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γ-Glutamyltransferase Is a *Helicobacter pylori* Virulence Factor but Is Not Essential for Colonization

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The contribution of glutamyl transpeptidase (GGT) (γ-glutamyltransferase [EC 2. 3. 2. 2]) to *Helicobacter pylori* virulence was investigated in piglets and mice using GGT-deficient isogenic strains. All animals became colonized. However, the bacterial load was significantly lower for mutant bacteria than for parent strains. These results suggest that GGT activity provides an advantage to *H. pylori* in colonization.

Helicobacter pylori is a gram-negative spiral bacterium that causes gastritis and ulcers and is associated with gastric cancer (14, 24, 28, 31). The mechanisms by which *H. pylori* colonizes and persists within the gastric mucosa are poorly understood. Elucidating the mechanisms involved in both survival and virulence of pathogenic bacteria is often facilitated through the use of animal models. Until recently, most *H. pylori* animal models were cost and space prohibitive for many researchers. These models included nonhuman primates (4, 5, 8, 13, 17), gnotobiotic piglets (11, 12, 21), and the domestic cat (16). Of these, the gnotobiotic piglet has been one of the most widely used and trusted models for *H. pylori* infection. Recently, the study of *H. pylori* immunity and pathogenesis has been greatly facilitated by the development of several murine models of *H. pylori* infection (19, 22, 23, 32).

The expansion of the number of animal models should facilitate the characterization of putative virulence and colonization factors. One such potential virulence factor is the glutamyl transpeptidase (GGT) (γ-glutamyltransferase [EC 2. 3. 2. 2]) enzyme. In mammalian tissues, GGT activity has been well studied and includes such functions as transpeptidation reactions and glutathione synthesis (35). However, GGT expression and activity in bacteria has been poorly characterized. Recently, the *ggt* gene for *H. pylori* was identified and sequenced by Chevalier et al. (7), making it one of only several bacterial species in which the *ggt* gene has been characterized (18, 34, 38). Chevalier et al. reported that deletion of GGT had no deleterious effect on the ability of *H. pylori* to grow in culture, but GGT is essential for *H. pylori* infection of the mouse (7). The present study was performed to extend the findings of Chevalier et al. by employing isogenic, GGT-deficient *H. pylori* mutants in the gnotobiotic-piglet model. However, we now report, using two distinct animal models, that although GGT activity may provide some advantage to *H. pylori* in colonization of the gastric mucosa, it is not essential for initial colonization or maintenance of chronic infection.

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The *ggt*::*aph* insertional mutation was constructed utilizing the sequence of the *ggt* gene (open reading frame 1118) from the database of The Institute for Genomic Research. The 5′ end of the gene was amplified with primer GTHPF4 (CATC GTCTCTTGTAATGAG) and primer GTHPR5 (CGACGAG ATCTCGCTGCCGAAGCGATGCG). The 3′ end of the gene was amplified with primer GTHPF5 (CGACGAGATCTCTC CCGAACTTGGCGGCG) and primer GTHPR4 (GCATCA TGTAAGTTATAAGCG). Each fragment was cloned into pCRscript plasmids (Stratagene, La Jolla, Calif.). These fragments were constructed to contain a *Bgl*II site at the 3′ end of the 5′ fragment and the 5′ end of the 3′ fragment to allow a 1-kb insertion of the kanamycin resistance gene *aph* from Tn*903* (29) (PCR amplified with the addition of flanking *Bam*HI and *Bgl*II sites), resulting in a 100-bp deletion and the loss of all enzyme activity. Confirmation of the mutant construction was performed by both GGT assay and PCR amplification utilizing primer GTHPF6 (GTGAAATCTTGGGGC TGAAACCGC) and primer GTHPR7 (CAAGGGCAAGTC GCTGAGC), which flanked the insertion. Transformation of *H. pylori* strains (the piglet-adapted strain KE26695 [12] and the mouse-adapted strain HpM5 developed by one of the authors [T.G.B.]) was performed by modification of the method described by Wang et al. (37). The DNA harboring the disrupted *ggt* gene was combined with *H. pylori* and spotted onto a brucella agar plate overnight. The cells were then resuspended in 100 μl of phosphate-buffered saline and plated on new plates containing 20 μg of kanamycin/ml. Kanamycin-resistant colonies were then assayed by PCR for the presence of the disrupted *ggt* gene and for lack of GGT activity. For development of a *ureB*-deficient strain, a plasmid containing a *ureB*::*aph* knockout, pEJ22, was obtained from the laboratory of Andrew Wright at Tufts University.

The assay for GGT activity was adapted from the method of Meister et al. (26). Briefly, reaction buffer consisting of 20 mM Gly-Gly, 300 μM L-γ-glutamyl-ρ-nitroanilide, and 60 mM Tris (pH 8.0) was prepared. One-tenth the volume of *H. pylori* cells at A_{600} of 1.0 was added for a final A_{600} of 0.1. Reactions proceeded at 37°C for 60 min, and the release of ρ-nitroanilide was monitored by the A_{405}. Additionally, a fluorimetric assay protocol was adapted from the method of Forman et al. (15).

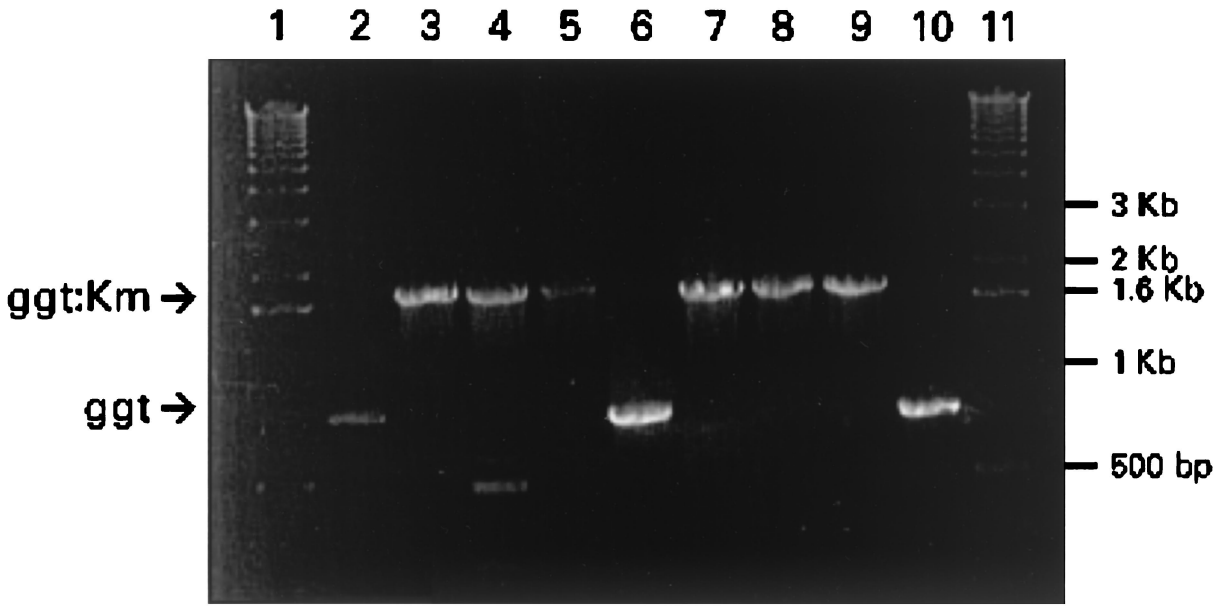

FIG. 1. PCR amplification of the *ggt* gene from *H. pylori* strains. Lanes 1 and 11, 1-kb ladder markers; lane 2, HpM5; lane 3, M5 *ggt*::*aph;* lane 4, M5 *ggt*::*aph* from mouse 31; lane 5, M5 *ggt*::*aph* from mouse 44; lane 6, KE26695; lane 7, KE26695 *ggt*::*aph;* lane 8, KE26695 *ggt*::*aph* from pig 312; lane 9, KE26695 *ggt*::*aph* from pig 313; lane 10, KE26695 from pig 316. The wild-type *ggt* 800-bp band and the *ggt*::*aph* 1.7-kb band are indicated by arrows.

Briefly, in each well of a 96-well plate, 70 μl of 70 μM γ-Glu-7-amino-4-methyl coumarin (AMC) was combined with 30 μl of *H. pylori* cells at an A_{600} of 0.05 for a final γ-Glu-AMC concentration of 50 μM and a final A_{600} for *H. pylori* of 0.015. The chemicals employed in these assays were purchased from Sigma (St. Louis, Mo.).

Caesarian-derived gnotobiotic piglets from date-mated specific-pathogen-free sows (21) were inoculated with 10^9 CFU of *H. pylori* in 2.0 ml of brucella broth at an age of 3 days and terminated either 6 or 20 days postinfection. The stomachs were opened, and a small strip from the lesser curvature, incorporating portions of the cardia, fundus, antrum, and pyloris, was removed for histopathological evaluation. The remaining stomachs were used for bacterial reisolation, in which the mucosa was removed from the muscularis, weighed, and homogenized in brucella broth (10% [wt/vol]). Quantitative 10-fold bacterial titrations were performed in triplicate (two replicates per dilution) onto Skirrow's agar plates. Gross lesions were recorded, and samples of formalin-fixed gastric cardia, fundus, antrum, and pyloris were prepared for histopathological evaluation. For histopathology, 6-μm-thick section replicates, stained with hematoxylin and eosin and the Warthin-Starry stain for demonstration of organisms, were examined by light microscopy. Each section was assigned a qualitative score of 0 to 4, as described previously (20).

Six- to 8-week-old C57BL/6 mice (Harlan Sprague Dawley, Inc., Indianapolis, Ind.), housed in microisolator cages, were inoculated by gastric intubation with 750 μl of culture grown to an OD_{450} of 0.1 on two consecutive days and terminated either 28 or 49 days postinfection. Two biopsy specimens (2 by 2 mm) were surgically removed from the gastric antrum of each mouse, homogenized in 200 μl of brucella broth, and cultured on Skirrow's agar plates under microaerobic conditions at 37°C for 96 h. Additionally, for histological evaluation, a narrow strip of tissue was surgically removed from the greater curvature of the stomach from the duodenum to the gastric cardia of each necropsied mouse. The tissues were fixed in 10% buffered formalin and processed for histological examination at University Hospitals in Cleveland, Ohio. For all experiments, differences in bacterial load between control and experimental groups were evaluated by analysis of variance. The differences were considered statistically significant if the *P* values were less than 0.05.

Cloning of the *ggt* gene into *Escherichia coli* resulted in a high level of GGT enzyme compared to that of *E. coli* without the plasmid (data not shown). Although *E. coli* normally carries the *ggt* gene, activity is not detectable. A kanamycin-resistant insertional *ggt* mutant of *H. pylori* was constructed as described above and analyzed by PCR. As shown in Fig. 1, lane 6, amplification of the *ggt* gene from strain KE26695 yielded the predicted fragment size of 750 bp. Amplification of the *ggt*::*aph* mutation, however, yielded a 1.7-kb fragment (Fig. 1, lane 7) corresponding to the size predicted for the *ggt* construct containing the kanamycin resistance cassette. *H. pylori* transformants created with plasmid containing the *ggt*::*aph* mutation were compared to parent strains to evaluate the levels of GGT activity. No GGT activity was detected in *H. pylori* strains harboring the *ggt* insertional mutation by either our colorimetric assay (Fig. 2) or the more sensitive fluorimetric assay (data not shown). The isogenic *H. pylori* control strains possessed the predicted wild-type levels of activity. The lack of GGT activity had no effect on the in vitro growth kinetics of the transformants compared to the parent strain. These data, combined with our PCR analysis, indicate successful deletion of GGT activity in our animal-adapted *H. pylori* strains.

Separately housed gnotobiotic piglets were orally inoculated

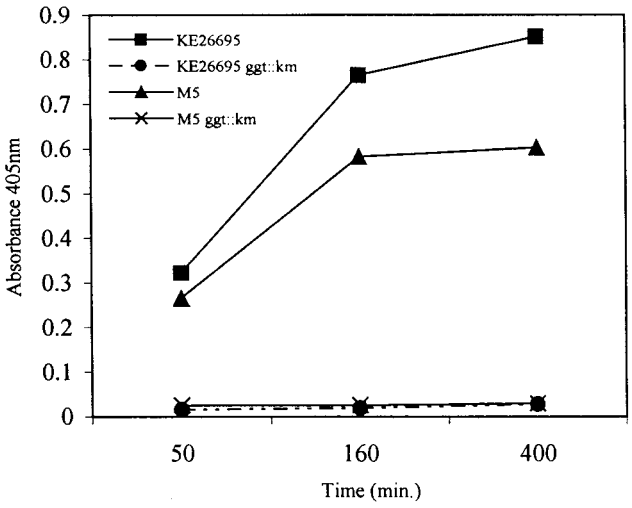

FIG. 2. GGT enzyme assay. The GGT activity was measured as the A_{405} of ρ-nitroanilide released by KE26695 and M5 and by *ggt* mutant derivatives.

with either KE26695 or KE26695 *ggt*::*aph* as described above. The piglets were terminated at either 6 or 20 days postinoculation, and the gastric tissue was analyzed for bacterial load and pathology as described above. Piglets inoculated with parental KE26695 yielded 9.9×10^6 to 18.6×10^6 CFU of bacteria per gram (mean, 14.25×10^6 CFU/g) (Table 1) at 6 days postinoculation, comparable to levels reported previously (20). The recovered organisms were confirmed to be *H. pylori* by colony morphology and biochemical assays for urease and catalase and were shown to express the parental-type GGT by PCR and enzyme activity (see below). In contrast, piglets inoculated with KE26695 *ggt*::*aph* had significantly reduced numbers of bacteria, with greater than 2-log unit reduction in colonization 6 days after inoculation ($<0.085 \times 10^6$ CFU/g) (Table 1). By 20 days after infection, the colonization levels in the KE26695 *ggt*::*aph*-inoculated piglets (1.64×10^6 CFU/g) approached those in the control groups from both 6 and 20 days after infection. The recovered organisms were confirmed to be *H. pylori* as described above and were shown to retain the *ggt* mutation, as detailed below. Histopathological evaluation revealed antral-dominant lymphoplasmacytic inflammation and lymphoid follicle formation typical of *H. pylori* gastritis (Fig. 3) in this species (20). The lesions, ranging from 0 to 2 (on a scale of 0 to 4) did not differ appreciably between the parental-strain-infected piglets and the *ggt* mutant-infected piglets.

A mouse model of *H. pylori* infection was also employed to determine if there was a significant difference in colonization requirements between animal models. Mouse-adapted HpM5 efficiently and reproducibly infects mice. This strain was chosen due to the difficulty in transforming other mouse-adapted strains, such as the Sidney strain (22). Kanamycin-resitant transformants were screened by PCR to verify the presence of the *ggt* insertional mutation (Fig. 1, lanes 2 and 3). As described above for KE26695, no GGT activity was observed in the HpM5 *ggt* mutant strain by either of our assays (Fig. 2). Mice were inoculated with the *H. pylori* strains as described above and terminated on days 28 and 49 postinoculation. These time points were chosen to increase the likelihood of recovering organisms from the gastric mucosa. All mice inoculated with mouse-adapted HpM5 were culture positive at both time points (Table 2). *H. pylori* was also recovered from 100% of the mice inoculated with HpM5 carrying the *ggt* knockout mutation at both 28 and 49 days postinoculation. However, while the bacterial counts per biopsy specimen increased over time for mice inoculated with either HpM5 or HpM5 *ggt*::*aph,* the number of bacteria in mice inoculated with the *ggt* knockout was significantly lower than in mice inoculated with wild-type HpM5. This observation was consistent among time points, although the disparity decreased (28 days, $P = 0.0002$; 49 days, $P = 0.024$). No bacteria were recovered from mice inoculated with M5 lacking the urease B subunit at either time point, verifying urease as a virulence factor for *H. pylori*. Two *H. pylori* isolates from the stomachs of piglets and two from mice infected with isogenic strains were tested both for the presence of the *ggt* mutation and for GGT activity. Both the genotype (Fig. 1) and phenotype (data not shown) of the mutants remained as expected during animal passage.

Our results indicate that the presence of GGT confers an advantage upon *H. pylori* in colonizing the gastric mucosa. Early identification and clinical classification of *H. pylori* showed GGT being expressed at unusually high levels (25). The presence of unusually high levels of this enzyme in *H. pylori* led Chevalier et al. to further examine the role of GGT in *H. pylori* viability and survival in the host (7). Our results are consistent with those of Chevalier et al. in showing that GGT is not essential for *H. pylori* viability, as both isogenic *ggt*-deficient *H. pylori* strains grew normally in vitro despite the demonstrated lack of GGT activity. However, our results differ from those in the previous report in that, although GGT activity did provide an advantage for increasing the bacterial load, GGT-deficient *H. pylori* strains successfully infected both gnotobiotic piglets and mice.

Animal models have been used to test the contributions of several *H. pylori* virulence factors in colonization and pathogenicity. Urease-deficient *H. pylori* generated via chemical mutagenesis fails to colonize gnotobiotic piglets (9), and a *ureA* mutant of *Helicobacter mustelae* was unable to colonize the ferret stomach (3). Similar studies using isogenic mouse-adapted *H. pylori* strains confirmed this observation in mice (33, 36). Motility has also been shown to be essential based on studies

TABLE 1. Recovery of *H. pylori* parental strain KE26695 and the isogenic mutant KE26695 *ggt*::*aph* from gastric mucosae of gnotobiotic piglets

Time and strain	Piglet no.	No. of bacteria (10^6 CFU/g)		% Parental recovery
		H. pylori	Group mean	
6 days postinoculation				
KE26695	1	18.6	14.25	100
	2	9.93		
KE26695 *ggt*::*aph*	3	<0.001	<0.085	0.05
	4	0.17		
20 days postinoculation				
KE26695	5	5.64		100
KE26695 *ggt*::*aph*	6	3.24	1.64	29.6
	7	0.5		

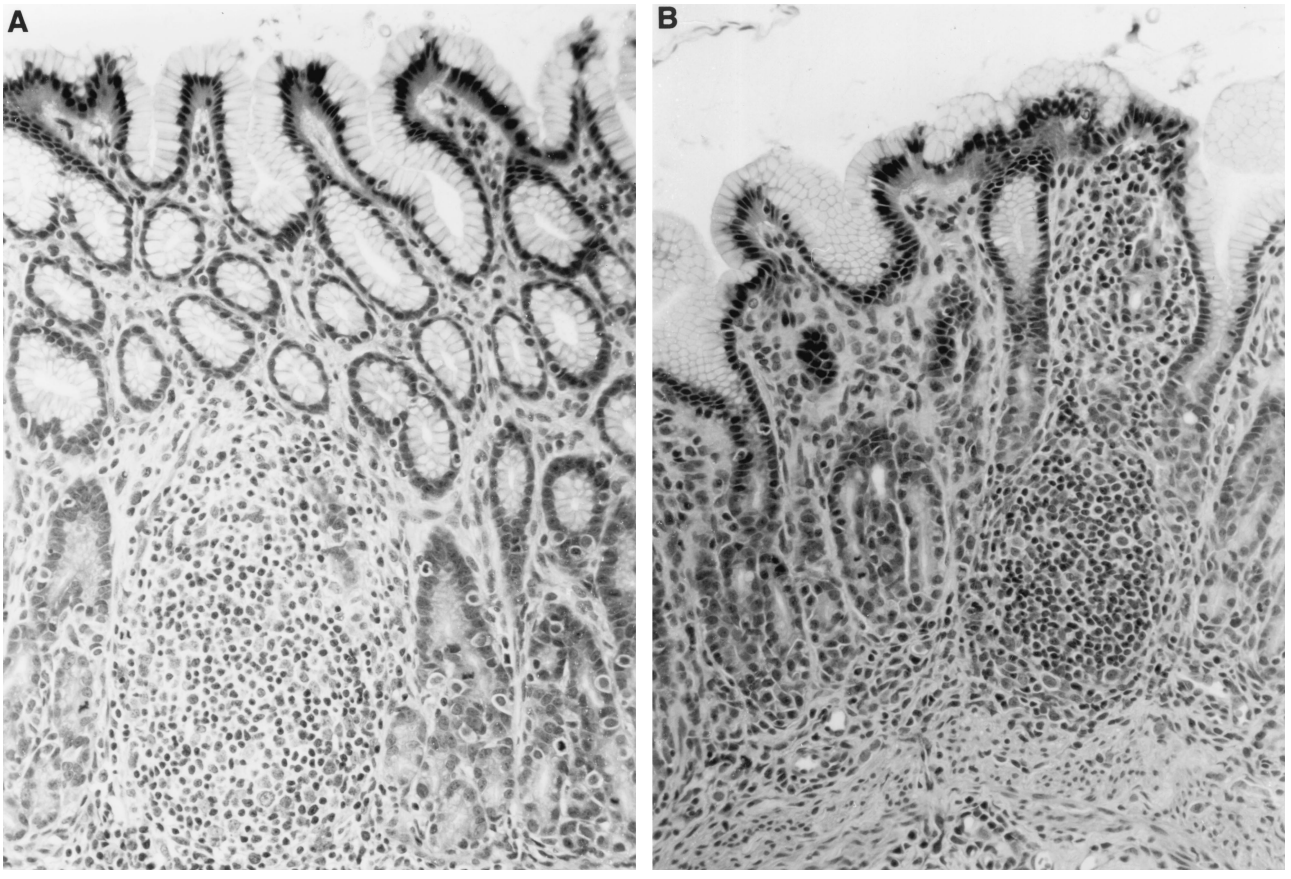

FIG. 3. Photomicrograph of gastric mucosa and lamina propria from a gnotobiotic piglet infected with KE26695 *ggt*::*aph* 20 days previously. (A) Developing lymphoid follicle. There are also lymphocytic infiltrates within the lamina propria (B). The tissues were stained with hematoxylin and eosin. Magnification, ×270.

demonstrating that strains with limited motility had poor colonization rates in the piglet model (12). A study performed using recombinant flagellin-negative *H. mustelae* confirmed the importance of motility in establishing chronic infection (2). The role of vacuolating toxin (VacA) in promoting more severe symptoms (reviewed in reference 30) was tested by using toxigenic and nontoxigenic isogenic *H. pylori* mutants in the piglet model, which showed that the presence of VacA does not enhance *H. pylori*-associated gastritis (10). Another protein believed to correlate with increased *H. pylori* pathogenicity in humans is the 120-kDa cytotoxin-associated protein (CagA), which is also a marker for a pathogenicity island, a 40-kb segment of DNA possessing up to 26 genes which is present in a large percentage of *H. pylori* strains (1, 6). Since strains lacking the *cag* pathogenicity island are isolated from a substantial percentage of *H. pylori*-infected individuals, this segment of DNA does not possess genes essential for colonization.

The present study used two distinct animal models of *H. pylori* infection to assess the importance of GGT activity in *H. pylori* virulence. Our results with gnotobiotic piglets indicated that after a 6-day infection with *ggt*-deficient *H. pylori,* colonization, although very much reduced in comparison to infection with the isogenic wild-type KE26695, was accomplished in 100% of our animals. Similar results were obtained for the *ggt* mutant after a 20-day infection, although the numbers were not as low as those observed at 6 days of infection. We extended our study by repeating it using a *ggt*-deficient mouse-adapted strain of *H. pylori*. The results complemented the piglet model. *H. pylori* could be isolated from 100% of the mice 28 days postinoculation with the HpM5 *ggt*::*aph* strain although the bacterial load was significantly lower than that from mice infected with the parent strain. When the mice were examined at 49 days, the *ggt* mutant was recovered at higher numbers than at 28 days but still significantly reduced in comparison to infection with HpM5.

The complementary results obtained in these two animal

TABLE 2. Recovery of *H. pylori* parental strain HpM5 and the isogenic mutant HpM5 *ggt*::*aph* from gastric mucosae of C57BL/6 mice

Strain	*H. pylori* organisms[a]			
	No. 28 days post-inoculation[b]	% Parental recovery	No. 49 days post-inoculation[c]	% Parental recovery
HpM5	879 (224)	100	3,811 (1,278)	100
HpM5 *ggt*::*aph*	37 (13)	8.4	1,191 (409)	31.2
HpM5 *ureB*::*aph*	0 (0)	0	0 (0)	0

[a] Data are presented as mean CFU per biopsy and standard error.
[b] Eight mice per group.
[c] Seven mice per group.

model systems are reassuring, since there is no ideal model for studying *H. pylori* infection other than humans. However, these results are in conflict with those observed by Chevalier et al. (7) in a murine model of *H. pylori* infection in which *ggt*-negative *H. pylori* was unable to infect the gastric mucosa. Although methods for bacterial culture and quantification from gastric biopsies were similar, several differences in experimental design may explain the difference in our results. First, Chevalier et al. used *H. pylori* SS1, a mouse-adapted strain recently used for vaccine models (22). We employed one of our own mouse-adapted isolates, HpM5, which is readily transformable and consistently colonizes C57BL/6 mice. Both *H. pylori* SS1 (22) and HpM5 (data not shown) are $cagA^+$ and $vacA^+$ by PCR analysis. Since deletion of GGT activity in our strain resulted in decreased bacterial loads, it is possible that in other strains of *H. pylori* such a deletion could completely ablate the ability to infect the host.

Second, Chevalier et al. inoculated outbred Swiss mice whereas we utilized inbred C57BL/6 mice. Although studies comparing mouse strain susceptibilities have focused primarily on disease (22, 27, 32), we have noticed differences among mouse strains with regard to the ease of infection with *H. pylori* when testing our other mouse-adapted strains. When seven different strains of mice were inoculated simultaneously with *H. pylori,* we were unable to subsequently culture organisms from gastric biopsy specimens from several mouse strains (unpublished observations). It is possible that Swiss mice are less amenable to hearty infection than C57BL/6 mice and that the loss of GGT activity that results in reduced bacterial loads in C57BL/6 mice may result in either a failure to colonize Swiss mice or colonization below the level of detection. These experiments underscore the concept that there is a considerable amount of variation among mouse-adapted *H. pylori* strains and *H. pylori* animal models in general.

Evidence obtained regarding the importance of specific virulence factors may be strengthened through the use of multiple models. The consistency in results that we observed in mice and piglets demonstrates that GGT activity provides at least some advantage to *H. pylori,* as *ggt* mutants colonized at levels that were significantly lower than those of wild-type isogenic strains of *H. pylori,* particularly at early time points. However, while GGT activity does provide some advantage, it is not essential for the establishment of chronic infection of the gastric mucosa.

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