Appendages of *Clostridium bifermentans* Spores

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Four distinct spore appendage types were detected in an electron microscope survey of 12 strains of *Clostridium bifermentans*. A smooth tubular appendage and a feather-like appendage are described in detail. In addition, hirsute tubular appendages and small pin-like appendages are depicted. Spores of four strains apparently lack appendages.

The subject of bacterial spore appendages promises to be an active field of investigation for some time to come. Following the pioneering survey reports of Krasil'nikov, Duda, and Sokolov (6-8), in which a variety of spore appendage types were described, several additional studies have appeared in the literature. Multiple tubular appendages have been described for spores of *Clostridium botulinum* (3), fine filaments emerging in tufts from coats of *Bacillus circulans* have been reported (1), and a brief study of ultrastructural features of several spore appendage types by the Russian workers (5) has appeared. More recently, Rode, Crawford, and Williams (11) described in considerable detail the ribbon-like appendages of *Clostridium* sp. N1 (*C. taeniosporum* nov. sp., V. I. Duda, personal communication). We have surveyed a number of strains of *C. bifermentans*, and thus far have detected four distinct appendage types. This report describes two of these, a smooth tubular appendage and a feather-like appendage, and notes the appearance of the two additional types.

**Materials and Methods**

*Organisms.* The cultures of *C. bifermentans* used in this study were obtained from the following sources: Strains 1A-SDH, 2A-SDH, 3A-SDH, 4A-SDH, and 9-SDH were obtained from Mary Jane Ashby, Division of Laboratories, Texas State Department of Health, and were classified by her as *C. bifermentans* according to the morphological and biochemical criteria set forth by the National Communicable Disease Center, Atlanta, Ga. Strain 431 was obtained from L. S. McClung, Division of Biological Science, Indiana University, Bloomington. Strain 4-W was obtained from E. Staten Wynne, School of Aerospace Medicine, Brooks Air Force Base, Texas. Strains FDA-1, U-11, U-15, U-27, and U-49 were sediment isolates obtained from Stanley M. Harmon, Division of Microbiology, Food and Drug Administration, Washington, D.C., and conformed biochemically to the description contained in *Bergey's Manual of Determinative Bacteriology*, but had not been otherwise studied in detail.

*Spore production.* Spores were produced on Brain Heart Infusion (Difco) medium supplemented with 0.3 g per liter of sodium thioglycolate and 2% agar (Difco). Surface-inoculated petri plates were incubated at 30 C in desiccator over wet oats for an anaerobic environment. Sporangia were usually formed within 3 to 4 days and free spores in 5 to 7 days.

*Preparation of specimens for electron microscopy.* Carbon replicas of thoroughly washed cells were prepared by conventional means (12).

Negative stain specimens were prepared with 2% phosphotungstic acid (PTA; pH 6.8), with 2% ammonium molybdate (pH 8.0), and with 0.5 to 1% uranyl acetate (pH 3.5). Specimens were taken directly from growth plates.

For sections, specimens were fixed 1 hr at 4 C in 0.5% gluteraldehyde (13) in Kellenberger's Veronal-acetate buffer at pH 6.1. After five rinses in buffer, the specimens were subjected to a routine Kellenberger osmium fixation at 4 C for 17 hr (4). In other cases, fixation was with 2% potassium permanganate, 30 min at room temperature (9). After fixation, specimens were dehydrated in graded alcohol series, followed by two changes of acetone. The cells were then embedded in a plastic mixture consisting of 70% decenyl succinic anhydride, 20% Araldite 6005, and 10% Epon 812 with 1 drop of accelerator DMP-30 (Rohm & Haas Co., Philadelphia, Pa.) added per ml of plastic used. Sections were cut on a Sorvall Porter-Blum MT-2 microtome with a diamond knife. All sections were stained with Reynolds' lead citrate (10).

*Electron microscopy.* Most specimens were viewed with a Hitachi HS-7S electron microscope with double condenser and 50 kv accelerating voltage. In other cases, an RCA EMU-3G electron microscope equipped with a double condenser, an objective aperture of approximately 30 , and an accelerating voltage of 100 kv was used. Initial magnifications ranged from 8,500 to 58,000. Micrographs were taken on Kodak projector slides, contrast grade, or on DuPont Cronar Ortho S Litho film.
RESULTS

Smooth tubular appendages of strain 9-SDH. Typically, the appendages appear flattened in both replica preparations (Fig. 1-4) and in negative stain preparations (Fig. 5 and 8) of free spores. Most commonly, two or three appendages are found at one end of the spore only (Fig. 1, 2, and 5), but they are occasionally detected projecting from both ends (Fig. 3). Visible appendage length in replicas is usually

FIG. 1-4. Strain 9-SDH, replicas.

FIG. 1. Typical free spore. The irregular surface of the spore body (B), the folded and collapsed exosporium (E), and three appendages (A) which project well beyond the exosporium margin are apparent. × 22,000.

FIG. 2. Free spore with flattened, intact exosporium enclosing two appendages which originate at the spore body and extend through an opening in the exosporium. × 22,000.

FIG. 3. Germinated spore with appendages present at both ends. The spore body has lost its rigidity (note absence of prominent shadow). × 22,000.

FIG. 4. Enlargement of typical appendage. The overall appearance is that of a flattened tube. × 66,000.
about 3 \( \mu \) (Fig. 1), and the width of tubes in the flattened state is approximately 600 A (Fig. 8, negative stain). The appearance of both replicas and negative stain preparations (Fig. 1-3 and 5) suggest that appendages originate at the spore body.

The exosporium is prominent and visible at both ends of the spore body (Fig. 1-3 and 5). Appendages are visible through the thin exosporium in both replicas (Fig. 2) and negative stain preparations (Fig. 5). The exosporium has an ultrastructure consisting of hexagonal repeating units (Fig. 6) and appears similar to those described for \( B. \) cereus (2) and \( B. \) circulans (1). The margins of exosporium fragments are often angular (Fig. 7), as described also for exosporia of \( B. \) cereus (2).

Sections through sporangia have provided little insight into the disposition of the tubular appendages within such cells. This failure may be due to the thinness of the tube walls (suggested in negative stains, e.g., Fig. 8) and the presence of background cytoplasm. The exosporium, however, is readily detected in such sections, and

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**Fig. 5.** Typical free spore. Note the dense spore body, the surrounding lucid exosporium, and two tubular appendages which emerge from the exosporium region. \( \times 17,600 \).

**Fig. 6.** Detail of exosporium with its hexagonal ultrastructural repeating pattern. \( \times 200,000 \).

**Fig. 7.** Exosporium fragment from which detail in Fig. 6 was taken. Note the angular margin. \( \times 100,000 \).

**Fig. 8.** Tubular appendages whose dimensions contrast sharply with flagella present also. \( \times 55,000 \).
an “opening” in the exosporium has been visualized (Fig. 9).

Sections of free spores have been more informative. Appendages in cross section, located between the spore coat and the exosporium, are present in Fig. 10. These tubes have a diameter of 350 to 400 A, a dimension which agrees well with an appendage width of 600 A in negative stain preparations of flattened appendages (Fig. 8).

The origin of appendages at the spore coat surface and extending through an opening in the exosporium is shown in Fig. 11. This exosporium opening is presumed to be identical with the exosporium opening visible in Fig. 9.

** Feather-like appendages of strain 1A-SDH.**

Designation of the spore appendages of this strain as feather-like is based on the superficial appearance of an individual appendage (Fig. 16).

These feather-like appendages originate at the spore body, most likely in the spore coat, and attachment of bundles of appendages to the spore is through common trunks at the spore ends (Fig. 12). Frequently, two such attachment trunks are present at a single spore end. Free coats may retain attached appendages (Fig. 13), and the attachment trunk appears continuous with a prominent band usually seen on the spore surface and present also on the free coat (Fig. 13). The appendages appear flaccid in replica preparations, and each consists of a central shaft and a surrounding material (Fig. 12 and see below).

A typical spore may have 12 or more appendages attached to each end (Fig. 14). These project from the spore body and penetrate the enveloping exosporium. The overall length of such a spore cell including appendages may be as great as 14 μ.

Each appendage has a central shaft (Fig. 15 and 16) which is 150 to 225 A in diameter and tapers somewhat toward the tip. Innumerable fine filaments, regularly spaced, radiate outward angularly from the entire shaft surface. These filaments have a beaded appearance (Fig. 16), are approximately 600 A in length, and are 20 to 30 A in diameter. The overall width of an appendage, central shaft plus surrounding filaments, is 1,100 to 1,200 A (Fig. 16).

Appendages are closely associated within sporangia as bundles (Fig. 17, 19, and 20). Presumably each appendage bundle is attached to the spore coat surface (Fig. 22) via a common trunk (Fig. 12), and from this origin passes through a natural opening(s) in the exosporium (Fig. 17 and 22) and coils about within the sporangial vegetative cytoplasm often in close association with the vegetative cytoplasmic membrane (Fig. 17-20).

A cross section of a bundle of appendages within a sporangium (Fig. 21) demonstrates that the appendage shaft is rod-like and possesses internal ultrastructure which may be tubular in nature (Fig. 21). Satisfactory resolution of the shaft ultrastructural features has not, however, been obtained. In sections of sporangia each appendage shaft is surrounded by an electron-transparent zone (Fig. 21) which is identical with the appendage filament region seen in negative stains (Fig. 15 and 16).

**Survey of 12 strains.** Table I summarizes the results of a survey wherein 12 strains of *C. bifermantans* were examined for the presence of spore appendages through the use of replicas and negative stain preparations. Appendages were not detected on the spores of four strains. One strain possessed the smooth tubular appendages and four strains possessed the feather-like appendages already described (Fig. 1-22).

In addition, one strain (FDA-1) possessed hairlike tubular appendages (Fig. 23), and two strains (U-11 and U-49) possessed pin-like appendages (Fig. 24). These latter two appendage types are being studied.

**Discussion.**

Despite the numerous electron microscope studies of many varieties of *Bacillus* and *Clostridium* spores, the existence of spore appendages was apparently not suspected until the recent Russian reports (6-8). It seems likely, therefore, that many spore types may lack appendages. On the other hand, spore appendages are not uncommon, particularly, it now appears, among clostridia (3, 5, 6-8, 11). Since not all clostridial spores possess appendages (this report and unpublished data), it seems that possession of appendages is not an indispensable property of the spores of these anaerobes.

Evidence in this study and elsewhere (11) suggests that spore appendages thus far examined may be mere, albeit elaborate, outgrowths of the outermost spore integument structures, presumably the spore coats. The proteinaceous coats of spores are assumed to be indispensable for the maintenance of spore properties, although no definitive experiments on this point have been performed. Clear evidence may be somewhat difficult to obtain; the dispensability or indispensability of spore appendages, on the other hand, should be readily amenable to investigation. For example, the ribbon-like appendages of *C. taeiniosporum* nov. sp. are readily removed with no apparent effect on spore structural integrity (11).
FIG. 9-11. Strain 9-SDH, sections, lead citrate poststain. Figure 9, glutaraldehyde-asmium fixation; Fig. 10-11, potassium permanganate fixation.

Fig. 9. Sporangia. Identifiable features include the vegetative cell wall (VW), the cytoplasmic membrane (CM), the spore exosporium (E), an opening (O) in the exosporium, spore coats (C), spore cortex (CX), the core wall (CW), and the spore core (SC). X 69,000.

Fig. 10. Free spore. Six tubular appendages cut in cross section are enclosed within the exosporium (E) and a longitudinally cut appendage fragment is visible outside the exosporium (appendages identified by unlabeled arrows). X 56,000.

Fig. 11. Free spore. Appendage (arrows), originating at spore coat, penetrates the exosporium through an opening. X 44,000.
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Fig. 12-13. Strain IA-SDH, replicas of free spores.

Fig. 12. Long, flaccid appendages, each surrounded by a layer of material (arrow), are attached to the spores by a common trunk (T). X 27,000.

Fig. 13. Appendages remain attached to free spore coat (C). The attachment trunk (T) appears continuous with a band (B) present on the spore coats. X 24,000.

The diversity of spore appendage types among clostridia is remarkable (3, 5, 6-8, 11). Perhaps, even more striking is the diversity detected within the single species, C. bifermantans. No less than five distinct spore types, including one type which apparently lacks appendages, have been observed (Table 1). In view of the limited number of strains examined, there appears no reason to assume that other types may not exist. If spore appendages of C. bifermantans are functional in some capacity, all serving an identical purpose, the requirement for a fixed structural configuration appears lacking. On the other hand, it is difficult to visualize a unique specific function for each distinct structural type.

The presence of spore appendages among Clostridium may be expected to have an impact on classification schema. Species distinctions are often based on relatively minor biochemical
FIG. 14-16. Strain 1A-SDH, negative stains with uranyl acetate, pH 3.5.

FIG. 14. Typical free spore with dense spore body, lucid exosporium, and numerous appendages extending from both ends of the spore body. × 11,500.

FIG. 15. Group of appendages extending from one end of a single spore. Each appendage consists of a central shaft (S) from which innumerable filaments (F) of uniform length project. × 135,000.

FIG. 16. Enlargement of the uppermost appendage in Fig. 15. The beaded appearance of the unstained filaments is apparent. × 233,000.
**Fig. 17**. Strain JA-SDH, sporangia, sections, glutaraldehyde-osmium fixation, lead citrate poststain. 

**Fig. 17.** The longitudinally cut spore, although mature, is still enclosed in the sporangial vegetative cytoplasm. Two bundles of appendages cut in cross section (A) lie external to the exosporium (E), and two appendages cut longitudinally (unlabeled arrow) extend through an opening in the exosporium at which site the exosporium margin (M) is characteristically coiled. × 81,000.

**Fig. 18.** Cross section. Appendages (A), one of which is labeled, are spaced between the exosporium and the vegetative cell cytoplasmic membrane. × 83,000.

**Fig. 19.** Cross section to illustrate two bundles of appendages in the sporangium at a site distal to the spore body. × 83,000.

**Fig. 20.** Oblique section through sporangium showing disposition of appendages (cut longitudinally) in sporangia vegetative cytoplasm. × 81,000.
FIG. 21–22. Strain IA-SDH, sections, glutaraldehyde-osmium fixation, lead citrate poststain.

FIG. 21. Sporangium. A bundle of eight appendages cut in cross section lies near the sporangial cytoplasmic membrane (CM) and cell wall (CW). Each appendage consists of a central shaft (S) and a surrounding transparent zone of filaments (F), not visible here, which corresponds to the filamentous region shown in detail in Fig. 15 and 16. X 284,000.

FIG. 22. Free spore. Section through the spore coat and exosporium. The feather-like appendages originate at the spore coat (C) and pass through an opening in the exosporium (E) with its characteristically coiled margin. X 62,000.


FIG. 24. Strain U-11, pin-like appendage, replica. X 68,000.
Table 1. Spore appendage types of 12 strains of Clostridium bifermentans

<table>
<thead>
<tr>
<th>Strains</th>
<th>Appendage appearance*</th>
<th>Electron micrographs</th>
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<tbody>
<tr>
<td>9-SDH</td>
<td>Smooth tubular</td>
<td>Fig. 1–11</td>
</tr>
<tr>
<td>1A-SDH</td>
<td>Feather-like</td>
<td>Fig. 12–22</td>
</tr>
<tr>
<td>2A-SDH</td>
<td>Feather-like</td>
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<td>3A-SDH</td>
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<tr>
<td>4A-SDH</td>
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<td></td>
</tr>
<tr>
<td>431</td>
<td>None detected</td>
<td></td>
</tr>
<tr>
<td>4-W</td>
<td>None detected</td>
<td></td>
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<tr>
<td>FDA-1</td>
<td>Hirsute tubular</td>
<td>Fig. 23</td>
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<tr>
<td>U-11</td>
<td>Pin-like</td>
<td>Fig. 24</td>
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<tr>
<td>U-15</td>
<td>None detected</td>
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<tr>
<td>U-27</td>
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<tr>
<td>U-49</td>
<td>Pin-like</td>
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* Appendage designations are based on the gross appearance of either or both replicas and negative stain preparations and are only for purposes of identification.

differences. It will be difficult to reconcile this with the spore morphological diversity described here. Although we have no reason to believe that the strains examined here are not all legitimate C. bifermentans by present criteria, a critical electron microscope appraisal of large numbers of diverse, rigorously classified strains of C. bifermentans seems indicated. In addition, it is not inconceivable that a situation similar to that described here may exist also for species other than C. bifermentans.

No evidence is at hand to indicate whether cultural conditions have any influence on the presence, absence, or types of appendages formed by spores of various strains of C. bifermentans. It is worthy of note, however, that the strains examined were all grown on the same medium under similar conditions.

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