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Intracellular *Campylobacter*-Like Organism from Ferrets and Hamsters with Proliferative Bowel Disease Is a *Desulfovibrio* sp.

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Proliferative bowel disease is an intestinal disorder of a variety of domestic animals associated with the presence of an intracellular *Campylobacter*-like organism (ICLO). We have identified the ICLO obtained from a ferret with proliferative colitis by 16S rRNA sequence analysis. In this ferret, proliferative bowel tissue containing the ICLO had translocated to the mesenteric lymph nodes, omentum, and liver. The 16S rRNA genes of the ICLO were amplified from an infected fragment of extraintestinal tissue by using universal prokaryotic primers. Approximately 1,480 bases of the amplified 16S rRNA gene were sequenced by cycle sequencing. Comparison of the sequence of the ICLO with those of over 400 bacteria in our data base indicated that the sequence of the ICLO was most closely related to that of *Desulfovibrio desulfuricans* (87.5% similarity). Phylogenetic analysis with 12 *Desulfovibrio* species and 20 species from related genera placed the ICLO in a subcluster within the genus *Desulfovibrio* with *D. desulfuricans* and 5 other *Desulfovibrio* species. We will refer to this organism as the intracellular *Desulfovibrio* organism (IDO). Specific primers were produced for PCR amplification of a 550-base fragment of the 16S rRNA gene of the IDO in proliferative intestinal tissue samples. This unique 550-base segment was amplified from samples of frozen intestinal tissue from nine ferrets and three hamsters with ICLO-associated disease but not in four intestinal tissue samples from animals without the ICLO-associated disease. The 550-base amplified products from the bowel tissues of one hamster and one ferret were fully sequenced. The ferret IDO partial sequence was identical to the previously determined 16S rRNA sequence over its length, and the hamster IDO sequence differed by a single base. The same intracellular organism has been identified in proliferative intestinal tissues of swine and that the organism has been successfully maintained in tissue culture. The availability of specific primers for PCR-based detection of this intracellular *Desulfovibrio* organism will aid in the determination of its role in the pathogenesis of proliferative bowel disease in a variety of infected hosts.

Proliferative bowel disease was first reported in pigs in the 1930s and has since been well characterized clinically and histopathologically in a variety of mammalian hosts, particularly swine and hamsters (2, 21, 27, 32). The illness has also been described in ferrets and rabbits, as well as other wild and domestic animals (6, 7, 10, 43). The disease manifests itself clinically as watery, sometimes bloody diarrhea, anorexia, and rapid weight loss. Mortality varies with the specific outbreak and species affected. Histologically, the intestinal lesions consist of proliferation of mucosal epithelia, sometimes accompanied by multiple foci of superficial epithelial necrosis, crypt abscesses, numerous macrophages, neutrophils, lymphocytes, and plasma cells in the lamina propria, and in certain cases, giant cells of both the Langerhans and foreign-body types (6, 10, 21, 32, 43). A universal feature of the disease is the presence of comma-shaped intracellular organisms within the apical parts of columnar epithelial cells, demonstrated by Warthin-Starry staining (7, 27, 43). These organisms have been referred to as intracellular *Campylobacter*-like organisms" (ICLO). Fluorescently labeled monoclonal and polyclonal antibodies directed against the ICLO isolated from pigs and hamsters also recognize ICLO present in infected epithelia of ferrets and rabbits (9, 27, 43). In this report, we use the term ICLO to refer to intracellular comma-shaped organisms that stain with Warthin-Starry stain and react with omega antiserum.

The disease has been studied extensively for several decades, and all early attempts to cultivate the intracellular organisms were unsuccessful. Experimental production of the disease in pigs and hamsters depended on oral inoculation of diseased intestinal tissue containing ICLO (21, 32). These intestinal homogenates were not infective after passage through filters which effectively retain bacteria but allow passage of viruses and rickettsiae (21, 32). Recently, however, intracellular organisms were isolated from hamsters and swine with proliferative bowel disease and grown in tissue culture (26, 47). When the hamster isolate was inoculated orally into healthy hamsters, the disease was successfully reproduced (47). The ICLO from swine with proliferative bowel disease was recently identified by 16S rRNA analysis as being closely related to *Desulfovibrio desulfuricans* (13). McOrist et al. reported that inoculation of the intracellular *Desulfovibrio* organism could reproduce the disease in conventional but not germfree pigs (31).

In this study, we used 16S rRNA sequencing to identify the ICLO present in extraintestinal tissue from a ferret with proliferative bowel disease (11). This organism was essentially identical to the swine ICLO identified by Gebhart et al. (13). We use the term intracellular *Desulfovibrio* organism (IDO) to refer to the organism identified by molecular sequence data. On the basis of 16S rRNA sequence information, highly specific PCR primers were designed and used to detect the organism in proliferative bowel tissues from nine ferrets and three hamsters.

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MATERIALS AND METHODS

Clinical samples from ferrets and hamsters. All of the ferrets examined in this study had clinical signs compatible with proliferative colitis: lethargy, rapid weight loss, dehydration, and diarrhea (10). Ferret 1, the index case with extraintestinal ICLO, had been treated with orally administered chloramphenicol, while all of the other ferrets were received from the vendor, examined, and then euthanized (11).

Necropsy and histology. Samples of normal-appearing and visibly thickened colonic tissue were excised from nine ferrets and frozen. Extraintestinal lymph nodes and omental nodules from ferret 1 were also excised and frozen (11). Frozen hamster ileal tissue from three previously studied outbreaks of ICLO-associated proliferative ileitis were provided by Robert Jacoby (Yale University, New Haven, Conn.) and Maria LaRegina (Washington University, St. Louis, Mo.). Additional fresh colonic sections from the nine ferrets with thickened colons were placed in 10% buffered formalin and processed for histologic evaluation. Tissue was processed by standard methods, embedded in paraffin, cut in 5-μm sections, and stained with hematoxylin and eosin and Warthin-Starry stain.

Fluorescein labeling with omega antiserum. Hyperimmune rabbit antiserum was prepared by using partially purified bacterial antigens of bacteria released from epithelial cells of intestines of swine with proliferative enteropathy. This serum reacts with intracellular organisms present in swine, hamster, ferret, and rabbit proliferative bowel disease (9, 27, 43). Fluorescein-labeled sheep anti-rabbit immunoglobulin G was used as the second antibody in all experiments. Deparaffinized sections of ferret colon and extraintestinal tissue from ferret 1 were processed for fluorescein tissue staining as previously described (11). Briefly, sections were incubated with polyclonal rabbit intracellular organism antiserum, followed by fluorescein-conjugated sheep anti-rabbit immunoglobulin and examined with a Leitz Orthoplan fluorescence microscope (9). Fluorescein labeling with omega antiserum was not performed with hamster tissue.

Extraction of DNA from ferret 1 extraintestinal tissue for PCR amplification and sequencing. Four small (3-mm-diameter) samples were cut from the ICLO-infected extraintestinal tissue of ferret 1 and placed in 1.5-ml Eppendorf tubes. One milliliter of 1% sodium dodecyl sulfate (SDS) in 10 mM Tris (pH 8.3), 10 μg of glycogen, and 505 μl of phenol were added to each tube. The remaining volume of each tube was filled with 0.1-mm-diameter glass beads, and the tubes were sealed with Parafilm. The tissue samples were disrupted by shaking the tubes on a 3110 BX mini-bead beater (Biospec Products) for 4 min. Glass beads and tissue debris were pelleted by centrifugation at 2,000 × *g* for 15 min. DNA was precipitated from the supernatant by adding 0.1 volume of 3 M sodium acetate (pH 5.4) and 2.5 volumes of absolute ethanol and incubating the mixture at −20°C for 24 h. DNA was pelleted by centrifugation for 30 min at 5,000 × *g* at 4°C. The pellets were rinsed with 70% ethanol and centrifuged again. The DNA pellets were vacuum dried for 15 min in a Speed-Vac and finally dissolved in 30 μl of distilled water. Universal prokaryotic primers (primers 1 and 2 [Table 1]) were used to amplify a 1,500-base fragment of the 16S rRNA gene. PCRs were performed with thin-walled tubes and a Perkin-Elmer 480 thermal cycler. Ampliwax PCR Gem100s (Perkin-Elmer) were used in a hot-start protocol suggested by the manufacturer. The conditions used for amplification were as follows: denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 45 s with an additional 5 s added for each cycle. A total of 25 cycles were performed followed by a final elongation step at 72°C for 15 min. The purity of the amplified product was determined by electrophoresis in a 1% agarose gel (FMC Bioproducts, Rockland, Maine). DNA was stained with ethidium bromide and viewed under short-wavelength UV light.

TABLE 1. Oligonucleotide primers used for amplification and sequencing of 16S rDNA

Primer	Type[a]	Sequence (5′–3′)	Positions[b]	Orientation
1	PCR/U	AGAGTTTGATYCTGGCT	8–24	Forward
2	PCR/U	TACGGYTACCTTGTTACGACT	1493–1513	Reverse
3	PCR/S, Seq	GATGATGGGTAGCCGAT	278–294	Forward
4	PCR/S, Seq	CCCAGCACCTAGCACC	822–837	Reverse
5	Seq	ACTGCTGCCTCCCGT	344–358	Reverse
6	Seq	CTACCAGGGTATCTAATC	786–804	Reverse
7	Seq	GGTTGCGCTCGTTGCGGG	1096–1113	Reverse
8	Seq	ACACGTACTACAATGGTG	1227–1245	Forward
9	Seq	ACGGGCGGTGTGTRC	1392–1406	Reverse

[a] Definitions: PCR, primer used for PCR; Seq, primer used for sequencing; U, universal bacterial primer; S, specific IDO primer.
[b] Numbering is based upon the *E. coli* sequence.

Purification of PCR products from ferret 1 extraintestinal tissue. The amplified DNA products from PCR were purified by precipitation with polyethylene glycol 8000 (Sigma) (25). After removal of Ampliwax, 0.6 volume of 20% polyethylene glycol 8000 in 2.5 M NaCl was added and the mixture was incubated at 37°C for 10 min. Samples were centrifuged for 15 min at 15,000 × *g*, and the pellet was washed with 80% ethanol and pelleted as before. The pellet was air dried, dissolved in 50 μl of distilled water, and used in cycle sequencing as described below.

Sequencing methods. DNA samples from PCR were directly sequenced with a TAQuence Cycle Sequencing Kit (United States Biochemical Corp.). The manufacturer's protocol was followed. Primers were end labeled with ^{33}P (NEN/Dupont) by using the manufacturer's protocol. A 110-ng sample of purified DNA from the PCR was used for sequencing. Reaction products were loaded onto 8% polyacrylamide–urea gels, electrophoresed, and detected by exposure of X-ray film for 24 h.

Phylogenetic analysis. A program set for data entry, editing, sequence alignment, secondary-structure comparison, similarity matrix generation, and phylogenetic tree construction for 16S rRNA data was written in Microsoft Quick BASIC for use on IBM PC-AT and compatible computers. rRNA sequences were read from the X-ray films, entered, and aligned as previously described (37). Similarity matrices were constructed by comparing only base positions for which there were data for 90% of the sequences. Distances were corrected for multiple base changes at a single position by the method of Jukes and Cantor (22). Phylogenetic trees were constructed by the neighbor-joining method (42, 49).

Extraction of DNAs from ferret and hamster intestinal tissues. DNAs were isolated from frozen diseased intestinal tissue of nine ferrets with proliferative colitis, from normal intestinal tissue of four healthy ferrets, from the extraintestinal tissue of ferret 1, and from the diseased ileal tissue of three hamsters previously diagnosed with proliferative ileitis. Tissue samples were centrifuged twice in phosphate-buffered saline at 13,000 × *g* for 10 min and suspended in buffer containing 8.0% sucrose, 50 mM Tris HCl (pH 8.0), 50 mM EDTA, and 0.1% Triton X-100. Lysozyme (from chicken egg white; Boehringer Mannheim, Indianapolis, Ind.) was added to a final concentra-

TABLE 2. Strains, sources, and accession numbers

Organism[a]	Culture collection[b] strain designation	GenBank accession no.	Reference
Desulfovibrio sp. Ferret	MIT 87-599	U07570	This report
Desulfovibrio sp. Ferret	MIT 92-472		This report
Desulfovibrio sp. Hamster	MIT 61890E	U07569	This report
Reference organisms			
"*Desulfoarculus baarsii*"[c]	ATCC 33931^T	M34403	4
Desulfobacter curvatus	DSM 3379^T	M34413	4
Desulfobacter hydrogenophilus	DSM 3380^T	M34412	4
Desulfobacter latus	DSM 3381^T	M34414	4
Desulfobacter postgatei	DSM 684	M26633	55
Desulfobacter sp. strain 3ac10	DSM 2035	M34415	4
Desulfobacter sp. strain 4ac11	DSM 2057	M34416	4
Desulfobacterium autotrophicum	DSM 3382^T	M34409	4
"*Desulfobacterium niacini*"[d]	DSM 2650^T	M34406	4
"*Desulfobacterium vacuolatum*"	DSM 3385^T	M34408	4
"*Desulfobotulus sapovorans*"	ATCC 33892^T	M34402	4
Desulfobulbus propionicus	ATCC 33891^T	M34410	4
Desulfobulbus sp. strain 3pr10	DSM 2058	M34411	4
Desulfococcus multivorans	ATCC 33890^T	M34405	4
"*Desulfomicrobium baculatus*"[e]	DSM 1743	M37311	5
Desulfomonile tiedjei	ATCC 49306^T	M26635	55
Desulfosarcina variabilis	DSM 2060^T	M34407	4
Desulfovibrio africanus	ATCC 19996^T	M37315	5
Desulfovibrio desulfuricans (1)	NCIB 8380	M37316	5
Desulfovibrio desulfuricans (2)	ATCC 27774	M34113	36
Desulfovibrio gigas	ATCC 19364^T	M34400	4
"*Desulfovibrio longus*"	SEBR 2582	X63623	29
"*Desulfovibrio pigra*"[f]	ATCC 29098^T	M34404	4
Desulfovibrio salexigens	ATCC 14822^T	M34401	4
Desulfovibrio vulgaris	ATCC 29579^T	M34399	4
Desulfovibrio sp. strain PT-2		M98496	23
Desulfuromonas acetoxidans	DSM 2030	M26634	55

[a] Names that have not been published in the approved lists of bacterial names are enclosed in quotation marks.

[b] Type strains are indicated by a capital T superscript. Culture collections are abbreviated as follows: ATCC, American Type Culture Collection (Rockville, Md.); DSM, Deutsche Sammlung von Mikroorganismen (Braunschweig; Germany); MIT, Massachusetts Institute of Technology (Cambridge) (these strains were not cultivated or deposited); NCIB, National Collection of Industrial Bacteria (Aberdeen, Scotland); SEBR, not known.

[c] Previously *Desulfovibrio baarsii.*

[d] Previously *Desulfococcus niacini.*

[e] Previously *Desulfovibrio baculatus.*

[f] Previously *Desulfomonas pigra.*

tion of 3 mg/ml, and the solution was incubated for 12 min at 37°C. SDS and RNase A (bovine pancreas; Boehringer Mannheim) were added to final concentrations of 1.0% and 50 μg/ml, respectively, and the mixture was incubated for 1 h at 37°C. Pronase and proteinase K (Boehringer Mannheim) were added to final concentrations of 0.8 and 0.5 mg/ml, respectively, and the samples were incubated overnight at 37°C. A 1:10 volume of CTAB-NaCl solution (5% [wt/vol] cetyl-trimethylammonium bromide [Sigma], 0.7 M NaCl) was added, and the solution was gently mixed and incubated at 65°C for 10 min. The DNA was extracted in an equal volume of phenol-chloroform (1:1) and precipitated overnight at −20°C in the presence of 0.3 M sodium acetate and 2.1 volumes of absolute ethanol. The DNA precipitates were then pelleted by centrifugation at 16,000 × *g* for 30 min and allowed to air dry. The pellets were suspended in sterile distilled water, and DNA was quantitated by measuring optical density at 260 nm.

Amplification of DNAs from ferret and hamster intestinal tissues. Specific primers for the ferret IDO (primers 3 and 4 [Table 1]) were used to amplify a 550-base segment of the 16S rRNA gene. From 0.1 to 100 ng of each DNA preparation was added to a 100-μl-final-volume reaction mixture containing 1× *Taq* polymerase buffer (2.25 mM $MgCl_2$, 50 mM KCl, Tris [pH 8.4]), each primer at 5 mM, and each deoxynucleotide triphosphate at 200 mM. Samples were heated to 95°C for 5 min to denature the DNA, briefly centrifuged, and cooled to 56.5°C, at which time 2.5 U of *Taq* polymerase enhancer (Perfect Match; Stratagene, La Jolla, Calif.) was added, followed by an overlay of 100 μl of mineral oil to prevent evaporation. The following conditions were used for amplification: denaturation at 95°C for 1 min, annealing at 56.5°C for 2.25 min, and elongation at 72°C for 1 min. A total of 30 cycles were performed and were followed by a final elongation step at 72°C for 7 min. The purity of the amplified product was determined by electrophoresis in a 3.5% NuSieve agarose gel (FMC Bioproducts). DNA was stained with ethidium bromide and viewed under short-wavelength UV light.

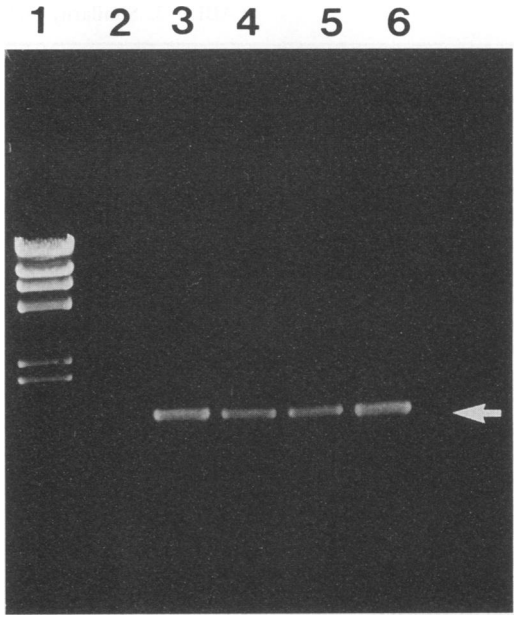

FIG. 1. Agarose gel electrophoresis of DNA amplified from four extraintestinal tissue fragments of ferret 1 by using universal prokaryotic primers 1 and 2 (Table 1). Lanes: 1, *Hin*dIII-digested molecular weight standard; 3 to 6, amplified product from tissue fragments. The arrow indicates a molecular size corresponding to 1,480 bases.

Southern blotting. Samples were electrophoresed in a 3.5% NuSieve agarose gel (FMC Bioproducts) for 5 h at 70 V. DNA was transferred onto a positively charged nylon membrane (Boehringer Mannheim) by using the manufacturer's protocol. A labeled probe was produced by the PCR method outlined previously, except that the final nucleotide mixture contained 200 mM dATP, dCTP, and dGTP, 130 mM dTTP, and 70 mM digoxigenin-11-dUTP (Boehringer Mannheim). Southern blots were prehybridized in a solution composed of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2.0% blocking reagent (Boehringer Mannheim), 0.1% *N*-laurylsarcosine, and 0.2% SDS for 1 h at 68°C. The prehybridization solution was then decanted and replaced with hybridization solution containing a denatured, digoxigenin-11-dUTP-labeled probe (the probe was denatured by heating at 95°C for 10 min followed by cooling for 10 min on a salt-ice slurry). Membranes

TABLE 3. Similarity matrix based on 16S rRNA sequence comparisons

	%Similarity and % difference[a]														
	D.spF	*D.sal*	*D.des*	*D.lon*	*D.afr*	*D.gig*	*D.des*	*D.pig*	*D.PT*	*D.vul*	*D.bac*	*D.lat*	*D.4ac*	*D.3ac*	*D.hyd*
Desulfovibrio sp. Ferret	.	84.6	83.1	83.9	82.6	85.2	87.5	87.7	87.3	86.8	85.1	79.5	78.9	78.9	79.5
Desulfovibrio salexigens	17.2	.	88.9	87.4	85.5	85.8	86.1	85.5	85.9	87.2	86.0	79.6	79.4	79.6	79.7
Desulfovibrio desulfuricans (1)	19.1	12.0	.	87.1	86.5	86.0	85.8	84.5	84.6	86.1	86.6	80.5	80.1	80.8	80.3
Desulfovibrio longus	18.2	13.8	14.2	.	86.1	86.4	87.6	86.0	86.2	86.5	85.4	79.6	78.3	78.7	78.9
Desulfovibrio africanus	19.8	16.1	14.9	15.4	.	86.0	86.8	85.9	85.8	87.0	86.0	80.1	78.9	79.2	79.4
Desulfovibrio gigas	16.5	15.7	15.4	14.9	15.4	.	87.1	87.2	87.5	86.1	85.7	79.5	79.2	78.9	79.4
Desulfovibrio desulfuricans (2)	13.7	15.4	15.8	13.5	14.5	14.2	.	94.7	90.5	90.3	87.6	80.5	80.0	80.2	80.5
Desulfovibrio pigra	13.5	16.1	17.4	15.5	15.6	14.1	5.5	.	90.0	89.7	86.6	80.5	79.9	79.8	80.1
Desulfovibrio sp. PT-2	13.9	15.6	17.3	15.3	15.8	13.7	10.2	10.7	.	94.3	86.7	79.0	78.5	78.3	78.7
Desulfovibrio vulgaris	14.5	14.1	15.4	14.9	14.3	15.4	10.4	11.1	6.0	.	86.5	79.8	79.2	79.2	79.6
Desulfomicrobium baculatus	16.6	15.5	14.8	16.3	15.5	15.9	13.6	14.7	14.6	14.9	.	82.4	82.1	82.5	82.9
Desulfobacter latus	24.0	23.8	22.5	23.8	23.2	23.9	22.6	22.6	24.6	23.5	20.1	.	97.7	97.2	97.0
Desulfobacter sp. 4ac11	24.8	24.1	23.1	25.7	24.7	24.4	23.3	23.4	25.3	24.3	20.4	2.4	.	96.7	96.8
Desulfobacter sp. 3ac10	24.8	23.8	22.2	25.0	24.4	24.8	23.0	23.6	25.7	24.4	19.9	2.9	3.4	.	96.6
Desulfobacter hydrogenophilus	23.9	23.6	22.9	24.8	24.1	24.1	22.6	23.1	25.0	23.8	19.4	3.1	3.2	3.5	.
Desulfobacter curvatus	24.6	23.7	22.7	24.8	24.2	24.0	22.4	22.6	24.7	24.1	19.9	4.6	4.8	4.6	4.3
Desulfobacter postgatei	23.2	22.6	21.5	23.2	22.8	22.2	21.4	21.5	23.8	23.8	19.1	3.6	4.4	3.3	4.2
Desulfobacterium autotrophium	21.7	22.1	22.1	23.4	22.8	22.6	21.8	22.3	23.3	22.3	18.3	11.9	12.4	12.5	11.2
Desulfobacterium niacini	22.6	22.9	22.1	23.2	24.1	24.3	22.9	22.4	23.6	23.3	21.9	13.8	14.2	14.2	12.9
Desulfobacterium vacuolatum	21.5	23.6	21.7	22.5	24.6	24.3	22.7	22.3	23.8	23.5	20.5	14.5	14.0	14.1	12.9
Desufobotulus sapovorans	20.6	23.2	21.7	24.1	23.2	23.2	22.2	22.8	22.0	20.8	19.8	18.8	18.1	18.5	17.6
Desulfosarcina variabilis	22.3	20.5	20.7	21.7	21.9	21.2	21.1	21.4	20.6	19.6	19.7	16.7	17.6	17.3	15.8
Desulfococcus multivorans	22.7	23.0	22.7	23.5	22.4	23.8	21.7	22.3	22.5	21.8	19.8	17.1	18.5	18.5	17.5
Desulfobulbus propionicus	21.8	22.3	21.5	23.3	23.4	21.2	22.6	22.1	22.1	21.6	20.6	19.5	20.6	20.3	19.9
Desulfobulbus sp. 3pr10	24.6	22.7	21.5	23.6	24.7	22.6	24.2	24.0	25.5	23.9	21.8	19.8	21.1	21.5	20.9
Desulfoarculus baarsii	23.0	21.7	20.4	21.6	21.3	20.4	21.7	22.4	21.5	20.1	21.1	21.5	21.9	22.4	21.0
Desulfomonile tiedjei	23.8	22.9	22.0	21.8	24.3	24.0	23.1	23.8	23.6	23.1	21.4	20.8	20.9	21.5	20.0
Desulfuromonas acetoxidans	21.5	19.7	21.5	21.5	22.9	20.6	20.6	20.5	21.4	20.4	19.1	18.8	19.6	19.2	18.7
Myxococcus xanthus	24.3	24.0	25.4	25.1	24.1	23.9	23.5	24.2	24.1	24.3	22.1	25.7	26.3	25.7	24.8
Bdellovibrio stolpii	24.0	24.0	24.3	23.9	25.7	24.2	23.9	24.4	24.7	24.7	20.3	25.0	25.3	25.0	24.4
Campylobacter coli	29.8	29.9	31.1	30.9	31.2	29.8	29.0	28.9	29.7	29.2	28.2	31.8	31.9	32.8	31.3
Arcobacter cryaerophilus	28.7	29.2	30.7	30.3	31.2	29.9	27.7	27.2	28.8	29.4	28.1	30.8	30.7	31.3	30.3
Helicobacter pylori	25.3	28.7	27.9	26.4	28.5	25.8	24.4	24.7	25.0	25.3	24.8	29.8	29.7	29.9	29.0
Wolinella succinogenes	24.3	27.8	28.3	25.7	25.3	24.4	23.6	24.5	25.1	23.9	25.0	29.4	29.8	29.9	28.9

[a] Numbers above the diagonal represent uncorrected percentages of similarity, and those below the diagonal are percentages of difference corrected for multiple base changes by the method of Jukes and Cantor (22).

were then incubated overnight at 68°C and washed as outlined by the manufacturer (Boehringer Mannheim). Membranes were incubated with anti-digoxigenin alkaline phosphatase-labeled antibody (1:1,000 dilution; Boehringer Mannheim) for 2 h and washed and developed in NBT/XPhosphate detection buffer (Boehringer Mannheim) until a color reaction developed (0.5 to 2 h).

Nucleotide sequence accession numbers. The species, strain number, sequence accession number, and literature reference for each organism examined by phylogenetic analysis are shown in Table 2.

RESULTS

Necropsy findings. The necropsy findings on ferret 1 have been described previously, but briefly, ferret 1 had enlarged mesenteric lymph nodes, omental nodules, an adherent mass on the liver, and a thickened distal colon (11). The remaining nine ferrets had segmental thickening of the colon, usually extending from the anus proximally for 10 to 15 cm.

Histology. In ferret 1, the microscopic changes in the intestines were similar to and characteristic of what we have seen in numerous other cases of proliferative bowel disease (10). The extraintestinal sites contained inflammatory infiltrates, as well as ICLO demonstrated by both Warthin-Starry staining and fluorescent labeling with omega antiserum (11). Histologic findings on hamsters were similar to those reported for ferrets, but the ileum was more commonly involved (21, 47).

Confirmation of proliferative colitis with the presence of ICLO in involved mucosal epithelium in the index case, ferret 1, and the other nine ferret colons was based on histological evaluation (10, 11). Histologic colon sections revealed a piled-up polypoid mucosa with numerous mitoses and focal atypia. With the Warthin-Starry stain, numerous comma- and rod-shaped intracellular organisms were present in the apical portion of mucosal epithelia. Sections of normal mucosa had no detectable intracellular organisms.

Fluorescein labeling with Ω antiserum. Fluorescent-antibody staining with polyclonal *Campylobacter* omega antiserum confirmed the presence of omega antigen in the epithelial cells in the proliferative bowel lesions of all of the ferrets, as well as

TABLE 3—*Continued*

%Similarity and % difference[a]																		
D.cur	*D.pos*	*D.aut*	*D.nic*	*D.vac*	*D.sap*	*D.var*	*D.mul*	*D.pro*	*D.3pr*	*D.baa*	*D.tie*	*D.ace*	*M.xan*	*B.sto*	*C.col*	*A.cry*	*H.pyl*	*W.suc*
79.0	80.0	81.1	80.5	81.3	82.0	80.7	80.4	81.1	79.0	80.2	79.6	81.3	79.2	79.4	75.4	76.2	78.5	79.2
79.7	80.5	80.9	80.3	79.7	80.0	82.1	80.2	80.7	80.4	81.1	80.2	82.7	79.5	79.5	75.3	75.8	76.1	76.8
80.4	81.3	80.9	80.9	81.1	81.2	81.9	80.4	81.3	81.3	82.1	80.9	81.3	78.4	79.2	74.5	74.8	76.7	76.4
78.9	80.0	79.9	80.0	80.6	79.4	81.1	79.8	80.0	79.8	81.2	81.1	81.3	78.6	79.5	74.7	75.0	77.7	78.3
79.3	80.3	80.3	79.4	79.0	80.1	81.0	80.7	79.9	79.0	81.5	79.3	80.3	79.4	78.2	74.5	74.5	76.3	78.5
79.5	80.8	80.5	79.3	79.3	80.0	81.5	79.6	81.5	80.5	82.1	79.5	82.0	79.6	79.3	75.4	75.4	78.2	79.1
80.6	81.4	81.1	80.3	80.4	80.8	81.6	81.2	80.5	79.3	81.2	80.1	82.0	79.9	79.5	75.9	76.8	79.2	79.7
80.5	81.3	80.7	80.6	80.7	80.3	81.4	80.7	80.9	79.5	80.6	79.6	82.1	79.4	79.2	76.0	77.2	78.9	79.1
78.9	79.6	80.0	79.7	79.6	81.0	82.0	80.5	80.8	78.4	81.3	79.8	81.4	79.4	78.9	75.5	76.1	78.7	78.7
79.4	79.6	80.7	80.0	79.8	81.8	82.7	81.0	81.2	79.5	82.4	80.1	82.2	79.2	79.0	75.8	75.7	78.5	79.5
82.5	83.1	83.7	81.0	82.1	82.6	82.7	82.6	82.0	81.0	81.6	81.4	83.2	80.8	82.2	76.5	76.6	78.9	78.8
95.5	96.4	89.0	87.4	86.8	83.4	85.1	84.7	82.8	82.6	81.3	81.8	83.3	78.2	78.7	74.1	74.8	75.4	75.6
95.3	95.7	88.6	87.1	87.2	83.9	84.3	83.6	82.0	81.6	81.0	81.8	82.7	77.8	78.5	74.0	74.8	75.4	75.4
95.6	96.7	88.5	87.1	87.1	83.6	84.5	83.6	82.2	81.3	80.6	81.3	83.1	78.3	78.7	73.4	74.4	75.3	75.4
95.8	95.9	89.6	88.1	88.1	84.3	85.7	84.4	82.5	81.8	81.7	82.5	83.5	78.9	79.2	74.4	75.1	76.0	76.0
.	95.6	89.0	87.9	87.5	83.6	85.3	84.1	82.2	81.3	81.7	82.2	83.0	78.6	79.0	73.9	74.7	75.6	75.3
4.5	.	89.0	87.2	87.6	84.7	86.4	85.2	84.1	82.6	81.7	83.1	84.6	79.9	80.4	75.5	76.2	76.5	76.9
11.9	11.9	.	92.5	92.3	86.5	87.1	86.3	82.8	83.4	81.6	83.4	84.0	80.1	79.6	75.4	76.8	77.1	76.7
13.2	14.1	7.9	.	95.9	85.9	86.7	85.5	81.1	81.5	80.2	81.3	81.6	78.8	77.5	74.3	76.7	75.8	75.9
13.6	13.6	8.1	4.2	.	85.2	86.0	85.1	80.8	81.2	80.8	82.0	81.9	78.8	78.4	74.6	76.6	77.0	77.4
18.5	17.1	14.9	15.6	16.4	.	86.9	87.5	82.2	80.0	83.3	83.1	83.1	79.8	80.0	76.4	76.5	76.7	76.7
16.4	15.0	14.2	14.7	15.5	14.4	.	90.7	84.7	83.1	84.0	85.8	84.8	81.0	80.2	76.0	78.9	77.8	78.6
17.8	16.5	15.2	16.1	16.6	13.7	9.9	.	82.5	82.1	83.6	85.5	83.6	81.0	79.5	75.9	76.7	77.2	77.3
20.3	17.8	19.6	21.8	22.2	20.3	17.1	19.9	.	90.5	82.2	84.5	84.7	81.6	80.1	76.0	76.2	75.8	77.3
21.5	19.8	18.8	21.2	21.7	23.3	19.1	20.5	10.2	.	81.4	83.0	83.3	79.9	78.7	74.7	75.7	75.1	77.2
21.0	21.0	21.1	23.0	22.1	18.8	18.0	18.5	20.3	21.3	.	86.0	84.1	80.9	79.0	73.6	74.3	75.4	76.5
20.3	19.1	18.7	21.5	20.6	19.2	15.8	16.1	17.3	19.3	15.5	.	87.3	83.1	81.0	76.1	77.6	77.6	77.8
19.3	17.2	18.0	21.2	20.7	19.1	16.9	18.5	17.2	18.9	17.8	13.9	.	84.4	81.1	77.4	78.2	78.4	78.1
25.1	23.3	23.1	24.9	24.9	23.6	21.9	22.0	21.1	23.3	22.1	19.1	17.5	.	79.8	74.6	75.7	75.7	77.3
24.6	22.7	23.8	26.8	25.5	23.3	23.0	23.9	23.1	25.1	24.7	21.9	21.8	23.5	.	74.8	75.7	76.8	76.8
32.0	29.7	29.8	31.4	30.9	28.3	29.0	29.1	28.9	30.9	32.5	28.7	27.0	31.0	30.7	.	86.4	85.3	86.0
30.9	28.7	27.7	27.8	28.1	28.2	24.8	27.9	28.6	29.4	31.5	26.6	25.8	29.4	29.3	15.0	.	85.4	85.9
29.6	28.2	27.4	29.2	27.5	28.0	26.3	27.2	29.2	30.2	29.8	26.5	25.5	29.4	27.7	16.3	16.3	.	90.7
29.9	27.6	27.9	29.0	26.9	27.9	25.3	27.0	27.1	27.1	28.2	26.3	25.9	27.0	27.8	15.5	15.7	9.9	.

in the extraintestinal sites of proliferative bowel disease in ferret 1, which has been previously reported (11).

Amplification of intracellular-organism 16S rRNA. By using the universal prokaryotic 16S rRNA primers, a 1,480-base fragment was amplified in each of the four extraintestinal tissue samples (Fig. 1).

16S rDNA sequences. An essentially complete (1,480-base) sequence was obtained for the amplified product from the ferret extraintestinal tissue. The primers used for sequencing are shown in Table 1 (primers 3 to 9).

By using specific primers, a 550-base segment was amplified and sequenced from one additional ferret colonic tissue and one hamster ileal tissue. The 550-base partial sequence from the ferret colon IDO was identical to that previously obtained. The sequence for the IDO from the hamster differed from the ferret sequence by a single base, A versus G, respectively, at position 458 (*Escherichia coli* numbering).

Phylogenetic analysis by 16S rRNA sequence comparisons. Comparison of the ferret IDO 16S rRNA sequence with over 400 other sequences in our data base indicated that it was closest to the sequence of *D. desulfuricans*, a member of the delta subdivision of the class *Proteobacteria*. The ferret IDO sequence was then compared with 30 delta and four epsilon (*Campylobacter-Helicobacter* branch) members of the class *Proteobacteria*. The similarity matrix for these comparisons is shown in Table 3. A phylogenetic tree based upon this similarity matrix is shown in Fig. 2. The ferret IDO sequence was 87.5% similar to that of *D. desulfuricans* and fell in the middle of a subcluster of *Desulfovibrio* species.

PCR analysis of intestinal tissue. Colonic tissue from eight of nine ferrets with ICLO-associated proliferative colitis was positive for amplification of a 550-base PCR product with primers for the ferret IDO. Results for five ferrets with proliferative bowel disease, three apparently healthy ferrets, and controls are shown in Fig. 3. The amplification product for the diseased ferrets corresponded in size to that obtained from the extraintestinal tissue containing intracellular organisms (lane 9). Tissue from the control ferrets (lanes 6 to 8) did not yield an amplified product. The lack of a positive response for one tissue sample from a diseased ferret may be due to the absence of organisms in this particular tissue sample. This could have occurred inadvertently at necropsy because of the characteristic segmental nature of the lesion. The frozen colonic tissue may have been collected proximal to where the

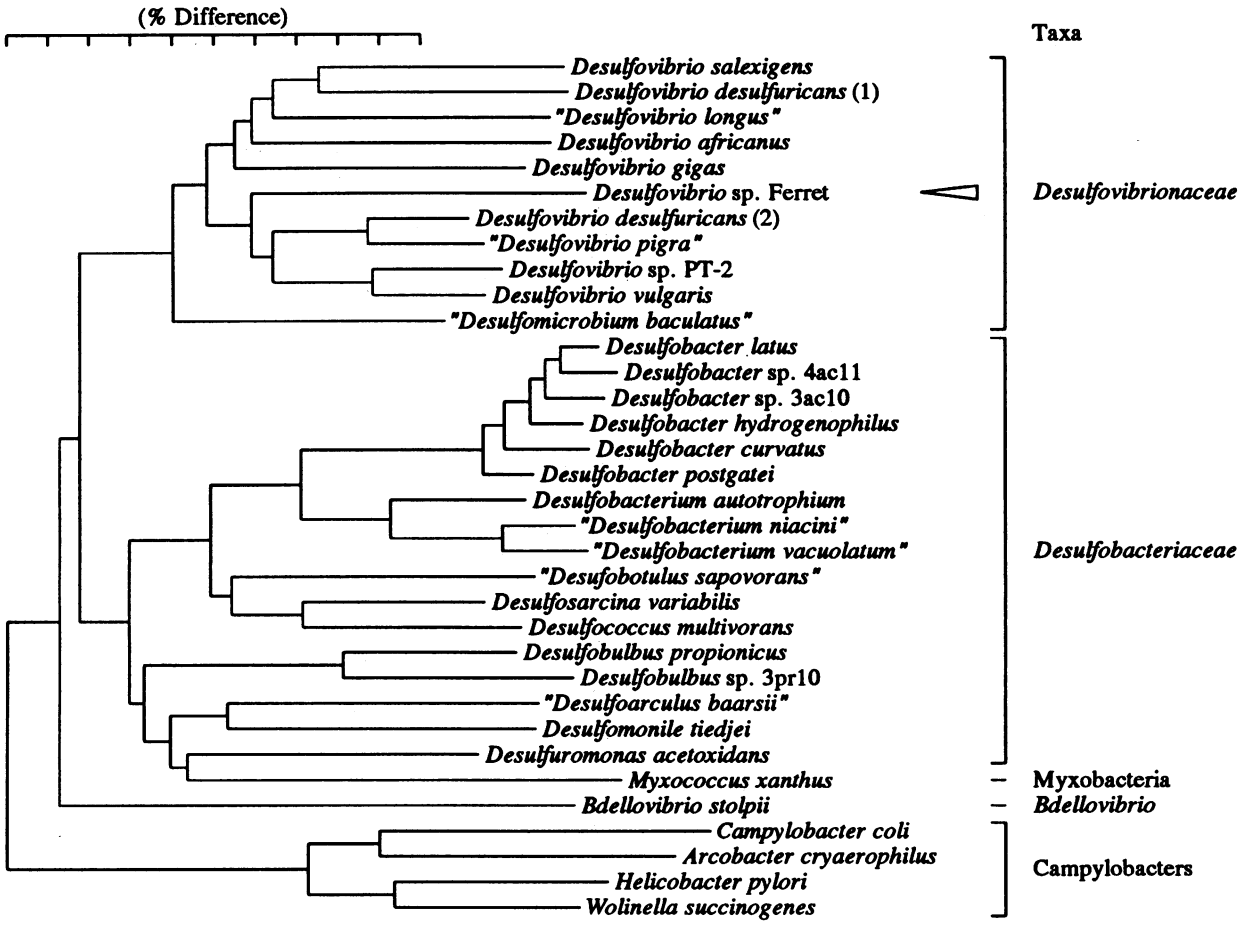

FIG. 2. Phylogenetic tree for 34 delta and gamma subgroup species of the class *Proteobacteria* based upon 16S rRNA sequence similarity. The scale bar represents a 10% nucleotide sequence difference as determined by measuring the lengths of the horizontal lines connecting any two species.

diseased colon (i.e., proliferative epithelium with ICLO) was present. The three hamster ileal samples obtained from outbreaks of proliferative ileitis also yielded correct-size amplification products (data not shown) when the primers for the ferret IDO were used.

Southern blot analysis. The digoxigenin-11-dUTP-labeled probe reacted with DNA from ferret 1 extraintestinal tissue (positive control), as well as with the PCR products isolated from seven disease-positive ferrets (data not shown). The probe did not react with any putative PCR product that may have been produced in two negative ferrets. These results, combined with the complete sequencing of two amplicons, indicate that the correct-size bands seen in agarose gels were from amplification of 16S rRNA genes from IDOs.

DISCUSSION

It is becoming routine to identify uncultivable organisms by 16S rRNA sequence analysis (18, 50). Examples involving pathogens include identification of the uncultured agent of bacillary angiomatosis (39), the bacillus of Whipple's disease (40), NIX, an intracellular pathogen which causes a high level of mortality in Pacific razor clams (24), human gastric spiral organisms related to *Helicobacter pylori* (45), and human oral spirochetes associated with periodontal disease (3).

Obtainment of the 16S rRNA sequence for the ferret intracellular organism was facilitated by the presence of the organism in otherwise sterile extraintestinal sites. This allowed direct sequencing of the PCR product rather than a more complex procedure involving cloning of the PCR product into *E. coli.*

Initially we were surprised to discover that the intracellular organism from ferrets and hamsters fell in the delta subdivision of the class *Proteobacteria*, a group perceived to contain mostly free-living sulfate-reducing bacteria (SRB). However, the morphological and staining characteristics of the ICLO are compatible with *Desulfovibrio* sp., i.e., gram negative and curved. Vacuolated inclusions observed in cytoplasm of ICLO in proliferative tissue of rabbits (34, 43) have been noted in *D. latus* (53).

Most SRB are members of the delta subdivision of the class *Proteobacteria* (4, 36, 54). The delta subdivision of the class *Proteobacteria* also contains chemotrophic non-SRB, including the obligately anaerobic *Pelobacter* species (46), the dissimilatory Fe(III) reducer *Geobacter metallireducens* (28), and the obligately aerobic *Bdellovibrio* and *Myxococcus* species (41, 54). Thus, there is precedence for metabolic diversity in the delta subdivision of the class *Proteobacteria*. While there are no other known intracellular pathogens of vertebrate hosts in the delta subdivision of the class *Proteobacteria*, *Bdellovibrio* species prey on other gram-negative bacteria and have biphasic

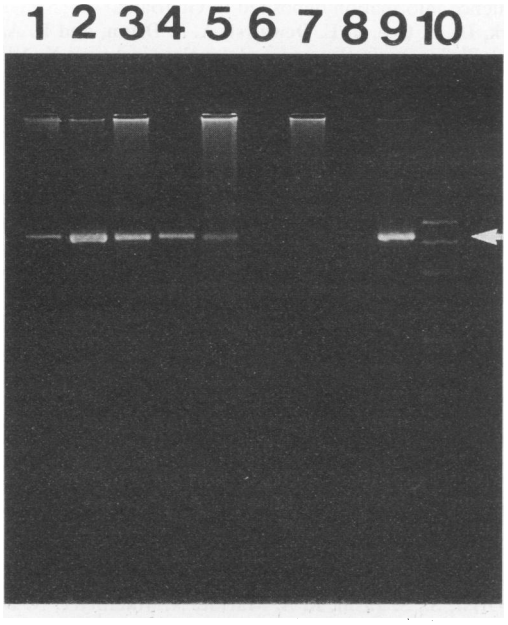

FIG. 3. Agarose gel electrophoresis of DNA amplified from ferret colonic tissue samples with ferret IDO-specific primers 3 and 4 (Table 1). Lanes: 1 to 5, ferrets with proliferative bowel disease; 6 to 8, ferrets without proliferative bowel disease; 9, extraintestinal tissue from ferret 1; 10, molecular weight standards (*Msp*I-digested pBR322 DNA; top bands, 622 and 527 bases). The arrow indicates a molecular size corresponding to 550 bases.

life cycles which include an intracellular growth-and-reproduction phase (41).

Most *Desulfovibrio* spp. inhabit subsurface parts of the aquatic environment, such as sediments which have turned anoxic or anoxic zones of fresh water which have anoxic microniches (38, 44, 53). These species appear to be important H_2 scavengers in the anaerobic degradation of organic matter in sediments. SRB and *Desulfovibrio* species have also been isolated from sheep rumens (20) and human intestines (1, 14, 35).

Because the phylogenetic diversity of the *Desulfovibrio* cluster is considerably greater than that which normally characterizes a genus, Devereux et al. defined this cluster of *Desulfovibrio* species as the family *Desulfovibrioaceae* (5). There has been no compelling taxonomic reason to divide the family *Desulfovibrioaceae* into five or more genera to reflect its genetic diversity. The ferret and hamster intracellular organism is clearly a member of the family *Desulfovibrioaceae*. We suggest that the organism be included in the genus *Desulfovibrio* until the genus is divided into additional genera or until more is known about the phenotype of the IDO.

A role for SRB, in particular the *Desulfovibrio* species, in the pathogenesis of ulcerative colitis has been proposed by Gibson et al. in a series of reports (14–17). Fifty percent of healthy individuals harbor significant populations of SRB (16). Among persons with ulcerative colitis, 96% harbor SRB. Most human gut SRB belong to the genus *Desulfovibrio*. These investigators noted that the H_2S produced by these bacteria is toxic to intestinal tissue and that mucin, a highly sulfated mucoprotein, stimulates growth of SRB at the expense of methanogens. Feeding of sulfate-containing polysaccharides, such as dextran sulfate, sulfated pectin, or degraded carrageenan, to hamsters, guinea pigs, rabbits, and rats causes ulcerative colitis and intestinal adenomas and carcinomas (19, 30, 51, 52). Thus, the IDO associated with proliferative bowel disease falls within a group of bacteria already implicated in bowel disease.

While this report was in preparation, reports by Lawson et al. and Gebhart et al. were published describing the isolation and cultivation of the comma-shaped intracellular bacterium of porcine proliferative bowel disease (26) and identification of this organism by 16S rRNA sequence methods similar to those described here (13). The organism we have identified in ferrets and hamsters is clearly the same as the organism identified in pigs. The 16S rRNA sequence reported here is essentially identical to that reported by Gebhart et al. and independently determined by Monckton (33). There are three base differences between the sequence we have reported and that reported by Gebhart et al. We found two Cs at positions 81 and 82 (*E. coli* numbering) rather than one and GC rather than CG at positions 214 and 215. On the sequencing gels, we found the GC bases at positions 214 and 215 compressed and in inverted order by using reverse primers but uncompressed and in correct order by using forward primers. We believe that these minor differences represent sequencing error rather than strain differences and that our reported sequence is correct on the basis of re-examination of sequencing gels, analysis of secondary structure, and homology with other *Desulfovibrio* species. The sequence reported by Gebhart et al. contains data on the 3′ and 5′ ends of the molecule which we did not obtain because of our use of primers internal to the 16S rRNA gene. The vernacular name ileal symbiont intracellularis has been suggested (13). We prefer the name IDO because it directly indicates the phylogenetic position of the organism.

There appears to be a consensus that the IDO we have identified in ferrets and hamsters, Monckton has identified in pigs (33), and Lawson et al., McOrist et al., and Gebhart et al. have isolated and identified in pigs (13, 26, 31) is the long-sought ICLO which reacts with Warthin-Starry stain and omega antiserum. Recently, McOrist et al. grew the intracellular organism in IEC18 rat enterocytes and inoculated the bacteria into conventional and germfree pigs (31). Conventional pigs developed proliferative ileal lesions, whereas germfree pigs did not, and the organism could not be reisolated from the intestines of germfree animals. The investigators surmised that the IDO required other intestinal flora to produce disease. *Serpulina hyodysenteriae*, the causative agent of swine dysentery, also requires lower-bowel anaerobic flora to cause marked intestinal lesions. This hypothesis is partially substantiated by an earlier study by McOrist in which partially purified ileal homogenates of affected ileal tissue containing a mixed flora reproduced proliferative ileitis in germfree pigs (32). Stills was able to transmit the disease in hamsters with INT407 cells containing an organism compatible by electron microscopy analysis as an ICLO (probably the IDO). *Chlamydia trachomatis* SFPD was subsequently isolated from the same cell culture (12, 47, 48, 56). Thus, while the IDO is clearly the ICLO associated with proliferative bowel disease, it may not have the ability to produce disease without other agents or insults. The presence of *C. trachomatis* SFPD in hamsters with proliferative bowel disease suggests the need for such a second organism (12, 56). We have also isolated *C. trachomatis* from the colons of ferrets with proliferative colitis (8). The specific primers for detection of the IDO should facilitate studies of the role of this organism in proliferative bowel disease.

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