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Ultrastructure of the Exosporium and Underlying Inclusions in Spores of *Bacillus megaterium* Strains[1]

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Spores of selected strains of *Bacillus megaterium* were prepared by various methods and examined with the electron microscope. An exosporium like that of *B. cereus*, with a nap and basal layer, was found in spores of a *B. megaterium* strain that reportedly contains a capsule-like exosporium. The exosporium occasionally appeared to be doubled or have an apical opening. Pili-like filaments were discerned on the surface. Beneath the exosporium were found large deposits of planar inclusions, which in cross section appeared laminated and in surface views consisted of a patchwork of striated packets with a periodicity of ~5 nm. The inclusions were usually attached to the exosporium, but in ultrastructure they differed from both the exosporium and coat. In two other strains of *B. megaterium*, one or two coats occurred but a typical exosporium was not present.

An exosporium described as a capsule-like slimy substance was detected by Tomcsik and Baumann-Grace (32) with the phase microscope on about half of 36 strains of *Bacillus megaterium* after reaction with homologous spore antiserum. In an effort to determine the ultrastructural basis for this seemingly unusual exosporium and for the difference in occurrence within the species, we obtained a representative reacting strain (Mg 19) and a nonreacting strain (Mg 13) from the late J. Tomcsik's collection in Switzerland. The reacting strain was shown by electron microscopy to contain a typical exosporium, but it was unusually distended by underlying deposits of planar inclusions which were structured as a laminar patchwork of striated packets. The nonreacting strain and the commonly used strain QM B1551 did not contain either of these structures.

MATERIALS AND METHODS

Organisms. Strains Mg 13 and Mg 19 of *B. megaterium* (32) were obtained from the Institute for Hygiene and Bacteriology, University of Basel, Switzerland. The spores were grown on potato-dextrose-agar and harvested after three to four days of incubation at 30 C. Strain QM B1551 was obtained from Arthur Kornberg of Stanford University, Palo Alto, Calif., and was grown by the method of Bertsch et al. (3).

Isolation of exosporium and inclusion bodies. Spores of strain Mg 19 were prepared as described by Matz et al. (21). A suspension of clean spores (50 mg of wet spores per ml) was repeatedly disrupted by expulsion at 50,000 psi through a refrigerated needle valve. The fragments were isolated by differential centrifugation at 1,200 × *g* for 30 min.

Electron microscopy. Specimens for sectioning were prepared by suspending spores in a few drops of 1% Noble agar or by collecting spores on a membrane filter (0.45 μm pore size) and adding a thin layer of the agar. Diced pieces were fixed in cold 3% glutaraldehyde in 0.1 M sodium-potassium phosphate buffer at *p*H 7.2 (27). After several buffer rinses, the pieces were postfixed overnight at room temperature in 1% OsO_4, *p*H 6.1 (26). The specimens were then stained for 2 hr in 0.5% buffered uranyl acetate. Alternatively, some samples were fixed in the presence of Luft's ruthenium red stain, as described by Pate and Ordal (23).

The agar blocks were dehydrated in graded concentrations of alcohol followed by propylene oxide and were embedded in Epon (19). Alternatively, some samples were embedded in Spurr (31) or D.E.R. 332-732 medium (Polysciences, Inc., Warrington, Pa.). Thin sections were cut with a du Pont diamond knife mounted on an Ultrotome III (LKB Instruments). The sections were post-stained for about 10 min with 2% aqueous uranyl acetate (34) and lead citrate (5), both of which contained a trace of Triton X-100 (2).

Samples for negative staining were placed on a

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collodion-carbon-covered grid. A drop of 2% phosphotungstic acid at *p*H 7.0 was added and withdrawn after 15 to 30 sec with filter paper.

Samples for shadowing were placed on Formvar-carbon-covered grids. Platinum-carbon was vaporized upon the sample at an incident angle of about 30 degrees.

For freeze-etch preparations, small pellets of cells were quick-frozen in Freon 12 and freeze-etched for 3 min at −100 C in a Balzers BA360M unit, and the carbon replicas were shadowed as described by Remsen and Lundgren (24).

Observations were made with a Philips EM-300 electron microscope. Photomicrographs of shadowed preparations were printed from the films as positives so that the shadows appear as light areas.

RESULTS

B. megaterium strain Mg 19. This strain from Tomcsik's collection is representative of spores which, when observed with the phase microscope, are surrounded by a halo in an India ink mount and a distinctly visible exosporium if treated with homologous spore antiserum. Tomcsik considered the exosporium to be a slimy layer, like the capsule of similarly reacting vegetative cells (32).

In electron micrographs of thin-sectioned spores, the exosporium of this strain proved to be typically structured, like that of *B. cereus* and *B. anthracis* spores (8), with a basal layer and a peripheral nap (Fig. 1). With negative staining, the nap had the characteristic mottled appearance, and the basal layer had hexagonal periodicity (~7 nm). With metal shadowing also, the appearance (Fig. 2) was like that of *B. cereus* spores (Fig. 2 in reference 8),

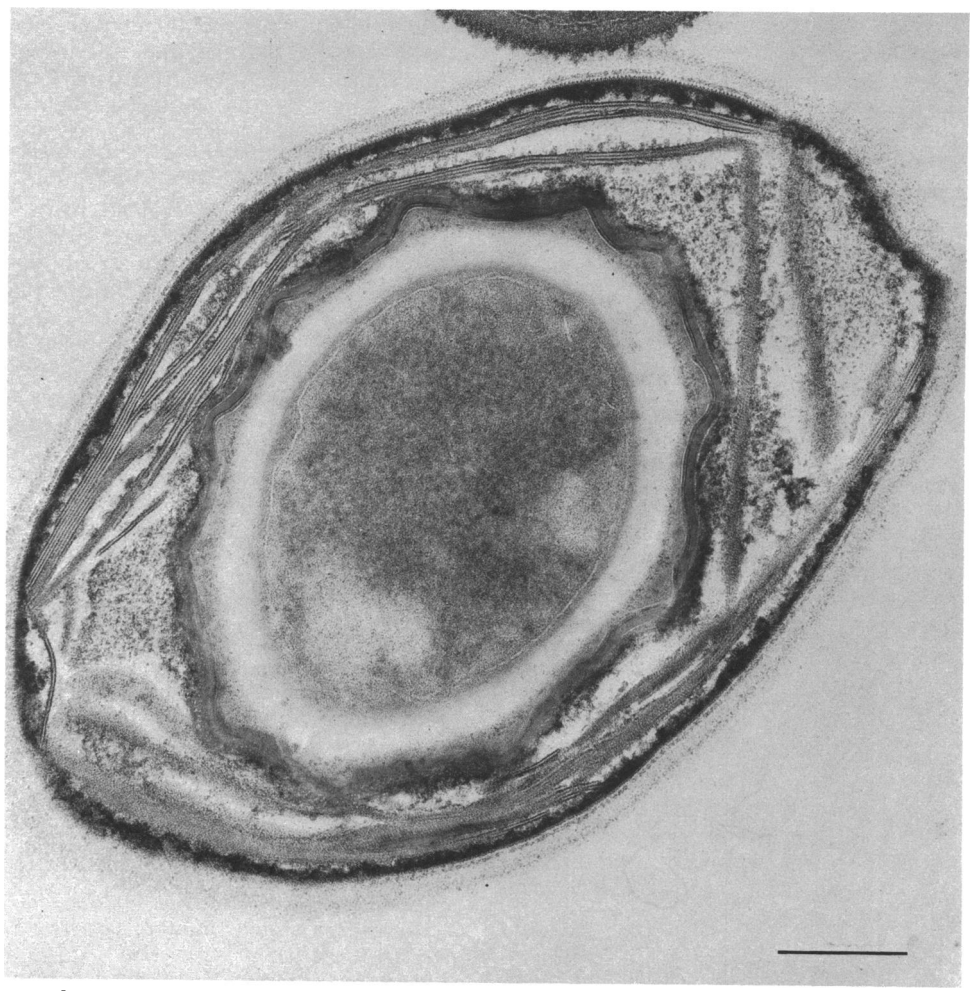

FIG. 1. *Cross-sectioned strain Mg 19 spore. The exosporium consists of a basal layer covered with a nap. The underlying planar inclusions consist of overlapping laminated packets and apparently terminate at the undersurface of the exosporium. Bar, 200 nm.*

including fibrillar material that seemingly emanates from the spore. Hodgkiss (*personal communication*) directed attention to the occurrence of these filaments, which resemble pili or fimbriae, on the exosporia of several *B. cereus* strains and to the fact that they are different from the larger and more elaborate appendages on spores of *Clostridium* strains (14). If the plane of sectioning chanced to intercept it, an apical hole or "pore" was infrequently observed in the exosporium (Fig. 3). Such openings occur frequently in the exosporium of *C. pasteurianum* spores (20). Figure 3 also illustrates the occasional occurrence of a double exosporium similar to that found in *B. anthracis* spores by Kramer and Roth (17).

FIG. 2. *Shadowed strain Mg 19 spores with surface filaments, several of which are wide and short and many of which are narrow and long. Bar, 500 nm.*

A remarkable feature of strain Mg 19 spores was the presence of many planar inclusions, which often seemed to distend the exosporium angularly, in the space beneath the exosporium and outside the coat (Fig. 1). The laminations which were evident in the sectioned inclusions varied in number from 1 to 20, measured ~5.5 nm from one dark line to another, and periodically overlapped, i.e., changed direction as in a patchwork. The elements in each line had a periodicity of ~5 nm.

The origin of the inclusions was not conclusively evident. One end of an inclusion was usually attached to the undersurface of the exosporium basal layer as if originating there (Fig. 1, 3, and 4), and the inclusions occurred with both of the double exosporia (Fig. 3). However, similar inclusions also seemed to be associated with doubled portions of the coat (Fig. 4) and sometimes seemed to be associated integrally with the primary coat (Fig. 5). The exosporium, inclusions, and coat appeared together during sporogenesis in the mother cell.

An effort to isolate fragments of exosporium and inclusions separately in pure states was unsuccessful. After extrusion of spores under high pressure through a needle valve, as for isolation of exosporium (8), the two structures were dislodged from spores but generally remained attached to each other (Fig. 6). The two structures were not separated by a variety of differential and gradient centrifugation techniques, either before or after various chemical and physical methods were used to effect dissociation or selective solubilization.

Fragments of exosporium and inclusions were not distinguished in metal-shadowed preparations of the mixture. With negative staining, however, the two structures appeared markedly different in ultrastructure. Fragments of exosporium had the characteristic mottling and hexagonal periodicity (Fig. 7). Fragments of the inclusions, in contrast, had a laminated patchwork of striated packets with a periodicity of ~5 nm (Fig. 8).

However, a similar laminar patchwork characterizes the spore coat (16). A freeze-etch preparation of strain Mg 19 yielded an informative micrograph (Fig. 9), showing a spore fractured around the coat, the typical striated

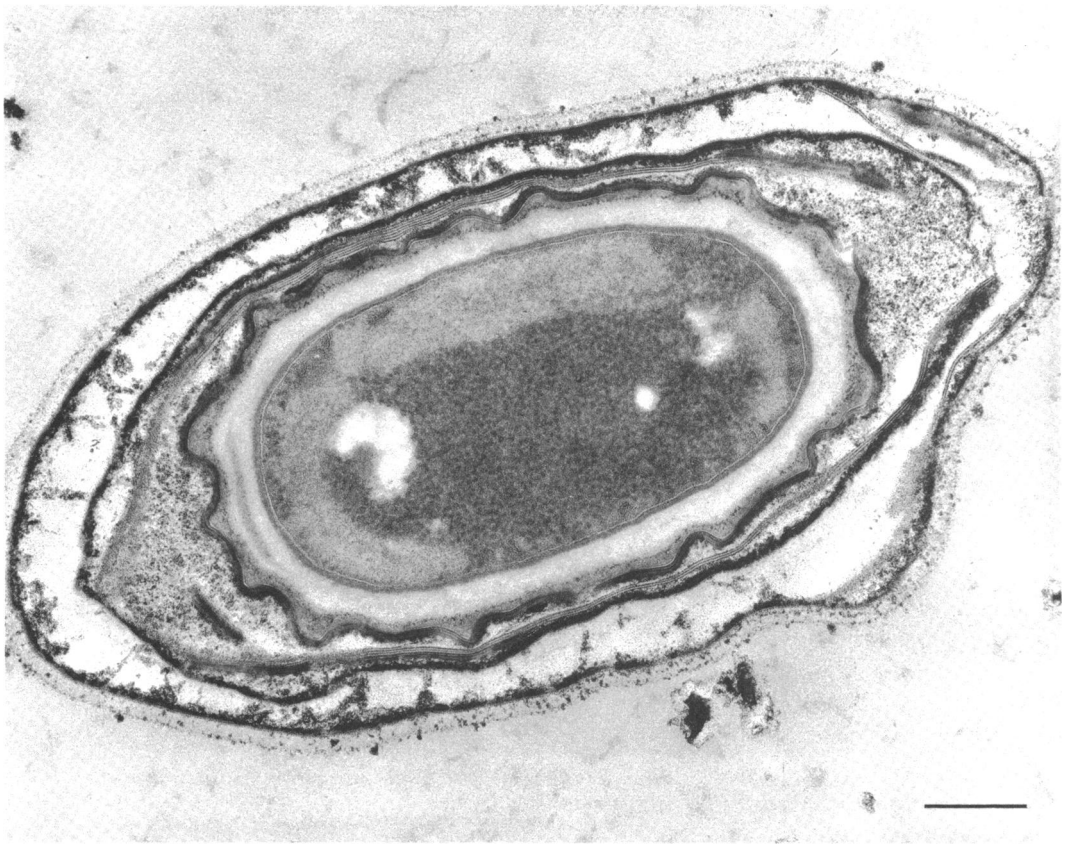

FIG. 3. *Cross-sectioned strain Mg 19 spore with a second exosporium. Each exosporium contains underlying laminar inclusions and has an opening at one end. Bar, 200 nm.*

patchwork of which had a periodicity of ~7 nm. The areas with a periodicity of only ~5 nm, the elements of which formed a hexagonal pattern, apparently corresponded to the inclusions. An inclusion is clearly distinguishable and differs in periodicity from the coat in a freeze-etch preparation of *B. cereus* T spores (Fig. 10 in reference 12).

The inclusions in strain Mg 19 also superficially resembled the spore coat in cross section, since both were laminar. In enlarged cross sections in which both structures were contiguous, however, their laminations had a distinctly different appearance (Fig. 5). In oblique sections, the inclusions often appeared comblike (Fig. 1) with periodicity of ~5 nm. In an almost planar section that contained all three structures (Fig. 10), the periodicity of the inclusions (~5 nm) was less than that in the exosporium and coat (~7 nm). A similar difference in periodicity was also found with the three structures in *B. cereus* T. Thus, the laminar inclusions apparently are ultrastructurally distinct from both the spore coat and exosporium.

B. megaterium strain Mg 13. Spores of this strain from Tomcsik's collection are representative of those in which an exosporium is not detected in the light microscope either by negative staining or the specific cytoimmune reaction (32).

With electron microscopy, a typical prominent exosporium was not found on spores of *B. megaterium* strain Mg 13 (Fig. 11). The spore was encased in a layered coat which was contiguous to the cortex and otherwise similar to the coat of strain Mg 19. On the outermost surface of the strain Mg 13 spore coat, there was a close-fitting, featureless layer which might be considered to be an atypical exosporium (16).

B. megaterium strain QM B1551. This strain is not in Tomcsik's collection, but it has been commonly used in metabolic studies and is said to possess an exosporium (3).

However, a typical exosporium was not

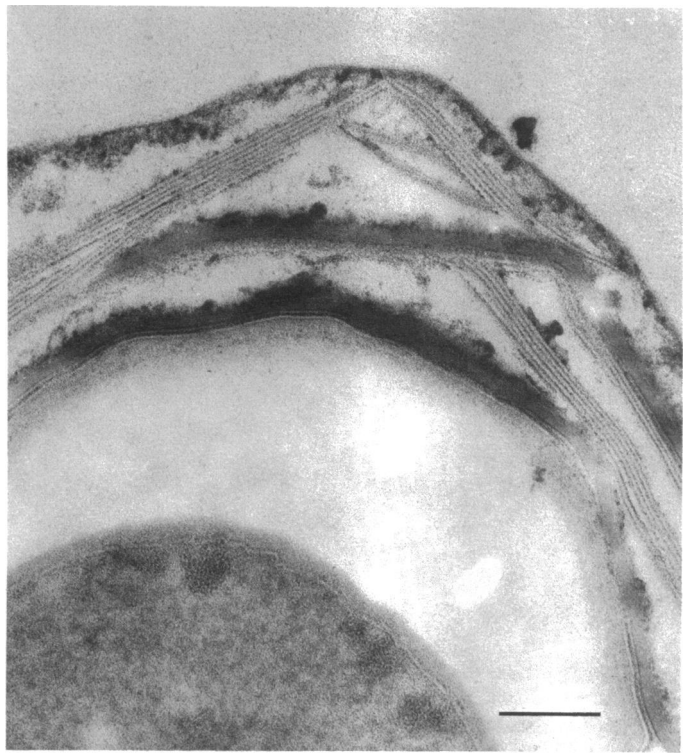

FIG. 4. *Cross-sectioned portion of a strain Mg 19 spore with a second segment of coat, under which is laminar material like that underlying the exosporium. Bar, 100 nm.*

found in *B. megaterium* strain QM B1551 (Fig. 12). In other micrographs, the periphery of the coat was occasionally distinguished as a thin layer, comparable to that in strain Mg 13, or possibly as an atypical close-fitting exosporium (16).

In cross sections, the peripheral surface of the coat of strain QM B1551 appeared to be studded at intervals with regular short protuberances; in oblique sections, the surface had a comblike appearance (Fig. 12). The periodicity of the elements in both was ~7 nm, which corresponds to the periodicity of the striated packets on the outer coat of this strain rendered visible by freeze-etch preparation (16).

A prominent outer coat fitted somewhat loosely around what usually is identified as an inner coat, with a greater interstice in spores washed with sodium dodecyl sulfate (3) and a lesser one in spores still encased in the mother cell. However, the inner coat of this strain is not greatly different in sectioned appearance from the gray or mottled layer contiguous with the electron-light (cortex) region in both strain Mg 13 (Fig. 11 B) and strain Mg 19 (Fig. 1). Thus the difference in the number of coats may be more apparent than real.

DISCUSSION

The results substantiated the hypothesis that a typical exosporium is present on spores of some strains of *B. megaterium* but not on others (6, 25, 32). The erroneous belief of Tomcsik and Baumann-Grace that the exosporium was constituted like the similarly reacting capsule of vegetative cells (32) was apparently caused by their observations in the light microscope of the exosporium after reaction with homologous antiserum. The accentuated halo they saw is explained by a typical exosporium extended by underlying inclusions. A hole existing in the exosporium might allow diffusion into and reaction of antibodies with antigens in the basal layer, inclusions, and coat surface, but the rapidity of the antibody-antigen reaction suggests that the hairy nap on the exosporium surface is primarily involved.

Differences exist in use of the term "exosporium." Because of its recognition and extensive characterization, the type of exosporium found on spores of *B. cereus* (i.e., loose-fitting and comprised of a laminar close-packed lattice inner layer and an outer layer with a nap of hairlike projections) was designated as typ-

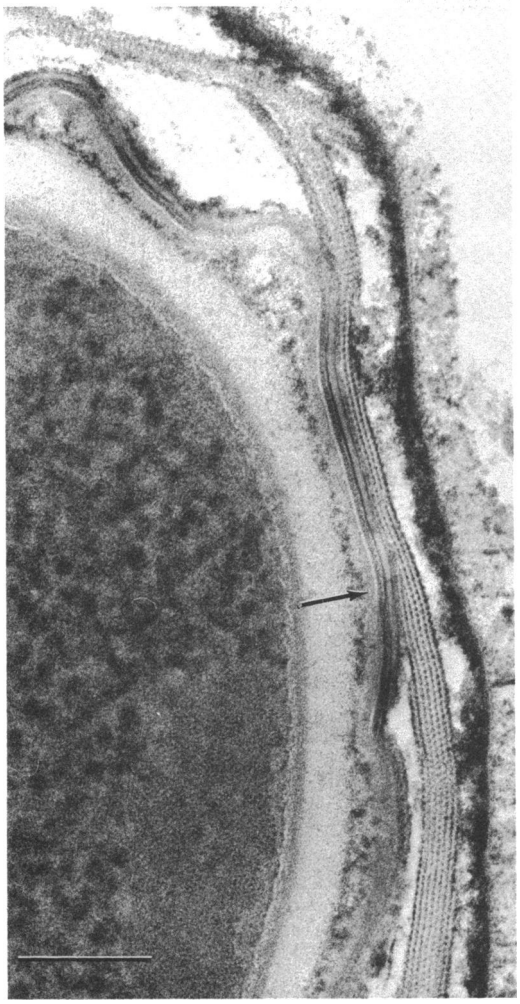

FIG. 5. *Cross-sectioned portion of a strain Mg 19 spore in which a laminar inclusion in part appears integral with the laminar spore coat (arrow), yet with different ultrastructure in each. Bar, 100 nm.*

ical. Some *Clostridium* strains possess this typical exosporium (15, 28), but others seem to have only the laminar inner layer varying in thickness (20, 30, 33). Hodgkiss, Ordal, and Cann (14) distinguished three types of exosporium in *Clostridium* strains. Holt and Leadbetter (16) advocated that the outermost surface of the spore be considered as an exosporium, whether loose-fitting and discrete or close-fitting and contiguous with the spore coat.

A closer look with the electron microscope helped to explain the nature of the laminated planar inclusions beneath the exosporium in *B. megaterium* Mg 19. These structures had been observed in *B. cereus* spores by Gerhardt and Ribi (8) but were miscalled "parasporal inclusion bodies" and were prematurely suggested to represent the same material as exosporium. Similar flakelike inclusions occur in spores of other bacilli (1, 4, 7, 9) and clostridia (15, 28, 29, 30). The inclusions in spores of *B. circulans* were shown by Abram (1) to be different in negatively stained ultrastructure than the exosporial envelope.

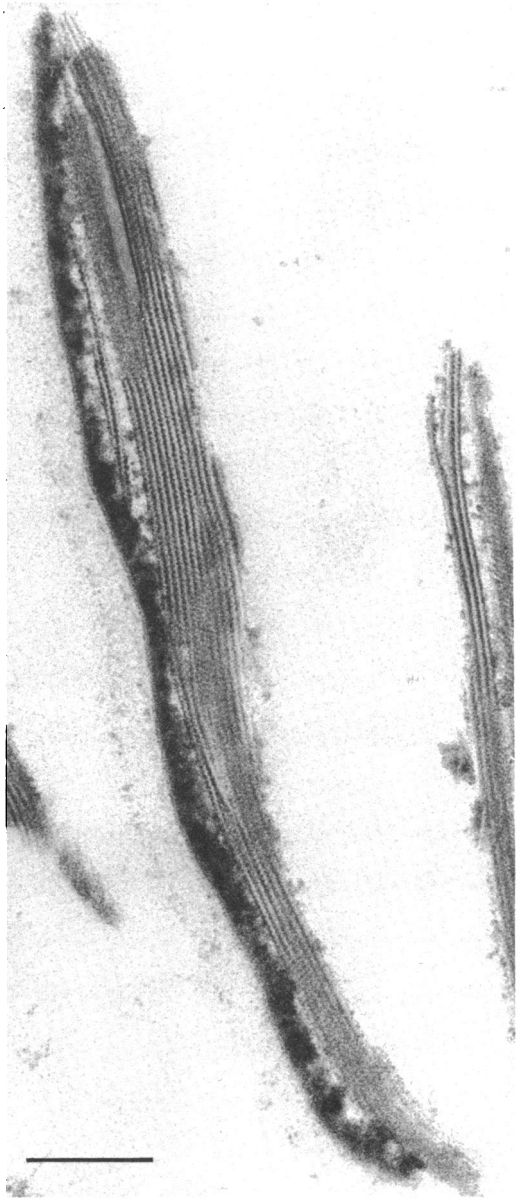

FIG. 6. *Cross-sectioned fragment of exosporium and attached laminar inclusion, isolated from strain Mg 19 spores. Bar, 100 nm.*

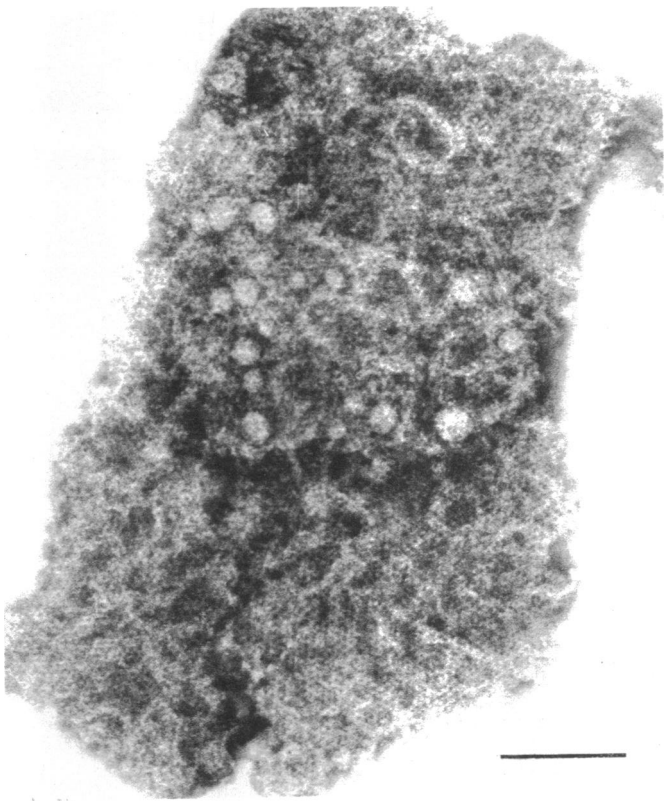

FIG. 7. *Negatively stained fragment of exosporium, isolated from strain Mg 19 spores. Bar, 100 nm.*

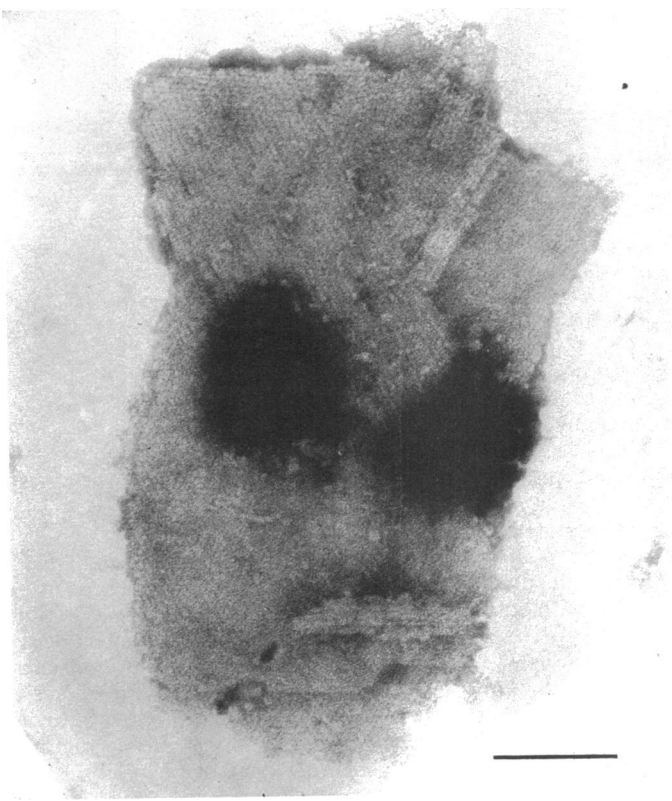

FIG. 8. *Negatively stained fragment of inclusion, isolated from strain Mg 19 spores. Bar, 100 nm.*

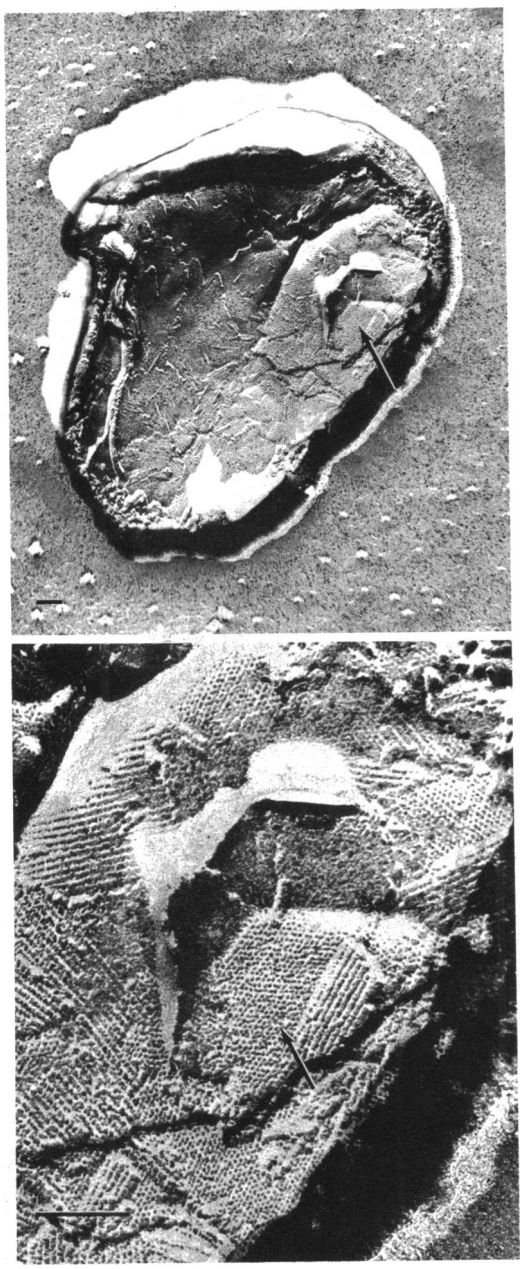

FIG. 9. *Freeze-etch preparation of a strain Mg 19 spore (entire spore at top, enlarged portion at bottom) with the fractured surface concave around the coat, which is identified by the patchwork of striated packets with a periodicity of ~7 nm. The underlying inclusions (arrows) are identified by a finer periodicity (~5 nm) and a hexagonal pattern of subunits. The arrows also indicate the direction of shadowing. Bar, 100 nm. This micrograph was provided by Charles E. Remsen.*

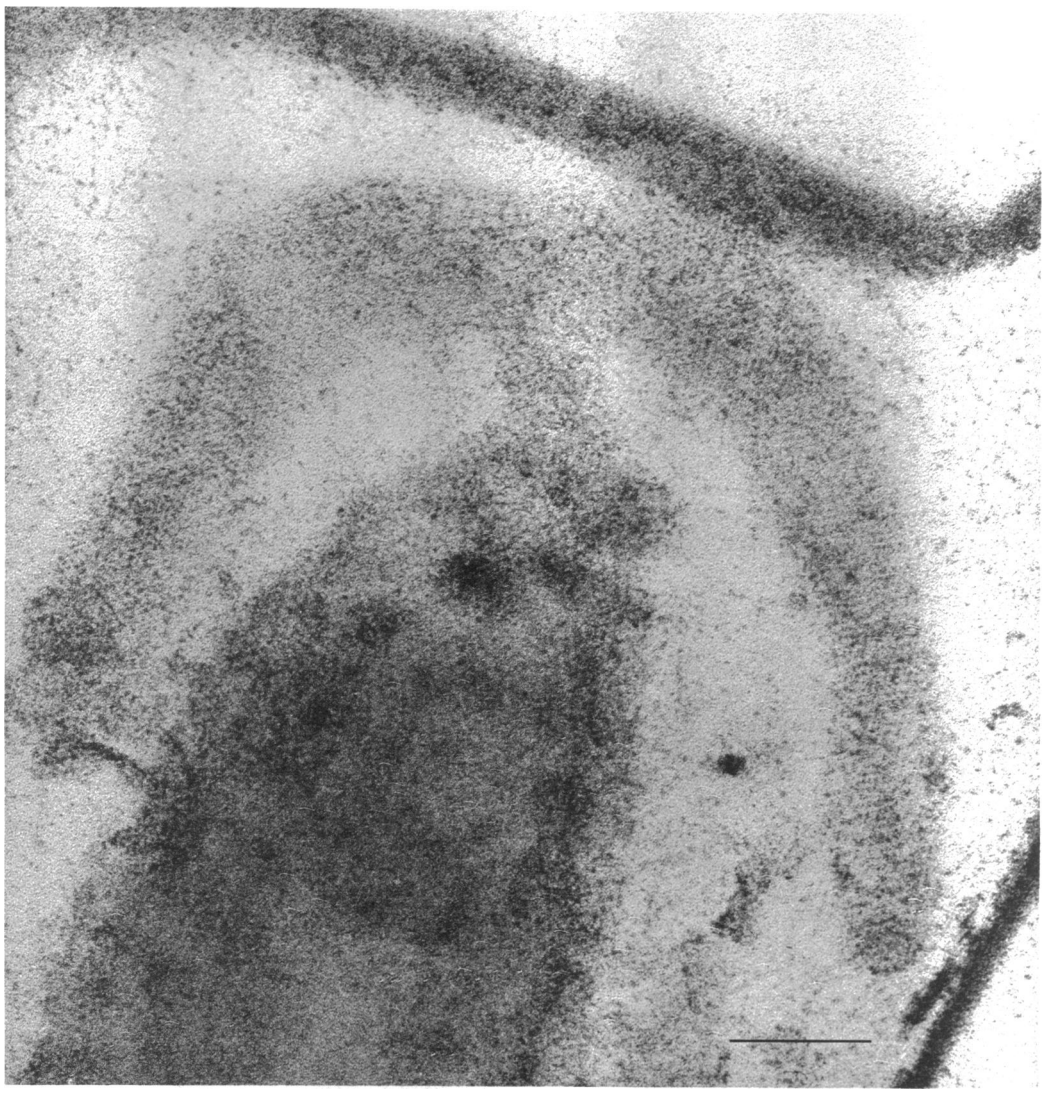

FIG. 10. *Planar-sectioned portion of a strain Mg 19 spore in which the periodicity of the inclusion (center) appears different from that of the exosporium (top) and coat (bottom). Bar, 100 nm.*

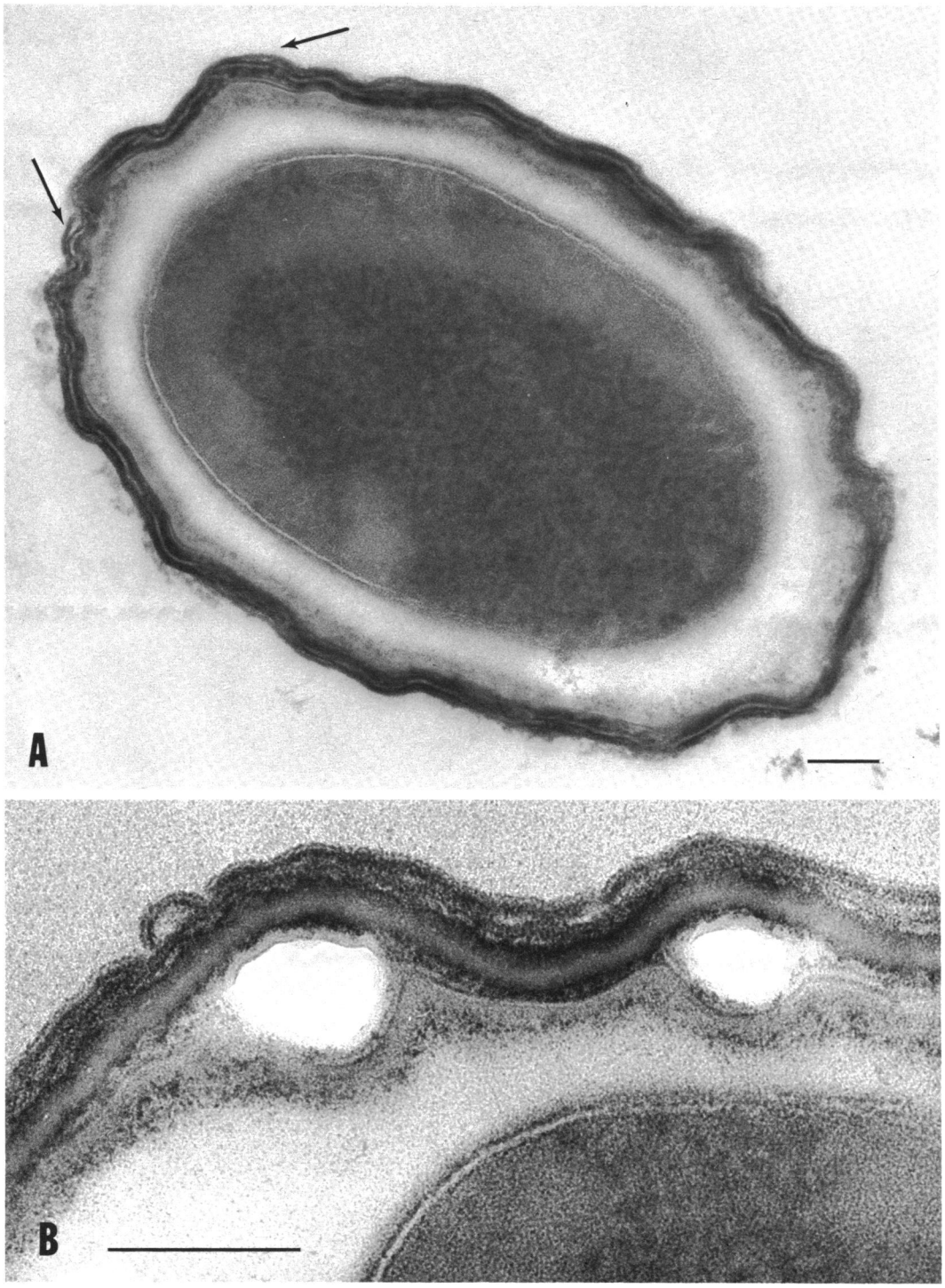

FIG. 11. *(A) Cross-sectioned spore of strain Mg 13, devoid of typical exosporium but with a close-fitting layer at the surface of the coat (arrows). (B) Enlarged portion of another strain Mg 13 spore in which the surface layer is thicker and more distinct. Bars, 100 nm.*

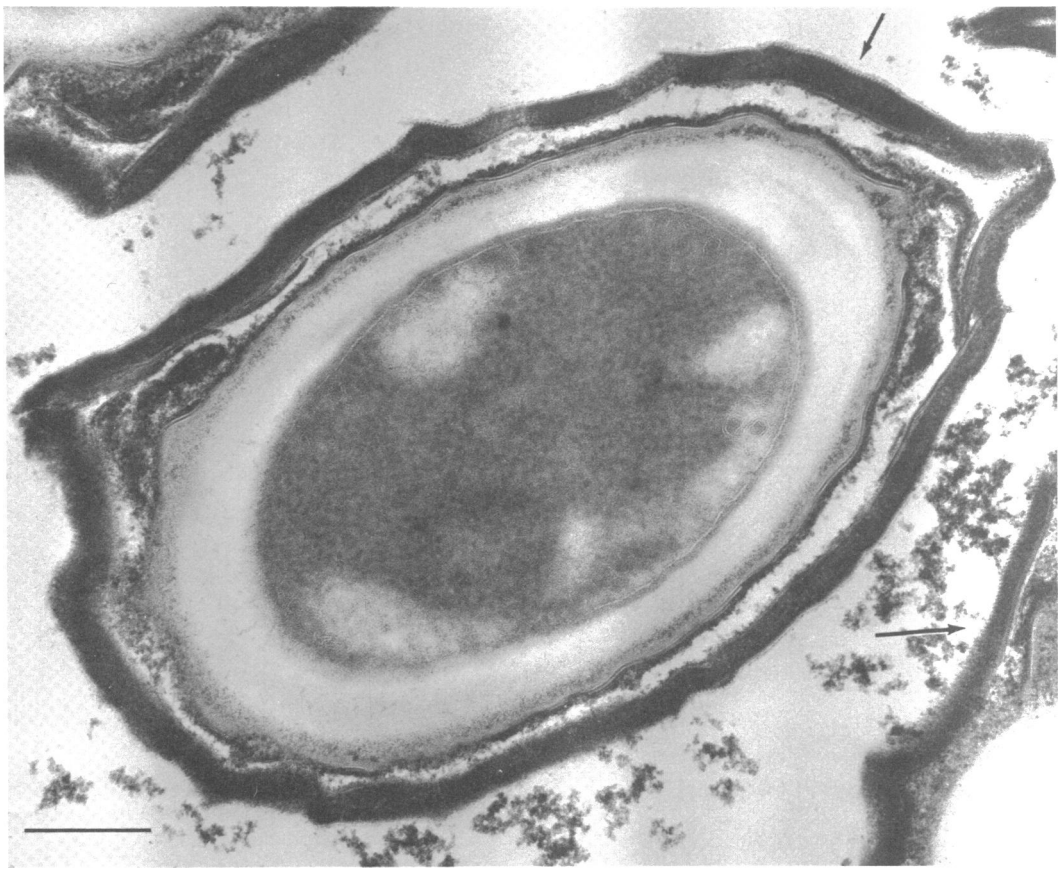

FIG. 12. *Sectioned spore of strain QM B1551, devoid of typical exosporium. The arrows designate portions of the outer coat surface, which in cross section appears studded with short protuberances and in oblique section appears comblike. Bar, 200 nm.*

Various species or varieties of *Bacillus* are classified in a crystalliferous group based on the formation of a three-dimensionally crystalline protein parasporal body, which is often toxic for insects (13, 22). However, the globular parasporal body of Fowler's bacillus [named *B. finitimus* var. *fowleri* by Heimpel (13)] is contained within the exosporium (10). This characteristic, physiological tests (10), and the absence of pathogenicity for insects (13) suggest that Fowler's bacillus is a variety of *B. cereus* (10). All of the crystalliferous bacteria should be compared, whether inclusions are formed inside or outside the exosporium. Apparently the characteristic is widely shared.

The similarity in appearance between cross sections of the laminar inclusions and the laminar spore coats has been noted (9, 15, 18). However, the superficial similarities between the inclusions and either the coat or the exosporium were not substantiated by examination of ultrastructure. The inclusions apparently represent distinct entities.

ACKNOWLEDGMENTS

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