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Helicobacter saguini, a Novel Helicobacter Isolated from Cotton-Top Tamarins with Ulcerative Colitis, Has Proinflammatory Properties and Induces Typhlocolitis and Dysplasia in Gnotobiotic IL-10$^{-/-}$ Mice

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A urease-negative, fusiform, novel bacterium named *Helicobacter saguini* was isolated from the intestines and feces of cotton-top tamarins (CTTs) with chronic colitis. *Helicobacter* sp. was detected in 69% of feces or intestinal samples from 116 CTTs. The draft genome sequence, obtained by Illumina MiSeq sequencing, for *H. saguini* isolate MIT 97-6194-5, consisting of ∼2.9 Mb with a G+C content of 35% and 2,704 genes, was annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline. *H. saguini* contains homologous genes of known virulence factors found in other enterohepatic helicobacter species (EHS) and *H. pylori*. These include flagellin, γ-glutamyl transpeptidase (*ggt*), collagenase, the secreted serine protease *htrA*, and components of a type VI secretion system, but the genome does not harbor genes for cytolethal distending toxin (*cdt*). *H. saguini* MIT 97-6194-5 induced significant levels of interleukin-8 (IL-8) in HT-29 cell culture supernatants by 4 h, which increased through 24 h. mRNAs for the proinflammatory cytokines IL-1β, tumor necrosis factor alpha (TNF-α), IL-10, and IL-6 and the chemokine CXCL1 were upregulated in cocultured HT-29 cells at 4 h compared to levels in control cells. At 3 months postinfection, all *H. saguini*-monoassociated gnotobiotic C57BL/129 IL-10$^{-/-}$ mice were colonized and had seroconverted to *H. saguini* antigen with a significant Th1-associated increase in IgG2c ($P < 0.0001$). *H. saguini* induced a significant typhlocolitis, associated epithelial defects, mucosa-associated lymphoid tissue (MALT) hyperplasia, and dysplasia. Inflammatory cytokines IL-22, IL-17a, IL-1β, gamma interferon (IFN-γ), and TNF-α, as well as inducible nitric oxide synthase (iNOS) were significantly upregulated in the cecal tissues of infected mice. The expression of the DNA damage response molecule γ-H2AX was significantly higher in the ceca of *H. saguini*-infected gnotobiotic mice than in the controls. This model using a nonhuman primate *Helicobacter* sp. can be used to study the pathogenic potential of EHS isolated from primates with naturally occurring inflammatory bowel disease (IBD) and colon cancer.

Cotton-top tamarins (CTTs) are New World primates indigenous to the rain forests of Colombia and were imported into the United States for biomedical research beginning in the 1960s, until their classification as endangered species in 1976 (1). Approximately 50% of colony-maintained tamarins develop idiopathic chronic colitis, with 20 to 40% of cases evolving into colonic adenocarcinomas (2). The clinical and histopathological manifestations of colitis in CTTs resemble human inflammatory bowel disease (IBD), particularly ulcerative colitis (UC), making the CTT an attractive animal model of naturally occurring IBD. The etiology of colitis in CTTs remains unknown but has been speculated to be caused by genetic predispositions and/or conditions related to captivity, such as husbandry, the environment (temperature, humidity, and sanitation), abnormal diet, stress, and infectious agents (*Escherichia coli*, *Campylobacter* spp., and *Helicobacter* spp.) (3–5).

In 1999, we detected and isolated a urease-negative, fusiform organism, for which we are proposing the name *Helicobacter saguini*, from the intestines and feces of CTTs with chronic colitis. Phylogenetic analysis by 16S rRNA gene sequencing classified this isolate as a novel enterohepatic helicobacter species (EHS) (5).

EHS are associated with the development of IBD in immunocompromised mice and have been identified in humans with diarrhea and in some individuals with IBD (6, 7). *H. hepaticus*, *H. bilis*, and other EHS infections in immunocompromised mice

cause chronic typhlocolitis, high-grade dysplasia, and progression to colitis-associated carcinoma (CAC) (8–11). Novel *Helicobacter* spp., including *H. macacae*, have been cultured from idiopathic colitis and colon adenocarcinomas in rhesus monkeys (12–14). *H. cinaedi* and *H. fennelliae* were first isolated from homosexual men (presumably immunocompromised by HIV infection) with proctitis (15). Experimentally, *H. cinaedi* and *H. fennelliae* infection in pigtail macaques induced diarrhea and inflammation of the lower bowel (16). Additionally, EHS prevalence in human UC patients was shown to be significantly greater than that in healthy individuals; however, the identification and isolation of a *Helicobacter* sp.

TABLE 1 *Helicobacter* sp. prevalence in a cotton-top tamarin colony

Helicobacter detection method	No. (%) of positive samples or animals			
	Anal swab ($n = 88$)	Fecal sample ($n = 29$)	Biopsy specimen ($n = 30$)	Total animal population ($n = 116$)
PCR[a]	57/88 (64.8)	22/29 (75.9)	24/30 (80)	80/116 (69)
Culture	2/18 (11.1)	1/9 (11.1)	8/30 (26.7)	11/57 (19.3)

[a] Select animals had more than one PCR analysis.

implicated in the pathogenesis of human IBD have remained elusive ([17](#)).

We hypothesized that *H. saguini* has a pathogenic potential similar to that of other EHS and could be associated with colitis and colon cancer in CTTs. However, the endangered status and predisposition toward colitis in captivity have precluded direct study of the causal relationship between *H. saguini* and IBD in CTTs. As an alternative, we used genome analysis, *in vitro* assays, and an interleukin-10 knockout (IL-10$^{-/-}$) mouse infection model to investigate the pathogenicity of *H. saguini*. We report herein that *H. saguini* harbors putative virulence factors and elicits proinflammatory responses *in vitro* and *in vivo*, thus demonstrating that *H. saguini* has the pathogenic potential to induce IBD in CTTs. This finding adds additional credence to the view that EHS may play a role in inducing IBD in humans ([6](#), [17](#)).

MATERIALS AND METHODS

Fecal and colonic biopsy samples. Thirty colonic biopsy samples, 88 rectal swabs, and 29 fecal samples were collected from 116 CTTs which were selected from a colony in which colitis was endemic. These samples were collected over a period of 5 years ([Table 1](#)).

Helicobacter sp. PCR. A High Pure PCR template preparation kit (Roche Molecular Biochemicals, Indianapolis, IN) was used for extraction of DNA from the bacterial isolates and the tissue samples; a QIAamp DNA Stool minikit was used for rectal swabs and fecal sample DNA extraction according to the manufacturer's directions (Qiagen, Valencia, CA). *Helicobacter* genus-specific primers C97 (5′-GCT ATG ACG GGT ATCC) and C05 (5′-ACT TCA CCC CAG TCG CTG) were used to amplify a 1.2-kb PCR product from the 16S rRNA gene ([18](#)). The 1,200-bp PCR products were sequenced using previously described techniques ([18](#)). A TOPO-TA Cloning kit was used to clone and sequence PCR products with mixed signals in the direct sequencing reactions (Life Technologies, Grand Island, NY) according to the manufacturer's instructions.

Culture and characterization of H. saguini. Eighteen rectal swabs, 9 fecal samples, and 30 biopsy samples were also subjected to microaerobic culture. Rectal swabs and fecal samples were diluted in brucella broth with 20% glycerol; biopsy samples were homogenized, and an aliquot of each slurry was placed on cefoperazone-vancomycin-amphotericin B (CVA) plates and passed through a 0.65-μm-pore-size syringe filter onto a Trypticase soy agar plate with 5% sheep blood (Remel Laboratories, Lenexa, KS). The plates were incubated at 37°C under microaerobic conditions in a vented jar containing N_2, H_2, and CO_2 (80:10:10) and evaluated for bacterial growth every 2 to 3 days for 3 weeks. Detailed biochemical characterization analysis was performed on eight individual isolates using a RapID NH System (Remel Laboratories, Lenexa, KS) and API Campy kits (bioMérieux, Boston, MA). Urease, catalase, and oxidase production, sensitivity to nalidixic acid and cephalothin, and growth in the presence of 1% glycine were determined as previously described by our laboratory ([19](#)). A disc assay was used for indoxyl acetate hydrolysis ([20](#)). Suspected bacterial growth was identified as *Helicobacter* spp. on the basis of colony morphology, phase microscopy, Gram staining, biochemical testing, *Helicobacter*-specific PCR, and 16S rRNA and 23S rRNA gene sequencing ([21](#)).

Electron microscopy. *H. saguini* isolate MIT 97-6194-5 was examined by electron microscopy. Cells grown on blood agar plates were centrifuged and gently suspended in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of about 10^8 cells per ml. Samples were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 20 to 30 s. The specimens were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

H. saguini whole-genome sequence analysis. Genomic DNA from *H. saguini* MIT 97-6194-5 was sequenced using Illumina MiSeq sequencing technology as described previously ([22](#)). The 250-bp paired-end sequencing reads generated by MiSeq were assembled into contigs using Velvet ([23](#)). Sequences were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline ([24](#)). The whole genomic distance between *H. saguini* MIT 97-9694-5 and its nearest neighbor, *H. jaachi* MIT 09-6949, was calculated using the Genome-to-Genome Distance Calculator (GGDC [http://ggdc.dsmz.de]) ([25](#)).

Interactions of H. saguini with human intestinal epithelial cell line HT-29. HT-29 cells (colorectal adenocarcinoma) were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Sigma) and 1% antibiotic-antimycotic (Thermo Fisher Scientific, Grand Island, NY) at 37°C with 5% CO_2. HT-29 cells were seeded into six-well cell culture plates and incubated at 37°C in a humidified incubator with 5% CO_2 for 48 h. *H. saguini* cells were passaged on blood agar under microaerobic conditions and collected after 48 h, which corresponded to the mid-exponential phase of the bacterial growth. Viable organisms were predominantly fusiform and demonstrated rapid movement when observed under phase-contrast microscopy. Gram staining was used to ensure the purity of the bacterial preparation. HT-29 cells (2.5×10^6 cells/well) were inoculated with *H. saguini* at multiplicities of infection (MOIs) of 1:100 and 1:25 in 2 ml of fresh culture medium containing 1% FBS without antibiotics. The plates were centrifuged at $200 \times g$ to facilitate bacterial cell adhesion and then incubated under 5% CO_2. After 4 and 24 h of incubation, cell supernatants were collected after centrifugation for determination of IL-8 concentration by enzyme-linked immunosorbent assay (ELISA; Qiagen). HT-29 cells were washed once with phosphate-buffered saline (PBS) and collected in TRIzol reagent for total RNA extraction.

Experimental infection of C57BL/129 IL-10$^{-/-}$ mice with H. saguini MIT 97-6194-5. Forty specific-pathogen-free (SPF) and 14 germfree (GF) C57BL/6 (B6.129P2-IL-10^{tm1Cgn} [IL-10$^{-/-}$]) mice, aged 6 to 8 weeks old, were used in the infection study, with similar numbers of male and female mice. All mice were from a breeding colony maintained at the Massachusetts Institute of Technology (MIT). SPF mice were maintained free of known murine viral pathogens, *Salmonella* spp., *Citrobacter rodentium*, ecto- and endoparasites, and known *Helicobacter* spp. in an AAALAC International-accredited facility under barrier conditions. Animals were housed in microisolator, solid-bottomed polycarbonate cages on hardwood bedding, fed a commercial pelleted diet (Prolab RMH 3000; PMI Nutrition International), and administered water *ad libitum*.

Germfree and monoassociated mice were housed in sterile isolators on autoclaved hardwood bedding in sterile, solid-bottomed polycarbonate cages and fed autoclavable Mouse Breeder Diet 5021 (PMI Nutrition International) and sterile water *ad libitum*. Isolators were surveyed biweekly and confirmed negative for microbial contaminants. The protocol was approved by the Committee on Animal Care of the Massachusetts Institute of Technology.

H. saguini MIT 97-6194-4 was grown under microaerobic conditions at 37°C on 5% sheep blood agar plates for 2 days. Bacteria were collected and resuspended in brucella broth with 20% glycerol and adjusted to a bacterial concentration of 1 optical density unit at 600 nm (OD$_{600}$)/ml. Half of the mice received 0.2 ml of fresh inoculum by gastric gavage every other day for three doses, and the other half were sham dosed with broth only. Colonization with *H. saguini* was confirmed 2 weeks postinoculation by PCR analysis of feces using *Helicobacter* genus-specific primers

(18). SPF mice were necropsied at 4 weeks postinfection (wpi), and mono-associated mice were necropsied at 3 months postinfection (p.i.). Serum and feces from SPF and monoassociated mice were collected, followed by liver, stomach, cecum, and colon, for culture, quantitative PCR, RNA isolation, and histology.

Detection of serum antibody responses to *H. saguini* by ELISA. Sera were collected at necropsy, and total IgG along with Th1-associated IgG2c and Th2-associated IgG1 responses to antigens of *H. saguini* were measured by ELISA (26). Sonicated antigen from *H. saguini* MIT 97-6194-5 was coated on Immulon II plates (Thermo Labsystems, Franklin, MA) at a concentration of 1 μg/ml (total IgG) or 10 μg/ml (subclass isotypes), and sera were diluted 1:100. Biotinylated secondary antibodies included goat anti-mouse IgG (Southern Biotechnology Associates, Pittsburgh, PA) and monoclonal anti-mouse antibodies (BD PharMingen, San Jose, CA) for detecting IgG1 and IgG2c. Incubation with ExtrAvidin peroxidase (Sigma, St. Louis, MO) was followed by avidin-biotin-peroxidase complex (ABTS) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for color development. Optical density (OD) development at a wavelength (λ) of 405 nm was recorded by an Epoch spectrophotometer (BioTek, Winooski, VT).

Histological evaluation. Formalin-fixed tissues were routinely processed, embedded in paraffin, cut at 4 μm, and stained with hematoxylin and eosin (H&E). Large bowel lesions were scored on the basis of size and frequency of hyperplastic and inflammatory lesions on a scale of 0 to 4 with ascending severity (0, none; 1, minimal; 2, mild; 3, moderate; and 4, severe). Epithelial dysplasia and neoplasia were graded using a scale of 0 to 4, as follows: 0, normal; 1, mild dysplastic changes; 2, moderate to severe dysplasia; 3, gastrointestinal intraepithelial neoplasia (GIN); and 4, invasive carcinoma as previously described (8, 9).

Fluorescent *in situ* hybridization (FISH) for *Helicobacter* species. Paraffin sections of colons were deparaffinized and rehydrated. *Helicobacter* genus-specific probes, HEL274 and HEL717, labeled with Cy3 were used (Integrated DNA Technologies) (27). Hybridization buffer (0.9 M NaCl, 100 mM Tris-HCl, 0.1% SDS, 30% formamide) with 5 ng of probe ml^{-1} was preheated for 10 min at 74.5°C; 80 μl of this solution was added to each slide. Slides were covered in Parafilm and placed in a dark humidification chamber overnight at 48°C. After incubation, slides were rinsed in double-distilled water and serially washed in prewarmed rinsing buffers for 15 min each (buffer 1 consisting of 0.9 M NaCl, 100 mM Tris-HCl, and 0.01% SDS; buffer 2 consisting of 0.9 M NaCl, and 100 mM Tris-HCl). Slides were air dried, mounted in Vectashield with 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories), and examined under a Zeiss Axioskop 2 fluorescence microscope. Tissues were considered positive for *Helicobacter* spp. if fluorescent spiral organisms were observed under the rhodamine filter (21).

Immunohistochemical analysis of γ-H2AX. The paraffin-embedded mouse cecum tissues (5 μm thick) were prepared, and after the tissue sections were deparaffinized and rehydrated in graded ethanol concentrations, the slides were immersed in low-pH target retrieval solution (Dako, Carpinteria, CA, USA) in a 95°C water bath for 20 min. The slides were washed in Tris-buffered saline (TBS) and blocked with 3% bovine serum albumin (BSA) in TBS overnight at 4°C. The slides were incubated with monoclonal rabbit anti-mouse γ-H2AX antibody (1:200 dilution; Cell Signaling, Danvers, MA, USA) for 2 h, followed by incubation for 90 min with Alexa Fluor 488-conjugated anti-rabbit F(ab′)^2 fragment (Cell Signaling). To stain the nuclei, the slides were mounted using 10 μl of Prolong Gold antifade reagent with DAPI (Cell Signaling). The tissue sections were observed using a Zeiss Axioskop 2 Plus microscope (Zeiss, Germany), and cells possessing one or more γ-H2AX$^+$ foci were counted as positive. The results are presented as the average percentage of positive epithelial cells observed in 8 to 10 high-power images.

Cytokine mRNA expression profiles in HT-29 cells and the cecum and colon of monoassociated C57BL/129 IL-10$^{-/-}$ mice. RNA was extracted from cells and mouse cecum tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA (2 μg) was converted into cDNA using

a High Capacity cDNA Archive kit according to the manufacturer's protocol (Applied Biosystems). cDNA levels for tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), IL-1β, IL-4, IL-6, IL-17α, IL-22, IL-23α, CXCL1, and inducible nitric oxide synthase (iNOS) mRNAs were measured by quantitative PCR using commercial primers and probes for each cytokine. Briefly, duplicate 20-μl reaction mixtures contained 5 μl of cDNA, 1 μl of a commercial 20× primer-probe solution, 10 μl of 2× master mix (all Applied Biosystems), and 4 μl of double-distilled H$_2$O. Relative expression of mRNA from infected and control tissues was calculated using the comparative threshold cycle (C_T) method with RNA input standardized between samples by expression levels of the endogenous reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results from duplicate samples were plotted as fold changes between cells or tissues from infected and uninfected controls.

Statistical analysis. Cecal and colonic lesion scores were analyzed using a Mann-Whitney U nonparametric test for ordinal data; levels of cytokine mRNA expression and serology results evaluated were compared by Student's *t* test. Statistical analysis was performed using GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla, CA). Results were considered significant at a P value of <0.05.

Nucleotide sequence accession numbers. The draft genome sequence of *H. saguini* has been submitted to GenBank under accession number JRMP00000000. The 16S rRNA sequence of the type strain of *H. saguini* MIT 97-6194-5 has been deposited in GenBank under accession number AF107494.1.

RESULTS

***Helicobacter saguini* is highly prevalent in cotton-top tamarins.** *H. saguini* was originally isolated from colony-maintained CTTs by our laboratory in 1999 (5), where 53% of colon biopsy samples were found to be positive for *Helicobacter* spp. by genus-specific *Helicobacter* sp. PCR. We further assessed the prevalence of *Helicobacter* infection in CTTs by surveying samples previously collected in 2001 and 2006 from animals living in the same colony in which colitis was endemic; 65% of anal swabs, 76% of feces, and 80% of colon biopsy samples were PCR positive for *Helicobacter* spp. In total, *Helicobacter* spp. were detected in 69% of the animals (Table 1). Representative PCR products from two anal swab samples, nine biopsy samples, and two fecal samples had 16S rRNA sequences identical to those of *H. saguini* strain MIT-97-6194-5. Eleven *Helicobacter* isolates successfully cultured from 57 samples had 16S rRNA sequences identical to those of *H. saguini* strain MIT 97-6194-5 (Table 1). PCR products from four biopsy samples had mixed sequences and were cloned into a pCR2.1-TOPO TA vector; eight colonies from each PCR-positive biopsy sample were sequenced. In addition to *H. saguini*, two other *Helicobacter* spp. were present. They had 97% sequence homologies with *Helicobacter* spp. previously identified in the feces of a rhesus macaque (MIT 01-3238) and the colon of a baboon (MIT 03-7674c), respectively.

Twenty-three CTTs had both anal swabs and biopsy samples submitted for testing in 2001. Overall, 20/23 (86.9%) of the animals were PCR positive. The *Helicobacter* PCR results for both types of samples correlated very well (overall, 21/23, or 91.3%, with the same result; both positive, 18/23, or 78.3%; both negative, 3/23, or 13%); only 2/23 (8.7%) had different results between sample types. These results suggest that anal swabs can be used as an alternative to the invasive biopsy sampling method for *H. saguini* detection. Feces from 4 of the 23 CTTs were retested on 2006; all remained *Helicobacter* positive, indicating a chronic, persistent *Helicobacter* infection in these animals.

Biochemical analysis. The biochemical characteristics of eight

TABLE 2 Comparison of biochemical tests for *Helicobacter saguini* and other *Helicobacte*r spp.[a]

Taxon	Catalase	Oxidase	NO$_3$	Urease	IAH	GGT	PO$_4$	Growth at 42°C	Growth with 1% glycine	Drug resistance[c]		DNA G+C content (mol%)
										NA	CE	
Helicobacter saguini[b]	+	+	−	−	−	+	−	+	+	R	R	34.6
Helicobacter jaachi	+	+	−	+	+	−	−	+	+	S	R	41
"*Helicobacter callitrichis*"	+	+	−	−	−	−	+	+	+	S	R	39.6
Helicobacter macacae	+	−	−	−	−	−	−	+	+	R	R	40.6
Helicobacter trogontum	+	+	+	+	−	+	−	+	ND	R	R	34
Helicobacter bilis	+	+	+	+	−	+	−	+	+	R	R	ND
Helicobacter marmotae	+	+	−	+	−	−	+	−	+	R	R	ND
Helicobacter canis	−	+	−	−	+	+	+	+	−	S	I	45
Helicobacter hepaticus	+	+	+	+	+	−	−	−	+	R	R	35.9
Helicobacter pylori	+	+	−	+	−	+	+	−	−	R	S	35–37

[a] NO$_3$, nitrate reduction; IAH, indoxyl acetate hydrolysis; GGT, γ-glutamyl transpeptidase; PO4, alkaline phosphatase hydrolysis.
[b] Data were modified from our 1999 paper (5) and represent 4 strains from the original study and 4 strains from 2006 isolates. In all cases except for GGT, the results represent 8/8 strains. For GGT, the result represents 5/8 strains.
[c] NA, nalidixic acid; CE, cephalothin; R, resistant; S, susceptible.

H. saguini isolates (four from original isolates in 1999 and four from the current study) were compared with those of other *Helicobacter* species (Table 2). All isolates were oxidase and catalase positive and urease negative. The isolates did not reduce nitrate to nitrite, did not hydrolyze alkaline phosphatase, and were not able to hydrolyze indoxyl acetate. Five of the eight isolates had γ-glutamyl transpeptidase activity, and all were resistant to nalidixic acid and cephalothin. The organism grew in 1% glycine and at 37 and 42°C, but not at 25°C. The type strain of *H. saguini* MIT 97-6194-5 has been deposited in the BCCM/LMG Bacteria Collection as LMG 28611.

Electron microscopy. By electron microscopy, cells of *H. saguini* had a fusiform appearance and measured approximately 0.5 by 4 to 5 μm (Figure 1). The cells possessed periplasmic fibers and 6 to 12 bipolar, sheathed flagella.

Phylogenetic analysis. Full 16S rRNA genes from six *H. saguini* strains were sequenced. They shared over 99% sequence similarity with each other. The most closely related species was *H. jaachi*, isolated from common marmosets (97% similarity with the type strain 16S rRNA sequence) (Fig. 2). The 23S rRNA gene from *H. saguini* MIT 97-6194-5 was also compared with the 23S rRNA sequences of other *Helicobacter* species. The closest related species was *H. jaachi*, which also had a sequence similarity of 97% to the *H. saguini* type strain 23S sequence (Fig. 2). Finally, the *hsp60* gene of *H. saguini* obtained from the whole-genome sequence of MIT 97-6194-5 was compared with the 600-bp conserved region of the *hsp60* gene in other *Helicobacter* spp. The most closely related species was *H. canis* (79% sequence identity to that of the *H. saguini* type strain *hsp60* sequence).

Draft genome of *H. saguini*. The *H. saguini* type strain MIT 97-6194-5 genome measures 2.92 Mb in size with a G+C content of 34.6%, and it contains 2,704 genes encoding 2,294 proteins, 5 rRNAs, 39 tRNAs, 1 other RNA, and 365 pseudogenes. Homologous genes of previously described virulence factors from *Helicobacter* spp. and related species were identified in the annotated genome, including flagellum components, flavodoxin *fldA*, γ-glutamyl transpeptidase (*ggt*), the secreted serine protease *htrA*, and the type VI secretion component *vgfG*. Unlike other selected EHS, *H. saguini* does not harbor genes for cytolethal distending toxin (*cdt*). However, similar to other EHS, *H. saguini* also lacks *vacA* or *cagA* (Table 3).

In silico comparison of the *H. saguini* MIT 97-6194-5 genome sequence with that of *H. jaachi* MIT 09-6949 (GenBank accession number JRPR00000000), its closest phylogenetic neighbor, revealed 12.90% ± 2.98%, 23.30% ± 2.38%, and 13.30% ± 2.55% similarity, using the three formulas offered by the Genome-to-Genome Distance Calculator (GGDC) (25), version 2.0. These data unambiguously demonstrate that both strains represent distinct species, thus confirming the 6.38% difference in mol% G+C content between the two genome sequences (28).

H. saguini induces proinflammatory cytokines and chemokines in the human intestinal epithelial cell line HT-29. Human
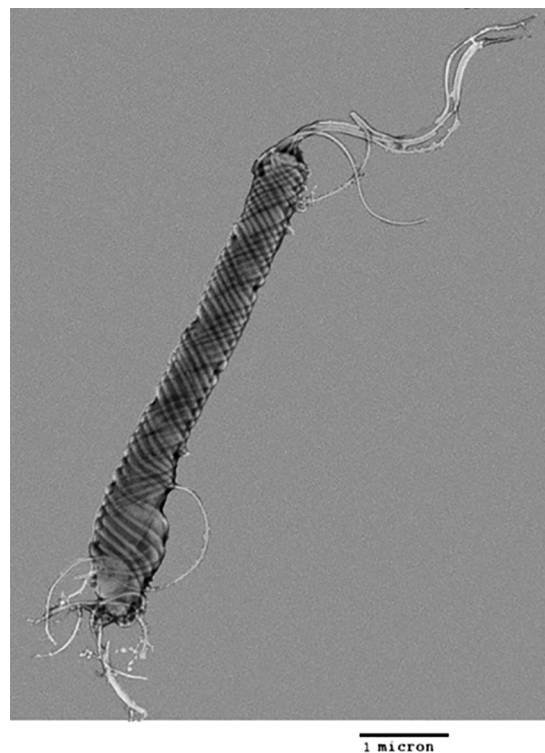

FIG 1 Transmission electron micrograph of negatively stained *H. saguini* MIT 97-6194-5.
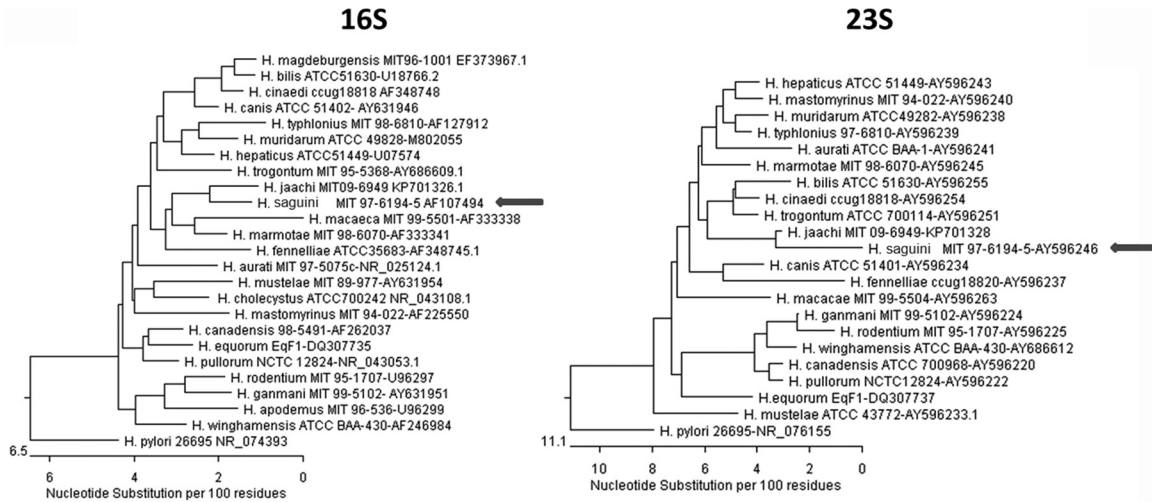
16S **23S**

FIG 2 Phylogenetic analysis of 16S rRNA and 23S rRNA gene sequences. Neighbor-joining trees were based on the comparison of genes from different *Helicobacter* species. Arrows indicate *H. saguini*.

intestinal epithelial cells (HT-29) were cocultured with *H. saguini* (MIT 09-6949-5) at an MOI of 25 or 100 for 4 and 24 h. Inflammatory cytokine gene expression of the HT-29 cells and secreted IL-8 in cell culture supernatant were quantified to determine if *H. saguini* is capable of inducing a proinflammatory response. *H. saguini* stimulated IL-8 production in HT-29 cells. IL-8 concentration in the supernatant of *H. saguini*-infected cells was significantly higher than that in the medium control at an MOI of 1:100 ($P < 0.001$). IL-8 levels were 150 pg/ml at 4 h and 900 pg/ml at 24 h in HT-29 cells infected with *H. saguini* (Fig. 3). mRNA expression levels of IL-8, TNF-α, IL-1β, IL-6, and CXCL1 were significantly increased ($P < 0.001$) compared to levels in the medium controls 4 h after infection at an MOI of 1:100 (Fig. 4).

Experimental *H. saguini* successfully colonizes germfree C57BL/129 IL-10$^{-/-}$ mice. Specific-pathogen-free (SPF) and germfree (GF) C57BL/129 IL-10$^{-/-}$ mice were dosed with 2×10^8 CFU of *H. saguini* by oral gavage every other day three times. *H. saguini* successfully colonized GF mice. Feces from all monoassociated mice infected with *H. saguini* were PCR positive for *Helicobacter* spp. at 2 weeks postinfection (wpi), and sequencing of the 16S rRNA PCR products confirmed the organisms to be *H. saguini*. Additionally, *H. saguini* was cultured from gnotobiotic mouse feces. Monoassociated mice euthanized at 3 months postinfection were also positive for *Helicobacter* spp. by PCR. In contrast, SPF IL-10$^{-/-}$ mice were not colonized by *H. saguini*, as determined by PCR-negative results for *Helicobacter* spp. at 2 wpi in fecal samples. None of the SPF mice at necropsy had gross changes at 4 wpi, and they were PCR negative for *Helicobacter* in colon samples.

FISH. A *Helicobacter* genus-specific probe was used to identify *H. saguini* in monoassociated cecal tissues. *H. saguini*, as demonstrated by fluorescence *in situ* hybridization (FISH), was present in the lumen and crypts of the colons and ceca of monoassociated mice (Fig. 5).

***H. saguini* induces significant typhlocolitis in monoassociated mice.** In monoassociated mice, *H. saguini* induced typhlocolitis characterized by moderate to severe infiltration of inflammatory cells, including lymphocytes, histiocytes, and neutrophils, into the mucosa and submucosa. Also present were epithelial defects, mucosa-associated lymphoid tissue (MALT) hyperplasia, and dysplasia (Fig. 6A to C). *H. saguini*-monoassociated mice had significantly higher inflammation, hyperplasia, and dysplasia scores than control mice in both the cecum and colon ($P < 0.05$). GF control mice did not have overt histologic signs of lower bowel inflammation and had normal crypts and epithelial surfaces in the colon and cecum.

***H. saguini* infection induces H2AX-positive epithelial cells.** Double-stranded breaks (DSBs) are defined as DNA damage in which two complementary strands of double-helix DNA are damaged simultaneously in a location close to each other. It is considered the most dangerous type of DNA damage, and unrepaired DSBs are sufficient for induction of cell death. DSBs can be initiated in response to a variety of stress signals that are encountered during physiological processes. When cells are exposed to ionizing radiation, DNA-damaging chemotherapeutic agents, or chronic infection by *Helicobacter* spp., DSBs are generated that rapidly result in the phosphorylation of histone H2A variant H2AX. Because phosphorylation of H2AX at Ser139 (γ-H2AX) correlates

TABLE 3 Profile of draft genomes of *H. saguini*, *H. pylori*, and *H. hepaticus*

Characteristic	*H. pylori* 26695	*H. saguini* LMG 28611	*H. hepaticus* ATCC 51449
Genome size (Mb)	1.67	2.9	1.8
G+C content (%)	39	34.6	35.9
No. of genes	1,590	2,704	1,875
Selected virulence genes[a]	*cagA*, *vacA*, *ggt*, *nap*, T4SS, *htrA*, *ure*	*ggt*, T6SS, *htrA*	T6SS, *ure*, *cdt*

[a] *vacA*, vacuolating cytotoxin A; *cagA*, cytotoxin-associated gene; *ggt*, γ-glutamyl transpeptidase; *nap*, neutrophil-activating protein; T4(6)SS, type IV(VI) secretion system; *htrA*, high-temperature requirement A protein-secreted serine protease; *ure*, urease.
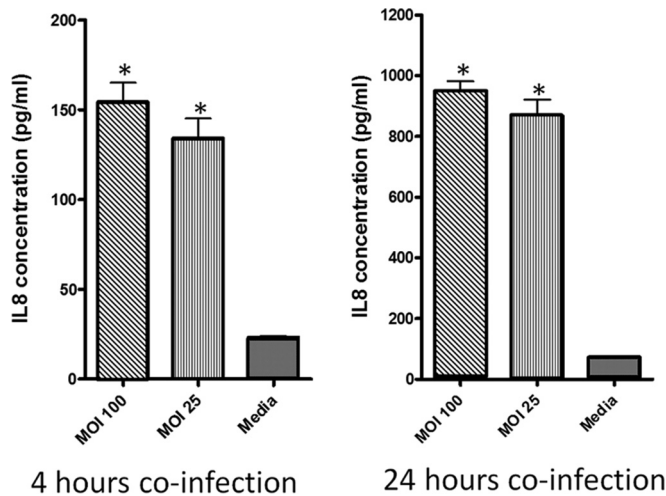
FIG 3 *H. saguini* stimulated IL-8 production by HT-29 cells. HT-29 cells were coincubated with *H. saguini* at an MOI of 25 or 100 or with medium alone for 4 and 24 h. IL-8 concentrations in the supernatants were measured by ELISA. *, $P < 0.001$.

FIG 4 Expression levels of proinflammatory genes in HT-29 colonic cell line in medium alone or coincubated with *H. saguini* (*H.s.*) for 4 h at an MOI of 100. Data represent means and standard errors of fold changes in mRNA expression levels. *, $P < 0.01$.

well with each DSB, it is the most sensitive marker that can be used to examine DNA damage and the subsequent repair of the DNA lesion (29–31).
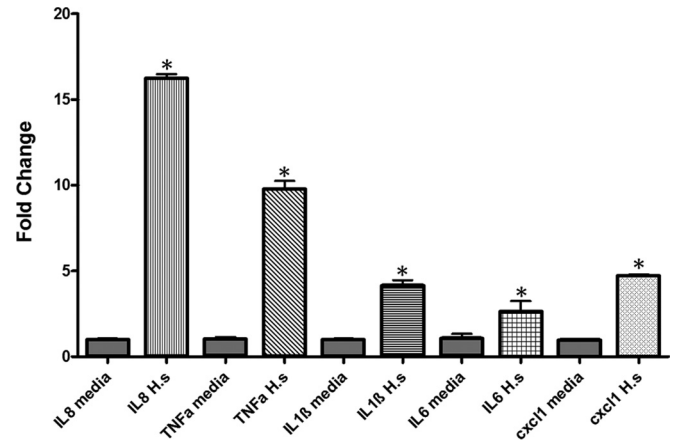
Immunofluorescence staining of γ-H2AX was performed on the ileo-cecal-colic junction of GF and monoassociated mice. In the *H. saguini*-monoassociated mice, significantly increased numbers of γ-H2AX-positive cells were observed in the intestinal epithelium, particularly within the regions of intestinal hyperplasia and dysplasia; the uninfected tissue had very few positively stained cells (Fig. 7).

H. saguini has in vivo proinflammatory properties. Consistent with histological evidence of inflammation, *H. saguini* induced mRNAs for proinflammatory cytokines and chemokines in the cecal tissue of infected mice compared to levels in control mice ($P < 0.001$) (Fig. 8). The most significantly increased cytokines were IL-22, IL-17a, IFN-γ, TNF-α, and IL-6; iNOS expression was increased as well; no significant changes were noted in IL-23a and IL-4 mRNA expression levels (data not shown).

H. saguini induces a systemic immune response. Serum antibody against *H. saguini* was measured by ELISA. Total IgG and Th1-associated IgG2c antibodies in the sera of infected *H. saguini*-monoassociated mice were significantly increased ($P < 0.0001$) (Fig. 9). The IgG2c response was significantly higher than the Th2-associated antibody IgG1 response ($P < 0.0001$), consistent with IgG isotype subclass responses of C57BL IL-10$^{-/-}$ mice to *Helicobacter* infections (26).

DISCUSSION

To further assess the prevalence of *H. saguini* infection in CTTs, we surveyed a collection of frozen anal swabs, feces, and colonic biopsy samples previously collected in 2001 and 2006 from a colony in which colitis was endemic. From the 147 samples analyzed from 116 animals, 69% were PCR positive for *Helicobacter* sp. infection. Representative PCR-positive samples were *H. saguini* based on 16S rRNA sequence analysis. Given the age of the frozen samples and the difficulty of culturing *Helicobacter* spp., *Helicobacter* spp. were successfully cultured only from 11 of the 57 sam-

ples; however, in all cases, the cultured *Helicobacter* sp. was identical to *H. saguini* by 16S rRNA sequencing. Sequential sampling of selected CTTs indicated that these animals were persistently colonized or, less likely, reinfected. Based on our knowledge of EHS infections in mice and *H. macacae* infection in rhesus macaques, we believe *H. saguini* is a persistent infection in CTTs. Whether *H. saguini* is also present in wild CTTs residing in South America is difficult to assess and remains unknown.

IL-10$^{-/-}$ mice develop spontaneous colitis when housed under conventional or SPF conditions but fail do to so when housed under germfree conditions (32, 33). Thus, without the anti-inflammatory actions of IL-10, an unregulated Th1-mediated immune response against selected enteric bacteria ensues and precipitates colitis characterized histologically by epithelial hyperplasia, mucosal and submucosal inflammation, and ulcerative lesions in the cecal-colonic junction (26, 34–38). EHS infection exacerbates the onset of disease in IL-10$^{-/-}$ mice and thus is a frequently used model to study pathogen-induced IBD and colon cancer. *H. hepaticus* and *H. bilis* are the most thoroughly studied EHS; however, other EHS, like *H. trogontum*, *H. typhlonius*, *H. rodentium*, *H. mastomyrinus*, and *H. cinaedi*, a human pathogen, can also cause IBD-like disease in immunocompromised mice (9, 11, 34, 38–40). Because EHS-induced colitis in IL-10$^{-/-}$ mice is well characterized, we chose this mouse model to investigate the pathogenic potential of *H. saguini*.

H. saguini induced typhlocolitis in monoassociated C57BL/ 129 IL-10$^{-/-}$ mice and elicited pathological lesions consistent with those previously described for EHS-induced colitis in SPF IL-10$^{-/-}$ mice, including cecal and colonic transmural inflammation and epithelial hyperplasia and dysplasia. Interestingly, *H. saguini* colonized and induced typhlocolitis in monoassociated, but not SPF, mice. This was unexpected considering previous reports suggesting that *H. hepaticus* infection required other gut microflora in IL-10$^{-/-}$ mice for the induction of colitis. Infection by *H. hepaticus* produced colitis in conventional or SPF mice, but not in germfree C57BL/129 IL-10$^{-/-}$ mice (41, 42). *H. saguini* may have failed to colonize SPF mice because of colonization re-
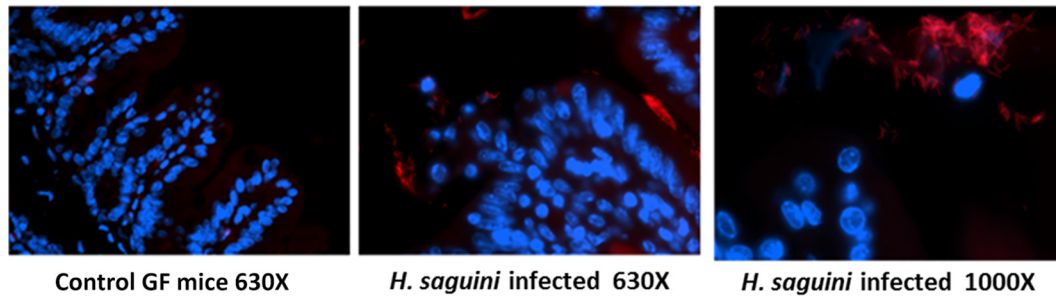
FIG 5 Fluorescence in situ hybridization (FISH) using a *Helicobacter* genus-specific probe in monoassociated mouse cecum. *H. saguini* cells are labeled in red. DAPI stained the nuclei blue.
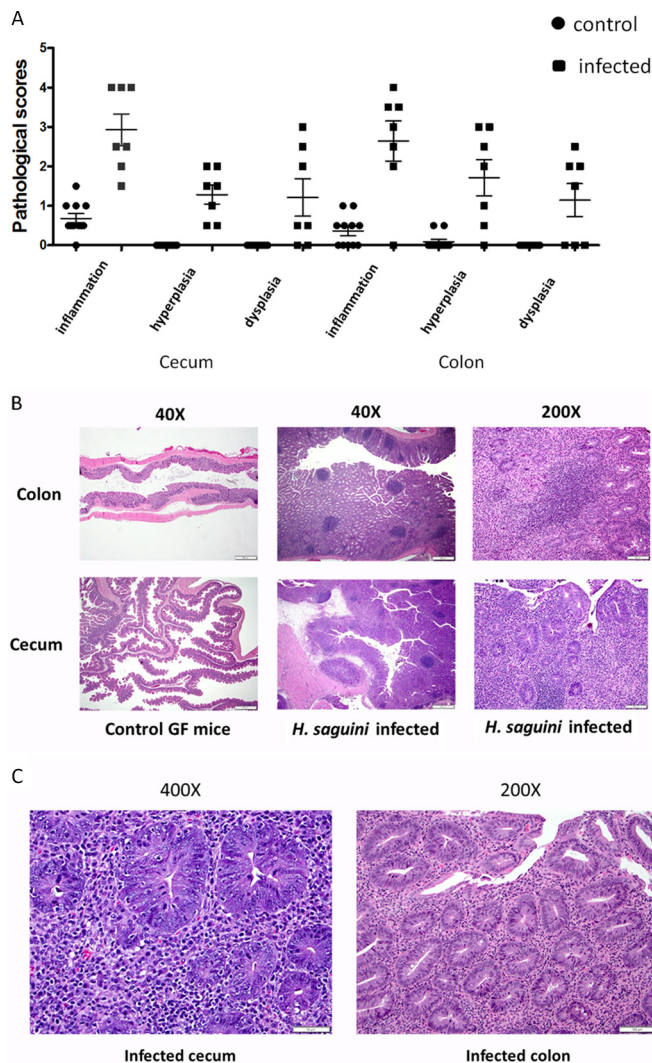

FIG 6 (A) *H. saguini*-infected monoassociated IL-10$^{-/-}$ mice had significantly higher inflammation, hyperplasia, and dysplasia scores than GF mice in both cecum and colon ($P < 0.05$). (B and C) *H. saguini*-induced typhlocolitis characterized by moderate to severe infiltration of inflammatory cells, including lymphocytes, histiocytes, and neutrophils, into the mucosa and submucosa, with epithelial defects, mucosa-associated lymphoid tissue (MALT) hyperplasia, and dysplasia (H&E staining).

sistance conferred by commensal flora. *H. saguini* was isolated from a primate and is not a natural colonizer in rodents. In our previous experiments, *H. macacae*, which was isolated from rhesus monkeys, was inoculated into SPF C57BL/129 IL-10$^{-/-}$ mice and failed to colonize the mice (unpublished data). The different gut flora of primates which allow colonization of *Helicobacter* spp. in primates, but not mice, could contribute to the inability of specific *Helicobacter* spp. to colonize mice. Importantly, *H. saguini* is sufficient by itself to cause IBD in monoassociated IL-10$^{-/-}$ mice, unlike *H. hepaticus* (41, 42), suggesting that the expression of virulence properties of *H. saguini* does not require cocolonization of the lower bowel with other flora to initiate an inflammatory response.

The genome of *H. saguini* with its fusiform morphology is rather large. Nevertheless, other enterohepatic *Helicobacter* spp. also have draft genome sizes of the same magnitude as that of *H. saguini*. *Helicobacter* spp. which have a fusiform morphology tend to have large genomes: *H. bilis* has a genome size of 2.5 Mb, and *H. trogontum* has a size of 2.77 Mb. In contrast, curved or spiral-shaped helicobacters have smaller-sized genomes, such as those of *H. hepaticus* and *H. rodentium*, which have 1.8-Mb genomes. There are 28 *Helicobacter* species which have complete or draft genomes available on NCBI. Pseudogene counts range from 16 genes in *H. mustelae* to 526 genes in *H. apodemus*. *H. saguini* has the third-highest pseudogene count at 365 genes, while *H. magdeburgensis* has the second highest at 398 genes. The average ± standard deviation of pseudogene counts from the 28 genomes is approximately 140 ± 120 genes. So although *H. saguini* falls into the upper range, having 365 pseudogenes, there is also substantial diversity in pseudogene counts overall within the *Helicobacter* genomes.

Interestingly, *H. saguini* is relatively devoid of known EHS virulence factors, particularly cytolethal distending toxin. However, the γ-glutamyl transpeptidase (*ggt*) gene was detected in the annotated genome of *H. saguini*. GGT genes from *H. pylori*, *H. suis*, *H. bilis*, and *Campylobacter jejuni* have been shown to be influential virulence factors required for persistence of the organism *in vivo*, as well as important in inducing inflammation *in vivo* (43–46). Also, *in vitro* data show that GGT inhibits gastrointestinal and lymphocyte cell proliferation as well as having a role in producing reactive free oxygen species (43–46). NF-κB activation and IL-8 secretion in gastrointestinal cell lines may have also been stimulated by GGT expression. Ongoing studies in our laboratory with *H. saguini*, which persistently colonizes the lower bowel of CTTs, are exploring whether GGT plays a role in *H. saguini*'s pathogenic
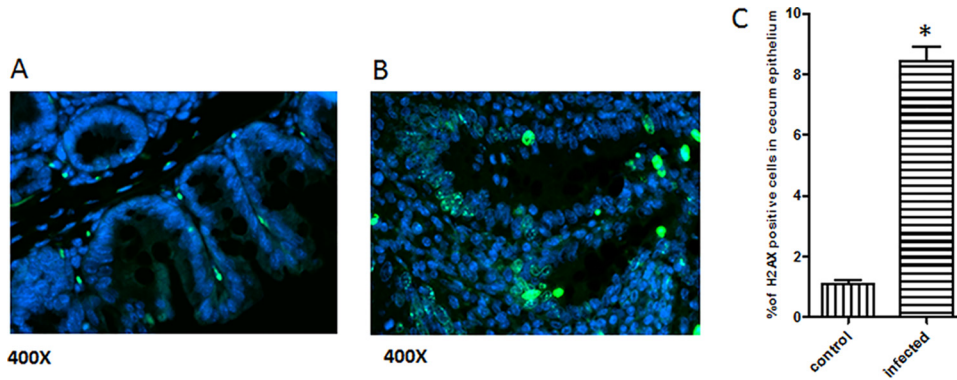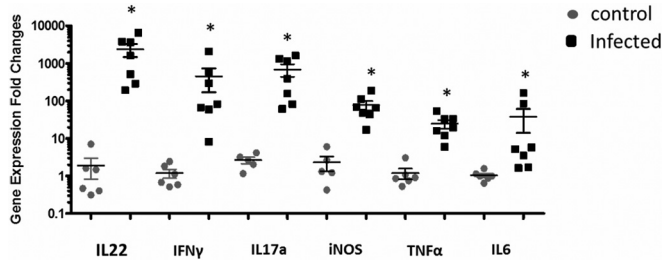
FIG 7 Immunofluorescence staining of gnotobiotic mouse cecum for γ-H2AX (in green). Five mice in each group were examined, with the following results: the uninfected tissue had very few positively stained cells in the cecum (A) and more γ-H2AX-positive cells were observed in the epithelium, particularly within areas of intestinal hyperplasia and dysplasia in the infected cecum (B). (C) The number of γ-H2AX-positive cells in the cecal epithelium of *H. saguini*-infected mice was statistically higher than that in the control. (*, $P < 0.05$).

potential by weakening the intestinal barrier integrity, modulating immune function, and sustaining chronic inflammation.

Chronic intestinal inflammation is well known to increase the risk of colon cancers (10, 47, 48). Chronically inflamed tissues which are continuously regenerating are at an increased risk for mutagenesis and tumor transformation. It has been reported that inflammation-induced cell proliferation greatly potentiates exposure-induced mutations; inflammation can act synergistically with DNA damage to induce mutations that promote cancer and cancer recurrence (49). Reactive oxygen species (ROS) are associated with the inflammatory response and frequently contribute to the tissue-damaging effects of inflammatory reactions. In *H. saguini*-monoassociated IL-10$^{-/-}$ mice, iNOS expression, a biomarker for nitrosative intestinal damage, also noted in *H. hepaticus*-induced IBD and colon cancer in Rag2 mice (9, 10, 50), was significantly higher in the IL-10$^{-/-}$ *H. saguini*-infected mice. Suspected DNA DSBs were detected in the inflamed cecum of monoassociated *H. saguini* IL-10$^{-/-}$ mice, especially in the regions with high hyperplasia and dysplasia scores. DNA damage in the epithelial cells may lead to mutations and cancer formation in *H. saguini*-infected mice, and a similar mechanism may be present in the *H. saguini*-infected CTT colonies with a high incidence of colon cancer.
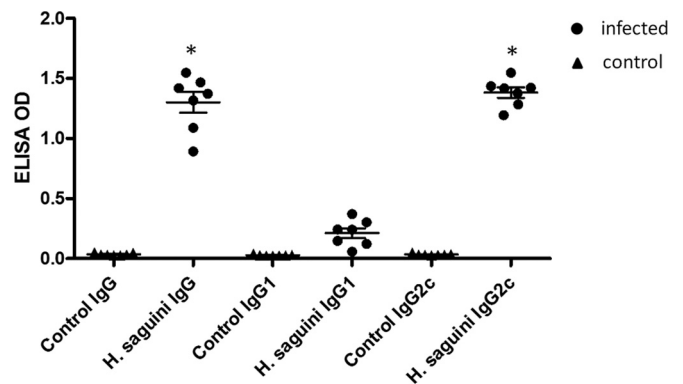
IBD in CTTs, similar to IBD in humans, is likely multifactorial in etiology but occurs at a higher incidence in CTTs maintained in captivity; CTTs in the wild have a low incidence of mild colitis (2). The precise inflammatory stimuli responsible for IBD in captive

CTTs are unknown but may be related to housing conditions, diet, social interaction, and infectious agents. Captivity can also elicit chronic stress, which is known to suppress immune function and increase susceptibility to pathogenic microbes. We have demonstrated that *H. saguini* persistently colonizes the lower bowel of captive CTTs maintained in a colony, with a high incidence of colitis and colon cancer. *H. saguini* is also capable of eliciting chronic colitis in monoassociated IL-10$^{-/-}$ mice, has robust *in vitro* proinflammatory properties, and induces DNA damage. Further, the immune response measured as total IgG and IgG2c isotype was robust and on a scale consistent with data from our prior publication (26). The IgG2c isotype reflects a proinflammatory Th1 response in C57BL/129 IL-10$^{-/-}$ mice, and there is typically an inverse relationship with the Th2-associated IgG1 isotype, which is the reason IgG1 measurements were low.

The role of *Helicobacter* sp.-induced IBD in humans is unknown, but EHS have been identified in human cases of IBD, as well diarrheal patients (17, 51, 52). Importantly, *H. fennelliae* and *H. cinaedi* were originally isolated from the colons of homosexual men with proctitis, who were presumably immunocompromised by HIV infection (15). The *H. saguini* mouse model described in this report can be used to study the pathogenic potential of EHS

FIG 8 Inflammatory gene expression in cecal tissue at 3 months postinfection. Expression of each inflammatory gene was significantly greater in infected than in control mice (*, $P < 0.001$). The data are presented as the fold change compared to mean gene expression of GAPDH.

FIG 9 Antibody responses against *H. saguini* in the sera of GF mice were measured by ELISA. In the *H. saguini*-monoassociated mice, the total IgG was significantly increased ($P < 0.0001$); the Th1-associated antibody IgG2c response was significantly higher than the Th2-associated antibody IgG1 response (*, $P < 0.0001$).

isolated from nonhuman primates with IBD. Although new insights into the cause of human IBD were not expressly studied, the isolation of a novel *Helicobacter* sp. from CTT historically used to study the role of UC and colon cancer serves as a surrogate nonhuman primate model of IBD. Isolation and characterization of EHS in CTT reinforce that there may be a connection between EHS infection and IBD in CTTs and, by extrapolation, also in humans. The phenotypic distinctiveness of *H. saguini* and the genomic distance between *H. saguini* and *H. jaachi* type strains demonstrate that the former is appropriately classified as a novel *Helicobacter* species.

Description of *Helicobacter saguini* sp. nov. *Helicobacter saguini* (sa′gui.ni. N. L. masc. gen. n. of *Saguinus* [*Oedipus*], taxonomic name of cotton-top tamarin from which the bacterium was first isolated). The organism is motile; cells are fusiform with periplasmic fibers, having multiple, bipolar sheathed flagella, and measure 0.5 by 5 μm. The bacterium is Gram negative and non-sporulating; it grows slowly and appears on solid agar as a spreading film on the surface. The bacterium grows at 37° and 42°C, but not at 25°C, under microaerobic conditions, but not aerobically or anaerobically. The bacterium is oxidase, catalase, and γ-glutamyl transpeptidase positive, but urease, indoxyl acetate hydrolysis, and alkaline phosphatase hydrolysis negative. It grows on 1% glycine and is resistant to cephalothin and nalidixic acid. The type strain MIT 97-6194-5 has been deposited in BCCM/LMG Bacteria Collection as LMG 28611. It has a DNA G+C content of 34.6%, and its genome is 2.92 Mb. The 16S rRNA sequence of the type strain has been deposited in GenBank under accession number AF107494.1. The draft genome has been submitted to GenBank under accession number JRMP00000000.

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FUNDING INFORMATION

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