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Citation: Ge, Zhongming, Yan Feng, Sureshkumar Muthupalani, Mark T. Whary, James Versalovic, and James G. Fox. " Helicobacter Hepaticus Cholesterol-α-Glucosyltransferase Is Essential for Establishing Colonization in Male A/JCr Mice ." Helicobacter 19, no. 4 (May 23, 2014): 280–288.

As Published: http://dx.doi.org/10.1111/hel.12135

Publisher: Wiley Blackwell

Persistent URL: http://hdl.handle.net/1721.1/99366

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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Helicobacter. Author manuscript; available in PMC 2015 August 01.

Published in final edited form as:
Helicobacter. 2014 August ; 19(4): 280–288. doi:10.1111/hel.12135.

Helicobacter hepaticus cholesterol-α-glucosyltransferase is essential for establishing colonization in male A/JCr mice

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Abstract

Background—*Helicobacter pylori* cholesterol-α-glucosyltransferase (*cgt*) is essential for survival of *H. pylori* in mice. Enterohepatic *H. hepaticus*, the cause of colonic and hepatocellular carcinoma in susceptible mouse strains, contains an ortholog of the *H. pylori cgt*. However, the role of *cgt* in in the pathogenesis of *H. hepaticus* has not been investigated.

Materials and Methods—Two *cgt*-deficient isogenic mutants of wild-type *H. hepaticus* (WT) 3B1 were generated and used to inoculate male A/JCr mice. Cecal and hepatic colonization levels of the mutants and WT 3B1 as well as select inflammation-associated cytokines were measured by qPCR at 4 months post-inoculation.

Results—Both mutants were undetectable in the cecum of any inoculated mice (10 per mutant) but were detected in two livers (one for each mutant); by contrast, 9 and 7 of 10 mice inoculated with WT 3B1 were qPCR-positive in the ceca and livers, respectively. The mice inoculated with the mutants developed significantly less severe hepatic inflammation ($P < 0.05$) and also produced significantly lower hepatic mRNA levels of proinflammatory cytokines Ifn-γ ($P < 0.01$) and Tnf-α ($P \leq 0.02$) as well as anti-inflammatory factors Il10 and Foxp3 compared to the WT 3B1-inoculated mice. Additionally, the WT 3B1-inoculated mice developed significantly higher Th1-associated IgG2a ($P < 0.0001$) and Th2-associated IgG1 responses ($P < 0.0001$) to *H. hepaticus* infection than mice dosed with isogenic *cgt* mutants.

Conclusion—Our data indicate that the cholesterol-α-glucosyltransferase is required for establishing colonization of the intestine and liver and therefore plays a critical role in the pathogenesis of *H. hepaticus*.

Introduction

Cholesterol is an essential component of mammalian cell membranes where it is required to establish proper membrane permeability and fluidity, but also plays a pivotal role in obesity and cardiovascular diseases. This molecule can be glycosylated into cholesteryl glucosides

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We have no competing conflict interest.

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by glucosyltransferases in plants, most fungi and some animals. Cholesteryl glucosides are also found in bacteria such as *Helicobacter pylori* and *Acholeplasma axanthum*, despite the fact that prokaryotes do not produce cholesterol. Three major cholesteyl glucosides, including cholesteryl-α-D-glucopyranoside (αCG) and its two derivatives αCAG (cholesteryl—6′-*O*-tetradecanoyl-α-D-glucopyranoside) and αCPG (cholesteryl—6′-*O*-phosphatidyl-α-D-glucopyranoside), have been identified and characterized in *H. pylori* cells using two-dimensional thin-layer chromatography (1). Subsequently, an *H. pylori* gene (HP0421) was identified to encode cholesterol-α-glucosyltransferase (Cgt) which catalyzes conversion of cholesterol and glucosides into cholesteryl glucosides such as αCG, αCAG and αCPG (2, 3). In C57BL/6 mice and gerbils, inactivation of *cgt* in *H. pylori* increased host phagocytosis and T cell activation against infection, and the *cgt* mutants did not sustain colonization (3, 4). It was reported that gastric mucosa-associated *O*-glycans inhibited the growth and motility of *H. pylori* by disrupting its biosynthesis of αCG. In the presence of exogenous cholesterol, increased cholesterol glucosylation by *H. pylori* enhances its resistance to 8 antibiotics and an antimicrobial peptide LL-37 cathelicidin *in vitro* (4). These lines of evidence indicate that cholesteryl glycosides protect *H. pylori* from the host immunity.

Helicobacter hepaticus, a prototype of enterohepatic *Helicobacter* species (EHS), induces chronic active hepatitis, hepatocellular carcinoma, colon cancer, and inflammatory bowel disease in susceptible mouse strains and has been widely used as infectious models for dissecting pathogenic mechanisms of similar human diseases (5, 6). *H. hepaticus* 16S rDNA are also detected in human subjects via PCR-based assays (7, 8). Approximately 50% of the genes predicted from the genome of *H. hepaticus* are orthologs of *H. pylori*, including a *cgt* ortholog (Hh0676) (9). In addition, the *cgt* gene is also present in several gastric helicobacters including *H. felis*, *H. mustelae* and *H. acinonychis*. Among the recently sequenced genomes of 5 species of EHSs known to infect humans, this gene is identified only in *H. bilis* but is absent from the genomes of *H. canadensis* MIT 98-5491, *H. cinaedi* CCUG 1881, *H. pullorum* MIT 98-5489, *H. winghamensis* ATCC BAA-430 (at www.broadinstitute.org/annotation/genome/Helicobacter_group). To explore the role of *cgt* in *H. hepaticus*-induced liver disease in a mouse model of infectious hepatitis, we generated 2 isogenic *H. hepaticus cgt* mutants and characterized how *cgt* inactivation influences intestinal and hepatic colonization, proinflammatory responses and induction of chronic hepatitis in male A/JCr mice.

Materials and Methods

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 antibiotics ampicillin (Amp, 50 μg/ml) and chloramphenicol (Cm, 25 μg/ml) when appropriate. Wild-type *H. hepaticus* (WT) 3B1 (ATCC 51449) was cultured on blood agar (Remel, Lexington, Ky) for 2–3 days under microaerobic conditions (10% H_2, 10% CO_2, 80% N_2) (10). Cm-resistant *H. hepaticus* mutants were selected on tryptic soy agar supplemented with 5% sheep blood and 25 μg/ml of Cm (all from Sigma, St. Louis, MO.).

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DNA preparation and PCR

Chromosomal DNA from murine tissues and cultured bacteria was isolated using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN). Plasmid DNA was prepared using the Aquick minipreparation kit (Qiagen Inc., Valencia, CA). PCR reactions were conducted in a 50-μl volume containing the following: 10 to 50 ng of DNA template, 1 X commercial, 100 μg/ml BSA, 500 nM each of forward and reverse primers and 2.5 units of High Fidelity DNA polymerase (Roche Applied Science). A thermocycling program of 35 cycles in a Thermocycler Genius (Technie Incorporated, Princeton, NJ) included denaturation at 94°C for 1 min, followed by annealing at 50–60°C (based on the respective primers) for 30 sec and extension at 72°C for 1 min.

Construction of isogenic *cgtΔcat* mutants

Approximately 600-bp fragments upstream and downstream of Hh0676 (*cgt*) were amplified from WT 3B1 genomic DNA using primer pairs ZMG169/170 and 171/172, respectively (Table I). PCR fragments were ligated with pBluescript II SK and subsequently inserted with a Cm acetyltransferase cassette lacking a transcriptional terminator (*catNT*) as illustrated in Figure 1. We previously demonstrated that the *catNT* has no polar effect on the downstream gene *ureI* (11). Authenticity of the ligation and inserted DNA sequences was confirmed by sequencing (the ABI Genetic Analyzer 3500). Isogenic *H. hepaticus cgtΔcat* mutants were generated as described previously (12). Cm^R isogenic mutants of *H. hepaticus* were characterized by PCR and DNA sequencing. Two mutants, *cgtΔcatNT1* (*cat* within the transcriptional orientation of *cgt*) and *cgtΔcatNT2* (*cat* opposite to the transcriptional orientation of *cgt*), were selected for further characterization *in vivo* (Figure 1).

In vivo experimental infection

Male A/JCr mice at 4 to 6 weeks of age free of known murine viruses, pathogenic bacteria including *Helicobacter* spp. and parasites, were obtained from NCI (Germantown, NY). The mice were maintained in an Association for Accreditation and Assessment of Laboratory Animal Care, International-accredited facility in static microisolater cages. Forty male A/JCr mice were divided into 4 groups of 10 mice dosed with either WT 3B1, *cgtΔcatNT1*, *cgtΔcatNT2* or sham-dosed with Brucella broth as a control. For oral gavage, bacteria were cultured on blood agar, suspended in Brucella broth, and adjusted to10^9 organisms/ml as estimated by spectrophotometry at OD_{600nm}. Mice received 0.2 ml of fresh inoculum by gastric gavage every other day for three doses.

Histopathology evaluation

All mice were euthanized at 4 months post-inoculation (MPI). Immediately after euthanasia, the ileocolic junction and two sagittal sections of each liver lobe were collected for routine histological processing and sectioning. Haematoxylin and eosin of the liver and intestine were examined by a veterinary pathologist (SM) blinded to sample identity as previously described (13). Hepatitis was graded on a 0–4 scale for lobular histologic activity (lobular hepatitis) and portal activity (portal and/or interface hepatitis).

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qPCR for *H. hepaticus* and hepatic mRNA levels of selected genes

Tissues for RNA/DNA isolation were frozen in liquid nitrogen immediately after sampling and stored at −70°C prior to processing. For enumerating *H. hepaticus* organisms, chromosomal DNA from the ceca and livers was prepared using a High Pure PCR Template kit according to the manufacturer's protocol (Roche Applied Science,). Levels of hepatic and cecal *H. hepaticus* were measured by qPCR in the 7500 Fast Sequence Detection System (Applied Biosystems) as described elsewhere (14). The colonization level of *H. hepaticus* was estimated by normalizing to μg of mouse chromosomal DNA measured by qPCR using the 18S rRNA gene-based primers and probe mixture (Life Technology).

For relative mRNA quantitation of selected genes, total RNA from livers of A/JCr mice was prepared using Trizol reagent according to manufacturer's recommendations (Invitrogen, Carlsbad, CA). Five μg of total RNA from each sample was converted into cDNA using the High Capacity cDNA Archive kit (Applied Biosystems). Levels of *Ifn-γ*, *Tnf-α*, *Il10* and *Foxp3* mRNA were measured by qPCR using commercial primers and probes (TaqMan Gene Expression Assays) in the 7500 FAST Sequence Detection System. Transcript levels were normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase mRNA (*Gapdh*), and expressed as fold change compared with sham-dosed control mice using the Comparative C_T method (Applied Biosystems User Bulletin no. 2).

IgG1 and IgG2a responses to *H. hepaticus* antigens

Sera was collected from all mice at necropsy (4 MPI) and evaluated by ELISA for Th1-associated IgG2a and Th2-associated IgG1 antibody responses to outer membrane antigens (OMPs) of WT *H. hepaticus* 3B1. Preparation of OMPs and ELISA were conducted as previously described (15).

Phylogenetic analysis of known and putative bacterial Cgts

To identify and compare orthologs of bacterial Cgts, the putative *H. hepaticus* Cgt (deduced based on Hh0676) was used as a query sequence for searching homologs currently available in public databases using the Blastp at the website of the National Center of Biotechnology Information. Amino acid sequences of genes with ≥30% identity over 300 amino acids compared to the *H. hepaticus* Cgt were selected for phylogenetic analyses. Where putative Cgt orthologs are present in multiple species within the genus such as *Lactobacillus*, *Bifidobacterium* or *Clostridium*, only 2 better characterized species were chosen. A phylogenetic tree was generated using Lasergene software (DNASTAR, Inc., Madison, WI).

Statistical analyses

All statistical analyses were performed using the GraphPad Prism 5 software package. Histopathological scores were compared using a Mann–Whitney non-parametric t-test. Data on the colonization levels of *H. hepaticus* and cytokine mRNA in the tissues were analyzed using two-tailed Student's t-test. Serology results were compared using regression, ANOVA and the Student's t-test. Values of $p<0.05$ were considered significant.

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Results

Cgt is required for intestinal colonization and is important for persistent hepatic colonization by *H. hepaticus*

It was previously reported that inactivation of the *H. pylori cgt* resulted in failure of isogenic mutants to colonize stomachs of C57BL/6 mice at 12 hours post inoculation and gerbils by one month PI (3, 4). To characterize a role for *cgt* in the colonization and pathogenesis of *H. hepaticus*, colonization efficiencies of 2 *cgt* isogenic mutants *cgt*Δ*catNT1* and *cgt*Δ*catNT2* of WT 3B1 in parallel with WT 3B1 were examined by qPCR in male A/JCr mice (10 per group), a mouse model of infectious hepatitis and hepatocellular carcinoma At 4 MPI, 9 of 10 WT 3B1-dosed mice were qPCR-positive for *H. hepaticus* in the ceca and 7 out of 10 livers (Figure 2). In contrast, all mice dosed with either mutant were negative for *H. hepaticus* in the ceca and qPCR-positive only in one of 10 livers for each *cgt* mutant.

A/JCr mice infected with *cgt*-deficient *H. hepaticus* mutants did not develop hepatic pathology

Infection with *H. hepaticus* leads to hepatic inflammation by 4 MPI in male A/JCr mice (11, 13, 16, 17). Consistent with these previous findings, 5 of 10 male mice infected with WT 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, 6–9 for hepatitis index scores). The observation that 5 WT 3B1-infected mice did not develop overt hepatitis is in agreement with our previous observation that a subset of A/JCr mice appears resistant to *H. hepaticus*-induced hepatic disease (13). This resistance was supported by relatively lower levels of *H. hepaticus* colonization in the liver (~100-fold) compared to mice with hepatitis (Fig. 2). In contrast, no overt hepatic pathology was evident in male A/JCr mice inoculated with the *cgt*-deficient *H. hepaticus* mutants, including one mouse from each group that was PCR positive for *H. hepaticus* in the liver (Figure 2). Hepatitis indices in WT 3B1-infected mice were significantly higher than in the *cgt* mutants-infected mice or the sham controls ($P < 0.05$, Fig. 3B); there were no significant differences in hepatitis indices between mice inoculated with the isogenic mutants or the sham-dosed controls. These data indicate that infection with the *cgt*-deficient *H. hepaticus* mutants did not lead to the development of chronic active hepatitis as observed in the WT 3B1-dosed male A/JCr mice. This was consistent with the inability of the mutants to colonize the intestine and liver. Histopathological evaluation of the intestines of all mice did not reveal significant lesions.

mRNA levels of hepatic *Tnf-α*, *Ifn-γ*, *Il10* and *Foxp3* were significantly higher in WT *H. hepaticus*-infected mice than mice dosed with *cgt* mutants

Previous studies showed that WT 3B1 infection significantly elevated expression of proinflammatory cytokines in splenocyte culture and in livers of male A/JCr mice (11, 15, 17). To ascertain how infection with the *cgt* mutants influence hepatic proinflammatory responses compared to WT 3B1 infection, mRNA levels of proinflammatory Ifn-γ and Tnf-α, and anti-inflammatory Il-10 as well as Foxp3, a marker for natural regulatory T cells, were measured in cDNA prepared from hepatic RNA (Figure 4). In the liver of mice infected with the *cgt* mutants, significantly lower mRNA levels of *Ifn-γ* ($P < 0.01$), *Tnf-α* (*P*

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< 0.05), *Il10* (P <0.05) and *Foxp3* (P < 0.05) were observed compared to mice infected with WT 3B1. Consistent with a lack of overt hepatic pathology, mRNA levels of these hepatic cytokines in the two mice, which were positive for *H. hepatics cgt* mutants, were similar to those in the *cgt* mutant-negative mice from the same groups. There were no significant differences in mRNA levels of these targets between the *cgt* mutant-infected mice and the sham-dosed controls (P > 0.2), except *Ifn-*γ mRNA was lower in the *cgt*Δ*cat1*-dosed mice compared to the sham controls (P<0.05) (Fig. 4).

Serum levels of IgG1 and IgG2a were significantly higher in WT *H. hepaticus* 3B1-infected mice

Th1-associated IgG2a and Th2-associated IgG1 responses to WT 3B1- and *cgt* mutants-infection were compared by ELISA. WT 3B1-dosed male A/JCr mice developed significantly higher levels of both IgG2a (P < 0.0001) and IgG1 (P < 0.0001) responses to infection than the *cgt* mutants-dosed mice, consistent with the lack of colonization by *cgt* mutants (Figure 2).

Cgt-like proteins are present in diverse bacterial species

Twenty-one Cgt-like bacterial proteins were retrieved from the databases and phylogenetically analyzed. These gene products belong to the cd03178 family whose members are UDP-glucose-diacylglycerol glucosyltransferase-like proteins and are most closely related to the GT1 super family of glycosyltransferases. Six gastric *Helicobacter* species and 2 EHS contain the Cgt-like enzymes (Figure 6). Interestingly, the sequence of the Cgt-like protein in gastric *H. mustelae* is more similar to those from EHS *H. hepaticus* and *H. bilis* than those from other gastric helicobacters (Figure 6). Additionally, the sequences of the Cgt-like proteins in *Clostridium thermocellum* and *Treponema brennaborenese* are distantly related to those from *Clostridium methhylpentosum* and *Treponema azotonutricium*, respectively, despite belonging to the same genus. It is also interesting to note that the genomes of multiple species in the genera *Lactobacillus* and *Bifidobacterium*, including probiotic *L. acidophilus* and *B. bifidum*, contain genes coding for Cgt-like proteins.

Discussion

Members of the genus *Helicobacter* are grouped into two groups based on their preferential colonization niches: gastric species and enterohepatic species (18). The human pathogen *H. pylori*, the prototype of gastric helicobacters, can extract cholesterol from the epithelial membrane of host cells and convert it into cholesteryl-α-glucosides via Cgt. The loss of Cgt function leads to clearance of *H. pylori* by the host within 12 hours post innoculation in C57BL/6 mice or one month in gerbils (3, 4). Intriguingly, orthologs of *cgt* are found in the genomes of 6 gastric *Helicobacter* species but only present in two of 6 EHS species analyzed, *H. hepaticus* and *H. bilis*. Our data demonstrate that *H. hepaticus* Cgt plays a pivotal role in establishing cecal colonization. In addition, only one of 10 mice infected with each of the two *cgt* mutants was PCR-positive for *H. hepaticus* in the liver, suggesting that this enzyme is also important for hepatic colonization of this bacterium. Reduced colonization efficiency of the *cgt* mutants in the liver may result from inefficient

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translocation of the bacteria from the intestine to the liver due to their clearance in the intestine (19). Alternatively, the *cgt* mutants may be cleared from the liver after their translocation. Interestingly, the *cgt* mutant-colonized livers, like other *H. hepaticus*-free cohorts in the same groups, did not develop hepatic inflammation nor up- regulate transcription of proinflammatory cytokines, Ifn-γ and Tnf-α or anti-inflammatory mediators Il10 and Foxp3; these two mice also induced less IgG1 and IgG2a responses, when compared to WT 3B1-dosed mice. These results were not apparently caused by a polar effect or spontaneous mutations elsewhere in the genome for the following reasons. Firstly, we previously showed that the allelic replacement of *ureA/B* with *catNT* lacking its transcription terminator did not down-regulate the transcription of its downstream gene *ureI* in the *ure* operon of *H. hepaticus* (11). Secondly, two *cgt*-deficient mutants containing a *catNT* with opposite orientations conferred similar clinical manifestations. Thirdly, a -35 (TTTATA), a 21-nucleotide spacer and a -10 (TAAAAT) elements conserved among bacterial promoters precede HH0677 which locates immediately downstream of the *H. hepaticus cgt* (Hh0676), indicating that transcription of Hh0677 is likely controlled under its own promoter (Figure 1A).

Pathogenic bacteria, which are likely tohave coevolved with their hosts, have developed multiple strategies for colonization success. Bacteria utilize various protein secretion systems, dynamic cell surface structures as well as virulence factors to evade host immunity, survive in complex ecosystems, and maintain pathogenicity. Cholesterol is present in the membranes of eukaryotic organisms and has various cellular functions including maintenance of the membrane structure, modulation of membrane fluidity and involvement in cell signaling (20, 21). However, the mechanisms underlying the importance of the biosynthesis of cholesteryl-α-glucosides for the gastrointestinal and hepatic colonization of helicobacters require further studies. Accumulated evidence suggests that Cgt-mediated cholesterol α-glucosylation confers several advantages for *H. pylori* to survive in the host. First, this bacterium utilizes this process to escape phagocystosis and eventual ingestion by antigen presenting cells including macrophages, a first line of defense in innate immunity (3). Second, this process helps *H. pylori* evade T cell-mediated immune responses of the host via reduction of T cell proliferation (3, 22). Third, α-glucosylation of cholesterol apparently increases resistance to bactericidal effects by the host mucus-associated *O*-glycans and cathelicidin LL-37 as well as select antibiotics (1, 4, 23). These advantages may be attributed to reduction of the availability of cholesterol for the host antibacterial response. We hypothesize that *H. hepaticus,* which preferentially colonizes the large intestine and the liver, also encounter cholesterol-mediated antibacterial effects similar to that described for *H. pylori* colonizing the stomach. It is known that approximately 80% of total body cholesterol is endogenously produced primarily in the liver and intestine (24).

Epidemiological data indicate there is an inverse association between *H. pylori* infection and prevalence of allergic asthma (25, 26). *In vivo* studies demonstrated that infection with *H. pylori* protected neonatal mice from experimentally induced asthma via expansion of regulatory T cells driven by dendritic cell-derived Il-18 or neutrophil activation protein-mediated downregulation of Th2 responses (27–29). Interestingly, a recent report indicated that treatment with a glycolipid P157, a chemically synthesized version of the *H. pylori*

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αCAG described previously (1, 3), protected suckling BALB/c mice against ovalbumin (allergen)-induced airway hyperreactivity (30). This protection resulted from activation of natural killer T cells mediated by CD1d (related to class I MHC molecules) (30). Given that production of the *H. pylori* αCAG requires Cgt, the presence of *cgt* orthologs in EHS such as *H. hepaticus* and *H. bilis* as well as other enteric bacteria implies that bacterial glycolipids catalyzed by Cgt may have broad immune regulatory effects in human health and disease.

Blastp search and phylogenetic analyses suggest that diverse bacterial species have the potential to synthesize Cgt-like proteins. Distribution of this gene in members of the same genus is not ubiquitous; for example, only some members of *Helicobacter* or *Clostridium* are positive for homologs of Cgt. Whether these predicted proteins have enzymatic activity to conduct α-glucosylation of cholesterol remains unknown. However, significant sequence homology between *H. pylori* and other Cgt-like proteins suggests that at least some of these proteins could be Cgts.

Taken together, the results from this study and previous reports demonstrate that cholesterol α–glucosylation plays a pivotal role in colonization of both gastric and enterohepatic helicobacters in the gastrointestinal and hepatic tissues of mice and gerbils. Given the role of *H. pylori* cholesteryl α-glucosides in phagocytosis, immune evasion and protection against allergic asthma, our results further highlight the physiological and pathogenic potential of these bacterial cholestoryl glucosydes warrant further investigations into expression and biological functions of cholestoryl glucosydes in medically important *cgt*-positive bacteria, including additional *Helicobacter*, *Clostridium*, *Lactobacillus* and *Bifidobacterum* species.

Acknowledgments

This study was partially supported by the NIH grants to JGF: R010D011141, P01CA026731, R01AT004326, P30-ES002109.

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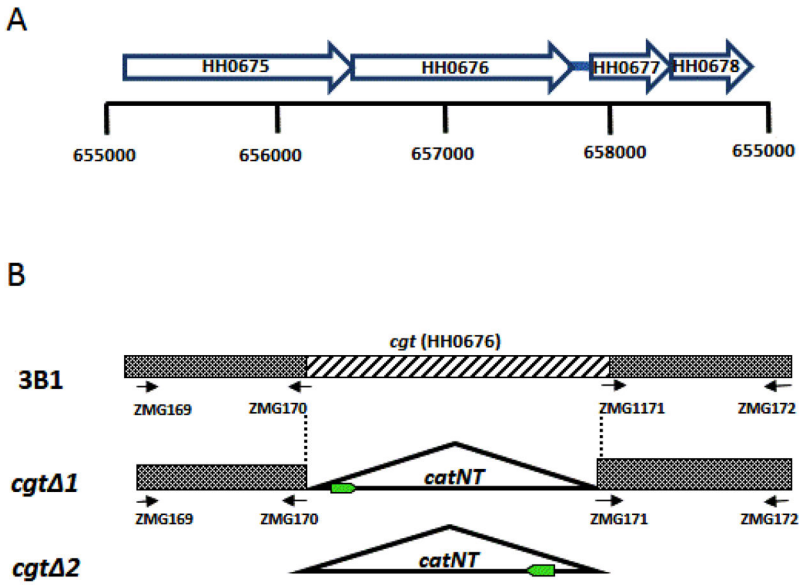

Figure 1.

Construction of *cgt*-deficient *H. hepaticus* mutants. A, Genomic locus (indicated by numbers) of *H. hepaticus cgt* (HH0676) and its flanking genes. B, Genotypes of WT 3B1 and 2 *cgt* mutants are schematically depicted. Locations of PCR primers used for generating and characterizing the mutants are denoted.

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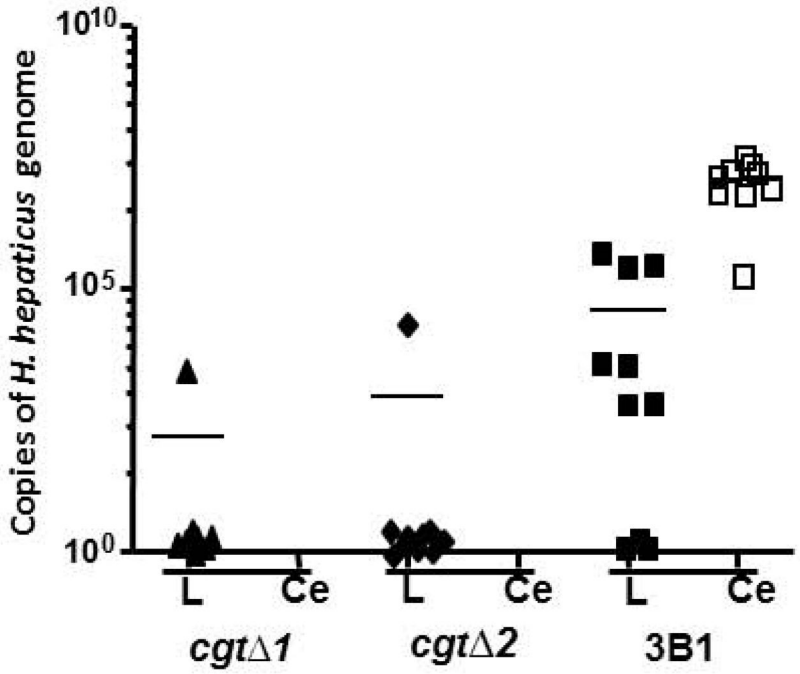

Figure 2.

Deficiency in *cgt* impaired the ability of *H. hepaticus* to persistently colonize the intestine and liver of male A/JCr mice (n=10 per group). Numbers represent the copies of the *H. hepaticus* genome per μg mouse DNA in the individual samples.

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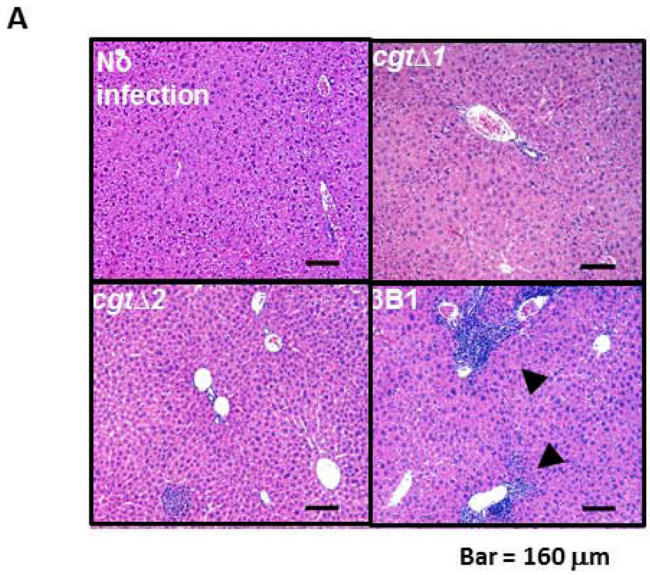

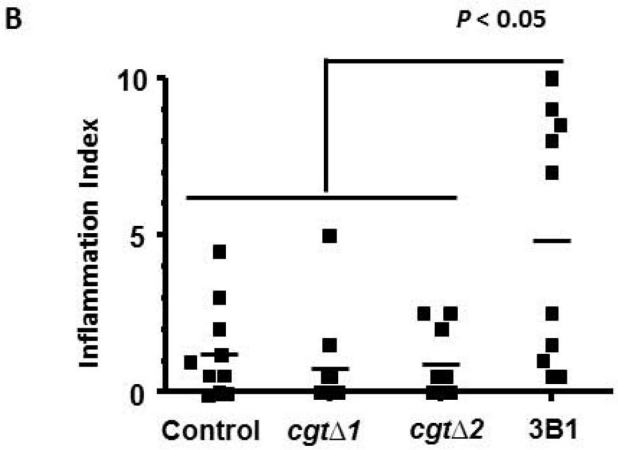

Figure 3.

Histology of the livers of male A/JCr mice dosed by WT 3B1 *or cgt*-deficient mutants *cgt*Δ*cat1* and *cgt*Δ*cat2* at 4 months post inoculation. A. Representative histopathology. (a) Normal liver, sham-inoculated mouse. (b) and (c) Minimal hepatic inflammation present in the livers of mice dosed with the isogenic mutants. (d) Subacute hepatitis with hepatocellular coagulative necrosis induced by WT 3B1. B. Hepatitis index for male A/JCr mice. The *cgt* mutant-infected mice developed significantly less severe hepatitis compared to those infected with WT 3B1.

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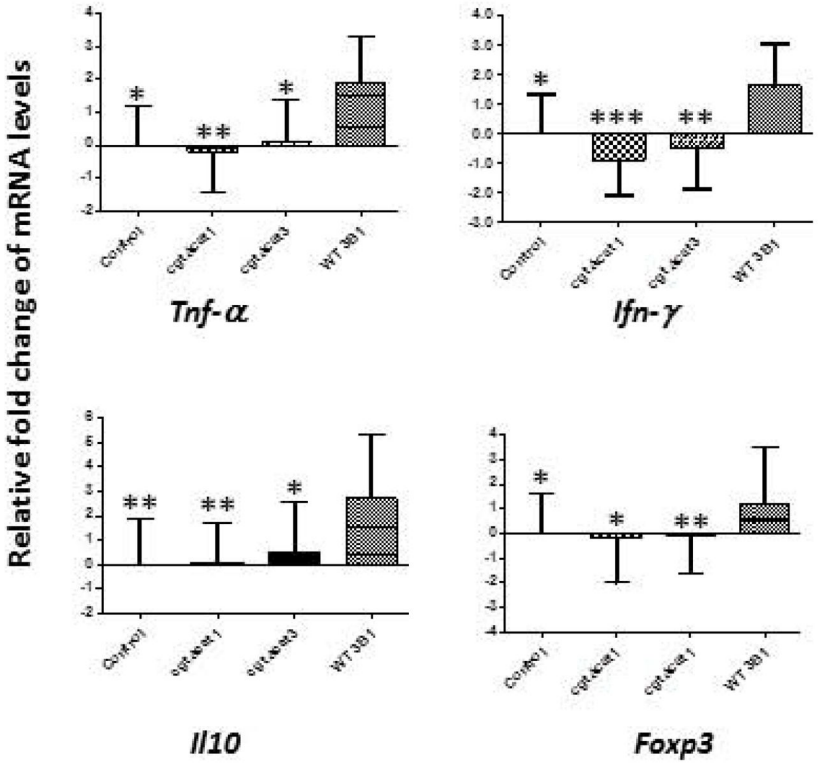

Figure 4.

Mice dosed with the *cgt* mutants expressed lower mRNA levels of hepatic proinflammatory Th1 cytokine Tnf-α and Ifn-γ as well as antiinflammatory mediators Il10 and Foxp3 compared to the WT 3B1-dosed mice. In each sample, the target gene mRNA was normalized to the transcript levels of *Gapdh*. Numbers on the y axis represent mean fold change of the individual mRNA levels in reference to the control group (0 defined as no change with its standard deviations denoted). P values versus WT 3B1-infected samples: * < 0.05, ** <0.01, *** <0.001.

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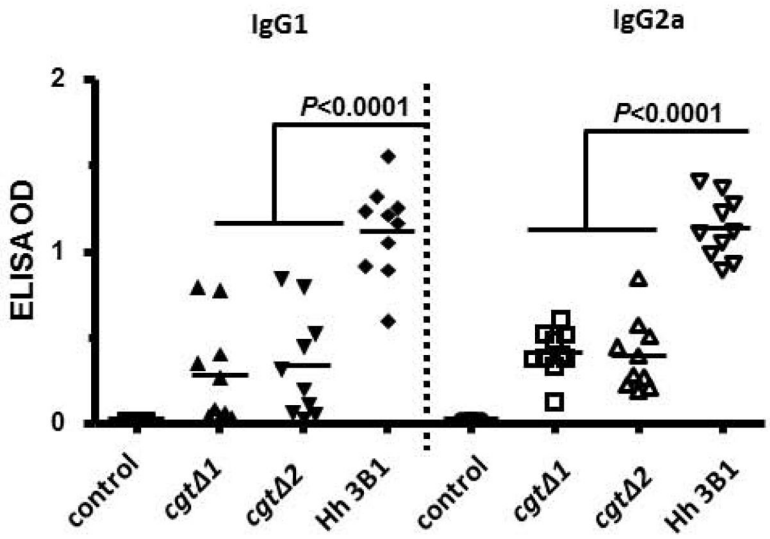

Figure 5.

Male A/JCr mouse Th1-associated IgG2a and Th2-associated IgG1 responses to WT 3B1 outer membrane antigens measured by ELISA. WT 3B1–dosed mice produced significantly higher levels of sera IgG2a and IgG1 compared to the *cgt* mutant-dosed mice, which is consistent with the failure of these *cgt* mutants to colonize or persist in the host.

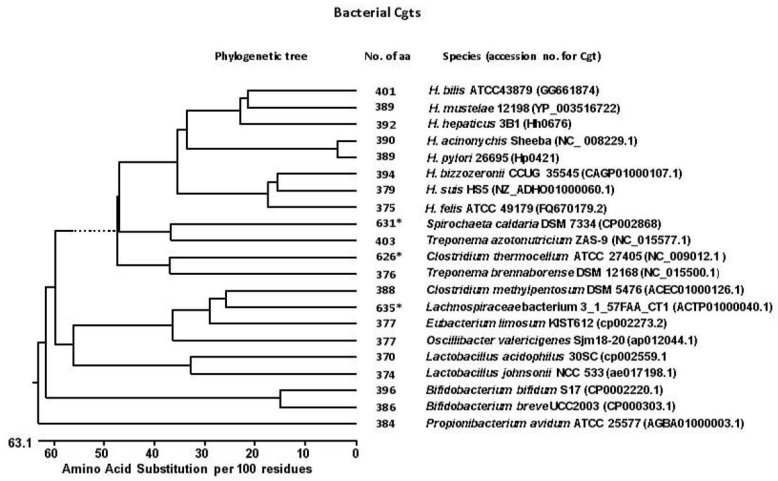

Figure 6.

Phylogenetic analyses of bacterial Cgts. Majority of Cgt protein sequences were deduced from genome sequences. The phylogenetic tree was constructed using the DNASTAR software package.

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Table I

Primer sequences used for generating *cgt* isogenic mutants of *H. hepaticus*

Primers	Sequence (5′ to 3′),	Strand
ZMG16	TTAA<u>GCGGCCGC</u>$_{(-580)}$ATATCCCACAGATGAAGCAGA, NotI	Sense
ZMG170	TTAA<u>GTCGAC</u>$_{(+13)}$TGGCTATAATCATTATTTGCACT HincII	Antisense
ZMG171	TTAA<u>GTCGAC</u>$_{(+1109)}$ACGCTTGATAAACGTTACACTCT HincII	Sense
ZMG172	TTAA<u>CTCGAG</u>$_{(+1677)}$TCGCATCGCAATTTTCAATTAGCA XhoI	Antisense

Numbers (+, downstream; −, upstream) denote starting nucleotide positions of primers in correspondence to the start codon of the *H. hepaticus cgt* (Hh0676).