Evidence for Conserved Function of $\gamma$–Glutamyltranspeptidase in Helicobacter Genus

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Introduction

$\gamma$-Glutamyltranspeptidase ($\gamma$GT) is a threonine N-terminal nucleophile (NtN) hydrolase that catalyses the transpeptidation and hydrolysis of the $\gamma$-glutamyl group of glutathione and related compounds [1]. $\gamma$GT is widely distributed in living organisms and is highly conserved, with mammalian and bacterial homologues often sharing more than 25% of sequence identity [2]. From the ~1000 of whole genome sequenced bacterial species available in the MEROPS databases [3], 540 (~200 genera) possess $\gamma$GT-like proteins belonging to protease family T03. Moreover, several bacterial species carry multiple copies of genes annotated as $\gamma$GT, but the majority of these genes lack functional verification.

$\gamma$GT is found in all gastric Helicobacter species. However, among the 20 validly published enterohalophilic Helicobacter species (EHS), only H. aurati, H. bilis, H. canis, H. muridarum and H. trogontum express this enzyme [4]. In H. pylori, $\gamma$GT represents an important virulence factor, playing a role in colonization [5,6] and pathogenesis [7–10]. It is constitutively expressed in vivo and in vitro [11] and enables the bacterial cells to use extracellular glutamine and glutathione as a source of glutamate [9,12]. Purified Hp-$\gamma$GT was shown to be involved in upregulation of growth factors in MKN-28 gastric cells [7], to induce apoptosis in AGS cells [10], to inhibit T-cell proliferation [9] and to contribute to H. pylori-mediated H2O2 generation [8]. By contrast, the biochemical properties of EHS $\gamma$GT and its role in the colonization of the gut and in the pathogenesis of gastrointestinal and hepatobiliary diseases are completely unknown. EHS is a phenotypically and genotypically heterogeneous phylogroup within the Helicobacter genus [4], including species usually colonizing the intestinal tract and/or the liver of mammals and birds. Although EHS could be considered part of the normal microbiota of rodents, some species cause diseases in these animals [13]. In particular, H. bilis, an endemic EHS in most experimental mouse colonies, induces disease in susceptible animals and may substantially confound interpretations of some research studies [14]. Natural H. bilis infection in inbred [15] or outbred mice [16] has been associated with multifocal hepatitis. Moreover, H. bilis has been used experimentally to induce inflammatory bowel disease (IBD) in mdr−/− and IL-10−/− knock-out mice [16], typhlocolitis in the

Abstract

The confounding consequences of Helicobacter bilis infection in experimental mouse populations are well recognized, but the role of this bacterium in human diseases is less known. Limited data are available on virulence determinants of this species. In Helicobacter pylori, $\gamma$-glutamyltranspeptidase ($\gamma$GT) contributes to the colonization of the gastric mucosa and to the pathogenesis of peptic ulcer. The role of $\gamma$GT in H. bilis infections remains unknown. The annotated genome sequence of H. bilis revealed two putative ggt genes and our aim was to characterize these H. bilis $\gamma$GT paralogues. We performed a phylogenetic analysis to understand the evolution of Helicobacter $\gamma$GTs and to predict functional activities of these two genes. In addition, both copies of H. bilis $\gamma$GTs were expressed as recombinant proteins and their biochemical characteristics were analysed. Functional complementation of Escherichia coli deficient in $\gamma$GT activity and deletion of $\gamma$GT in H. bilis were performed. Finally, the inhibitory effect of T-cell and gastric cell proliferation by H. bilis $\gamma$GT was assessed. Our results indicated that one gene is responsible for $\gamma$GT activity, while the other showed no $\gamma$GT activity due to lack of autoprocessing. Although both H. bilis and H. pylori $\gamma$GTs exhibited a similar affinity to L-Glutamine and $\gamma$-Glutamyl-p-nitroanilide, the H. bilis $\gamma$GT was significantly less active. Nevertheless, H. bilis $\gamma$GT inhibited T-cell proliferation at a similar level to that observed for H. pylori. Finally, we showed a similar suppressive influence of both H. bilis and H. pylori $\gamma$GTs on AGS cell proliferation mediated by an apoptosis-independent mechanism. Our data suggest a conserved function of $\gamma$GT in the Helicobacter genus. Since $\gamma$GT is present only in a few enterohelopatic Helicobacter species, its expression appears not to be essential for colonization of the lower gastrointestinal tract, but it could provide metabolic advantages in colonization capability of different niches.
C3H/HeN mice strain [17] and cholesterol gallstone formation in C57L mice [18]. *H. bilis* is able to infect and cause diseases in different animal hosts, showing one of the broadest host spectrums in the *Helicobacter* genus [19]. It was isolated from the aborted fetus of sheep and pig [19] and from chronic hepato-biliary diseases in hamsters [20]. *H. bilis* has been also isolated from human patients with chronic diarrhoea [21] and pyoderma gangrenosum-like ulcers [22]. In addition, several studies have reported an association of this species with chronic liver diseases [23,24] or biliary tract and gallbladder cancers [25,26] in human, using either PCR or serological tests. Limited data are available on virulence determinants of *H. bilis* [27–29], and no studies to date have described the biochemical and biological properties of *H. bilis* γGT (Hb-γGT).

In contrast to observations in gastric *Helicobacter* spp., the genome sequence of *H. bilis* ATCC 43879 revealed the presence of two *gtt* copies. In this study, we used a phylogenetic and a functional approach to analyse both *H. bilis* γGT paralogues. Although both genes were phylogenetically related to other *Helicobacter* γGTs, analysis of the recombinant proteins, western blot using specific antibodies, complementation of *H. bilis* clearly showed that only one gene was responsible for *H. bilis* γGT activity. The γGT of *H. bilis* exhibited a similar affinity as *H. pylori* to γ-Glutamyl-p-nitroanilide and to L-Glutamin; however, it was significantly less active. Nevertheless, *H. bilis* γGT inhibited T-cell and gastric cell proliferation at a similar level to that observed for *H. pylori* γGT. The inhibition observed was mediated by an apoptosis-independent mechanism and suggested a conserved function of γGT in *Helicobacter* genus.

**Results**

Sequence analysis revealed marked differences between two γGT paralogues of *H. bilis* ATCC 43879

The *gtt* paralogues HRAG_01341 and HRAG_01828 of the human-associated *H. bilis* strain ATCC 43879 genome (NCBI ACIDN0000000), were named *bgh1* (*H. bilis* γGT homologue 1) and *bgh2* (*H. bilis* γGT homologue 2), respectively. Nucleotide and amino acid similarity between the two *H. bilis* γGT paralogues, and between each homologue and *H. pylori* γGT (Hp-γGT; HP1110) were analysed by pairwise global alignment. The two *H. bilis* γGT paralogues showed 65.3% and 62.0% of nucleotide and amino acid identity, respectively. Moreover, in comparison with Hp-γGT, Bgh2 showed 65.2% of amino acid identity, while Bgh1 showed 53.4%. To detect amino acid positions potentially involved in functional change, conserved sites of both Bgh1 and Bgh2 genes were evaluated on the basis of γGT structural data available for *H. pylori* [30,31] and *E. coli* [32]. A multi-alignment including all *Helicobacter* γGT sequences, other bacterial γGTs and class IV cephalosporin acylase (CA) of *Pseudomonas* sp. strain SE82, was constructed (Figure 1). In Bgh2, all the functional sites described for *H. pylori* were conserved. In Bgh1, by contrast, amino acid substitutions in the 20 kDa subunit potentially involved in functional change were observed. The substitutions were as follows: Asp21Glu, Asp22Asn, Leu342Arg, Tyr433His, Ser452Thr and Gly473Ser (Hp-γGT numeration).

To predict the localization of both *H. bilis* γGT paralogues, the sequences were submitted to SignalP 3.0, using both Neural networks and Hidden Markov Models (HMM) methods [33], PSLpred (Hybrid Approach Based; [34]), PSORTb v3.0.2 [35] and CELLO v2.5 [36]. These tools were unable to uniformly predict the sub-cellular localization of both Bgh1 and Bgh2. To verify the presence of other potential start codons, we analysed the upstream region of both genes. We identified a second putative start codon 34 bp upstream of the annotated methionine of Bgh2, but no other potential start codons for Bgh1. Using the new predicted start codon, all of the analysis strongly predicted Bgh2 as a periplasmic protein, and SignalP detected the presence of a signal sequence with a potential cleavage site between positions 30 and 31 (VFA-AS). The same prediction was obtained for all γGTs of *Helicobacter* spp. and *C. jejuni*. No twin-arginine signal peptide cleavage site was detected in any γGTs of *Helicobacter* spp. and *C. jejuni*.

Figure 1. Multi-alignment of amino acid sequences of different bacterial γGTs, *Helicobacter bilis* Bgh1 and Bgh2, and class IV Cephalosporin Acylase (CA). Multi-alignment between residues 379 and 477 (*Helicobacter pylori* γGT numeration) is shown. Sequences of γGTs of *H. pylori* (26695; HP1118), *H. acinonychis* (Sheeba; Hac_0598), *H. bizzozeronii* (CIII-1; HBZC1_08080), *H. salomonii* (O6A; EMBL FR821684), *H. suis* (HS1; HSUH51_0265), *H. felis* (ATCC 49179; Hfelis_06880), *H. mustelae* (12198; HMU08020), *Esherichia coli* (K12; SwissProt P18956), *Campylobacter jejuni* (81–176; CJ81176_0067), *Pseudomonas* sp. (A14; Swiss-Prot P36267), *Bacillus subtilis* (168; Swiss-Prot P54422), *Arcobacter nitrofigilis* (DSM 7299; Arnit_0203), *Arcobacter butzleri* (RMA018; Abu_0961), *H. bilis* (Bgh1 = HRAG_01341; Bgh2 = HRAG_01828) as well as class IV CA of *Pseudomonas* sp. (SE82; Swiss-Prot P15557) are shown. Residues completely conserved among the sequences are indicated with a black background. The catalytic dyad is highlighted with a red box, and the conserved motif GXXGGXXI is enclosed in a black box. The Lid loop consists of the residues G428 to G438 of *Helicobacter* spp. and *C. jejuni*. Residues involved in the substrate recognition and the catalytic centre are highlighted in grey. Amino acid substitutions in Bgh1 potentially involved in functional change are indicated by arrows below the sequences.

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Phylogenetic analysis of Helicobacter \(\gamma\)GTs

Phylogenetic relationships among epsilon proteobacteria and other bacterial \(\gamma\)GTs and Pseudomonas spp. CA were analysed (Figure 2). The Minimum Evolution tree based on amino acid sequence alignment showed that both H. bilis \(\gamma\)GT paralogues cluster with Helicobacter and C. jejuni \(\gamma\)GTs, forming, however, two distinct branches: Bgh2 and C. jejuni \(\gamma\)GT belonged to a single clade, while Bgh1 formed a separate branch.

To examine the phylogenetic relationships among Helicobacter \(\gamma\)GTs in more detail and to identify other Helicobacter species potentially carrying multiple \(\gamma\)GT sequences, consensus degenerate hybrid oligonucleotide primers were designed on the basis of highly conserved amino acid motifs (Table 1). A fragment of about 1300 bp, corresponding to an almost complete ggt sequence, was successfully amplified from H. canis, and two fragments, corresponding to an almost complete sequence of two distinct ggt copies, were amplified from H. trogontum and H. bilis genomospecies FL56. From H. aurati and H. muridarum, amplification of only the N-terminal part of a single ggt copy was possible. An unrooted Minimum Evolution tree was built on the basis of an almost complete \(\gamma\)GT pro-enzyme of Helicobacter spp. and C. jejuni (Figure 3a). Phylogenetic analysis showed that the two ggt copies amplified from H. trogontum and H. bilis genomospecies FL56 corresponded to orthologues of bgh1 and bgh2, whereas the sequences obtained from H. canis, H. aurati and H. muridarum (Figure 3a,b) clustered with other Helicobacter ggt and bgh2. In Figure 3, the phylogeny of the pro-enzyme was compared with those of the single sub-units. The trees showed differences in both the topology and the bootstrap supporting nodes, suggesting different evolution of the two parts. The same results were obtained comparing the maximum likelihood trees of the nucleotide sequences (data not shown). Finally, to provide a measure for the selective pressure that the gene pair bgh1 and bgh2 was subjected to, the ratio between non-synonymous/synonymous substitution (Ka:Ks) was evaluated by sliding window analysis (window size 50 bp) using SWAKK [37]. A Ka:Ks ratio smaller than one was observed in all positions analysed. The average Ka:Ks ratio was calculated to be 0.175, indicating that the genes are under purifying selection. A similar Ka:Ks ratio was observed also for the bgh1 and bgh2 pairs of H. trogontum and H. bilis genomospecies FL56 (data not shown).

Frequency of bgh1 and bgh2 genes in H. bilis strains

To determine the frequency of bgh1 and bgh2 in H. bilis, 33 H. bilis strains from our collection [38], including H. bilis type strain ATCC 51630^T, H. bilis ATCC 49314 and H. bilis ATCC 49320, were subjected to a specific BclI RFLP-PCR for bgh1 and a specific PCR for bgh2. All strains were positive for the presence of both genes. Furthermore, bgh1 orthologues of H. bilis type strain ATCC 51630^T and three canine strains [38] were sequenced and uncorrected distance matrices were constructed on the basis of both nucleotide and amino acid sequences. The overall nucleotide sequence identity observed varied from 94.7% to 97.3%, while the amino acid identity ranged from 97.5% to 100%.

Transcription of bgh1 and bgh2 by H. bilis CCUG 23435

The transcription of both bgh1 and bgh2 was evaluated in H. bilis CCUG 23435 at different time points. Bacterial growth was
monitored by measuring OD \textsc{600} up to 24 h, corresponding approximately to the end of the exponential phase. At 8, 12 and 24 h both genes were transcribed (data not shown).

**Expression, purification and autoprocessing of recombinant Bgh1 and Bgh2 proteins**

To further analyse the biochemical characteristics of both *H. bilis* γGT paralogues, we expressed recombinant His-tagged Bgh1 and Bgh2 proteins in *E. coli*. Bgh2 was expressed without the signal peptide, while for Bgh1 the entire sequence was used. Recombinant expression of Bgh2 resulted in a soluble protein that was directly purified by Ni-affinity chromatography. By contrast, Bgh1 was expressed as an insoluble protein of \textsc{70 KDa} and was purified after refolding. The purity of the proteins on SDS-PAGE was \textsc{90%} (Figure 4a). Bgh2 showed a catalytic activity for the substrate analogue L-\textsc{c-glutamyl-p-nitroanilide (gGpNA) and was synthesized as a pro-form of \textsc{60 KDa, which undergoes autocatalytic processing to generate two subunits of \textsc{40 and \textsc{20 KDa (Figure 4a). By contrast, after solubilisation and refolding, recombinant Bgh1 showed no significant maturation after 24 h of incubation at \textsc{37°C and no activity for gGpNA (Figure 4b). To estimate the secondary structure of Bgh1 and to verify the absence of a random folding, a Circular Dichroism (CD) spectrum of the recombinant protein was calculated. The spectrum minimum calculated for recombinant Bgh1 was \textsc{206 nm}, indicating the absence of random coil. The secondary structure of Bgh1 was predicted by K2D3 [39] to contain \textsc{30.23% of alfa helix and \textsc{16.82% of beta sheet. The CD spectrum for Bgh1 resulted similar to the spectrum predicted by K2D3 and to those for proper folded proteins, e.g. lysozyme [40], indicating that recombinant Bgh1 has a defined secondary structure (Figure S1).}

**Autoprocessing of Bgh1 and Bgh2 in H. bilis**

In order to evaluate the maturation of both Bgh1 and Bgh2 in *H. bilis*, antisera against both recombinant proteins were produced in mice and western blot analysis on *H. bilis* whole lysate was performed. The antisera showed high specificity for the corresponding *H. bilis* γGT parologue in ELISA test at \textsc{1:900 dilution selected for the subsequent western blots (data not shown). The western blot performed on *H. bilis* whole lysate using the antiserum against Bgh1 clearly showed a single band without indication of autoprocessing (Figure 5a). On the contrary, the western blot

| Table 1. Oligonucleotides used in this study. |
|------------------------------------------------|
| **Oligonucleotides** | **Sequence** |
| ggt\textsc{CODEHOPfw-b} | GATGAAGGCGGGAATGCTaagcngc (conserved a.a. IDAA) |
| ggt\textsc{CODEHOPrw-k} | CAATTCTAATTTCATCAGGTAGCcaytgcatrtg (conserved a.a. HMQW) |
| ggt\textsc{CODEHOPfw-g} | ATCCATATTAGCTAGTGAGTCaagmgncargc (conserved a.a. MRQA) |
| ggt\textsc{CODEHOPrw-g} | ACGGCTTCATACGAGTCatgmgncargc (conserved a.a. MRQA) |
| ggt\textsc{seqCODEHOPfw-b} | GATGAAGGCGGGAATGCT |
| ggt\textsc{seqCODEHOPrw-k} | CAATTCTAATTTCATCAGGTAGC |
| ggt\textsc{seqCODEHOPfw-g} | ATCCATATTAGCTAGTGAGTC |
| ggt\textsc{seqCODEHOPrw-g} | ACGGCTTCATACGAGTC |
| 1341fw | AAGTGGACCAAAAGGAGGCTAGTC |
| 1341rw | ATCCATATTAGCTAGTGAGTC |
| 1828fw | AAGTGGACCAAAAGGAGGCTAGTC |
| 1828rv | GGTGATCTACGGTTTTGTC |
| 1341fw-RFLP | CGAAAAGAAGGGAAGGAGAGG |
| 1341rw-RFLP | TGAAAGCGTGGAGGAGAGG |
| HRAG1341pBADKpnI | AGCAGGTACGCTATATCATATCTAAACCGCTAG |
| HRAG1341fwNdel | AGCAGGTACGCTATATCATATCTAAACCGCTAG |
| HRAG1341fwHindIII | CCCAAGCTTATATCTCTCTCTGATGTCATATCG |
| HRAG1828pBADKpnI | AGCAGGTACGCTATATCATATCTAAACCGCTAG |
| HRAG1828fwNhel | CTAGTGATCTAGGAGGGTTTTAAGCGTGAG |
| HRAG1828fwHindIII | CCCAAGCTTATATCTCTCTCTGATGTCATATCG |
| HRAG1828up5PstI | ATCTCGCATATCAGGGTTTTAAGCGTGAG |
| HRAG1828up3XbaI | ATCTCGCATATCAGGGTTTTAAGCGTGAG |
| HRAG1828up5KpnI | ATGCTGCAATCGAGGGTTTTAAGCGTGAG |
| HRAG1828up3XbaI | ATCTCGCATATCAGGGTTTTAAGCGTGAG |
| U1catF2 | ATCTCGCATATCAGGGTTTTAAGCGTGAG |
| U1catR | ATGCTGCAATCGAGGGTTTTAAGCGTGAG |
| up1828 | CGTCAATTTTCATATCTCGAGCC |
| dw1828 | CTAAAGGGGAGGAGGGTTTTAAGCGTGAG |
| CatR | CTTAATTTTGAGGAGGAGGGTTTTAAGCGTGAG |
| CatL | TATGCTGAATTCATATCTCTCTCTG |

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carried out using antiserum against Bgh2 revealed the expression of the pro-form and its corresponding two subunits (Figure 5b). These results confirmed that both proteins are expressed in H. bilis and that only Bgh2 undergoes autocatalytic processing.

Complementation of E. coli Δggt

To evaluate the capability of H. bilis γGT paralogues to functionally complement an E. coli γGT-deficient strain, Bgh1 or Bgh2 (carried on plasmid pMRg3 and pMRg5, respectively) were introduced in E. coli CY128 [41]. E. coli DH5α grown in LB overnight at 25°C was used as positive control in γGT assay. Only Bgh2 was found to successfully complement E. coli CY128, while Bgh1 was unable to restore γGT activity (data not shown). The induction of pMRg3 in E. coli CY128 resulted in a clear inhibition of growth. This finding was consistent with formation of inclusion bodies, as a consequence of the overexpression of Bgh1. However, when the protein was expressed overnight at a lower temperature (15°C with low amount of inducer (0.002% instead of 0.2%), there was no inhibition of the growth rate of E. coli, suggesting a low accumulation of insoluble proteins. The successful expression of Bgh1 under improved conditions was confirmed by SDS-PAGE (data not shown). Nevertheless, Bgh1 was not able to complement E. coli Δggt, while Bgh2, expressed under the same conditions, restored γGT activity in the mutant strain.

Construction of a H. bilis mutant deficient in γGT activity

To confirm that Bgh2 is responsible of the γGT activity in H. bilis, a mutant of bgh2 was constructed in CCUG 23435 strain. The bgh2 gene was disrupted by insertion of a chloramphenicol cassette between positions 1182 and 1246, in order to delete the threonine-threonine catalytic dyad. The successful insertion of the chloramphenicol resistance cassette and the deletion of bgh2 were confirmed by PCR. RT-PCR and western blot analysis showed the expression of bgh1 in both wild-type and mutant strains (data not shown), indicating that the replacement of bgh2 had no effect on the expression of the bgh1 paralogue gene. Since complementation approaches are currently unavailable for H. bilis, another independent Δbgh2 mutant was used as a control for secondary mutations. Neither of the H. bilis mutants were able to hydrolyse the substrate analogue gGpNA. Moreover, in the supernatant of H. bilis wild-type γGT activity was determined with a gGpNA turnover of 0.48 μM/min at a concentration of 50 μg/mL of total protein. No activity was detected in the supernatant of the mutant strain MR9 (Figure S2). These results indicate that only bgh2 encodes a functional γGT in H. bilis CCUG 23435 (Hb-γGT). The mutants grew normally in vitro, as described for H. pylori γGT-deficient strains [9], indicating that γGT is not essential for survival and growth of both H. pylori and H. bilis in vitro.

Figure 3. Unrooted tree based on the amino acid sequences of Helicobacter spp. γGTs, Campylobacter jejuni γGTs and Bgh1 and Bgh2 homologues of Helicobacter trogontum and Helicobacter bilis. The evolutionary history was inferred using the Minimum Evolution method, and the evolutionary distances were computed using the Dayhoff matrix-based method. Bars indicate amino acid substitutions per position. Numbers at the nodes indicate support for the internal branches within the tree obtained by bootstrap analysis (>70%; percentages of 500 bootstraps). (A) Phylogeny of almost complete pro-enzyme sequences (447 AA). (B) Phylogeny of almost complete N-terminal γGT sequences (Heavy chain; 333 AA). (C) Phylogeny of the almost complete C-terminal γGT sequences (Light chain; 190 AA).

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Figure 4. Purity and autoprocessing of recombinant Bgh1 and Bgh2. (A) SDS-page of the purified proteins after gel filtration: Bgh1 (Lane 1) and fully autoprocessed Bgh2 (Lane 2); (B) time-line for autoprocessing of Bgh1.

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Biochemical characterization of recombinant Bgh2 (Hb-\(\gamma\)GT) and comparison with \(H.\) \(pylori\) \(\gamma\)GT (Hp-\(\gamma\)GT)

The kinetic parameters of recombinant Bgh2 (Hb-\(\gamma\)GT) were measured. The initial experiments were performed at pH 8.0. The \(K_M\) was determined by titration of the gGpNA concentrations between 1 and 1000 \(\mu\)M. The processed Hb-\(\gamma\)GT had a \(K_M\) of 7.7 ± 1.2 \(\mu\)M and a \(k_{cat}\) of 1.12 ± 0.03 \(\times\) 10^3 sec\(^{-1}\). Compared with \(K_M\) and \(k_{cat}\) values calculated for Hp-\(\gamma\)GT (\(K_M = 9.8 \pm 1.5 \mu M\) and \(k_{cat} = 17.7 \pm 0.5 \times 10^3 \) sec\(^{-1}\)), the apparent \(K_M\) value for Hb-\(\gamma\)GT was 20% lower, while the \(k_{cat}\) value was 10-fold reduced (Table 2).

The pH dependence of the Hb-\(\gamma\)GT activity was analysed and compared to Hp-\(\gamma\)GT (Figure 6). The pH profile of Hp-\(\gamma\)GT activity was similar to reported data [30] (data not shown), whereas the activity optimum of Hb-\(\gamma\)GT was shifted to a more acidic pH between 6.0 and 7.0. By contrast, the \(K_M\) was increased more than 2-fold at this pH range. Initial experiments for comparing the substrate specificity of Hp-\(\gamma\)GT and Hb-\(\gamma\)GT for glutathione, D-glutamine, L-glutamine and L-glutamic acid were performed by a competition assay. A better binding affinity to the active \(\gamma\)GT and therefore a competitive inhibition of the gGpNA-reaction would be expected for its native substrates. As previously described [9,12], Hp-\(\gamma\)GT substrate inhibition was observed for both by glutathione and L-glutamine, whereas Hb-\(\gamma\)GT was only inhibited by L-glutamine (Figure S3).

**Table 2.** Comparison of the kinetic constants for \(Helicobacter\) \(bilibis\) \(\gamma\)GT (Hb-\(\gamma\)GT) and \(Helicobacter\) \(pylori\) \(\gamma\)GT (Hp-\(\gamma\)GT).

|          | \(k_{cat}\) \([10^2 \text{ min}^{-1}]\) | \(K_M\) [gGpNA] \([\mu\text{M}]\) |
|----------|--------------------------------------|-------------------------------|
| Hp-\(\gamma\)GT | 17.7 ± 0.5                            | 9.8 ± 1.5                    |
| Hb-\(\gamma\)GT | 1.12 ± 0.03                           | 7.7 ± 1.2                    |

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Inhibition of human T-cell and AGS cell proliferation

To evaluate biological properties of recombinant Hb-\(\gamma\)GT and compare these with effects already observed for Hp-\(\gamma\)GT, the ability of Hb-\(\gamma\)GT to inhibit human T-cell and AGS cell proliferation was assessed. Recombinant Hb-\(\gamma\)GT efficiently inhibited the proliferation of Jurkat cells in a dose-dependent manner (Figure 7a). Concentrations of Hb-\(\gamma\)GT as low as 0.1 \(\mu\)g/mL induced inhibition of T-cell proliferation. Comparison of the inhibitory potential of supernatants of wild-type and \(A_\Gamma\)gt strains confirmed the results obtained with the recombinant proteins. The supernatants of \(H.\) \(bilis\) wild type reduced T-cell proliferation up to 60%, while the supernatant of \(A_\Gamma\)gt strain had no significant inhibitory activity. When Jurkat cells were infected with acivicin pre-treated supernatant of \(H.\) \(bilis\) wild type, no significant reduction in proliferation of T-cells was observed, indicating that only Hb-\(\gamma\)GT is responsible for the inhibition of T-cell proliferation in our system (Figure 7b).

Moreover, both recombinant Hb-\(\gamma\)GT and Hp-\(\gamma\)GT showed inhibitory effect on AGS cell proliferation in a dose-dependent manner as well, although the minimum concentration of protein required was 25 time higher (2.5 \(\mu\)g/mL) compared to that able to inhibit the proliferation of human T-cells (Figure 8). No statistically significant differences were observed between the two proteins.

Apoptosis in AGS cell line

The ability of recombinant Hp-\(\gamma\)GT and Hb-\(\gamma\)GT to induce apoptosis in AGS cells was evaluated using AnnexinV-Propidium iodide double staining and FACS analysis. After 24 h of incubation, concentrations of up to 5 \(\mu\)g/mL of both recombinant proteins did not induce apoptosis in AGS cells (Figure S4). The average of percentage of apoptosis observed after treatment with Hp-\(\gamma\)GT and Hb-\(\gamma\)GT was 6.9 ± 1.2% and 6.8 ± 0.9%, respectively. Those values were not statistically different from the percentage of apoptosis measured in the untreated cell (7.3 ± 1.9).

Discussion

Two \(Helicobacter\) \(\gamma\)GT genes, identified in the shotgun genome sequence of the human strain \(H.\) \(bilis\) ATCC 43879 on the basis of sequence homology, were subjected to functional analysis to
confirm their annotations. Although the two genes bgh1 and bgh2 (H. bilis ggt homologue 1 and 2) showed significant homology and both are classified as T03.01 peptidases in MEROPS, the evaluation of conserved amino acid residues indicated a marked difference between these two paralogues, especially in the substrate-binding pocket. In fact, all critical functional residues are present in Bgh2, as well as in all the other Helicobacter γGTs, but not in Bgh1. The amino acid substitutions observed in Bgh1 could potentially affect the enzyme maturation, catalytic activity and substrate specificity, suggesting different biochemical properties. Moreover, the absence of a signal peptide in Bgh1, in contrast to observations for Bgh2 and for the other Helicobacter γGTs, suggests a different sub-cellular localization of this protein.

We first evaluated the phylogeny of Helicobacter γGTs in relation with T03 proteases of other bacterial species. The Minimum Evolution tree clearly showed that both H. bilis T03.01 peptidase paralogues were phylogenetically associated with other e-proteobacteria γGTs. Nevertheless, Bgh1 formed an independent branch, indicating a different evolution. Moreover, among Helicobacter species, only H. trogontum showed two distinct γGT paralogues, as observed in H. bilis. In addition, the data suggested that both genes are not restricted to H. bilis ATCC 43879, but are common features among H. bilis genomospecies and universally shared by all members of the taxon.

A more detailed phylogenetic analysis further revealed that the two subunits of γGTs have followed different evolutionary paths. The tree based on the C-terminal part resembled the topology of the tree of 16S rRNA and other housekeeping genes and includes H. bilis in the same branch as other enterohepatic Helicobacter spp. [38]. On the contrary the phylogenetic analysis of the N-terminal part separates the γGTs of H. bilis and related species (H. aurati, H. muridarum and H. trogontum) from others enterohepatic Helicobacter species. On the basis of our phylogenetic analysis we can hypothesise that differing phylogeny of the subunits reflects a possible divergence of substrate specificity between the γGTs of H. bilis and related species and the other Helicobacter or Campylobacter γGTs. More studies are needed to verify this hypothesis.

In the trees based on the sequences of both subunits, the position of the clade including the orthologues of bgh1 indicated that the paralogues potentially evolved by gene duplication from a common ancestral sequence probably before the speciation event of H. bilis and related species. The hypothesis of gene duplication is well supported also by the low Ka:Ks ratio between the two paralogues (0.175), indicating that the two genes are under strong purifying selection [42].

Both sequence and phylogenetic analyses reveal clear differences between the two H. bilis T03.01 peptidases paralogues. To compare their biochemical properties, both genes were overexpressed in E. coli and the recombinant proteins were purified by affinity chromatography. The overexpression of Bgh1 resulted in formation of inclusion bodies due to the accumulation of an insoluble protein of ~70 KDa. After solubilisation and refolding, recombinant Bgh1 showed no significant maturation into two subunits and, subsequently, no γGT activity. In contrast, recombinant Bgh2 was expressed in soluble form as a pro-enzyme of ~60 KDa, which undergoes autocatalytic processing to generate two subunits of ~40 and ~20 KDa and showed γGT activity. No clear molecular evidence explains the absence of an autocatalytic process for Bgh1 since all of the amino acid residues critical for Hp-γGT [2,43] and Ec-γGT [44,45] autoprocessing...
are also conserved in Bgh1. We, therefore, cannot exclude that the absence of maturation could be due to misfolding in vitro. However, the CD spectrum of purified Bgh1 showed that the protein has a defined set of secondary structures indicating a non-random folding of the recombinant protein. Moreover, Bgh1 was unable to restore γGT activity after complementation of E. coli Δgtt mutant. Furthermore, the H. bilis strain in which bgh2 was disrupted but still able to transcribe bgh1 resulted in deficient γGT activity. This data clearly indicate bgh1 does not encode a functional H. bilis γGT. As reported previously, the single amino acid substitution D433N converts Ec-γGT to a class IV cephalosporin acylase [46]. Since in Bgh1 the same substitution having more than 10-fold less kcat. Moreover, in contrast to that of Hp-γGT, the inhibitory effect of T-cell proliferation was completely abolished in the γGT-deficient mutants of H. bilis. Our data demonstrate that this activity is not limited to H. pylori but is conserved among the genus, confirming the potential immune suppressive role of secreted Helicobacter γGTs. The conserved function of this enzyme among Helicobacter spp. with different niches and host specificity opens a new scenario on the role of γGT in the pathogenesis of Helicobacter associated diseases.

We detected AGS cell proliferation inhibition after treatment with both recombinant γGTs, as observed for T-cells, but only when a high concentration was used (2.5 μg/mL). However, in contrast to what was previously described [10], we showed that the suppressive influence of both Hb-γGT and Hp-γGT on AGS cells was mediated by an apoptosis-independent mechanism. The discrepancy between our results and those of Shibayama and colleagues [10] could attribute to different methodologies applied, in particular to the use of serum starvation by these authors. An increase of apoptosis was verified in AGS cells treated with both Hp-γGT and Hb-γGT under the same conditions used by these authors (data not shown), apparently confirming the apoptosis inducing potential of these enzymes. However, if this phenomenon is only detectable upon stress conditions (i.e. serum starvation), we cannot exclude that the observed apoptosis is a consequence of a nonspecific activity of γGT. Further studies are needed to establish the real role of γGT in the apoptosis of epithelial cells.

Methods

Bacterial strains, cell line, culture condition and DNA extraction

The bacterial strains and plasmids used in this study are listed in Table 2 and 4. All Helicobacter strains were routinely grown on HP medium (LabM Limited, Lancashire, UK), containing 5% bovine blood at 37°C in a microaerobic atmosphere (10% CO2; 5% O2). (Thermo Forma, Series II Water JACKETED INCUBATOR; Thermo Fisher Scientific, Waltham, MA USA). E. coli strains were cultivated on Luria-Bertani (LB) agar or broth supplemented with 100 mg/L of ampicillin, 15 mg/L of kanamycin or 20 mg/L of chloramphenicol when needed. Jurkat T-cells (DSMZ, ACC 282, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were cultured in RPMI 1640 (Invitrogen Corporation, Carlsbad, CA, USA) with 10% of foetal bovine serum (FBS; Gibco, Invitrogen). AGS cells (CLS, 300408; Cell Lines Service, Eppelheim, Germany) were grown in Dulbecco’s Modified Eagle's growth medium (DMEM high glucose, 11971, Invitrogen) supplemented with 10% FBS and 200 U/mL of penicillin and 0.2 mg/mL of streptomycin (Pen Strep, Gibco, Invitrogen). Cell lines were incubated at 37°C with 5% CO2. Bacterial DNA extraction was performed as described earlier [19].

PCR, cloning and sequencing

Consensus DEgenerate Hybrid Oligonucleotide Primers [iCO-DEHOP [52]] were designed on the basis of highly conserved amino acid sequences of available Helicobacter and C. jejuni γGTs. Standard PCR was done in a 50 μL reaction mixture containing 50 ng of DNA template, 25 pmol of each primer and 1.25 U of DyNAzyme™ (Finzymes Oy, Espoo, Finland). The 5’ consensus
### Table 3. Helicobacter species used in this study.

| Species          | Strain designation and genotype | Hosts                  | Reference |
|------------------|---------------------------------|------------------------|-----------|
| **Gastric Helicobacter spp.** |                                 |                        |           |
| *H. acinonychis*  | Sheeba [ggt, Hac_0598]          | Cheetah; Big Felines   | [60]      |
| *H. bizzozeronii* | CIII-1 [ggt, HBZC1_08080]       | Dog; Human             | [61]      |
| *H. felis*       | ATCC 49179 [ggt, Hfelic_06880]  | Cat                    | [62]      |
| *H. mustelae*    | 12198 [ggt, HMU08020]           | Ferret                 | [63]      |
| *H. pylori*      | 26695 [ggt, HP1118]             | Human                  | [64]      |
| *H. salomonis*   | O6A [ggt, EMBL FR821684]        | Dog                    | [38]      |
| *H. suis*        | HS1 [ggt, HSUH51_0265]          | Pig                    | [65]      |
| **Enterohepatic Helicobacter spp.** |                              |                        |           |
| *H. aurati*      | ATCC BAA-1T [ggt EMBL FR821682] | Hamster               | [38]      |
| *H. bilis*       | CCUG 23435 (= ATCC 43879) [ggt, HRAG_01828; bgh1, HRAG_01341] | Mouse; Human; Dog; Cat; Pig; Sheep | [19] |
| MR9              | (= CCUG 23435 D bgh2)          |                        |           |
| ATCC S1630T      | [bgh1 homolog EMBL FR821680]   |                        |           |
| ATCC 49314       |                                 |                        |           |
| ATCC 49320       |                                 |                        |           |
| KO220            | [bgh1 homologue EMBL FR821686]  |                        |           |
| KO214            | [bgh1 homologue EMBL FR821687]  |                        |           |
| KO794            | [bgh1 homologue EMBL FR821688]  |                        |           |
| FL56             | [ggt EMBL FR821679] [bgh1 homologue EMBL FR821676] | |           |
| *H. canis*       | NCTC 12739T [ggt EMBL FR821681] | Dog; Cat               | [66]      |
| *H. muridarum*   | CCUG 29262 (= ATCC 49282) [ggt EMBL FR821683] | Mouse                | [38]      |
| *H. trogontum*   | ATCC 700114T [ggt EMBL FR821678] [bgh1 homologue EMBL FR821677] | Rat; Mouse; Pig       | [38]      |

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### Table 4. E. coli strains and plasmids used in this study.

| Strains or plasmids | Relevant characteristics or genotype | Reference |
|---------------------|-------------------------------------|-----------|
| **Escherichia coli strains** |                                       |           |
| DH5s                | F− endA1 glnV44 thi-1 relA1 gyrA96 deoR nupG Φ80d lacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK mK) Δλ− | [67]      |
| BL21 Rosetta 2     | F− ampT hsdS(rK mK)− gal dcm (DE3) pLysSα and (CamF) | Novagen   |
| JM109              | endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB Δ(lac-proAB) e14- [F− traD36 proAB ΔlacZΔM15 hsdR17(rK mK)−] | Promega   |
| CY128              | DH5s but Δggt ΔampC                    | [41]      |
| **Plasmids**       |                                       |           |
| pGEMH-T            | Promega                              |           |
| pet28b(+)          | Novagen                              |           |
| pBAD24             | pMB1/M13 replicon, Ap+, AraC+, terminator (mB8), promoter ParaBAD | [57]      |
| pUC119             | pMB1/M13 replicon, Ap+, lacZΔM15      | [68]      |
| pUOA14             | pMB1/M13 replicon, pIP1445 replicon, lacZΔM15, Ap+, Km+ | [58]      |
| pMRg1              | pet28b(+), His6-Bgp1Δ                 | this study |
| pMRg2              | pet28b(+), His6-Bgp2Δ                | this study |
| pMRg3              | pBAD24, Bgp1Δ                        | this study |
| pMRg5              | pBAD24, Bgp2Δ                        | this study |
| pMRg9              | pUC119, bgp2::cam+                    | this study |

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clamp region contains the sequencing primers used for direct sequencing of the PCR products. If secondary peaks or multiple sequences were present, the PCR products were inserted into pGEM®-T and sequenced on both strands using M13 primers. The area corresponding to nucleotides 702–1569 (amino acids 234–525) of bg1 was used to design specific primers for RFLP-PCR protocol. The PCR was done in a 50 µL reaction mixture, as described above, and then the products were digested by BclI (NEB, New England Biolabs, Ipswich, MA, USA). BclI specifically cuts the PCR fragment in two pieces of 606 and 260 bp. EMBL accession numbers are listed in Table 3. All oligonucleotides used in this study are listed in Table 1.

Phylogenetic analysis
Phylogenetic analyses of γGT amino acid sequences were conducted in MEGA4 [53] using the Minimum Evolution (ME) method. The amino acid sequences were aligned using MAFFT [54]. The evolutionary distances were computed using the Dayhoff matrix-based method, and the ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (level 1). All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons [53]. A multi-alignment of γγ sequences of Helicobacter spp and C. jejuni was built in MEGA4 on the basis of the amino acid sequence alignment. Maximum likelihood (ML) trees were constructed using PHYML [55] with a nucleotide evolution model selected by FindModel (http://www.hiv.lanl.gov/). Trees were visualized by iTOL [56]. Non-synonymous/synonymous substitution (Ka:Ks) was evaluated by sliding window analysis (window size of 50 bp) using SWAKK [37].

Expression of Bgh1 and Bgh2 and purification of recombinant proteins
Primers were designed to amplify complete sequences of bg1 and bg2 genes using Phusion® High-Fidelity DNA Polymerase (Finzymes). Bgh1 and Bgh2 were expressed as 6×His-tagged proteins by inserting the genes respectively in Ndel and HindIII or Ndel and HindIII restriction sites of pet28b+ vector (Novagen®, Merck KGaA, Darmstadt, Germany). The 6×His-tag was fused in the N-terminal part of the proteins. The resulting expression constructs pMRg1 and pMRg2 were sequenced and confirmed to be identical to bg1 and bg2, respectively. Both expression constructs were used to transform E. coli Rosetta 2 (Novagen®). Expression of recombinant protein was induced with 0.5 mM IPTG for 4 h at 27°C. After centrifugation, pellets were solubilized in ice-cold binding buffer (50 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, pH 7.4) containing protease inhibitors and then lysed by subsequent sonication (5 times for 30 s) on ice. The supernatant was loaded on the HisTrap™ column (GE Healthcare Biosciences, Pittsburgh, PA, USA) at 1 mL/min at 4°C, and bound protein was eluted with an imidazole-gradient (0–

1000 mM) using HEPES buffer with 1000 mM imidazole. Eluate was collected and then tested for purity by SDS-PAGE. For further purification, fractions with the recombinant proteins from Ni-Sepharose affinity chromatography were pooled, dialysed overnight against 100 mM HEPES, 140 mM NaCl, 2.5% Sucrose pH 7.0, at 4°C and processed to the second and final purification step. The dialysed samples were loaded on a SuperdexTM 75 column (GE Healthcare) and eluited with 100 mM HEPES, 140 mM NaCl, 2.5% Sucrose pH 7.0. All fractions collected were analysed by SDS-PAGE for the presence of recombinant proteins. Fractions containing the protein were pooled, aliquoted and stored at −80°C until further use.

Circular Dichroism (CD) spectrum of the recombinant Bgh1
To estimate the secondary structure of Bgh1 a CD spectrum of the recombinant protein was calculated. The measurements were performed on a JASCO J-810 spectropolarimeter equipped (Jasco) according to the manufacturer's instructions. Non-synonymous/synonymous substitution (Ka:Ks) was evaluated by sliding window analysis (window size of 50 bp) using SWAKK [37].

Immunization of mice and Western-Blot on whole H. bilis lysate
Balb/c mice (3 mice per group) were immunized i.p. with 30 µg of antigen and 10 µg Cholera toxin (CT) (Sigma-Aldrich) as adjuvant at days 0, 7 and 14 (control group with CT only). Sera were taken 21 days after the last immunization and stored at −20°C. Animal experiments were performed according to the guidelines of the Bavarian Ministry of Animal Wealth Fare. For evaluation of Bgh-specificity, ELISA plates were coated with 2 µg/mL recombinant Bgh1 and Bgh2 proteins in PBS pH 7.4 overnight at 4°C. After washing (4×0.01% Tween-20 in PBS) and blocking (1% BSA in PBS), different dilutions of sera (in blocking buffer) were incubated for 1 h at 37°C. HRP-conjugated anti-mouse IgG antibody (1:3000) (Promega Corporation, Fitchburg, WI, USA) was incubated (1 h at 37°C) and detected with ELISA Opt ELATM (BD, Becton, Dickinson and Co., NJ, USA) according to the manufacturer’s instructions.

For the western blot, H. bilis overnight culture was resuspended in 1 mL BHI (BD). Following lysis (sonication 4×30 sec), the protein-concentration in clarified lysates was determined by BCA-Assay and adjusted to 1 mg/mL. Per lane 5 µg of total protein were loaded. After blocking with 5% BSA in Tris-buffered saline (TBS), the Whatman Protran® Nitrocellulose membranes (GE Healthcare) were incubated for 1 h at RT with the sera of the immunized mice (1:800 diluted in TBS). Following washing, HRP-conjugated secondary anti-mouse IgG antibody (Promega) (diluted 1:3000 in TBS) were added and detected by the acidic-based ECL-system (Thermo Fisher Scientific).

Complementation of E. coli Δggt
bg1 and bg2 were amplified as described above and inserted between KpnI and HindIII restriction sites of pBAD24 expression vector [57], resulting in pMRg3 and pMRg5,
respectively. Both expression constructs were used to transform E. coli CY128 Δggt. The recombinant strains were grown in LB medium with ampicillin at 37 °C to OD600=0.4, and the expression of the corresponding gene was induced with 0.2% L-arabinose for 4 h at 25 °C. The basal expression of pMRg5 and pMRg5 vectors was inhibited by using 0.2% of glucose instead of L-arabinose. Expressions were verified by SDS-PAGE electrophoresis and Coomassie Blue stain. A total of 10^9 cells were harvested for renaturation and subsequent SDS-PAGE analysis. 

Construction of *H. bilis* isogenic mutant

Chromosomal inactivation of *bgh2* was performed in *H. bilis* CCUG 23435. Deletion was introduced by allelic exchange using vector pUC119 in which ~1100 bp of the 3’-end and ~500 bp of the 5’-end of the target gene and the chloramphenicol resistance gene from pUOA14 [58] were cloned. The resultant plasmid, pMRg5, was constructed and amplified in *E. coli* DH5α and used as a suicide plasmid in *H. bilis* CCUG 23435. *H. bilis* mutant was obtained by electroporation as described for *H. heparicus* [59]. The mutant strain, *H. bilis* MR9 (*bgh2*:cam), was selected on an HP medium containing 5% bovine blood supplemented with chloramphenicol (20 mg/mL). The site of recombination was verified by PCR.

RNA extraction and RT-PCR

To analyse the expression of both *bgh1* and *bgh2* during growth, *H. bilis* wild-type CCUG 23435 was cultivated in Brain Heart Infusion (BHI; BD, NJ USA) containing 20% FBS and Vitox supplement (Oxoid Ltd., Cambridge, UK) at 37 °C microaerobically by continuous shaking at 150 rpm. OD600 was measured at 8, 16 and 24 h and the same amount of cells was treated with RNAprotect Bacteria Reagent (Qiagen GmbH, Hamburg Germany). RNA was extracted using RNeasy Mini Kit (Qiagen) and treated with TURBO DNase™ (Applied Biosystems/Ambion, Austin, TX, USA). Moreover, *H. bilis* CCUG 23435 and the mutant MR9 were cultivated in YT×2 medium (16 g/L Tryptone, 10 g/L Yeast Extract, 5 g/L NaCl) containing 10% FBS at 37 °C in a microaerobic atmosphere. After 24 h RNA was extracted as described above. cDNAs were synthesized from 1 μg of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen) using random hexamers (Finzymes). RT-PCR reactions were carried out in a total volume of 50 μL as described above, using 1 μL of cDNA and 1 μL of RNA as negative control and 20 pmol of specific primers for *bgh1* and *bgh2*. Thermocycling conditions were 94 °C for 2 min followed 40 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min. The PCR products were sequenced using the same primers.

T-cell and AGS proliferation assay

For measurement of the effect of *H. bilis* γGT on T-cell proliferation, Jurkat cells were seeded at 5 x 10^4 per well in a complete culture medium in 96-well microtiter plates and a variable amount of protein (0.001 to 10 μg/mL) or sterile-filtrated culture supernatant (1 μg/mL total protein) was added in a total volume of 100 μL. As a control for the inhibition of T-cell proliferation, the recombinant γGT from *H. pylori* 26695 (HP-γGT) was used. The HP-γGT was produced as previously described [9]. Additionally, supernatant were preincubated with 50 μM Acivicin for 30 min at 37 °C to inactivate the γGT. Cells were incubated at 37 °C for 48 h and analysed for proliferation as described previously [9]. Results are expressed as the mean±SEM from three experiments.

For the measurement of the effect of recombinant *H. bilis* and *H. pylori* γGT on proliferation of AGS cells, 5 x 10^5 cells per well were seeded in culture medium in a 96-well plate and treated with 0.5–5 μg/mL of protein in a total volume of 100 μL. Cells were incubated at 37 °C for 48 h and analysed for proliferation as described above.

**Apoptosis in AGS cells**

Apoptosis in gastric epithelial cells was determined by Fluorescence-activated cell sorting (FACS). AGS cells were seeded in a 6-well plate till they reached a confluence of 60–70%, and treated for 24 hours with 0.5–5 μg/mL of purified recombinant γGT proteins from *H. pylori* and *H. bilis* in DMEM with 10% of FBS. Untreated cells were used as a negative control and cells treated with 10 μM of Staurosporine (LC laboratories, Woburn, MA, USA) as a positive control for apoptosis. Cells were harvested with 0.25% Trypsin-EDTA (Invitrogen) and 2 x 10^5 cells were washed once with PBS. The pellets were resuspended in 10 μg/mL of AnnexinV–FITC antibody (BD, Becton, Dickinson and Co., NJ USA) in FACS buffer and incubated for 20 minutes in dark at room temperature. Cell suspensions were then diluted with FACS binding buffer and 10 μl of 50 μg/mL of Propidium Iodide (Sigma-Aldrich, Inc. St. Louis, MO, USA) was added before acquiring samples. Cell debris was excluded by scatter gating (forward vs. side scatter). Approximately 50,000 events were collected per sample using CyAn ADP FACS cytometer (Beckman Coulter, Inc., CA, USA) and data was analysed with FloJo software (Tree Star, Inc, Oregon, USA). The percentage of apoptotic cells is expressed as the double positive cell population (AnnexinV+PI) and pre-apoptotic cells stained only for AnnexinV.

**γGT enzymatic and competition assays**

For determination of γGT activity, ~10^9 bacteria were suspended in 400 μL of reaction buffer containing 1 mM of L-γ-glutamyl-p-nitroanilide (gGpNA) as described previously [6]. Additionally, culture supernatants, containing 50 μg/mL protein were analysed by diluting 50 μL of supernatant in 150 μL of reaction buffer. The kinetic parameters for the enzymatic reaction of the recombinant proteins were measured by following the cleavage of gGpNA. To determine the exchange rate of the substrate to 4-p-nitroaniline, the reaction was monitored at 205 nm and calculated by using the reported extinction coefficient of 8800 M/cm. Enzymatic activity was measured in 0.1 M Tris HCl with a pH range from 5.0 to 9.0, 20 mM glycyl-glycine and gGpNA at a concentration range from 1 μM to 2.5 mM. The experiments were carried out at 37 °C for 30 min using a Mithras LB940 Well-Reader. The initial rates were determined at each gGpNA concentration and entered in the Michaelis-Menten equation. To investigate the affinity of the postulated γGT-substrates (i.e. L-Glutamine, D-Glutamine, L-Glutamic acid and Glutathione), we made a competition experiment in which the hydrolysis and transpeptidation of gGpNA was analyzed by measuring the absorption at 405 nm in the presence of γGT-substrates. For this assay we used 250 ng of the Hb-γGT or Hp-γGT per reaction with 100 μM gGpNA, 10 mM GlyGly and the substrates in a concentration-range between 0.5–5 mM in 100 mM Tris/HCl pH 8.0. The measurement was performed for 15 min at 37 °C in a total volume of 200 μL.

**Supporting Information**

**Figure S1** CD spectrum and photomultiplier voltage for recombinant Bgh1. (A) CD spectrum for recombinant Bgh1 (black line), the predicted spectrum for Bgh1 obtained by K2D3 (red line) and lysozyme (blue line). The spectrum for lysozyme was taken from the Protein Circular Dichroism Data Bank (http://
pcddb cryst.bbb.ac.uk/~. (B) Photomultiplier voltage of the spectrum for recombinant Bglh.

**Figure S2** Rate of γGpNA turnover of culture supernatant (50 μg/mL total protein) of Helicobacter bilis wild-type CCUG 23435 (WT) and H. bilis Aggt MR9 (Aggt). The white bars show the results for untreated culture supernatants, while the black bars show the results after treatment of culture supernatants with 50 μM of Acivicin.

(TIF)

**Figure S3** γGT-activity competition with glutathione, L-/D-Glutamine and L-Glutamic Acid. Error bars in the graph were calculated as SEM. The analysis was performed using Prism v.4.03 (GraphPad Software). Inhibitory effect with various amounts of substrate analogues on (A) Helicobacter bilis γGT (Hb-γGT) and (B) Helicobacter pylori γGT (Hp-γGT).

(TIF)

**Figure S4** Apoptosis analysis of AGS cells after 24 hour treatment with γGT recombinant proteins. AnnexinV and PI staining of AGS cells analysed by Flow cytometer treated with 0.5, 0.25 and 5 μM of recombinant proteins. Helicobacter bilis (Hb-γGT), and Helicobacter pylori γGT (Hp-γGT), compared to untreated cells and cells treated with staurosporin.

(TIF)

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