Cholangiohepatitis and Inflammatory Bowel Disease Induced by a Novel Urease-Negative *Helicobacter* Species in A/J and Tac:ICR:Ha*scidf*RF Mice

NIRAH H. SHOMER,* CHARLES A. DANGLER,* MARK D. SCHRENZEL,* MARK T. WHARY,* SHILU XU,* YAN FENG,* BRUCE J. PASTER,† FLOYD E. DEWHIRST,† AND JAMES G. FOX*,[1]

**Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; †Forsyth Institute, Boston, Massachusetts 02115*

Helicobacter bilis and *H. hepaticus*, both urease-positive intestinal helicobacters of mice, have been shown experimentally to induce proliferative typhlocolitis in *scid* mice. We recently isolated a urease-negative Helicobacter sp. (*H.* sp.) that also induced proliferative typhlocolitis in pilot studies in *scid* mice. To determine the pathogenic potential of *H.* sp. in immunocompromised and immunocompetent mice, 5-week old male A/J or Tac:Icr:Ha(ICR)-*scidf*RF mice were inoculated by intraperitoneal (IP) injection with ~ 3×10^7 colony-forming units (CFU) of *H.* sp. Mice were necropsied at various time points postinoculation (PI). Sham-inoculated mice had no clinical, gross, or histopathological lesions. In contrast, *scid* mice inoculated IP with *H.* sp. had severe hemorrhagic diarrhea and decreased weight gain at 2, 7, and 18 weeks postinoculation (PI), with severe proliferative typhlocolitis, phlebothrombosis, and hepatitis. A/J mice had no clinical signs, but had mild to moderate proliferative typhlocolitis and moderate to marked cholangiohepatitis at 7 and 24 weeks PI. A/J mice infected with *H.* sp. developed robust immune responses of a predominant Th1 type. This report demonstrates that infection with a urease-negative helicobacter can cause inflammatory bowel disease (IBD) and hepatitis in *scid* and immunocompetent A/J mice. These results provide a new model of IBD and cholangiohepatitis associated with a specific urease-negative, novel *H.* species. [Exp Biol Med Vol. 226(5):420–428, 2001]

Key words: *Helicobacter;* mouse; typhlocolitis; cholangiohepatitis

This work was funded in part by NIH (grants R01CA67529, R01DK52413, P01CA26731, and RR01046).

[1] To whom requests for reprints should be addressed at Division of Comparative Medicine, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Building 16, Room 825C, Cambridge, MA 02139. E-mail: jgfox@mit.edu

Received July 21, 2000.
Accepted December 15, 2000.

0037-9727/01/2265-0420$15.00

There has been considerable interest in intestinal *Helicobacter* spp. (*H.* sp.) of mice because of the association with hepatitis and inflammatory bowel disease (1–8). These organisms are of particular relevance for three reasons. First, they may be important models implicating bacterial infections in carcinogenesis. *Helicobacter hepaticus*, an intestinal helicobacter that has been shown to cause persistent hepatitis in A/JCr mice, has been linked with hepatitis and hepatic tumors in susceptible strains of mice, and has been proposed as a model of helicobacter-induced tumorogenesis (1, 3, 6–9). Second, many of these enterohepatic *H.* species are widespread in rodent colonies and may significantly confound the interpretation of some research studies (10, 11). For example, some transgenic rodent strains that have been proposed as spontaneous models of inflammatory bowel disease (IBD) are infected with rodent intestinal helicobacters (10) that are known to potentiate the development of IBD in both unreconstituted and $CD45RB^{hi}$- reconstituted *scid* mice (9, 12, 13). Experimental infection with *H. bilis* has also been shown to induce inflammatory bowel lesions in *scid* mice (9, 12). Furthermore, a mixed infection of *H. bilis* and the recently characterized *H. rodentium* (14) has been associated with severe necrotizing proliferative typhlocolitis and diarrhea in a colony of immunodeficient mice (5). However, urease-negative *H.* species, until recently, have been considered nonpathogenic (2). Third, they may have important zoonotic implications; recently, *H. bilis*, *H. pullorum*, and "*H. rappini*" were identified by PCR in the gallbladders and bile of humans with chronic cholangitis (15), and novel helicobacters were identified in the livers of humans with hepatocellular carcinomas (16).

The purpose of this study was to determine the pathogenic potential of a novel urease-negative *H.* species in both immunocompromised and immunocompetent mice, and to

determine if infection with this organism in these mice would provide a suitable model for enterohepatic disease.

Materials and Methods

Animals. Five-week-old, male Tac:Icr:Ha(ICR)-*scid*fRF *scid* mice were obtained from Taconic Farms (Germantown, NY). The mice had a restricted flora and were colonized with a proprietary cocktail (altered Schaedler's flora) of eight anaerobic bacteria (17), plus several other nonpathogenic bacteria. All husbandry materials for *scid* mice (microisolator caging, bedding, pelleted rodent chow [Purina Mills, Richmond, IN], and water bottles) were autoclaved before use; all *scid* mouse manipulations took place in a biosafety cabinet. Four- to 5-week-old *H.* spp.-negative, male A/J mice were obtained from a commercial vendor (Jackson Laboratories, Bar Harbor, ME). The A/J mice were maintained in nonautoclaved filter-top microisolator caging for the duration of the experiment. The mice were fed pelleted rodent chow (Purina Mills) and were provided water *ad libitum*. All mice were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Animals were maintained at a constant temperature of 70° to 72°C, 40% to 70% humidity, with 10 to 15 air changes per hour. All manipulations performed on the mice were approved by the Animal Care Committee of the Massachusetts Institute of Technology.

Bacteria. The novel urease-negative *H.* sp. (MIT 96-1001) was originally isolated from the cecum of a clinically normal feral mouse. It was grown under microaerobic conditions in vented jars containing N_2, H_2, and CO_2 (80:10:10) at 37°C in Brucella broth supplemented with 5% fetal calf serum. The bacteria were harvested after 24 to 48 hr of growth, resuspended in phosphate-buffered saline (PBS), and visualized by gram stain and phase microscopy for purity, morphology, and motility. The optical density (OD_{600}) was adjusted to 1.0 and 0.3 ml of this inoculum (~3×10^7 colony-forming units [CFU]) was used for each intraperitoneal (IP) injection or oral dose.

Bacterial Isolation. Bacteria were cultured from fecal pellets (*ante mortem*) or from cecal contents or ground liver at necropsy. At scheduled intervals, pooled samples of three to five fresh fecal pellets were obtained from each cage of mice. Fecal pellets were suspended in 1 ml of sterile PBS, and the resulting slurry was plated directly onto CVA plates (cefaperazone, vancomycin, amphotericin B; Remel, Lenexa, KS) for microaerobic isolation of the novel *H.* sp. At necropsy, cecal contents were applied directly to CVA plates. Aseptically collected liver was ground and applied directly to CVA plates for isolation of *H.* sp., and to blood agar plates for isolation of aerobic bacteria. Although growth of *H.* sp. was generally evident within 5 days, plates were maintained for 2 weeks before a determination of no growth was made.

Electron Microscopy. The novel *H.* sp. was examined by electron microscopy. Cells were grown on blood agar plates (Remel) at 37°C under microaerobic conditions for 48 h. Then they were gently resuspended in 10 mM Tris buffer (pH 7.4) at a concentration of approximately 10^8 cells per milliliter. Samples were negatively stained with 1% (w/v) phosphotungstic acid (pH 6.5) for 20 to 30 sec and were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

Genomic DNA Extraction for 16S rRNA Gene Sequencing. Bacteria were cultured on blood agar plates and the cells were harvested and washed twice with 1 ml of double-distilled H_2O. The pellets were suspended in STET buffer (8% sucrose, 50 mM EDTA, 0.1% Triton X-100, and 50 mM Tris HCl, pH 8.0) and lysozyme (hen egg white, Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to a final concentration of 3 mg/ml. The suspension was incubated for 12 min at 37°C and was then lysed with sodium dodecyl sulfate. RNAse A (bovine pancreas; Boehringer Mannheim) was added to a final concentration of 0.05 mg/ml and the solution was incubated for 1 hr at 37°C. Then 0.1 volume of a 5% cetyltrimethylammonium bromed-0.5 M NaCl solution (Sigma Chemical, St. Louis, MO) was added and the solution was gently mixed and incubated at 65°C for 10 min. DNA was extracted with an equal volume of phenol-chloroform (1:1, v/v), precipitated overnight in 0.3 M sodium acetate with 2 volumes of absolute ethanol at –20°C, and pelleted by centrifugation at 13,000*g* for 1 hr at 4°C. The ethanol was decanted and the pellet was air dried and suspended in distilled water.

16S rRNA Gene Sequencing. The sequence of the 16S rRNA gene of the novel urease-negative helicobacter isolate was determined. For amplification of the 16S rRNA cistrons, 16S rRNA gene sequencing, and 16S rRNA data analysis, we used the methods described by Fox *et al.* (4). Briefly, primers C60 and B37 (4) were used to amplify the 16S rRNA genes. The amplicons were purified and directly sequenced by using a TAQeunce cycle sequencing kit (US Biochemica, Cleveland, OH). The 16S rRNA gene sequences were entered into a program for analysis of 16S rRNA data in Microsoft QuickBASIC for use on PC-compatible computers and were aligned as previously described (18). The sequence database used contains approximately 100 helicobacter, wolinella, arcobacter, and campylobacter sequences and more than 900 sequences for other bacteria. Similarity matrices were constructed from the aligned sequences by using only those base positions for which 90% of the strains had data and were corrected for multiple base changes by the method of Jukes and Cantor (19). Phylogenetic trees were constructed by using the neighbor-joining method of Saitou and Nei (20).

ELISA for Anti-*H.* sp. IgG in Serum and IgA in Feces. An outer membrane antigen preparation (OMP) of *H.* sp. was obtained by methods previously described for preparing *H. hepaticus* antigen (21). Briefly, *H.* sp. was cultured in trypticase soy broth containing 5% fetal bovine serum for 48 hr under microaerobic conditions as detailed above. After three washes in PBS and examination for bac-

terial contaminants using gram stain and phase microscopy, the pellet was resuspended in 4 ml of 1% N-octyl-beta-glucopyranoside (Sigma) for 30 min at room temperature. Insoluble material was removed by ultracentrifugation at 100,000*g* for 1 hr. After dialysis against PBS for 24 hr at 4°C, supernatant protein concentration was measured by the Lowry technique (Sigma). For serum IgG measurement, 96-well plates were coated with 100 μl per well of 1 μg/ml of *H.* sp. OMP protein in carbonate buffer (pH 9.6) overnight at 4°C. Coating concentration of antigen was increased to 10 μg/ml for measurement of IgA in fecal extracts. Biotinylated secondary antibodies included goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL), α-chain-specific goat anti-mouse IgA (Sigma), and monoclonal rat anti-mouse antibodies produced by clones G1-6.5 and R19-15 (Pharmingen, San Diego, CA) for detecting IgG1 and IgG2a, respectively. Incubation with extravidin peroxidase (Sigma) was followed by ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for color development. OD development at λ = 405 mμ was recorded by an ELISA plate reader (Dynatech MR7000, Dynatech Laboratories, Chantilly, VA).

Sample Preparation and Analysis. Sera were collected from all mice prior to dosing with *H.* sp. and then again at necropsy, and were stored at −20°C prior to analysis. Serum IgG results are reported as mean OD values at a sample dilution of 1:100, with all samples run in triplicate. Feces were collected from all mice prior to dosing with *H.* sp. and then monthly until necropsy. Four freshly voided fecal pellets (approximately 100 mg) were suspended in a protease inhibitor cocktail (1 μg/ml aprotonin, 10 μM leupeptin, 3.25 μ M bestatin, and 0.2 mM 4-[2-aminoethyl]-benzene sulfonylfluoride [Sigma] in 5% nonfat dry milk) as previously described (1). The fecal slurry was microcentrifuged at 10,000 rpm (Microcentrifuge 235C, Fisher Scientific, Pittsburgh, PA) for 10 min to yield supernatant for IgA measurement. Fecal extracts were frozen at −70°C pending analysis, and were assayed undiluted. Because of an unknown dilution factor inherent in sample preparation, the OD measurement of IgA specific for *H.* sp. in fecal extracts was standardized against total IgA concentration of the sample. A standard curve was generated on each ELISA plate by applying known amounts of purified mouse IgA-κ (Sigma) precoated with α-chain-specific sheep anti-mouse IgA.

Histology. Sections of liver and gastrointestinal tissues were fixed in neutral buffered 10% formalin and in Carnoy's fixative. Tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) stain for assessment of histopathology, Masson's trichrome stain for assessment of hepatic fibrosis, or Warthin-Starry silver stain to visualize bacteria in tissues (formalin-fixed sections only).

Statistics and Analysis. Statistics reported throughout the text are based on a two-tailed Student's *t* test.

Experimental Design. Pooled feces from the mice were analyzed by PCR and were cultured microaerobically upon arrival to ensure that they were not colonized with any *H.* sp. After 1 week of acclimatization, baseline blood samples were obtained from each mouse. Mice were randomly assigned to inoculation groups. The mice were inoculated IP with 0.3 ml of PBS (sham-dosed controls) or 0.3 ml of PBS containing 3×10^7 CFU of the novel *H.* sp. Two uninoculated *scid* cage-contact mice were housed with IP-inoculated *scid* mice for the purpose of establishing whether natural fecal-oral transmission of the novel *H.* sp. occurred. In addition, to determine whether the IP route of infection influenced the severity of clinical signs, five *scid* mice were inoculated orally with 0.3 ml PBS containing 3×10^7 CFU of *H.* sp. Mice were observed daily and weighed at scheduled intervals. Mice were necropsied at time points listed in Table I. Unless otherwise stated, all data presented in the Results section pertains to IP-inoculated mice only.

Results

Characterization of the Novel *H.* sp. *Bacterial morphology and growth characteristics.* Urease-negative, catalase- and oxidase-positive, gram-negative motile bacteria grew at 37°C under microaerobic conditions. Colonies appeared as a thin spreading layer on blood agar media, with a characteristic feathery iridescent leading edge. When grown in broth culture, cells could attain lengths exceeding 10 μ m. Cells exhibited microaerobic, but not aerobic, growth at both 37° and 42°C.

Ultrastructure. The novel *H.* sp. was spiral and it measured 0.15 by 2 to 10 μm with three to seven spiral turns (Fig. 1). The bacterium possessed single bipolar, sheathed flagella, but did not have periplasmic fibers.

16S rRNA analysis. The 16S rRNA sequences determined for the initial isolate (MIT 96-1001) and from two isolates (MIT 98-5356 and MIT 98-5357) recovered from experimentally infected mice were entered into our database, aligned, and compared with the over 100 *Helicobacter*

Table I. Experimental Design

Group	Number of mice necropsied at various time points			
	2 WPI	7 WPI	18 WPI	24 WPI
scid Control	2	9[a]	4	4
scid Oral *H.*sp.	—	4[b]	—	—
scid IP *H.* sp.	2	8[c]	5[d]	0
A/J Control	—	10	—	10
A/J IP H. sp	—	10	—	10

Note. Control mice were sham-inoculated with PBS only. Inoculated mice were inoculated ip or orally with the 3×10^7 CFUs of *H.* sp.—no animals were analyzed. WPI, weeks postinoculation.
[a] One control died of anesthetic complications during blood collection.
[b] One out of five animals died.
[c] Two out of 10 animals necropsied early (at 2 WPI).
[d] Five out of 10 animals died prior to necropsy; one died immediately prior to necropsy and was included in gross, but not histological, examinations.

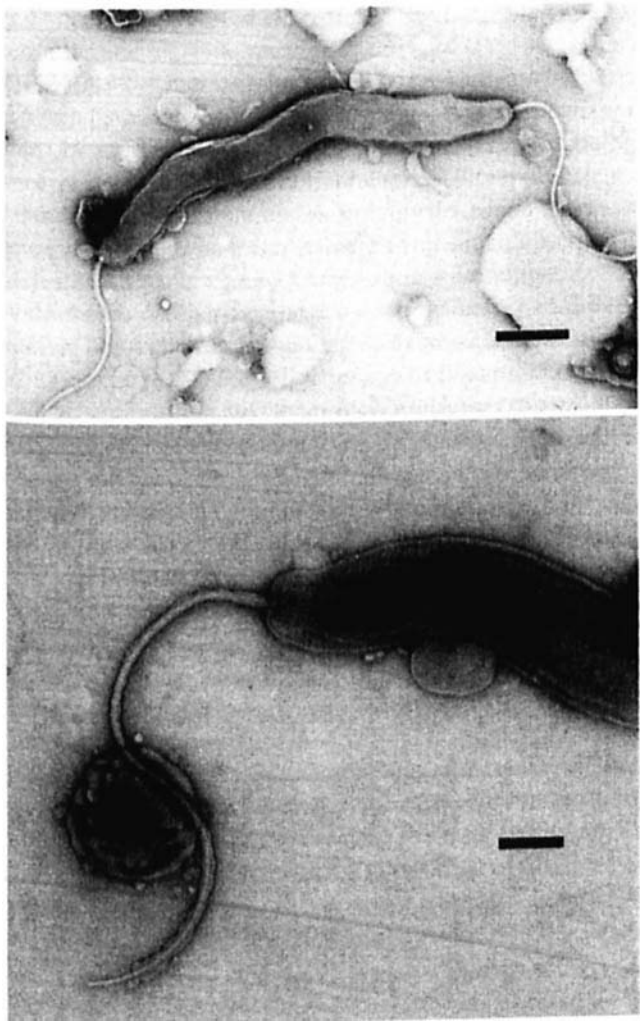

Figure 1. Ultrastructure of the novel *Helicobacter* species. (Top) Transmission electron micrograph (bar = 0.5 μ m). The novel *H.* sp. is motile and spiral, and measures 0.15 by 2 to 10 μm. with two to seven spiral turns. (Bottom) (bar = 0.2 μm) The bacterium has single bipolar, sheathed flagella. but does not have periplasmic fibers.

sequences in the database to determine similarity. The essentially complete sequence (from base 28–1524 using *Escherichia coli* numbering) was determined for three strains: 96-1001, 98-5356, and 98-5357. A neighbor-joining phylogenetic tree showing the consensus sequence for the novel strains and selected helicobacters is shown in Fig. 2. The novel *H.* sp. sequence fell in the helicobacter cluster, which includes *H. canis*, *H. bilis*, *H. cinaedi*, *H.* sp. Group 8, and *H. westmeadii*. The three sequences were identical and were most closely related to that of *H.* sp. Flexispira Group 8 (GenBank AF047851), and "*H. ulmiensis*" (GenBank AJ007931). The novel *H.* sp. strains contain an intervening sequence (IVS) that is essentially identical to that in *H. bilis* and *H.* sp. Flexispira Group 8. The novel *H.* sp. sequence differs by 12 bases from that of the "*F. rappini*" (GenBank AF047851) and differs from the "*H. ulmiensis*" sequence (AJ007931) by seven bases in the ribosomal portion of the sequence (data not shown); however, "*H. ulmiensis*" contains a uniquely different IVS.

Clinical and Gross Findings. *A/J mice.* There were no clinical signs or significant difference in the body weight of control and *H.* sp.-inoculated A/J mice at any time point (Fig. 3). Control mice had no gross lesions at necropsy at 7 or 24 weeks PI. Multiple 1- to 5-mm pale yellow foci in the liver parenchyma (corresponding to hepatic necrosis) were observed in two of 10 inoculated mice at 7 weeks PI. Translucent nodules and tracts (corresponding to mononuclear cell accumulations seen by light microscopy) were apparent on cut sections of the livers of nearly all (eight of 10) inoculated mice at 24 weeks PI.

scid mice. Control mice weighed significantly more than the mice inoculated with the novel *H.* sp. at every time point (Fig. 3). Control mice had no clinical signs and no grossly apparent lesions at necropsy at 2, 7, 18, or 24 weeks PI. In contrast, all mice inoculated IP with *H.* sp. developed

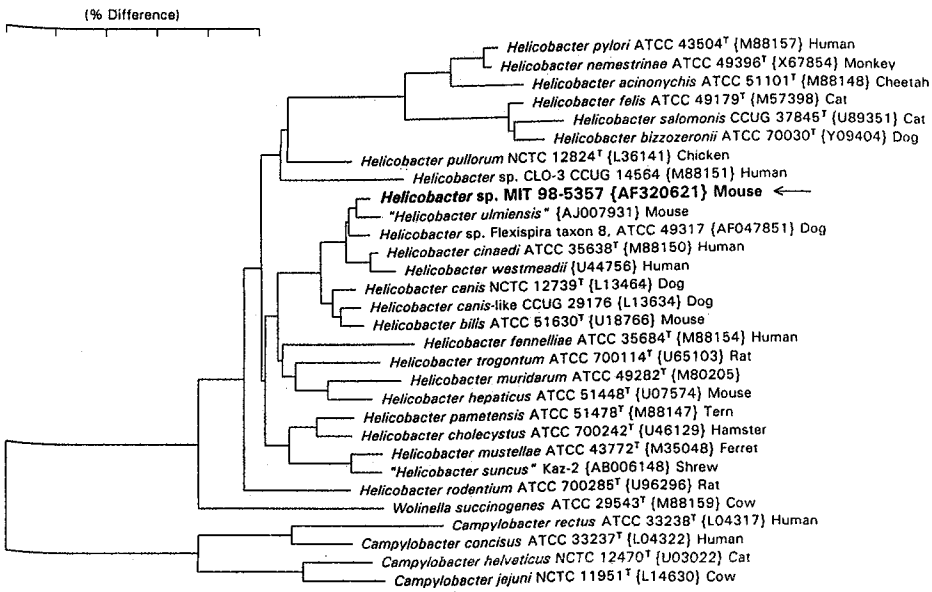

Figure 2. 16SrRNA analysis. Neighbor-joining phylogenetic tree based on 16S rRNA sequence comparisons (20). Scale bar represents 5% difference in nucleotide sequences, as described by measuring the lengths of horizontal lines connecting any two species. Species names are followed by strain number, GenBank accession number (in curly brackets), and animal species from which strain was isolated.

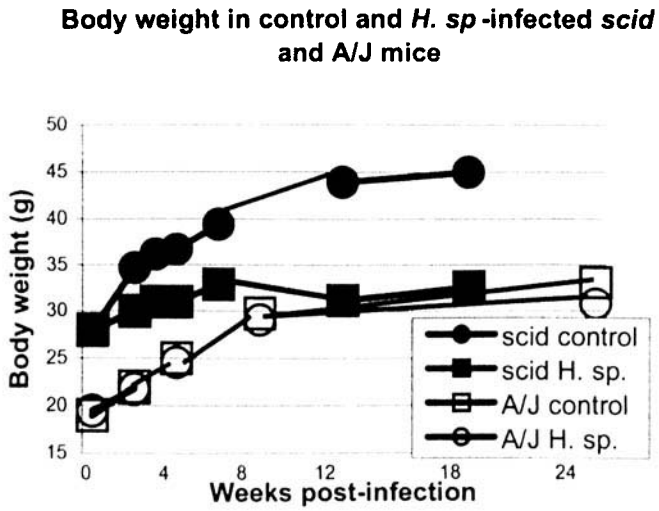

Figure 3. Effect of intraperitoneal inoculation with the novel *H.* sp. on body weight. Mean body weight of infected and control *scid* and A/J mice at the indicated time points: Infected *scid* mice weighed significantly less ($P < 0.004$) than the control mice at every PI time point. There was no significant difference in the weights of control and Helicobacter-infected A/J mice at any time point.

intermittent severe bloody diarrhea at 2 weeks PI. Diarrhea episodes typically lasted 1 week, with frequent relapses noted. Eight IP-inoculated *scid* mice died or were euthanized by 18 weeks PI. Multiple 1- to 5-mm pale yellow foci in the liver parenchyma (corresponding to hepatic necrosis) were seen in five of 10 IP-inoculated mice at 7 weeks PI. At 18 weeks PI, there was gross evidence of massive hepatic necrosis and remodeling of the livers of three of four surviving *H.* sp.-inoculated *scid* mice. They had abnormally shaped fused liver lobes indicative of prior massive necrosis and subsequent repair.

Orally dosed scid mice. To determine if the severe clinical signs were due to the IP route of inoculation, five *scid* mice were orally inoculated with *H.* sp. These mice developed bloody diarrhea within 1 week of inoculation, an even earlier time point than in the IP inoculated *scid* mice, indicating that the clinical signs were not an artifact of IP inoculation.

Uninoculated scid cage contact mice. To determine if fecal-oral transmission could occur, two uninoculated *scid* cage contacts were co-housed with IP-inoculated infected mice 2 weeks after inoculation. The cage contacts also developed intermittent severe bloody diarrhea within 1 week of exposure.

Colonization by the Novel *H.* sp. All of the sham-dosed *scid* ($n = 20$) and A/J mice ($n = 20$) remained culture-negative for the novel *H.* sp. The *H.* sp.-dosed *scid* and A/J mice were culture positive within 2 weeks of dosing by the IP route. *H.* sp. was also isolated from the feces of orally inoculated *scid* mice and the uninoculated *scid* cage contacts within 2 weeks of exposure. The novel *H.* sp. was readily cultured from the livers of selected IP-inoculated *scid* or A/J mice with gross liver lesions. A few slender spiral argryophilic organisms consistent with *H.* sp. were seen in selected livers stained with Warthin-Starry silver stain.

Histopathology. *IP-inoculated scid mice* No lesions were observed in the livers or gastrointestinal tracts of the control *scid* mice at 7 weeks PI. At 18 weeks PI, mild mucosal hyperplasia and limited focal inflammation were observed in the rectums of two control mice. Mild focal hyperplasia of the gastric antral mucosa was also observed in two control mice at this time point. In contrast, infection with *H.* sp resulted in moderate-to-marked proliferative typhlocolitis accompanied by hepatic necrosis and inflammation both mice necropsied at 2 weeks PI. At 2 weeks PI, IP-inoculated *scid* mice had moderate proliferative typhlocolitis with multifocal epithelial necrosis. Hyperplastic crypts were elongated and lined by densely packed, hyperchromic epithelium with diminished goblet cell differentiation (Fig. 4a). The proximal colon and cecum were further characterized by submucosal edema and infiltration by histiocytes, granulocytes, and small mononuclear cells (Fig. 4b). Both of the mice necropsied at the 2-week time point developed phlebothrombosis within the portal circulation, accompanied by extensive, multifocal coagulative necrosis and inflammation (Fig. 4c). Subacute periportal inflammation and multifocal hepatic microabscessation were prominent in the affected livers.

At 7 and 18 weeks PI, moderate-to-marked prolifera-

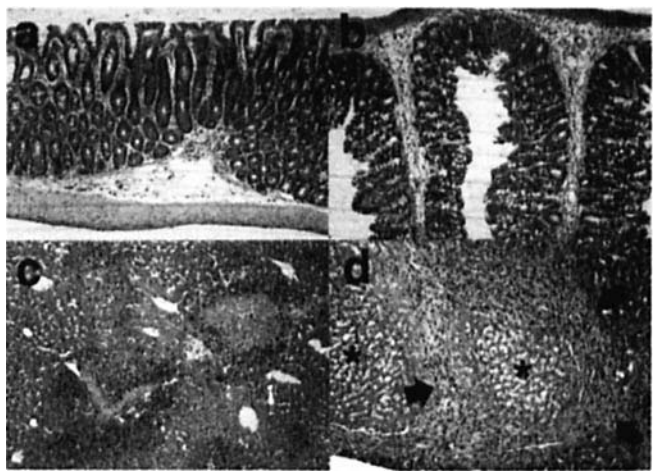

Figure 4. Histopathology of *H.* sp. infection in *scid* mice. (a) Proliferative typhlitis in a *scid* mouse inoculated IP with *H.* sp. (18 weeks PI). Characteristic features of proliferative typhlitis were observed at 2, 7, and 8 weeks PI in *scid* mice. Cecal hyperplasia is characterized by focal areas of increased crypt length, with increased mucosal epithelial cell hyperchromicity and increased crypt cell density. The mucosal cellular infiltrate consists primarily of mononuclear cells morphologically characterized as macrophages, with neutrophils and occasional eosinophils. Similar lesions were seen at 7 weeks PI. 100×. (b) Proliferative colitis in a *scid* mouse inoculated IP with *H.* sp. (2 weeks PI). (proximal colon) The colonic inflammatory infiltrate was similar to the cecal infiltrate (Fig. 4a). At 7 and 18 weeks PI, the proximal colon was normal, and only the distal colon and cecum were affected. 100×. (c) Severe hepatitis with extensive coagulative necrosis in a *scid* mouse inoculated IP with *H.* sp. (2 weeks PI). Similar lesions were seen at all time points. Thrombi were frequently present in veins. 40×. (d) Coagulative hepatic necrosis in a *scid* mouse inoculated IP with *H.* sp. (18 weeks PI). A fibrous capsule (arrow) surrounds areas of coagulative necrosis (asterisk). 400×.

tive typhlitis was consistently observed in eight out of eight and four out of four mice, respectively (Fig. 4a). Cecal epithelial necrosis was observed in several mice (not shown). The intensity of colonic inflammation was diminished to mild intensity present in only seven out of eight mice at 7 weeks PI, and colitis was manifested as moderate hyperplasia and inflammation polarized toward the distal colon and rectum in four out of four mice at 18 weeks PI. Multifocal hepatic phlebothrombosis was common, associated with moderate-to-marked hepatic necrosis and inflammation. Remodeling of the hepatic tissue was manifested grossly as large, abnormally contoured lobes. Portal inflammation, multifocal necrosis, fibroplasia, organization of thrombi, and multifocal suppurative inflammation were observed in hepatic tissue of eight out of eight mice at 7 weeks PI and three out of four mice at 18 weeks PI (Fig. 4d). Extrahepatic thrombi were also rarely observed in veins of the cecum and pancreas in mice from the 7-weeks PI group.

IP-inoculated A/J mice. No significant lesions were observed in the livers or gastrointestinal tracts of the control A/J mice at 7 and 24 weeks PI. Infected A/J mice typically developed less intense inflammation and proliferation of the cecum and colon, as compared with *scid* mice. At 7 weeks PI, proliferative typhlitis (present in 10/10 mice) was mild in intensity, and was characterized by hyperplasia and multifocal or diffuse infiltration by lymphocytes. At 24 weeks PI, typhlitis progressed toward mild (four out of 10 mice) or moderate (five out of 10 mice) intensity (Fig. 5a), and in one case developed a severe inflammation and mucosal hyperplasia with multifocal cecal ulceration. Moderate focal gastric antral hyperplasia and inflammation and mild ileitis were also observed in several mice. Mild-to-moderate colitis was observed infrequently (three out of 10 mice), limited primarily to the distal colon or rectum.

A unique pattern of hepatic lesions, progressing to chronic cholangiohepatitis, developed in response to experimental infection in A/J mice. At 7 weeks PI, moderate-to-marked portal hepatitis and multifocal necrosis were observed in six out of 10 mice (Fig. 5b). Multifocal hepatic phlebitis, characterized by prominent lymphocytic perivascular infiltrates, was also present. At 24 weeks PI, in eight out of 10 mice, the portal inflammation was marked in intensity and was manifested by large lymphoplasmacytic infiltrates that formed thick cuffs around portal structures, especially bile ducts (Fig. 5c). Scattered bile ducts contained suppurative exudate and necrotic cells (Fig. 5d). Hepatic fibrosis was observed as a pattern of mild-to-moderate collagen deposition, extending from the portal areas into the periportal parenchyma.

Serology. *Serum IgG responses.* A/J mice experimentally infected with the novel *H.* sp. developed significant ($P < 0.001$) serum IgG responses to the infection by 24 weeks PI (Table II). Using an isotype-specific ELISA for discrimination between IgG2a (Th1-like) and IgG1 (Th2-like) antibody responses, mice infected with the novel *H.* sp. developed a predominant IgG2a serum response to *H.* sp. infection. The IgG2a response was significantly higher than the IgG1 response in all mice tested ($P < 0.0005$) with a mean ratio of IgG2a to IgG1 of 2.8 ± 0.6.

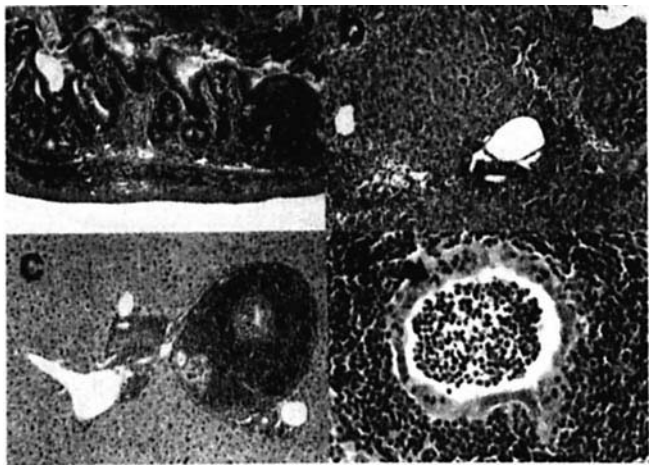

Figure 5. Histopathology of novel *H.* sp. infection in A/J mice (a) Proliferative typhlitis in an A/J mouse infected with *H.* sp. for 24 weeks. Cecal hyperplasia is characterized by focal areas of increased crypt length, increased mucosal epithelial cell hyperchromicity, and increased crypt cell density. Cecal inflammation consisting primarily of mononuclear cells with neutrophils and occasional eosinophils was present. 100×. (b) Necrosis and hepatitis in an A/J mouse infected with *H.* sp. for 7 weeks. Hepatitis, characterized by pericholangial and perivascular inflammatory cell infiltrates and mild-to-moderate areas of coagulative necrosis (asterisk), was present in the majority of the Helicobacter-infected mice at 7 weeks PI. 100×. (c) Severe cholangiohepatitis in an A/J mouse infected with the novel *H.* sp. for 24 weeks. Infiltrates were concentrated around biliary tracts, and necrosis and perivascular inflammation were minimal. Bile duct reduplication was observed. 40×. (d) Chronic suppurative cholangitis in an A/J mouse infected with the novel *H.* sp. for 24 weeks. A dense lymphoplasmacytic and histiocytic cell infiltrate surrounds the bile duct, which is filled with a suppurative exudate. A neutrophil (PMN) can be seen translocating across the bile duct wall (arrow). 200×.

Fecal IgA responses. The total IgA concentration in the fecal extract samples was equivalent across experimental groups (data not shown). The A/J mice infected with *H.* sp. developed significant IgA responses compared to controls by 24 weeks PI ($P < 0.007$; Table II).

Discussion

The results of this study show that experimental infection with a novel urease-negative helicobacter, *H.* sp., can cause severe proliferative typhlocolitis, hepatic phlebothrombosis, and hepatitis in outbred *scid* mice, and moderate proliferative typhlocolitis and marked cholangiohepatitis in male A/J mice. We have previously reported an outbreak of severe diarrhea and proliferative necrotizing ulcerative typhlitis in inbred (C.B17) *scid* mice associated with a dual infection of *H. bilis* and *H. rodentium*, (5) and moderate typhlocolitis in female outbred ICR *scid* mice infected with *H. bilis* (12). Others have reported mild typhlocolitis and hepatitis in male C.B17 *scid* mice infected with *H. bilis* (9). Several factors, including strain and sex, have been shown to modulate the severity of helicobacter-induced disease (22). Lesions from infection with *H. hepaticus* have been

Table II. Mean OD ± SE of ELISA for Serum IgG and Fecal IgA

Infection status	IgG	IgA	IgG1	IgG2a
Control	0.069 ± 0.009	0.092 ± 0.015	0.004 ± 0.006	0.000 ± 0.005
Novel *H.* sp.	2.056 ± 0.034[a]	0.295 ± 0.032[a]	0.665 ± 0.065[a]	1.822 ± 0.133[a,b]

Note. Serum IgG and Fecal IgA at 24 weeks PI. Sera and fecal extracts from A/J mice ($n = 10$) infected for 24 weeks with the novel *H. sp.* were tested in a serum IgG and fecal IgA ELISA against *h.* sp. antigen. Sera from a subset of five A/J mice infected for 24 weeks with the novel *H.* sp. were tested in an isotype-specific ELISA for discrimination between IgG2a and IgG1 serum antibody responses. The IgG, IgA, IgG1, and IgG2a responses were significantly elevated compared with controls and the IgG2a (Th1-like) response was significantly higher than the IgG1 (Th2-like) response in the mice infected with *H.* sp.

[a] Significant compared to controls at $p < 0.001$

[b] Significant compared to IgG1 response at $p < 0.001$

reported to vary with mouse strain and sex. (6, 8). The enterohepatic lesions observed in *H.* sp-inoculated mice in this study were considerably more severe than those seen in our previous pilot study, which used female defined flora ICR *scid* mice (23). Also, the clinical signs in the *scid* mice were so severe that the original experimental design (necropsy at 7 and 24 weeks) had to be modified (necropsy at 2, 7, and 18 weeks). It is unlikely that the severe hepatic necrosis, which resulted in massive remodeling of the livers of some mice, was simply due to the IP route of exposure, as this route has been used in other studies (8, 23, 24) with no similar consequences. Also, the orally exposed *scid* mice and even the *scid* mice exposed only by cage contact developed severe lesions and developed clinical signs at an earlier time point than the IP-inoculated mice.

Our interpretation is that the clinical signs were due to colonization of the gut by the *H.* sp., and that this colonization takes place faster when the bacteria are inoculated directly *per os* than when they have to reach the gut via the IP route. The increased severity of the enterohepatic lesions noted in mice in the current study may be due to a gender predilection or to the presence of other bacterial species in the gut of "restricted" rather than "defined" flora mice. Finally, there may be bacterial-specific factors that are important determinants of the severity of disease. For instance, strains of *H. pylori* that elaborate vacuolating cytotoxin have been reported to be more pathogenic than strains without the cytotoxin (25, 26). *H. hepaticus* has been reported to produce a granulating cytotoxin (27) and a newly reported cytolethal distending toxin (28). Urease is an essential colonization factor for gastric helicobacters, allowing for the organism's survival in an acidic environment. Urease is produced by the established enterohepatic pathogens *H. hepaticus* and *H. bilis,* but not by the novel *H.* sp. reported on here, or by "*H. typhlonicus*," which has been reported to cause disease in IL10$^{-/-}$ and *scid* mice (2, 29). Thus, our results confirm that urease is not a required virulence factor for enterohepatic helicobacters to cause hepatic or enteric disease. It remains to be determined whether there are virulence factors unique to the novel *H. sp.*, or strains of the novel *H.* sp. that are more or less pathogenic.

The liver lesions reported in this study are similar to those seen in aged mice naturally infected with *H. hepaticus* (8) and to IL-10$^{-/-}$ mice naturally or experimentally infected with another recently described urease-negative *H.* sp. (*H. typhlonicus*), which is taxonomically distinct from the novel *H.* sp used in this study (2, 29). The early onset of these lesions in our study (apparent at 7 weeks) may be due to the route of exposure (IP versus oral) or may indicate that the novel urease negative *H.* sp. is more virulent. The severe cholangitis and pericholangitis may be the direct result of colonization of the liver and bile ducts with *H.* sp. or may reflect an autoimmune response directed against an antigen common to intestinal helicobacters and the biliary epithelium (30). Prolonged (1–2 years) infection with *H. hepaticus* is associated with the development of hepatic tumors (31). Although the development of inflammatory lesions was greatly accelerated compared to infection with *H. hepaticus*, no hepatic tumors were seen in mice infected with the novel *H.* sp. The study was of insufficient duration to determine the carcinogenic potential of *H.* sp. and further studies with longer-term infection are clearly needed. Infection with *H. hepaticus* also causes a significant hepatic vasculitis (3, 31). It has recently been theorized that *H. pylori* exerts some pathogenic effects on the vasculature, and it has been epidemiologically and anecdotally linked with vascular disorders such as migraine and Reynaud's syndrome (32).

The significant serum IgG and fecal IgA responses of the A/J mice to the novel *H.* sp. are consistent with the marked inflammatory response to the infection. Mice infected with the novel *H.* sp. developed a serum IgG2a antibody response that was greater then the IgG1 response, indicating that a pro-inflammatory Th1 response to the infection predominated over a Th2 response, as previously observed in A/JCr mice infected with *H. hepaticus* (21) and in C57BL/6 mice infected with *H. felis* (33).

It has been hypothesized that some human IBD is triggered by bacterial antigens and that the IBD is a dysregulated immune response to unspecified bacterial insult (34–36). Several mouse models of IBD have been reported to result from the elimination or overexpression of cytokines involved in the balance between a Th1 and Th2 response (37, 38). For example, Kullberg *et al.* (37) showed that *H. hepaticus* induces chronic colitis in SPF-reared IL-10$^{(-/-)}$ mice, and the disease is accompanied by a type 1 cytokine response (IFNγ, TNFα, and nitric oxide). In contrast, wild-type C57BL-background animals infected with the same

bacteria did not develop disease and they produced IL-10 as the dominant cytokine in response to Helicobacter antigen. *In vivo* neutralization of IFNγ or IL-12 resulted in a significant reduction of intestinal inflammation in *H. hepaticus*-infected IL-10$^{-/-}$ mice, suggesting an important role for these cytokines in the development of colitis in the model. Apparently, in immunocompetent hosts, IL-10 stimulated in response to intestinal flora is important in preventing IBD. The A/J mice in this study exhibited a Th1 response to the novel *H.* sp. and a robust inflammatory response in the liver. However, the *scid* mice also developed severe enterohepatic lesions, similar in quality to those in the A/J mice, despite a lack of T cells. The inflammatory infiltrate in the *scid* mice was primarily mononuclear, predominantly consisting of cells morphologically identified as macrophages. Although macrophage activation is enhanced by T cells, it has recently been shown that *H. pylori* alone can stimulate macrophages and induce iNOS (39). The severe histiocytic lesions in the intestines and livers of the *scid* mice in this study certainly suggest that novel *H.* sp. shares this property, although this needs to be confirmed by *in vitro* testing.

Von Freeden-Jeffry *et al.* (38) found that *H. hepaticus*-infected mice mutant for both IL-7 (a macrophage activator) and RAG-2 (IL-7/RAG-2$^{-/-}$) did not develop myeloid responses or colitis whereas RAG2$^{-/-}$ did, indicating that IL-7 plays a critical role in exacerbating a non-T cell/non-B cell-mediated chronic inflammatory response. Therapy with recombinant IL-10 protein (which inhibits antigen presentation and macrophage production of IL-1, IL-6, and TNFα) was able to prevent the occurrence of colitis in susceptible RAG-2$^{(-/-)}$ mice, suggesting a pivotal role for macrophages (38). The present study, as well as our earlier studies using helicobacter-infected defined flora *scid* mice, using B and T-cell free *scid* mice, is additional evidence for an important role for macrophages in IBD (13, 24).

There is some evidence that intestinal helicobacters may play a role in IBD in humans; *H. cinaedi* (40) and *H. fennelliae* (41) were first identified as agents causing proctitis and colitis in immunocompromised humans (41). In this study, a novel urease-negative murine *H.* sp. closely related to a cluster of enteric helicobacters by 16S rRNA sequence analysis produced mild-to-moderate IBD in both outbred *scid* mice and inbred A/J mice. Importantly, in this study the *H.* sp. also caused hepatitis with cholangitis. In some human patients with IBD, primary sclerosing cholangitis is a clinically important sequelum of unknown etiology. An animal model with similar pathological changes could prove valuable for investigating hypotheses related to bacterial/autoimmune etiology of this disease process.

Intestinal helicobacters may play an important role as factors or cofactors in the development of IBD in many species. It is particularly important to consider the murine intestinal helicobacters as potential pathogens. Some genetic mouse models of IBD may now be attributable to *H.* sp. infection (10), and some long-term toxicology studies using B6C3F1 mice have been compromised by *H.* sp. infection (3, 31). This study both confirms the pathogenicity of *H.* sp. in immunocompetent and immunocompromised mice, and offers a potential murine model for studies of IBD and associated liver disease.

1. Fox JG, Dewhirst FE, Tully JG, Paster BJ, Yan L, Taylor NS, Collins MJ Jr., Gorelick PL, Ward JM. *Helicobacter hepaticus* sp. nov, a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. J Clin Microbiol **32:**1238–1245, 1994.
2. Fox JG, Gorelick PL, Kullberg MC, Ge Z, Dewhirst FE, Ward JM. A novel urease-negative *Helicobacter* species associated with colitis and typhlitis in IL-10-deficient mice. Infect Immunol **67:**1757–1762, 1999.
3. Fox JG, Li X, Yan L, Cahill RJ, Hurley R, Lewis R, Murphy JC. Chronic proliferative hepatitis in A/JCr mice associated with persistent *Helicobacter hepaticus* infection: A model of *helicobacter*-induced carcinogenesis. Infect Immunol **64:**1548–1558, 1996.
4. Fox JG, Yan LL, Dewhirst FE, Paster BJ, Shames B, Murphy JC, Hayward A, Belcher JC, Mendes EN. *Helicobacter bilis* sp. nov., a novel Helicobacter isolated from bile, livers, and intestines of aged, inbred mice. J Clin Microbiol **33:**445–454, 1995.
5. Shomer NH, Dangler CA, Marini R, Fox JG. *Helicobacter bilis/Helicobacter rodentium* co-infection associated with diarrhea in a colony of *scid* mice. Lab Anim Sci **48:**455–459, 1998.
6. Ward JM, Anver MR, Haines DC, Benveniste RE. Chronic active hepatitis in mice caused by *Helicobacter hepaticus*. Am J Pathol **145:**959–968, 1994.
7. Ward JM, Anver MR, Haines DC, Melhorn JM, Gorelick P, Yan L, Fox JG. Inflammatory large bowel disease in immunodeficient mice naturally infected with *Helicobacter hepaticus*. Lab Anim Sci **46:**15–20, 1996.
8. Ward JM, Fox JG, Anver MR, Haines DC, George CV, Collins MJ, Gorelick PL, Nagashima K, Gonda MA, Gilden RV, Tully JG, Russell RJ, Benveniste RE, Paster BJ, Dewhirst FE, Donovan JC, Anderson LM, Rice JM. Chronic active hepatitis and associated liver tumors in mice caused by a persistent bacterial infection with a novel *Helicobacter* species. J Natl Cancer Inst **86:**1222–1227, 1994.
9. Franklin CL, Riley LK, Livingston RS, Beckwith CS, Besch-Williford C, Hook RRJ. Enterohepatic lesions in SCID mice infected with *Helicobacter bilis*. Lab Anim Sci **48:**334–339, 1998.
10. Foltz CJ, Fox JG, Cahill RJ, Murphy RC, Yan L, Shames B, Schauer DB. Spontaneous inflammatory bowel disease in multiple mutant mouse lines: association with colonization by *Helicobacter hepaticus*. Helicobacter **3:**69–78, 1998.
11. Shames B, Fox JG, Dewhirst FE, Yan L, Shen Z, Taylor NS. Identification of widespread *Helicobacter hepaticus* infection in feces in commercial mouse colonies by culture and PCR assay. J Clin Microbiol **33:**2968–2972, 1995.
12. Shomer NH, Dangler CA, Schrenzel MD, Fox JG. *Helicobacter bilis*-induced inflammatory bowel disease (IBD) in defined flora *scid* mice. Infect Immunol **65:**4858–4864, 1997.
13. Cahill RJ, Foltz CJ, Fox JG, Dangler CA, Powrie F, Schauer DB. Inflammatory bowel disease: An immune mediated condition triggered by bacterial infection with *Helicobacter hepaticus*. Infect Immunol **65:**3126–3131, 1997.
14. Shen Z, Fox JG, Dewhirst FE, Paster BJ, Foltz CJ, Yan L, Shames B, Perry L. *Helicobacter rodentium* sp. nov., a urease negative *Helicobacter* species isolated from laboratory mice. Int J Syst Bacteriol **47:**627–634, 1997.
15. Fox JG, Dewhirst FE, Shen Z, Fen Y, Taylor NS, Paster B, Erickson RL, Lau CN, Correa P, Araya JC, Roa I. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. Gastroenterology **114:**755–763, 1998.
16. Avenaud P, Marais A, Monteiro L, Le Bail B, Bioulac P, Megraud F. Detection of *Helicobacter* sp. in liver from patients with or without hepatocarcinoma. Gastroenterology **116:**A1186, 1999.

17. Dewhirst FE, Chien CC, Paster BJ, Ericson RL, Orcutt RP, Schauer DB, Fox JG. Phylogeny of the defined murine microbiota: Altered schaedler flora. Appl Environ Microbiol **65:**3287–3292, 1999.
18. Paster BJ, Dewhirst FE. Phylogeny of *Campylobacter,* wolinellas, *Bacteroides gracilis,* and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing. Int J Syst Bacteriol **38:**56–62, 1988.
19. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, Ed. Mammalian Protein Metabolism. New York: Academic Press, pp21–132, 1969.
20. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol **4:**406–425, 1987.
21. Whary MT, Morgan TJ, Dangler CA, Gaudes KJ, Taylor NS, Fox JG. Chronic active hepatitis induced by *Helicobacter hepaticus* in the A/JCr mouse is associated with a Th1 cell-mediated immune response. Infect Immunol **66:**3142–3148, 1998.
22. Fox JG, Yan L, Shames B, Campbell J, Murphy JC, Li X. Persistent hepatitis and enterocolitis in germfree mice infected with *Helicobacter hepaticus.* Infect Immunol **64:**3673–3681, 1996.
23. Shomer NH, Dangler CA, Whary MT, Xu S, Fox JG. A novel urease-negative *Helicobacter* species induces cholangitis, hepatic vasculitis, and inflammatory bowel disease (IBD in A/J and Tac:ICR:HA*scid* mice (abstract). Gastroenterology **41:**G1138, 1999.
24. Shomer NH, Dangler CA, Fox JG. *Helicobacter bilis*-induced inflammatory bowel disease (IBD) in defined flora *scid* mice. Infect Immunol **65:**4858–4864, 1997.
25. Ghiara P, Marchetti M, Blaser MJ, Tummuru MKR, Cover TL, Segal ED, Tompkins LS, Rappuoli R. Role of the *Helicobacter pylori* virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. Infect Immunol **63:**4154–4160, 1995.
26. Tee W, Lambert JR, Dwyer B. Cytotoxin production by *Helicobacter pylori* from patients with upper gastrointestinal tract diseases. J Clin Microbiol **33:**1203–1205, 1995.
27. Taylor NS, Fox JG, Yan L. *In vitro* hepatotoxic factor in *Helicobacter hepaticus, H. pylori* and other *Helicobacter* species. J Med Microbiol **42:**48–52, 1995.
28. Young VB, Knox KA, Schauer DB. Cytolethal distending toxin sequence and activity in the enterohepatic pathogen *Helicobacter hepaticus.* Infect Immunol **68:**184–191, 2000.
29. Franklin CL, Riley LK, Livingston RS, Beckwith CS, Hook RR, Jr, Besch-Williford C, Huziker R, Gorelick PL. Enteric lesions in SCID mice infected with "*Helicobacter typhlonicus,*" a novel urease-negative *Helicobacter* species. Lab Anim Sci **49:**496–505, 1999.
30. Ward JM, Benveniste RE, Fox CH, Battles JK, Gonda MA, Tully JG. Autoimmunity in chronic active *Helicobacter hepatitis* of mice: Serum antibodies and expression of heat shock protein 70 in liver. Am J Pathol **148:**509–517, 1996.
31. Hailey JR, Haseman JK, Bucher JR, Radovsky AE, Malarkey DE, Miller RT, Nyska A, Maronpot RP. Impact of *Helicobacter hepaticus* infection in B6C3F1 mice from twelve National Toxicology Program two-year carcinogenesis studies. Toxicol Pathol **26:**602–611, 1998.
32. Gasbarrini A, Serricchio M, Tondi P, Franceschi F, Ojetti V, Sanz Torre E, Candelli M, Gabrielli M, Fiore G, Pola R, Santoliquido A, Giacovazzo M, Gasbarrini G, Pola P. *Helicobacter pylori* infection and vascular diseases. Ital J Gastroenterol Hepatol **30:**S307–S309, 1998.
33. Mohammadi M, Czinn S, Redline R, Nedrud J. Helicobacter-specific cell-mediated immune responses display a predominant Th1 Phenotype and promote a delayed-type hypersensitive response in the stomachs of mice. J Immunol **156:**4729–4738, 1996.
34. Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K, Buschenfelde KHMZ. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol **102:**448–455, 1995.
35. Duchmann R, Marker-Hermann E, Buschenfelde KHMZ. Bacteria-specific T-cell clones are selective in their reactivity towards different enterobacteria or *H. pylori* and increased in inflammatory bowel disease. Scand J Gastroenterol **44:**71–79, 1996.
36. Podolsky D. Inflammatory bowel disease. N Engl J Med **325:**928–937, 1991.
37. Kullberg MC, Ward JM, Gorelick PL, Caspar P, Hieny S, Cheever A, Jankovic D, Sher A. *Helicobacter hepaticus* triggers colitis in specific pathogen-free interleukin-10- (IL-10) deficient mice through an IL-12 and gamma interferon-dependent mechanism. Infect Immunol **66:**5157–5166, 1998.
38. von Freeden-Jeffry U, Davidson N, Wiler R, Fort M, Burdach S, Murray R. IL-7 deficiency prevents development of a non-T cell non-B cell-mediated colitis. J Immunol **15:**5673–5680, 1998.
39. Mannick EE, Bravo LE, Zarama G, Realpe JL, Zhang XJ, Ruiz B, Fontham ETH, Mera R, Miller MJS, Correa P. Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in *Helicobacter pylori* gastritis: Effect of antibiotics and antioxidants. Cancer Res **56:**3238–3243, 1996.
40. Gebhart CJ, Fennell CL, Murtaugh MP, Stamm WE. *Campylobacter cinaedi* is normal intestinal flora in hamsters. J Clin Microbiol **27:**1692–1694, 1989.
41. Totten PA, Fennel CL, Tenover FC. *Campylobacter cinaedi* (sp. nov.) and *Campylobacter fennelliae* (sp. nov.): Two new *Campylobacter* species associated with enteric disease in homosexual men. J Infect Dis **151:**131–139, 1985.