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# Isolation of an Intracellular Bacterium from Hamsters (*Mesocricetus auratus*) with Proliferative Ileitis and Reproduction of the Disease with a Pure Culture

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**An intracellular bacterium was isolated from hamsters (*Mesocricetus auratus*) with proliferative ileitis. The organism was isolated in Intestine 407 and GPC-16 cell cultures (incubated in a microaerophilic atmosphere) from isolated and lysed epithelial cells from hamsters with proliferative ileitis. The bacterium measured 1.4 to 1.7 μm in length by 0.26 to 0.34 μm in width, was slightly curved, and had an irregular trilaminar cell wall. Inoculation of hamsters with a cell culture lysate containing the organism or a 0.65-μm-pore-size filtrate of an infected-cell lysate resulted in the typical lesions of proliferative ileitis in approximately 50% of the animals in 28 days. Hamsters inoculated with uninfected cells or a 0.2-μm-pore-size filtrate of an infected-cell lysate remained uninfected. Attempts to propagate the organism on cell-free media have been unsuccessful.**

Proliferative ileitis (transmissible ileal hyperplasia, wet tail) is a serious infectious disease of hamsters characterized by hyperplasia of the ileal epithelium and pyogranulomatous inflammation. Light microscopic studies have revealed a gram-negative, slightly curved bacillus within the hyperplastic ileal epithelial cells (6–8, 19). Ultrastructurally, the intracellular bacterium resembles members of the genus *Campylobacter* (2, 9, 20).

*Campylobacter jejuni* has been repeatedly associated with proliferative ileitis by microbiologic studies (10, 11). Experimental production of proliferative ileitis by inoculation with *C. jejuni* orally (1, 10, 11) or by surgically inoculating the ileum (6) has been uniformly unsuccessful. Proliferative ileitis has also been experimentally produced by using an infected homogenate free of *C. jejuni* (17). *Campylobacter hyointestinalis* (3, 5), *Campylobacter cinaedi* (4), and several additional *Campylobacter*-like organisms (18) have also been isolated from hamsters, but attempts to reproduce the disease with these organisms, as well as with *Campylobacter mucosalis* and *Campylobacter coli*, have also been unsuccessful (15). Porcine proliferative enteropathy is a disease of pigs with similar lesions and possibly the same etiology as hamster proliferative ileitis (15). Antigenic (14) and DNA (16) analysis of a preparation of intracellular organisms recovered from pigs with porcine proliferative enteropathy has suggested that the intracellular organism is unrelated to the presently described *Campylobacter* spp. and may not be a member of the genus *Campylobacter*.

In this paper we report the isolation of an organism in cell culture from hamsters with proliferative ileitis, reproduction of proliferative ileitis in hamsters following oral inoculation with the organism, and reisolation of the organism. Serological evidence of infection and immunohistochemical stains supporting the role of this organism in the pathogenesis of proliferative ileitis of hamsters are also provided.

## MATERIALS AND METHODS

**Isolation procedures.** The aseptically removed ilea of three hamsters with experimentally induced proliferative ileitis (21 days postinoculation of infected ileal homogenate) provided the source material. The ilea were cut into 2.5-cm sections, opened longitudinally, and repeatedly washed with Hank's balanced salt solution (HBSS) without $Ca^{2+}$ or $Mg^{2+}$ with 1 mM EDTA (HBSS-EDTA) to remove the intestinal contents. The mucus was removed by incubating the ileal sections in 20% (wt/vol) *N*-acetyl-cysteine (Sigma Chemical Co., St. Louis, Mo.) for 45 min at 37°C with agitation. The supernatant was decanted, and the sections were washed twice in HBSS-EDTA and then three times in HBSS. The sections were then transferred to 20 ml of hyaluronidase (1 mg/ml) in HBSS with 5% (wt/vol) bovine serum albumin (BSA) and incubated at 37°C for 2 h with agitation to free the epithelial cells. The resulting solutions were successively filtered through a sterilized glass bead-filled syringe, a 105-μm-pore-size polypropylene mesh, and a 52-μm-pore-size nylon mesh (Spectra/Mesh; Spectrum Medical Industries, Inc., Los Angeles, Calif.) to remove the larger tissue fragments. The crude cell suspensions were centrifuged (400 × *g* for 10 min) and resuspended in HBSS twice. Following a final centrifugation, the cell pellets were resuspended in 50 ml of Leibovitz L-15 media (Sigma) with 10% fetal calf serum, 100 μg of gentamicin sulfate (Sigma) per ml, and 2.5 μg of amphotericin B (Sigma) per ml and incubated overnight at 4°C with agitation.

The cells were separated by centrifugation (400 × *g* for 10 min) and washed three times in HBSS-EDTA with 5% BSA and resuspended in a minimal volume of HBSS-EDTA with 5% BSA. The cell suspensions were then layered onto preformed (15 min at 15,000 × *g* and at 4°C) 30% Percoll (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) gradients in HBSS-EDTA with 5% BSA and centrifuged at 400 × *g* for 15 min. The epithelial cell bands (density, 1.035 g/ml) were suspended in HBSS with 5% BSA and washed four times in HBSS with 5% BSA followed by centrifugation (400 × *g*, 10 min). The cell pellets were resuspended in HBSS with 5% BSA and 0.2% Triton X-100 and incubated at 37°C for 30 min with agitation to effect cell lysis. The suspensions were then centrifuged (400 × *g*, 10 min) to remove cell fragments, and the supernatants were filtered through stacked 20-μm-pore-size nylon (Micron Separations, Inc., Westboro, Mass.) and 0.65-μm-pore-size nitro-

cellulose filters (Whatman Co., Clifton, N.J.). The filtrates were centrifuged at 10,000 × *g* for 15 min at 4°C, and the resulting bacterial pellets were washed once in HBSS, recentrifuged, and resuspended in HBSS.

**Microbiologic procedures.** Three Trypticase soy agar–5% sheep blood plates were inoculated with 250 μl of each bacterial suspension. All plates were incubated at 37°C. One plate was incubated at ambient atmosphere, one was incubated in an atmosphere consisting of approximately 6% $O_2$, 7% $CO_2$, 7% $H_2$, and 80% $N_2$ obtained as described previously (5), and the remaining plate was incubated anaerobically. The aerobic and microaerobic plates were examined daily for growth, while the anaerobic plate was examined every 3 days for growth. All plates were held for a minimum of 12 days prior to being discarded.

**Cell culture isolation procedures.** All cell lines were obtained from the American Type Culture Collection (Rockville, Md.). Culture media, heat-inactivated fetal calf serum, media supplements, and additives were obtained from Sigma.

Intestine 407 human embryonic intestinal cells (ATCC CCL 6) and GPC-16 guinea pig colonic adenocarcinoma cells (ATCC CCL 242) were grown in Dulbecco's modified Eagle's medium with 4,500 mg of glucose per liter, 110 mg of sodium pyruvate per liter, 584 mg of L-glutamine per liter, and 10% fetal calf serum (DME). HaK Syrian hamster kidney cells were grown in Eagle's minimum essential media with Hank's salts and 10% fetal calf serum. Vero African green monkey kidney cells were grown in medium 199 containing Hank's salts and 10% fetal calf serum. All cell lines were plated onto 75-$cm^2$ culture flasks (Corning Glass Works, Corning, N.Y.) in the respective media and incubated at 37°C in a 5% $CO_2$ atmosphere until approximately 50% confluent. Each cell line was then inoculated with 500 μl of each isolated bacterial suspension and incubated at 37°C in a 6% $CO_2$, 6% $O_2$, 88% $N_2$ atmosphere. Cell cultures were split 2:1 by scraping on days 4 and 9 of incubation and examined daily by phase microscopy (Olympus INT; Olympus Corp., Lake Success, N.Y.) for evidence of bacterial growth. All cell cultures were maintained for a minimum of 14 days before negatives were discarded.

Cell cultures with intracellular bacterial growth were continued in cell culture by scraping and splitting through four doublings. The cell culture supernatant was used to infect additional flasks of the same cell line to expand the culture. Infected cells were frozen at −80°C in the respective media with 10% dimethyl sulfoxide for reference and later use.

**Inocula preparation.** Four inocula were prepared for the infection studies. The inoculum for the control group (group A) was prepared from two 75-$cm^2$ flasks of confluent uninfected Intestine 407 cells. The cells were mechanically separated from the flask by scraping, concussion, and vortexing. Triton X-100 was then added to the cell media suspension to a concentration of 0.2% (vol/vol), and the suspension was incubated at 37°C with agitation for 20 min. The suspension was then centrifuged (400 × *g*, 20 min), and the supernatant was filtered through a 20-μm-pore-size nylon filter (Micron Separations, Inc.). The resulting filtrate was centrifuged at 11,000 × *g* for 20 min at 4°C, and the resulting pellet was resuspended in 10 ml of DME.

The infected inocula were prepared in a similar manner except that a total of six 75-$cm^2$ flasks were processed. Following the filtration through a 20-μm-pore-size nylon filter, the infected inocula were divided into three portions. The group B inoculum was processed identically to the group A control inoculum. The group C inoculum was filtered through a 0.65-μm-pore-size cellulose filter (Whatman) prior to final centrifugation, and the group D inoculum was similarly filtered through a 0.2-μm-pore-size cellulose filter (Whatman) prior to final centrifugation. All inocula were held at 4°C after preparation and were utilized within 3 h of preparation.

**Animals.** Four adult Syrian hamsters (Hsd:SYR) with 17-day-old litters were purchased from a commercial supplier whose colony had no previous history of proliferative ileitis. The weanling hamsters were separated from their dams, sexed, weighed, and ear tagged (National Band and Tag Co., Newport, Ky.) upon arrival at our facility and placed into group cages by gender. Postinoculation, hamsters were housed in conventional polycarbonate rodent cages and provided a commercial rodent diet (RMH 3000; Agway, Inc., Syracuse, N.Y.) and water ad libitum. Each hamster was weighed at 3- to 5-day intervals by placing the animal in a clean pretared container.

**Animal inoculation.** The weanling hamsters were divided into four groups with a conscious attempt to equalize the sex and weight distribution among the groups. Each hamster was given 7.5 mg of cimetidine (Tagamet; SK&F Lab Co., Cidra, Puerto Rico) intraperitoneally 30 min prior to inoculation to induce temporary achlorhydria. Following oral inoculation by gavage, each hamster was given 0.2 mg of dexamethasone (Azium; Schering Corp., Kenilworth, N.J.) intramuscularly.

Control group A hamsters (five male and three female) were inoculated with 1 ml of a cell-free filtrate from uninfected Intestine 407 cells. Group B hamsters (five male and three female) were inoculated with 1 ml of a cell-free filtrate from infected Intestine 407 cells. Groups C and D hamsters (four male and four female [each]) were inoculated with 0.65-μm- and 0.2-μm-pore-size filtrates from infected Intestine 407 cells.

**Necropsy and tissue processing.** Hamsters were killed by an intraperitoneal injection of 25 mg of sodium pentobarbital at day 28 postinoculation. A terminal blood sample of 2 ml was drawn from the right ventricle for serum titer determination by enzyme-linked immunosorbent assay (ELISA), and the ileum was aseptically removed. A small section of the ileum was fixed in McDowell-Trump fixative (13) for light and electron microscopy. The remainder of the ileum was either frozen (−80°C) for later use or further processed for organism reisolation. Sections for light microscopy were routinely processed, embedded in paraffin, cut at 4 μm, and stained with hematoxylin and eosin and with Warthin-Starry silver stains (12) for microscopic evaluation. Light microscopic sections were coded and evaluated without knowledge of experimental group assignment.

**Reisolation procedures.** Reisolation procedures were identical to those utilized in the initial isolation with the exception that only Intestine 407 cells were inoculated for organism reisolation. The ilea of three hamsters from group C (0.65-μm-pore-size filtrate inoculated) and two hamsters from group A (control cell inoculated) were independently processed for reisolation of the organism.

**Transmission electron microscopy.** Hamster ileal sections fixed in McDowell-Trump fixative were washed twice in 0.122 M Millonig's buffer, immersed in 1.33% osmium tetroxide in 0.122 M Millonig's buffer, dehydrated in graded ethanol solutions, cleared in propylene oxide, and embedded in Epon 812. Alkaline toluidine blue-stained thick sections were examined for selection of thin sections. Thin sections were mounted on 300-mesh copper grids, stained with lead

citrate-uranyl acetate, and examined with a Philips 300 electron microscope.

Infected Intestine 407 cells (passage 13) for electron microscopy were removed from the culture flask by scraping, pelleted by centrifugation (400 × *g*, 10 min), and fixed in 2.5% glutaraldehyde in Millonig's buffer. The cells were then processed as described above except for the necessity of pelleting the cells by centrifugation between steps.

**ELISA technique.** The terminal serum samples, day 28 postinfection, were evaluated for serum titer to the organism by ELISA. A crude whole-cell preparation was used as the antigen in the ELISA procedures. Supernatant from infected Intestine 407 cell cultures (passages 25 to 32) was centrifuged at 400 × *g* for 10 min to remove large particulates and cells. The supernatant was then centrifuged at 12,000 × *g* for 15 min at 4°C to pellet the bacteria. The pellet was washed once with phosphate-buffered saline (PBS; 0.01 M., pH 7.4, with 0.02% $NaN_3$), recentrifuged, resuspended in PBS to a density corresponding to a McFarland standard of 2, and refrigerated until used. *C. jejuni* (ATCC 29428), isolated from brucella broth, was similarly processed as a control antigen.

Ninety-six-well assay plates (E.I.A. Microtiter Plates; Flow Laboratories, McLean, Va.) were coated with a 1:64 dilution of both the isolated bacterial antigen and *C. jejuni* at 100 μl per well and dried overnight at 37°C. Wells were incubated with 100 μl of absolute methanol containing 0.3% $H_2O_2$ for 30 min at 37°C to inactivate endogenous peroxidase activity. The plates were then washed twice in ELISA wash buffer (0.15 M NaCl, 0.1 M Tris-HCl, 0.02% Tween 20 [pH 7.4]) and incubated for 1 h at room temperature with 200 μl of PBS containing 0.20% (vol/vol) Tween 20 and 2.5% (wt/vol) nonfat powdered milk per well. Plates were washed twice with ELISA wash, and 100 μl of test sera diluted from 1:100 to 1:12,800 in PBS was applied for 1 h at room temperature. After being washed three times, plates were incubated for 1 h at room temperature with 100 μl of peroxidase-labeled goat anti-hamster immunoglobulin G (IgG) diluted 1:2,500 in ELISA wash per well. Following an additional three washes, bound antibody was detected by the addition of 100 μl of 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid) substrate (Sigma) for 15 min, and the $A_{405}$ was determined with an automated ELISA reader (Titertek Multiscan; Flow Laboratories). All assays were performed in duplicate and were repeated if values differed by greater than 10%. Absorbance values greater than 0.2 absorbance units above that of the control (*C. jejuni*) were considered positive. Pooled sera from adult female hamsters from a closed colony without a history of proliferative ileitis were included as a negative control with all tests.

**Polyclonal antibody production.** The bacterial isolate was purified from Intestine 407 cell culture (passage 15 and 16) for antibody production. Culture supernatant was centrifuged at 400 × *g* for 10 min to remove cell debris, and the resulting supernatant was filtered through a 0.65-μm-pore-size cellulose acetate filter (Whatman). The filtrate was then centrifuged (12,000 × *g* for 15 min at 4°C), and the pelleted organisms were washed once in PBS, recentrifuged, and resuspended in 10 ml of PBS containing 4% formalin. Following overnight incubation at 4°C, the organisms were again isolated by centrifugation, washed once in Dulbecco's PBS, recentrifuged, and resuspended in Dulbecco's PBS to a concentration approximating a McFarland standard of 0.5. The organism suspension was then emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co.).

Two male rabbits were utilized for antibody production. Each rabbit was injected with a total of 0.5 ml of the adjuvant-organism mixture divided into 10 sites intradermally on the back. A booster injection of 1.0 ml of the organism in Dulbecco's PBS was given subcutaneously at 40 days postinjection, and sera were collected 7 days later.

The IgG fraction of the rabbit antisera was purified by precipitation with ammonium sulfate (33% final concentration) followed by ion-exchange chromatography on DEAE cellulose (Whatman DE-52) equilibrated and eluted with 0.0175 M phosphate buffer, pH 6.3. The resulting IgG fractions were then reprecipitated with ammonium sulfate, isolated by centrifugation, and desalted on a Sephadex G25 column (Pharmacia LKB Biotechnology, Inc.) equilibrated and eluted with PBS.

**Immunohistochemistry.** Fixed ileal sections embedded in paraffin and cut to 4 μm were utilized for immunohistochemistry. Sections were routinely deparaffinized in xylene and rehydrated through decreasingly concentrated ethanol solutions. The sections were then incubated for 1 h in 0.03% $H_2O_2$ in absolute methanol to remove endogenous peroxidase activity. Following washing in PBS, the sections were incubated for 1 h in a blocking solution of 0.2% (vol/vol) Tween 20 and 2.5% (wt/vol) nonfat dry milk in PBS. Following an additional wash, the rabbit anti-organism antibody was applied at a dilution of 1:100 in PBS, and the slides were incubated overnight at 4°C in a humidified chamber. The slides were then washed four times in ELISA wash and incubated for 3 h with affinity-purified peroxidase-labeled goat anti-rabbit IgG (Jackson Immunoresearch) diluted 1:500 in ELISA wash. The sections were then washed four times in ELISA wash, and bound antibody was detected with a commercial peroxidase detection system for immunohistochemistry (HistoMark Black; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) by following the manufacturer's directions. Controls to ensure specificity included both infected and uninfected Intestine 407 cells, preimmunization rabbit sera, and organism-adsorbed immune rabbit antibody.

## RESULTS

**Isolation and propagation of the organism.** Inoculation of cell cultures with two of the bacterial suspensions resulted in heavy bacterial contamination and discarding of the cell cultures after 18 h of incubation. Cell cultures inoculated with the remaining bacterial suspension appeared to be free of bacterial contamination, incubation was continued, and the flasks were split (2:1) on day 4. Intracellular organisms were first observed in both the Intestine 407 and GPC-16 cell lines by phase microscopy on day 8 following inoculation. Clusters of minute organisms within the cells were readily identified by their rapid motility. The clusters of organisms were extremely variable in size, from barely discernible within the cell cytoplasm at a ×400 magnification to large clusters completely filling the distended cells (Fig. 1). Occasional cells with small clusters of organisms were observed in mitosis with the cell cluster apparently transferring to one of the daughter cells. No evidence of intracellular bacteria was observed in either the HaK or Vero cell lines.

The organism continued to multiply in the original cell cultures (with additional 2:1 splittings on days 14, 18, and 20) through day 19 postinoculation (Intestine 407) and day 21 postinoculation (GPC-16), when the cultures were contaminated. The organism was readily and continually (more than 40 passages) propagated by infecting confluent Intestine 407 cell cultures with the supernatant from infected cultures (1

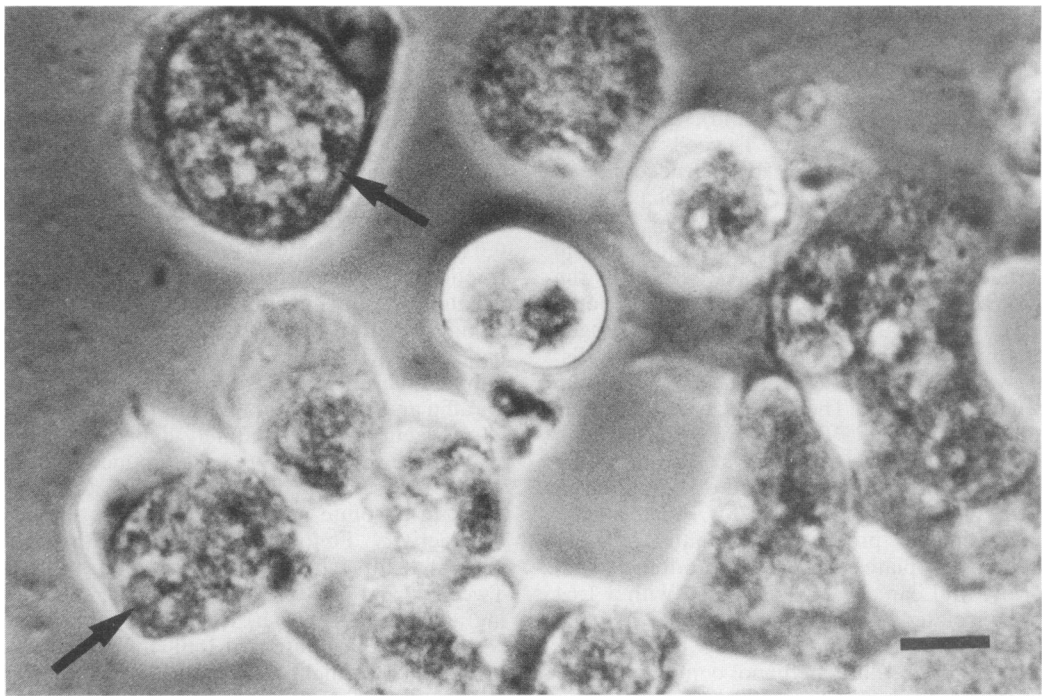

FIG. 1. Intestine 407 cells 48 h after infection with the intracellular organism. Approximately 90% of the cells are infected, and the monolayer has begun to degenerate. Numerous motile organisms are present within vacuoles in the cells (arrows). Phase-contrast microscopy. Bar = 10 μm.

ml of infected supernatant per 25-cm$^2$ surface area). During the first five passages in cell culture, intracellular organisms could be detected by phase microscopy within 72 h of infection, with the interval between infection and microscopic detection decreasing in later passages. Cell culture monolayer viability decreased from 10 days (passage 1) to 6 days by passage 5. Following the initial five passages in cell culture, organisms were detected by phase microscopy at 24 h post-cell culture infection, and monolayer destruction was evident in 4 to 6 days following infection. No changes in bacterial morphology were evident following up to 40 passages in cell culture. Growth of the organism appeared to be substantially greater on the Intestine 407 cell line, and passage 12 of this line was utilized for inoculum preparation.

**Necropsy results.** The gastrointestinal tract of all control-inoculated (group A) and 0.2-μm-pore-size filtrate-inoculated (group D) hamsters appeared grossly normal at necropsy (Fig. 2). No evidence of epithelial cell proliferation or intracellular bacteria was present on light microscopic examination of hematoxylin- and eosin-stained or Warthin-Starry-stained ileal sections from hamsters in either of these groups.

In four (two male and two female) of the eight group B hamsters (cell-free infected inoculate) and four (three male and one female) of the eight group C hamsters (0.65-μm-pore-size filtrate), the ileum was grossly thickened (Fig. 3). Ileal wall thicknesses varied from 2 to 5 mm in these cases. Ileal wall necrosis and abscessation with secondary fibrinopurulent peritonitis was present in three of these hamsters (one from group B and two from group C). Light microscopic examination of ileal sections from hamsters with grossly evident ileal thickening revealed immature and disorganized villus epithelial cells with mitotic figures extending from the crypt region up the entire length of the villus (Fig. 4). Numerous small curved bacteria were visible in the apical cytoplasm of the epithelial cells in silver-stained ileal sections of all hamsters with grossly evident ileal thickening (Fig. 5). A mixed inflammatory cell infiltrate of neutrophils and macrophages was present in the lamina propria of all hamsters with gross lesions. Mild focal epithelial cell proliferation and intracellular bacteria were present in an addi-

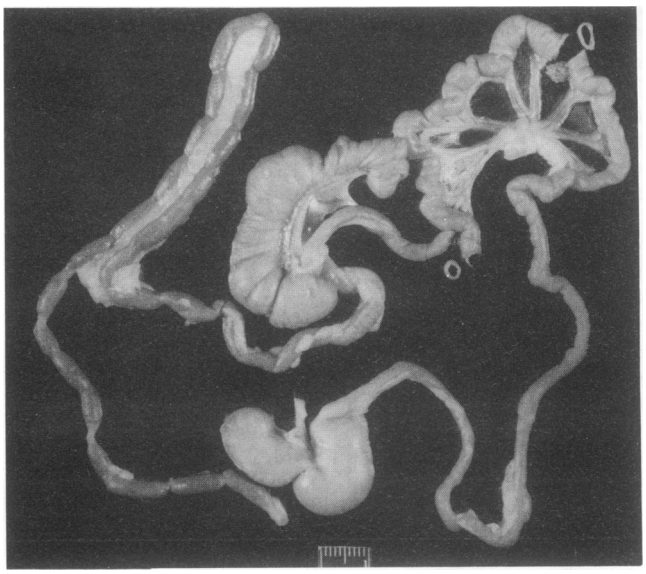

FIG. 2. Gastrointestinal tract from hamster inoculated with the 0.2-μm-pore-size filtrate (group D). No thickening is present. A 1-cm scale is shown.

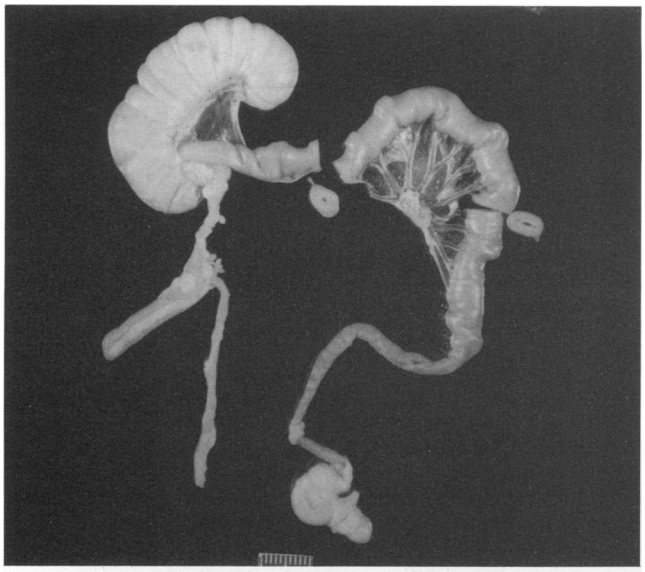

FIG. 3. Gastrointestinal tract from group C hamster inoculated with a 0.65-μm-pore-size filtrate. Ileum and terminal jejunum are severely thickened with lumenal constriction. A 1-cm scale is shown.

tional two (one from group B and one from group C) of the eight hamsters that appeared normal at gross necropsy.

**Reisolation results.** Three of the five bacterial suspensions inoculated into Intestine 407 cell cultures resulted in heavy bacterial contamination at 18 h of incubation, and these cultures were discarded. The remaining two cell cultures (one group A and one group C) remained free of bacterial contamination through day 7 of incubation when intracellular organisms were detected by phase microscopy in the group C (0.65-μm-pore-size filtrate) inoculated cell culture. Intracellular bacteria were not detected in the group A (control) inoculated cell culture through 14 days of incubation.

**Microbiologic results.** Of the eight bacterial suspensions, three from the original isolation and five from reisolation, only those resulting in contamination of the cell cultures produced any growth on Trypticase soy agar–5% sheep blood plates. *Pseudomonas aeruginosa*, one to eight colonies per 250 μl, was isolated from all five contaminated suspensions. No other isolates were detected by any of the incubation conditions.

**Transmission electron microscopy results.** Numerous intracellular bacteria were observed within the cytoplasm of ileal epithelial cells from hamsters inoculated with the 0.65-μm-pore-size filtrate (group C). The bacteria were free within the epithelial cell cytoplasm (Fig. 6). Macrophages with intracellular bacteria both free in the cytoplasm and in phagosomes were present adjacent to epithelial cells. The bacteria were typically slightly curved on longitudinal sections and measured 1.4 to 1.6 μm in length by 0.25 to 0.38 μm in width. The bacterial cell wall appeared trilaminar and irregular. No flagella were observed.

Examination of infected Intestine 407 cells revealed the presence of bacteria morphologically identical to those seen in the ileal cells of infected hamsters (Fig. 7). The bacteria in the Intestine 407 cells measured 1.4 to 1.7 μm in length by 0.26 to 0.34 μm with a similar trilaminar irregular cell wall. A majority of the bacteria in the Intestine 407 cells were free within the cell cytoplasm, although some were membrane bound within vacuoles.

**ELISA results.** Serum titers of hamsters from group A (control inoculated) ranged from <1:100 to 1:1,600, with a median titer of 1:400. The serum titers from the group D (0.2-

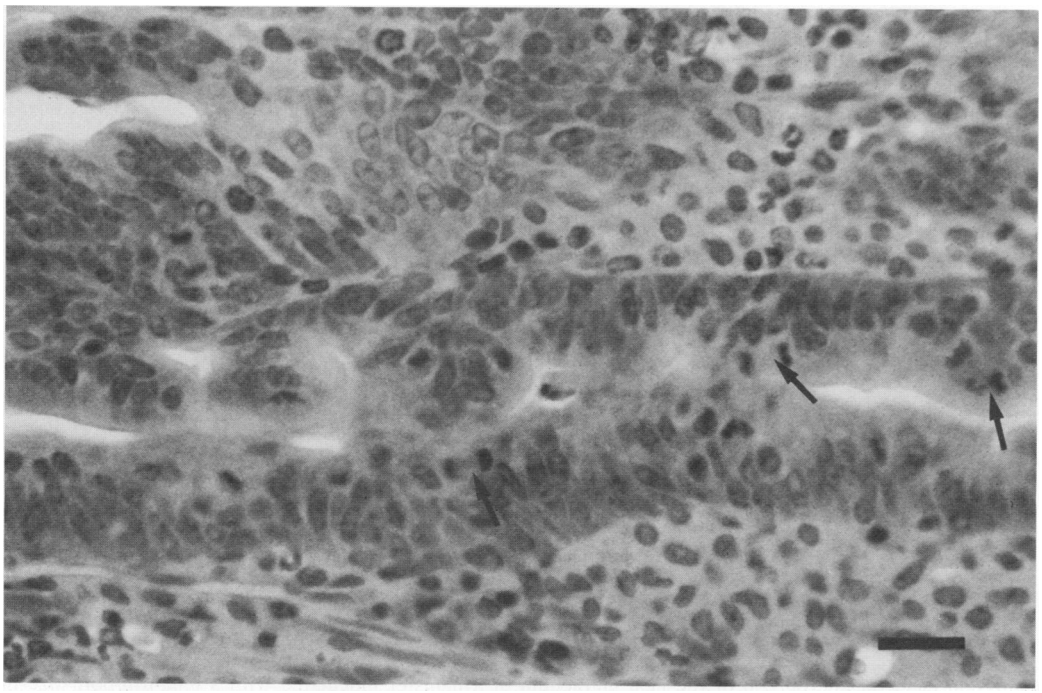

FIG. 4. Ileal section from hamster infected with a 0.65-μm-pore-size filtered inoculate. Immature epithelial cells with numerous mitotic figures (arrows) are visible extending the length of the villus. Hematoxylin and eosin stain. Bar = 20 μm.

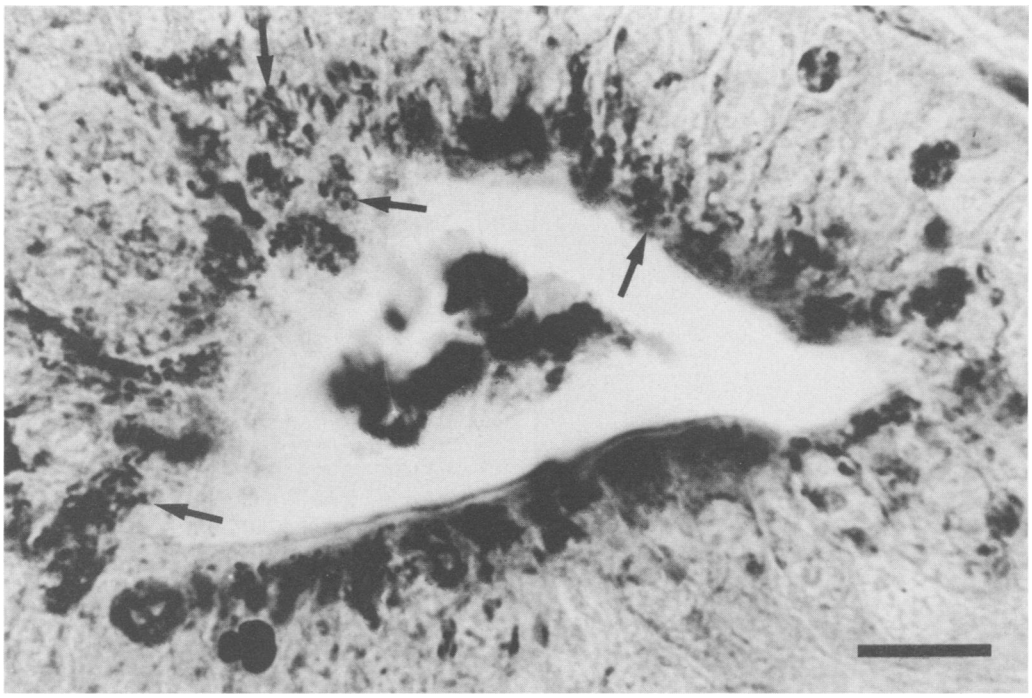

FIG. 5. Ileal section from hamster infected with a 0.65-μm-pore-size filtered inoculate. Numerous slightly curved bacteria are present in the apical cytoplasm of the epithelial cells and extend into the lumen (arrows). Warthin-Starry silver stain. Bar = 10 μm.

μm-pore-size filtrate inoculated) hamsters ranged from <1:100 to 1:800, with a median titer of 1:400. Serum titers of hamsters from group C (0.65-μm-pore-size filtrate inoculated) ranged from 1:1,600 to >1:12,800, with a median titer of 1:6,400, while the titers of the group B hamsters ranged from 1:1,600 to >1:12,800, with a median of 1:3,200. The serum titers of the group B and group C hamsters were significantly ($P < 0.01$, Mann-Whitney test) higher than the

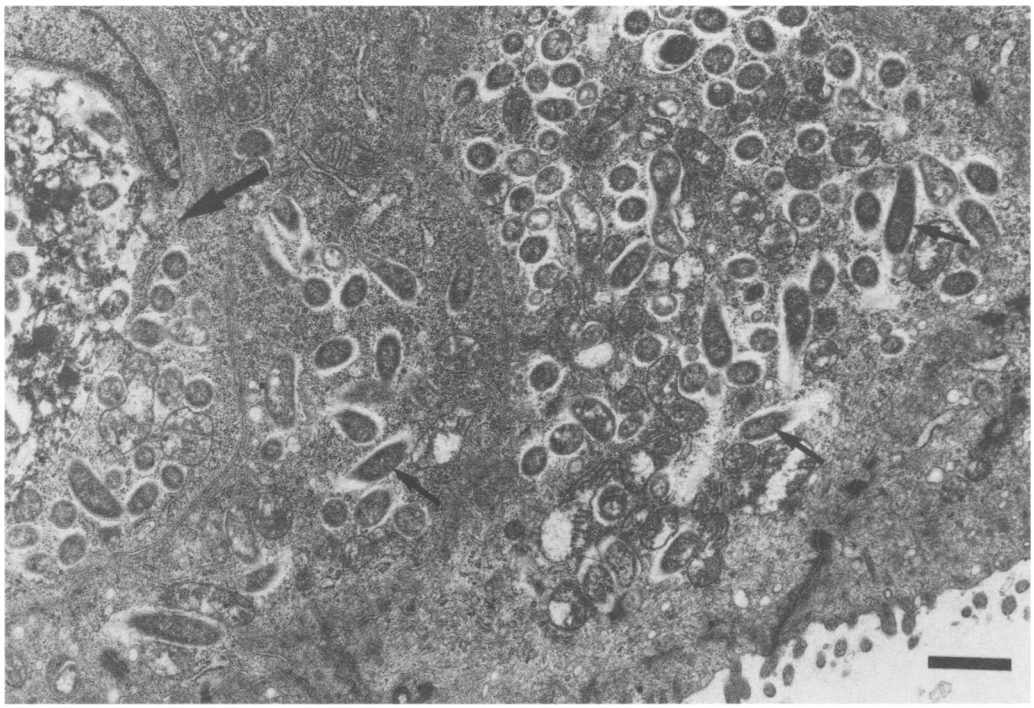

FIG. 6. Transmission electron micrograph of the ileal epithelium of a hamster inoculated with a 0.65-μm-pore-size filtrate (group C). Numerous slightly curved bacteria are present within the cytoplasm of the epithelial cells (small arrows). A macrophage with both free intracytoplasmic bacteria and bacteria within a phagosome is also present (large arrow). Bar = 1 μm.

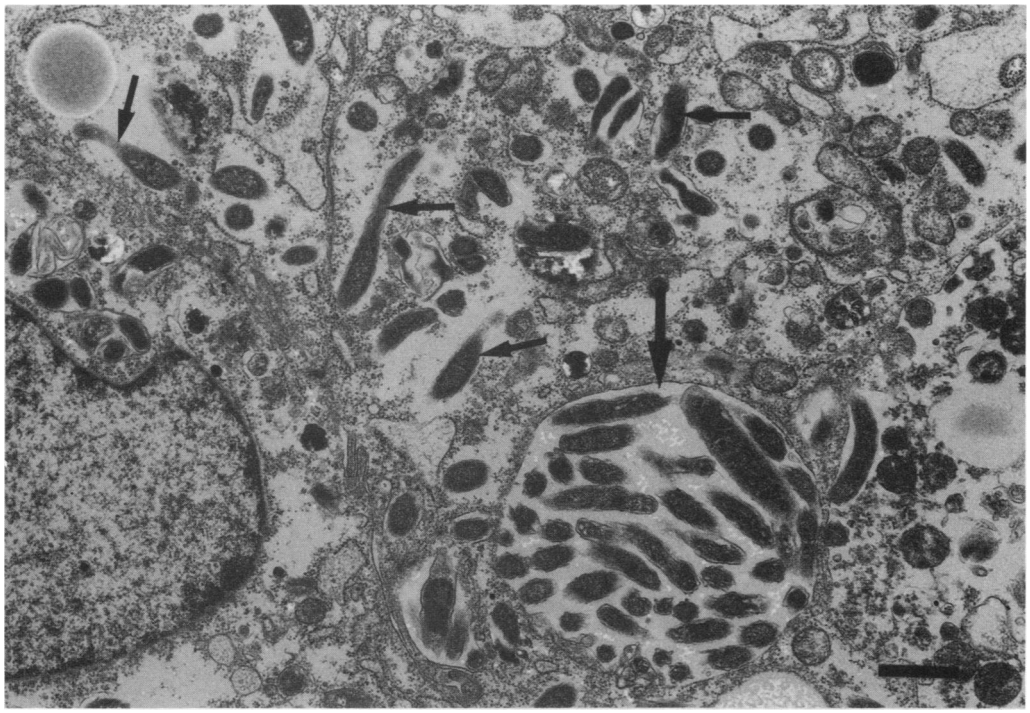

FIG. 7. An electron micrograph of infected Intestine 407 cells. Numerous slightly curved bacteria are free within the cell cytoplasm (small arrows). A membrane-bound vacuole filled with bacteria is also present (large arrow). Bar = 1 μm.

titers of either group A or group D hamsters (Fig. 8). The highest titers, ranging from 1:6,400 to >1:12,800, were recorded from hamsters with grossly evident ileal thickening at necropsy. The pooled negative control sera consistently titered at <1:100.

**Immunohistochemistry results.** Organisms labeled with the polyclonal rabbit anti-organism antibody were present in the ileal sections from all 10 hamsters with histologic evidence of proliferative ileitis. Organisms were primarily confined to the apical portions of ileal epithelial cells, with few organisms being present in the lamina propria or within the lumen (Fig. 9). The distribution of organisms corresponded to the distribution of silver-stained organisms seen on the Warthin-Starry-stained sections from the same hamster. No stained organisms were present within the ileal epithelial cells from any of the eight group A or eight group D hamsters (Fig. 10).

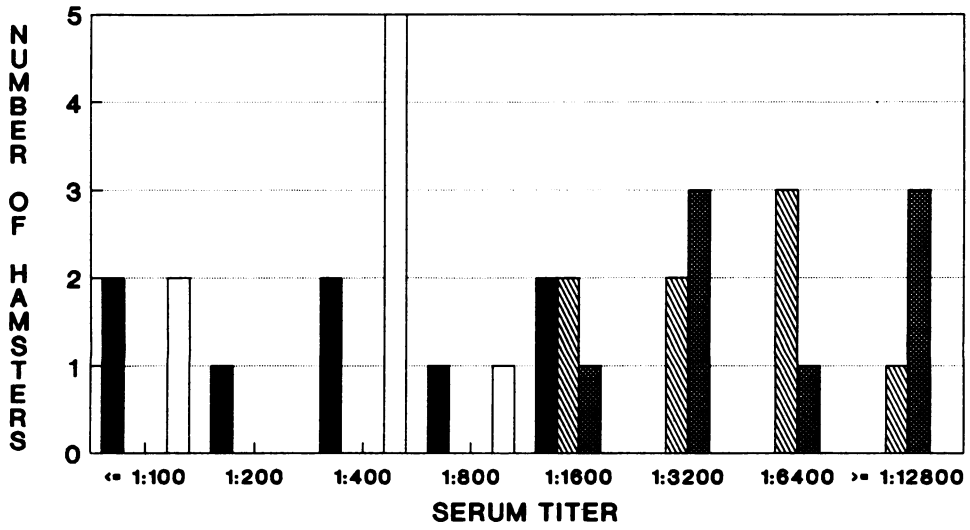


FIG. 8. The distribution of serum titers to the intracellular organism for the four experimental groups is shown. Serum titers for group A (■; control filtrate) and group D (□; 0.2-μm-pore-size filtrate) range from ≤1:100 to 1:1,600, while titers from group B (▨; infected filtrate) and group C (▩; 0.65-μm-pore-size filtrate) range from 1:1,600 to ≥1:12,800.

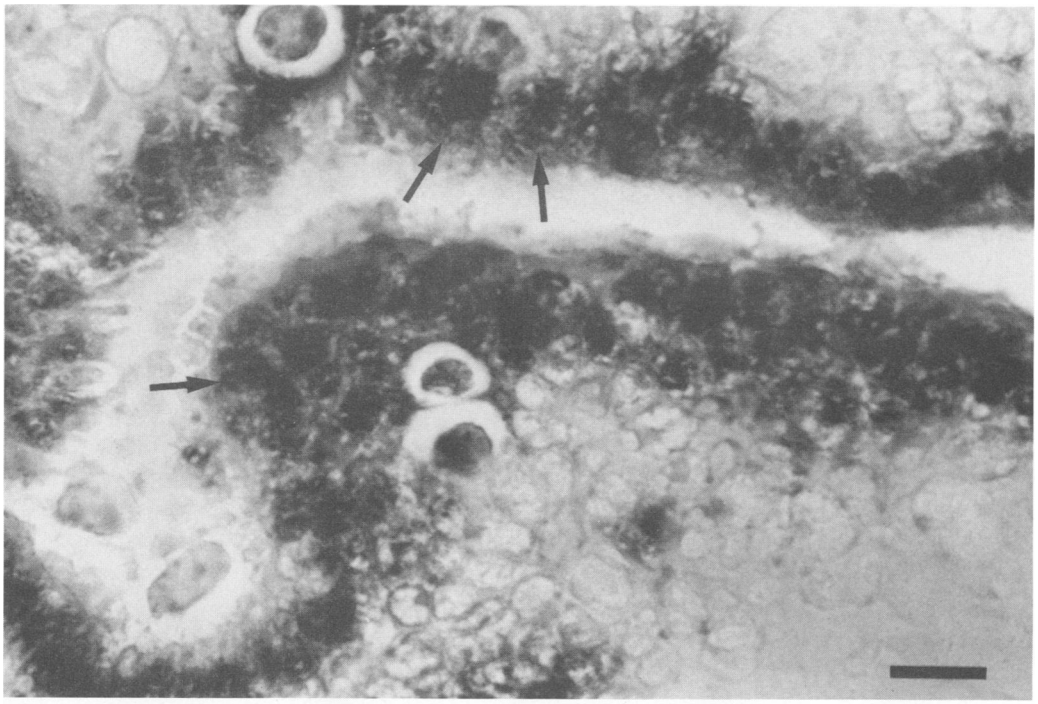

FIG. 9. An immunoperoxidase-stained ileal section from a group C (0.65-μm-pore-size filtrate inoculated) hamster. Intracellular bacteria specifically labeled with the rabbit anti-organism antibody are primarily confined to the apical portions of the epithelial cells (arrows), corresponding to the location of silver-stained bacteria on Warthin-Starry-stained sections (from the hamster in Fig. 5). Bar = 10 μm.

Organisms specifically labeled with the polyclonal rabbit anti-organism antibody were also present in the infected Intestine 407 cells (Fig. 11). No reactivity was present with uninfected Intestine 407 cells, and reactivity was completely eliminated by adsorption of the rabbit antisera with formalin-fixed organisms.

## DISCUSSION

The organism isolated in this study appears to fulfill the criteria to be considered the etiologic agent of hamster proliferative ileitis. The organism was isolated from hamsters with proliferative ileitis and was propagated by utilizing cell culture methodology. The typical lesions of proliferative ileitis were successfully reproduced in hamsters inoculated with the infected cell-free filtrate and the 0.65-μm-pore-size filtrate, while littermates given the uninfected control cell-free filtrate and 0.2-μm-pore-size filtrate remained free of disease. The organism was then reisolated from one of the 0.65-μm-pore-size filtrate-inoculated hamsters which developed proliferative ileitis. The organism was not recovered from hamsters inoculated with uninfected control cell filtrate or the 0.2-μm-pore-size filtrate.

The possible involvement of a virus or other organism coisolated with the bacteria as the etiologic agent is unlikely. The 0.2-μm-pore-size filtrate did not reproduce the disease, suggesting that the causative organism is larger than 0.2 μm and is probably not a virus. Extensive transmission electron microscopic examinations have also failed to detect any viral particles or other bacteria within the infected Intestine 407 cells. The nearly identical ultrastructural morphology of the bacterium within the infected Intestine 407 cells and infected hamster ileal epithelium supports the role of the bacterium in disease pathogenesis.

The significantly elevated serum titers in hamsters infected with the organism, compared with those of hamsters given the uninfected cell-free filtrate or the 0.2-μm-pore-size filtrate and the association of the highest titers with gross lesions of proliferative ileitis support the involvement of this organism in disease pathogenesis. The immunohistochemistry studies using a specific polyclonal antibody confirm that the curved organisms seen in the epithelial cells of the hamsters with experimentally induced proliferative ileitis are the same that were present in the cell culture, further supporting the role of this organism in the disease pathogenesis. The lack of cross-reactivity (data not shown) of the rabbit antibody with members of the genus *Campylobacter* (*C. jejuni*, *C. hyointestinalis*, and *C. coli*) suggests that this organism is antigenically distinct and probably not a member of the genus *Campylobacter*.

As reported by other investigators (9, 14, 16, 20), flagella were not observed in any of the light or electron microscopic preparations. The organism exhibited a dramatic motility within the cytoplasm of infected Intestine 407 cells examined by phase microscopy, indicating the presence of some mechanism for motility. Further studies are necessary to more fully define the overall bacterial morphology and to elucidate the mechanism of motility.

The inability, to date, to propagate this organism on artificial media presents difficulties. The isolation procedures utilized in recovering this organism are lengthy and labor intensive and result in low levels of success because of contamination with other bacterial organisms present in the intestinal lumen. Improved isolation and propagation proce-

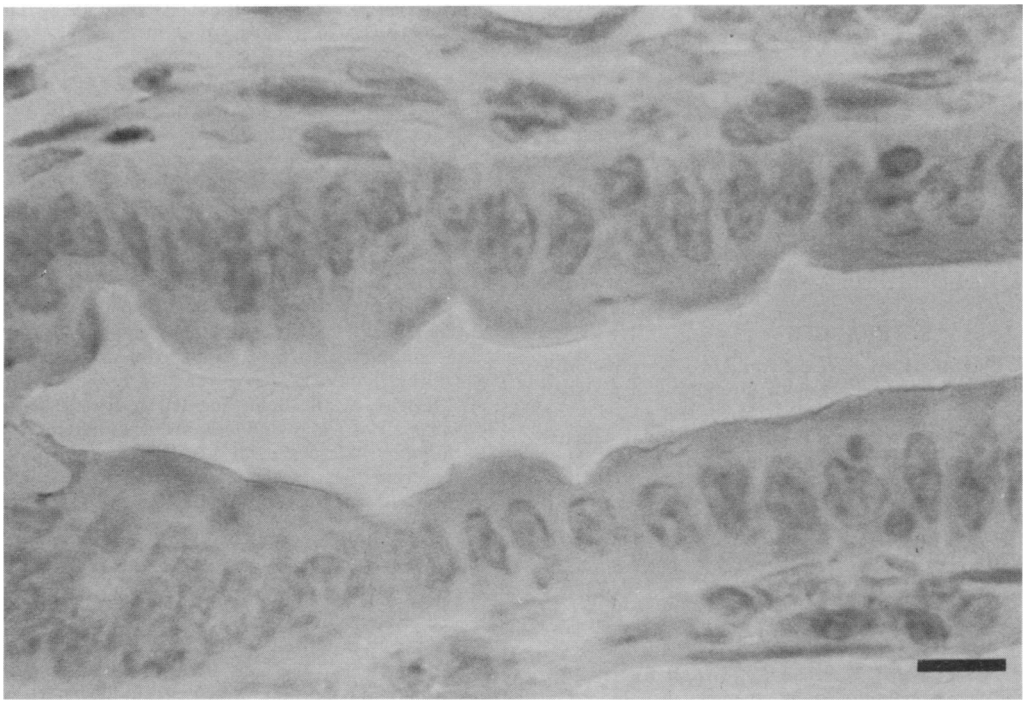

FIG. 10. An immunoperoxidase-stained ileal section from a group D (0.2-μm-pore-size filtrate inoculated) hamster. Section was stained identically to section shown in Fig. 9. No staining of intracellular bacteria is present. Bar = 10 μm.

dures are necessary to fully evaluate the role of this organism in the pathogenesis of proliferative ileitis.

Standard biochemical tests for characterization and identification are presently unfeasible, necessitating the use of other methods for classification. Genomic DNA homology and 16S rRNA analyses are needed for further classification of this organism. Information gained from these studies may also lead to successful artificial medium isolation strategies

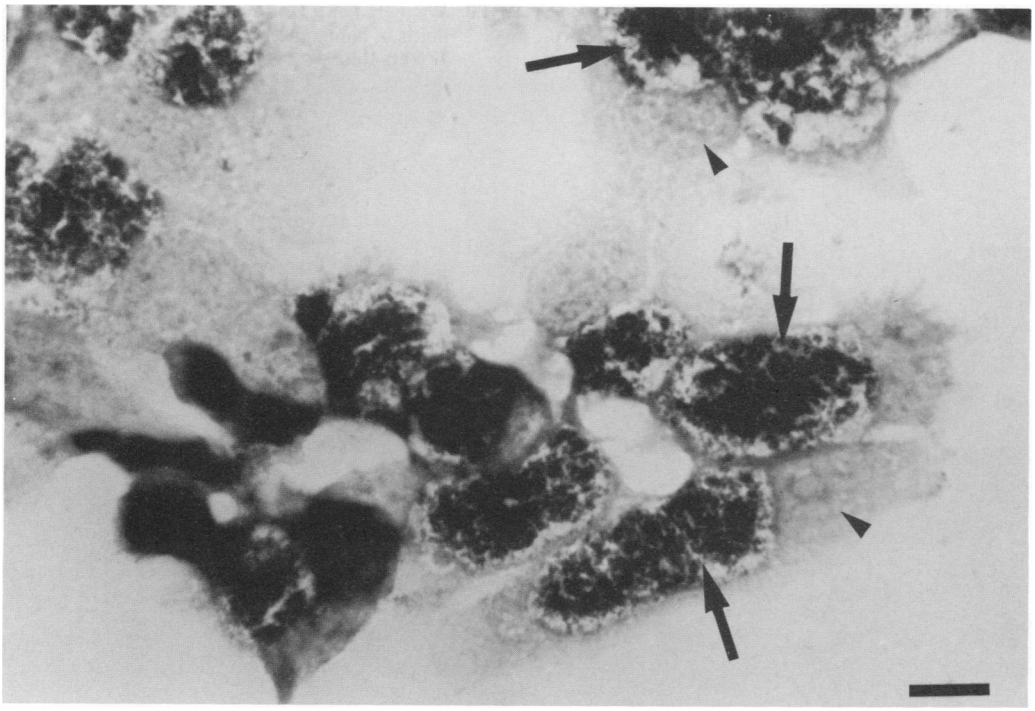

FIG. 11. Infected Intestine 407 cells stained with the rabbit anti-organism antibody and the immunoperoxidase technique. Clusters of stained intracellular bacteria are present within the cell cytoplasm (arrows). The cell nuclei (arrowheads) are displaced to the outer margin of the cells. Bar = 10 μm.

which would dramatically reduce the difficulty in studying the role of this organism in the proliferative intestinal diseases of many species.

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