Helicobacter bilis sp. nov., a Novel *Helicobacter* Species Isolated from Bile, Livers, and Intestines of Aged, Inbred Mice

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A fusiform bacterium with 3 to 14 multiple bipolar sheathed flagella and periplasmic fibers wrapped around the cell was isolated from the liver, bile, and lower intestine of aged, inbred mice. The bacteria grew at 37 and 42°C under microaerophilic conditions, rapidly hydrolyzed urea, were catalase and oxidase positive, reduced nitrate to nitrite, did not hydrolyze indoxyl acetate or hippurate, and were resistant to both cephalothin and nalidixic acid but sensitive to metronidazole. On the basis of 16S rRNA gene sequence analysis, the organism was classified as a novel helicobacter, *Helicobacter bilis*. This new helicobacter, like *Helicobacter hepaticus*, colonizes the bile, liver, and intestine of mice. Although the organism is associated with multifocal chronic hepatitis, further studies are required to ascertain whether *H. bilis* is responsible for causing chronic hepatitis and/or hepatocellular tumors in mice.

Microaerophilic spiral to curved bacteria isolated from the stomachs of humans and animals have become a subject of intense research because of their association with gastric disease (17, 30). These bacteria were originally classified in the genus *Campylobacter* but are now recognized as belonging to the genus *Helicobacter* (47). The type species of the genus, *Helicobacter pylori*, causes chronic gastritis and peptic ulcer disease in humans (18, 26, 30). It has recently been linked to the development of gastric adenocarcinoma and gastric mucosa-associated lymphoma (13, 32, 33, 51). The helicobacters *H. acinonyx*, *H. nemestrinae*, *H. felis*, and *H. mustelae* have been isolated from the stomachs of several mammalian species (2, 8, 16, 27, 36) and cause various degrees of gastritis in their hosts (11, 12, 17). The helicobacters *H. cinaedi*, *H. fennelliae*, *H. canis*, *H. hepaticus*, *H. pametensis*, and *H. pullorum* have been isolated from the intestinal tracts of humans (9, 43, 44, 46), mammals (6, 15, 44), and birds (6, 43). *Helicobacter muridarum* has the potential to colonize both the stomach and the intestinal tract of rodents. It naturally colonizes the ileum and cecum but can also elicit a gastritis after colonizing the gastric mucosa of older animals (28, 39). With 11 *Helicobacter* species now formally named, it is clear that *Helicobacter* species can infect several animal species, as well as colonize different anatomical regions of the gastrointestinal system (17).

Our laboratory has recently described a new helicobacter, *H. hepaticus*, which normally colonizes the cecum and colon of mice, but which in certain strains of mice can induce a persistent hepatitis (15). In A/JCr mice, *H. hepaticus* is associated with hepatoma and hepatocellular carcinoma (49, 50). *H. hepaticus* cells are long, narrow, slightly curved rods with two

TABLE 1. Oligonucleotide primers used for PCR amplification and sequencing of 16S genes coding for rRNA

Primer	Identification	Type	Sequence (5′ to 3′)[a]	Position[b]	Orientation
1	C70	PCR	AGAGTTTGATYMTGGC	8–23	Forward
2	B37	PCR	TACGGYTACCTTGTTACGA	1495–1513	Reverse
3	B12	Sequencing	TGGCGCACGGGTGAGTAA	103–120	Forward
4	X88	Sequencing	GTATTAATCACCGTTTC	159–175	Reverse
5	B34	Sequencing	RCTGCTGCCTCCCGT	344–358	Reverse
6	B35	Sequencing	GTRTTACCGCGGCTGCTG	519–536	Reverse
7	B36	Sequencing	GGACTACCAGGGTATCTA	789–806	Reverse
8	C01	Sequencing	GGTTGCGCTCGTTGCGGG	1096–1113	Reverse
9	C31	Sequencing	GGAATCGCTAGTAATCG	1337–1353	Forward
10	X91	Sequencing	CCCGGGAACGTATTCACCG	1369–1387	Reverse
11	C62	PCR	AGAACTGCATTTGAAACTACTTT	627–649	Forward
12	C12	PCR	GGTATTGCATCTCTTTGTATGT	1244–1265	Reverse
13	C14	PCR	GGGGCTTTCAATAAAGAATT	IVS	Both

[a] Base codes are standard International Union of Biochemistry codes for bases and ambiguity.
[b] Numbering is based upon *E. coli* positions.

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TABLE 2. Sources and accession numbers of strains studied

Organism	Strain examined[a]	Culture collection(s)[b]	GenBank accession no.[c]
Isolate			
H. bilis	Fox Hb1[T]	ATCC 51630[T]	U18766
	Fox Hb2	ATCC 51631	U18767
	Fox Hb3	ATCC 51632	U18768
Reference species			
A. cryaerophilus	CCUG 17801[T]	ATCC 43158[T]	L14624
A. butzleri	CCUG 10373		L14626
A. skirrowii	CCUG 10374[T]		L16625
C. coli	CCUG 11238[T]	ATCC 33559[T]	L04312
C. concisus	Tanner 484[T]	ATCC 33237[T]	L04322
C. fetus subsp. *fetus*	ATCC 27374[T]		M65012
C. helveticus	NCTC 12470[T]		U03022
C. jejuni	CCUG 11284[T]		L04315
C. rectus	Tanner 371[T]	ATCC 33238[T]	L04317
"*F. rappini*"	NADC 1893[T]	ATCC 43966[T]	M88137
"*Gastrospirillum hominis*" 1	Uncultivable		L10079
"*Gastrospirillum hominis*" 2	Uncultivable		L10080
H. acinonyx	Eaton 90-119-3[T]	ATCC 51101[T], CCUG 29263[T]	M88148
H. canis	NCTC 12739[T]		L13464
H. cinaedi	CCUG 18818[T]	ATCC 35683[T]	M88150
H. felis	Lee CS1[T]	ATCC 49179[T]	M37642
H. felis	Lee DS3	Not deposited	M37643
H. fennelliae	CCUG 18820[T]	ATCC 35684[T]	M88154
H. hepaticus	Fox Hh-2[T]	ATCC 51448[T]	U07574
H. mustelae	Fox R85-13-6[T]	ATCC 43772[T]	M35048
H. muridarum	Lee ST1[T]	CCUG 29262[T], ATCC 49282[T]	M80205
H. nemestrinae	ATCC 49396[T]		X67854
H. pametensis	Seymour B9[T]	CCUG 29255[T], ATCC 51478[T]	M88147
H. pylori	ATCC 43504[T]		M88157
Helicobacter sp. CLO 3	CCUG 14564		M88151
Helicobacter sp. Bird-B	Seymour B10[T]	CCUG 29256[T], ATCC 51480[T]	M88139
Helicobacter sp. Bird-C	Seymour B52[T]	CCUG 29561[T], ATCC 51482[T]	M88144
W. succinogenes	Tanner 602W[T]	ATCC 29543[T]	M88159

[a] Strains from which sequences were determined were obtained from the following individuals or culture collections: K. A. Eaton, Department of Veterinary Pathobiology, Ohio State University, Columbus; J. G. Fox, Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge; A. Lee, Department of Microbiology and Immunology, University of New South Wales, Sydney, Australia; C. Seymour, Department of Microbiology, Boston University School of Medicine, Boston, Mass.; A. Tanner, Department of Microbiology, Forsyth Dental Center, Boston, Mass.; American Type Culture Collection (ATCC), Rockville, Md.; Culture Collection, University of Göteborg (CCUG), Göteborg, Sweden; National Animal Disease Center (NADC), Ames, Iowa; and National Collections of Type Cultures (NCTC), London, United Kingdom.

[b] Alternate culture collection sources for sequenced strains. Abbreviations are as described above.

[c] 16S rRNA sequences for these strains are available for electronic retrieval from GenBank under the accession numbers given above. Through cross-distribution of databases, these sequences should also be available from EMBL and DDBJ.

bipolar sheathed flagella. *H. hepaticus* is morphologically similar to three other intestinal helicobacters, *H. fennelliae*, *H. cinaedi*, and *H. canis* (15). While examining aged, inbred mice with chronic hepatitis for infection with *H. hepaticus*, we isolated organisms with a different ultrastructural morphology. These organisms were fusiform slightly curved rods, had helical periplasmic fibers, and possessed 3 to 14 bipolar sheathed

TABLE 3. Recovery of *H. bilis* from inbred strains of mice

Strain	No. of *H. bilis*-positive samples/total no. of mice			
	Ceca and/or colon	Liver	Bile	Spleen
DBA/2	3/4	1/5	NA[a]	NA
C57BL/6	6/6	1/6	1/1	1/6
CBA/CA	3/3	1/6	1/1[b]	NA
BALB/c	4/5	0/5	NA	NA
Total	16/18	3/22	2/2	1/6

[a] NA, not attempted.
[b] From a mouse positive for *H. bilis* in the liver.

flagella. Organisms isolated from sheep, mice, and dogs which possess this morphology have been called "*Flexispira rappini*" (1, 21, 40, 42). 16S rRNA sequence analysis has demonstrated that "*F. rappini*" strains isolated from humans, dogs, and mice represent a *Helicobacter* species closely related to *H. canis* (44). Other "*F. rappini*" isolates represent additional *Helicobacter* species (35). In this paper, we describe *Helicobacter bilis*, a novel *Helicobacter* species distinct from "*F. rappini*," *H. hepaticus*, and *H. muridarum* (also found in mice), which we have isolated from the bile, liver, and intestine of aged, inbred mice, some of which had chronic hepatitis and hepatomas.

MATERIALS AND METHODS

Inbred mice. Twenty-two mice (11 male, 11 female) representing of four inbred strains, C57BL, CBA/CA, DBA/2, and BALB/c, were housed in a commercially maintained barrier colony specifically maintained for investigators interested in studying various biomedical aspects of aging in the mouse model. The mice varied in age from 19 to 27 months: the CBA/CA mice were 24 to 27 months old, the C57BL mice were 19 to 22 months old, the DBA/2 mice were 20 to 24 months old, and the BALB/c mice were 22 to 24 months old. Upon arrival at our institution, the mice were maintained in an isolation cubicle for 1 to 5 days, during which time the animals were serially euthanized for culture and histopathological study.

FIG. 1. Transmission electron micrograph of *H. bilis* Hb1 illustrating different cell morphologies. Note that flagella are sheathed. Bar, 0.5 μm.

Culture techniques. In brief, the bacterial strains were isolated from the liver and/or intestine of mice by streaking liver tissue or intestinal scraping onto either moist tryptic soy blood agar plates or brucella blood agar with TVP (trimethoprim, vancomycin, polymyxin; Remel Laboratories, Lenexa, Kans.) and incubating them at 37°C under microaerophilic conditions in vented jars containing N_2, H_2, and CO_2 (90:5:5).

Biochemical characterization. Detailed biochemical characterization was performed for six strains, Hb1 to Hb6, as previously described (28, 36). For the remaining 25 strains, motility; Gram stain reaction; oxidase, catalase, and urease activities; and sensitivity to nalidixic acid and cephalothin were determined.

Histopathology. Specimens of liver tissue from each mouse were fixed in neutral buffered 10% formalin and processed by standard methods, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin and Warthin-Starry stains. These sections were examined by light microscopy for evidence of lesions and for the presence of a bacterium with a morphology compatible with that of *H. bilis*.

Electron microscopy. Cells grown on tryptic soy blood agar plates were gently suspended in 10 mM Tris buffer (pH 7.4) at a concentration of approximately 10^8 cells per ml. Samples were negatively stained with 1% (wt/vol) phosphotungstic

acid (pH 6.5) for 20 to 30 s. Specimens were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

Crude DNA isolation for 16S sequencing. Bacteria were cultured on tryptic soy blood agar plates. A loopful of cells was harvested and suspended in 100 μl of lysis buffer (50 mM Tris-HCl [pH 7.6], 1 mM EDTA, 0.5% Tween 20, 200 μg of proteinase K per ml) and incubated at 55°C for 2 h. The proteinase K was inactivated by heating to 95°C for 10 min. Crude DNA was then precipitated with 2 volumes of cold absolute ethanol.

Amplification of 16S rRNA cistrons. The 16S rRNA cistrons were amplified with primers 1 and 2 in Table 1. PCRs were performed in thin-walled tubes with a Perkin-Elmer 480 thermal cycler. Ten microliters of the crude DNA and 1 μM primers were added to the reaction mixture, which had a final volume of 82 μl. Ampliwax PCR Gem 100's (Perkin-Elmer) were used in a hot-start protocol as suggested by the manufacturer. The following conditions were used for amplification: denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 45 s, with 5 s added for each elongation step. 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
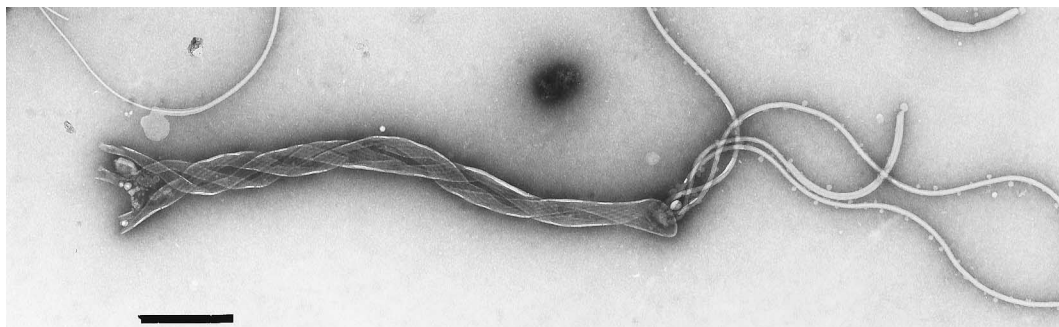

FIG. 2. Transmission electron micrograph of unraveled periplasmic fibers. Bar, 0.5 μm.

(FMC Bioproducts). DNA was stained with ethidium bromide and viewed under short-wavelength UV light.

Purification of PCR products for 16S sequencing. The amplified DNA was purified by precipitation with polyethylene glycol 8000 (25). After removal of Ampliwax, 0.6 volume of 20% polyethylene glycol 8000 (Sigma) in 2.5 M NaCl was added and the mixture was incubated at 37°C for 10 min. The sample was 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 and dissolved in 30 µl of distilled water and used for cycle sequencing as described below.

Sequencing methods. The DNA sample from PCR was directly sequenced with a cycle-sequencing kit (TAQuence Cycle Sequencing Kit; United States Biochemical Corp.). The manufacturer's protocol was followed. The eight sequencing primers are given in Table 1. Primers were end labeled with ^{33}P (Dupont, NEN) by the manufacturer's protocol. Approximately 100 ng of purified DNA from the PCR was used for sequencing. Reaction products were loaded onto 8% polyacrylamide–urea gels, electrophoresed, and detected by exposure to X-ray film for 24 h.

16S rRNA data analysis. A program set for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and dendrogram construction for 16S rRNA data was written in Microsoft Quick BASIC for use on IBM PC-AT and compatible computers (34). RNA sequences were entered and aligned as previously described (34). Our sequence database contains approximately 300 sequences determined in our laboratory and another 200 obtained from GenBank or the Ribosomal Database Project (29). Reference strains used in the 16S rRNA analysis are given in Table 2. Similarity matrices were constructed from the aligned sequences by using only those sequence positions from which 90% of the strains had data. The similarity matrices were corrected for multiple base changes at single positions by the method of Jukes and Cantor (19). Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (41).

Identification of strains by PCR with specific primers. Subsequent to obtaining the full sequences for three strains of *H. bilis*, PCR primers 11 and 12 (Table 1) were designed for identification of *H. bilis* strains and *H. bilis*-infected intestinal and liver tissues. PCR primer 13 (Table 1) was designed to identify *H. bilis* strains and closely related "*F. rappini*" strains that contain a 187-base intervening sequence in their 16S rRNA genes (see Discussion below). Primer 13 acts as a forward primer at the 5′ end of the intervening sequence (IVS) and a reverse primer at the 3′ end (on the complementary strand with three G-U matches) to produce a 186-base amplicon.

The DNA from bacteria and tissue homogenate was prepared as described previously (14). DNA preparations were added to a 100-µl (final volume) reaction mixture containing 1× *Taq* polymerase buffer (2.25 mM MgCl$_2$, 50 mM KCl, 10 mM Tris [pH 8.4], 0.01% gelatin), 0.5 mM (each) primer, 200 µM (each) nucleotide, and 200 µg of bovine serum albumin per ml. For primers 11 and 12, samples were heated to 94°C for 4 min and cooled to 56°C, at which time 2.5 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.) and 1 U of the polymerase enhancer Perfect Match (Stratagene, La Jolla, Calif.) were added, followed by an overlay of 75 µl of mineral oil. Thirty-three cycles, each comprising 1 min at 94°C, 1.45 min at 56°C, and 2.3 min at 72°C, were performed. For primer 13, the annealing temperature was lowered to 48°C. After the reactions were completed, 10-µl fractions were removed from each reaction tube and electrophoresed on a 6% Visigel separation matrix (Stratagene).

Nucleotide sequence accession numbers. The GenBank and culture collection accession numbers for the strains examined are given in Table 2. The complete 16S rRNA sequence of the type strain is available for electronic retrieval from the GenBank, EMBL, and DDBJ databases under accession number U18766.

RESULTS

Isolation and growth characteristics. After 3 to 7 days of incubation under microaerophilic conditions, a thin spreading film was noted on the agar surface. Bacteria were gram negative. Examination with phase microscopy revealed the presence of rod-shaped, motile bacteria. While the bacteria grew at 42°C, incubation at 37°C was routinely used to isolate bacteria from the liver and intestine.

Culture. In aggregate, 5 strains (Hb1 to Hb5) were isolated from the livers and/or bile of four mice, 11 strains (Hb6 to Hb16) were isolated from the ceca, 14 strains (Hb17 to Hb30) were isolated from the colon, and 1 strain (Hb31) was isolated from the spleen (Table 3).

Ultrastructure. Cells measured 0.5 µm by 4 to 5 µm and had a fusiform to slightly spiral appearance, with 3 to 14 bipolar sheathed flagella (Fig. 1). The organism had periplasmic fibers, which are found in two other mouse intestinal helicobacters, "*F. rappini*" and *H. muridarum*, as well as a gastric helicobacter, *H. felis*, which experimentally causes gastritis in mice

(11). The periplasmic fibers were generally tightly coiled around the cell, which gave a crisscross appearance in negatively stained preparations because both sides of the cell were seen simultaneously (Fig. 1). Occasionally, the coiling of the periplasmic fibers loosened and the fibers appeared paired (Fig. 1). This is believed to be an unraveling of the fibers, either as an artifact or as a process of aging of the bacteria. Some cells, or the end portion of cells, did not have any fibers (Fig. 1). Occasionally, unraveled fibers with flagella still attached at the ends were observed (Fig. 2). Coccoid cells, often seen in older cultures, usually possessed periplasmic fibers and flagella (Fig. 1).

Biochemical and physiological characteristics. Six strains of *H. bilis* were analyzed by a number of biochemical and physiological properties and then compared with known helicobacter species (Table 4). Another four strains of *H. bilis* isolated from outbred mice also had identical biochemical profiles. *H. bilis*, like *H. muridarum*, "*F. rappini*," and *H. hepaticus*, had strong urease activity and was oxidase and catalase positive. *H. bilis* strains reduced nitrate to nitrite. The bacteria grew microaerophilically at 37 and 42°C but not at 25°C. The bacteria also grew in 1, 3, 6, and 20% bile and had grown in 1% glycine. *H. bilis* did not hydrolyze indoxyl acetate or hippurate and did not produce hemolysis in blood agar or produce yellow pigment. The remaining 25 strains were also gram negative, rod to slightly spiral shaped, and motile, as well as catalase, oxidase, and strongly urease positive. All of the strains were resistant to both cephalothin and nalidixic acid but sensitive to metronidazole.

In this series of mice, we did not isolate any other known *Helicobacter* species either in the intestine or in the liver.

Phylogenetic analysis. The PCR amplification products for Hb1, Hb2, and Hb3 were about 200 bases larger than those for typical *Helicobacter* species. Sequencing demonstrated the presence of 187-base IVS inserted at base 210 by using *Escherichia coli* numbering in each of the strains. We analyzed approximately 95% of the total RNA sequence (1,450 bases plus 187 bases for IVS) for each of the three strains. The sequences for the IVS are included in the 16S rRNA sequences submitted to GenBank. The sequences for the three strains were identical, including those of the IVS region. Comparison of the *H. bilis* sequence with sequences for other bacteria in our database indicated that it was most closely related to the sequences for *H. canis*, *H. cinaedi*, and "*F. rappini*" (99% similarity). The degree of sequence and phenotypic difference identifies *H. bilis* as a novel species. Hb1 was compared with 28 reference species in the genera *Helicobacter*, *Wolinella*, *Arcobacter*, and *Campylobacter*. The similarity matrix for these comparisons is presented in Table 5. A phylogenetic tree constructed by the neighbor-joining method is presented in Fig. 3. *H. bilis* falls in a cluster of intestinal helicobacters which includes *H. canis*, *H. cinaedi*, and "*F. rappini*."

PCR identification of strains. PCR amplification with the *H. bilis*-specific primers 11 and 12 (Table 1) produces a 638-base fragment for *H. bilis* strains. Shown in Fig. 4 are the appropriately sized bands for three isolates cultured from presumptively identified *H. bilis* isolates and from DNA extracted from cecal tissue of infected animals. This specific band was sequenced from two additional strains of *H. bilis* and compared with the sequence of the *H. bilis* type strain. The 638-base partial sequences from the two strains were identical to that of the type strain. Moreover, no bands were discerned with the control helicobacter species. Appropriately sized PCR bands were obtained with six isolates of *H. bilis* and five and four DNA samples isolated from *H. bilis*-infected (i.e., *H. bilis*-cultured) cecal and liver tissues, respectively. An additional 12

TABLE 4. Characteristics which differentiate *H. bilis* from other *Helicobacter* and *Campylobacter* species[a]

Taxon	Catalase production	Nitrate reduction	Urease	Alkaline phosphatase hydrolysis	Gamma-glutamyl transpeptidase	Indoxyl acetate hydrolysis	Growth at 42°C	Growth on 1% glycine	Susceptibility to		Periplasmic fibers	No. of flagella/cell	Distribution of flagella	G+C content (mol%)
									Nalidixic acid (30-μg disc)	Cephalothin (30-μg disc)				
H. bilis	+	+	+	ND	ND	−	+	+	R	R	+	3–14	Bipolar	ND
Helicobacter sp. 29176	+	+	+	−	+	+	ND	ND	ND	ND	−	2	Bipolar	ND
H. canis	−	−	−	+	ND	+	+	ND	S	I	−	2	Bipolar	48
H. cinaedi	+	−	−	−	−	−	−	+	S	S	−	1–2	Bipolar	37–38
"*F. rappini*"	+	−	+	ND	ND	ND	−	−	R	R	+	10–20	Bipolar	34
H. hepaticus	+	+	+	ND	ND	+	−	+	R	R	−	2	Bipolar	ND
H. muridarum	+	−	+	+	+	+	−	−	R	R	+	10–14	Bipolar	34
H. fennelliae	+	−	−	+	−	+	−	+	S	S	−	2	Bipolar	35
Helicobacter sp. CLO3	+	−	−	+	−	+	+	+	I	R	−	ND	ND	45
H. pylori	+	−	+	+	+	−	−	−	R	R	−	4–8	Bipolar	35–37
H. nemestrinae	+	−	+	+	ND	−	+	−	R	S	−	4–8	Bipolar	24
H. acinonyx	+	−	+	+	+	−	−	−	R	S	−	2–5	Bipolar	30
H. felis	+	+	+	+	+	−	−	−	R	S	+	14–20	Bipolar	42
H. pametensis	+	+	−	−	−	−	+	+	S	S	−	2	Bipolar	38
Helicobacter sp. Bird-C	+	+	+	+	−	+	+	+	S	S	−	2	Bipolar	30
Helicobacter sp. Bird-B	+	+	+	+	+	+	+	+	S	R	−	2	Bipolar	31
H. mustelae	+	+	+	+	+	+	+	−	S	R	−	4–8	Lateral	36
C. coli	+	+	−	ND	ND	+	+	+	S	R	−	1	Polar	30–33
C. jejuni subsp. *jejuni*	+	+	−	ND	ND	+	+	+	S	R	−	1	Polar	30–33
C. jejuni subsp. *doylei*	V	−	V	ND	ND	+	−	−	S	S	−	1	Polar	30–31
C. lari	+	+	−	ND	ND	−	+	+	R	R	−	1	Polar	30–32

[a] Data were obtained from references 5, 8, 36, 43, and 44 and this study. +, positive reaction; −, negative reaction; S, susceptible; R, resistant; I, intermediate; ND, not determined; V, variable results.

TABLE 5. Similarity matrix based on 16S rRNA sequence comparisons

Species or strain	% of similarity to [a]:										
	H. bilis	*"F. rappini"*	*H. cinaedi*	*H. canis*	*Helicobacter* sp. CCUG 29176	*H. hepaticus*	*H. muridarum*	*H. fennelliae*	*Helicobacter* sp. CLO-3	*H. pylori*	*H. nemestrinae*
Helicobacter bilis		98.9	98.5	98.8	99.2	97.4	96.4	95.8	95.4	93.4	93.7
"Flexispira rappini"	1.1		98.8	98.0	98.2	97.4	96.1	95.3	95.5	93.1	93.3
Helicobacter cinaedi	1.5	1.2		97.8	98.0	97.0	95.9	95.7	95.2	92.7	93.2
Helicobacter canis	1.2	2.0	2.2		99.2	97.3	96.5	95.3	95.9	93.7	93.7
Helicobacter sp. CCUG 29176	0.8	1.8	2.0	0.8		97.5	96.6	95.5	95.7	93.6	93.7
Helicobacter hepaticus	2.6	2.6	3.1	2.8	2.5		97.8	95.4	95.2	93.4	93.5
Helicobacter muridarum	3.7	4.0	4.2	3.6	3.4	2.3		94.9	94.3	93.0	93.0
Helicobacter fennelliae	4.3	4.9	4.4	4.9	4.7	4.8	5.2		94.6	92.9	92.6
Helicobacter sp. CLO-3	4.7	4.7	4.9	4.2	4.4	5.0	6.0	5.6		93.8	93.3
Helicobacter pylori	6.9	7.2	7.7	6.5	6.7	7.0	7.3	7.5	6.5		98.2
Helicobacter nemestrinae	6.6	7.0	7.2	6.6	6.6	6.8	7.4	7.8	7.0	1.8	
Helicobacter acinonyx	7.7	8.0	8.1	7.1	7.4	7.2	8.0	8.3	7.3	2.7	3.4
"Gastrospirillum hominis" 1	8.4	8.3	8.5	8.2	8.2	8.2	8.7	7.9	7.6	5.3	5.5
"Gastrospirillum hominis" 2	8.1	8.2	8.1	7.7	8.0	7.6	8.1	7.6	7.5	5.1	5.3
Helicobacter felis	7.6	7.8	7.8	7.2	7.4	7.0	7.8	7.3	7.1	4.7	4.7
Helicobacter pametensis	3.3	3.6	4.6	3.6	3.6	3.8	4.5	4.8	5.1	5.8	5.7
Helicobacter sp. Bird-C	3.4	3.7	4.5	3.2	3.1	3.6	3.8	5.7	4.7	6.2	5.9
Helicobacter sp. Bird-B	3.8	4.3	4.8	3.2	3.5	3.6	4.1	5.8	5.1	6.6	6.3
Helicobacter mustelae	4.0	4.4	5.0	3.7	3.7	3.7	4.4	6.0	4.9	6.5	6.3
Wolinella succinogenes	7.1	7.4	7.6	7.1	7.1	7.7	7.1	8.1	8.2	9.9	9.7
Arcobacter cryaerophilus	16.6	16.8	16.9	16.3	16.8	17.0	16.9	17.7	16.2	17.5	16.8
Arcobacter skirrowii	16.9	17.3	17.4	16.8	17.2	17.4	17.3	17.9	16.7	18.0	17.5
Arcobacter butzleri	15.9	16.2	16.3	15.8	16.0	15.9	15.9	16.3	15.3	16.4	16.0
Campylobacter rectus	16.1	15.5	15.7	15.9	15.9	16.1	17.0	16.4	16.5	18.0	17.1
Campylobacter concisus	14.3	13.9	14.2	14.1	14.3	14.3	15.2	14.4	14.8	16.7	15.6
Campylobacter fetus	15.3	14.8	15.2	15.1	15.2	15.5	16.2	15.4	15.8	16.7	16.0
Campylobacter helveticus	14.6	14.2	15.2	14.1	14.3	15.0	15.2	15.7	15.9	16.3	15.5
Campylobacter coli	14.8	14.4	15.2	14.5	14.6	15.3	16.0	15.0	15.9	16.8	16.4
Campylobacter jejuni	15.2	14.8	15.6	14.9	14.9	15.5	16.0	15.4	15.9	16.5	16.1

[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 (19).

strains subsequently isolated from outbred mice also had identically sized PCR bands and were therefore presumptively identified as *H. bilis*. The band was not present in 12 tissue homogenates (2 *H. hepaticus*-infected mice and 10 uninfected control mice) or in four other *Helicobacter* species and five other enteric bacterial species. Furthermore, *H. hepaticus*-specific primers were utilized to assay DNA isolated from two ceca and four livers in which *H. bilis* PCR product was previ-
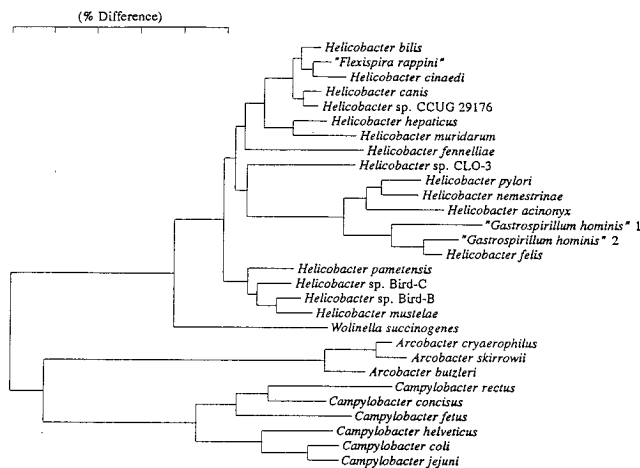

FIG. 3. Phylogenetic tree for strains representing 28 *Helicobacter*, *Arcobacter*, and *Campylobacter* species and related bacteria on the basis of 16S rRNA sequence similarity data. The scale bar represents a 5% difference in nucleotide sequences, as determined by measuring the lengths of horizontal lines connecting any two species.
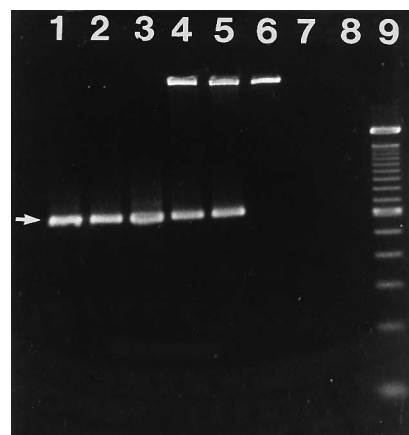

FIG. 4. Electrophoresis of DNA amplified by PCR on a Visigel separation matrix by using the *H. bilis*-specific primers listed in Table 1. Lanes: 1 to 3, DNA isolated from cultures of the presumptively identified *H. bilis*; 4 and 5, DNA isolated from tissue infected with *H. bilis*; 6, DNA isolated from *H. hepaticus*-infected tissue; 7, DNA isolated from *H. muridarum*; 8, DNA isolated from *H. pylori*; 9, DNA molecular size standards (150-bp ladder [GIBCO Bethesda Research Laboratories, Gaithersburg, Md.]). The arrow indicates a band of 638 bp.

TABLE 5—*Continued*

|	% of similarity to [a]:																
H. acinonyx	*"G. hominis"* 1	*"G. hominis"* 2	*H. felis*	*H. pametensis*	*Helicobacter* sp. Bird-C	*Helicobacter* sp. Bird-B	*H. mustelae*	*W. succinogenes*	*A. cryaerophilus*	*A. skirrowii*	*A. butzleri*	*C. rectus*	*C. concisus*	*C. fetus*	*C. helveticus*	*C. coli*	*C. jejuni*
92.7	92.0	92.3	92.8	96.8	96.7	96.3	96.1	93.2	85.1	84.9	85.7	85.5	87.0	86.2	86.7	86.5	86.3
92.4	92.1	92.2	92.6	96.5	96.4	95.8	95.8	93.0	85.0	84.6	85.4	86.0	87.3	86.6	87.0	86.9	86.6
92.4	92.0	92.4	92.6	95.5	95.6	95.4	95.1	92.8	84.9	84.5	85.3	85.8	87.1	86.3	86.2	86.2	85.9
93.2	92.2	92.7	93.2	96.5	96.9	96.8	96.4	93.3	85.3	84.9	85.8	85.7	87.1	86.3	87.1	86.8	86.5
93.0	92.2	92.4	93.0	96.5	97.0	96.6	96.4	93.3	85.0	84.6	85.6	85.7	87.0	86.3	86.9	86.8	86.5
93.1	92.3	92.7	93.3	96.3	96.5	96.5	96.4	92.7	84.8	84.5	85.7	85.5	86.9	86.0	86.4	86.2	86.0
92.4	91.8	92.3	92.6	95.7	96.3	96.0	95.7	93.3	84.8	84.5	85.7	84.8	86.2	85.4	86.2	85.6	85.6
92.1	92.5	92.7	93.0	95.4	94.5	94.4	94.2	92.3	84.2	84.1	85.3	85.3	86.9	86.0	85.8	86.4	86.1
93.0	92.7	92.8	93.2	95.1	95.4	95.1	95.2	92.2	85.4	85.0	86.2	85.2	86.6	85.8	85.7	85.7	85.7
97.4	94.9	95.1	95.4	94.4	94.0	93.7	93.8	90.7	84.4	84.0	85.3	84.0	85.0	85.0	85.4	85.0	85.2
96.7	94.7	94.9	95.5	94.5	94.4	93.9	94.0	90.9	84.9	84.4	85.6	84.7	85.9	85.6	86.0	85.3	85.5
	94.7	96.0	96.4	93.9	93.4	93.5	93.5	90.4	84.1	83.9	85.0	83.6	84.6	84.4	85.0	84.6	84.8
5.5		96.5	96.6	92.5	92.1	92.1	92.0	89.3	83.4	83.2	83.7	83.1	84.5	84.4	84.9	84.0	84.3
4.1	3.6		98.8	93.6	92.8	93.0	93.2	89.9	83.6	83.2	84.1	82.8	84.4	84.4	84.9	84.0	84.5
3.7	3.4	1.3		93.9	93.2	93.4	93.5	90.1	83.5	83.3	84.3	83.0	84.5	84.5	85.0	84.2	84.6
6.3	7.9	6.7	6.4		98.1	97.9	97.1	94.0	85.9	85.5	86.3	86.0	87.5	86.4	87.1	86.9	87.1
6.9	8.3	7.5	7.2	2.0		98.3	97.9	94.5	85.4	85.1	86.2	85.5	86.7	86.4	87.2	86.6	87.0
6.9	8.3	7.4	6.9	2.1	1.7		98.6	94.1	86.0	85.8	86.8	85.1	86.4	86.0	86.8	86.4	86.7
6.8	8.4	7.2	6.8	3.0	2.1	1.4		93.6	85.9	85.5	86.4	84.8	85.9	85.6	86.3	86.1	86.4
10.3	11.6	10.8	10.6	6.2	5.7	6.2	6.7		85.6	85.3	86.1	86.2	86.3	85.8	85.9	86.1	86.2
17.9	18.7	18.5	18.6	15.6	16.2	15.5	15.6	16.0		98.9	97.4	85.9	87.5	86.1	86.4	86.6	87.2
18.2	19.1	19.0	18.9	16.1	16.7	15.7	16.1	16.4	1.1		97.2	85.5	87.6	86.0	86.1	86.5	87.1
16.7	18.4	17.9	17.7	15.1	15.3	14.5	15.1	15.4	2.6	2.8		86.4	88.0	86.2	86.3	86.2	86.8
18.5	19.2	19.5	19.3	15.5	16.1	16.6	17.0	15.2	15.6	16.1	15.0		95.9	93.8	91.7	93.5	92.7
17.2	17.3	17.5	17.3	13.7	14.6	15.0	15.6	15.1	13.6	13.5	13.1	4.3		95.4	92.1	94.3	93.6
17.5	17.4	17.5	17.4	15.1	15.0	15.5	16.0	15.7	15.4	15.5	15.3	6.4	4.7		93.1	95.4	94.3
16.7	16.8	16.8	16.7	14.2	14.0	14.6	15.1	15.6	15.1	15.4	15.2	8.8	8.3	7.2		96.1	97.1
17.3	18.0	17.9	17.8	14.4	14.8	15.0	15.3	15.3	14.7	14.9	15.2	6.8	6.0	4.8	4.0		98.6
16.9	17.6	17.4	17.2	14.2	14.3	14.6	15.0	15.2	14.0	14.2	14.6	7.6	6.7	5.9	3.0	1.5	
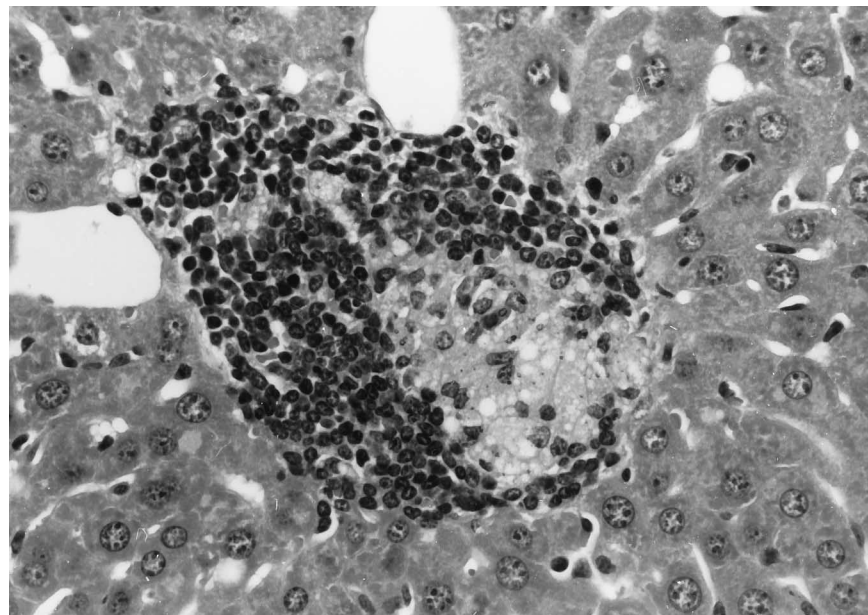
FIG. 5. High-power view of a focus of inflammation in a mouse liver depicting the lymphocyte and lipid- and pigment-laden macrophage composition of these foci. The stain used was hematoxylin and eosin. Magnification, ×300.
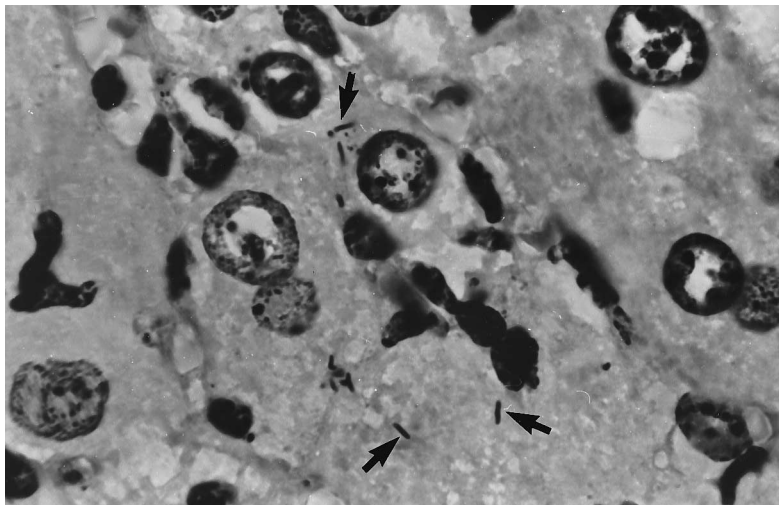
FIG. 6. An example of *H. bilis*-like organisms (arrows) observed in mouse liver. The stain used was Warthin-Starry. Magnification, ×750.

ously identified. All six DNA samples failed to yield an amplicon when the *H. hepaticus*-specific PCR primers were used (unpublished observation).

PCR primer 13 (Table 1) successfully produced the expected 186-base amplicons for *H. bilis* Hb1 and Hb2, the only strains tested with this primer. Because some strains of "*F. rappini*" also contain this IVS, the probe is not species specific. However, the probe can be used to differentiate *H. bilis* from *H. hepaticus*.

Histopathology. Liver lesions in the four different strains of mice varied from no lesions to disseminated focal aggregates of leukocytes located in close proximity to portal triads, central and collecting veins, or both and were occasionally randomly distributed in the parenchyma. The cell infiltrates were composed primarily of lymphocytes and macrophages, with a few polymorphonuclear leukocytes associated with the lesions in one strain of mice. Small clusters of pigment- and lipid-laden macrophages were generally found in association with the cell infiltrates but in some cases were independent of the cell infiltrates. The most severe lesions were in the CBA/CA strain, where the cell infiltrate included both mononuclear and polymorphonuclear leukocytes (Fig. 5). The lesions found in the DBA/2 strain were less severe and consisted of cell infiltrates which included both mononuclear and polymorphonuclear leukocytes. The C57BL/6 strain had very mild cell infiltrates, and the BALB/c strain had no inflammatory liver lesions. Hepatomas were observed in three mice—two male CBA/CA mice and one male BALB/c mouse. Bacteria were identified by Warthin-Starry stain in liver sections at the periphery of the hepatic lesions in four (one DBA/2, one CBA/CA, and two C57BL/6) mice and the lumen of bile ducts in one C57BL mouse (Fig. 6). In all of these cases, *H. bilis* was isolated from the livers and/or bile. No bacteria were observed in the BALB/c livers.

DISCUSSION

The epizootiology of *H. hepaticus* and *H. bilis* infections is unknown. However, like other *Helicobacter* species, these two species probably are normal colonizers of the gastrointestinal tract. Prior to the association of *H. hepaticus* and *H. bilis* with hepatitis in mice, two other helicobacters, *H. muridarum* and "*F. rappini*," were cultured from the mouse gastrointestinal tract (28, 38, 42). Both have their ecological niche in the lower gastrointestinal tract, where their presence normally does not elicit an inflammatory response (38, 42). *H. muridarum* can colonize the gastric tissue of both mice and rats and induce a gastritis (38). While not completely understood, the gastric colonization is probably related to subtle pH changes in the stomach of rodents as they age. On the basis of culture results, *H. hepaticus* can be isolated from the ceca and colon of mice with and without *H. hepaticus*-associated liver lesions (15). This appears to be the case in older inbred mice, in which *H. bilis* is more commonly isolated from the ceca and colon than from the liver. There is precedence for *Helicobacter* species causing hepatitis in other mammals under certain circumstances: "*F. rappini*" can cross the placenta of pregnant sheep, induce abortions, and cause acute hepatic necrosis in sheep fetuses (3, 21). Also, an organism with "*F. rappini*" morphology (possibly *H. bilis*) was observed in the common bile duct of rats experimentally infected with the liver fluke *Fasciola hepatica* (10). The authors speculated that the fluke infection altered the biochemical properties of the rats' bile and allowed the bacteria to colonize this normally bacteriostatic milieu. Most recently, "*H. pullorum*" has been isolated from livers of chickens with hepatitis and from humans with gastroenteritis (43). *H. bilis* and *H. hepaticus* not only are efficient colonizers of the gastrointestinal tract but in addition colonize the liver and bile, and in certain strains of mice, *H. hepaticus* induces a persistent hepatitis.

The intestinal (and hepatic) helicobacters of mice are closely related phylogenetically and are similar biochemically. Unlike *H. canis*, *H. fennelliae*, and *H. cinaedi*, which are recovered from other mammals and humans, the mouse helicobacters produce high levels of urease. With the exception of *H. hepaticus*, all have periplasmic fibers and bipolar tufts of sheathed flagella. "*F. rappini*" and *H. bilis* are fusiform, whereas *H. muridarum* has two to three spiral turns and *H. hepaticus* is a smaller, slightly curved to spiral rod with single bipolar flagella.

The presence of an IVS in the 16S rRNA genes for *H. bilis* at *E. coli* position 210 is common to several species of campylobacters and helicobacters (5). The identical IVS is present in strains representing two species with "*F. rappini*" morphology: ATCC 49314 and ATCC 49317. Because the sequences of the IVS from these three species are more conserved than the 16S rRNA sequences into which they are inserted, it seems prob-

able that the IVS were not present in the common ancestor of the three species but rather were acquired recently by horizontal transfer. A full discussion of IVS in campylobacters and helicobacters is beyond the scope of this report and will be the subject of a separate report.

One notorious pathogen, *Salmonella typhi*, is known for its ability to colonize bile. In patients with gallstones, long-term colonization results in the typhoid carrier state. Because *S. typhi* is resistant to high concentrations of bile, selective media containing high concentrations of bile salts are used for its isolation. The high concentrations of bile salts impede the growth of most gram-negative and gram-positive bacteria normally present in fecal flora (20). Similar strategies may be useful for the isolation of *H. bilis*, which is resistant to 20% bile salts, and may also prove useful for isolating *H. hepaticus* from feces of mice. It is interesting that, with the exception of *H. muridarum*, the intestinal helicobacters are resistant to 1% bile salts, whereas the gastric helicobacter species are sensitive. Sensitivity to bile salts that normally transit the lower gastrointestinal tract may help explain the difficulty in isolating gastric helicobacters from feces. Whether helicobacter colonization of the bile helps confer carrier status in selected strains of mice or whether crypt colonization with these bacteria is sufficient for persistent infection requires further studies.

While there are no reports of helicobacter-associated hepatitis in humans, there are occasional reports of hepatitis and cholecystitis in humans caused by *Campylobacter jejuni* (4, 7, 31, 37, 48). Little has been done experimentally on the role of *Helicobacter* species in hepatic disease; however there has been substantial research with *C. jejuni*-associated hepatitis in mice (22–24). Focal necrotic hepatitis in the absence of diarrhea also has been noted in mice 1 to 2 months after oral dosing with selected strains of *C. jejuni* (22, 24). Ten micrograms of the purified hepatotoxic factor, isolated from selected strains (in 4 of 20 tested) of *C. jejuni*, reproducibly caused acute hepatic necrosis in specific-pathogen-free dd-Y mice when inoculated intravenously. The hepatotoxin also caused cell death in cultured mouse hepatocytes.

A soluble hepatotoxic factor may be involved in the pathogenesis of the liver lesions induced by helicobacter species. Indeed, the first description of the necrotic hepatitis in mice, before the discovery of *H. hepaticus*, referred to the lesion as "toxic" hepatitis (49). Considerable effort was expended in search of a toxic substance in food, water, and bedding of the mice with hepatitis until the organism was demonstrated in liver tissue with a silver stain. We recently determined that culture supernatants from several strains of *H. hepaticus* and selected strains of *H. pylori*, *H. mustelae*, and *H. felis* cause distinct cytopathic effects in a rodent hepatocyte cell line (45). Studies are under way to further purify and characterize this toxin and to ascertain whether *H. bilis* strains also have this toxin.

The types of hepatitis associated with *H. hepaticus* and liver lesions observed in the aged mice with *H. bilis* infection are similar in some ways. In both *H. hepaticus* and the aged mouse livers, the lesions consisted of multifocal hepatitis with a mixed-cell inflammatory infiltrate. However, livers from aged mice did not exhibit oval cell hyperplasia and cholangitis observed in some strains of mice with *H. hepaticus*-associated hepatitis (50). The fact that we cultured *H. bilis* and demonstrated *H. bilis*-like bacteria in the livers of several mice by histology and PCR does not prove that *H. bilis* is the cause of hepatitis in these mice; however, its presence in livers with hepatitis certainly indicates that further studies are warranted to prove or disprove its association with hepatitis.

Description of *H. bilis* sp. nov. *H. bilis* (L. *bilis*, relating to the bile). Cells are fusiform to slightly spiral and measure 0.5 by 4 to 5 μm. In older cultures, coccoid forms with overlapping periplasmic fibers are common. Cells are gram negative and nonsporulating. Cells are motile by means of tufts of sheathed flagella numbering 3 to 14 at each end. Colonies are pinpoint, but cultures often appear as a thin spreading layer on agar media. There is microaerophilic growth at 37 and 42°C but not at 25°C. There is growth in 20% bile and 0.4% TTC (triphenyltetrazolium chloride), variable growth in 1% glycine, but no growth in 1.5% NaCl. The organism possesses urease, catalase, and oxidase activities. Nitrate is reduced. H_2S is detected on lead acetate discs. Indoxyl acetate and hippurate were not hydrolyzed. The organism is resistant to cephalothin and nalidixic acid but sensitive to metronidazole. Cells have been isolated from the colons and ceca of mice and the bile and livers of mice with hepatitis. The type strain, Hb1, was isolated from the bile and liver of a mouse with active chronic hepatitis and hepatoma. The type strain has been deposited with the American Type Culture Collection as ATCC 51630.

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