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Ultrastructural Changes Associated with Germination and Outgrowth of an Appendage-Bearing Clostridial Spore

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The sequence of events occurring during the germination and outgrowth of appendage-bearing spores of *Clostridium bifermentans* was studied by phase-contrast and electron microscopy. The mature spore was characterized ultrastructurally as having the normal spore components as well as long tubular appendages which originated from the surface of the spore coat. Spores were incompletely enclosed by a distinctly laminated exosporium which possessed hairlike projections on its outermost layer. During germination, structural changes were observed in the core, core wall, cortex, and spore coat layers. Cortical material was extruded from the spore during outgrowth, which usually occurred from the pole opposite the appendages. The subunits comprising the structure of the appendages and the morphology of the mature appendages were observed. No discernible changes could be observed in the spore appendages during germination and outgrowth.

It has been shown that spores of some members of the genus *Clostridium* possess unique structures termed appendages (9). Although the fine structure of appendages has been studied (2, 3, 13, 17, 21), and the ultrastructural changes occurring during the germination of clostridial spores have been followed (4, 5, 14, 20), the changes which take place during the germination and outgrowth of an appendage-bearing spore have not been reported. We have observed some of these changes with a strain of *C. bifermentans* which produces an appendage-bearing spore.

MATERIALS AND METHODS

Organism. *C. bifermentans* strain UK-A1003 was obtained from the culture collection of the Department of Microbiology, University of Kentucky.

Medium and growth conditions. Mature spores were produced after 96 hr of anaerobic incubation at 37 C on Tryptic Soy Agar (Difco) plates; a GasPak unit (BioQuest, Cockeysville, Md.) was used to obtain anaerobic conditions. Spores were harvested with distilled water, washed six times, and stored at −10 C.

Germination and outgrowth. Germination was achieved by heat-shocking spores at 65 C for 3 hr and incubating them anaerobically in Tryptic Soy Broth at 37 C. The presence of 5 to 10% carbon dioxide in the gas phase was found to accelerate germination of these spores.

Phase-contrast microscopy. Samples were taken directly from the germination medium at various time intervals over a 2-hr period and were viewed with a Zeiss Universal phase-contrast microscope. Photomicrographs were taken on Kodak Plus-X film by use of a Nikon camera attachment.

Electron microscopy. Mature spores and spores undergoing germination were harvested by centrifugation and suspended in 2% glutaraldehyde prepared in 0.09 M cacodylate buffer at *p*H 6.1 (1). After 2 hr of fixation at room temperature, the cells were centrifuged and washed free of glutaraldehyde with 0.09 M cacodylate buffer (*p*H 6.1) containing 0.25 M sucrose and 3 mM calcium chloride (1). The spores were then fixed for 18 hr at 4 C with a 1% osmium tetroxide solution prepared in *p*H 6.1 veronal acetate buffer (6). After fixation, the cells were washed in 0.5% uranyl acetate prepared in the same buffer, dehydrated in a graded acetone series, embedded in Epon, and polymerized at 60 C for 22 hr.

Ultrathin sections were cut with an LKB Ultrotome using glass knives, mounted on 300-mesh copper grids, and stained with lead citrate (15).

Formvar-coated grids were mounted with a drop of spore suspension and negatively stained with a 2% solution of sodium phosphotungstate at *p*H 7.0 or shadowed with carbon-platinum. All electron microscope preparations were observed in an Hitachi HU-11B or a Philips EM 200 electron microscope.

RESULTS

Phase microscopy. Phase-contrast microscope observations revealed that dormant spores were refractile and that appendages projected from

only one end of the spore (Fig. 1a). The initial event observed during the transformation of a spore into a vegetative cell was the darkening of the spore (Fig. 1b). The spore subsequently swelled and elongated (Fig. 1c), and the vegetative cell emerged from the pole opposite appendage attachment (Fig. 1d). As outgrowth proceeded, the emerging cell was compressed by the rigid spore coat (Fig. 1e); the transformation process culminated with the liberation of the vegetative cell from the spore integument (Fig. 1f).

Electron microscopy. The ultrastructure of a dormant spore is illustrated in Fig. 2a. The core is surrounded by a cortex which is enclosed by the spore coat.

The most exterior structural component of the spore observed is the exosporium which is laminated in the lateral regions of the spore (Fig. 2a). The laminations consist of 2.5-nm wide electron-dense bands interposed between 4-nm wide bands of less electron-dense material. Hairlike projections extend approximately 25 nm from the outer surface of the exosporium (Fig. 3).

The exosporium is prevented from completely enveloping the spore by the presence of long tubular appendages (AP, Fig. 2a and 2b) which originate from the surface of the spore coat (Fig. 4) and emanate from only one pole of the spore.

The ultrastructure of the germinated spore was quite different from that of the dormant spore. In the germinated spore the core is readily stained with heavy metals, and the nucleus and densely packed ribosomes within the cytoplasm are evident (Fig. 2b). The core wall is clearly visible, and the cortex appears to consist of amorphous material. The only change observed in the spore coat during germination was an increased affinity of the material surrounding the electron-dense spore coat for heavy metals.

The initial phase of outgrowth is characterized by elongation of the germinated spore and a degradation of the spore integument in the polar regions, thereby forming a germination pore (Fig. 2c). Although breakdown of the integument

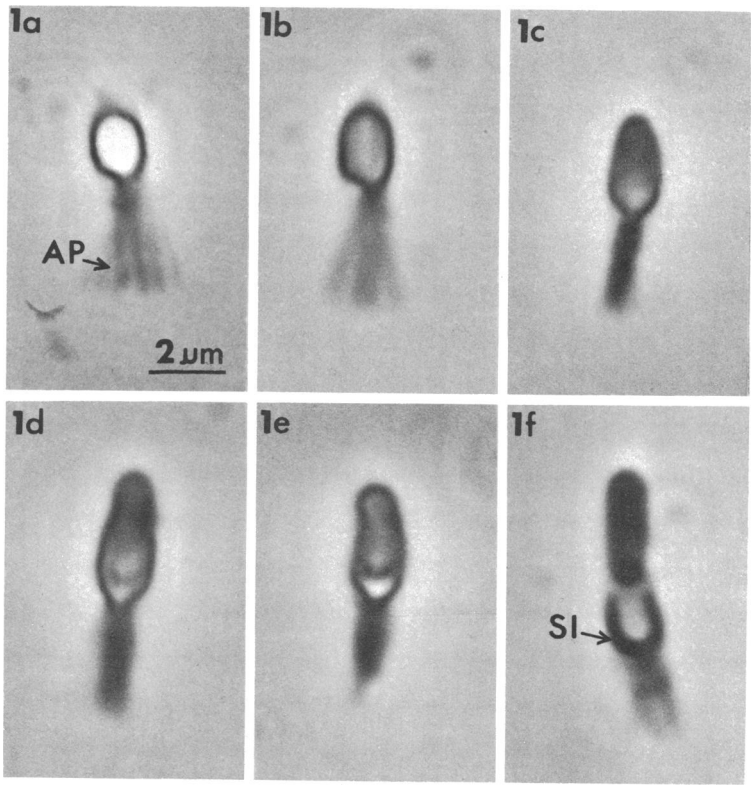


FIG. 1. *Phase-contrast micrographs of C. bifermentans UK-A1003 spores undergoing germination and outgrowth illustrating: a refractile dormant spore with appendages (AP, 1a); loss of refractility (1b); elongation (1c); emergence of the cell (1d and 1e); and release of the vegetative cell leaving behind spore integument (SI) and appendages (1f).*

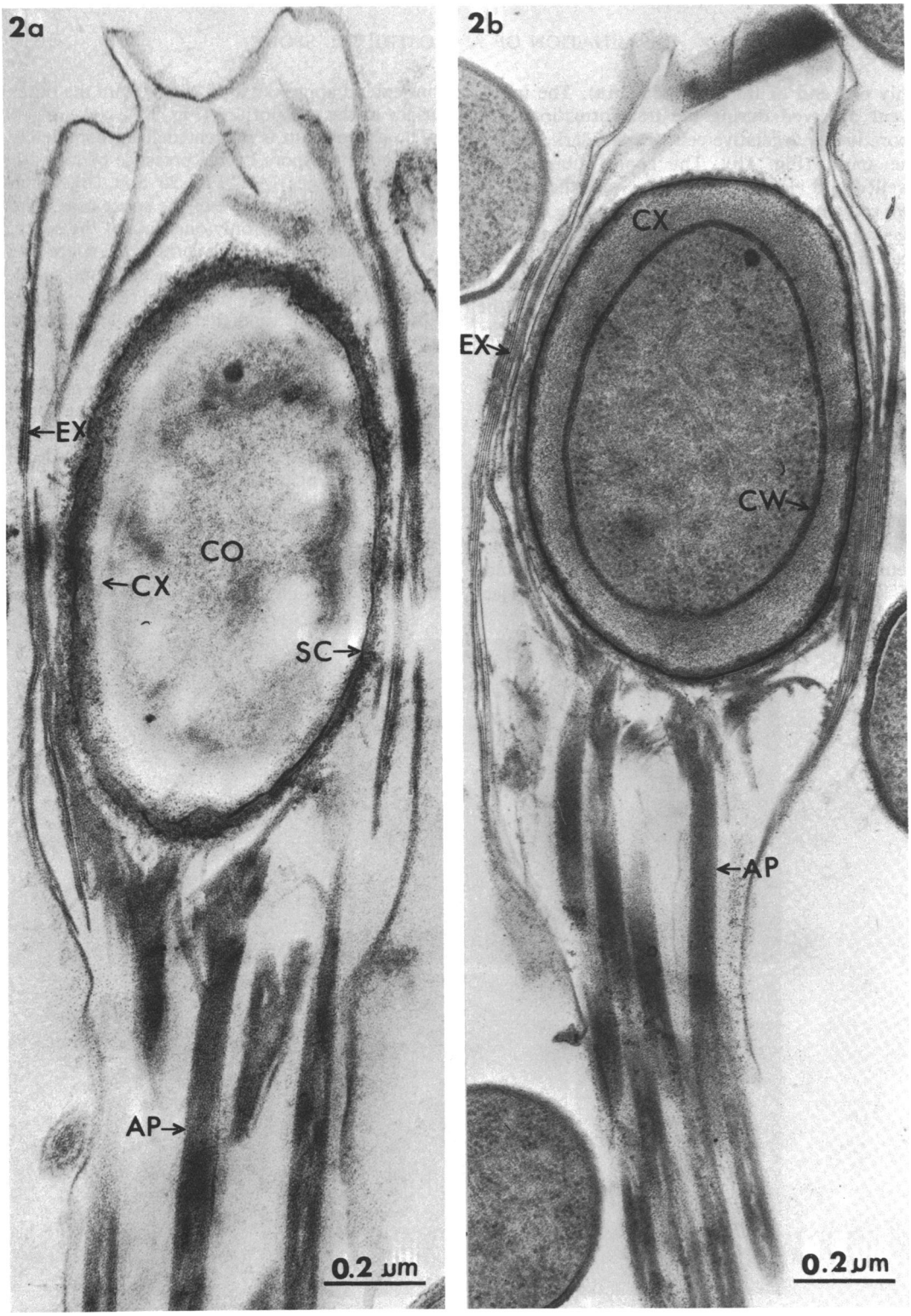


FIG. 2a. *Section of a dormant spore of C. bifermentans illustrating the core (CO) cortex (CX), spore coat layers (SC), exosporium (EX), and appendages (AP).*

FIG. 2b. *Section of a spore during an early stage of germination. Preservation of the ultrastructure is enhanced as compared to the dormant spore (Fig. 2a). The transformation of the cortex (CX) into a region containing amorphous material is readily observed, and the core wall (CW) is distinct.*

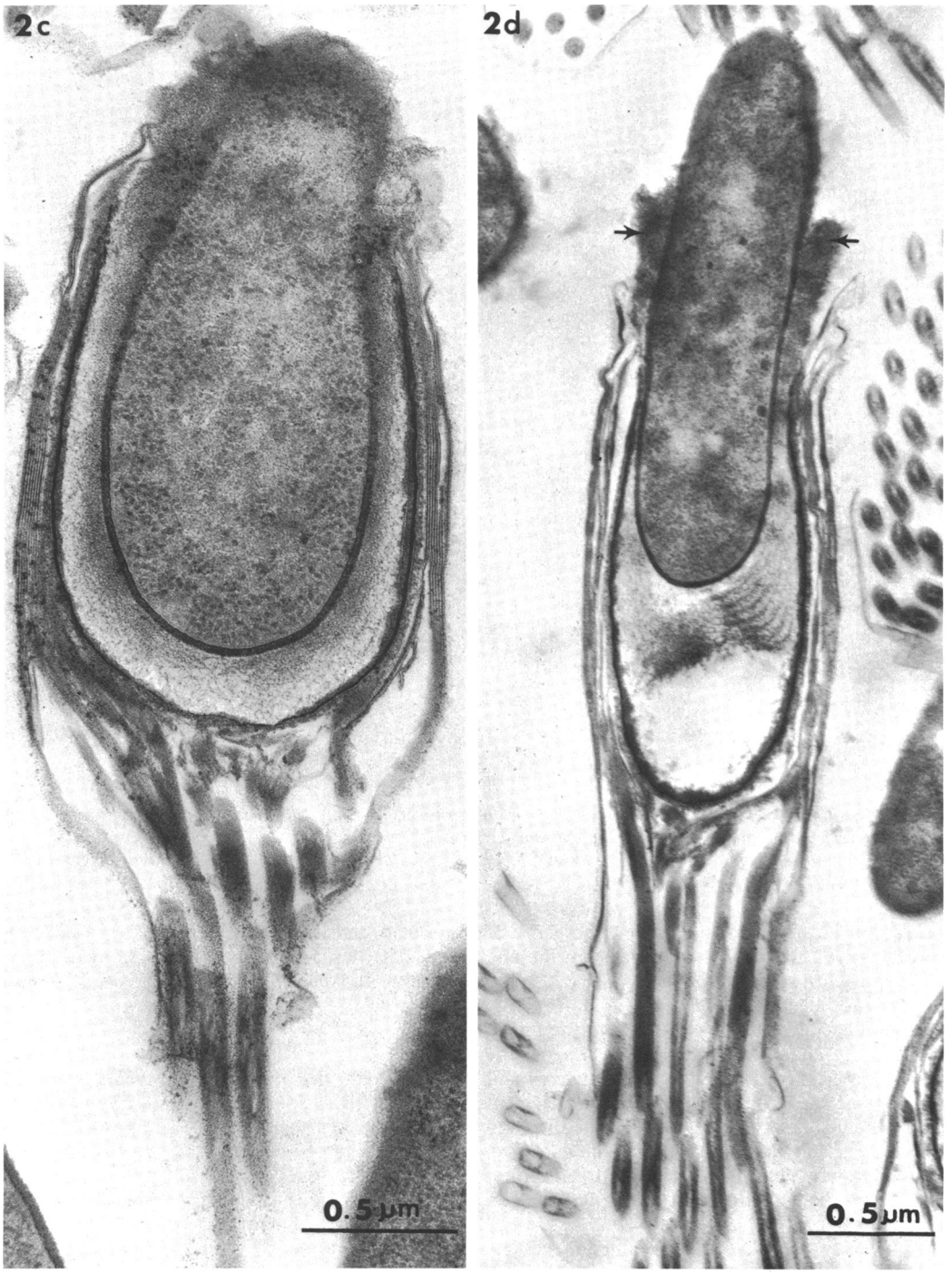


FIG. 2c and 2d. *Sections of germinating spores undergoing elongation and outgrowth. Elongation and outgrowth from the pole opposite appendage attachment (2c); constriction of the emerging cell by the spore coat and extrusion of cortex material (arrows) during outgrowth (2d); material left behind (spore integument and appendages) by emerging vegetative cell (2d).*

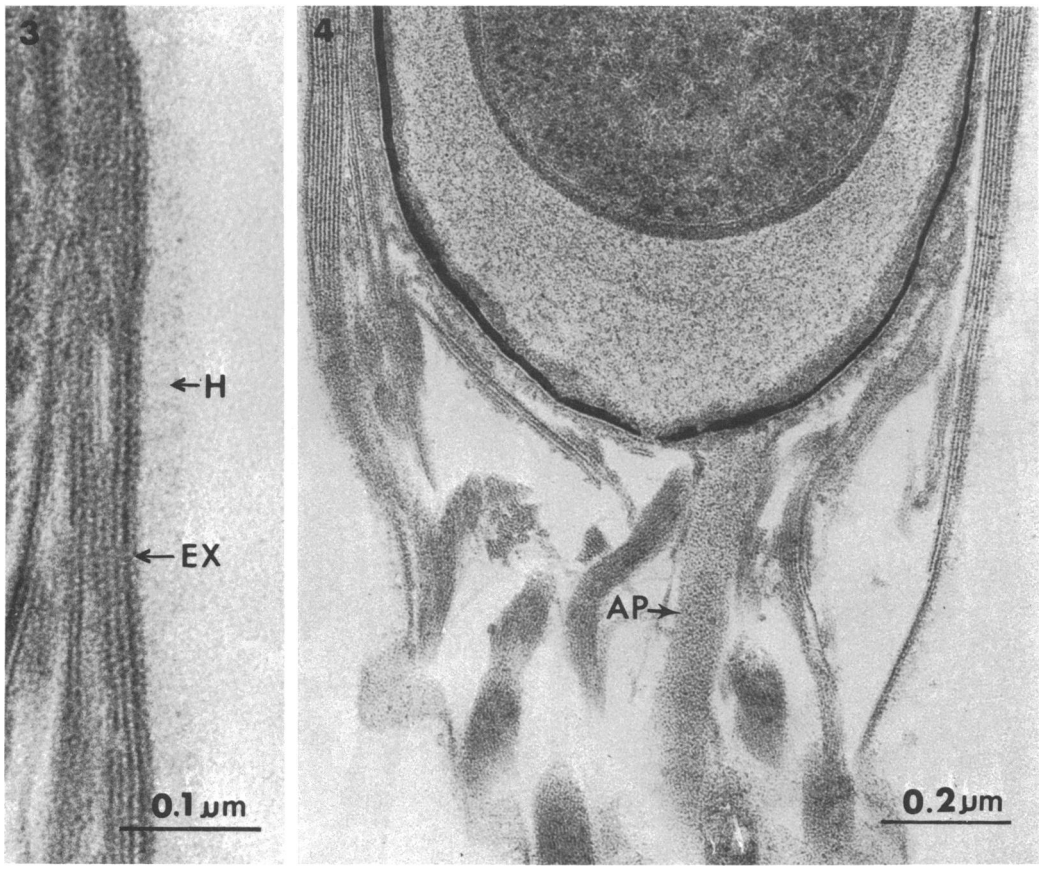


FIG. 3. *Section through the exterior region of a germinating spore illustrating the presence of hirsute projections (H) on the outer surface of the exosporium (EX).*

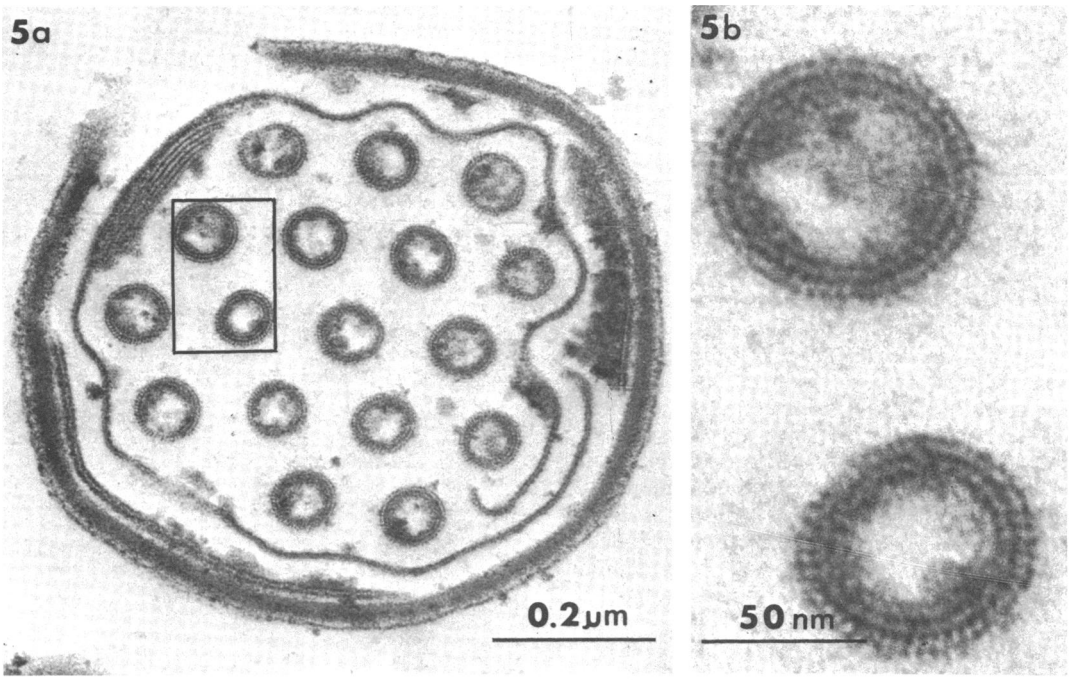
FIG. 4. *Section through a portion of a germinated spore; the appendages (AP) appear to originate from the outer surface of the spore coat.*

occurred at both ends of the spore, emergence of the vegetative cell usually occurred from the end of the spore not bearing appendages (Fig. 2c and 2d). As the vegetative cell is released from the germination pore, it appears to be compressed by the spore coat layers (Fig. 2d). Some of the cortical material is extruded into the environment (Fig. 2d) as the vegetative cell emerges. Except for the events described, the new cell leaves behind a relatively intact spore integument (Fig. 2d). We are unable to observe any ultrastructural changes in the appendages during germination and outgrowth.

Cross sections through these appendages revealed that each appendage consisted of three concentric layers of small electron-dense subunits (2.5 nm diameter, Fig. 5a and 5b). The fibrous appearance of the appendages was best revealed by negative staining (Fig. 6). In addition, each tubule was sheathed by a substance of low electron density; the presence of this substance was best observed in negatively stained (Fig. 6, arrows) and shadowed (Fig. 7, arrows) preparations.

DISCUSSION

Although the sequence of events associated with the formation of a vegetative cell from this appendage-bearing spore is similar to that described for some members of the *Bacillaceae* (4, 5, 12, 14, 16, 18, 20), it did not follow the pattern described for any one species. During germination the electron-transparent core was transformed into a readily stainable cytoplasm replete with ribosomes and a nuclear area (4, 16). There was, however, no period of vesiculation within the core as described for *C. pectinovorum* (4) and *B. subtilis* (18). Mesosome-like structures were observed only during the latter stages of outgrowth.



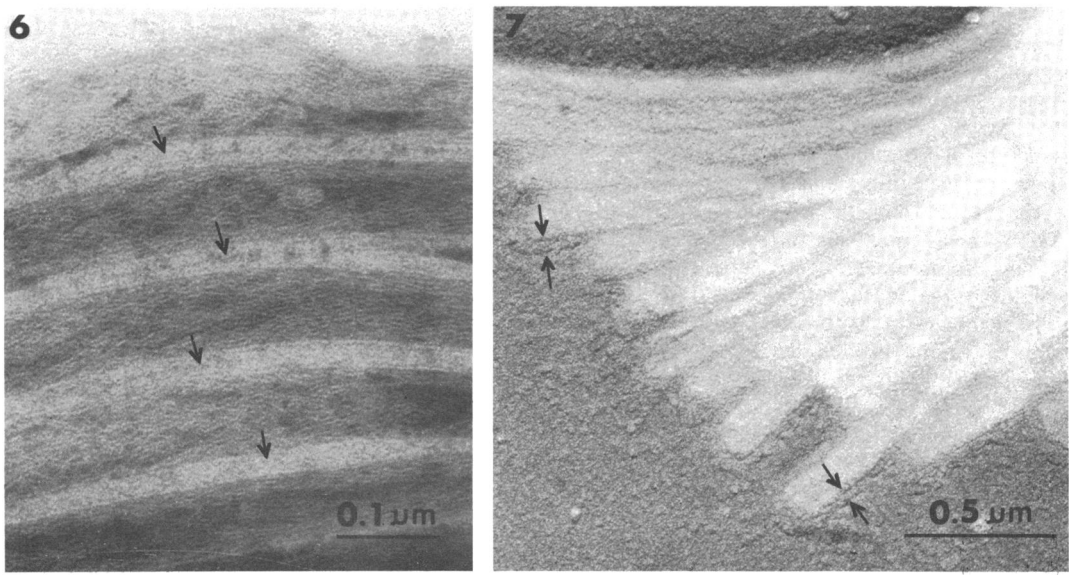


FIG. 5a and 5b. *A cross section through developing appendages within the sporangial wall reveals their tubular nature. Each appendage tubule is composed of subunits arranged in three parallel concentric layers. Fig. 5b. Enlargement of the designated area of Fig. 5a.*

FIG. 6. *Negative stain of the appendages showing their fibrillar structure. Less electron-dense material (arrows) separates the tubules.*

FIG. 7. *Shadowed preparation of appendages showing the sheath material which surrounds each tubule (arrows).*

During germination the structureless cortex swelled and contained amorphous material similar in appearance to the spongelike material observed in the cortex of germinated spores of *B. subtilis* (18) and *C. sporogenes* (5). Occasionally the cortical material was arranged in concentric bands reminiscent of that shown in resting spores of *B. megaterium* (11). Although the cortex of *C. pectinovorum* (4) and *B. megaterium* (11) was reported to be dissolved away during germination, a portion of the cortical residue of *C. bifermentans* strain UK-A1003 appeared to be extruded into the environment during outgrowth.

Among the *Bacillaceae* two principal types of outgrowth have been described. One is the ready escape of the vegetative cell from its spore integument as exemplified by some *Bacillus* species (7, 8, 16), and the other is the restricted emergence of the new cell through a narrow opening in the rigid spore integuments as shown in certain clostridia (4, 14). The latter was demonstrated in *C. bifermentans* strain UK-A1003. In addition, outgrowth was almost always from the pole opposite the appendages.

Although tubular appendages of *C. bifermentans* have been characterized electron microscopically (3, 13, 21), the ultrastructure of the appendages of strain UK-A1003 appears to differ significantly from that described for other strains. A cross section through the free appendages of *C. bifermentans* strain FDA-1 showed that the substructure of the tube walls consisted of 30 subunits arranged in a single row (21). In contrast, we have found that the tube walls of strain UK-A1003 consist of at least three rows, each containing approximately 60 subunits. Negative staining revealed that the appendage subunits were arranged in a fibrous pattern which is in contrast to the beaded pattern reported in *C. bifermentans* strain 9-SDH (21). In addition, we were able to observe material of low electron density surrounding each appendage. Previous reports of such material surrounding developing tubular appendages have associated these areas with hirsute regions of the mature appendage (13, 21). This is not the situation in strain UK-A1003 since there are no hirsute regions on the mature appendages.

The structure of the exosporium is also unique for clostridial spores in its highly laminated nature only in the lateral regions of the spore (19), and the presence of hirsute structures emanating from the outer surface of the exosporium. This latter feature has been observed in spores of *Bacillus* (10, 12) and *C. sprorogenes* (5).

We were unable to detect any structural changes in the appendages during germination and outgrowth, which suggests that they do not play a significant role in these processes.

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