Morphology of the Spore of Some Strains of 
Clostridium botulinum Type E

W. HODGKISS AND Z. JOHN ORDAL

Ministry of Technology, Torry Research Station, Aberdeen, Scotland

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ABSTRACT

HODGKISS, W. (Torry Research Station, Aberdeen, Scotland), AND Z. JOHN ORDAL. Morphology of the spore of some strains of Clostridium botulinum type E. J. Bacteriol. 91:2031–2036. 1966.—The spores of four strains of C. botulinum type E show an unusual and elaborate morphology. Numerous tubular appendages radiate from the surface of the spore. The spore and its appendages are enclosed in a delicate exosporium. The electron microscopic morphology of the spores, as seen in metal-shadowed and negatively stained preparations, and by the carbon-replica technique, is described.

The morphology of the bacterial endospore has been the subject of a number of studies. A review of the information then available on the formation and morphology of spores was presented by Robinow (16). The spores of different species vary in size and in shape and may exhibit an irregular surface structure. Van den Hooff and Aninga (9), by electron microscopy, substantiated the observations of Meyer (15) on the ribbed or starlike structure of Astasia asterosporus (Bacillus polymyxa). This spore has longitudinal ribs which make it appear as a multi-pointed star when examined in cross section. Bradley and Williams (1) and Franklin and Bradley (6) introduced the use of the carbon-replica technique to study the surface characteristics of the spores of several Bacillus spp. and of Clostridium welchii (C. perfringens). These studies demonstrated that the outer spore surface was not always smooth and that the spores of certain species exhibited a characteristic sculptured effect. More recently Krassil’nikov, Duda, and Sokolov (11) presented electron micrographs of the spores of 20 soil isolates of Clostridium spp. These spores possessed varied and elaborate protrusions which the investigators considered to be significant enough to place the strains in six groups made up of 13 new species.

Our studies were initiated following a routine electron microscopic examination of spores of a strain of C. botulinum type E (NCIB 4248). Attempts were being made to produce a clean spore preparation, one free of vegetative and sporangial debris. When dark contrast phase optics were employed, it was noted that the spores possessed a distinct halo and that frequently bits of debris appeared to cling tenaciously to this halo. Our initial electron microscope observations prompted us to undertake a more detailed study of the morphology of spores of C. botulinum type E.

MATERIALS AND METHODS

The cultures used were transfers of two well-known strains (Beluga and Nanaimo) and two recent isolates that were obtained from sea bottom deposits off the Scandinavian coast. These strains are from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, and are identified by the following NCIB numbers: 4240 (Nanaimo), 4248 (Beluga), 4256 and 4275 (Torry isolates).

Spores were produced in the medium of Schmidt, Nank, and Lechowich (17) or in this medium modified by the addition of calcium and manganese. The spores were removed from the culture medium by centrifugation and washed three times in 0.75% (w/v) sodium chloride solution. As sporulation was never complete, enzymes were used to free further the sample of vegetative debris (trypsin alone, or trypsin, pepsin, ribonuclease, and lysozyme). The spores were then washed again with the saline solution, and the preparation was examined under dark contrast phase optics. After three additional washings with distilled water, the spores were suspended in distilled water for electron microscopic examination.

Metal-shadowed preparations were made by placing droplets of the spore suspension on Formvar-coated grids, which were then shadowed with gold-palladium (40:60) at 20°. Negatively stained preparations were made on carbon-coated grids. A droplet of the spore suspension was allowed to air-dry on the grid, which
was subsequently treated for 1 min with 1% aqueous ammonium molybdate or 1% aqueous phosphotungstic acid adjusted to pH 7.0 (2). Carbon replicas were prepared by the method of Bradley and Williams (1) and were shadowed with gold-palladium (40:60) at 20°.

Preparations were examined in a Siemens Elmiskop I with a single or double condenser, a 200-μ condenser aperture, a 50-μ objective aperture, and an accelerating voltage of 80 kv. Micrographs were taken at initial magnifications of 10,000 to 80,000 × on Ilford N50 plates.

RESULTS

In all, four strains of C. botulinum type E have been examined by the techniques described. In principle, the morphology of the spores of the several strains is very similar; the minor differences observed do not appear to be significant. For the sake of brevity, we will therefore confine our remarks to the morphology and structure of the Beluga strain (NCIB 4248).

The appearance of a spore lacking an exosporium, as seen in a shadow-cast preparation, is shown in Fig. 1. Radiating from the well-defined spore are numerous stalklike appendages which are slightly swollen at the ends. A carbon-replica preparation (Fig. 2) demonstrates that the appendages are randomly distributed over the entire surface of the spore. Such a preparation also suggests that the coat of the spore is not perfectly smooth but has surface irregularities which may be the result of a folding of the outer layer. Additional detail of the structure of the appendages is brought out in a negatively stained spore (Fig. 3). This electron micrograph suggests that the appendages are variable in length and have a characteristic substructure. The fine structure of an appendage, as seen in negatively stained preparations, is represented diagrammatically in Fig. 4 and illustrated by the micrograph in Fig. 5. The appendages are tubular in structure, and, although of varying length (up to

Fig. 1. Trypsin-treated spore, gold-palladium shadow. The appendages are seen radiating from the surface of the electron-dense spore. At the distal end of each appendage is an enlargement (the cap). All the electron micrographs are of preparations of the Beluga strain, NCIB 4248. Figures 1 to 4 are electron micrographs of spores lacking an exosporium. The fragile exosporia of these specimens have been lost during the cleaning of these spore suspensions.

Fig. 2. Trypsin-treated spore; carbon replica, gold-palladium shadow. The appendages project from the surface of the spore.
0.56 μ in the Beluga strain), they are of quite uniform diameter. The overall diameter is of the order of 200 A, and the diameter of the lumen is 80 A. The wall of the tubule is 60 A thick and appears to be composed of spherical subunits of that order of diameter. The distal end of each tubule appears to be solid or to have a plug of material closing it. The lumen terminates about 400 A from the distal end of the appendage, and the tip of each tubule is surmounted by a hemispherical cap. The cap is not a continuous membrane but is composed of several spherical subunits each approximately 40 A in diameter. Between the subunits in the cap and the distal end of the tubular appendage is a gap of the order of 40 A which is filled with a material that takes up the negative stain.

The presence of a discernable exosporium was variable and could not be related to any particular spore preparation technique. It was usual to find spores with and spores without an exosporium on the same microscope grid. In many cases the exosporium was obviously disintegrating, and in others only residual fragments of the exosporium remained. Figure 6 illustrates the delicate nature of the exosporium in which the spore and its appendages are enclosed. Additional detail on the structure of the exosporium is presented in Fig. 7. The exosporium is in the process of disintegration, and the micrograph demonstrates that it is composed of macromolecular fibrils which are essentially parallel to each other. This electron micrograph also shows some of the minute debris that characteristically clings to the exosporium.

**DISCUSSION**

As far as we are aware, no description of the gross morphology of the spore of *C. botulinum* type E has yet been published. Stewart (18) made an electron microscopic study of spores of *C. botulinum* type A. She reported that spores of this organism possess an exosporium but that the spore itself does not contain any unusual structures attached to its surface. Takagi, Kawata, and Yamamoto (19) examined ultrathin sections of spores of a strain of *C. botulinum* type E during the sporulation process, but did not demonstrate the presence of an exosporium or any unusual structures attached to the spore. They did, however, demonstrate a pronounced intermediate space between the inner and outer spore coats.

Krassil’nikov et al. (11) were the first to describe clostridial spores possessing a variety of structural

![Figure 3](http://jb.asm.org/)

**Fig. 3.** Trypsin-treated spore, ammonium molybdate preparation. The tubular nature of the appendages is apparent.

![Figure 4](http://jb.asm.org/)

**Fig. 4.** Diagrammatic representation of the fine structure of an appendage as revealed by the negative-stain procedure. C = cap; L = lumen.
FIG. 5. Appendages of a trypsin-treated spore, ammonium molybdate preparation. Fine detail of the tubular appendages is revealed by the negative stain. The distal end of each tubule is surmounted by a hemispherical cap composed of several spherical subunits.

FIG. 6. Spore and exosporium from a mixed enzyme preparation, gold-palladium shadow. The delicate nature of the exosporium is shown by the narrow shadow and the fact that the detached spore appendages are clearly visible within it.

FIG. 7. Exosporium from a mixed enzyme preparation, gold-palladium shadow. The fibrillar structure of the exosporium is particularly clear in the portion that is fragmenting. The intact portion demonstrates that the microfibrils are parallel to each other (arrow).
appendages or protrusions. They considered that the spore morphology of the 20 soil strains which they examined was constant enough to be of taxonomic value. Our results with the spores of four strains of *C. botulinum* type E would at first appear to support this suggestion. However, our investigations have not yet covered enough strains to confirm entirely this viewpoint.

It is well established that the spores of certain *Bacillus* spp. have a characteristic surface structure (1, 6). A parallel is to be found in the spore morphology of *Streptomyces* spp. (21). A cautionary note should be made of the fact that the spore morphology of the *Streptomyces* may vary with the composition of the culture medium and the period of incubation (8). Similar factors may equally apply to the morphology of spores of the genera *Bacillus* and *Clostridium*, although this aspect has not yet been adequately examined.

Of the many morphological spore types described by Krassil'nikov et al. (11), the morphology of the spores of the four strains of *C. botulinum* type E is most similar to their strain 80, *Clostridium sporosporum* nov. sp. Unfortunately, their electron micrographs do not permit us to make a detailed comparison between the spores of their strain 80 and those of *C. botulinum* type E. Our electron micrographs demonstrate a degree of detailed structure in the tubular appendages as well as for the delicate exosporium.

A variety of spores, members of the genera *Bacillus* and *Clostridium*, have been shown to possess an exosporium. The most detailed study so far presented is that of Gerhardt and Ribi (7) on the exosporia of *B. cereus* and *B. anthracis*. They demonstrated that the exosporium of *B. cereus* consists of two main layers. The outer layer has a nap of hairlike projections and is about 250 A thick. The inner basal layer is highly organized, with a perforate hexagonal surface pattern of holes on 76-A centers and consisting of four lamellae. The lamellae of the inner basal membrane can be fragmented into crystal-like fragments. The entire basal membrane has a thickness of 190 A. Between the two layers, and essentially a part of the outer layer, is an intermediate covering which adds another 60 A to the overall thickness of the exosporium (500 A). The exosporium of spores of *B. anthracis* is somewhat thicker (720 A), but the extra thickness is largely due to its deeper hairlike nap. Although we have not as yet conducted a detailed study of the exosporium of the spores of *C. botulinum* type E, our electron micrographs do clearly indicate that its ultrastructure is quite different from that described for *B. cereus*. It would appear that this exosporium consists of a single layer of subunits arranged in filamentous threads lying side by side. The overall thickness is of the order of 60 A.

The morphological description that we have presented does not provide a satisfactory clue as to the function of the tubular appendages of this unusual spore. One is tempted to speculate that they may serve either as attachment processes or as chemo-sensory organs with which might be advantageous to the spore in the germination process. In the vegetative cells of certain gram-negative bacteria, pil or fimbiae are considered to act as attachment processes (3, 4, 5, 10). However, fimbiae do not possess the elaborate structure of the tubular appendages (20). The diameter of the tubular appendages is of the same order of magnitude as the diameter of the ubiquitous cytotubules or microtubules of the cytoplasm of plant and animal cells. These cytoplasmatic microtubules were noted by Manton (13, 14) and first described in detail by Ledbetter and Porter (12). The precise function of these cytoplasmatic microtubules is still a matter for conjecture. Speculation upon the function of the appendages of the spore can be substantiated or disproved only by knowledge gained from additional study of these structures.

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**Literature Cited**


