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Taxonomic Implications of Spore Fine Structure in *Clostridium bifermentans*

L. J. RODE AND LOUIS DS. SMITH

Department of Microbiology, University of Texas, Austin, Texas 78712, and Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg, Virginia 24060.

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Thirty-five strains of *Clostridium bifermentans* were, in most part, culturally homogeneous by conventional taxonomic criteria but were heterogeneous with respect to spore fine structure. Fourteen of the strains produced spores with appendages, distributed among four distinct ultrastructural types. No consistent correlation existed between spore type and other variable properties of these strains. It is proposed, therefore, that these spore appendage-type strains be considered as "varieties" of *C. bifermentans* and that they should not be designated as new species.

Gross spore features which are readily detectable with the light microscope have historically played an important and proper role in speciation within the *Bacillus* and *Clostridium* genera. Distinctive attributes such as spore shape, spore size, and location in the sporangium are considered relevant in taxonomy of the *Bacillaceae* (*Bergey's Manual*, 7th ed.)

Spore morphological diversity which extends beyond such general gross features is now recognized, though not generally utilized, for taxonomic purposes. The elegant, early electron microscope study of Franklin and Bradley (1) using carbon replicas documented such diversity for some *Bacillus* species, and additional detail has been provided through use of the freeze-etch technique (3).

Additional, even more pronounced, spore morphological diversity became evident with the report by Krasil'nikov, Duda, and Sokolov (5) of elaborate protrusions (appendages) on spores of *Clostridium* isolates. Two ensuing developments in this immediate area appear to have important taxonomic implications: (i) the creation of new *Clostridium* species with spore ultrastructure (i.e., spore appendage status), the apparent sole or primary criterion for speciation (6) and (ii) the discovery that multiple spore appendage types occur within presently established species such as *C. botulinum* (2) and *C. bifermentans* (2, 8, 14). Thus, what role, if any, should properly be assigned to spore ultrastructure features, such as the possession of appendages, in the taxonomy of sporeforming bacteria?

Since at least five distinctive spore morphological types were already known for *C. bifermentans* (8, 14), we have, in the present study, chosen this species of known diversity for more intensive study. Thirty-five strains from diverse sources, including those for which electron microscope studies had already been performed (2, 8, 14), have been characterized by both conventional taxonomic methods and by electron microscope appraisal of spore fine structure. Our objective has been to provide experimental rationale for proper use of the details of spore ultrastructure in the taxonomy of this species.

MATERIALS AND METHODS

C. bifermentans strains. Thirty-five strains of *C. bifermentans* were studied. Strain designations, strain identifications, and strain sources are listed in Table 1.

Electron microscope appraisal of spores. The organisms were grown to sporulation at 30 C on the surface of 2% agar plates of Brain Heart Infusion (Difco) medium, supplemented with 0.5 g per liter of sodium thioglycolate, in desiccators with wet oats to provide an anaerobic environment (10).

For carbon replicas, free spores from growth plates were washed six times with demineralized water, placed on mica squares, shadowed with platinum, and coated with carbon. After flotation onto water and dissolution of the cellular matter with 0.5% sodium hypochlorite, the replica films were washed and transferred to copper grids for examination.

For thin sections, specimens were used directly from growth plates. Preliminary fixation was for 1 hr at 4 C in 0.5% glutaraldehyde (11) and was followed by routine Kellenberger osmium fixation at 4 C for 17 hr (4). The specimens were embedded in a plastic mixture of dodecenyl succinic anhydride, Araldite 6005, and Epon 812. Sections, cut on a Porter-Blum MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn.) fitted with a diamond knife, were stained for 5 to 60 sec with Reynolds lead citrate (19).

An Hitachi HS-7S electron microscope with double

TABLE 1. *Clostridium bifermentans strain designations, identification, and source*

Strain no.[a]	Other or prior strain designation[b]	Source
2012	ATCC 638	Sterility test of tuberculin
2035	CDC 258	Human infection
2036	CDC 352	Human infection
2450		Canned tomatoes
2658	McClung collection 134	
2659	McClung collection 135	
2708		Lagoon mud
2825A		Isolated from Beerens collection 544
2825-1		Isolated from Beerens collection 544
2829		Dust on hardware parts
2895	ATCC 638	Sterility test of tuberculin
3036B		Marine sediment
3131		Marine sediment
4131A		Stomach ulcer of goat
4406	SDH 9[c]	
4407	SDH 1A[c]	
4408	SDH 2A[c]	
4409	SDH 3A[c]	
4410	SDH 4A[c]	
4411	FDA 1[c]	Sediment
4412	McClung collection 431[c]	
4413A	FDA U-11[c]	Sediment
4414	FDA U-15[c]	Sediment
4415	FDA U-27[c]	Sediment
4416	FDA U-49[c]	Sediment
4438		Human infection
4669A	TRS 288B[d]	Vacuum-packed smoked fish
4670	TRS 244B[d]	Vacuum-packed smoked fish
4671	TRS 247B[d]	Vacuum-packed smoked fish
4672A	NCIB 6800[d]	
4673	NCIB 506[d]	
4674	NCIB 1341[d]	
4675A	NCIB 2929[d]	
4676A	NCIB 6928[d]	
4677A	TRS 275B[d]	Vacuum-packed smoked fish

[a] Accession numbers of the Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg, Va., which identify the *C. bifermentans* strains for the present work.

[b] Absence of designation indicates that other or prior designation does not exist or is not known. ATCC, American Type Culture Collection; CDC, Center for Disease Control; SDH, Texas State Department of Health; FDA, Food and Drug Administration; TRS, Torry Research Station; NCIB, National Collection of Industrial Bacteria.

[c] Strain designations used in two prior spore appendage studies (8, 14).

[d] The spore appendage status of these strains has been investigated (2).

condenser and 50-kv accelerating voltage was used for specimen examination. Micrographs were taken on Kodak contrast process Ortho film. Spore dimensions were determined by measurements performed on prints of suitable magnification.

Conventional taxonomic appraisal of strains. With a few minor exceptions, the media and methods used for studying these strains were those of Moore, Cato, and Holdeman (7), and Smith and Holdeman (13).

RESULTS

Conventional taxonomic appraisal: constant features. The 35 strains were identical in the following respects: All strains were gram-positive rods with central to subterminal spores (light microscopy), liquefied gelatin, digested casein in milk, digested cooked-meat medium with the production of ammonia, formed indole, fermented glucose, and produced lecithinase on egg yolk agar. No strain fermented sorbose, galactose, xylose, arabinose, rhamnose, sucrose, lactose, trehalose, cellobiose, raffinose, dextrin, starch, glycogen, inulin, cellulose, erythritol, adonitol, mannitol, dulcitol, salicin, esculin, amygdalin, or inositol. No strain reduced nitrate, produced acetylmethylcarbinol, produced urease or lipase, formed pigment, or produced toxin detectable in mice. The diaminopimelic acid in the vegetative cell wall was of the DL form. Fermentation products from a peptone, yeast extract glucose medium included acetic, propionic, isobutyric, isovaleric, and isocaproic acids, with or without small amounts of butyric or heptanoic acids.

Conventional taxonomic appraisal; variable features. Variability among the 35 strains was observed with respect to the following: fermentation of fructose, mannose, ribose, maltose, glycerol, and sorbitol; the production of H_2S; motility; cell wall sugars. These data are shown in Table 2.

Electron microscopy; constant spore features. The spores of all 35 strains possessed an exosporium which was detected both in carbon replicas of free spores (Fig. 1–5) and in sections of sporangia (Fig. 6–10). The spores were located centrally to subterminally in sporangia (Fig. 6–10) and were oval to elongated (Fig. 1–10). In no case was a terminal spore observed. Measurements of spore size carried out on electron micrographs indicated that spore size, both length and width, was variable over a fairly narrow range; however, size variability between strains did not exceed size variability of spore populations within strains.

Electron microscopy; variable spore features. Significant ultrastructural variability among spores of different strains was limited to the spore appendage aspect (Fig. 1–5; Table 2). On this basis, five spore morphological types were

TABLE. 2. *Variable properties of thirty-five strains of Clostridium bifermentans*

Strain no.	Type of spore appendage	Fermentation of								Vegetative cell wall sugars
		Fructose	Mannose	Ribose	Maltose	Glycerol	Sorbitol	H_2S production	Motility	
4413A	Pinlike (Fig. 2)[a]	+	−	−	−	−	−	+	+	Glucose
4416	Pinlike	−	+	−	−	−	−	+	+	
4406	Smooth, tubular (Fig. 3)[b]	−	+	−	−	−	−	+	+	Glucose
2450	Smooth, tubular	−	+	−	+	+	−	+	+	
2658	Smooth, tubular	+	+	−	−	−	−	+	+	
4411	Hirsute, tubular (Fig. 4)[a]	−	−	−	−	−	+	+	+	Glucose, rhamnose, mannose
4669A	Hirsute, tubular	−	−	−	−	+	+	+	+	
4670	Hirsute, tubular	−	+	−	−	+	+	+	+	
4671	Hirsute, tubular	−	−	−	+	+	−	+	+	
4677A	Hirsute, tubular	−	−	−	−	+	+	+	+	
4407	Featherlike (Fig. 5)[b]	−	−	−	−	−	−	+	−	Glucose, rhamnose, mannose
4408	Featherlike	−	−	−	−	−	−	+	−	
4409	Featherlike	−	−	−	−	+	+	+	−	
4410	Featherlike	−	−	−	−	−	−	+	+	
4412	None (Fig. 1)	−	−	−	−	−	−	+	+	
2012	None	+	−	−	−	−	−	−	+	
2035	None	+	−	+	−	−	+	−	+	
2036	None	+	−	−	−	−	−	−	+	
2659	None	+	+	−	−	−	−	−	+	
2708	None	−	+	−	−	−	−	−	+	
2825A	None	+	+	−	−	−	−	+	+	
2825-1	None	+	+	−	−	−	+	−	+	
2829	None	−	+	−	−	−	+	+	+	Glucose, rhamnose, galactose
2895	None	+	−	−	−	−	−	−	+	
3036B	None	+	−	−	−	−	+	−	+	
3131	None	+	−	−	−	−	−	−	+	
4131A	None	−	+	+	−	−	−	+	+	
4414	None	−	+	−	−	−	−	+	+	
4415	None	−	−	−	−	−	−	+	−	
4438	None	+	+	−	−	−	−	+	+	Glucose, rhamnose, mannose
4672A	None	+	+	−	−	−	−	+	+	
4673	None	+	+	+	−	+	+	+	+	
4674	None	−	−	−	−	−	+	+	+	
4675A	None	+	+	−	−	+	+	+	+	
4676A	None	+	+	−	−	+	−	+	+	

[a] For ultrastructure characterization see Yolton et al. (14).
[b] For ultrastructure characterization see Pope et al. (8).

observed: (i) spores lacking appendages (Fig. 1; 21 strains); (ii) spores with pinlike appendages (Fig. 2; 2 strains); (iii) spores with smooth tubular appendages (Fig. 3; 3 strains); (iv) spores with hirsute tubular appendages (Fig. 4; 5 strains); (v) spores with featherlike appendages (Fig. 5; 4 strains). These five spore types, which correspond in ultrastructure and in descriptive terminology to the five spore types described in previous studies of 12 strains of *C. bifermentans* (8, 14), are distinctive and readily distinguishable. Experience to date indicates that spore appendage status is a constant strain property. Multiple appendage types within single strains have not been observed. No "new" spore appendage types were detected in the present study.

Lack of correlation between conventional taxonomic variables and spore appendage status. Those features which were found to be variable for the 35 strains are summarized in Table 2. No corre-

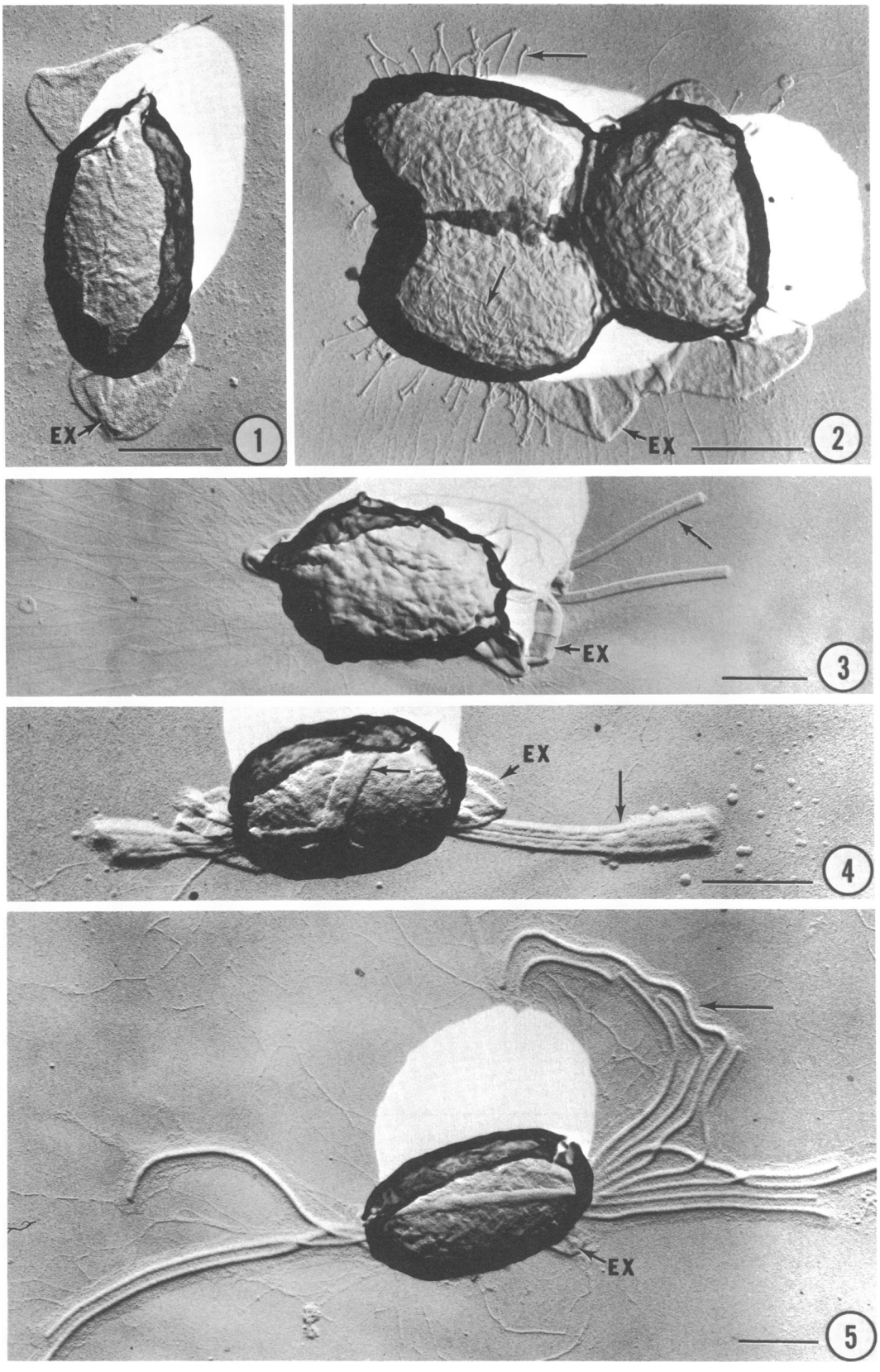

FIG. 1–5. *Replicas of the free spores of five strains of C. bifermentans showing exosporium (EX) and appendages (unlabeled arrows). Fig. 1, strain 4412, spore lacks appendages; Fig. 2, strain 4413A, spore has pinlike appendages; Fig. 3, strain 4406, spore has smooth tubular appendages; Fig. 4, strain 4411, spore has hirsute tubular appendages; Fig. 5, strain 4407, spore has featherlike appendages. Bars indicate 0.5 μm.*

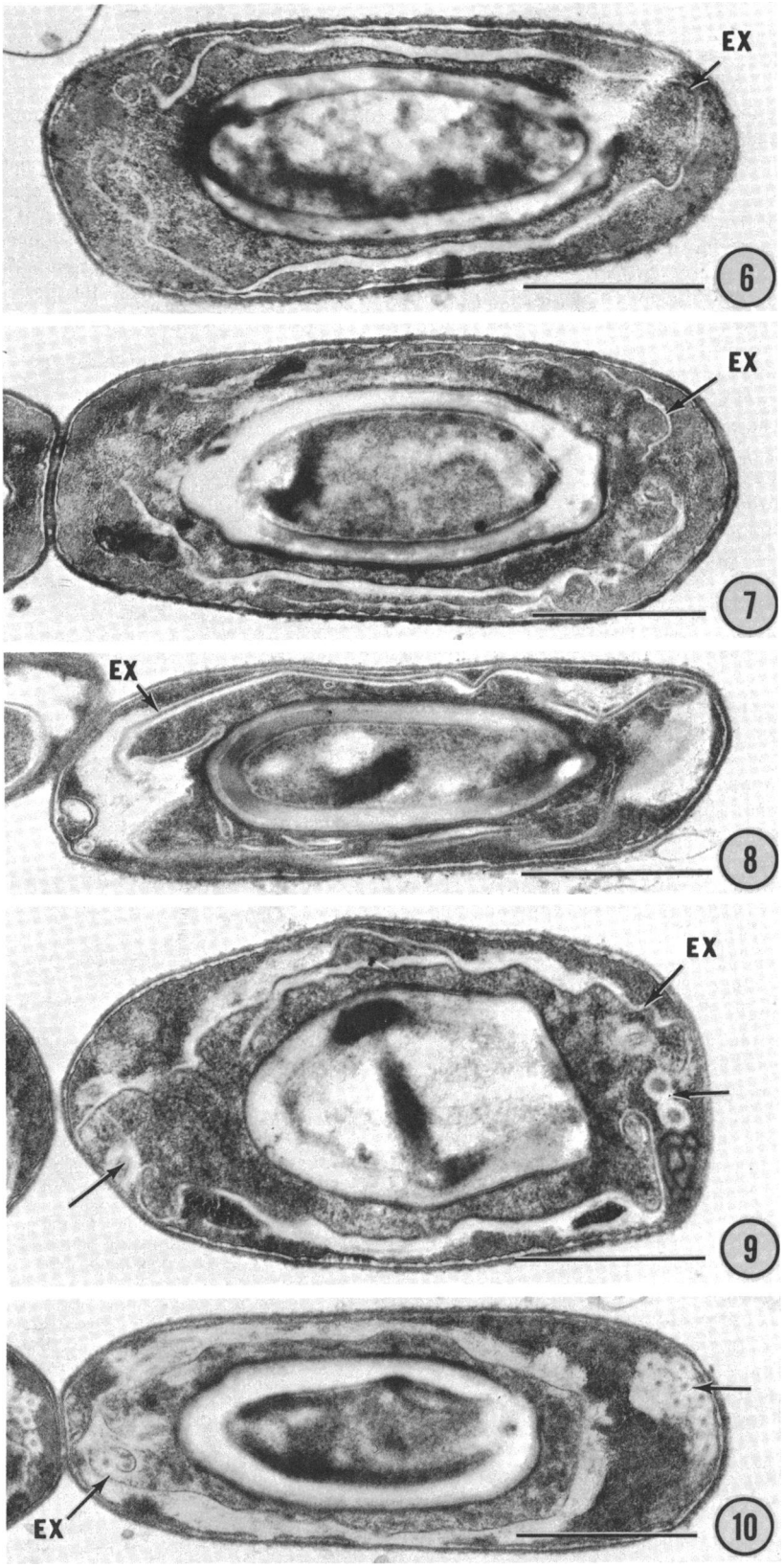

FIG. 6–10. *Sections of sporangia of five strains of C. bifermentans which illustrate spore size, shape, location, the presence of an exosporium (EX), and sectioned appendages (unlabeled arrows, Fig. 9, 10). Fig. 6, strain 4412; Fig. 7, strain 4413A; Fig. 8, strain 4406; Fig. 9, strain 4411; Fig. 10, strain 4407. Bars indicate 0.5 μm.*

lation seems apparent between spore appendage status and other strain properties.

DISCUSSION

The 35 *Clostridium* strains, appraised by conventional taxonomic methods, exhibited marked homogeneity; variable properties were few and did not serve to exclude any of the strains from the *C. bifermentans* species. This homogeneity extended to the gross spore features of the 35 strains: uniformity of spore size, spore shape, exosporium status, and location of spores within sporangia. These features are consistent with current characterization of the species (*Bergey's Manual*, 7th ed.).

On the ultrastructural level, marked spore differences were encountered among the strains. These differences were limited, however, to the spore appendage aspect; 21 strains lacked appendages, whereas 14 strains possessed appendages (Table 2), and these were distributed among four ultrastructurally distinct appendage types (Table 2, Fig. 1–5).

Two extreme approaches to the taxonomic problem posed by the spore appendage variability come to mind. The first of these is to ignore spore appendage status in the taxonomy of *C. bifermentans* and possibly of other clostridia. A second approach, used by the investigators at Moscow State University (6), is to create new *Clostridium* species with spore appendage status as the basis for speciation. This approach makes spore fine structure paramount in speciation, ignores all other natural areas of relatedness, and risks the possibility that very similar or identical spore appendage types may be found in several otherwise distinct species.

A more workable approach at this time is to accommodate this new knowledge of spore fine structure within the framework of existing taxonomic schemes. This finds support in our observation that variation in cultural characteristics does not correlate with spore appendage morphology. Although spore appendage morphology is highly reliable as a strain characteristic, we see nothing to be gained by an attempt to subdivide a species as generally accepted and as uniform, on the whole, as *C. bifermentans*.

Inherent in this approach is a designation of the several spore appendage types as "varieties" of *C. bifermentans*. This can be done by the application of appropriate nomenclature. For the present, we prefer to retain the simple descriptive terminology we have used in this report (Table 2, Fig. 1–5) and in earlier reports (8 and 14).

Since the conclusion of our experiments, an additional spore appendage type of *C. bifermentans* has been described by Samsonoff, Hashimoto, and Conti (12). Although additional diversity is not unanticipated, the basic problem of taxonomy remains the same.

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