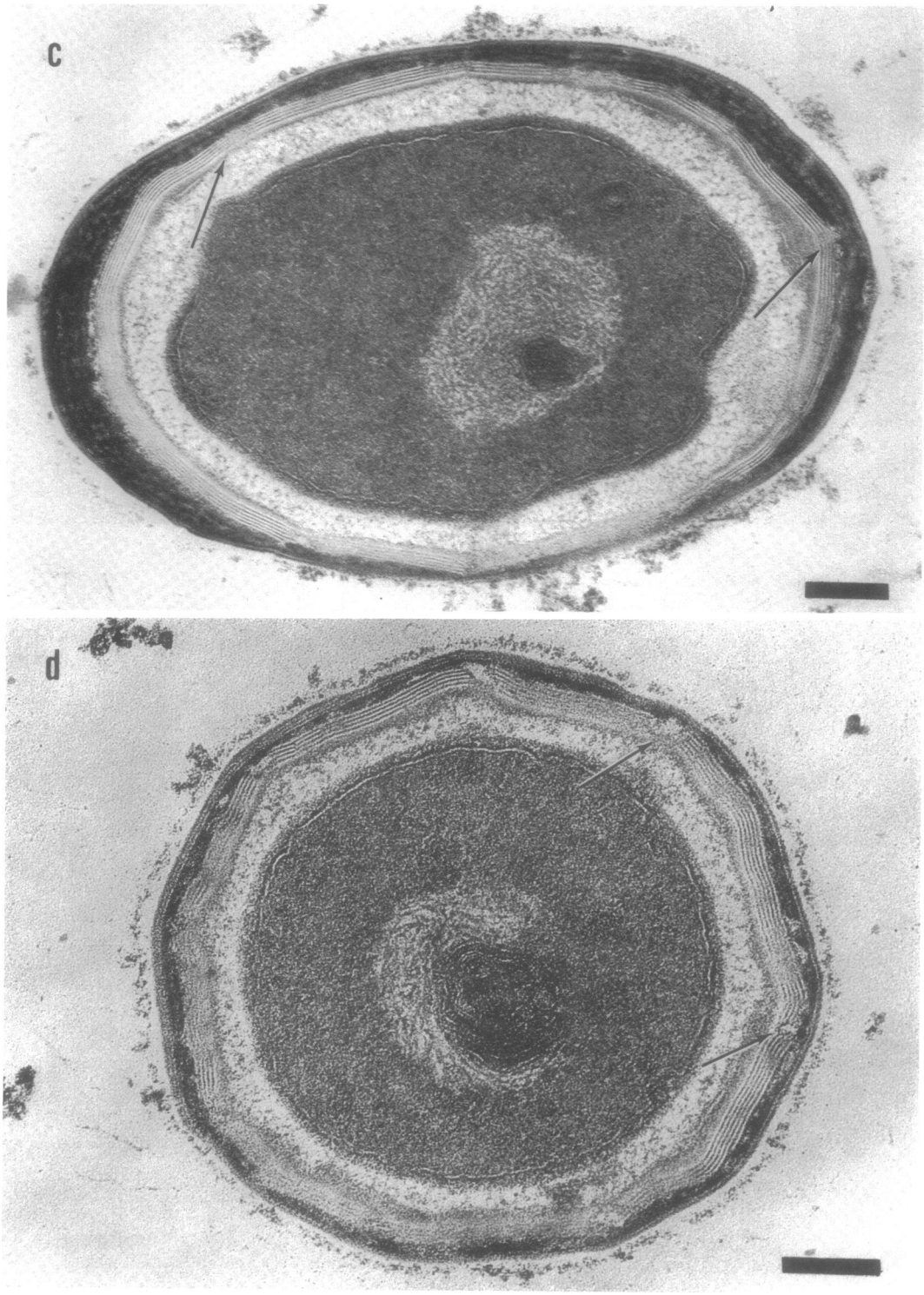

FIG. 2. *c and d*

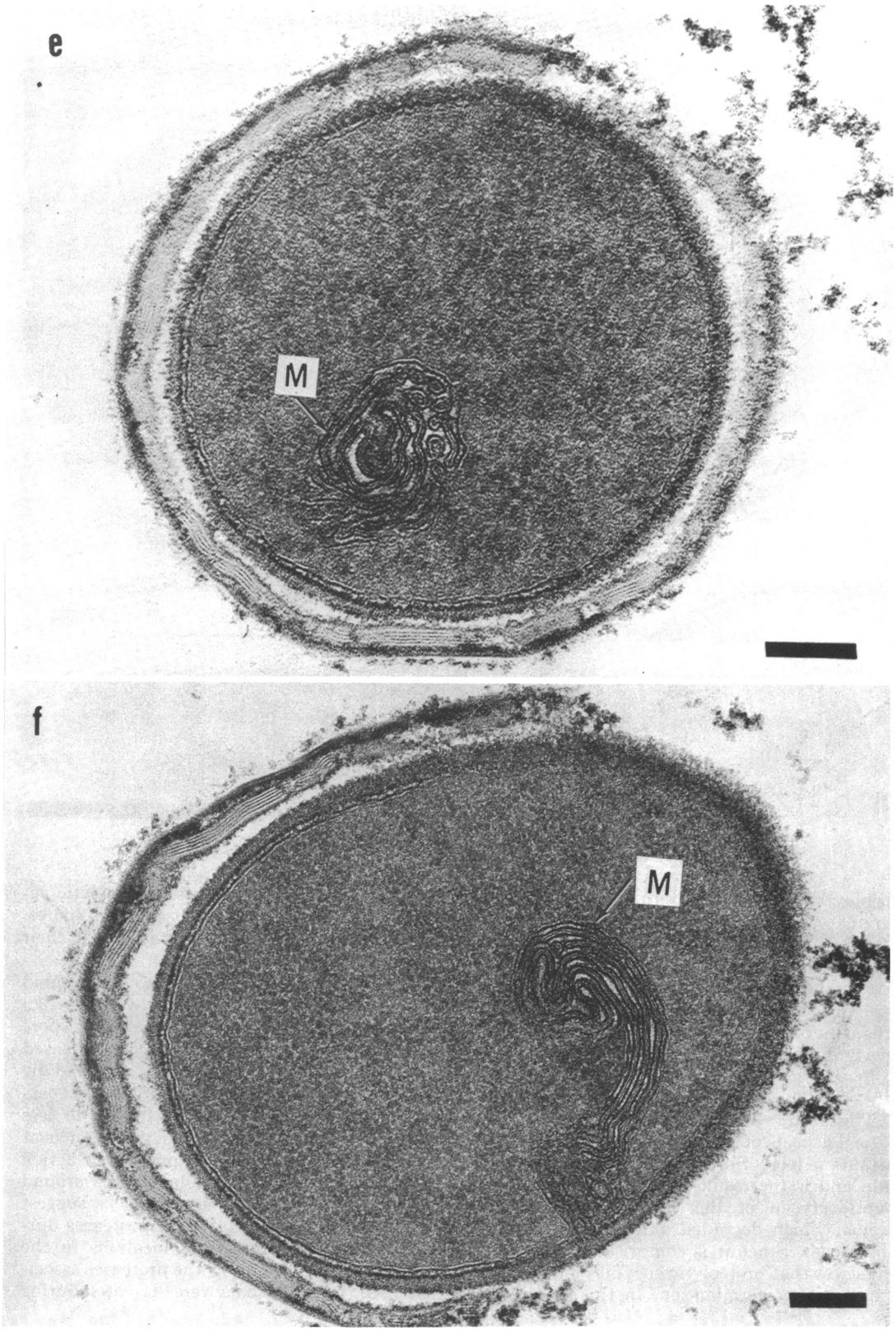

FIG. 2. *e and f*

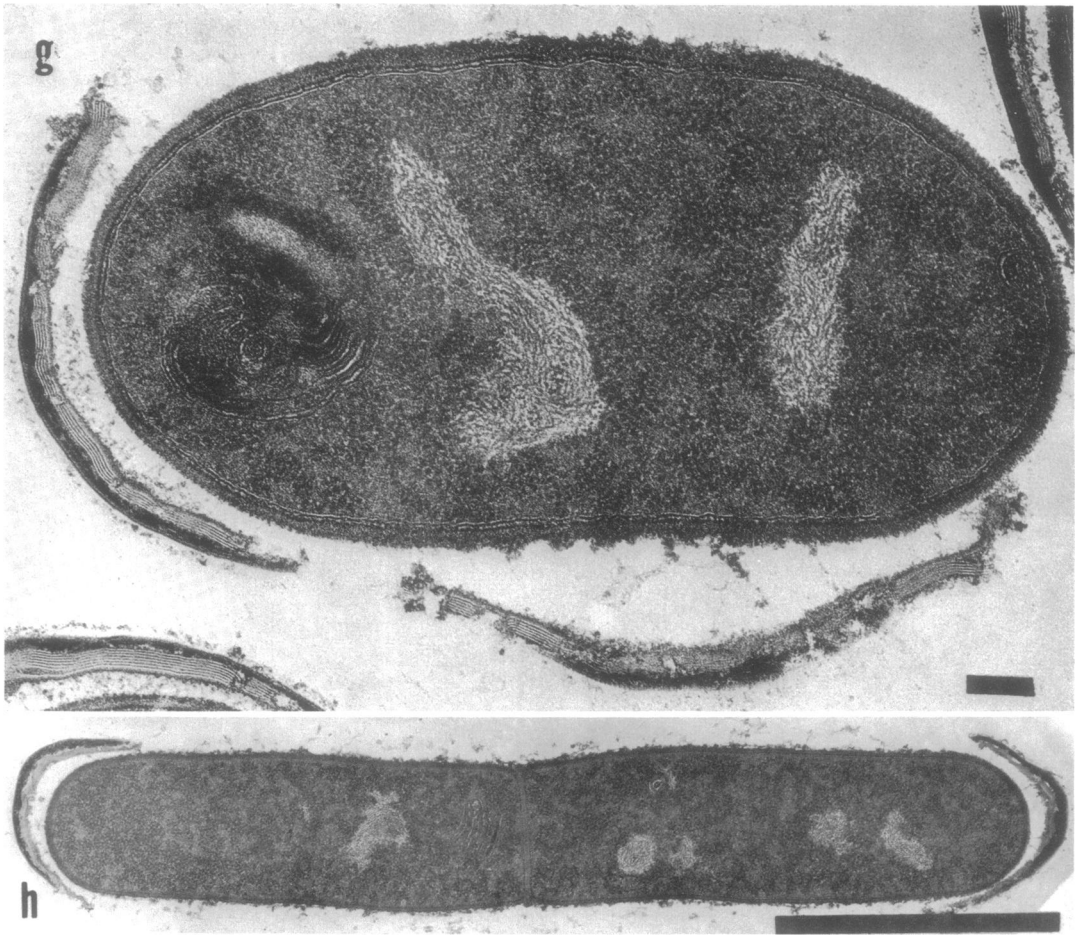

FIG. 2. *g and h*

displayed a regular pattern of nicking in the laminated inner spore coats. Two nuclei were evident. The elongating cell prepared for cell division, which occurred at 90 min under these conditions (Fig. 2h). Mesosome activity was still present after 90 min, and the spore coat fragments then associated with one end of the newly divided cell.

DISCUSSION

This study revealed several interesting cytological aspects of spore germination. The cortex attains a loose fibrillar appearance within 10 min and is the result of the breakdown of the peptidoglycan of the cortex (3). The other region, which degraded quickly was the outer spore coat. Since it is comprised of protein, it indicates that proteolytic enzymes may be activated during germination. In this regard, our earlier studies have shown that proteolytic activity was released from heat-shocked spores, indicating the presence of protease near their surface (1).

At 20 min after germination, nicks appeared in the fine striated inner spore coat (Fig. 2c) accompanied by a thinning of the outer spore coat. The nicks in the inner spore coat increased in number and size by 30 min, followed by disintegration of the inner and outer spore coats by 40 min. The outer coat was more or less stripped away evenly, whereas the inner coat was nicked and weakened by proteolytic action at discrete and almost regular spaces around the spore. The regularly spaced nicks suggest the possibility of localization of proteases during sporulation near certain locations in the inner spore coat. Somehow the proteases associated with the spore coats were activated during

germination, so that a rapid attack on these outer layers ensued.

An increasing amount of mesosome activity occurred starting at about 20 min after incubation, and mesosomes became very prominent just prior to net DNA synthesis, which occurs at 45 min under these germination conditions (14). Large mesosome structures of various configurations have been observed next to the DNA from 30 to 90 min, suggesting that they may be involved in initiating DNA replication as proposed previously (17).

Emergence, which was quite evident by 50 min, was a key stage in outgrowth, since elongation was now possible to prepare the cell for the first cell division. The polar ends of the dividing cell at the first division may contain caps of spore coat fragments (Fig. 2h). We were amazed at the strong adherence of these cell wall fragments, since the cells were washed quite vigorously during the staining steps in preparation for electron microscopy.

These results with *B. subtilis* are generally similar to that reported for other *Bacillus* species (25, 26). However, these studies provide a detailed picture of the sequential removal of the complex spore coat envelope and of the developing mesosomal structures prior to DNA synthesis. Further analysis on the enzyme systems involved in the degradative processes of spore coats should be revealing.

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