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Helicobacter typhlonius sp. nov., a Novel Murine Urease-Negative *Helicobacter* Species

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Over the past decade, several *Helicobacter* species have been isolated from rodents. With the advent of PCR for the diagnosis of infectious agents, it has become clear that several previously uncharacterized *Helicobacter* species also colonize rodents. In this report, we describe a novel urease-negative helicobacter, *Helicobacter typhlonius* sp. nov., which was isolated from colonies of laboratory mice independently by two laboratories. Infection of immunodeficient mice by this bacterium resulted in typhlocolitis similar to that observed with other helicobacter infections. *H. typhlonius* is genetically most closely related to *H. hepaticus*. Like *H. hepaticus*, it is a spiral bacterium with bipolar sheathed flagella. However, this novel species contains a large intervening sequence in its 16S rRNA gene and is biochemically distinct from *H. hepaticus*. Notably, *H. typhlonius* does not produce urease or H_2S nor does it hydrolize indoxyl-acetate. Compared to other *Helicobacter* species that commonly colonize rodents, *H. typhlonius* was found to be less prevalent than *H. hepaticus* and *H. rodentium* but as prevalent as *H. bilis. H. typhlonius* joins a growing list of helicobacters that colonize mice and are capable of inducing enteric disease in various strains of immunodeficient mice.

The genus *Helicobacter* has rapidly expanded since the discovery of the type species, *Helicobacter pylori*, in 1982. This genus currently contains 24 named species and numerous provisionally named species. It is likely that several novel *Helicobacter* species await discovery. Members of this genus are microaerobic, have a fusiform or curved to spiral rod morphology and are motile by flagella that vary in number and location among different species (44). All known helicobacters live in human and animal hosts, where colonization occurs primarily in the gastrointestinal tract. The type species, *H. pylori*, was isolated from the stomach of humans and has been associated with a variety of gastric anomalies including gastritis, peptic ulcer disease, gastric carcinoma, and gastric mucosa-associated lymphoma (25, 26, 28, 29, 48). Like *H. pylori*, other species of *Helicobacter* have also been shown to colonize the stomach and cause disease in animals. Gastric colonizers include *H. felis*, *H. mustelae*, *H. acinonychis*, *H. bizzozeronii*, *H. heilmannii*, *H. salomonis*, and a recently isolated novel *Helicobacter* sp. of dolphins (5, 19–21, 30). Several species of *Helicobacter* have been identified in rodents, including the species *H. hepaticus*, *H. bilis*, *H. muridarum*, *H. aurati*, *H. cinaedi*, *H. cholecystus*, *H. trogontum*, *H. rodentium*, and a bacterium morphologically resembling *Helicobacter* Flexispira taxon 8 (formerly *Flexispira rappini*) (9, 13, 14, 17, 24, 27, 31, 32, 36, 37). Of these, only *H. muridarum* and *H. aurati* have been demonstrated to colonize the rodent stomach. Instead, most rodent helicobacters colonize the large intestine and in some cases translocate to the liver and colonize the biliary system.

Several rodent *Helicobacter* spp., including *H. hepaticus*, *H. bilis*, and *H. rodentium*, have been associated with enterohepatic disease. *H. hepaticus* has been shown to cause inflammatory bowel disease and chronic active hepatitis in both immunocompetent and immunodeficient mice (2, 12, 45–47). Conversely, *H. bilis* and *H. rodentium* have only been convincingly associated with disease in immunodeficient rodents (15, 18, 38). Lesions caused by these rodent *Helicobacter* spp. often mimic those seen in human idiopathic enterohepatic diseases, especially inflammatory bowel diseases. Therefore, rodent helicobacter infections have attracted the attention of scientists interested in the study of these diseases. Not only have these infections been used as models for disease, but inadvertent infections by rodent *Helicobacter* spp. have confounded interpretation of results from established models of inflammatory bowel disease (2, 7, 10, 23). As a result, it has become increasingly important to ascertain the *Helicobacter* status of experimental animals prior to the initiation of studies using rodent inflammatory bowel disease models.

With the advent of molecular diagnostic techniques such as PCR designed to detect known *Helicobacter* species, it is becoming increasingly evident that rodents are colonized by numerous novel *Helicobacter* species. Our laboratories have been screening rodents by PCR and culture for several years. As a result, several uncharacterized *Helicobacter* spp. have been identified (L. K. Riley, unpublished results; J. G. Fox, unpublished results). Whether or not these novel species of *Helicobacter* cause disease awaits further systematic study of infected animals.

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Recently, Fox et al. described a novel urease-negative helicobacter, designated *Helicobacter* sp. strain MIT 97-6810, that was isolated from interleukin-10 (IL-10)-deficient mice with typhlocolitis (10). IL-10-deficient and SCID/NCr mice experimentally infected with this *Helicobacter* strain also developed typhlitis, colitis, and proctitis, and experimentally infected A/JCr mice developed minimal to mild typhlitis. Shortly thereafter, Franklin et al. described a disease syndrome characterized by proliferative typhlocolitis in C.B-17 *scid/scid* mice naturally and experimentally infected with a novel species of *Helicobacter* that was provisionally named "*Helicobacter typhlonicus*" (16). *H. typhlonicus* was genetically and morphologically identical to *Helicobacter* sp. strain MIT 97-6810. In this report, we propose to use the more appropriate neo-Latin adjective "*typhlonius*" and formally name this novel helicobacter *Helicobacter typhlonius*. Furthermore, we demonstrate that *H. typhlonius* is a common intestinal colonizer of research mice.

MATERIALS AND METHODS

Bacterial isolates. Isolates MIT 97-6810 and MIT 97-6811 were isolated from IL-$10^{-/-}$ knockout mice on a C57BL6/129-Ola background (10). One mouse had a rectal prolapse, while the second was clinically normal. Isolates MU 96-1 and MU 96-2 were cultured from feces of BALB/c mice (16). There was no history of clinical signs, abnormal necropsy findings, or significant histologic lesions in mice from this colony. Isolate MU 96-3 was recovered from the feces of a FOX CHASE SCID C.B-17/IcrCrl-*scid*BR (SCID) mouse that had been inoculated with MU 96-1 (16). Isolation procedures were similar to those previously described for the isolation of *H. hepaticus* and *H. bilis* (9, 13, 14). Briefly, fecal material was homogenized in either phosphate-buffered saline (PBS) or brain heart infusion broth containing horse serum and yeast extract. The fecal slurries were filtered through either a 0.45- or 0.80-μm (pore-size) filter and placed on plates of Trypticase soy or brucella agar that contained 5% sheep blood. In some cases, blood agar plates also contained trimethoprim, vancomycin, and polymyxin. Cultures were incubated in a microaerobic environment at 37 and 42°C for up to 2 weeks.

DNA sequencing. Sequence analyses of bacterial 16S rRNA genes were performed as previously described (13, 14). Briefly, bacterial DNA was extracted from samples, and sequencing templates were prepared by PCR with one of the following primer sets: (i) C70f and B37r, (ii) H35f and H587r, (iii) C70f and H676r, (iv) H276f and cg4r, and (v) H276f and p13Br (3, 13, 33, 34). Templates were purified on 3.5% polyacrylamide gels, and the gene sequence was determined by using either the *Taq* Dye Deoxy Termantory Cycle Sequencing Kit (Applied BioSystems, Inc., Foster City, Calif) or the TAQuence Cycle Seq Kit (U.S. Biochemicals, Cleveland, Ohio) (10, 16). Sequence analyses were performed by using the Sequence Analysis Software Package (Wisconsin Package, version 10.0; Genetics Computer Group, Inc., Madison, Wis.). The sequences (1,307 bp) of the five novel *Helicobacter* isolates were aligned with sequences of 32 bacteria representing formally and provisionally named species of *Helicobacter*, and select species of *Wolinella*, *Campylobacter*, *and Arcobacter*. A matrix of pairwise evolutionary distances between aligned sequences (similarity matrix) was constructed; both uncorrected distances and corrected distances made using the Jukes-Cantor method (22) were calculated. A phylogenetic tree was created by the neighbor-joining method (35, 42, 43). Sequence data used for comparisons were obtained from GenBank (accession numbers are provided in Fig. 1).

Specific identification of *H. typhlonius* by PCR. Two PCR primer sets were generated from sequence data. Primer set 1 consisted of the forward primer JGF-F1 (5′-GAA ACT ATC ACT CTA GAG TAT G-3′) and the reverse primer JGF-R1 (5′-TGC TCC TCA TTG TAT GCC-3′). This primer set amplified a 622-bp fragment from nucleotide positions 639 to 1260 (*Escherichia coli* numbering). The following conditions were used: 50 μl of reaction mixture with 50 ng of template, 200 μg of bovine serum albumin ml^{-1}, 5 pmol of primer, 1× reaction buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3; Roche Molecular Biochemicals, Indianapolis, Ind.), and 2.5 U of *Taq*, with 40 cycles of 1-min denaturation at 94°C, 1-min annealing at 52°C, and 1-min extension at 72°C. Primer set 2 consisted of the forward primer Ht184f (5′-TTA AAG ATA TTC TAG GGG TAT AT-3′) and the reverse primer Ht640r (5′-TCT CCC ATA CTC TAG AGT GA-3′). The forward primer was designed from the intervening sequence. This primer set amplified a 455-bp fragment from base position 131 of the intervening sequence to nucleotide position 665 (*E. coli* numbering). The following conditions were used for this PCR: 50 μl of reaction mixture with 1.25 μg of template, 2.5 pmol of primer, 10× reaction buffer, and 1.25 U of *Taq*, with 45 cycles of 2-s denaturation at 94°C, 10-s annealing at 53°C, and 30-s extension at 72°C. Samples were analyzed by PCR as previously described (1, 10, 34).

Biochemical characterization. To further characterize isolates, phenotypic tests commonly used to characterize helicobacters were performed (14). Growth was examined at 25, 37, and 42°C. Tolerances to 1% (wt/vol) glycine and 1.5% NaCl were determined as previously described (9). Bacteria were tested for urease activity by a selective rapid urea test (Remel, Lenexa, Kans.) and examined for oxidase and catalase activity by standard microbiological methods (6). Alkaline phosphatase activity and hydrolysis of indoxyl-acetate were analyzed by using the Ani-Ident system (bioMerieux Vitek, Inc., Hazelwood, Mo.). Gamma-glutamyltransferase and alkaline phosphatase activities, hydrolysis of hippurate, reduction of nitrate to nitrite, and production of hydrogen sulfide were analyzed by using the Campy identification system (bioMerieux Vitek). Reduction of nitrate was also determined by using nitrate impregnated disks (Remel). Susceptibilities to cephalothin and nalidixic acid were determined by culturing isolates in the presence of antibiotic impregnated disks (Remel).

Electron microscopy. Bacteria were collected from blood agar plates, placed in PBS (pH 7.4), and centrifuged at 12,000 × *g*. Bacteria were washed, resuspended, stained with phosphotungstic acid for 20 to 30 s, and examined with either a JEOL model JEM-1 200EX or an Hitachi H-6700 transmission electron microscope.

Nucleotide sequence accession numbers. The type strains of *H. typhlonius* sp. nov., MIT 97-6810 and MU 96-1, isolated from the intestinal contents of an IL-$10^{-/-}$ knockout mouse and a BALB/c mouse, respectively, have been deposited with The American Type Culture Collection (ATCC) and are designated strains ATCC BAA-367^T and ATCC BAA-368. The 16S rRNA sequences of the type strains are available for electronic retrieval from GenBank under accession numbers AF127912 and AF061104.

RESULTS

Bacterial analyses. Five isolates of the *H. typhlonius* (MIT 97-6810, MIT 97-6811, MU 96-1, MU 96-2, and MU 96-3), were examined by 16S rRNA gene sequence analysis. Sequences were obtained from a 1,614-bp fragment or a 1,448-bp fragment internal to the 1,614-bp fragment. The 1,448-bp fragments from all five isolates were 100% identical. A 166-bp intervening sequence (IVS) was identified following position 198 in all isolates. This IVS occupied the area normally occupied by a seven-base stem-loop centered on position 210 in several other species of *Helicobacter*. Because not all helicobacters contain an IVS, this sequence was removed for genetic comparisons. The consensus sequence from the novel *Helicobacter* sp. was aligned with sequences from bacteria of the genera *Helicobacter*, *Campylobacter*, and *Wolinella* as previously described (13, 14). A similarity matrix was generated (data not shown), and a phylogenetic tree was constructed (Fig. 1). Sequences of all known rodent helicobacters were included in this comparison. On the basis of this comparison, *H. typhlonius* was most closely related to *H. hepaticus* but was a distinct species exhibiting 97.64% similarity and containing the aforementioned intervening sequence.

Six additional isolates obtained from mice inoculated with MIT 97-6810 were analyzed by PCR with primer set 1. Isolates 98-6781 and 98-6782 were from 4-week postinoculation mice, and isolates 98-784, 98-6785, 98-7686, and 98-6787 were from 18-week postinoculation mice. All isolates were found to be positive by this PCR. DNA from *H. rodentium*, another urease-negative *Helicobacter* sp., did not amplify with this primer set.

Phenotypic tests commonly used to characterize helicobacters were performed on MU 96-1, MU 96-2, MIT 97-6810, and MIT 97-6811. All isolates had identical biochemical phenotypes (Table 1). Cells were motile and helical and grew on

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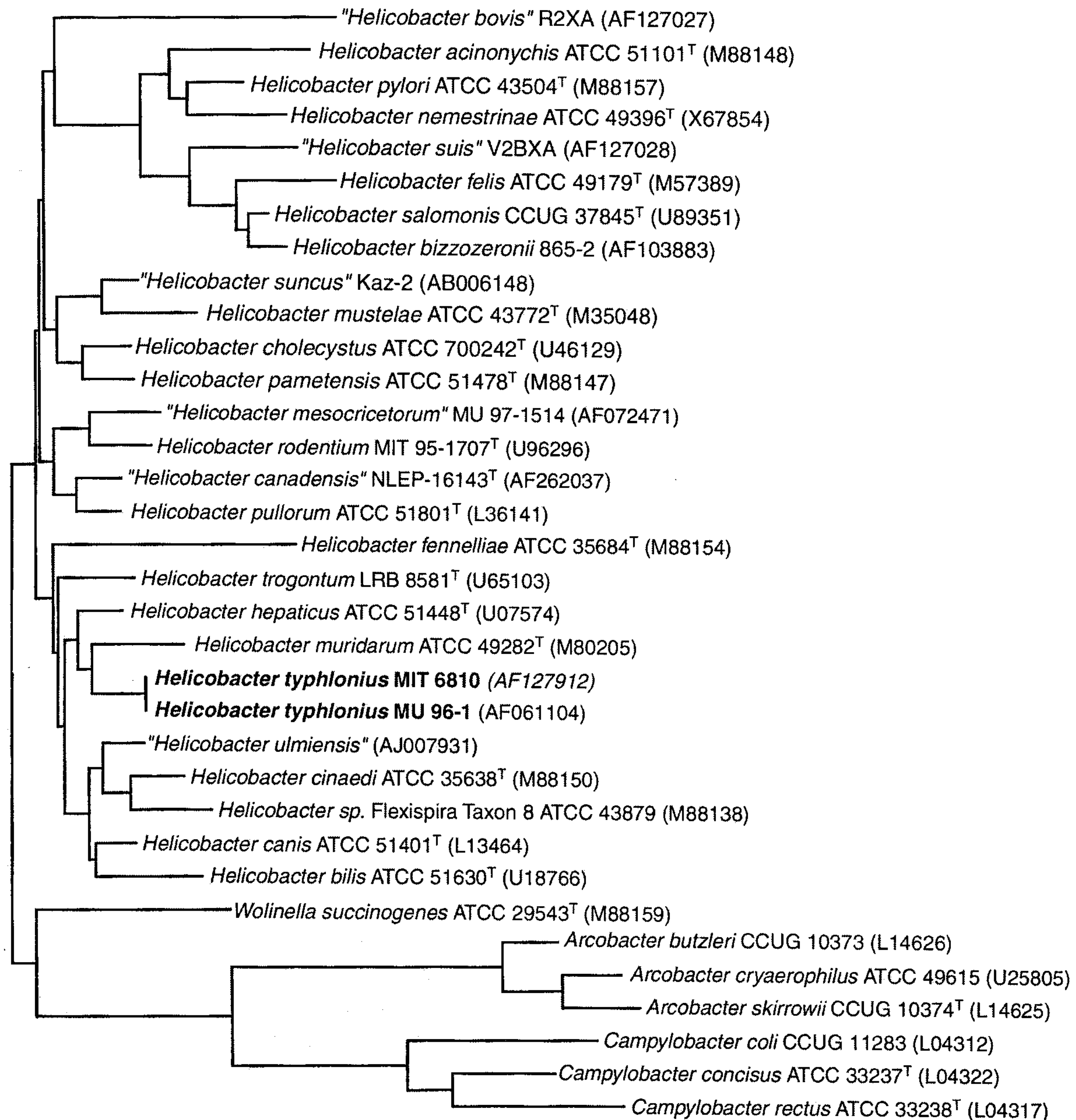

FIG. 1. Phylogenetic tree of members of the genera *Helicobacter, Campylobacter, "Flexispira," Wolinella*, and *Arcobacter* based on 16S ribosomal DNA sequences and prepared by using the neighbor-joining method. Phylogenetic distances between bacteria are calculated by totaling horizontal branches between bacteria. Bar, 1 nucleotide substitution per 100 nucleotides.

moist blood agar as transparent pinpoint colonies. Upon ultrastructural examination, isolates MIT 97-6810 and MU 96-1 were identical. The majority of bacteria were 0.3-μm by 2- to 3-μm curved to spiral rods, with single sheathed bipolar flagella and no periplasmic fibers (Fig. 2).

***H. typhlonius* is common in research mice.** Fecal samples submitted to the University of Missouri Research Animal Diagnostic and Investigative Laboratory for helicobacter testing were examined by PCR with primer sets designed to detect (i) all species of *Helicobacter* (generic test), (ii) *H. hepaticus*, (iii) *H. bilis*, (iv) *H. rodentium*, and (v) *H. typhlonius* (with primer set 2 [Ht184f and Ht640r]). Samples that were found to be positive by the generic helicobacter PCR but negative by all species tests were designated as positive for *Helicobacter* spp. Of 1,271 samples tested from November 1999 through April 2000, 4.88% were positive for *H. typhlonius*, 16.44% were positive for *H. hepaticus*, 4.33% were positive for *H. bilis*, 15.11% were positive for *H. rodentium*, and 10.54% were positive for *Helicobacter* spp.

DISCUSSION

In this report we describe the phylogenetic characterization of a novel urease-negative spiral bacterium that was isolated

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TABLE 1. Biochemical and morphologic characteristics of *H. typhlonius* compared to closely related and other rodent *Helicobacter* spp.

Characteristic	Results with[a]:					
	H. typhlonius[b]	*H. hepaticus*	*H. trogontum*	*H. muridarum*	*H. rodentium*	*H. bilis*
Enzyme activities						
Catalase	4/4	+	+	+	+	+
Urease	0/4	+	+	+	−	+
Oxidase	4/4	+	+	+	+	+
Alkaline phosphatase	0/2	−	−	+	−	−
Gamma-glutamyltransferase	0/2	−	+	+	−	+
H_2S production	0/4	+	−	+	ND	+
Indoxyl-acetate hydrolysis	0/2	+	−	+	−	−
Hippurate hydrolysis	0/4	−	−	−	ND	ND
Nitrate reduction	2/4	+	+	−	+	+
Flagellar morphology						
Periplasmic fibers	0/2	−	+	+	−	+
Number	2	2	5–7	10–14	2	3–14
Location	Bipolar	Bipolar	Bipolar	Bipolar	Bipolar	Bipolar
Growth conditions						
1% Glycine	4/4(wk)[c]	+	−	−	+	+
1.5% NaCl	0/4	+	−	−	+	−
25°C	0/4	−	−	−	−	−
37°C	4/4	+	+	+	+	+
42°C	4/4	−	+	−	+	+
Anaerobic	0/4	+	ND	+	+	ND
Aerobic	0/2	−	ND	−	−	ND
Antibiotic sensitivities						
Nalidixic acid	S (4/4)	R	R	R	R	R
Cephalothin	R (4/4)	R	R	R	R	R

[a] Symbols: +, positive result; −, negative result; ND, not determined; R, resistant; S, susceptible. Information on *H. hepaticus* is based on references 4 and 9; information on *H. trogontum* is based on references 4 and 27; information on *H. muridarum* is based on references 13 and 24; information on *H. rodentium* is based on reference 37; and information on *H. bilis* is based on references 4 and 13.

[b] Number of positive strains/number tested.

[c] Most of the cells from isolates MU 96-1 and MU 96-2 harvested in these conditions had coccoid morphology.

independently by two laboratories. This bacterium merits a formal name as a novel *Helicobacter* species because it is genetically distinct from other *Helicobacter* spp. and exhibits important biochemical differences compared to its closest relatives. We propose the name *H. typhlonius* because this bacteria was isolated from inflamed intestinal contents of naturally infected IL-$10^{-/-}$ mice and has, under defined conditions, produced inflammation of the ceca and colon of both IL-$10^{-/-}$ and SCID mice (10, 16).

H. typhlonius is most closely related to *H. hepaticus* (97.44%), and it clusters with *H. muridarum* and *H. trogontum*. While *H. typhlonius* is on a genetic basis closely related to these species, it also possesses a unique IVS in its 16S rRNA sequence, and it is morphologically and/or biochemically distinguishable from its closest phylogenetic relatives. Unlike *H. muridarum* and *H. trogontum*, *H. typhlonius* lacks the periplasmic fibers and multiple flagella and does not have gamma-glutamyltransferase activity. *H. typhlonius* is morphologically similar to *H. hepaticus*; however, unlike *H. hepaticus*, it does not produce H_2S or hydrolyze indoxyl-acetate. Perhaps the most dramatic difference between *H. typhlonius* and its three closest relatives is its lack of urease activity. Preliminary data obtained by both of our laboratories suggest that *H. typhlonius* lacks the gene for urease (10; C. S. Beckwith, unpublished results).

The role of urease in species of *Helicobacter* that colonize the large intestine is unknown. Both urease-positive (*H. hepaticus* and *H. bilis*) and two urease-negative helicobacters have been shown to cause proliferative disease of the cecum and colon (10, 15, 16, 39, 46). Because the novel urease-negative helicobacter described by Shomer et al. produces significant hepatic and intestinal disease in A/JCr mice, as well as in immunodeficient mice, it is unlikely that urease plays a major role in the pathogenesis of enteric disease (40). Interestingly, only mild portal inflammation in the liver has been seen in *H. typhlonius*-infected immunocompromised mice (10, 16). This is in contrast to the severe necrotizing liver disease seen in SCID mice infected with either *H. hepaticus* and *H. bilis*. There was no evidence of colonization of the liver by *H. typhlonius*, as evidenced by uniformly negative PCR results (16). Also, *H. rodentium*, another urease-negative helicobacter, has not been isolated from livers nor is it associated with liver disease (37). This may be due to a lack of undetermined virulence factors in both of these urease-negative *Helicobacter* spp.

Several other species of *Helicobacter* also lack urease activity. These include *H. pullorum*, *H. canis*, *H. fennelliae*, *H. cinaedi*, and *H. canadensis* (8, 11, 41, 44). Interestingly, all of have been isolated from the feces of diarrheic humans, supporting the premise that urease is not critical to the pathogenesis of enteric disease. Also, *H. pullorum*, *H. cinaedi*, and *H. canis* have been isolated from inflamed livers (11, 41). The only other known rodent helicobacters that lack urease are an un-

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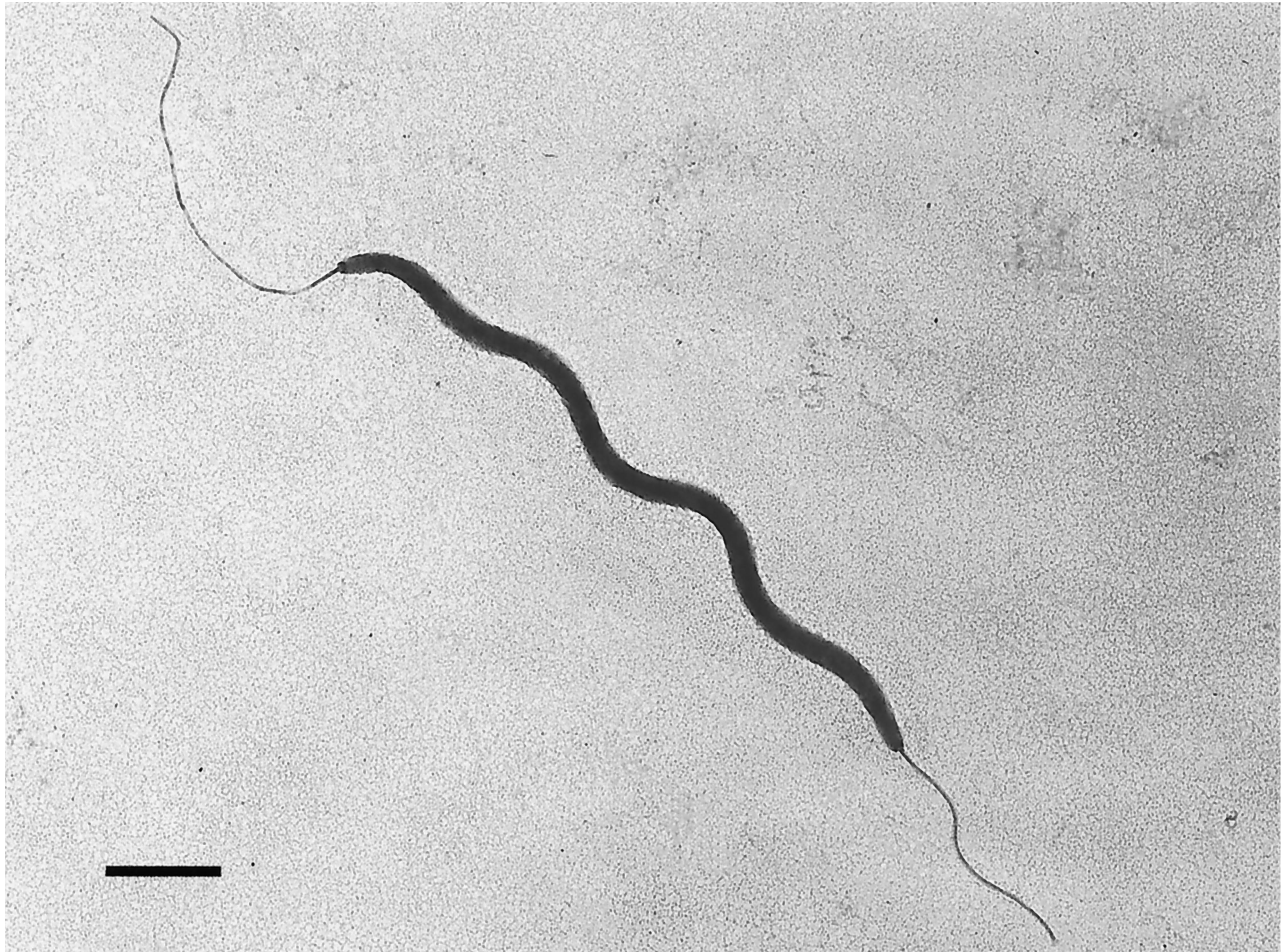

FIG. 2. Negatively stained preparation of *H. typhlonius* MU 96-1 demonstrating single sheathed bipolar flagella and no periplasmic fibers. Bar, 1 μm.

named novel species of *Helicobacter* that can cause cholangiohepatitis and inflammatory bowel disease (40) and *H. rodentium* (37). Coinfection with *H. rodentium* and *H. bilis* has been associated with diarrhea in SCID mice, suggesting that, like *H. typhlonius*, *H. rodentium* may be pathogenic to immunocompromised mice (38). Although *H. typhlonius* is genetically distinct from *H. rodentium* (94.28% identity), these bacteria are biochemically and phenotypically very similar. Both agents produce catalase, reduce nitrate, and have bipolar flagella, and both are negative for indoxyl-acetate hydrolysis and gamma-glutamyltranspeptidase and alkaline phosphatase activities. Like *H. rodentium*, *H. typhlonius* grows in the presence of 1% glycine, but the majority of the bacteria grown in these conditions are degenerative coccoid forms. The only other known phenotypic differences are growth in anaerobic conditions, susceptibility to nalidixic acid, and flagellar morphology; *H. rodentium* grows in anaerobic conditions, is resistant to nalidixic acid, and has nonsheathed flagella, whereas *H. typhlonius* does not grow in anaerobic conditions, is susceptible to nalidixic acid, and has sheathed flagella. Another previously described, unnamed novel urease-negative *Helicobacter* sp. is also characterized by single polar sheathed flagella (40).

Screening of over 1,000 fecal samples submitted to the University of Missouri Research Animal Diagnostic and Investigative Laboratory demonstrated *H. typhlonius* colonization in 4.88% of the samples. This finding suggests that *H. typhlonius* is prevalent in laboratory rodent colonies in the United States. *H. typhlonius* was not as prevalent as *H. hepaticus* (16.44%) or *H. rodentium* (15.11%) but was slightly more prevalent than *H. bilis* (4.33%). While these data must be interpreted cautiously because some samples were likely submitted from colonies known or suspected to be contaminated by *Helicobacter* spp., they highlight that multiple *Helicobacter* spp. are common in research mouse colonies. Furthermore, the IL-10 knockout mice from which the MIT isolates were obtained originated in Germany, suggesting that *H. typhlonius* has a worldwide distribution (10).

In conclusion, geographically disparate laboratories independently identified a novel *Helicobacter* sp. that is capable of causing enteric disease in immunodeficient mice (10, 16). We propose to name this species *H. typhlonius*. Routine screening of rodent colonies throughout the United States suggests that *H. typhlonius* is prevalent in rodent colonies. Its importance in causing naturally occurring gastrointestinal disease in immunocompetent mice, as well as enteric disease in other hosts, will require further studies.

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Description of *H. typhlonius* sp. nov. *Helicobacter typhlonius* (ti.flo′ ni.us Gr. n. *typhlon*, cecum; N.L. adj. *typhlonius*, pertaining to the cecum). Filamentous cells are 0.3- by 2- to 3-μm curved to spiral rods, with no periplasmic fibers. Cells do not form spores and are motile by single sheathed bipolar flagella. Colonies are pinpoint. Cells grow in microaerobic but not in anaerobic or aerobic conditions. There is growth at 37 or 42°C but not at 25°C nor in the presence of 1.5% NaCl. There is growth in the presence of 1% glycine; however, the majority of cells harvested in these conditions have coccoid morphology. *H. typhlonius* is oxidase and catalase positive but does not have urease, alkaline phosphatase, or gamma-glutamyltransferase activities. Nitrate is reduced to nitrite. Indoxyl-acetate and hippurate are not hydrolyzed. Cells are resistant to cephalothin and sensitive to nalidixic acid.

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