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Intestinal Spirochetosis: Morphological Characterization and Cultivation of the Spirochete *Brachyspira aalborgi* gen. nov., sp. nov.

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The ultrastructure of spirochetes obtained from rectal biopsies of patients with intestinal spirochetosis was studied by means of negative staining and ultrathin sectioning. The cells were sigmoidal with tapered ends, 2 to 6 μm long, with a wavelength of 2 μm. Four flagella were inserted at each end of the cells. The maximal cell width was about 0.2 μm. The spirochetes were cultured on tryptose soy blood agar plates. They were anaerobic and grew, although very slowly, at 37 to 38.5°C in an atmosphere of 5% CO_2–95% H_2. Two types of colonies could be distinguished. The growth characteristics and the morphology of the isolated spirochetes differ from those of previously isolated spirochetal strains. Consequently, it is proposed that the present strains constitute a new genus, *Brachyspira*, of the family *Treponemataceae*. The type species is *Brachyspira aalborgi*, the type strain of which is 513A (NCTC 11492).

Since the 19th century, spirochetes have been known to be present in the human intestinal tract (3, 4). The interest in intestinal spirochetosis has, however, shown great variations, and the pathological and clinical significance of this condition has been debated and is still considered uncertain (1, 5–7, 9, 15, 16, 21). A massive infestation of spirochetes of the colonic epithelium may induce diarrhea (2, 5), but some investigators have been unable to relate any symptoms to the presence of intestinal spirochetes (6, 15). During the last few years, we have investigated the prevalence of intestinal spirochetosis among patients requiring sigmoidoscopy who were hospitalized in a gastroenterological department (clinical aspects to be published elsewhere). In the present paper, we report on the morphology of the spirochetes studied by electron microscopy of sections of rectal biopsies and by negative staining of the organisms obtained by elution from another biopsy of the same patient. Our attempts to cultivate the spirochetes are also described, as are some of the biological characteristics of the strains we obtained.

MATERIALS AND METHODS

Electron microscopy. (i) Negative staining. Biopsies were obtained from five patients in whom intestinal spirochetosis had been confirmed by electron microscopy of sectioned material. The biopsies were placed in phosphate-buffered saline (pH 7.2) and sent air freight from Aalborg to the Copenhagen laboratory, where the liquid was pipetted off and centrifuged at 20,000 × *g* for 20 min. The resultant pellet was suspended to a suitable density in a few drops of SMC (0.03% sucrose in redistilled water with 0.01 M $MgCl_2$ and 0.01 M $CaCl_2$ added). Specimen grids were prepared by the multiple drop technique (11). Some cells were treated on the grids with 0.2% Teepol in redistilled water for 10 min. All specimens were negatively stained with 1% ammonium molybdate.

Cultivated spirochetes were washed off the plates with 1 to 2 ml of SMC and gentle scraping with a glass triangle. Specimen grids were prepared from the resulting suspension as described above.

(ii) Sectioned material. Sigmoidal rectal biopsies were prefixed for 4 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in alcohol and propylene oxide, and embedded in Epon 812. Some biopsies were fixed with ruthenium red added to the fixatives (17).

The ultrathin sections were stained with uranyl acetate and lead citrate.

Electron microscopy was carried out with a JEOL 100 B electron microscope at primary magnifications of ×1,000, ×6,600, and ×33,300 or with a Philips EM 200 electron microscope at primary magnifications of ×9,000 and ×16,000. Prints were obtained after photographic enlargement as desired. Approximately 400 micrographs were studied for the present paper.

Cultivation. (i) Media. Prereduced plates of tryptose soy agar to which was added 10% calf blood, and 400 μg of spectinomycin and 5 μg of polymyxin per ml were used for isolation of the spirochetes. The same

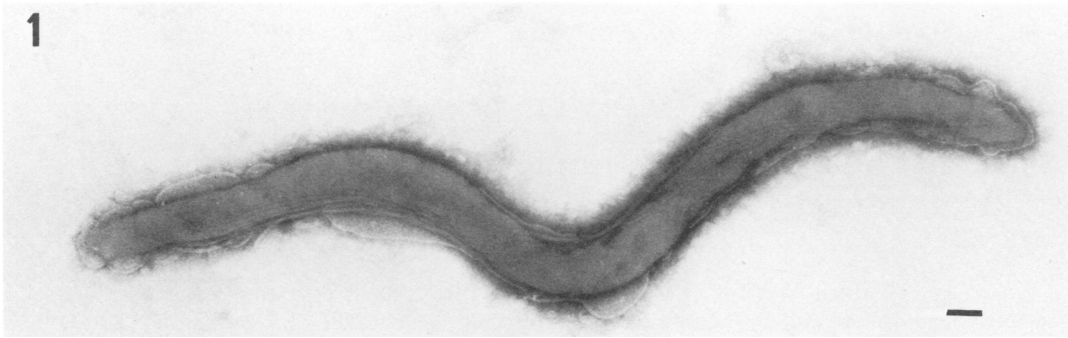
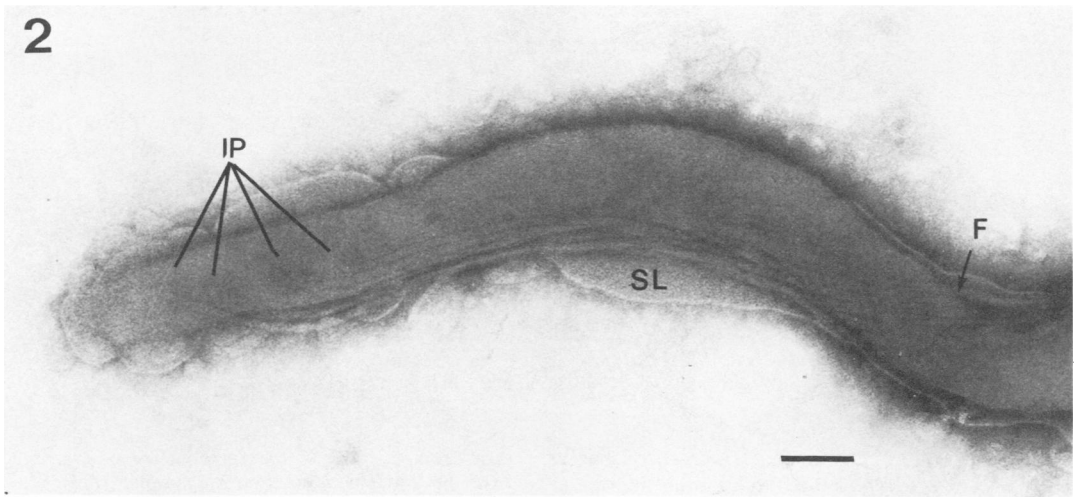


FIG. 1. A regularly waved spirochete with tapered ends. Obtained directly from colon biopsy; negatively stained with 1% ammonium molybdate. ×45,000. Bar, 100 nm.



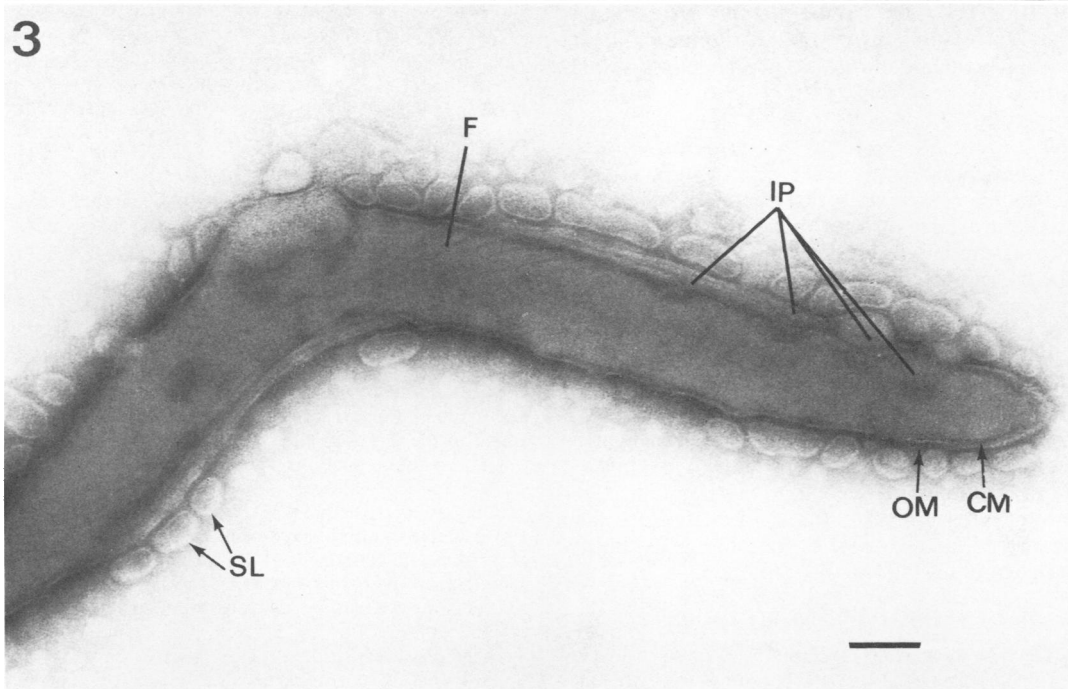
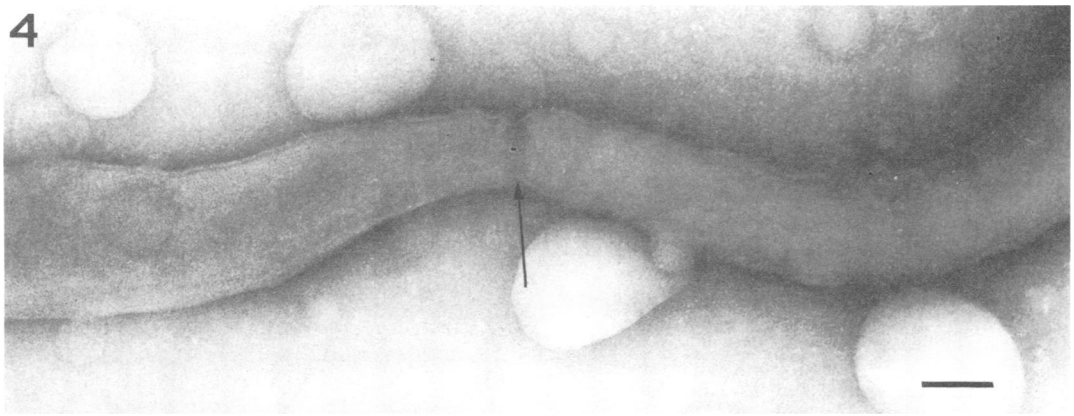


FIG. 2 and 3. Tapered ends of the cells. The cells are covered by a surface layer (SL). The insertion points (IP) of the four flagella (F) are well resolved. Outer membrane and cytoplasmic membrane of the cells are marked OM and CM, respectively. ×90,000. Obtained directly from colon biopsy; negatively stained with 1% ammonium molybdate. Bar, 100 nm.



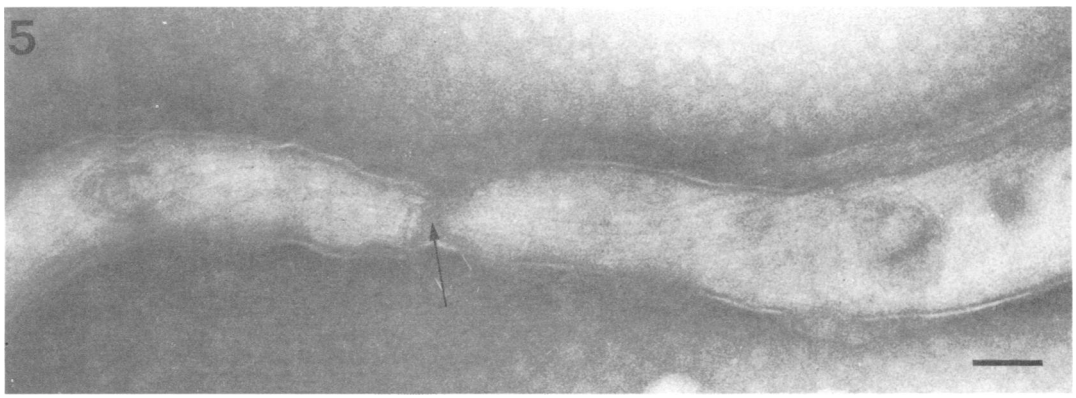


FIG. 4 and 5. Dividing cells. Note the truncated ends at the division site (Fig. 4 arrow) and that the cells are still enclosed in the mutual outer membrane. Flagella can be seen to pass the division site (Fig. 5 arrow). ×90,000. Bar, 100 nm. (4) Obtained directly from colon biopsy; negatively stained with 1% ammonium molybdate. (5) Obtained from plate culture; negatively stained with 1% ammonium molybdate.

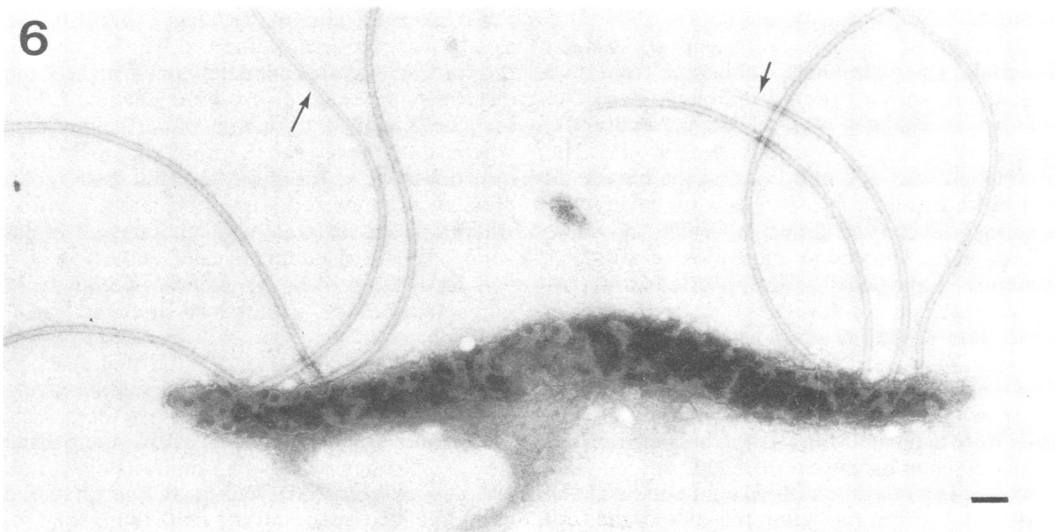


FIG. 6. Cell on which the outer cell wall was removed by Teepol treatment. The four flagella at each end are still attached to the cell body. Note that the distal part of the flagella are unsheathed (arrows). ×45,000. Bar, 100 nm. Obtained directly from colon biopsy; negatively stained with 1% ammonium molybdate.

medium without spectinomycin and polymyxin was used for maintenance of the isolates, as well as for determination of optimum temperature and composition of atmosphere for growth of the cells.

(ii) Isolation technique. A few drops of the buffer in which the biopsies were sent was streaked onto each agar plate. The cultures were incubated anaerobically at 38.5°C in an atmosphere of 95% H_2–5% CO_2 for at least 2 weeks.

Isolation was attempted with biopsies from six patients and was accomplished in all cases.

(iii) Enzymatic reactions. The enzymatic reactions of the cultivated spirochetes were tested with the API ZYM system (API System S.A., La Balme les Grottes, France), a commercially available semiquantitative micromethod designed to allow a rapid, systematic study of 19 enzymatic reactions. A dense suspension of organisms was made in 5 ml of sterile deionized water. The optical density was estimated with a spectrophotometer and adjusted to give an extinction of about 0.30 at 550 nm (1-cm lightpath). Two drops of the standardized suspension were placed in each cupule of the API ZYM test strip and incubated at 37°C for 4 h. The reagents for detection of the reaction products were added, and the colors were allowed to develop for 20 min. The test strips were then read by comparing the color intensities obtained with those shown on a color chart supplied with the kit; a value of 0 to 5 was estimated according to the intensity of the color.

RESULTS

Morphology. (i) Light microscopy. Live spirochetes were examined by light microscopy with the dark-field technique. The organisms were short, thin, and flexible. Some cells were comma-shaped, whereas others were helical with one to two complete turns. Some of the spirochetes showed a serpentine swimming type of motility, whereas others were attached to the slide with one end, around which they rapidly gyrated.

(ii) Electron microscopy. Negatively stained material. The spirochetes obtained from five patients all showed the same appearance. The cells were regularly waved, had tapered ends, and were 1.7 to 6.0 μm long (Fig. 1). The wavelength was about 2 μm, and the maximum cell width was 0.2 μm. All cells were covered by a surface layer (Fig. 2 and 3), which, on some micrographs, showed an indication of a regular structure. Four flagella were inserted subterminally at each end of the cells (Fig. 2 and 3), from which they wound around the cytoplasmic bodies, with overlap in the mid region of the cells. The cells divided by binary fission of their cytoplasmic membranes (Fig. 4 and 5). The new ends were truncated (Fig. 4) and became tapered as the division process proceeded.

Treatment of cells with Teepol removed the surface layer and the outer cell membrane, but generally the four flagella at each end were still attached to the cell body (Fig. 6). Some flagella were, however, completely detached from the cells by the action of Teepol. The individual flagellum consisted of a shaft which was covered by a sheath, and the insertion part possessed a hook and a basal complex (Fig. 7 and 8). The sheath was frequently found to be shorter than the core, so that approximately one-fourth of the distal part of the core was left unsheathed (Fig. 6). The sheathed flagellum was 18 nm wide, whereas the core was 12 nm (Fig. 6–8). The insertion part of the flagellum, the basal complex, was connected to the shaft by a hook, which was approximately 40 nm long and 14 nm wide. The basal complex itself consisted of a pair of disks in close opposition (diameter, 33 nm) connected to the hook by a short rod (Fig. 7 and 8).

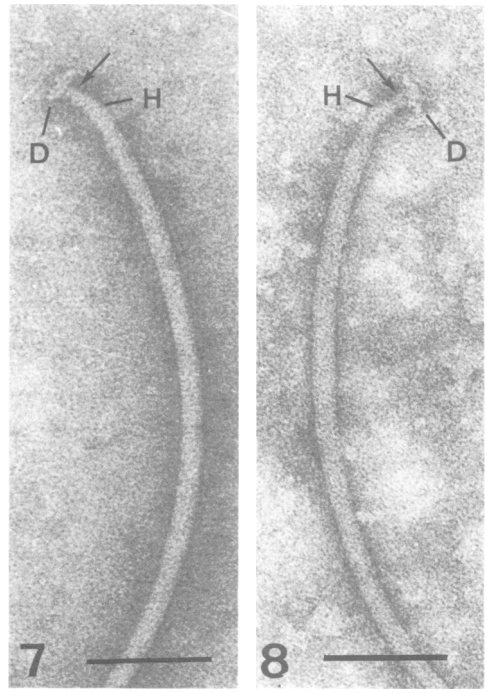


FIG. 7 and 8. Flagella released by treatment with Teepol. Note that the basal complex consists of a pair of disks (D), connected to the hook (H) by a thin rod (arrow). ×160,000. Bar, 100 nm. Obtained directly from colon biopsy; negatively stained with 1% ammonium molybdate.

Sectioned material. Despite the fact that the luminal border of the epithelium was massively infested with spirochetes, the cells of the colonic epithelium appeared remarkably unaffected (Fig. 9). No sign of inflammation was observed, although an occasional leukocyte was observed in intercellular spaces of the epithelium (Fig. 9). Over large regions, spirochetes could be seen to be situated between and parallel to the microvilli

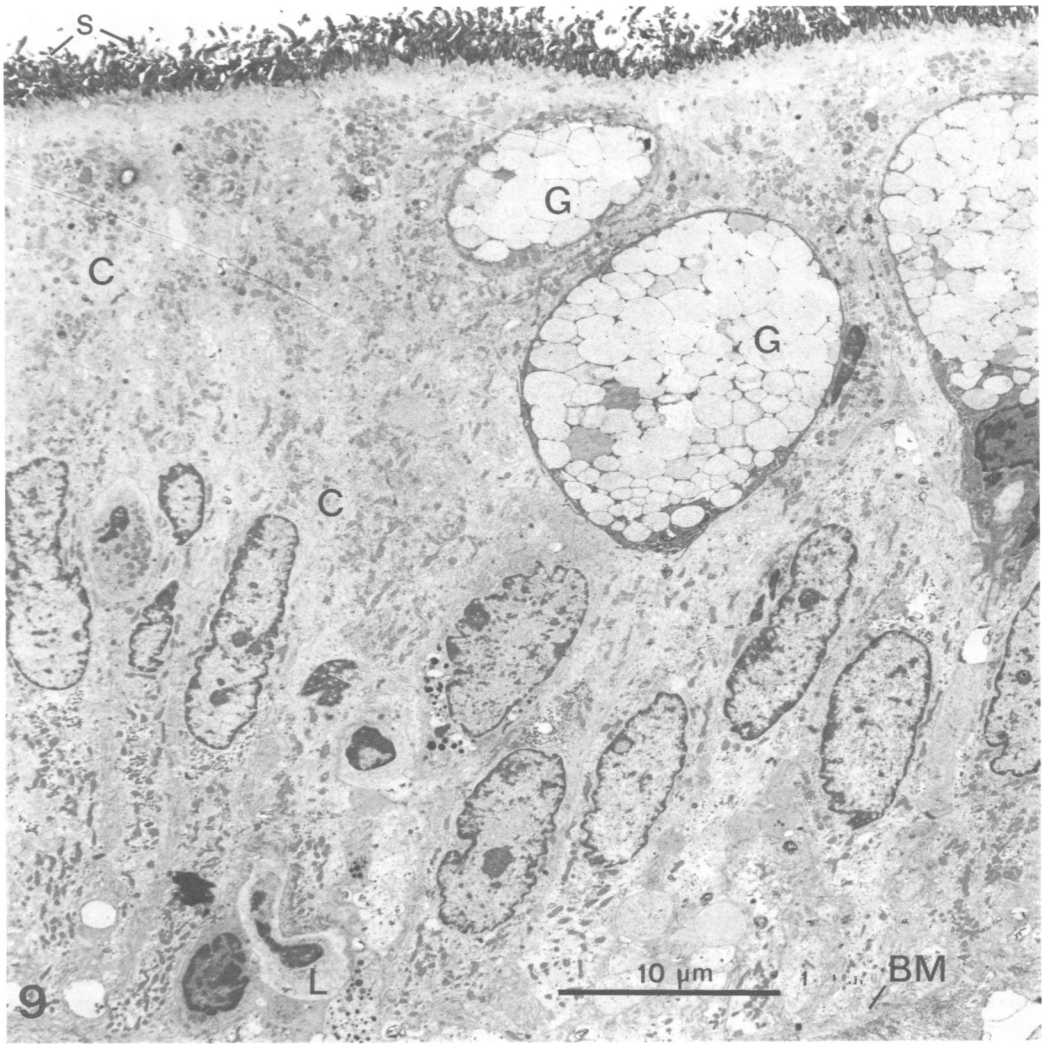


FIG. 9. Part of a section showing the colonic epithelium massively infested with spirochetes (S). The epithelial cells appear remarkably unaffected. An occasional leukocyte (L) is present between the columnar cells (C). BM, Basal membrane; G, goblet cells. ×2,940. Obtained directly from colon biopsy; ruthenium red added to fixative.

of the luminal cells (Fig. 9 and 10). No ruptures of the epithelial cell membranes were observed, and no spirochetes were present intracellularly in the epithelium. The spirochetes were always attached end on to the luminal cell membrane (Fig. 11–13). Microvilli adjacent to spirochetes seemed to be shorter and appeared to originate from a lower position than microvilli in regions devoid of spirochetes.

A depression in the epithelial cell surface was observed at the attachment site of the spirochete, and a small electron lucent pit was usually present between the tip of the organism and the cell membrane (Fig. 11). A zone of electron-dense material was visible just beneath the cell membrane at the point of contact (Fig. 11). This zone was not seen in tissue fixed with ruthenium red added to the fixative. In these preparations, however, the interspace between the spirochete and the epithelial cell membrane appeared to be filled with an electron-dense precipitate (Fig. 12).

The spirochetes were enveloped by two unit membranes, both of which were asymmetric, with the outer leaflets slightly wider and more electron dense than the inner leaflets (Fig. 11–14). These asymmetric contours probably reflect the presence of the surface layer and the pepti-

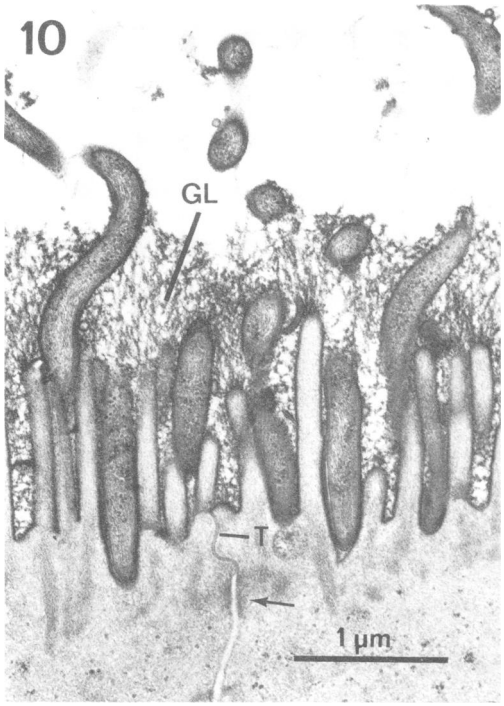


FIG. 10. Spirochetes attached end on to the luminal cells. A tight junction (T) and a desmosome (arrow) are seen. GL, Glycocalyx. ×20,000. Sectioned material; obtained directly from colon biopsy; ruthenium red added to fixative.

doglycan layer on the exterior of the outer membrane and the cytoplasmic membrane, respectively. The asymmetric densities were especially pronounced on organisms which were fixed with ruthenium red added to the fixative (Fig. 12 and 14).

Transversally as well as longitudinally sectioned spirochetes presented ribosomes and nuclear regions in the interior of the cells and flagella in the periplasmic space (Fig. 11–14). In transverse sections of the middle parts of the spirochetes, the flagella of the two bundles were seen to interdigitate (Fig. 13 and 14). Ribosomes were usually found to be scanty at the outermost tapering part of the ends of the organisms (Fig. 11 and 12). On rare occasions, nuclear strands were present at the tips of a spirochete.

Cultivation. After 2 weeks of incubation, a thin haze could be seen at the site of inoculation on the agar plates with oblique illumination and by use of a hand lens. The organisms were routinely transferred to fresh prereduced media every 2 weeks, but they survived if they were transferred only every 4 weeks.

The spirochetes grew at 38.5 and 37°C in an atmosphere of 5% CO_2–95% H_2 or 20% CO_2–80% H_2. Optimal growth was obtained at 38.5°C in an atmosphere of 5% CO_2–95% H_2. No growth was observed at 30 or 42°C or in an atmosphere of 50% CO_2–50% H_2 or 100% H_2.

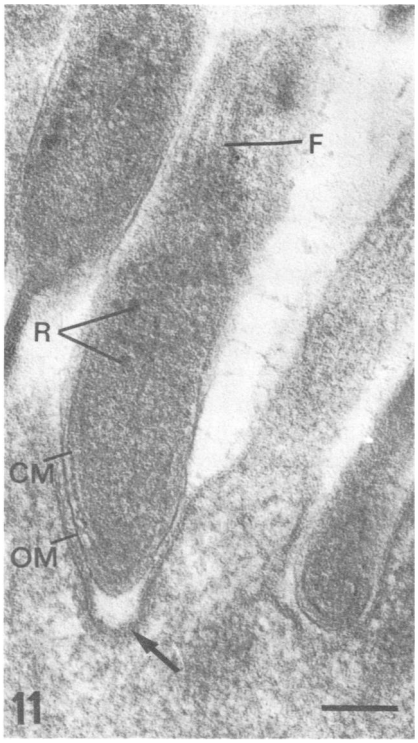
Growth did not occur at any of the combinations of temperature and atmosphere used when tested in Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy broth supplemented with 10% rabbit serum inoculated with small agar blocks showing confluent growth of spirochetes.

Stock cultures could be maintained at −80°C by freezing agar blocks with confluent growth in phosphate-buffered saline (pH 7.2).

Two types of colonies were observed after three to four subcultivations. Type A consisted of clear, flat, rough-edged colonies with weak hemolytic activity (Fig. 15) and type B of clear, smooth-edged, pinpoint convex colonies which lacked hemolytic activity (Fig. 16). The two colony types were isolated and were found to be stable; i.e., no conversion between the two types was observed. These two colony types were isolated from five of the six biopsies examined, whereas only type A colonies were obtained from one biopsy.

The ultrastructure of negatively stained cells from type A colonies was identical to that of organisms eluted directly from the biopsies, as described above. The spirochetes in type B

FIG. 11–14. (11) Narrow zone of electron-dense material present below an electron-lucent pit in the region of contact between the spirochete and the epithelial cell (arrow). The spirochete is enveloped by an outer membrane (OM) and a cytoplasmic membrane (CM). R, Ribosomes; F, flagella. ×100,000. Bar, 100 nm. Sectioned material; obtained directly from colon biopsy. (12) Field of view similar to that of (11). Ruthenium red-positive material (arrow) is present in the interspace between the spirochetes and the epithelial cell membrane (E). The outer membranes (OM) and cytoplasmic membranes (CM) of the spirochetes are clearly asymmetric in appearance. The cell to the left is atypical in that it presents ribosomes (R) at the outermost tip of the cell. N, Nuclear material. ×100,000. Bar, 100 nm. Sectioned material; obtained directly from colon biopsy; ruthenium red added to fixative. (13) and (14). These fields of view show parts of sections in which the spirochetes were cross-sectioned. The triple-layered outer membranes (OM) and cytoplasmic membranes (CM) of the organisms are clearly visible. The flagella (F) are present in the periplasmic space between these two membranes. Note that the flagella interdigitate where the two bundles of flagella overlap, i.e., where more than four flagella can be seen. The peptidoglycan layer (P) is prominent in (14). Ribosomes (R) and nuclear material (N) are present in the cell interior. ×100,000. Bar, 100 nm. Obtained directly from colon biopsy. Ruthenium red added to fixative for (14).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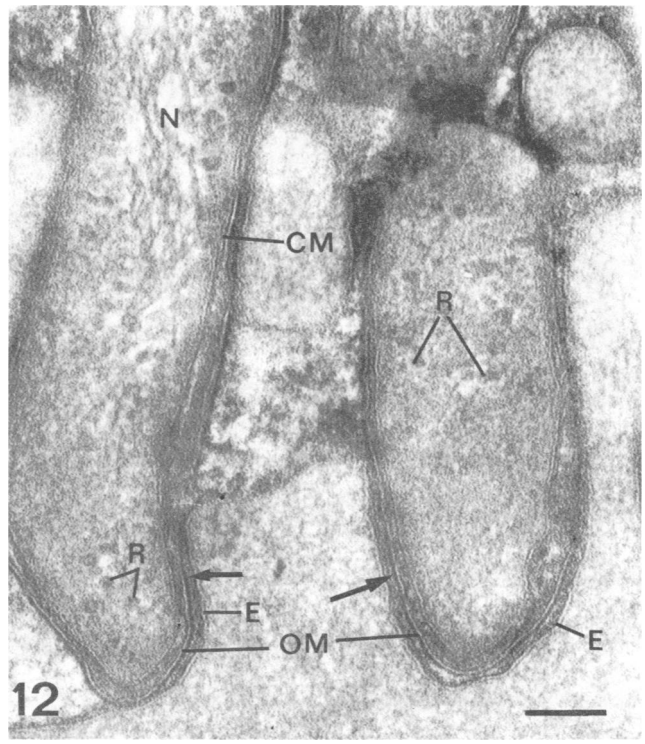
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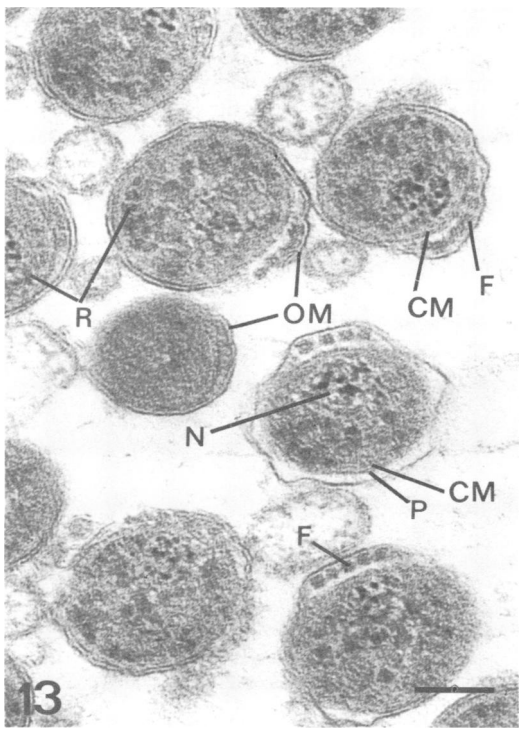
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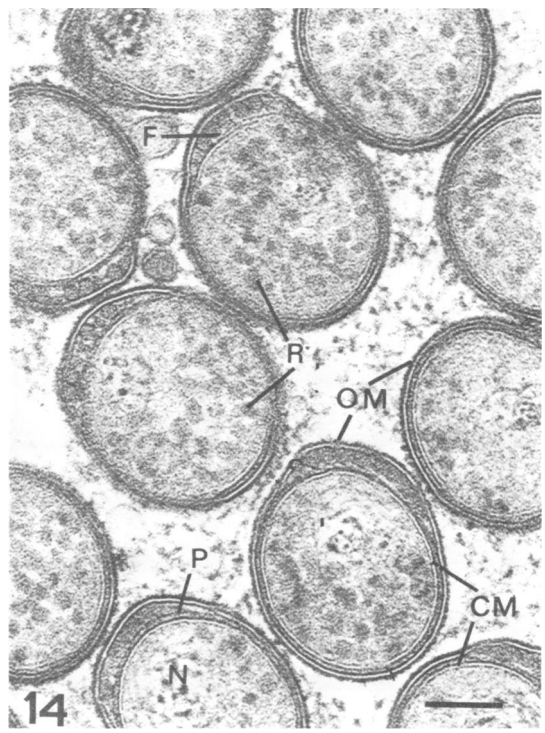
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colonies appeared somewhat different in that they possessed a looser outer envelope and showed a segmentation of the cytoplasm of the cells (Fig. 17). The two types of cells could not be distinguished with respect to morphology or motility by dark-field microscopy.

Enzymatic reactions. The enzymatic reactions of the cells of the two colony types were identical (Table 1).

DISCUSSION

The ultrastructure of the spirochetes described here, which infest the human colonic epithelium, is similar to the description previously given for the spirochetes observed in sections of intestinal epithelium from humans (1, 5, 15, 18). Their ultrastructure seems to be rather similar to that of some of the previously cultivated spirochetes from human stool (13, 19), but differs from others, which were longer and wider, had a larger wavelength, and possessed five flagella at each cell end (T. Lambert, F. Peter, G. Gourfort, P. Bourlioux, and F. Goldstein, Abstr. Int. Symp. Recent Advances in Enteric Infections, Brügge, 1981). Also, with respect to the biological characteristics, our spirochetes are different from the spirochetes isolated by Kaplan and Takeuchi (13) and Lambert (Abstr. Int. Symp. Recent Advances in Enteric Infections, Brügge, 1981), especially in that they grow more slowly. Furthermore, our isolates differ from those isolated by Lambert in that they do not possess α-galactosidase.

Werner (21) reported on the presence of two morphologically different spirochetes in human stool: one was 3.5 to 6.1 μm long, with a wavelength of 1.3 μm, and the other was 4.6 to 7.3 μm long, with a wavelength of about 3 μm (calculated from his illustrations). He proposed the names *Spirochaeta stenogyrata* and *Spirochaeta eurygyrata* for these spirochetes, and until 1922 these names were used by various authors to designate different helical and coiled microorganisms observed in the human intestinal tract.

A review of intestinal spirochetes was published by Hogue (9) in 1922, and it is obvious from this that the names *S. stenogyrata* and *S. eurygyrata* were used for a variety of helically shaped microorganisms observed in human stool, including the aerobic helical microorganisms she cultivated from stool (9).

The present state of taxonomy of spirochetes is imperfect. In practice, anaerobic, saprophytic, and pathogenic spirochetes are classified as *Borrelia* if they are known to be transmitted by vectors, whereas those that do not fulfill this requirement are classified as *Treponema*. Thus, the genus *Treponema* consists of a very inhomogeneous group of spirochetes. Furthermore, because anaerobic spirochetes are difficult to cultivate under conditions generally available in the laboratory, very little is known of their biological characteristics.

The spirochetes we have isolated and cultivated do not seem to belong to any of the presently described species of *Treponema*. Our organisms are, for instance, definitely shorter than any previously described treponemes. Furthermore, they do not possess cytoplasmic tubules, which is regarded as a morphological criterion for treponemes (10). The majority of anaerobic spirochetes do not grow on tryptose soy-blood agar, but those that do so form visible colonies within 48 to 72 h, in contrast to the 2 weeks needed for our material to form barely visible colonies. Except for the spirochete *Treponema hyodysenteriae*, which causes swine dysentery (8, 20), very little is known about the other intestinal spirochetes, and to the best of our knowledge no comparative studies with these

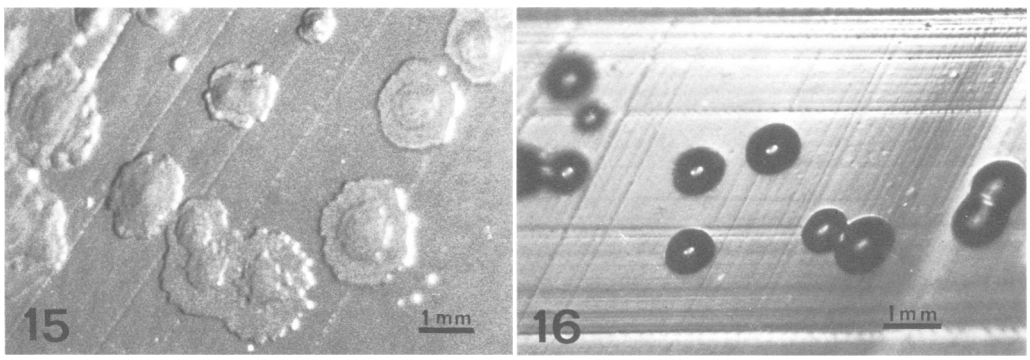

FIG. 15 and 16. (15) Type A colonies: effuse, transparent colonies with lobated edges and prominent growth zones. ×70. Plate culture. (16) Type B colonies: convex, shiny, transparent colonies with smooth edges. ×70. Plate culture.

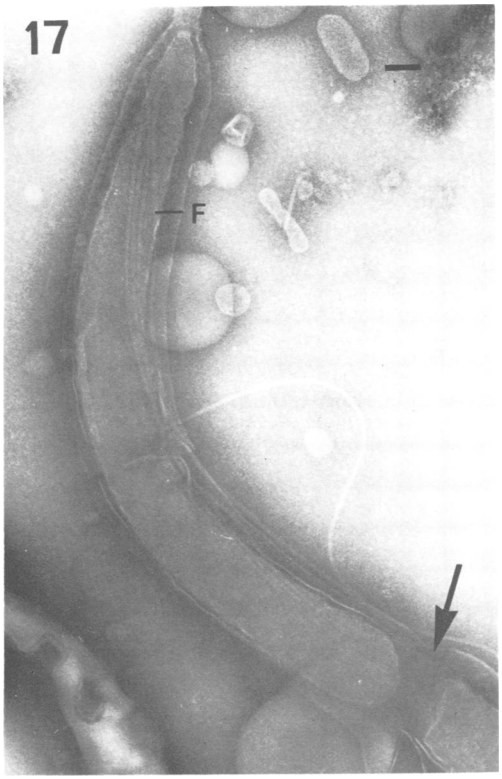

FIG. 17. Cell obtained from a culture of type B colonies. The cell has a looser outer envelop than that found on cells from biopsies and type A colonies (cf. Fig. 2–5). Most of the cells present a segmented appearance (arrow). F, Flagella. ×45,000. Bar, 100 nm. Negatively stained with 1% ammonium molybdate.

have yet been performed. The spirochetes we have isolated clearly differ morphologically from those of *T. hyodysenteriae*, as well as from those of a related species, *Treponema innocens* (14), by being shorter and thinner and by having only 4 flagella inserted at each end, whereas cells from the latter two organisms have 13 flagella at each end. Also, the wavelengths and amplitudes of the cells of our isolates differ from those of the spirochetes isolated from swine (K. Hovind-Hougen, unpublished data). Finally, there is a difference in the enzymatic reactions of our spirochetes and those related to swine dysentery, as revealed by the API ZYM system. Spirochetes related to swine dysentery always produce α-glucosidase and β-glucosidase (12). Consequently, the spirochetes we have isolated and cultivated must constitute a new genus of the family *Treponemataceae*, and for this genus we propose the name *Brachyspira*.

We thought of using the species name *eurygyrata* or *stenogyrata* (21) for our spirochetes,

TABLE 1. Presence of enzymes in cells of *Brachyspira* and spirochetes associated with swine dysentery tested by the API ZYM system

Enzyme	Reaction[a]	
	Brachyspira	Swine dysentery
Alkaline phosphatase	0	1–4
Esterase (C4)	0	1–3
Esterase lipase (C8)	1	1–3
Acid phosphatase	0	1–4
Phosphoamidase	1	0 (1)
α-Galactosidase	0	0
β-Galactosidase	4	3–5
β-Glucuronidase	0	0
α-Glucosidase	0	2–4
β-Glucosidase	0	3–5

[a] Values of 0 to 5 were assigned according to the intensity of color as compared with the colors on a chart provided with the API ZYM kit.

but because these names have already been used to designate other spirochetes (9) and in order not to introduce further confusion, we propose the species name *aalborgi* for the organisms of the present study.

Brachyspira gen. nov. (Bra·chy·spi·ra; Gr. adj. brachy, short; Gr. n. spira, a coil, a helix; Brachyspira, a short helix). Unicellular, motile, helicoidal, gram-negative organisms which measure 0.2 by 1.7 to 6 μm, with a wavelength of about 2 μm. The organisms have four flagella inserted at each end. They do not possess cytoplasmic tubules.

The cells are anaerobic but will tolerate normal atmosphere for at least 6 to 8 h. They can be cultivated on solid tryptose soy medium supplemented with 5% calf blood. They possess β-galactosidase and traces of esterase (lipase C8), acid phosphatase, and phosphoamidase, but are catalase and oxidase negative. Some strains show hemolytic activity. Parasitic to humans.

The type species is *Brachyspira aalborgi* (aal·bor·gi. M. L. mase. n. aalborgi, for the Danish town Aalborg, in which the biopsies were taken). The morphological, cultural, and physiological characteristics of this species are those of the genus *Brachyspira*.

The type strain of *B. aalborgi* is strain 513A, a strain whose characteristics are those given above for the species and genus. The type strain is deposited with the National Collection of Type Cultures, London, England, under the number NCTC 11492.

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