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Isolation and Characterization of a Spiral Bacterium from the Crypts of Rodent Gastrointestinal Tracts

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Spiral-shaped bacteria with a distinctive morphology were isolated from the intestinal mucosa of rats and mice on a campylobacter selective medium using microaerophilic incubation. These bacteria have been shown by other authors to be present in the intestinal tracts of several animal species but have not been cultured previously. The results of electron microscopic examinations and biochemical testing have shown that these organisms do not correspond to any known genus. Colonization experiments with pure cultures in gnotobiotic rodents have shown these bacteria to be mucosa associated, with a particular affinity for intestinal crypts. The pattern of colonization of the intestinal crypts in gnotobiotes known to be free of other mucosa-associated organisms differed from the colonization occurring in conventional animals that possess a normal mucosa-associated flora.

Spiral-shaped microorganisms have been found in the digestive tracts of many animals, often in close association with the host tissue. These bacteria are seen directly attached to the tissue surface (A. Takeuchi, Am. J. Pathol. **59**:46, 1970), colonizing the mucous layers between the surface and the lumen (8) and filling the crypts of the mucosal surface (9). The crypts of Lieberkuhn in rats and mice have been shown to contain large populations of curved and spiral-shaped bacteria (9, 21).

One of these organisms is a morphologically distinct spiral-shaped bacterium characterized by the presence of 9 to 11 helically coiled periplasmic fibers and bipolar tufts of flagellum-like apendages. The occurrence of such organisms in the intestinal tracts of rodents has been reported by a number of authors. Erlandsen and Chase (10) observed variable numbers of these organisms in the rat ileal crypts, while Davis et al. (9) found them to be one of three morphological types seen in the cecal crypts of conventional rats. Similarly, Phillips et al. (21) found these distinctive bacteria in the ileal, cecal, and colonic crypts of rats.

The organisms are of interest for reasons other than their unusual morphology. While other species of mucosa-associated organisms are localized in either the ileum, cecum, or colon, these organisms can be found in each tissue. Another feature of these bacteria is their ability to invade the mucosa of compromised hosts (13, 22). All of the above reports were based solely on histological observations, as it was not known how to culture the organisms on artificial media. The aim of the present study has been to develop techniques for the isolation and cultivation of this interesting microorganism. These methods are described as well as the results of basic identification tests and experiments on the colonization of gnotobiotic rats and mice with pure cultures.

MATERIALS AND METHODS

Animals. Conventional Wistar rats and BALB/c mice were obtained from a colony kept at the University of New South Wales. These animals were maintained on a diet of commercial pellets (Mecon) and water. Gnotobiotic animals, derived originally from germfree Wistar rats and BALB/c mice, were obtained from the Australian Atomic Energy Commission Animal Breeding Unit. Cultures of *Lactobacillus* and *Bacteroides* spp. were established in rats, and *Lactobacillus*, *Bacteroides*, and *Fusobacterium* spp. were established in mice. These organisms were originally isolated from conventional animals of the same species. The gnotobiotic colonies were maintained in plastic isolators by germfree techniques. For the colonization experiments, gnotobiotic animals were transferred from the isolators to sterilized plastic cages and given a diet of presterilized food cubes (Mecon) and water. Mouse cages were covered with Isocaps (Carworth). Test and control animals were kept separately in a laminar flow cabinet and away from conventional animals.

Media. The spiral-shaped organisms were isolated from tissue specimens on lysed blood agar (Oxoid Blood Agar Base no. 2 and 7.5% lysed horse blood) containing an antibiotic supplement, Campylobacter Selective Supplement SR69 (Oxoid Ltd.), added per the manufacturer's instructions (CSA 69). The routine cultivation of the organism was carried out with slopes or plates of lysed blood agar. Biphasic media were

used to grow the organisms in liquid culture, as no growth occurred in broth culture. Slopes were inoculated and incubated for 24 h to obtain surface growth before peptone water (1% peptone, 0.5% NaCl [pH 7.4]) was added. After an initial microaerophilic incubation (24 h), the overlaid slopes were incubated in air.

Incubation conditions. Media were incubated at 37°C in microaerophilic conditions. Two methods were used to reduce the oxygen tension. Initially, the method of Butzler and Skirrow (4) was used, i.e., a partial vacuum of 500 mmHg (66.7 kPa) was drawn in a Brewer gas jar without a catalyst and replaced with 460 mmHg (61.3 kPa) of nitrogen (N_2) and 40 mmHg (5.3 kPa) of carbon dioxide (CO_2), giving a final oxygen (O_2) concentration of 7% with 5% CO_2. Alternately, the organisms were grown in gas jars without a catalyst with GasPak H_2-CO_2 generators (BBL Microbiology Systems).

Isolation of the spiral-shaped organisms from rats and mice. Animals were killed by spinal dislocation, 3-cm lengths of proximal ileum were removed and cut open to expose the inner surface, and mucosal scrapings were prepared as previously described (21). The scrapings were inoculated onto CSA 69 plates and incubated for 4 to 6 days.

Biochemical characterization. Hydrolysis of casein, starch, and tributyrin was tested and interpreted by the methods described by Roach et al. (23) though 5% serum agar (5% horse serum [Commonwealth Serum Laboratories], 1% peptone, 0.5% NaCl, and 1.2% agar [Oxoid], pH 7.4) was used as the basal medium. Lipase and lecithinase activities were tested on egg yolk agar (7) supplemented with 5% horse serum. *Vibrio cholerae* was used as a positive control for each of these tests. Catalase and oxidase tests were carried out as described by Roach and Tannock (24) from growth on 10% serum agar plates. In all other tests the organisms were grown on 10% serum slopes to which liquid overlays were added and were then incubated for a further 6 days. Fermentation tests were done with peptone water containing 1% arabinose, cellobiose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, raffinose, salicin, sorbitol, starch, sucrose, or xylose. After incubation, the pH of the solutions was determined with a pH electrode. Fermentation was considered to have occurred if the pH was less than 6.0. Bile sensitivity was tested by growth in an overlay of peptone water containing 2% bile salts (Oxoid). The overlays used for gelatin liquefaction, indole production, litmus milk, meat digestion, and nitrate reduction were prepared and interpreted as previously described (7). The biphasic tests were found reliable when results for control organisms such as *Escherichia coli* and *Pseudomonas aeruginosa* were compared with those obtained by conventional methods.

The production of volatile and nonvolatile fatty acids after 14 days of growth in 1% glucose–peptone water overlaying a 10% serum slope was determined by gas-liquid chromatography (15).

Gaseous requirements. The growth of the isolates in various atmospheres was tested in Brewer gas jars. A partial vacuum was drawn and replaced with measured amounts of oxygen, nitrogen, or carbon dioxide. The ability of the organisms to use oxygen was tested by the method of Carter and Bull (5) using a Clark-type electrode mounted in a 2.5-ml reaction chamber. As a control, the uptake of an *E. coli* K-12 strain growing exponentially in nutrient broth (Oxoid) was also measured.

Electron microscopy. (i) Animals. Animals were killed by spinal dislocation, and 1-cm lengths of ileum, cecum, and colon were removed, fixed in Karnovsky's combined fixative (16) containing 0.05% ruthenium red (20), and embedded as previously described (21).

(ii) Cultures. Four-day-old plate cultures were harvested and fixed with Karnovsky's combined fixative with 0.05% ruthenium red. After 4 h, the suspensions were centrifuged, and the pellets were washed with cacodylate buffer and either embedded for sectioning or negatively stained with 2% sodium phosphotungstate containing 0.01% bacitracin. When embedding cultures, the washed pellets were resuspended in a small volume of 3% agar, cut into 1-mm^3 cubes, and embedded in the same manner as tissue specimens. Thick sections (0.5 μm) and thin sections (60 to 90 nm) were cut on a Huxley Ultramicrotome (LKB Instruments). Thick sections stained with methylene blue containing 1% borax were examined by light microscopy. Thin sections stained with uranyl acetate and lead citrate and negative stains were examined with a Philips 300 transmission electron microscope.

RESULTS

Isolation and cultivation. The spiral-shaped bacteria were isolated on CSA 69 plates inoculated with intestinal scrapings from either rats or mice. The organisms were fastidious, growing only on media enriched with blood or serum. Optimum growth occurred on plates with a moist surface. If the condensation present on the surface of the medium after pouring was removed by air drying, the organism grew poorly or not at all. The organisms grew as a fine, spreading film on media with either 1.2 or 3% agar (24). The spreading growth became apparent after 2 to 3 days of incubation for the rat isolate and 3 to 5 days for the mouse isolate. Individual spreading colonies were initially present; however, with longer incubation these merged into a continuous film of growth. For subculturing, material was transferred from an isolated focus of growth. Lactobacilli and *Campylobacter* spp. were isolated regularly from ileal scrapings on CSA 69.

Attempts to grow the organisms in broth culture have been unsuccessful; however, large numbers of viable organisms were found to be present in the liquid phase of biphasic cultures. When incubated at 37°C, cultures on solid media lost viability within 6 to 8 days, while those on overlaid slopes remained viable for up to 30 days. The organisms died after 1 to 2 days when kept at 4°C and have not been successfully lyophilized. Long-term retrieval was achieved by storing cultures in liquid nitrogen.

Morphology and ultrastructure. In 2- to-3-day-old cultures, cells were spiral shaped, rigid, and gram negative. They were 3.5 to 5 μm long and 0.5 to 0.6 μm wide with two to three spiral turns (Fig. 1). The cells were motile by a rapid cork-

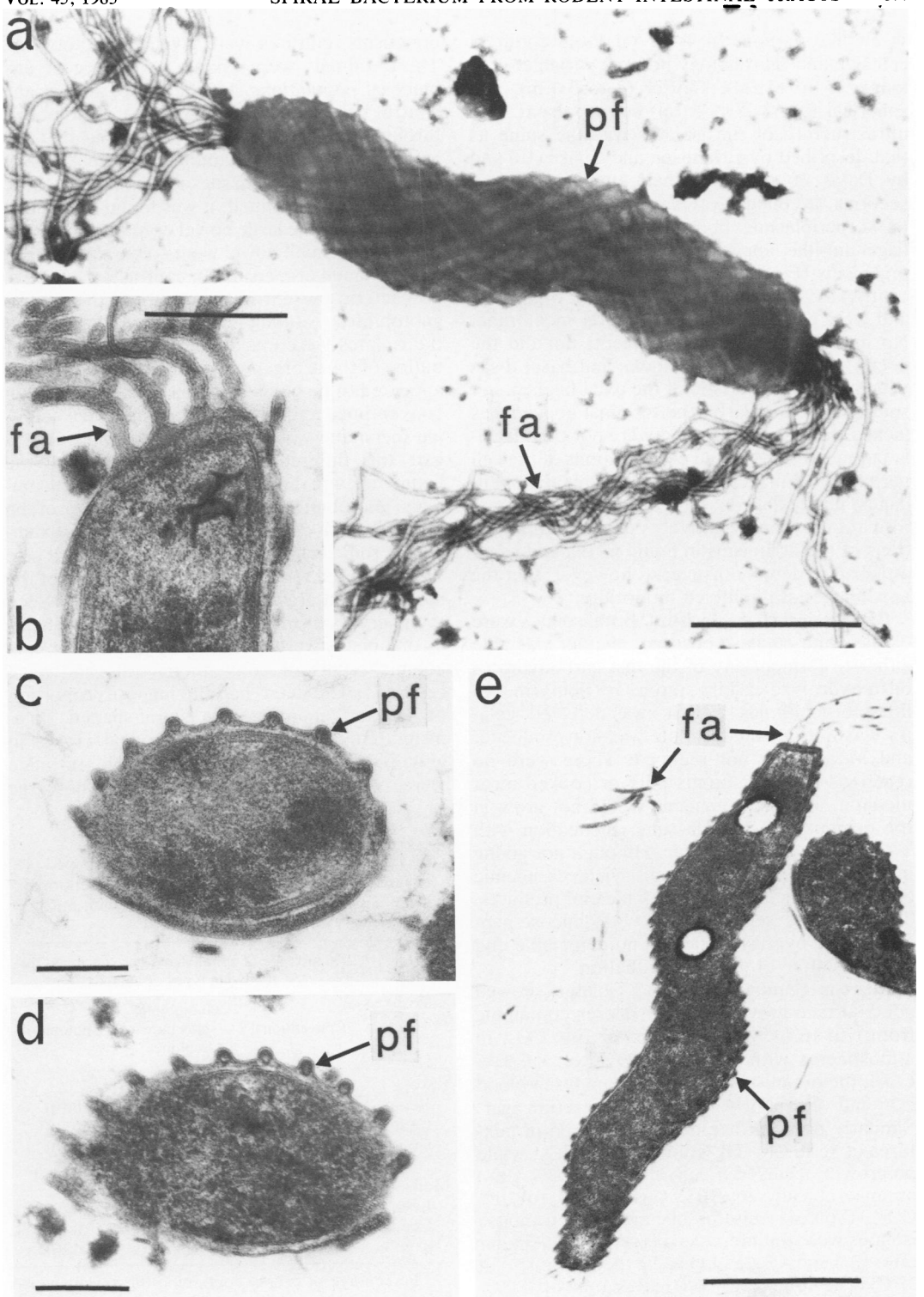

FIG. 1. Transmission electron micrographs of the spiral-shaped isolates. (a) Negative stain of the mouse isolate showing the presence of periplasmic fibers (pf) and bipolar tufts of flagellum-like appendages (fa). (b) Longitudinal section, polar region of the rat isolate showing the membrane-bound flagellum-like appendages. (c) Cross section of the mouse isolate. (d) Cross section of the rat isolate. (e) Longitudinal section of the mouse isolate. Bar = 1 μm in (a) and (e) and 0.2 μm in (b), (c), and (d).

screw-like motion. In 4- to 5-day-old cultures, cell size and morphology became variable, with longer (5 to 6 μm), shorter (2 to 3 μm), and spherical forms (2 to 4 μm) being present. The ultrastructure of the isolates was the same as that described by Erlandsen and Chase (10) and by Davis et al. (9) in their studies of tissue sections, a coiled protoplasmic cylinder with 9 to 11 periplasmic fibers and having 10 to 14 flagellum-like appendages at the ends of the organisms (Fig. 1). The fibers were coiled concentrically around the length of the organisms and were contained within an outer membrane. No insertion points were evident, nor did the fibers have the proximal hooks and basal disks which are characteristic of the axial filaments of spirochetes. Similarly, the terminal appendages lacked these structures and were not true flagella but membrane-bound continuations of the cell periplasm. Furthermore, the terminal appendages, unlike flagella, did not retain a sine wave formation when negatively stained. Observations of the organisms in liquid media and when attached to debris do suggest, however, that the appendages are involved in motility.

Biochemical characteristics. Both isolates were oxidase and catalase positive. Neither was able either to ferment any of the test carbohydrates or to hydrolyze casein, starch, or tributyrin. No lipase or lecithinase activity was detected, gelatin was not liquefied, indole was not produced, and nitrate was not reduced. There were no reactions in either litmus milk or cooked meat medium, and the organisms would not grow in the presence of 2% bile salts. Incubation with 1% glucose–peptone water, although not giving a positive fermentation result, yielded succinic acid and acetic acid as detectable end products. The controls, serum slopes with glucose-peptone water overlays, showed no detectable end products after 14 days of incubation.

Gaseous requirements. The isolates showed good surface growth in atmospheres containing from 1 to 16% O_2 with either 5 or 10% CO_2. In atmospheres without added CO_2 (i.e., <0.03% CO_2), the organisms grew poorly on lysed blood agar and very poorly or not at all on serum agar. Similarly poor surface growth occurred in mixtures of 16 to 21% O_2 with 5 or 10% CO_2, while no growth occurred in air (21% O_2, 0.03% CO_2) or anaerobically in BBL Gas Jars (4 to 10% CO_2). Biphasic cultures of both rat and mouse isolates were found to use oxygen and at similar rates (3.5 and 4.3 μg of O h^{-1} g [dry weight]$^{-1}$ at 37°C). The control, *E. coli*, had an uptake of 29.1 μg of O h^{-1} g^{-1}, which was similar to the uptake previously reported for this organism (14).

Colonization of gnotobiotic animals with the spiral-shaped isolates. To verify that the isolates from the rat and mouse were mucosa-associated organisms, cultures were given to gnotobiotes. These animals were known to be free of any mucosal populations. Furthermore, all test and control gnotobiotes remained free of coliforms throughout the experiment, indicating that the animals were not contaminated by material of fecal origin. When tissue sections from these animals were examined, it was found that a high proportion of the large bowel crypts and a few of those in the small bowel were colonized with the spiral-shaped organisms. In contrast, there were no bacteria present in the crypts of the control gnotobiotes. Although this result confirmed that the isolates were mucosa associated, the distribution of these organisms in the intestinal crypts appeared to be quite different from that found in conventional animals. A semiquantitative counting technique was developed to further investigate this difference. Thick sections from the ileum, cecum, and colon of conventional animals, gnotobiotes inoculated with cultures of the isolates, and control gnotobiotes were examined. From each section 20 crypts of similar size were assessed for the presence or absence of the spiral-shaped organisms. No bacteria were seen in the crypts of the gnotobiotic controls, whereas organisms were found in the ileal, cecal, and colonic crypts of conventional rats and mice (Table 1). The ileum had the highest proportion of crypts containing the spiral-shaped organisms. In contrast, few of the ileal crypts in gnotobiotic rats and none in gnotobiotic mice were colonized with the bacteria. The large

TABLE 1. Occurrence of the spiral-shaped organisms in the intestinal crypts of conventional animals and gnotobiotes inoculated with pure cultures

Tissue	Occurrence of spiral-shaped organisms in the following types of animals		
	Conventional	Gnotobiotic plus pure culture	Gnotobiotic
Rat			
Ileum	70[a] (5)[b]	23 (4)[c]	0 (0)
Cecum	12 (3)	81 (5)[c]	0 (0)
Colon	13 (3)	10 (4)[c]	0 (0)
Mouse			
Ileum	63 (5)	0 (0)[d]	0 (0)
Cecum	38 (5)	100 (5)[d]	0 (0)
Colon	42 (4)	81 (5)[d]	0 (0)

[a] Percentage of crypts containing the spiral-shaped organism (100 crypts per tissue).
[b] Number of animals with crypts containing the spiral-shaped organism (five animals per group).
[c] Gnotobiotes were inoculated with the rat isolate.
[d] Gnotobiotes were inoculated with the mouse isolate.

bowel crypts of these animals, however, with the exception of those in the rat colon, were more heavily populated by organisms than the corresponding crypts in conventional animals. The cecal crypts in particular were densely packed with organisms in the gnotobiotic animals (Fig. 2).

The spiral-shaped organisms were seen within the cells lining the crypts of both the gnotobiotic rats and mice, with none being seen in the cells of conventional animals. The organisms were found free in the cytoplasm, and the cells containing them often appeared degenerate with numerous vacuoles, swollen mitochondria, and diffuse cytoplasm (Fig. 3).

DISCUSSION

While the occurrence of spiral-shaped organisms in the intestinal tracts of many animals has been well documented, the role of these organisms in the gut ecosystem is poorly understood. One of the main reasons for this lack of information has been an inability to grow these organisms on artificial media. In a previous publication, we reported on the isolation of spiral-shaped organisms from the mucosa of the rodent large bowel (18). By similar methods it has been possible to culture organisms from the ileum. Plates were incubated microaerophilically, as it was expected that the ileum would be less reduced than the large bowel and hence more suited to oxygen-tolerant organisms. The selective medium CSA 69 used for the isolation of the spiral-shaped organisms in this study was originally designed for the isolation of *Campylobacter jejuni* using an incubation temperature of 42°C; however, it was found that when incubated at 37°C, this medium was also useful for the isolation of other spiral-shaped organisms such as those reported in this paper.

The requirements of the spiral-shaped organisms for oxygen and carbon dioxide were very similar to those reported for *Campylobacter jejuni* strains (27) which can also be found in the intestine. The isolates grew optimally in reduced partial pressures of oxygen and increased partial pressures of carbon dioxide. The toxicity of oxygen to the isolates was dependent on the carbon dioxide concentration. Growth in atmospheric concentrations of oxygen, i.e., 21%, occurred only if the concentration of carbon dioxide was increased substantially from the level in air, 0.03%, to higher concentrations, e.g., 5 to 10%. Furthermore, the isolates, like many other organisms that require CO_2 for growth, produce succinic acid as an end product. Presumably the sites from which the organisms were isolated had environmental parameters similar to those giving optimum growth in vitro. The intestinal crypts populated by the spiral-shaped organisms would therefore be expected to contain appreciable amounts of both carbon dioxide and oxygen. Similarly, the ability of the organisms to colonize various sites in the gut may be influenced by the availability of these gases.

Organisms similar to the rodent isolates have been found in the digestive tracts of a number of other animal species. Spiral-shaped bacteria with concentric periplasmic fibrils and flagellum-like appendages have been observed in the fundic glands of dogs and cats (28), the stomachs of monkeys (A. Takeuchi, Am. J. Pathol. **59**:46, 1970), and the colonic washings of mice (8). Recently, we have also observed this type of organism associated with the intestinal mucosa of both cats and dogs. Although the subject of many reports, the taxonomy of this morphologically similar group of organisms has remained ambiguous. For example, when Erlandsen and Chase (10) first observed these organisms, they suggested that the organisms "bear a close resemblance to members of the order *Spirochaetales*." A later report on their research, however, identifies the organism as being *Spirillum* species (17).

The spiral-shaped organisms from the fundic glands of dogs and cats were originally classified as *Spirillum* species in *Bergey's Manual of Determinative Bacteriology (6th ed.)* (2). Subsequent editions have, however, omitted these organisms as a result of the paucity of accurate information relevant to their classification. After a detailed ultrastructural study of the organisms from the gastric mucosa of dogs and a survey of the literature, Lockard and Boler (19) were of the opinion that the organisms were "more clearly related to the order *Spirochaetales* than to the genus *Spirillum*."

If the current edition of *Bergey's Manual of Determinative Bacteriology*, i.e., the eighth edition (3), is referred to, the rodent isolates cannot be included in any of the presently defined genera of bacteria. The possession of periplasmic fibers within an outer membrane is suggestive of the family *Spirochaetaceae*. In other respects the organisms resemble *Spirillaceae* species, being S shaped, gram negative, microaerophilic, oxidase positive, catalase positive, and unable to ferment carbohydrates. The ultrastructure of the organisms, however, is distinctly different from either *Spirillaceae* or *Spirochaetaceae* species. The periplasmic fibers are unlike the filaments of the spirochetes in both structure and arrangement on the protoplasmic cylinder and are inconsistent with the genus *Spirillum*. Similarly, the possession of terminal, membrane-bound appendages is not consistent with the definitions of either group. The morphological studies of the organisms

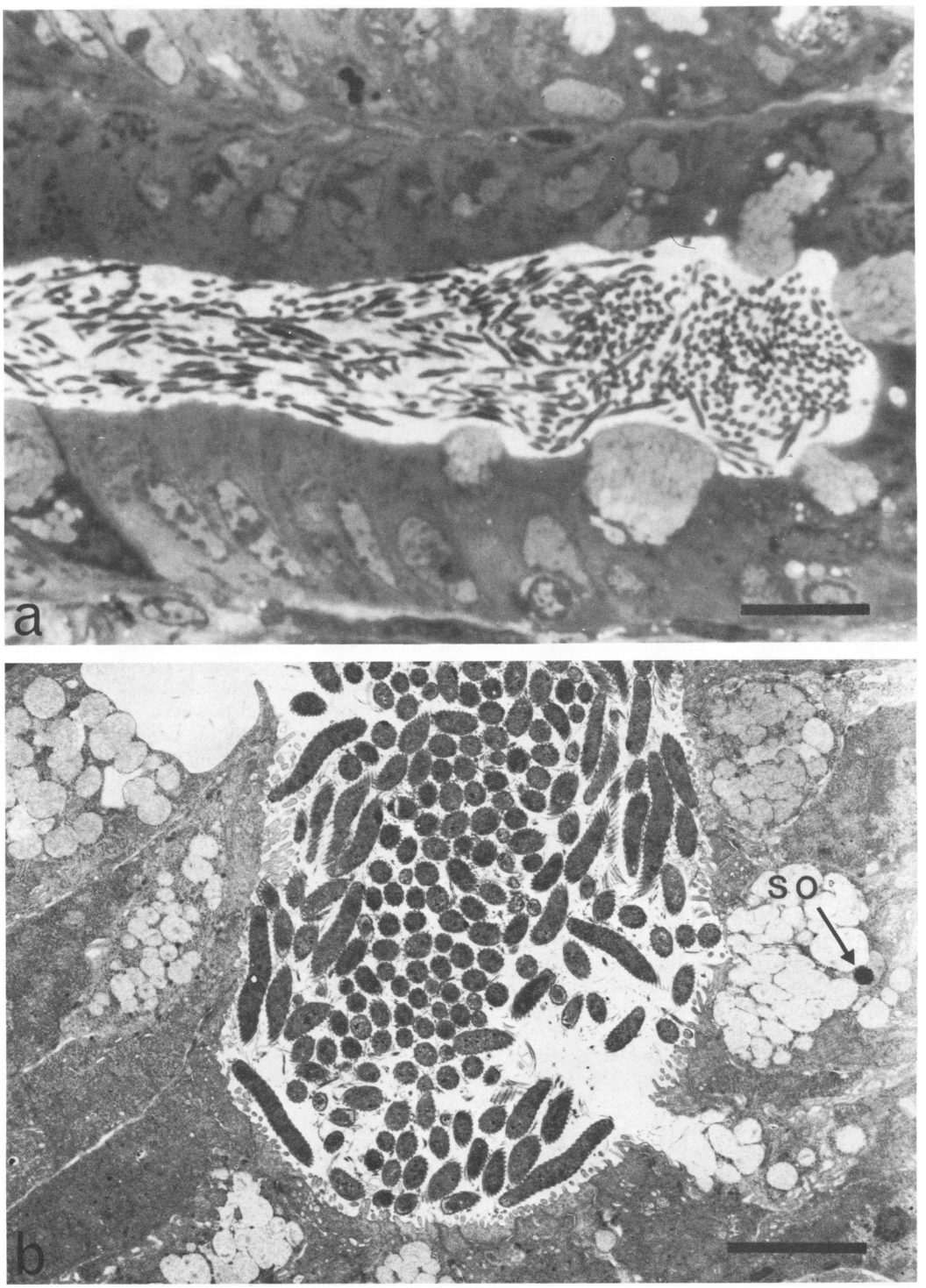

FIG. 2. Colonization of the cecal crypts of gnotobiotic rodents after oral inoculation of pure cultures of spiral-shaped organisms. (a) Light micrograph of a mouse cecal crypt showing heavy colonization by the mouse isolate. Bar = 10 μm. (b) Transmission electron micrograph of a rat cecal crypt colonized with the rat isolate. A spiral shaped organism (so) can be seen within a goblet cell. Bar = 3 μm.

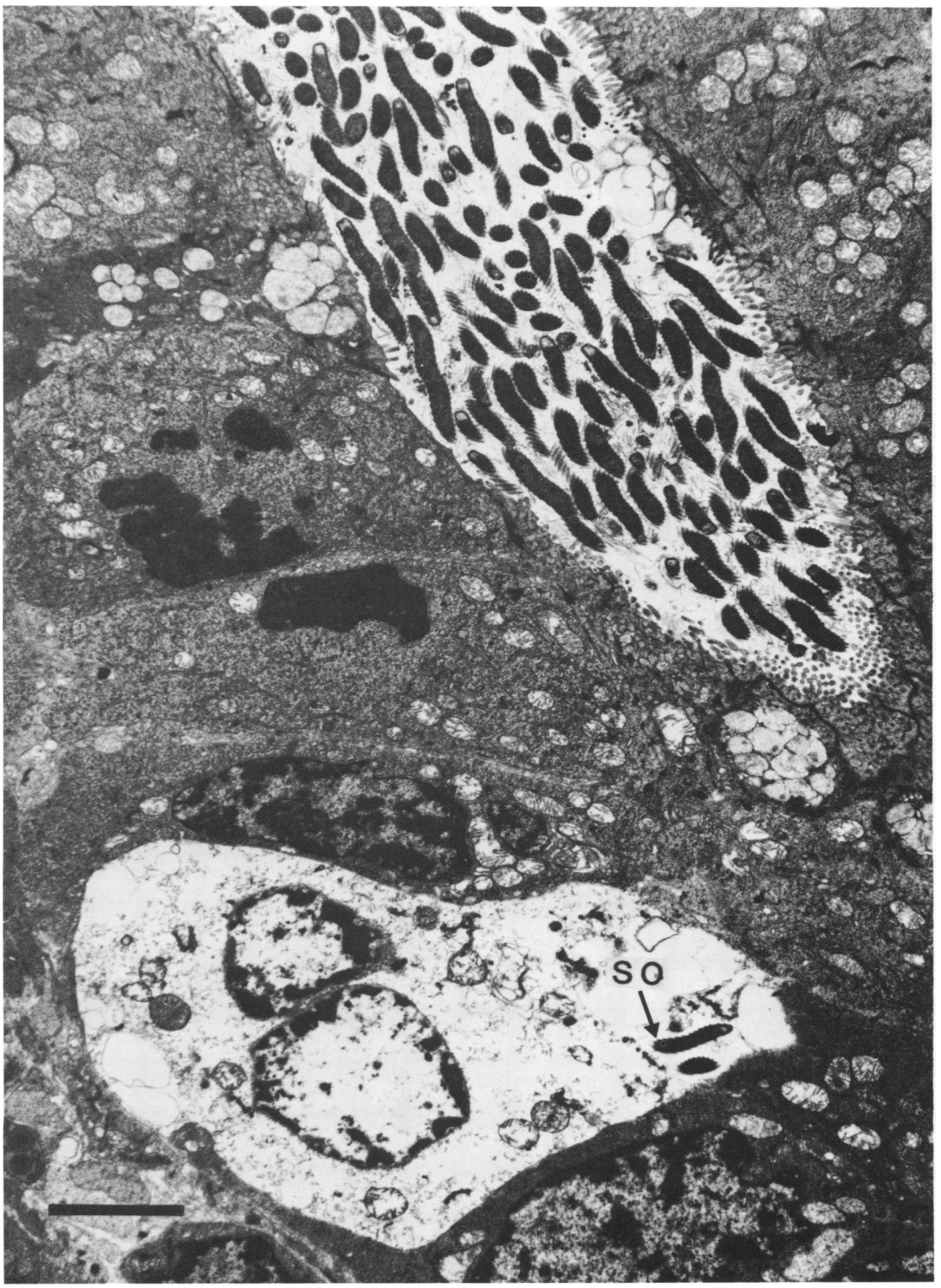

FIG. 3. Transmission electron micrograph of gnotobiotic rat cecal tissue after oral inoculation of a pure culture of the rat isolate, showing the presence of spiral-shaped organisms (so) within a degenerate epithelial cell. Bar = 3 μm.

from the cat, dog, and monkey suggest that they also do not belong to any of the known genera for the same reasons, and it is possible that they, along with the rodent isolates, belong to a new, as yet unnamed genus of bacteria. More detailed studies of the rodent isolates in comparison with other spiral-shaped bacteria are underway.

Gnotobiotes with a limited, defined flora of lumen organisms were used for the colonization experiments as they were known to lack mucosa-associated populations. Spiral-shaped organisms with the very distinctive morphology could therefore be clearly identified as originating from the inoculated culture. The presence of these organisms in the intestinal crypts of the gnotobiotes confirmed that they were mucosa associated. No organisms were seen associated with the mucosa of the control animals maintained under the same conditions.

The pattern of colonization of the spiral-shaped organisms in the inoculated gnotobiotes with a restricted microbial flora was different from that found naturally in conventional animals with their complex flora. Thus, the organisms in conventional animals were found mainly in the ileal crypts, while in the inoculated gnotobiotes, they were seen in the crypts of the cecum and to a lesser extent in those of the colon.

A probable explanation for the limited colonization of the conventional large bowel by the spiral-shaped organisms is competition from other microorganisms. The cecal crypts normally contain large numbers of bacteria other than the organisms reported in this paper (9, 21). When these competing populations are absent, as in the gnotobiotes, the spiral-shaped organisms are able to colonize more extensively. An explanation for the low numbers of the spiral-shaped organisms in the ileal crypts of the gnotobiotic animals in comparison to conventional animals appears to be less obvious. It is possible that other bacteria in the small or large bowel of the conventional animal create conditions in the local environment which are more favorable for the spiral-shaped organisms, e.g., changes in surface chemistry (1), production of growth factors (12), or alterations in redox potentials more suitable for microaerophilic growth (6). Alternatively, the presence of normal bacterial populations may affect the intestine itself and hence alter crypt conditions, e.g., by increasing peristalsis (25), affecting cell turnover (1), altering the structure of the ileal mucosa (11), or changing the fluid balance of the mucosa (26). Studies with the less complex gnotobiotes are more likely to reveal the role of these factors than are studies of the more complex conventional animals.

The spiral-shaped organisms have been described in two papers which reported on the appearance, under the electron microscope, of tissue specimens from immunologically compromised hosts, namely, irradiated mice (22) and mice treated with nitrogen mustard (13). In these papers the bacteria were seen to have passed from the intestinal crypts into the tissue cells. In our studies on normal animals, this phenomenon has only been seen very rarely. However, in the recolonized gnotobiotic animals, intracellular organisms were consistently seen within the tissues. Whether this is simply a consequence of larger numbers of bacteria colonizing the crypts leading to increased tissue invasion or due to the reduced defense mechanisms in the gnotobiotes is not known.

An interesting observation is that there does appear to be some evidence of cell damage by these bacteria. It has been postulated that the mucosa-associated microflora, due to their excellent adaption to the local environment of the intestinal surfaces, play an important role in protection against invasion by intestinal pathogens (12). The findings reported in this study raise the interesting possibility that changes in the intestinal environment might allow these surface-associated bacteria to harm the host. The ability to culture these organisms will permit an experimental approach to this phenomenon and perhaps a determination of the role of these interesting organisms in intestinal ecology.

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