γ-glutamyl transpeptidase has a role in the persistent colonization of the avian gut by *Campylobacter jejuni*

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Abstract

The contribution of γ-glutamyl transpeptidase (GGT) to *Campylobacter jejuni* virulence

and colonization of the avian gut has been investigated. The presence of the *ggt* gene in *C. jejuni* strains directly correlated with the expression of GGT activity as measured by cleavage and transfer of the γ-glutamyl moiety. Inactivation of the monocistronic *ggt* gene in *C. jejuni* strain 81116 resulted in isogenic mutants with undetectable GGT activity, nevertheless these mutants grew normally *in vitro.* However, the mutants had increased motility, a 5.4-fold higher invasion efficiency into INT407 cells *in vitro* and increased resistance to hydrogen peroxide stress. Moreover, the apoptosis-inducing activity of the *ggt* mutant was significantly lower than that of the parental strain. *In vivo* studies showed that, although GGT activity was not required for initial colonization of one-day-old chicks, the enzyme was required for persistant colonization of the avian gut

Keywords: Campylobacter jejuni; γ-glutamyl transpeptidase (*ggt*); avian colonization

1. Introduction

Campylobacter jejuni is found in the intestinal tracts of a large number of food-producing animals, but appears to be adapted to the avian gut [1,2,3]. Colonization with *C. jejuni* in poultry is asymptomatic. However, infection with this organism in humans is a common cause of acute bacterial enteritis particularly in industrialized countries [4,5]. It is commonly assumed that the handling of contaminated poultry carcasses, and the consumption of undercooked poultry meat, are major sources of campylobacteriosis.

Quantitative risk assessment has demonstrated that the control and prevention of human campylobacteriosis will be best achieved by intervention to reduce or eradicate colonization at the poultry flock level [6]. Although improved biosecurity may contribute to this, it seems likely that complementary methods, such as vaccination or decontamination, may also be required [2, 7]. Such targeted intervention strategies require fundamental understanding of those bacterial factors associated with the campylobacter properties enabling colonization and survival. To date approaches to the identification of such factors have largely been pragmatic with genes selected for characterization on the basis of previously published observations in other enteric bacteria. However, with the availability of the genome sequence of the *C. jejuni* reference strain NCTC11168 [8], post-genomic approaches are now being adopted [9, 10, 11]. In one such approach genomic subtractive hybridization between two strains, 81116 and NCTC11168 was undertaken to identify genes present in 81116 but absent in NCTC11168 [11], which is poorly able to colonise chickens. More recent studies have shown that the ability of the NCTC11168 isolate to colonize birds is highly dependent on

the variant used [9, 10]. Thus colonization is not only due to the presence or absence of genes but also whether those genes are transcribed. Nevertheless, given the difference in colonization potential between strains 81116 and NCTC11168, it has been speculated that such novel genes could contribute to aspects of colonization in chicks [12, 13, 14, 15, 16]. One such novel genes was a 369 bp DNA fragment (insert 236), unique to strain 81116, and which was identified and predicted to be part of a *C. jejuni* γ-glutamyl transpeptidase (*ggt*) gene [11].

The enzyme γ-glutamyl transpeptidase (GGT) is present in both prokaryotes and eukaryotes. Most of our knowledge is based on studies in mammalian tissues where GGT is a membrane-bound enzyme that plays a major role in glutathione (L-γ-glutamyl-L-cysteineglycine) degradation in the γ-glutamyl cycle [17]. GGT cleaves and transfers the γ-glutamyl moiety from glutathione to amino acids or peptides. Glutathione is an antioxidant molecule, which plays an important part in providing vital cellular protection against the reactive oxygen species (ROS), such as hydrogen peroxide, generated by aerobic respiration [18]. Thus in eukaryotes, GGT-dependent breakdown of glutathione aids maintenance of cellular glutathione levels and increased cellular resistance to hydrogen peroxide-induced injury [19]. Not surprisingly, eukaryotic cells, once depleted of glutathione, have an increased susceptibility to oxidant-mediated killing [20, 21]. Therefore GGT appears to have an important role in combating oxidative stress.

A number of GGT-encoding genes from both mammalian and bacterial species have been sequenced and share extensive amino acid homology. However, there are two major taxa-

associated differences. Firstly, the N-termini of the bacterial GGTs are signal peptides, and consequently the enzyme is thought to be either periplasmic or associated with the inner membrane [22]. In contrast, the N-termini of mammalian GGTs are anchor domains, which interact with plasma membranes [17, 23]. Secondly, mammalian GGT is glycosylated [17]. In contrast bacterial GGTs are not known to be glycosylated. However, *C. jejuni* is unusual in that protein glycosylation pathways are active [24] and therefore the *C. jejuni* GGT could be glycosylated. Both bacterial and mammalian GGTs are first translated as precursor proteins, which in prokaryotes then undergo two proteolytic cleavages, the first of which results in cleavage of the signal peptide and the second processes the GGT to form the two subunits of the enzyme.

In *Helicobacter pylori*, GGT is involved in colonization of the gastric mucosa of mice [25, 26, 27], potentially by participating in the *de novo* synthesis of essential amino acids and thus enabling survival *in vivo*. In addition, GGT also possesses an apoptosis-inducing activity [28] and upregulates COX-2 and EGF-related peptide expression in human gastric cells [29]. Interestingly, in *Neisseria meningitidis,* GGT appears to provide an advantage for bacterial multiplication during environmental cysteine shortage by supplying cysteine from environmental peptides [30]. GGT is also involved in osmoadaptation in *Escherichia coli* [31].

In this study, the *ggt* gene from *C. jejuni* strain 81116 has been identified, fully sequenced and an isogenic mutant generated. The effect of this mutation on GGT activity and bacterial growth rate, motility, resistance to hydrogen peroxide stress, rate of

84 internalization into INT407 cells, apoptosis and avian gut colonization, have been
85 investigated.
86
87 **2. Materials and Methods**
88 ***2.1 Bacterial strains***
89 *C. jejuni* strain 81116 was isolated from a patient with diarrhea during a water-borne
90 outbreak [32]. Strains NCTC11168 and 81-176 were both derived from human cases of
91 campylobacteriosis [8, 33]. Other isolates included in this investigation were randomly
92 selected from the collection of *C. jejuni* strains held at the Veterinary Laboratories
93 Agency (Weybridge), including isolates from the faecess of humans (n=32), broilers
94 (n=34), cattle (n=32), pigs (n=15), sheep (n=18), ostrich (n=1), and dog (n=6), as well as
95 isolates from in and around poultry broiler houses (n=4).
96
97 ***2.2 Media and growth conditions***
98 All *C. jejuni* strains were cultured on 10% (vol/vol) sheep blood agar plates containing
99 250 μg actidione ml^{-1} and Skirrow's supplement (10 μg of vancomycin ml^{-1}, 2.5 IU of
100 polymyxin B ml^{-1}, and 5 μg of trimethoprim) (BASA) [34] at 42^{o}C in a microaerobic
101 environment (7.5% [vol/vol] CO_2, 7.5% [vol/vol] O_2, and 85% [vol/vol] N_2 for 20 or 40
102 hours. *Escherichia coli* strain TOPO10F' (Invitrogen) was grown on Luria-Bertani (LB)
103 (Difco) agar or LB broth at 37 ^{o}C under atmospheric conditions. When required, the
104 media was supplemented with 100 μg ampicillin ml^{-1} or 50 μg kanamycin ml^{-1}. *C. jejuni*
105 and *E. coli* were stored as frozen cultures at -80 ^{o}C in 1% (w/v) proteose peptone water

(Difco) containing 10 % (v/v) glycerol or LB broth containing 50 % (v/v) glycerol [35], respectively.

For assessing growth rates, strains were grown in biphasic broths (100 ml of brain heart infusion agar with 1 % (w/v) yeast extract (BHI/YE) overlaid with 200 ml of BHI/YE broth). Viable counts of bacteria were taken after 0, 4, 7, 22, 30 and 50 hours of growth.

2.3 PCR and DNA sequencing

PCR amplifications were performed using HotStar*Taq* master mix (Qiagen) as recommended by the manufacturer. For colony PCR, a loopful of freshly grown bacteria was suspended in 200 µl of water and 1 µl of the suspension was directly used (without prior boiling) as template with HotStar*Taq* (Qiagen). Degenerate PCR and chromosomal walking were performed as previously described [36, 37]. Sequencing was carried out on duplicate samples with BigDye Terminator mix (Applied Biosystems) according to the manufacturer's instructions, the sequences analyzed using an ABI 3700 DNA sequencer (Applied Biosystems). Contigs were assembled using the SeqMan program and protein molecular mass calculated by the Protean Program (Lasergene version 5; DNAstar Inc., Madison USA). The SignalP program at www.cbs.dtu.dk/services/signalP/ was used to identify the signal sequence.

2.4 Construction of ggt mutant

The standard and recombinant vectors and oligos used in this study are listed in Table 1. All standard methods of DNA manipulation were performed according to the protocols of

Ausubel *et al.* [35]. The *ggt* gene in *C. jejuni* strain 81116 was disrupted by insertional mutagenesis with a kanamycin cassette. Briefly, *ggt* was amplified from genomic DNA isolated from *C. jejuni* strain 81116 using oligo primers If50 and If66 (Table 1b). The 1.5 kbp DNA fragment generated was cloned into pCR-Script^{TM}SK(+). The *ggt* gene was mutated using inverse PCR oligos If67 and If68 (Table 1b), which introduced a unique *Bgl*II restriction site [38] 532 bp downstream from the *ggt* start codon. The PCR fragment was digested with *Bgl*II and self ligated. The resulting plasmid was digested with *Bgl*II and a kanamycin resistance gene with *Bam*HI ends from pJMK30 (kindly provided by J. Ketley, University of Leicester) was inserted and the kanamycin cassette was in the opposite orientation to *ggt*. The suicide vector pIHA-*ggt* was introduced into *C. jejuni* strain 81116 by electroporation, as previously described [38].

2.5 In vitro phenotypic studies

All the phenotypic assays were carried out in triplicate. Experiments were performed three times for verification. Statistical analysis of the data was carried out using GraphPad Prism™ software version 2.01 (San Diego, CA, USA).

Thermotrace method. The 'GGT reagent' (Alpha Laboratories Ltd) was used, as recommended by the manufacturer, to spectrophotometrically determine the specific activity for GGT. The protein concentration of whole *C. jejuni* cells (~10^7-10^9 cfu), disrupted by freeze-thawing in 1 M Tris pH 8.0, was predetermined using the 'Protein Assay Dye Reagent' (Bio-Rad Laboratories Ltd). The sample was then diluted 1 in 10 with water and a 50 µl aliquot was mixed with 950 µl of prewarmed GGT reagent. The

spectrophotometer was maintained at the appropriate temperature (32, 37, 42 or 47^{o}C). Cleavage of the substrate (L-γ-glutamyl-3-carboxy-4-nitroanilide) due to GGT activity induced the appearance of a yellow color due to the formation of 5-amino-2-nitrobenzoate, which was quantified by absorbance at 405nm. The final activity value was determined as U/ml/mg of whole bacterial cell protein. One unit was defined as the quantity of enzyme that released 1 μmol of 5-amino-2-nitrobenzoate per min per mg of protein at the relevant temperature. For the colorimetric GGT assay *C. jejuni* isolates were incubated at 37^{o}C for 30 min and visually monitored. In the presence of GGT activity the solution changed color from clear to yellow.

In vitro invasion assay. Bacterial invasion into INT407 cells was studied using the gentamicin protection assay [38] using a range of MOIs (50-1200) as previous studies have indicated that invasion efficiency varies with the inoculum [39]. Briefly, the INT407 cells were cultured in a 24-well tissue culture plate at 37^{o}C with 5 % (v/v) CO_2 until confluent monolayers of approx. 5 x 10^5 cells per well were established. The cells were rinsed with Hank's Balanced Salt Solution (HBSS) and inoculated with *C. jejuni*. Tissue culture plates were centrifuged at 600 g, 22^{o}C for 15 min to eliminate variations in motility between strains. Infected monolayers were incubated at 37^{o}C in 5 % (v/v) CO_2 for three hours to allow the bacteria to invade the cells. The monolayers were washed three times with HBSS and incubated for a further two hours in maintenance media containing gentamicin at 250 μg/ml. Finally, the monolayers were washed in HBSS and lysed with 1 ml of 1 % (v/v) Triton X-100 in PBS. The suspensions were serially diluted

and the number of viable, internalized bacteria were determined by counting the resultant colonies on blood plates.

Gentamicin susceptibility assay. Bacterial susceptibility to gentamicin was determined on 10% (vol/vol) sheep blood agar plates supplemented with serial two-fold dilutions of gentamicin (0 to 64 μg/ml).

Motility Assay. The motility of *C. jejuni* strains and mutants was determined as a measure of swarming on semisolid motility (SSM) media [40]. Briefly, a blunt hypodermic needle was dipped into the strain to be tested and then stabbed into the center of a SSM plate. The plates were incubated in microaerobic conditions at 37^{o}C and the diameter of the colony in mm was determined after 24 and 48 hrs. Statistical significance was assessed using the two-tailed- T-Test.

Hydrogen peroxide susceptibility. Hydrogen peroxide (final concentration of 0.5 mM) was added directly to bacterial cells grown in Mueller Hinton broth (10^{9} cfu ml^{-1}) and incubated under microaerobic conditions at 42^{o}C. Viable counts of bacteria were taken after 0, 20, 40, 60, 80, 100 and 120 mins.

Apoptosis assay. The normal colonic epithelial cell line, CCD 841 CoN was obtained from the American Type Culture Collection (ATCC, Manassas, VA; no. CRL-1790™). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and cultured in 6-well plates. Once the cells were 40-60%

confluent, *C. jejuni* grown to an OD_{600} of 1.0 were added and incubated at 37°C in 5 % (v/v) CO_2 for 4 days. The cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) and used to assess both apoptosis and relative cell density by fluorescence quantitation. Nuclei with highly condensed and fragmented chromatin were considered apoptotic. Apoptotic cells were enumerated by counting 400 cells in multiple randomly selected fields.

2.6 Chick colonization

The one-day-old chick model of colonization [41] was used to determine colonization potential. Briefly, eggs from specific-pathogen-free chickens (Lohmann's) were hatched in isolators. Groups of eight chicks were maintained in separate isolators with unlimited food and water. At 1 day old, chicks were each dosed orally, by gavage, with approximately 10^4 cfu of *C. jejuni* in 0.1 ml of 0.1 M PBS, pH 7.2. Bacteria were grown overnight on blood agar plates under microaerobic conditions at 42°C. Chicks were sacrificed 5 days or 3 weeks after dosing, and bacterial colonization levels were determined by plating serial dilutions of caecal contents. The non-parametric Mann-Whitney test was used to assess the statistical significance of differences in colonization levels.

3. Results

3.1 Identification of the genetic location of the ggt gene in C. jejuni

Insert 236, previously identified by subtractive hybridization [11], encoded a putative polypeptide with 72% amino acid identity to the *H. pylori* γ-glutamyl transpeptidase

(GGT) polypeptide (GenBank accession no. **AE000511**). There was also a significant degree of similarity with GGT from *E. coli* (GenBank accession no. **P18956**) and *Salmonella enterica* serovar Typhimurium (GenBank accession no. **NP_462452**) and therefore a degenerate primer, If66 (Table 1) was designed to a conserved amino acid region, as near as possible to the N-terminus of these aligned proteins. The degenerate primer was used with a primer (If50) designed to the known *C. jejuni ggt* DNA sequence [11], which aligned to the amino acids at the C-terminus of the GGT sequence. Degenerate PCR was performed using genomic DNA from strain 81116 with the aforementioned primers and sequencing of the resulting 1.5 kbp PCR product identified a DNA sequence, which encoded a putative polypeptide with significant amino acid similarity (76%) to GGT from *H. pylori*.

Chromosomal walks on genomic DNA from strain 81116, using primer If49 identified the remaining *ggt* sequence and additional flanking DNA, which had no similarity to any sequence in strain NCTC11168, but had 54% amino acid identity and 67% similarity (Blast P value $2e^{-62}$) to a cytochrome c-type biogenesis protein (YCF5) from *Wolinella succinogenes* (GenBank accession no. **NP_908253.1**). The DNA sequence of *ggt* and this flanking DNA, obtained from strain 81116, was compared to the *ggt* genetic region (30 kbp) identified and kindly provided by Emily Kay, Sanger Center, Cambridge, from strain 81-176. Comparison of the *ggt* sequences from strains 81-176 and 81116 revealed 99.5% similarity at the DNA level (data not shown). In addition, the cytochrome c biogenesis DNA sequence obtained from strain 81116 was 99% identical to that in 81-176 (data not shown). This indicated that the genomic location of *ggt* in strain 81116 was

similar to that in strain 81-176 (Fig. 1). Following the identification of the full *ggt* sequence from strain 81116 (GenBank accession no. **AY623656**), this gene was also identified in another *C. jejuni* strain (GenBank accession no. **AJ786772**) which was isolated from chicken caecal contents. Comparison of the GGT amino acid sequences from three strains (GenBank accession no. **AY623656**, **AJ786772** and from strain 81-176), confirmed the high degree of conservation of *ggt* within the *C. jejuni* species at both the DNA and amino acid levels. Bioinformatical analysis of the *C. jejuni ggt* gene from strain 81116 revealed a 1671 base-pair open reading frame encoding a polypeptide with a calculated mass of 60300 Da. The first 17 amino acids at the N-terminus of GGT exhibit a typical signal sequence. A second post-translational cleavage site is predicted between positions Asn-370 and Thr-371, which would subsequently result in the formation of the large and small subunits of the mature enzyme, consistent with other bacterial and mammalian GGT orthologues [17, 22].

3.2 The GGT-specific activity for strain 81116

Strain 81116 was assayed for GGT activity by the rate of formation of 5-amino-2-nitrobenzoate, using the Thermotrace method (Alpha Laboratories) at exponential and stationary phases of growth (20 and 40 hours from the predetermined growth curve, respectively). The GGT activity at 32, 37, 42 and 47^{o}C, was also determined. The results are summarized in Table 2. GGT activity was observed at both growth phases but higher (approx. 1.7 to 1.9-fold) in the exponential phase, regardless of temperature. GGT activity was also observed at all the temperatures tested but appeared to be maximal at 42^{o}C, though this was not statistically significant.

3.3 Prevalence of ggt and GGT activity among C. jejuni isolates

One hundred and thirty four *C. jejuni* strains, from various sources, were tested for the prevalence of the *ggt* gene by colony-PCR using the primers If50 and If100 (Table 1b). These primers were designed to detect the regions of highest conservation observed within the sequenced *ggt* genes. Overall 19.4% of the strains generated the 1.5 kbp *ggt* PCR product (Table 3). This prevalence was considerably lower than the 54.5% indicated by a similar previously published study [11] but represents a substantially larger strain sample size. However, because the level of conservation of the primer regions is only known over a few strains, false negative PCRs are possible. Therefore, 19.4% must be considered as the minimal prevalence of positivity for *ggt*. Nevertheless, it is interesting to note that *ggt*-positive strains predominated in human isolates (37.93%).

The *C. jejuni* isolates previously screened for the presence of *ggt* [11] were tested in the colorimetric GGT assay. The yellow color change was only detected in strains positive for *ggt* (data not shown). Thus the presence of the gene, as determined by PCR, correlated directly with the expression of the GGT activity.

3.4 Construction of a C. jejuni mutant deficient in GGT activity

To determine the function of GGT in *C. jejuni*, a mutant was constructed in strain 81116. The *ggt* gene was disrupted by insertion of a kanamycin resistance cassette at 532 bp downstream from the *ggt* start codon, using the suicide vector pIHA-*ggt*. PCR and Southern blot analysis (data not shown) confirmed a double crossover and successful

insertion of the kanamycin resistance cassette. Southern blot analysis also showed that *C. jejuni* strain 81116 carried only one copy of the *ggt* gene. In strain 81116, *ggt* gene is monocistronic and cannot be transcriptionally-linked with its downstream gene, which is transcribed in the opposite direction. It is therefore extremely unlikely that gene replacement would have any effect on the expression of flanking genes (Fig. 1). However, in order to prevent any effect on the flanking genes, the PCR primers were designed within the *ggt* gene so that flanking genes and intergenic regions including potential promoters would remain undisrupted in the mutant. Nevertheless, as complementation is difficult with *Campylobacter*, another independently isolated *ggt* mutant was used as a control for secondary mutations. Neither of the kanamycin-resistant mutants had detectable GGT-specific activity. Comparison of the growth curves in biphasic broth or morphologies as observed by electron microscopy indicated no differences between the wild-type and mutant strains (data not shown).

3.5 Invasion assay using INT407 cells

The potential role of the GGT of *C. jejuni* 81116 in virulence was investigated by comparing the mutant and wild-type strains in an *in vitro* assay of invasion (Fig. 2). At all MOIs tested, higher numbers of the *ggt* mutant had internalized INT407 cells. At the optimal MOI of 200 the apparent invasion efficiency was 5.4-fold higher for the *ggt* mutant (0.0092%) compared to the wild-type (0.0017%).

3.6 Gentamicin resistance

An alteration in the gentamicin resistance may have influenced the invasion assay results and therefore was tested. Both the *ggt* mutant and wild-type strains were susceptible to 0.125 μg ml^{-1} gentamicin and were unable to grow on 0.25 μg ml^{-1} gentamicin (data not shown). This showed that the increased invasion efficiency of the *ggt* mutant was not due to increased gentamicin resistance.

3.7 Motility

Motility may also have been a factor influencing the INT407 invasion phenotypes. The average diameter of the zone of motility of the wild-type was 25.42 mm ±3.95 at 24 hours and 58.85 mm ±6.89 at 48 hours. The *ggt* mutant was significantly more motile than the parent strain; at 24 hours the diameter was 32.85 mm ±2.61 (P=0.0018) and 48 hours it was 69.86 mm ±2.91 (P=0.0045). This difference may have caused the increased invasiveness but any such effect should have been minimized by centrifugation of the bacteria onto the monolayer.

3.8 Hydrogen peroxide susceptibility

GGT activity has been shown to be associated with hydrogen peroxide resistance [19]. The sensitivity of the *ggt* mutant to hydrogen peroxide, at a final concentration of 0.5 mM, was considerably less than the wild-type strain (Fig. 3). After 20 mins, levels of viable mutant cells were consistently around 3 $\log_{10}$ higher than those of the wild-type strain, indicating that the inactivation of GGT increases the bacterium's resistance to hydrogen peroxide stress. Such differences could also influence the invasion efficiencies.

3.9 Apoptosis-inducing activity of GGT

Previous studies in *H. pylori* have shown that GGT is involved in the induction of apoptosis [28]. Therefore the ability of the *C. jejuni* wild-type strain to induce apoptosis of CCD 841 CoN epithelial cells was compared with that of the *ggt* mutant. The percentage of CCD 841 CoN cells with either highly condensed or fragmented nuclei was dose-dependently higher following incubation with the wild-type strain. (Fig. 4(a)). However, the relative CCD 841 CoN cell number decreased following incubation with either *C. jejuni* strain but to a much higher degree with the wild-type strain (Fig. 4(b)). Furthermore, phase contrast microscopy analysis showed that incubation with both the *ggt* mutant and wild-type strain altered the normal cell morphology and significantly reduced the density of adherent cells (Fig. 4(c-h)) compared with the control monolayer. Only the control cells become 95-100% confluent following the incubation period. Interestingly, nuclear DAPI staining revealed an increased population of cells with highly condensed and fragmented nuclei following infection with the wild-type strain. In contrast, a greater proportion of enlarged cell nuclei were observed following incubation with the *ggt* mutant. Overall, these results indicate that GGT plays a significant role in *C. jejuni*-mediated apoptosis.

3.10 Chick colonization model

The colonization potential of the *ggt* mutant was determined using a well established one-day-old chick model of colonization. Five days post-oral-challenge, with a dose of approximately 10^4 cfu, both the wild-type strain and *ggt* mutant had similar colonization potentials with 88% of chicks colonized (Fig. 5(a)). The geometric mean level of

colonization by the *ggt* mutant was 4.74 $\log_{10}$ cfu $g^{-1}$of caecal contents, which was lower than the wild-type strain at 6.11 $\log_{10}$ cfu g^{-1}. However, this was not statistically significant (P = 0.1049). Nevertheless, after three weeks post-oral challenge, the wild-type strain colonized 100% of chicks to maximal levels of 8-9 $\log_{10}$ cfu g^{-1} (Fig. 5(b)) while the colonization level in all birds by the *ggt* mutant was undetectable (P=0.0002). These results indicate a lack of persistence of the GGT mutant in the avian gut.

4. Discussion

Although the importance of γ-glutamyl transpeptidase in glutathione metabolism and amino acid transport in mammalian tissues has been recognized for many years [17], there is little information about the role of this enzyme in bacteria. To date, several prokaryotes, including *E. coli, Pseudomonas aeruginosa, Bacillus subtilis, H. pylori,* and *T. denticola,* have been shown to exhibit GGT activity [22, 25, 42, 43, 44] but the physiological role of GGT in these bacteria remains unclear.

In this study, the γ-glutamyl transpeptidase (*ggt*) gene has been identified and characterized in *C. jejuni* strain 81116. The role of GGT in *C. jejuni* was investigated using two defined *ggt* mutants generated in *C. jejuni* strain 81116. Both mutants had identical properties. Neither *ggt* mutant expressed any detectable GGT activity. Two-dimensional protein analysis also confirmed that GGT was no longer present (data not shown). Interestingly, in the wild-type strain, two adjacent GGT protein spots of the same molecular mass, but different isoelectric points, were observed by the 2-dimensional electrophoresis method (data not shown), suggesting that GGT may be post-

translationally modified, and possibly glycosylated, which would be consistent with the known post-translational glycosylation system in *C. jejuni*.

Results with the mutants demonstrated that GGT is not essential for *in vitro* growth, which also agrees with a recently published study using *C. jejuni* strain 81-176 [45]. Although characterization of *ggt* mutants in *C. jejuni* and in *N. meningitidis* [46] indicates that GGT is not required for bacterial growth under normal *in vitro* conditions, the expression of active GGT in *E. coli* is essential for the utilization of exogenous γ-glutamyl peptides [47]. It therefore seems likely that, although not essential, GGT may contribute to the organism's ability to cope with growth limiting factors during environmental survival.

Only 19.4% of 134 randomly selected *C. jejuni* strains contained the *ggt* gene and prevalence was highest in human isolates (37.93%). This suggests that there may be some advantage for those *C. jejuni* possessing GGT to colonize the human host. In contrast, in the closely related *Helicobacter* species all strains synthesize a catalytically active GGT, regardless of host sources, even though this was also inessential for growth [25].

In order to assess the role of GGT, a representative *ggt* mutant was investigated in a number of assay systems, reflecting properties related to virulence, survival and colonization potential. To date there are no acceptable models of virulence for *C. jejuni* [48]. However, surrogate models, such as invasion, have been utilized as indicators of potential of this organism to cause enteric disease.

The apparent higher recovery of *ggt* mutant cells, as compared to the wild-type, during the INT407 cell invasion assay, was initially surprising and contradicts the study on a *ggt* mutant in strain 81-176 in which the ability to invade T84 intestinal epithelial cells was indistinguishable [45]. This discrepancy is most likely due to the differences in cell lines used and genetic background of strains. Nevertheless, the combined effects of increased motility and resistance to hydrogen peroxide of the *ggt* mutant in strain 81116 most likely contributed to the enhanced invasion phenotype. Previous reports have shown motility as a major factor influencing invasion [38, 49]. In addition, prolonged intracellular survival within host cells has been linked with increased hydrogen peroxide resistance [50]. Previous studies [51] have indicated that loss of GGT activity in bacteria is initially accompanied by a rise in intracellular glutathione levels, potentially enhancing the organism's resistance to host cell-mediated oxidative stress. Although the association of GGT with that of the antioxidant gluthathione in prokaryotes has not been clearly established, in some bacteria protection against peroxides is related to the ability to acquire glutathione [52]. Conversely, an essential role for GGT in glutathione metabolism has been reported in the periodontal bacterial pathogen, *Treponema denticola* [42, 53], in which GGT-mediated hydrolysis of glutathione is a source for the H_2S necessary to reduce injury or death as a consequence of some environmental stresses.

Additional results indicated that the *C. jejuni* GGT enzyme has an apoptosis-inducing activity, which may be involved in pathogenesis. Clearly, the induction by *C. jejuni* of apoptotic cell death and changes to cell morphology is multifactorial [54, 55]. The *C.*

426 *jejuni* cytolethal distending toxin (CDT) causes epithelial cells to become blocked in the
427 G_2/M phase, where the cells appear enlarged and obvious mitotic cells are absent [54,
428 56]. Coincidently, a greater proportion of enlarged cells were observed following
429 infection with the *ggt* mutant and whether this is due an indirect effect on CDT has yet to
430 be investigated. The role of such effects on host cells is unclear but apoptotic cells may
431 be a source of essential nutrients to *C.jejuni*, thus contributing to pathogen survival in the
432 gut.
433
434 The colonization potentials of the wild type and mutant strains in the avian intestine were
435 also investigated. Interestingly, although initial colonization was not markedly different
436 between the variants, the *ggt* mutant was unable to sustain colonization of the chicken
437 gut. The dose response curve for colonization with *C. jejuni* strain 81116 wild type is
438 highly reproducible, and with the dose level of 10^4 cfu, colonization once established
439 consistently persists for at least 40 days after which it may decline [2]. A similar effect
440 was also observed for the *ggt* mutant in strain 81-176 in mixed-infection mouse
441 colonization experiments [45]. Why GGT should be required for only persistent, but not
442 initial, intestinal mucosal colonization is unclear and confusing when considering the *in*
443 *vitro* phenotypes. Nevertheless, the presence or absence of GGT may, at least in part,
444 explain differences in colonization phenotype reported by others [57].
445
446 In contrast to the avian studies, GGT-negative mutants of *H. pylori* tested in adult
447 mammalian *in vivo* models were either non-colonizing [25] or initiated poor but sustained
448 gastric mucosal colonization [26]. Such differences may reflect the separate ecological

niches of these closely-related organisms. It, therefore, seems possible that the presence of GGT in some *C. jejuni* strains confers an advantage in the *in vivo* environment by possibly sustaining glutathione levels (via recycling) to reduce the sensitivity to host inflammatory responses or as a means of providing a more effective supply of the essential amino acids, glycine and cysteine [58].

In conclusion, this study has identified the *ggt* gene in *C. jejuni* strain 81116 and partly characterized the function of GGT in this strain using an isogenic mutant. Although genetic tools for *C. jejuni* remain somewhat limited, future analysis involving attempts to complement *ggt* need to be undertaken to confirm these observations. Nevertheless, a number of interesting indicators of the role of GGT in *C. jejuni* infections have been provided. Of particular interest was the role in sustaining avian gut colonization. Although, colonization by *C. jejuni* is clearly multifactoral in nature, the identification of bacterial colonization factors, which enable persistent avian gut colonization, such as *ggt*, may in the future enable the development of targeted strategies for intervention to control and prevent this major public health problem.

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638 **Fig. Legends**

639

640 **Fig. 1**

641 The arrangement of *ggt* and adjacent genes in strains 81116 and 81-176. Arrows denote
642 predicted direction of transcription.

643

644 **Fig. 2**

645 Comparison of the capacity of the *ggt* mutant to invade INT407 cells compared to the
646 wild-type strain 81116. Invasion is represented as the total number of bacteria surviving
647 after the gentamicin treatment. The MOI at 200 was compared for the mutant and wild-
648 type strain. Symbols: ▲, *ggt* mutant; ■, wild-type.

649

650 **Fig. 3**

651 Susceptibility of the *ggt* mutant to hydrogen peroxide compared to the wild-type strain
652 81116. This was determined as the numbers of bacteria (log_{10} c.f.u. ml^{-1}) that survived
653 exposure to 0.5mM H_2O_2 over a period of 120 minutes. The assay was performed three
654 times and the results from one assay using triplicate samples is shown. Symbols: ▲, *ggt*
655 mutant; ■, wild-type.

656

657 **Fig. 4**

658 (a) DAPI assay for *C. jejuni* infection induced apoptosis and (b) the relative number of
659 adherent colonic epithelial (CCD 841 CoN) cells. The assay was performed three times.
660 Symbols: ▲, *ggt* mutant; ■, wild-type. Phase-contrast microscopy analysis of colonic

epithelial (CCD 841 CoN) cells: (c) control uninfected cells, (d) infection with wild-type strain and (e) *ggt* mutant. DAPI stained epithelial nuclei: (f) control uninfected cells, (g) infection with wild-type strain and (h) *ggt* mutant.

Fig. 5

Chick caecal colonization at **(a)** five days post-challenge and **(b)** three weeks post-challenge by the *ggt* mutant compared to the wild-type strain 81116. Colonization was determined as the number of bacteria recovered from the caecum, expressed as c.f.u. per gram of caecal contents. The geometric mean level of colonization for each group is shown by a +. The dotted line represents the limit of detection of 100 c.f.u. Both wild-type and mutant were given to the chicks at a dose of ~ 1 x 10^4 cfu per 100µl 0.1 M PBS pH 7.2. Symbols: ▲, *ggt* mutant; ■, wild-type.

Fig. 1

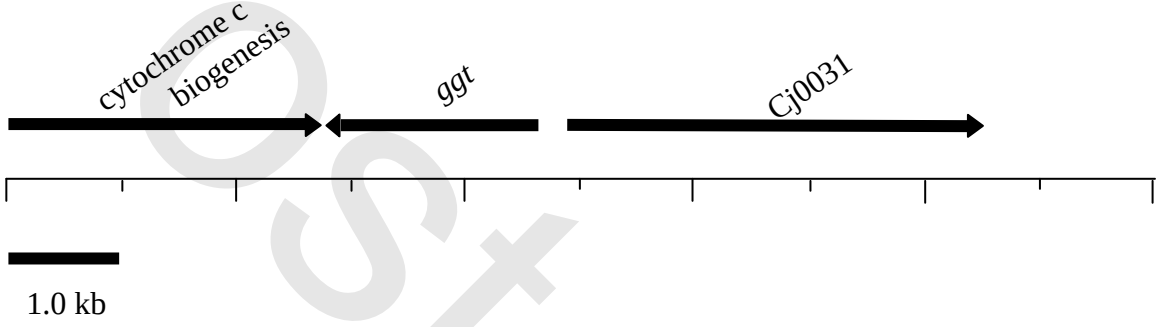

Fig. 2

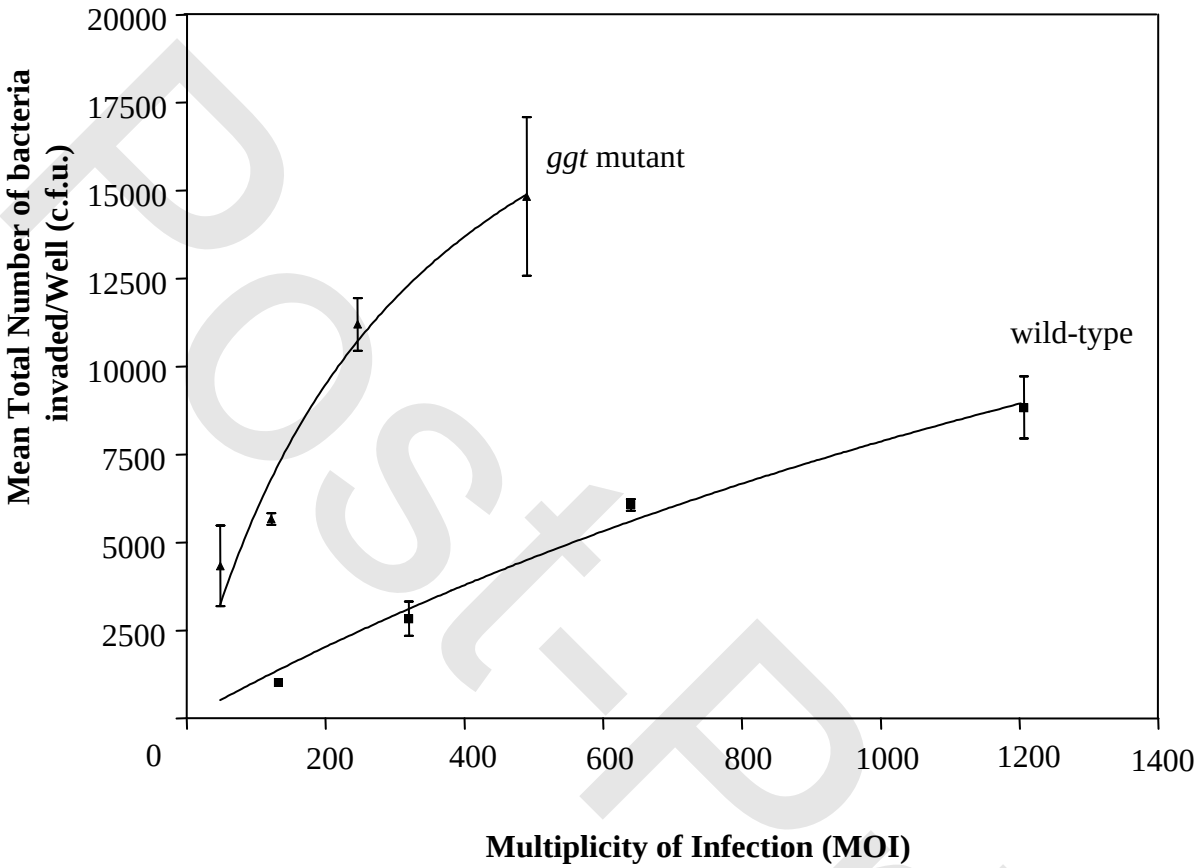

Fig. 3

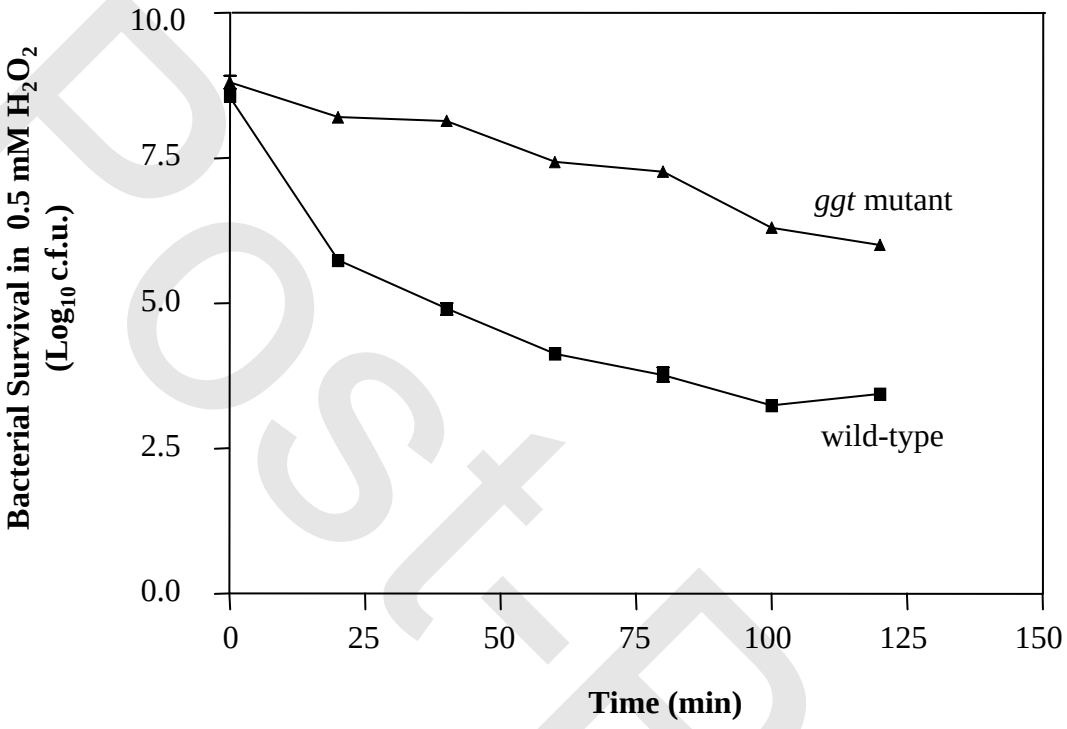

Fig. 4

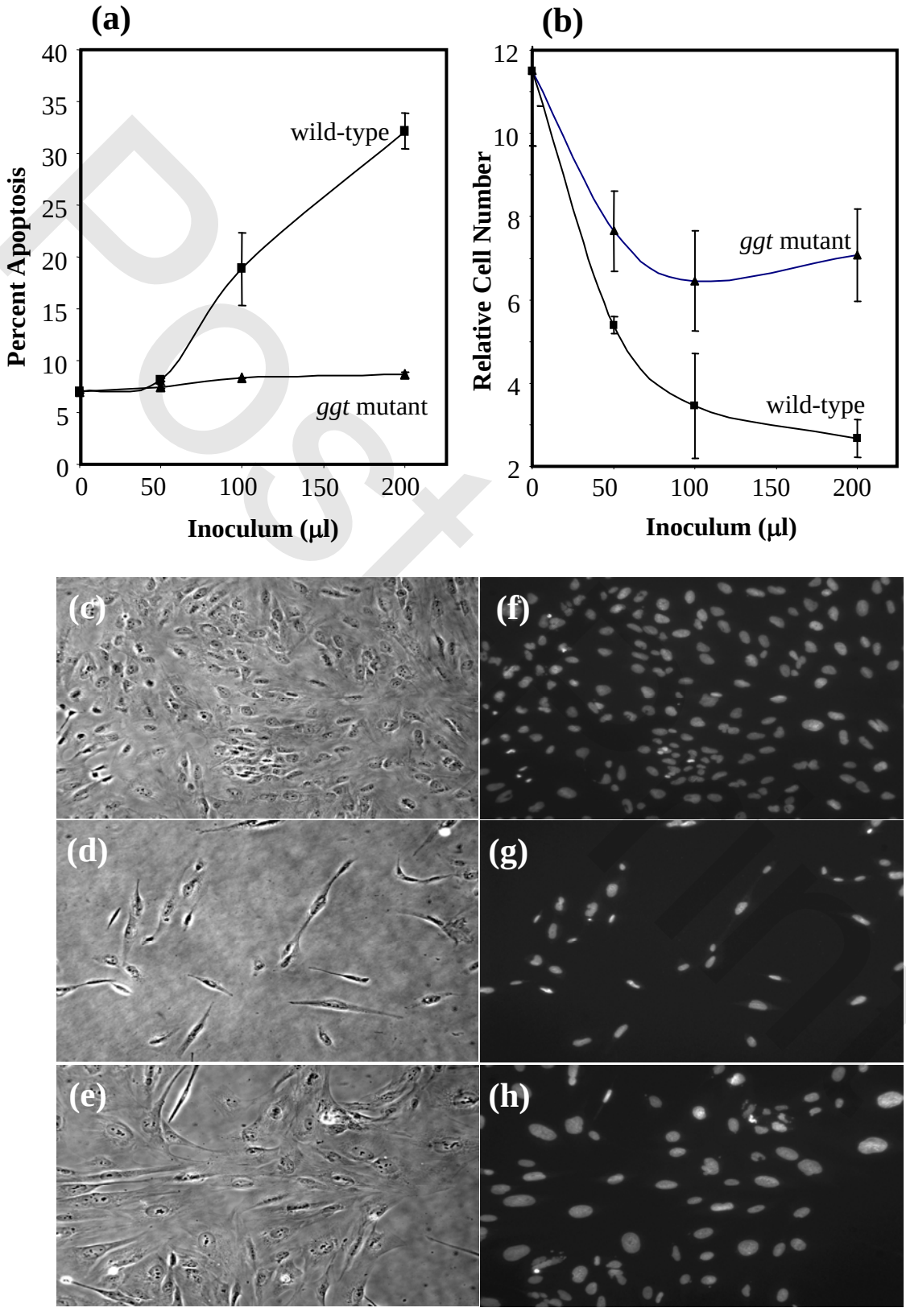
(a)
Percent Apoptosis
40
35
30
25
20
15
10
5
0
0
50
100
150
200
Inoculum (µl)
wild-type
ggt mutant
(b)
Relative Cell Number
12
10
8
6
4
2
0
50
100
150
200
Inoculum (µl)
ggt mutant
wild-type
(c)
(d)
(e)
(f)
(g)
(h)

Fig. 5

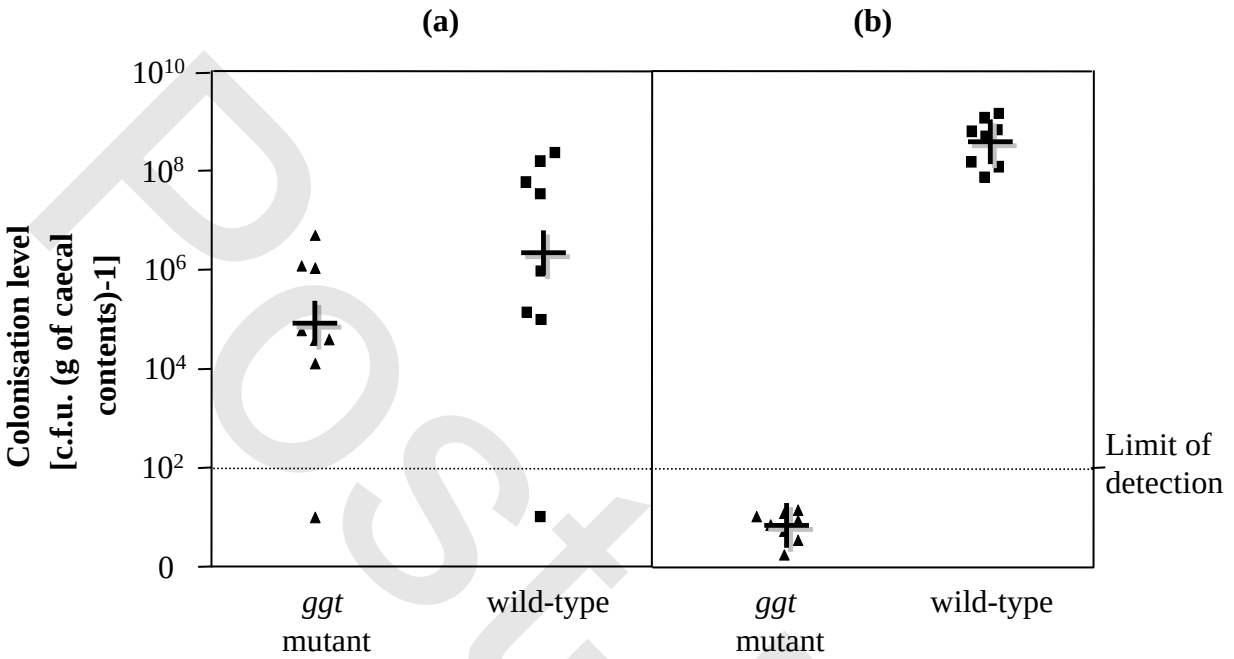

Table 1

(a) Standard and recombinant vectors used in this study. (b) Primers used in this study (Tm, temperature in °C; CW, chromosomal walking; Kan, kanamycin resistance; Amp, ampicillin resistance).

(a)

Vectors	Comment	Reference
pCR-Script™SK(+)	Cloning vector, Amp	Stratagene Europe
pJMK30	*C. coli* Kan cassette in pUC19	van Vliet *et al.*, 1998
pCR-Script-*ggt*	ggt in cloning vector, Amp	This study
pIHA-ggt	*ggt* suicide vector, Amp, Kan	This study

(b)

Oligo	Tm	Sequence 5' to 3'	Comment
If49	54.9	gtc tct atg cca act atc a	standard/CW primer
If50	53.7	ggg taa ata aga agt tag aat tc	standard primer
If66	62.7	ggn ggn aay gcn rtn gay gcn	degenerate PCR primer
If67	64.2	gaa gat ctc ata tct tta gct tct agc atg	inverse PCR primer 1
If68	65.6	gaa gat cta aga tgg cag cac tta taa aag	inverse PCR primer 2
If69	63.4	ggc agc act tat aaa agc gg	sequencing primer 1
If70	63.1	cct ggt ttg att gaa aaa tca tc	sequencing primer 2
If81	56.9	gtg atg taa atg cta tca tgg	sequencing primer for CW product
If100	66.3	ctt gat aaa ggc gga aat gcc	standard primer

Table 2

The GGT specific activity of strain 81116 at different temperatures and at exponential phase or stationary phase. Quantitative determination of γ-glutamyltranspeptidase activity expressed as Units per ml per mg of total protein.

	Exponential Phase (20 hours)	**Stationary Phase (40 hours)**
32 ºC	21.40 U/ml/mg	12.49 U/ml/mg
37 ºC	22.95 U/ml/mg	12.12 U/ml/mg
42 ºC	25.63 U/ml/mg	13.76 U/ml/mg
47 ºC	22.25 U/ml/mg	12.01 U/ml/mg

The sensitivity of the assay is 0.001 ΔAbs/min per U/L

Table 3
Presence of γ-glutamyl transpeptidase gene in *C. jejuni* from various sources
The number (percentage) of isolates from a particular source carrying *ggt* gene is give.

C. jejuni Source	Numbers (percentage) positive
Poultry	7/31 (22.58%)
Bovine	2/31 (6.45%)
Human	11/29 (37.93%)
Ovine	3/18 (16.67%)
Porcine	1/15 (6.67%)
Other	2/10 (20.00%)
Total	**26/134 (19.40%)**