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***Helicobacter hepaticus* urease is not required for intestinal colonization but promotes hepatic inflammation in male A/JCr mice**

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Abstract

Urease activity contributes to bacterial survival in the acidic environment of the stomach and is essential for persistent infection by known gastric helicobacters such as the human pathogen *Helicobacter pylori*. Several enterohepatic *Helicobacter* species (EHS) that primarily infect the less acidic intestine also have very active urease enzymes. The importance of urease and its contribution to pathogenesis for these EHS are poorly understood. In this study, we generated a urease-deficient, isogenic mutant (*HhureNT9*) of *Helicobacter hepaticus* 3B1 (Hh 3B1), an EHS that possesses a urease gene cluster similar to that of *H. pylori*. Lack of urease activity did not affect the level of cecal colonization by *HhureNT9* compared to Hh 3B1 in male A/JCr mice ($P = 0.48$) at 4 months post-inoculation (MPI). In contrast, there was no *HhureNT9* detected in the livers of any infected mice, whereas all livers from the Hh 3B1-infected mice were PCR-positive for Hh 3B1. The mice infected with *HhureNT9* developed significantly less severe hepatitis ($P = 0.017$) and also produced significantly lower hepatic mRNA levels of proinflammatory cytokines IFN- γ ($P = 0.0007$) and TNF- α ($P < 0.0001$) compared to the Hh 3B1-infected mice. The Hh 3B1-infected mice developed significantly higher total IgG, Th1-associated IgG2a and Th2-associated IgG1 responses to infection. These results indicate that *H. hepaticus* urease activity plays a crucial role in hepatic disease but is not required for cecal colonization by *H. hepaticus*.

Keywords

H. hepaticus; urease; isogenic mutant; virulence factor; animal model

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1. Introduction

The genus *Helicobacter* comprises a group of gram-negative, microaerophilic bacteria isolated from human and a variety of animals, most commonly the gastrointestinal tract of mammals [1]. Since the discovery of its type species, *Helicobacter pylori*, by Marshall and Warren in the early 1980s [2], the *Helicobacter* genus has expanded and currently includes at least 26 formally named species [3]. The *Helicobacter* species have been sub-grouped into gastric and enterohepatic *Helicobacter* species (EHS) based on their primary colonization niches [4]. The best known gastric helicobacter is *H. pylori*, a human pathogen which causes chronic active gastritis, peptic ulcer disease, gastric adenocarcinoma, and MALT lymphomas [5]. *H. hepaticus*, the prototype EHS, is a murine pathogen which induces chronic active hepatitis, hepatocellular carcinoma, colon cancer, inflammatory bowel disease, and promotes formation of cholesterol gallstones in susceptible mouse strains [6-8]. Natural and experimental *Helicobacter* infections in animal models have been widely used to dissect underlying mechanisms of infectious diseases in humans, since these infections reproducibly recapitulate important pathological features of human diseases [9, 10].

All known gastric helicobacters produce urease which catalyzes the hydrolysis of urea into ammonia and carbon dioxide. It has been proposed that production of ammonia not only is beneficial for the bacteria to survive in acidic microenvironments, but also serves as a nitrogen source for the bacteria [11, 12]. This enzyme is essential for gastric infection by *H. pylori* in nude mice and gnotobiotic piglets, and by *H. mustelae* in ferrets [13, 14]. Among known EHS, approximately 40% of the species, including *H. hepaticus*, are urease-positive. Interestingly, the genome of *H. hepaticus* contains an *ure* gene cluster similar to that of *H. pylori*, including *ureA* and *ureB* that code for catalytic subunits, *ureI* that encodes a urea channel protein, and *ureEFGH* that encode accessory proteins necessary for enzyme activity [15, 16]. However, it is unclear if urease plays a role in *H. hepaticus* colonization similar to that in *H. pylori*. In vitro urease activity in *H. hepaticus*, in contrast to *H. pylori*, is not induced at acidic pH and *H. hepaticus* does not grow or survive at pH 3.0, suggesting that the biological functions of urease may differ between gastric helicobacters and EHS [17]. In this study, we investigated the in vivo role of *H. hepaticus* urease in intestinal and hepatic infection, proinflammatory responses and induction of chronic hepatitis using an *ureAB*-defective *H. hepaticus* mutant in male A/JCr mice.

2. Results

2.1. The deletion mutants were non-polar and did not affect urel transcription

The urease-deficient *H. hepaticus* mutant, *HhureNT9*, contained a *catNT* cassette which replaced a ~2.3-kb region representing the large portion of *ureA* and the entire *ureB*. In contrast to *Helicobacter hepaticus* strain 3B1 (Hh 3B1), *HhureNT9* had no detectible urease activity completely disappeared from as measured by incubating the organisms on urea agar. *catNT*, lacking the predicted transcriptional terminator of the original *cat*, was used to avoid polar effects on downstream genes, particularly *ureI*, which is immediately downstream of *ureB* and is also essential for gastric colonization of *H. pylori* [18]. As visualized on an ethidium bromide-stained agarose gel (Figure 1B), quantities of the PCR products amplified

from the *ureI* cDNA equivalent to 1 ng of total bacterial RNA are comparable between *Hh* 3B1 (lane 2) and *HhureNT9* (lane 4); RNase treatment prior to cDNA synthesis abolished PCR amplification (lanes 1 and 3), demonstrating no DNA contamination in the RNA preparations. Additionally, QPCR assays were performed to quantify mRNA levels of *ureI*; the average copy number of *ureI* transcripts (per ng of total RNA) in 3 independent assays from *HhureNT9* (3915 ± 322) were higher by ~2.4-fold than from *Hh* 3B1 (1604 ± 152). These data indicate that the replacement of *ureAB* with *catNT* did not reduce the transcription of *ureI* within the mutant *HhureNT9*.

2.2. Absence of urease genes did not affect colonic infection but abolished liver infection as measured by Q-PCR

All 5 male A/JCr mice infected with *Hh* 3B1 were positive by Q-PCR for *H. hepaticus* in both cecum and liver at 4 MPI; cecal and hepatic colonization levels of *Hh* 3B1 in one mouse were relatively lower than the remaining 4 mice in the same group (Figure 2). These results were consistent with our previous observations that selected A/JCr mice are relatively resistant to *H. hepaticus* infection in the cecum and liver [10, 19]. However, the mice dosed with *HhureNT9* were negative for *HhureNT9* by Q-PCR in the liver (Figure 2), suggesting that urease activity is essential for hepatic colonization of *H. hepaticus*. All *HhureNT9*-infected mice were PCR-positive for *H. hepaticus* in the cecum at 4 MPI; there was no significant difference in cecal colonization levels between the *HhureNT9* and the *Hh* 3B1-infected groups (Figure 2, $P = 0.48$).

2.3. No hepatic lesions were detected in mice infected with the urease mutant

It has been established that *H. hepaticus*-infected male A/JCr mice develop chronic active hepatitis [7, 10, 19]. In agreement with previous results from our group, at 4 MPI, 4 out of 5 male mice infected with wild-type *Hh* 3B1 developed subacute-to-chronic hepatitis characterized by multifocal lobular hepatitis with coagulative necrosis, and mild to moderate lymphocyte-predominant portal and interface hepatitis ((Fig. 3A) [10, 19]. One *Hh* 3B1-infected mouse did not develop hepatitis which may be due to a relative low level of *H. hepaticus* in the liver (~100-fold) compared to the remaining mice in the group (Figure 2). In contrast, *HhureNT9*-infected mice that did not have *H. hepaticus* in the liver by PCR, did not develop hepatic lesions. The hepatitis indices in the *Hh* 3B1-infected mice were significantly higher than in the *HhureNT9*-infected mice ($P < 0.05$, Figure 3B); there was no significant difference in hepatitis indices between the *HhureNT9*-infected and the sham dosed mice. Therefore, our data indicate that urease-deficient *HhureNT9* was incapable of promoting chronic active hepatitis in A/JCr mice. Gross and histopathological evaluation of the intestines of all mice did not reveal significant lesions.

2.4. There was lower induction of inflammatory cytokines in liver, but not in colon with the urease mutant

Our previous studies showed that *Hh* 3B1 infection significantly elevated expression of proinflammatory cytokines in splenocyte culture and in livers of male A/JCr mice [19, 20]. To ascertain whether *HhureNT9* infection induced proinflammatory responses comparable to *Hh* 3B1 infection, mRNA levels of proinflammatory cytokines, IFN- γ and TNF- α , and

anti-inflammatory cytokine IL-10 as well as Foxp3, which is a marker for natural regulatory T cells, were measured in cDNA prepared from cecal and hepatic RNA. In the liver of mice infected with *HhureNT9*, significantly lower mRNA levels of IFN- γ ($P=0.0007$), TFN- α ($P<0.0001$), IL-10 ($P=0.0003$) and Foxp3 ($P=0.0005$) were observed compared to mice infected with Hh 3B1. There were no significant difference in mRNA levels of these targets (all $P>0.37$) between the *HhureNT9*-infected group and the sham controls (Figure 4). In the ceca, there were no significant differences in mRNA levels of all the target genes (all P values >0.1) between the Hh 3B1-infected, *HhureNT9*-infected and the sham dosed groups except for IFN- γ mRNA which was significantly higher in mice infected with Hh 3B1 or *HhureNT9* than the sham controls ($P<0.003$, Figure 4). However, there was no significant difference in the cecal mRNA levels of IFN- γ between the two infected groups ($P=0.99$).

2.5. IgG1 and IgG2a levels were higher in wild type than in mutant infected animals but had similar reactivity with the outer membrane proteins of *H. hepaticus*

Th1-associated IgG2a and Th2-associated IgG1 responses to Hh 3B1- and *HhureNT9* infection were compared by ELISA. Hh 3B1-infected male A/JCr mice developed significantly higher specific levels of both IgG2a ($P<0.008$) and IgG1 ($P<0.0005$) responses to infection than *HhureNT9*-infected mice (Figure 5I). Anti-*H. hepaticus* IgG1 or IgG2a from the Hh 3B1-infected mice had similar reactivity against Hh 3B1 and *HhureNT9* outer membrane antigens ($P=0.16$ for IgG1, $P=0.17$ for IgG2a); the same result was obtained from the IgG2a responses from the *HhureNT9*-infected mice ($P=0.26$). However, IgG1 responses from the *HhureNT9*-infected mice expressed significantly stronger reactivity with the outer membrane antigens from *HhureNT9* than Hh 3B1 ($P=0.02$).

2.6. No anti-UreA or anti-UreB antibodies were detected with infection with either strain and the antigenic profile was similar except for one band

By using Western blotting, mouse IgG responses to Hh 3B1 or *HhureNT9* protein antigens were characterized and compared. No apparent UreA (~25 kDa) and UreB (~61 kDa)-specific bands in the whole-cell lysates (Figure 5II.A, lane 3) or the outer membrane proteins (lane 4) of Hh 3B1 were detected using the sera from Hh 3B1-infected mice when compared to sera from *HhureNT9*-infected mice (Figure 5II.A), indicating that *H. hepaticus* UreA and UreB were not immunodominant proteins. Sera from the *HhureNT9*-infected mice had similar overall IgG reactivity to the protein antigens from Hh 3B1 and *HhureNT9* (Figure 5II, B). In addition, there were no significant differences in the profiles of the overall *H. hepaticus* immunogenic proteins (Figure 5II, A) from the outer membrane preparations (lanes 1, 3) or from whole-cell lysate (lanes 2, 4) between *HhureNT9* (lanes 1, 2) and Hh 3B1 (lanes 3, 4) except for one protein band (denoted by arrows, Figure 5II) that was present in the whole-cell lysate of *HhureNT9* but absent from Hh 3B1. Consistent with the ELISA results, sera from Hh 3B1-infected mice showed higher IgG responses to antigens from both Hh 3B1 and *HhureNT9* (Figure 5I, A) than those from *HhureNT9*-infected mice (Figure 5II, B).

3. Discussion

Many pathogenic bacteria, including the gastric pathogen *H. pylori* and a urinary pathogen *Proteus mirabilis*, produce urease that plays a central role in pathogenesis [21, 22]. *H. hepaticus* persistently colonizes the mucosal surface of the murine large intestine and is capable of colonizing bile canaliculi in the liver and inducing chronic active hepatitis and hepatocellular carcinoma in males of susceptible mouse strains such as A/JCr mice [6, 7, 9, 10, 23]. In this study, our data demonstrated that *H. hepaticus* urease is not required for cecal infection of male A/JCr mice but is essential for infection of the liver. Inability of the urease-deficient isogenic *H. hepaticus* mutant to infect the liver is further supported by the observation that *HhureNT9* infection did not lead to the development of hepatitis and did not induce transcriptional up-regulation of proinflammatory cytokines IFN- γ and TFN- α , in contrast to the *Hh* 3B1-infected mice. These results were not caused by a polar effect on the genes downstream of *catNT*, since the level of *ureI* transcripts within *HhureNT9* was not lower but rather, on a relative basis, higher than that in *Hh* 3B1. Because the *Hh ureI* is located immediately downstream of *ureB*, its transcription is likely controlled by the *ureAB* promoter [15, 16]. Thus, the higher level of *ureI* transcripts in *HhureNT9* is possibly attributable to stronger transcription initiated by a combination of the promoters for both the *Hh ureAB* and the *catNT*, a stronger promoter efficiency of *catNT*, or both.

Within the *Helicobacter* genus, all known gastric helicobacters demonstrate strong urease activity and approximately 40% of characterized EHS are urease-positive (Table II). The genomes of *H. hepaticus* (EHS) and *H. pylori* (gastric) contain similar urease gene clusters [15, 16]. The critical role of urease in gastric helicobacters infection of the gastric mucosa, an extremely acidic environment (pH < 3), has been demonstrated in piglets and mice [14]. It has been proposed that this enzyme releases ammonia by hydrolyzing urea, which enables gastric helicobacter to establish colonization by neutralizing gastric acid [21]. In contrast, the pH for the large intestine of mice, a primary site for *H. hepaticus* colonization, is approximately 6.1 ([24]; at this pH, according to our findings, urease was not essential for *H. hepaticus* infection of the ceca of mice. In contrast, urease is essential for *H. hepaticus* colonization of the liver of mice where the pH is ~7.4 [25]. The indispensable role of urease in hepatic infection of *H. hepaticus* could be related to nitrogen assimilation by the bacteria in the urea-rich liver. The presence of glutamine synthetase (*glnA*, HH0561) in the *H. hepaticus* genome supports this possibility [16]. Glutamine synthetase (GlnA) plays an essential role in the metabolism of nitrogen by catalyzing the reaction $\text{NH}_3 + \text{glutamate} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{P}_i$; glutamine thus serves as the nitrogen donor for various nitrogenous compounds [26]. This hypothesis could be tested by experimental infection with *glnA*-deficient *H. hepaticus* mutants. However, it should be noted that *H. pylori* GlnA appears to be essential for its viability, because isogenic *glnA* mutants could not be generated, making in vivo experimental infection infeasible [11].

It has been documented that *H. pylori* UreA and UreB are strongly immunogenic in humans and mice and have been used to develop serological tests or vaccine strategies against *H. pylori* infection [27]. By contrast, our results indicate that *H. hepaticus* UreA and UreB are not as immunodominant as those from *H. pylori*, in spite of the fact that these urease subunits display extensive amino acid sequence similarities (identity: 65% for UreA, 76%

for UreB). The relatively low immunogenicity of Hh 3B1 UreA and UreB could result from the lower expression of these proteins expressed in Hh 3B1 compared to *H. pylori*, particularly in vivo. Indeed, it has been reported that *H. hepaticus* urease activity, unlike the acid-induced urease system of *H. pylori*, is acid-independent, and does not increase acidic survivability in the presence of urea *in vitro* [17]. One would expect that in humans or in mice, *H. pylori* produces a large quantity of urease in response to an acidic environment of the stomach during establishment of colonization, whereas expression of urease in *H. hepaticus* colonizing the less acidic intestine may be limited in vivo, because urease activity is not required for intestinal infection by *H. hepaticus*.

Antibody responses of total IgG, Th1-associated IgG2a, and Th2-associated IgG1 in the infected male A/JCr mice were significantly higher in mice infected with Hh 3B1 infection than to in mice infected with *HhureNT9*. No significant differences in the profiles or cross-activities of immunogenic proteins between *HhureNT9* and Hh 3B1 were observed, indicating that allelic replacement of *ureAB* with *catNT* did not significantly alter immunogenic components within the mutant compared to Hh 3B1. Since bacterial levels in the ceca were comparable between Hh 3B1 and *HhureNT9*, the enhanced IgG responses to Hh infection which developed in the Hh 3B1-infected mice likely resulted from hepatic colonization by Hh 3B1 that induced hepatitis and thereby triggered a more robust host humoral immune responses.

Both pro-inflammatory and anti-inflammatory mediators were up-regulated in the livers of male A/JCr mice infected with Hh 3B1 compared to *HhureNT9* infection or the sham controls. The up-regulation of hepatic Th1-type, pro-inflammatory cytokines IFN- γ and TFN- α is consistent with our previous results [19, 20]. IL10 and Foxp3-positive regulatory T cells play a pivotal role in suppressing *H. hepaticus*-induced intestinal tumors in *Rag2*^{-/-} and *Apc*^{min/+} mice [28]. Enhanced transcription of *IL-10* and *Foxp3* genes observed in this study were likely due to host responses elicited to suppress *H. hepaticus*-induced hepatitis. This notion is supported by the lack of up-regulation of these cytokines in the ceca where typhlitis was not noted, despite *H. hepaticus* colonization.

In conclusion, *H. hepaticus* urease was essential for hepatic infection but dispensable for intestinal colonization by *H. hepaticus*. This study represents the first *in vivo* characterization of the role of urease from an enterohepatic helicobacter during chronic infection. In addition, use of urease-deficient Hh mutants will allow us to further elucidate the role of urease in helicobacter-induced diseases such as promotion of cholesterol gallstone formation and induction of inflammatory bowel disease in mouse models.

4. Materials and Methods

4.1. Bacterial strain, growth media and conditions

Escherichia coli strain Top10 was used as a recipient for cloning, mutagenesis, and plasmid propagation and was cultured in LB broth or agar supplemented with ampicillin (50 μ g/ml) and chloramphenicol (Cm) (25 μ g/ml), where indicated. Wild-type *H. hepaticus* strain 3B1 (Hh 3B1) [6] was cultured on blood agar (TSA with sheep blood, Remel, Lexington, KN) for 2-3 days under microaerobic conditions (10% H₂, 10% CO₂, 80% N₂).

Chloramphenicol-resistant, *ureAB*-deficient *H. hepaticus* mutants were selected on tryptic soy agar supplemented with 5% sheep blood and 25 µg/ml of chloramphenicol (all from Sigma, St. Louis, MO.).

4.2. Molecular biology techniques and reagents

Chromosomal DNA from cultured bacteria was prepared using the High Pure PCR Template kit according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). Total DNA and RNA from murine cecum and liver were prepared using Trizol Reagents following the supplier's instructions (Invitrogen). cDNA from cecal and hepatic mRNA (5 µg) or from *H. hepaticus* RNA (1 µg) was reverse-transcribed using the High Capacity cDNA Archive kit following the supplier's instructions (Applied Biosystems, Foster City, CA). Real-time quantitative PCR (Q-PCR) was performed in the 7500 Fast Real-Time PCR System (Applied Biosystems). All reagents, primers and probes of murine genes of interest for Q-PCR were purchased from Applied Biosystems. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). The number of *H. hepaticus* in murine tissues was determined using Q-PCR as described elsewhere [29].

PCR primers used in this study are presented in Table I. PCR was performed in a 50 µl-volume containing: 10 to 50 ng of DNA template, 1 X commercial buffer (Roche Applied Science), 100 µg/ml BSA, 500 nM each of forward and reverse primers and 2.5 units of High Fidelity DNA polymerase (Roche Applied Science). Thermocycling for 35 cycles in a Thermocycler Genius (Technie Incorporated, Princeton, NJ) consisted of: denaturation at 94°C for 1 min, followed by annealing at 50°C-60°C (based on the respective primers) for 1 min and extension at 72°C for 1 min.

4.3. Construction of a urease-negative isogenic mutant

For creating a urease-deficient *H. hepaticus* mutant, PCR products were amplified with primers ZMG120/ZMG121 (the upstream fragment of the *Hh* 3B1 *ureAB*, 951 bp) or with ZMG122/ZMG123 (the downstream fragment of the *Hh* 3B1 *ureAB*, 521 bp.) (Fig. 1A). The upstream and downstream PCR fragments were then digested with BamHI/HincII and HincII/XhoI, respectively. The digested products were ligated with BamHI/XhoI-digested vector pBluescript II KS (Stratagene, La Jolla, CA). Recombinant plasmids containing the inserts of interest were screened using PCR with primers ZMG120/ZMG123. The insert-harboring plasmid DNA was digested with HincII, following by ligation with HincII-digested chloramphenicol acetyltransferase cassette without its putative transcriptional terminator (designated as *catNT*) ([30]). The *catNT* was generated via PCR using primers ZMG54/ZMG55; ZMG55 was derived from the sequence preceding the predicted transcription terminator [30]. Cm-resistant (Cm^r) *E. coli* clones were selected on LB agar supplemented with ampicillin and Cm. Recombinant plasmids containing a *catNT* cassette flanking by the upstream and downstream DNA fragment of *ureAB* were verified using PCR and DNA sequencing. A recombinant plasmid, designated as pBKSHhureAB⁹ (Fig. 1A), was used to transform *H. hepaticus* by high-voltage electroporation, and Cm^r isogenic *H. hepaticus* mutants were selected as described previously ([29]). Genetic authenticity of the mutants was confirmed with PCR and sequencing and one clone, designated as *HhureNT9*, was selected for further characterization. Urease activity in *Hh* 3B1 and *HhureNT9* was

determined using urea agar (Remel). Potential polar effects of the *catNT* on the downstream gene *ureI* were analyzed using RT-PCR and the TaqMan 7500 Fast detection system with primers ZMG174 and ZMG175 as described below.

4.4. Reverse transcription and Q-PCR of *H. hepaticus ureI* and selected cytokines

The mRNA levels for GAPDH, IL-6, IL-10, TFN- α , IFN- γ and the transcription factor Foxp-3 were measured by Q-PCR using commercial primers and probes (Applied Biosystems). Briefly, a 20- μ l mixture contained 5 μ l of cDNA (in duplicate), 1 μ l of a commercial 20 $\times$ primer/probe solution (Applied Biosystems), 10 μ l of 2 $\times$ master mix (Applied Biosystems), 4 μ l of ddH₂O. Transcript levels were normalized to the endogenous control *GAPDH* transcripts, and expressed as fold change in reference to sham-dosed control mice using the Comparative C_T method (Applied Biosystems User Bulletin no. 2).

To determine if an allelic replacement of Hh *ureAB* with *catNT* caused a polar effect on downstream genes, transcript levels of *H. hepaticus ureI*, which is located immediately downstream of the Hh *ureAB* [16], were measured as described above for murine cytokine quantification with some modifications. Q-PCR was carried out using 2X SyBr Green I PCR Master Mix (Applied Biosystems) and 400 nM of each primer ZMG174 and ZMG 175 (Table 1). To ensure there was no DNA contamination of the prepared RNA samples, 1 μ g of RNA from Hh 3B1 or *HhureNT9* was treated with 20 units of DNase-free RNase prior to reverse transcription. Quantities of *ureI* transcripts in the samples were determined using a standard curve of 10 to 10⁷ copies of Hh 3B1 DNA and reported as copy number per ng of total RNA. In addition, amplicons from the cDNA templates were also produced by PCR with the same set of primers and visualized on a 3% agarose gel to further confirm purity of the RNA preparations and to verify that the amplicons were the correct size.

4.5. Experimental infection and sampling

Fifteen, 4 to 6-week-old male A/JCr mice free of known murine viruses, pathogenic bacteria including *Helicobacter* spp. and parasites, were obtained from the National Cancer Institute (Frederick, MD). The mice were maintained in an Association for Accreditation and Assessment of Laboratory Animal Care, International-accredited facility in static microisolator cages. The mice were divided into 3 groups of 5 mice and were dosed with Hh 3B1, *HhureNT9* or sham-dosed with Brucella broth as a control, respectively. For oral gavage, bacteria were cultured on blood agar, suspended in Brucella broth, and adjusted to 10⁹ organisms/ml as estimated by spectrophotometry at OD_{600nm}. Mice received 0.2 ml of fresh inocula by gastric gavage every other day for a total of three doses.

All mice were necropsied at 4 months postinoculation. Immediately after euthanasia, contents in the intestine were removed by rinsing with sterile saline. Liver sections (one sample per liver lobe) and approximately 1 cm segments of cecum were collected for RNA/DNA isolation. Tissues for RNA/DNA isolation were frozen in liquid nitrogen immediately after sampling and stored at -70°C prior to use. Representative tissue sections were fixed in 10% buffered formalin for histology.

4.6. Histopathology evaluation

Tissues were routinely processed, sectioned at 4 μ m, stained with hematoxylin and eosin, and scored for inflammation by a veterinary pathologist blinded to sample identity. A hepatitis index comprised of the sum of scores for lobular, portal and interface inflammation as well as lobar distribution was generated as previously described [10] .

4.7. IgG responses to *H. hepaticus* infection

Sera were collected from individual mice infected with Hh 3B1 or *HhureNT9* at 4 MPI and evaluated by ELISA for Th1-associated IgG2a and Th2-associated IgG1 antibody responses. Outer membrane antigens of Hh 3B1 or *HhureNT9* were prepared and used as antigens as previously described ([20]). The protein concentration was determined by the BCA protein assay (Pierce, Rockford, Ill.) using bovine serum albumin as a standard.

IgG antibody responses to Hh 3B1 or *HhureNT9* infection were further characterized by Western blot using 1 μ g of outer membrane antigens or whole-cell lysate of Hh 3B1 and *HhureNT9* as described previously [31] . Whole cell lysate of Hh 3B1 or *HhureNT9* was prepared by sonication in 20 mM Tris-HCl (pH 8.0.) on ice 4 times for 30 s each time at intervals of 1 min. Unbroken cells and debris were removed by centrifugation at $10,000 \times g$ for 10 min at 4°C. Pooled sera from 5 sham-dosed, Hh 3B1- or *HhureNT9*- infected mice were used as primary antibody (1:500); the secondary antibody was a goat anti-mouse IgG conjugated with horseradish peroxidase (1:5000) from Zymed (San Francisco, CA). Antigen-antibody reactivity was detected using ECLTM Western Blotting Detection reagents following the supplier's instructions (Amersham Biosciences, Piscataway, NJ) and exposure on X-ray films.

4.8. Statistical analyses

Data on the levels of *H. hepaticus*, serology, and cytokine mRNA in the tissues were analyzed using the Student's t test, whereas the histopathological index was analyzed using a Mann-Whitney non-parametric *t*-test. Values of $p < 0.05$ are considered significant.

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Figure 1. Construction and characterization of urease-deficient *H. hepaticus* mutant *HhureNT9*. A, Schematic depiction of the genotype of *HhureNT9*. Primers used for generating and characterizing the mutant are denoted. B. Reverse-transcription PCR of the *H. hepaticus ureI* RNA with primers ZMG174 and ZMG175. Lanes: 1, (RNase treatment), 2 for *Hh 3B1*; 3 (RNase treatment), 4 for *HhureNT9* RNA; 5 for no template control and 6 for *Hh 3B1* chromosomal DNA.

Figure 2.

Q-PCR-based measurement of Hh 3B1 and *HhureNT9* in cecum and liver using the primers and probe derived from the Hh distending cytolethal toxin gene B [33]. The levels of *H. hepaticus* are expressed as genomic copies per μ g of mouse DNA.

Figure 3. Pathology in the livers of male A/JCr mice infected by Hh 3B1 or the urease-deficient Hh mutant *HhureNT9*. A. Liver histopathology. B. Pathological index for male A/JCr mice. No chronic hepatitis developed in the *HhureNT9*-infected mice at 4 MPI. In contrast, 4 out of 5 mice infected with Hh 3B1 developed severe hepatic inflammation.

Figure 4.

Relative mRNA levels of proinflammatory and anti-inflammatory cytokines in the murine livers and ceca. For comparison of mRNA levels, the target mRNA was normalized to that of the house-keeping gene *GAPDH*. Numbers represent mean fold change of target mRNA levels in reference to the control levels (defined as 0, standard deviation represented by hatched bars).

I

II

Figure 5.

Male A/JCr mouse IgG responses to Hh 3B1 or *HhureNT9* infection. **I**, Th1-associated IgG2a and Th2-associated IgG1 to the OMPs of Hh 3B1 or *HhureNT9*. Sera from the individual mice: C for the control mice; NT9 for *HhureNT9*-infected mice; Hh for Hh 3B1-infected mice. **II**, Western blotting for total IgG responses to antigens from Hh 3B1 (lanes: 1, outer membrane proteins [OMP]; 2, whole-cell lysate) and *HhureNT9* (lanes: 3, OMP; 4, whole-cell lysate). A, pooled sera from Hh 3B1-infected mice; B, pooled sera from *HhureNT9*-infected mice.

Table I

Origin and sequences of primers

Primers	Sequences (5' to 3')	Orientation ^A	Source ^B
ZMG120	TATAGGATCC ₍₋₈₄₇₎ TAGTGATGGTCCTATCATATTCGTA <i>Bam</i> HI	(sense)	HH0407 ^B
ZMG121	TATAGTCGAC ₍₈₄₎ AATACCTCGTTCTTTGCGA <i>Hinc</i> II	(antisense)	HH0407 ^B
ZMG122	TATAGTCGAC ₍₂₄₁₈₎ TATGCTAGGAATTGTATTAATGTA <i>Hinc</i> II	(sense)	HH0407 ^B
ZMG123	TATACTCGAG ₍₂₉₁₉₎ TCCATAAGGATAAGCCACGCT <i>Xho</i> I	(antisense)	HH0407 ^B
ZMG174	₍₂₆₂₎ GCCTATGGGTGGTATAGTTTATTTTGTT	(sense)	HH0409 ^B
ZMG175	₍₄₁₁₎ TCCATAAGGATAAGCCACGCT	(antisense)	HH0409 ^B
ZMG54	TATAGTCGAC ₍₋₉₆₎ GTGATATAGATTGAAAAGTGG <i>Hinc</i> II	(sense)	M35190 ^C
ZMG55	TATAGTCGAC ₍₆₄₃₎ AGTGCGACAAACTGGGATT <i>Hinc</i> II	(antisense)	M35190 ^C

The numbers in parentheses denote the 5' nucleotide of each primer in reference to the start codon of the respective genes: -, upstream of a start codon; +, downstream of a start codon.

^A Orientation of the primer to the coding strand of each gene.

^B Sequences of these genes were published by Suerbaum et al. [16].

^C The sequence of the *cat* gene was published by Wang and Taylor [30].

Table II

Association between hepatic infection ability and urease activity in enterohepatic helicobacters

<i>Non-gastric Helicobacter</i> Taxon ^a	Primary/Secondary site	Urease
<i>H. bilis</i>	Intestine/Liver	+
<i>H. hepaticus</i>	Intestine/Liver	+
<i>H. marmotae</i>	Intestine/Liver	+
<i>H. mastomyrinus</i>	Intestine/Liver	+
<i>H. muridarum</i>	Intestine/Stomach	+
<i>H. muricola</i>	Intestine	+
<i>H. rappini</i> ^b	Intestine	+
<i>H. trogontum</i>	Intestine	+
<i>H. canadensis</i>	Intestine	-
<i>H. canis</i>	Intestine	-
<i>H. cholecystus</i>	Gallbladder	-
<i>H. cinaedi</i>	Intestine	-
<i>H. fennelliae</i>	Intestine	-
<i>H. gammani</i>	Intestine	-
<i>H. mesocricetorum</i>	Intestine	-
<i>H. pullorum</i>	Intestine	-
<i>H. pametensis</i>	Intestine	-
<i>H. rodentium</i>	Intestine	-
<i>H. typhlonius</i>	Intestine	-

^aEnterohepatic *Helicobacter* species with known colonization sites are listed; the data are summarized from two recently published book chapters [3, 32].

^bFormerly regarded as '*Flexispira rappini*'; now sub-grouped into 10 taxa.