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# Anaerobic Bacteria on the Mucosal Epithelium of the Murine Large Bowel

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Anaerobic bacteria can be detected at population levels of $10^{11}$ organisms per g of cecum or colon in adult mice from four different colonies widely spaced in the United States. Most of these microorganisms are oxygen-intolerant fusiform-shaped bacteria. At least one type of these tapered, rod-shaped bacteria can be seen in layers in the epithelial mucin in frozen-section histological preparations of the large bowels of mice. In addition, such microorganisms can be seen within 0.5 $\mu$m of the epithelium in ultrathin sections of colon or cecum examined in an electron microscope. These fusiform-shaped bacteria predominate in the mucin layers. However, spiral-shaped microorganisms can be found as well near the mucosal epithelia in ultrathin sections of colon. Also, such organisms can be seen in negatively-stained preparations of washings of the colonic mucosal epithelia examined in an electron microscope. At least three types of spiral-shaped organisms, including both spiral-shaped bacteria and spirochetes, can be found in preparations from mice from three of the four colonies. Such spiral-shaped microorganisms can be detected at population levels as great as $10^9$ organisms per g of cecum or colon in anaerobic cultures of the large bowels of mice from all four colonies. One anaerobic spiral bacterium was isolated in pure culture. This particular organism was found by immunofluorescence to be intermingled with the fusiform-shaped bacteria in the mucin on the mucosal epithelium in the mouse large bowel.

Anaerobic bacteria, and in particular oxygen-intolerant anaerobes, predominate in the gastrointestinal tracts of mice (5). These anaerobic bacteria normally colonize only the cecum and colon (2, 12, 15). In frozen-section histological preparations of segments of these areas of the bowel, fusiform-shaped bacteria can be seen in layers in the mucin on the mucosal epithelium (12).

We studied these microbial layers more closely by using fluorescent-antibody techniques and the electron microscope. In this report, we report in detail our findings of spiral-shaped microorganisms that appear in the layers in addition to the fusiform-shaped bacteria.

## MATERIALS AND METHODS

**Animals.** Four- to seven-week-old specific pathogen-free (SPF) mice were purchased from the Ha/ICR colony of A. R. Schmidt Co. (Madison, Wis.), the CF-1 colony of Carworth-BioQuest (New City, N.Y.), the CD-1 colony of Charles River (Wilmington, Mass.), and the Swiss colony of Bioscience (Oakland, Calif.). The animals were housed in plastic cages with paper tops (Isocage, Carworth) with Ab-Sorb-Dri (Allied Mills, Chicago, Ill.) for bedding, and given Lab Blox (Allied Mills) and acid-water (15) ad lib. Random-bred rabbits weighing 3.0 to 3.5 kg (H. D. Bredthauer, Round Rock, Tex.) were housed in metal cages on wire grids and given Rabbit Chow (Purina, St. Louis, Mo.) and water ad lib.

**Anaerobic chamber.** Fusiform-shaped bacteria and probably most of the spiral-shaped microorganisms can be cultured only when the intestinal material or cultures containing the organisms are never exposed to oxygen or oxidized culture media (1, 5, 6, 11). All techniques, from autopsy of the animals through final incubation of the bacteriological media, must be accomplished in an oxygen-free environment. We used a chamber containing a deoxygenated nitrogen atmosphere for culturing these microorganisms (13). The chamber is similar to systems described previously (5, 6).

**Preparation of specimens for bacteriological culturing.** Animals were killed with chloroform, introduced into the chamber through an air-lock, and autopsied in the nitrogen atmosphere. Segments of the large bowel, always with contents intact, were homogenized in 5 ml of sterile deoxygenated charcoal water (13, 14). The homogenates were diluted in charcoal water in 10-fold steps. Calibrated loopfuls of each dilution were then spread on the surface of various agar media. For certain purposes, these steps from autopsy

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to culturing were also carried out in an identical manner outside of the anaerobic chamber.

**Bacteriological media.** O and D medium (8) and E agar (3) were incubated at 37 C in a nitrogen-carbon dioxide atmosphere (15) for 5 days (13). Only after such prereduction were the media introduced into the anaerobic chamber or used outside of the chamber. Media inoculated in the anaerobic chamber were incubated in an $H_2$-$CO_2$ atmosphere (Gas Pak, Bioquest, Baltimore, Md.). Media inoculated outside the chamber were incubated either in the $H_2$-$CO_2$ (Gas Pak) atmosphere or in the nitrogen-carbon dioxide atmosphere (15).

**Histological methods.** Segments of colons or whole ceca from mice were frozen with contents intact in 2% methyl cellulose in saline. Such blocks were sectioned at 4 to 8 μm with a microtome-cryostat. Sections fixed in absolute methanol were stained with hematoxylin and eosin or with a modified Gram stain (12).

**Electron microscopy.** Sectioned preparations were prepared from segments of mouse colon fixed for 1 hr at 4 C in 0.5% gluteraldehyde (10) in Kellenberger's Veronal-acetate buffer at *p*H 6.0. The fixed tissues were then washed in the buffer for 36 hr, exposed to 1% osmium tetroxide for 18 hr, and stained with 0.5% uranyl acetate. The stained segments then were dehydrated in a graded alcohol series followed by two changes of acetone and embedded in plastic mixture consisting of 70% dodecinyl succinic anhydride, 20% Araldite 6005, and 10% Epon 812 with one drop of DMP-30 (Rohm and Haas, Philadelphia, Pa.) added per ml of plastic used. Sections were cut on a Sorvall Porter-Blum MT-2 microtome with a diamond knife.

Negatively stained preparations were made from pure cultures of bacteria or from washings with the Veronal-acetate buffer of the epithelium of the mucosa of mouse colon. Such cultures or washings were centrifuged to sediment the microorganisms. The microorganisms in the pellets from the centrifuged washings or cultures were negatively stained with 2% phosphotungstic acid (*p*H 6.0) and then were sprayed onto 200-mesh grids with carbon-coated butvar films.

Both the ultrathin sections and the negatively stained preparations were examined in an Hitachi HS-7S electron microscope.

**Indirect immunofluorescence test.** A vaccine was prepared from a spiral-shaped microorganism isolated from a pooled homogenate of ceca from five Ha/ICR mice. The microorganism was grown in quantity on the surface of E agar (3), and washed from the surface of the agar in 0.033 M phosphate-buffered saline (0.075 M NaCl), *p*H 7.0. The microbial suspension was diluted to approximately $4 \times 10^9$ cells per ml of the buffered saline and exposed to 60 C for 1 hr. The heat-treated microorganisms were used as a vaccine.

Rabbits were bled from the marginal ear vein for normal sera and then given weekly intravenous injections of 0.25 ml of the vaccine containing $10^9$ of the heat-killed cells. After the third and the fourth such injections, the animals were bled for immune sera. All sera were tested for agglutinating antibody to the spiral-shaped microorganisms in suspension at about $10^9$ organisms/ml in phosphate-buffered saline. The agglutination titer in the normal sera was 1:2. In contrast, the immune sera agglutinated the microbes to a titer of 1:512. The normal and immune sera and a preparation of goat anti-rabbit immunoglobulin G labeled with fluorescein isothiocyanate (FITC goat anti-rabbit IgG; kindly supplied by W. J. Mandy) were absorbed twice with mouse liver powder (4) and once with a suspension of fusiform-shaped bacteria in phosphate-buffered saline. Just before being used in the test as described below, all of these immunoglobulin reagents were absorbed once again with mouse liver powder.

For the immunofluorescence test (4), frozen ceca or colons from Ha/ICR mice were sectioned at 4 μm on a microtome cryostat. The sections were fixed on slides in absolute methanol and stored overnight at 4 C. After the storage, the sections were flooded with either normal or immune rabbit serum and then incubated for 30 min at 37 C. After the incubation, the sections were washed well with buffered saline and flooded with the FITC goat anti-rabbit IgG. The slides were again incubated for 30 min at 37 C and washed well thereafter with buffered saline. Cover slips then were mounted on the sections with phosphate-buffered glycerin. They were then examined in a Leitz Ortholux fluorescence microscope fitted with an ultraviolet light source, 3-mm BG12 excitor filter, and K460 barrier filter.

## RESULTS

**Estimates of the populations of oxygen-sensitive anaerobic bacteria in the mouse cecum and colon.** Adult Ha/ICR, CF-1, CD-1, and Swiss mice were killed and autopsied in a deoxygenated nitrogen atmosphere. Cultures for viable counts of the oxygen-sensitive anaerobes then were made in that atmosphere on prereduced O and D medium from dilutions of homogenates of the ceca or colons of the mice (Table 1). Over $10^{11}$ oxygen-intolerant anaerobic bacteria per g of fresh tissue were estimated to reside in the ceca and colons of animals from all but the Bioscience Swiss colonies. Even in one of the animals of the latter type, over $10^{11}$ bacteria per g of whole organ were estimated to be viable in the large bowels. Similar findings have been reported for CD-1 mice (5).

**Fusiform-shaped anaerobic bacteria on the mucosal epithelium of the mouse large bowel.** Most of the oxygen-intolerant anaerobic microorganisms that colonize the large bowels of mice are fusiform-shaped bacteria of at least three genera: *Fusobacterium*, *Eubacterium*, and *Clostridium* (5). One or more types of these tapered rod-shaped microorganisms can be seen (Fig. 1) in layers on the epithelium in frozen-section histological preparations of the mouse colon (12). Similar but less striking layers can be found also

in the epithelial mucin in such preparations of mouse cecum (12). In electron microphotographs of sections of mouse colon, tapered rods can be seen within 0.5 μm of the mucosal epithelium (Fig. 2). In addition, the microorganisms are observed frequently in the mucin in close proximity to discharging goblet cells (Fig. 3a). These tapered rods were found colonizing the intestinal mucin in dense populations in the large bowels of mice from all four colonies. However, the bacteria in the layers remain unidentified at this time.

TABLE 1. *Estimates of microbial populations culturable under oxygen-free conditions from the ceca and colons of adult specific pathogen-free mice*[a]

| Mice | | Bacteria cultured | |
|---|---|---|---|
| Type[b] | No. | Cecum[c] | Colon[c] |
| Ha/ICR | 100 | 11 | 11 |
| CF-1 | 10 | 11 | ND |
| CD-1 | 15 | 11 | ND |
| Swiss | 10 | 10 | ND |

[a] Autopises and culturing procedures were carried out in an atmosphere of deoxygenated nitrogen. Homogenates of organs were diluted in 10-fold steps. Calibrated loopfuls of the dilutions were plated on prereduced O and D medium (9). The medium was incubated in a $CO_2$-$H_2$ atmosphere (Gas Pak, Bioquest) for up to 1 week at 37 C.

[b] Ha/ICR, A. R. Schmidt, Madison, Wis.; CF-1, Carworth-Bioquest, New City, N.Y.; CD-1, Charles River, Framingham, Mass.; Swiss, Bioscience, Oakland, Calif. Mice ranged in age from 4 to 7 weeks.

[c] $Log_{10}$ of the median number of bacteria cultured per g of whole cecum of colon. ND, not done.

**Spiral-shaped anaerobic bacteria on the mucosal epithelium of the mouse large bowel.** Spiral-shaped microorganisms also are known to colonize the large bowels of certain mice (5, 6). At least four types of such microbes have been found in the cecal contents of CD-1 mice (5). Some types of spiral-shaped microorganisms can be seen in the mucin near the mucosal epithelium in ultrathin sections of colon examined in the electron microscope (Fig. 3b). Sectioning of the tissues is not, however, the most advantageous method for locating the spiral-shaped organisms. These microbes are far less numerous than the tapered rods (5) and thus are hard to locate with the electron microscope in thin sections. They proved to be more easily found in negatively-stained preparations of pellets from centrifugal concentration of washings of the mucosal epithelium. Therefore, epithelial washings from the large bowels of mice from the four colonies were examined for the spiral-shaped microbes.

In the negatively stained specimens, we saw at least three morphological forms in washings of the epithelium of the colonic mucosa from mice from three of the four colonies. Usually, more

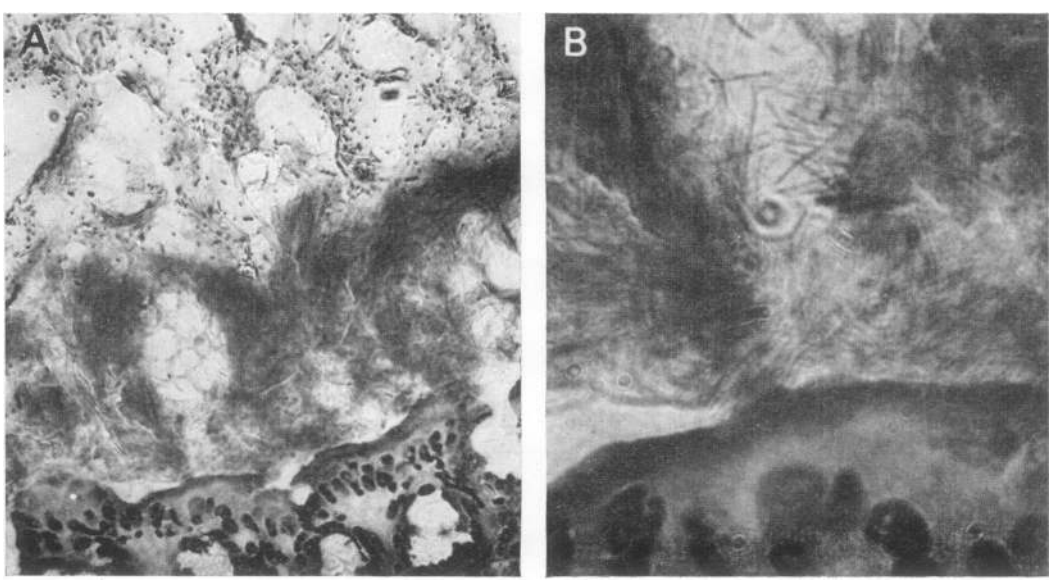


FIG. 1. *Frozen-section histological preparations stained with tissue Gram stain showing layers of fusiform-shaped bacteria in the colons of normal adult Ha/ICR. (A) Low-power magnification. × 775. (B) High-power magnification of same field shown in A. × 2,945.*

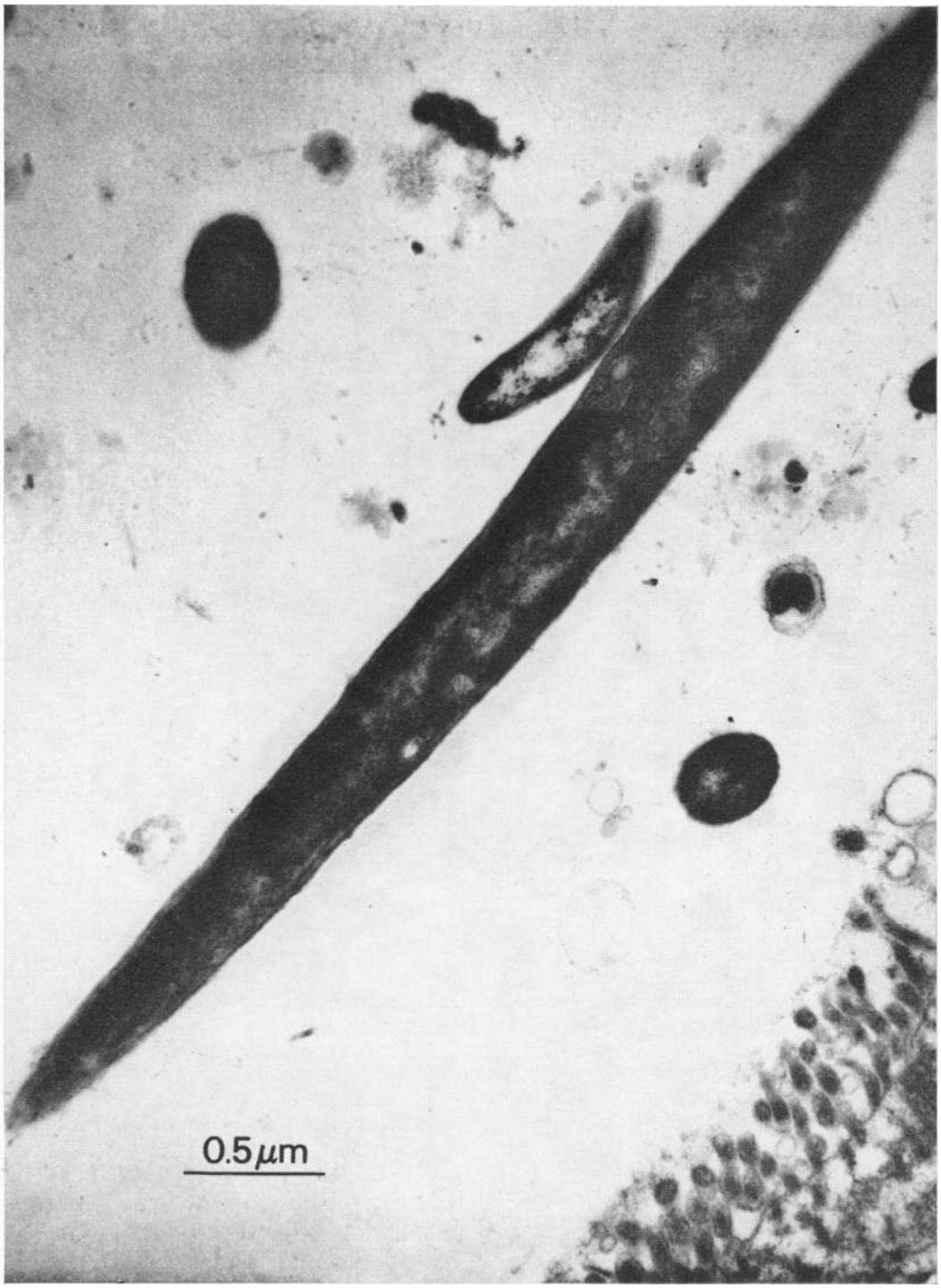


FIG. 2. *Ultrathin section of the colon of a normal adult Ha/ICR mouse showing fusiform-shaped bacteria in close proximity to the mucosal epithelium.* × *42,000.*

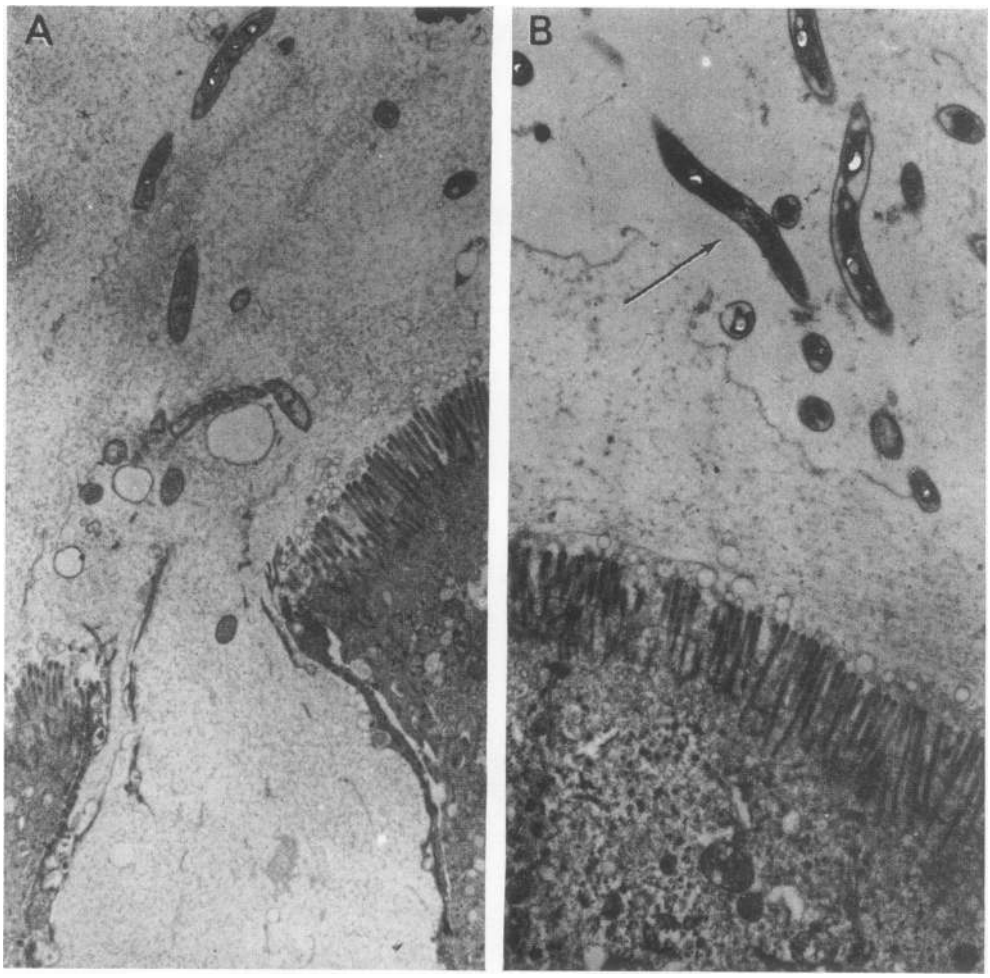


FIG. 3. *Ultrathin sections of the colon of a normal adult Ha/ICR mouse. (A) Fusiform-shaped bacteria in close proximity to a discharging goblet cell. × 10,500. (B) A spiral-shaped microorganism in close proximity to the mucosal epithelium. × 11,000.*

than one of the three forms could be seen in preparations from mice of one type. However, mice from a particular colony always had a predominant form.

For example, at least two spiral-shaped forms could be seen in washings from Ha/ICR mice (Fig. 4). Tightly folded forms (Fig. 4a, b, and c) predominated, although occasionally a straighter, much less tightly folded form could be seen as well (Fig. 4c). Both of these morphological forms possess single polar flagella at each end of the cell. The tightly folded cells have a mottled surface, however, whereas the cells of the straighter microorganism are granular in appearance. This difference may indicate at least two varieties of the same type if not two different types of microorganism. This question can be answered only when the organisms are isolated and identified.

Negatively stained washings of the epithelium of the colonic mucosa of CF-1 mice contain at least two clearly different morphological forms of spiral-shaped microorganisms (Fig. 5c). The predominating form is a microbe that appears to have an axial membrane system and bipolar tufts of flagella (Fig. 5a, b). It is possible that the flagella-like strands are actually frayed, broken axial filaments (7). However, this possibility does not seem likely; the strands appear to be thinner than the filaments in the axial membrane (Fig. 5b). Moreover, these organisms always appear as shown in the figures in washings from mice from the CF-1 colony. If the terminal flagella are real,

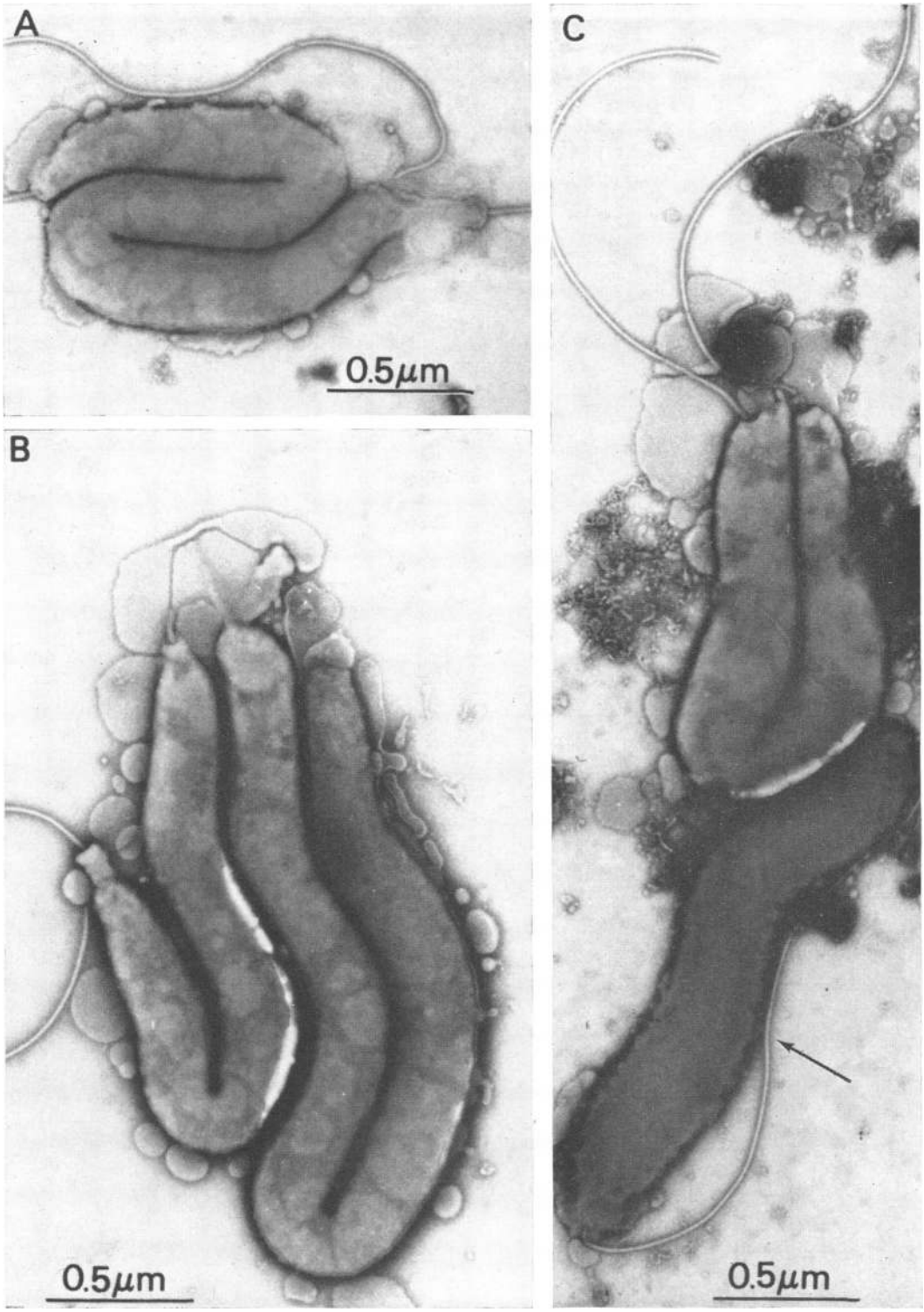


FIG. 4. *Spiral-shaped microorganisms found in negatively stained washings of the mucosal epithelia of the colons of normal adult Ha/ICR mice.* × *43,500.* (*A*) *Folded form seen frequently in the washings.* (*B*) *Another form also commonly seen in the washings. This form may be another configuration of the organism shown in A.* (*C*) *Spiral form with a granular surface near a form with a surface and configuration similar to the ones shown in B. The organism with the granular surface is seen less commonly than the other forms in the washings.*

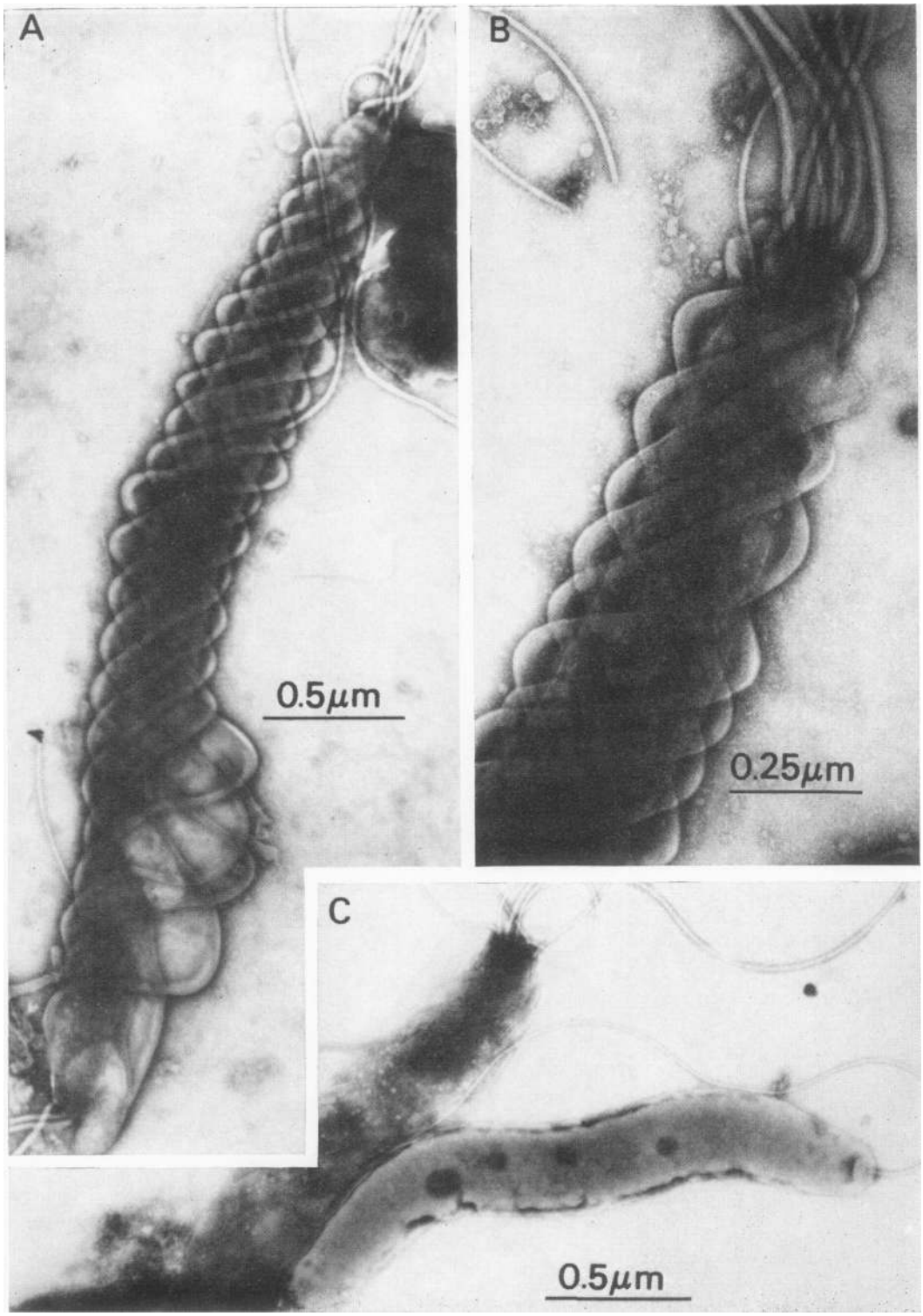


FIG. 5. *Spiral-shaped microorganisms seen in negatively stained washings of the colons of normal adult CF-1 mice. (A) Form seen most commonly in these washings. × 43,500. (B) Higher magnification of organism shown in A. × 80,000. (C) The organism shown in A and a polarly flagellated form also seen commonly in these washings. × 43,500.*

the organism may be an unusual type of spirochete.

In addition to the organism with the axial membrane, the CF-1 mice contain a large population of a spiral-shaped microbe with a single polar flagellum at each end (Fig. 5c). This morphological form is similar to the least tightly spiraled organism seen in washings from Ha/ICR mice (Fig. 4c).

Washings from CD-1 mice contain spiral-shaped microorganisms similar to those seen in Ha/ICR mice (Fig. 4).

TABLE 2. *Results of anaerobic cultures for spiral-shaped microorganisms from the ceca and colons of adult specific pathogen-free mice*[a]

| Type of mice[b] | Microorganisms cultured from | |
|---|---|---|
| | Cecum[c] | Colon[c] |
| Ha/ICR | 17/20 | 14/14 |
| CF-1 | 9/10 | 8/10 |
| CD-1 | 6/10 | 5/10 |
| Swiss | 4/10 | 4/10 |

[a] Homogenates of organs were diluted in 10-fold steps; loopfuls of the dilutions were streaked on plates of E medium (3); the plates were incubated in an atmosphere of nitrogen and carbon dioxide (15) at 37 C for at least 7 days.

[b] See Table 1 for commercial sources of animals; mice were 5 to 8 weeks of age.

[c] Data are given as the number of mice from which were cultured spiral-shaped organisms/number of mice cultured.

**Frequency of occurrence of spiral-shaped microorganisms in the mouse large bowel.** Spiral-shaped microbes were observed with the electron microscope in nearly every negatively stained mucosal washing taken from the colons of numerous mice of the three types. In addition, spiral-shaped microorganisms could be cultured from the large bowels of a high percentage of individuals from all four of the mouse colonies (Table 2). The populations of these microorganisms usually appeared to be at high levels in the organs. Unfortunately, accurate estimates of the populations proved to be difficult. The organisms are extremely motile; they spread readily over the surface of agar plates making colony counts difficult. Our crude estimates indicate that the populations approach $10^9$ organisms per g of intestine in most of the mice. This figure compares well with an estimate of $10^9$ to $10^{10}$ spiral-shaped microbes in the ceca of CD-1 mice (5).

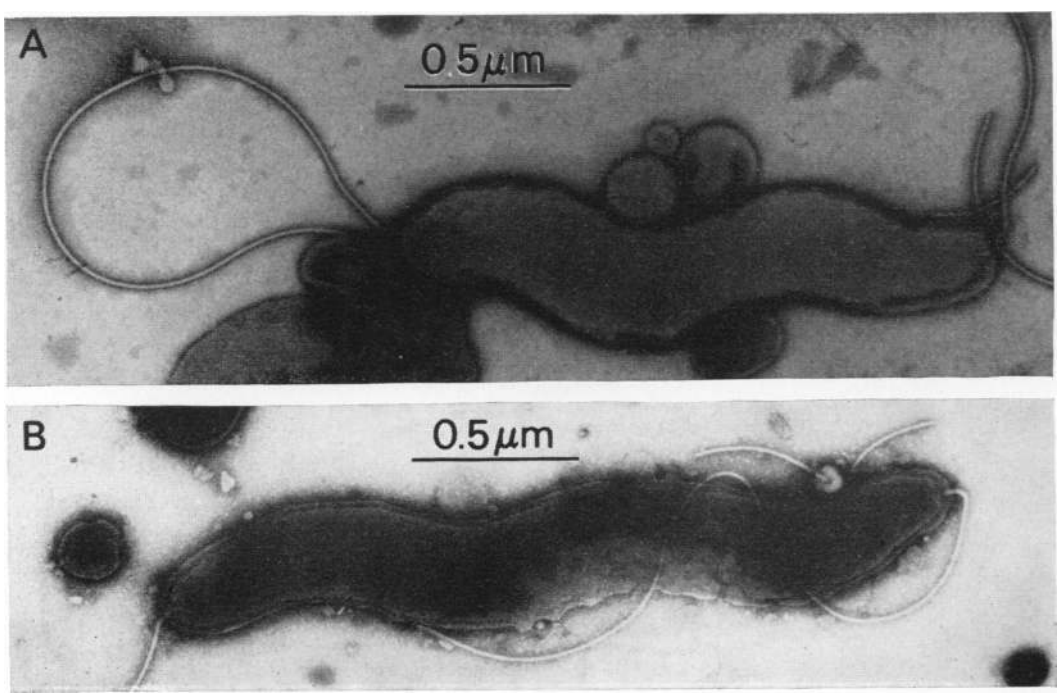


FIG. 6. *(A) Spiral-shaped microorganism with polar flagella grown on E medium, washed from the agar surface, and negatively stained. × 43,500. (B) An organism from the same culture as the one seen in A showing what appears to be a rigid cell wall. × 43,500.*

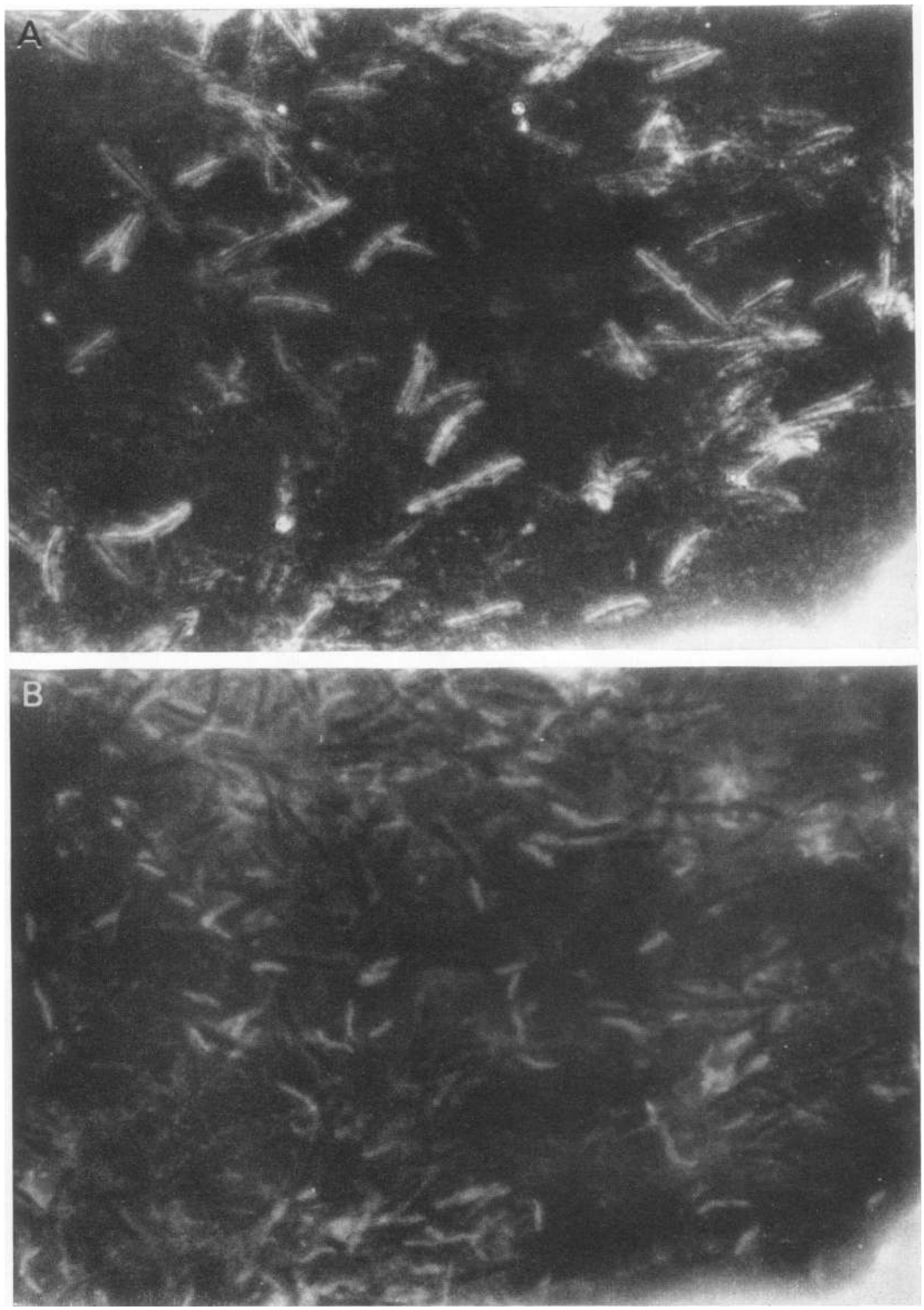


FIG. 7. *Microbial layer on the mucosal epithelium in a frozen section of the colon of an Ha/ICR mouse. The section has been stained for spiral-shaped bacteria by the indirect immunofluorescence method. The mucosal epithelium is the white area on the lower right corner of each photograph. (A) Section as seen in dark-field illuminated with incandescent light; fusiform-shaped bacteria can be seen. (B) Same field shown in A but now illuminated with ultraviolet light; fusiform-shaped bacteria appear as dark shadows; many fluorescing spiral-shaped microbes can be seen intermingled with the mass of fusiform-shaped rods.* × *4,300.*

**Isolation in culture of spiral-shaped bacteria.** To date, we have not been markedly successful in achieving transferrable cultures of most of the types of spiral-shaped microorganisms that can be cultured from the mouse large bowel. We have succeeded, however, in isolating and carrying in pure culture on E agar (3) one such microorganism (Fig. 6). This organism was isolated from Ha/ICR mice and is very similar in appearance to the least tightly curved form seen in washings of the colonic epithelium from those animals (Fig. 4c). It has a single polar flagellum at each end (Fig. 6a) and appears to have a rigid cell wall (Fig. 6b). It is gram-negative and anaerobic, but can be transferred in an air atmosphere, and has never been observed to form spores.

**Localization with fluorescent antibody of spiral-shaped microorganisms in microbial layers on colonic epithelia.** Spiral-shaped microorganisms cannot be seen in frozen-section histological preparations (Fig. 1). They seem to be hidden in dense populations of the other types of microorganisms. However, successful culturing of a spiral-shaped bacterium made it possible for us to try to demonstrate directly those particular organisms in the frozen sections. Accordingly, we examined for spiral-shaped cells stained by an indirect immunofluorescence technique in sections of large bowels from Ha/ICR mice. Fluorescing spiral-shaped bacteria could be found scattered among fusiform-shaped bacteria in the mucin on the mucosal epithelium (Fig. 7).

## DISCUSSION

The indigenous microbiota of the alimentary canals of mammals is composed of the normal and the autochthonous biotas (2). The normal biota consists of microorganisms that seem to have only a transient association with the animals. Consequently, the normal biota can vary considerably from one group to another of a particular type of animal. In contrast, the autochthonous biota consists of microorganisms with a much closer, more stable relationship with the animal. The autochthonous flora seems not to vary significantly from group to group of a particular type of animal.

The composition of the autochthonous microbial flora of laboratory mice has been understood more fully only recently. Most such autochthonous microorganisms are now known to be anaerobic. In fact, most of these microorganisms are bacteria that are extremely intolerant of oxygen and cannot multiply after even brief exposure to that gas or to oxidized culture media (5, 6). When proper anaerobic conditions are used, however, these oxygen-intolerant anaerobes can be cultured and shown to outnumber by a ratio of from 10 to 1 to 1,000 to 1 all other bacteria in the alimentary microflora (5, 6, 13).

These anaerobic bacteria are not just randomly distributed throughout the gut lumen; many of them localize in layers in the mucin on the epithelium of the cecal and colonic mucosa. In the light microscope, such layers appear to consist of only one type of microorganism. The most prominent in the layers are long rod-shaped bacteria with tapered ends (12). Our present work demonstrates, however, that the layers often contain not only such fusiform-shaped bacteria, but also substantial numbers of spirochetes and spiral-shaped bacteria. These spiral-shaped microbes could be seen in the mucin layers in the large bowels of mice from four colonies widely spaced in the United States.

Spirochetes and spiral-shaped bacteria of various types are known to reside in the gingival crevices in the mouths of normal humans (7, 9) and some animals (9) and are seen frequently in close association with fusiform-shaped bacteria (9). Spiral-shaped and fusiform-shaped microorganisms apparently enjoy more than just casual ecological relationships in the mammalian alimentary canal.

It now seems clear that mammals are dependent for certain metabolic functions upon their indigenous microorganisms and, perhaps more importantly, their autochthonous microorganisms (2). Oxygen-intolerant and other anaerobic microorganisms vastly outnumber aerobic microorganisms in the alimentary canal (5). Ecological interactions with these anaerobes may be of considerable importance to the mammalian host.

### ACKNOWLEDGMENTS

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