

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

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1 Abstract

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

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

13
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15 **1. Introduction**

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

22
23 Quantitative risk assessment has demonstrated that the control and prevention of human
24 campylobacteriosis will be best achieved by intervention to reduce or eradicate
25 colonization at the poultry flock level [6]. Although improved biosecurity may contribute
26 to this, it seems likely that complementary methods, such as vaccination or
27 decontamination, may also be required [2, 7]. Such targeted intervention strategies
28 require fundamental understanding of those bacterial factors associated with the
29 campylobacter properties enabling colonization and survival. To date approaches to the
30 identification of such factors have largely been pragmatic with genes selected for
31 characterization on the basis of previously published observations in other enteric
32 bacteria. However, with the availability of the genome sequence of the *C. jejuni* reference
33 strain NCTC11168 [8], post-genomic approaches are now being adopted [9, 10, 11]. In
34 one such approach genomic subtractive hybridization between two strains, 81116 and
35 NCTC11168 was undertaken to identify genes present in 81116 but absent in
36 NCTC11168 [11], which is poorly able to colonise chickens. More recent studies have
37 shown that the ability of the NCTC11168 isolate to colonize birds is highly dependent on

38 the variant used [9, 10]. Thus colonization is not only due to the presence or absence of
39 genes but also whether those genes are transcribed. Nevertheless, given the difference in
40 colonization potential between strains 81116 and NCTC11168, it has been speculated
41 that such novel genes could contribute to aspects of colonization in chicks [12, 13, 14, 15,
42 16]. One such novel genes was a 369 bp DNA fragment (insert 236), unique to strain
43 81116, and which was identified and predicted to be part of a *C. jejuni* γ -glutamyl
44 transpeptidase (*ggt*) gene [11].

45

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

58

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

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

71

72 In *Helicobacter pylori*, GGT is involved in colonization of the gastric mucosa of mice
73 [25, 26, 27], potentially by participating in the *de novo* synthesis of essential amino acids
74 and thus enabling survival *in vivo*. In addition, GGT also possesses an apoptosis-inducing
75 activity [28] and upregulates COX-2 and EGF-related peptide expression in human
76 gastric cells [29]. Interestingly, in *Neisseria meningitidis*, GGT appears to provide an
77 advantage for bacterial multiplication during environmental cysteine shortage by
78 supplying cysteine from environmental peptides [30]. GGT is also involved in
79 osmoadaptation in *Escherichia coli* [31].

80

81 In this study, the *ggt* gene from *C. jejuni* strain 81116 has been identified, fully
82 sequenced and an isogenic mutant generated. The effect of this mutation on GGT activity
83 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 faeces 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°C in a microaerobic
101 environment (7.5% [vol/vol] CO₂, 7.5% [vol/vol] O₂, and 85% [vol/vol] N₂ 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 °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 °C in 1% (w/v) proteose peptone water

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

108

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

112

113 ***2.3 PCR and DNA sequencing***

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

125

126 ***2.4 Construction of ggt mutant***

127 The standard and recombinant vectors and oligos used in this study are listed in Table 1.

128 All standard methods of DNA manipulation were performed according to the protocols of

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

140

141 ***2.5 In vitro phenotypic studies***

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

145

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

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

161

162 *In vitro* invasion assay. Bacterial invasion into INT407 cells was studied using the
163 gentamicin protection assay [38] using a range of MOIs (50-1200) as previous studies
164 have indicated that invasion efficiency varies with the inoculum [39]. Briefly, the INT407
165 cells were cultured in a 24-well tissue culture plate at 37°C with 5 % (v/v) CO₂ until
166 confluent monolayers of approx. 5 x 10⁵ cells per well were established. The cells were
167 rinsed with Hank's Balanced Salt Solution (HBSS) and inoculated with *C. jejuni*. Tissue
168 culture plates were centrifuged at 600 g, 22°C for 15 min to eliminate variations in
169 motility between strains. Infected monolayers were incubated at 37°C in 5 % (v/v) CO₂
170 for three hours to allow the bacteria to invade the cells. The monolayers were washed
171 three times with HBSS and incubated for a further two hours in maintenance media
172 containing gentamicin at 250 μg/ml. Finally, the monolayers were washed in HBSS and
173 lysed with 1 ml of 1 % (v/v) Triton X-100 in PBS. The suspensions were serially diluted

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

176

177 Gentamicin susceptibility assay. Bacterial susceptibility to gentamicin was determined on
178 10% (vol/vol) sheep blood agar plates supplemented with serial two-fold dilutions of
179 gentamicin (0 to 64 µg/ml).

180

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

187

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

192

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

197 confluent, *C. jejuni* grown to an OD₆₀₀ of 1.0 were added and incubated at 37°C in 5 %
198 (v/v) CO₂ for 4 days. The cell nuclei were stained with 4', 6-diamidino-2-phenylindole
199 (DAPI) and used to assess both apoptosis and relative cell density by fluorescence
200 quantitation. Nuclei with highly condensed and fragmented chromatin were considered
201 apoptotic. Apoptotic cells were enumerated by counting 400 cells in multiple randomly
202 selected fields.

203

204 ***2.6 Chick colonization***

205 The one-day-old chick model of colonization [41] was used to determine colonization
206 potential. Briefly, eggs from specific-pathogen-free chickens (Lohmann's) were hatched
207 in isolators. Groups of eight chicks were maintained in separate isolators with unlimited
208 food and water. At 1 day old, chicks were each dosed orally, by gavage, with
209 approximately 10⁴ cfu of *C. jejuni* in 0.1 ml of 0.1 M PBS, pH 7.2. Bacteria were grown
210 overnight on blood agar plates under microaerobic conditions at 42°C. Chicks were
211 sacrificed 5 days or 3 weeks after dosing, and bacterial colonization levels were
212 determined by plating serial dilutions of caecal contents. The non-parametric Mann-
213 Whitney test was used to assess the statistical significance of differences in colonization
214 levels.

215

216 **3. Results**

217 ***3.1 Identification of the genetic location of the ggt gene in C. jejuni***

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

220 (GGT) polypeptide (GenBank accession no. AE000511). There was also a significant
221 degree of similarity with GGT from *E. coli* (GenBank accession no. P18956) and
222 *Salmonella enterica* serovar Typhimurium (GenBank accession no. NP_462452) and
223 therefore a degenerate primer, If66 (Table 1) was designed to a conserved amino acid
224 region, as near as possible to the N-terminus of these aligned proteins. The degenerate
225 primer was used with a primer (If50) designed to the known *C. jejuni ggt* DNA sequence
226 [11], which aligned to the amino acids at the C-terminus of the GGT sequence.
227 Degenerate PCR was performed using genomic DNA from strain 81116 with the
228 aforementioned primers and sequencing of the resulting 1.5 kbp PCR product identified a
229 DNA sequence, which encoded a putative polypeptide with significant amino acid
230 similarity (76%) to GGT from *H. pylori*.

231

232 Chromosomal walks on genomic DNA from strain 81116, using primer If49 identified
233 the remaining *ggt* sequence and additional flanking DNA, which had no similarity to any
234 sequence in strain NCTC11168, but had 54% amino acid identity and 67% similarity
235 (Blast P value $2e^{-62}$) to a cytochrome c-type biogenesis protein (YCF5) from *Wolinella*
236 *succinogenes* (GenBank accession no. NP_908253.1). The DNA sequence of *ggt* and this
237 flanking DNA, obtained from strain 81116, was compared to the *ggt* genetic region (30
238 kbp) identified and kindly provided by Emily Kay, Sanger Center, Cambridge, from
239 strain 81-176. Comparison of the *ggt* sequences from strains 81-176 and 81116 revealed
240 99.5% similarity at the DNA level (data not shown). In addition, the cytochrome c
241 biogenesis DNA sequence obtained from strain 81116 was 99% identical to that in 81-
242 176 (data not shown). This indicated that the genomic location of *ggt* in strain 81116 was

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

256

257 **3.2 The GGT-specific activity for strain 81116**

258 Strain 81116 was assayed for GGT activity by the rate of formation of 5-amino-2-
259 nitrobenzoate, using the Thermotrace method (Alpha Laboratories) at exponential and
260 stationary phases of growth (20 and 40 hours from the predetermined growth curve,
261 respectively). The GGT activity at 32, 37, 42 and 47°C, was also determined. The results
262 are summarized in Table 2. GGT activity was observed at both growth phases but higher
263 (approx. 1.7 to 1.9-fold) in the exponential phase, regardless of temperature. GGT
264 activity was also observed at all the temperatures tested but appeared to be maximal at
265 42°C, though this was not statistically significant.

266

267 **3.3 Prevalence of *ggt* and GGT activity among *C. jejuni* isolates**

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

278

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

283

284 **3.4 Construction of a *C. jejuni* mutant deficient in GGT activity**

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

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

302

303 ***3.5 Invasion assay using INT407 cells***

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

309

310 ***3.6 Gentamicin resistance***

311 An alteration in the gentamicin resistance may have influenced the invasion assay results
312 and therefore was tested. Both the *ggt* mutant and wild-type strains were susceptible to
313 $0.125 \mu\text{g ml}^{-1}$ gentamicin and were unable to grow on $0.25 \mu\text{g ml}^{-1}$ gentamicin (data not
314 shown). This showed that the increased invasion efficiency of the *ggt* mutant was not due
315 to increased gentamicin resistance.

316

317 **3.7 Motility**

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

325

326 **3.8 Hydrogen peroxide susceptibility**

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

333

334 **3.9 Apoptosis-inducing activity of GGT**

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

351

352 **3.10 Chick colonization model**

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

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

363

364 **4. Discussion**

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

371

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

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

382

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

391

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

397

398 In order to assess the role of GGT, a representative *ggt* mutant was investigated in a
399 number of assay systems, reflecting properties related to virulence, survival and
400 colonization potential. To date there are no acceptable models of virulence for *C. jejuni*
401 [48]. However, surrogate models, such as invasion, have been utilized as indicators of
402 potential of this organism to cause enteric disease.

403

404 The apparent higher recovery of *ggt* mutant cells, as compared to the wild-type, during
405 the INT407 cell invasion assay, was initially surprising and contradicts the study on a *ggt*
406 mutant in strain 81-176 in which the ability to invade T84 intestinal epithelial cells was
407 indistinguishable [45]. This discrepancy is most likely due to the differences in cell lines
408 used and genetic background of strains. Nevertheless, the combined effects of increased
409 motility and resistance to hydrogen peroxide of the *ggt* mutant in strain 81116 most likely
410 contributed to the enhanced invasion phenotype. Previous reports have shown motility as
411 a major factor influencing invasion [38, 49]. In addition, prolonged intracellular survival
412 within host cells has been linked with increased hydrogen peroxide resistance [50].
413 Previous studies [51] have indicated that loss of GGT activity in bacteria is initially
414 accompanied by a rise in intracellular glutathione levels, potentially enhancing the
415 organism's resistance to host cell-mediated oxidative stress. Although the association of
416 GGT with that of the antioxidant glutathione in prokaryotes has not been clearly
417 established, in some bacteria protection against peroxides is related to the ability to
418 acquire glutathione [52]. Conversely, an essential role for GGT in glutathione
419 metabolism has been reported in the periodontal bacterial pathogen, *Treponema denticola*
420 [42, 53], in which GGT-mediated hydrolysis of glutathione is a source for the H₂S
421 necessary to reduce injury or death as a consequence of some environmental stresses.

422

423 Additional results indicated that the *C. jejuni* GGT enzyme has an apoptosis-inducing
424 activity, which may be involved in pathogenesis. Clearly, the induction by *C. jejuni* of
425 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 G₂/M phase, where the cells appear enlarged and obvious mitotic cells are absent [54,
428 56]. Coincidentally, 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 10⁴ 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

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

454

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

465

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472

473 **References**

474 [1] Stanley KN, Jones K. High frequency of metronidazole resistance among strains of
475 *Campylobacter jejuni* isolated from birds. Lett Appl Microbiol 1998;27:247-250.

476 [2] Newell DG, Wagenaar JA. Poultry infections and their control at the farm level.
477 *Campylobacter* 2nd Edition. Nachamkin I & Blaser MJ. Washington D. C., ASM Press,
478 2000;497-509.

479 [3] Petersen L, Nielsen EM, Engberg J, On SL, Dietz HH. Comparison of genotypes and
480 serotypes of *Campylobacter jejuni* isolated from Danish wild mammals and birds and
481 from broiler flocks and humans. Appl Environ Microbiol 2001;67:3115-3121.

482 [4] Tompkins DS, Hudson MJ, Smith HR, Eglin RP, Wheeler JG, Brett MM, Owen RJ,
483 Brazier JS, Cumberland P, King V, Cook PE. A study of infectious intestinal disease in
484 England: microbiological findings in cases and controls. Commun Dis Public Health.
485 1999;2:108-113.

486 [5] Friedman CR, Neimann J, Wegener HC, Tauxe R. Epidemiology of *Campylobacter*
487 *jejuni* infection in the United States and other industrialized nations. In Nachamkin I &
488 Blaser MJ (ed.), *Campylobacter*. ASM Press, Washington D. C. 2001;121-138.

489 [6] Rosenquist H, Nielsen NL, Sommer HM, Norrung B, Christensen BB. Quantitative
490 risk assessment of human campylobacteriosis associated with thermophilic
491 *Campylobacter* species in chickens. Int J Food Microbiol 2003;83:87-103.

492 [7] Newell DG, Fearnley C. Sources of *Campylobacter* colonization in broiler chickens.
493 Appl Environ Microbiol 2003;A69:4343-4351.

494 [8] Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth
495 T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ,
496 Penn CW, Quail MA, Rajandream MA, Rutherford KM, van Vliet AH, Whitehead S,
497 Barrell BG. The genome sequence of the food-borne pathogen *Campylobacter jejuni*
498 reveals hypervariable sequences. *Nature* 2000;403:665-668.

499 [9] Carrillo CD, Taboada E, Nash JHE, Lanthier P, Kelly J, Lau PC, Verhulp R,
500 Mykytczuk O, Sy J, Findlay WA, Amoako K, Gomis S, Willson P, Austin JW, Potter A,
501 Babiuk L, Allan B, Szymanski CM. Genome-wide Expression Analyses of
502 *Campylobacter jejuni* NCTC11168 Reveals Coordinate Regulation of Motility and
503 Virulence by *flhA*. *J. Biol. Chem* 2004;279:20327-20338.

504 [10] Gaynor EC, Cawthraw S, Manning G, MacKichan JK, Falkow S, Newell DG. The
505 Genome-Sequenced Variant of *Campylobacter jejuni* NCTC 11168 and the Original
506 Clonal Clinical Isolate Differ Markedly in Colonization, Gene Expression, and
507 Virulence-Associated Phenotypes. *J Bacteriol* 2004;186:503-517.

508 [11] Ahmed IH, Manning G, Wassenaar TM, Cawthraw S, Newell DG. Identification of
509 genetic differences between two *Campylobacter jejuni* strains with different colonization
510 potentials. *Microbiology* 2002;148:1203-1212.

511 [12] Dingle KE, Colles FM, Wareing DRA, Ure R, Fox AJ, Bolton FE, Bootsma HJ,
512 Willems RJJ, Urwin R, Maiden MCJ. Multilocus Sequence Typing System for
513 *Campylobacter jejuni*. *J Clin Microbiol* 2001;39:14-23.

514 [13] Wassenaar TM, Blaser MJ. Pathophysiology of *Campylobacter jejuni* infections of
515 humans. *Microbes Infect* 1999;1:1023-1033.

516 [14] Poly F, Threadgill D, Stintzi A. Identification of *Campylobacter jejuni* ATCC
517 43431-specific genes by whole microbial genome comparisons. J Bacteriol
518 2004;186:4781-4795.

519 [15] Poly F, Threadgill D, Stintzi A. Genomic diversity in *Campylobacter jejuni*:
520 identification of *C. jejuni* 81-176-specific genes. J Clin Microbiol. 2005;43:2330-2338.

521 [16] Stintzi A, Marlow D, Palyada K, Naikare H, Panciera R, Whitworth L, Clarke C.
522 Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle
523 of *Campylobacter jejuni*. Infect Immun 2005;73:1797-1810.

524 [17] Tate SS, Meister A. Gamma-glutamyl transpeptidase: catalytic, structural and
525 functional aspects. Mol Cell Biochem 1981;39:357-368.

526 [18] O'Donovan DJ, Fernandes CJ. Mitochondrial glutathione and oxidative stress:
527 implications for pulmonary oxygen toxicity in premature infants. Mol Genet Metab
528 2000;71:352-358.

529 [19] Shi M, Gozal E, Choy HA, Forman HJ. Extracellular glutathione and gamma-
530 glutamyl transpeptidase prevent H₂O₂-induced injury by 2,3-dimethoxy-1,4-
531 naphthoquinone. Free Radic Biol Med 1993;15:57-67.

532 [20] Izawa S, Inoue Y, Kimura A. Oxidative stress response in yeast: effect of
533 glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*.
534 FEBS Lett 1995;368:73-76.

535 [21] Meister A. On the antioxidant effects of ascorbic acid and glutathione. Biochem
536 Pharmacol 1992;44:1905-1915.

537 [22] Xu K, Strauch MA. Identification, sequence, and expression of the gene encoding
538 gamma-glutamyltranspeptidase in *Bacillus subtilis*. J Bacteriol 1996;178:4319-4322.

539 [23] Ikeda Y, Fujii J, Anderson ME, Taniguchi N, Meister A. Involvement of Ser-451
540 and Ser-452 in the catalysis of human gamma-glutamyl transpeptidase. J Biol Chem
541 1995;270:22223-22228.

542 [24] Szymanski CM, Yao R, Ewing CP, Trust T, Guerry JP. Evidence for a system of
543 general protein glycosylation in *Campylobacter jejuni*. Mol Microbiol 1999;32:1022-
544 1030.

545 [25] Chevalier C, Thiberge JM, Ferrero RL, Labigne A. Essential role of *Helicobacter*
546 *pylori* gamma-glutamyltranspeptidase for the colonization of the gastric mucosa of mice.
547 Mol Microbiol 1999;31:1359-1372.

548 [26] McGovern KJ, Blanchard TG, Gutierrez JA, Czinn SJ, Krakowka S, Youngman P.
549 Gamma-glutamyltransferase is a *Helicobacter pylori* virulence factor but is not essential
550 for colonization. Infect Immun 2001;69:4168-4173.

551 [27] Gong M, Ho B. Prominent role of gamma-glutamyl-transpeptidase on the growth of
552 *Helicobacter pylori*. World J. Gastroenterol 2004;20:2994-2996.

553 [28] Shibayama K, Kamachi K, Nagata N, Yagi T, Nada T, Doi Y, Shibata N, Yokoyama
554 K, Yamane K, Kato H, Iinuma Y, Arakawa Y. A novel apoptosis-inducing protein from
555 *Helicobacter pylori*. Mol Microbiol 2003;47:443-451.

556 [29] Busiello I, Acquaviva R, Di Popolo A, Blanchard TG, Ricci V, Romano M, Zarrilli
557 R. *Helicobacter pylori* gamma-glutamyltranspeptidase upregulates COX-2 and EGF-
558 related peptide expression in human gastric cells. Cell Microbiol 2004;6:255-267.

559 [30] Takahashi H, Hirose K, Watanabe H. Necessity of meningococcal gamma-glutamyl
560 aminopeptidase for *Neisseria meningitidis* growth in rat cerebrospinal fluid (CSF) and
561 CSF-like medium. J Bacteriol 2004;186:244-247.

562 [31] Smirnova GV, Krasnykh TA, Oktyabrsky ON. Role of glutathione in the response of
563 *Escherichia coli* to osmotic stress. *Biochemistry (Mosc)* 2001;66:973-978.

564 [32] Palmer SR, Gully PR, White JM, Pearson AD, Suckling WG, Jones DM, Rawes JC,
565 Penner JL. Water-borne outbreak of *Campylobacter* gastroenteritis. *Lancet* 1983;1:287-
566 290.

567 [33] Korlath JA, Osterholm MT, Judy LA, Forfang JC, Robinson RA. A point-source
568 outbreak of campylobacteriosis associated with consumption of raw milk. *J Infect Dis*
569 1985;152:592-596.

570 [34] Skirrow MB. *Campylobacter* enteritis: a 'new' disease. *Br Med J* 1977;2:9-11.

571 [35] Ausubel SF, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K,
572 Albright LM, Coen DM, Varki AE (ed.). *Current Protocols in Molecular Biology*. John
573 Wiley & Sons Inc, New York, N.Y. 1994.

574 [36] Sachadyn P, Sobiewska G, Kur J. Thermal profile with alternately raised and
575 lowered annealing temperature improves the PCR amplification using highly degenerate
576 primers. *Acta Biochim Pol* 1998;45:691-694.

577 [37] Sreaton GR, Bangham CR, Bell JI. Direct sequencing of single primer PCR
578 products: a rapid method to achieve short chromosomal walks. *Nucleic Acids Res*
579 1993;21:2263-2264.

580 [38] Wassenaar TM, Bleumink-Pluym NM, van der Zeijst BA. Inactivation of
581 *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that
582 *flaA* but not *flaB* is required for invasion. *Embo J* 1991;10:2055-2061.

583 [39] Hu L, Kopecko DJ. *Campylobacter jejuni* 81-176 associates with microtubules and
584 dynein during invasion of human intestinal cells. *Infect Immun* 1999;67:4171-4182.

585 [40] Goossens H, Vlaes L, Galand I, Van den Borre C, Butzler JP. Semisolid blood-free
586 selective-motility medium for the isolation of *Campylobacters* from stool specimens. J
587 Clin Microbiol 1989;27:1077-1080.

588 [41] Wassenaar TM, van der Zeijst BA, Ayling R, Newell DG. Colonization of chicks by
589 motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A
590 expression. J Gen Microbiol 1993;139:1171-1175.

591 [42] Chu L, Dong Z, Xu X, Cochran DL, Ebersole JL. Role of glutathione metabolism of
592 *Treponema denticola* in bacterial growth and virulence expression. Infect Immun
593 2002;70:1113-1120.

594 [43] Ishiye M, Yamashita M, Niwa M. Molecular cloning of the gamma-
595 glutamyltranspeptidase gene from a *Pseudomonas* strain. Biotechnol Prog 1993;9:323-
596 331.

597 [44] Suzuki H, Kumagai H, Echigo T, Tochikura T. DNA sequence of the *Escherichia*
598 *coli* K-12 gamma-glutamyltranspeptidase gene, *ggt*. J Bacteriol 1989;171:5169-5172.

599 [45] Hofreuter D, Tsai J, Watson RO, Novik V, Altman B, Benitez M, Clark C, Perbost
600 C, Jarvie T, Du L, Galán JE. Unique Features of a Highly Pathogenic *Campylobacter*
601 *jejuni* Strain. Infect Immun 2006;74:4694-4747.

602 [46] Riou JY, Buissiere J, Richard C, Guibourdenche M. Gamma-glutamyl-transferase
603 activity in the family "*Neisseriaceae*". Ann Microbiol (Paris) 1982;133:387-392.

604 [47] Suzuki H, Hashimoto W, Kumagai H. *Escherichia coli* K-12 can utilize an
605 exogenous gamma-glutamyl peptide as an amino acid source, for which gamma-
606 glutamyltranspeptidase is essential. J Bacteriol 1993;175:6038-6040.

607 [48] Newell DG. (2001). Animal models of *Campylobacter jejuni* colonization and
608 disease and the lessons to be learned from similar *Helicobacter pylori* models. Symp Ser
609 Soc Appl Microbiol 2001;57S-67S.

610 [49] Golden NJ, Acheson DWK. (2002). Identification of Motility and Autoagglutination
611 *Campylobacter jejuni*. Infect Immun 2002;70:1761-1771.

612 [50] Wu H, Seib KL, Edwards JL, Apicella MA, McEwan AG, Jennings MP. Azurin of
613 pathogenic *Neisseria* spp. is involved in defense against hydrogen peroxide and survival
614 within cervical epithelial cells. Infect Immun 2005;73:8444-8448.

615 [51] Nakayama R, Kumagai H, Tochikura T. Leakage of glutathione from bacterial cells
616 caused by inhibition of gamma-glutamyltranspeptidase. Appl Environ Microbiol
617 1984;47:653-657.

618 [52] Vergauwen B, Pauwels F, Van Beeumen JJ. Glutathione and catalase provide
619 overlapping defenses for protection against respiration-generated hydrogen peroxide in
620 *Haemophilus influenzae*. J Bacteriol 2003;185:5555-5562.

621 [53] Chu L, Xu X, Dong, Z, Cappelli D, Ebersole JL. Role for recombinant gamma-
622 glutamyltransferase from *Treponema denticola* in glutathione metabolism. Infect Immun
623 2003;71:335-342.

624 [54] Hickey TE, Majam G, Guerry P. Intracellular survival of *Campylobacter jejuni* in
625 human monocytic cells and induction of apoptotic death by cytolethal distending toxin.
626 Infect Immun 2005;73:5194-5197.

627 [55] Zhu J, Meinersmann RJ, Hiatt KL, Evans DL. Apoptotic effect of outer-membrane
628 proteins from *Campylobacter jejuni* on chicken lymphocytes. Curr Microbiol
629 1999;38:244-249.

630 [56] Whitehouse CA, Balbo PB, Pesci EC, Cottle DL, Mirabito PM Pickett CL.
631 *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block.
632 Infect Immun 1998;66:1934-40.

633 [57] Ringoir DD, Korolik V. Colonisation phenotype and colonization potential
634 differences in *Campylobacter jejuni* strains in chickens before and after passage *in vivo*.
635 Vet Microbiol. 2003;92:225-35.

636 [58] Fang FC. Perspectives series: host/pathogen interactions. Mechanisms of nitric
637 oxide-related antimicrobial activity. J Clin Invest 1997;99:2818-2825.

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 H₂O₂ 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

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

664

665 **Fig. 5**

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

Fig. 1

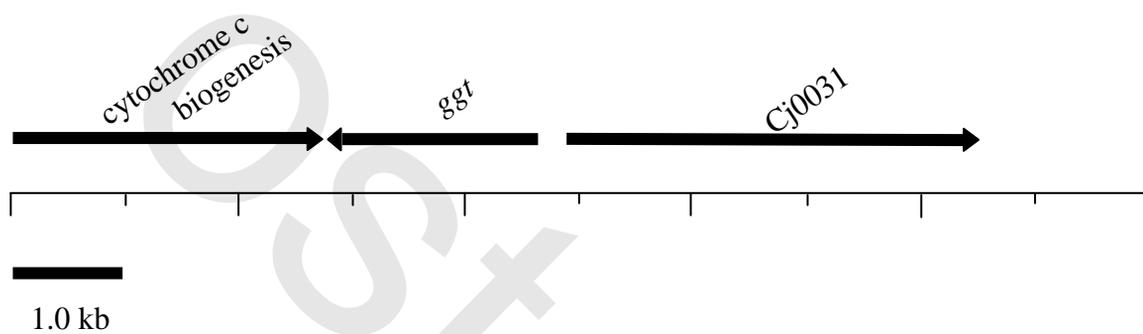


Fig. 2

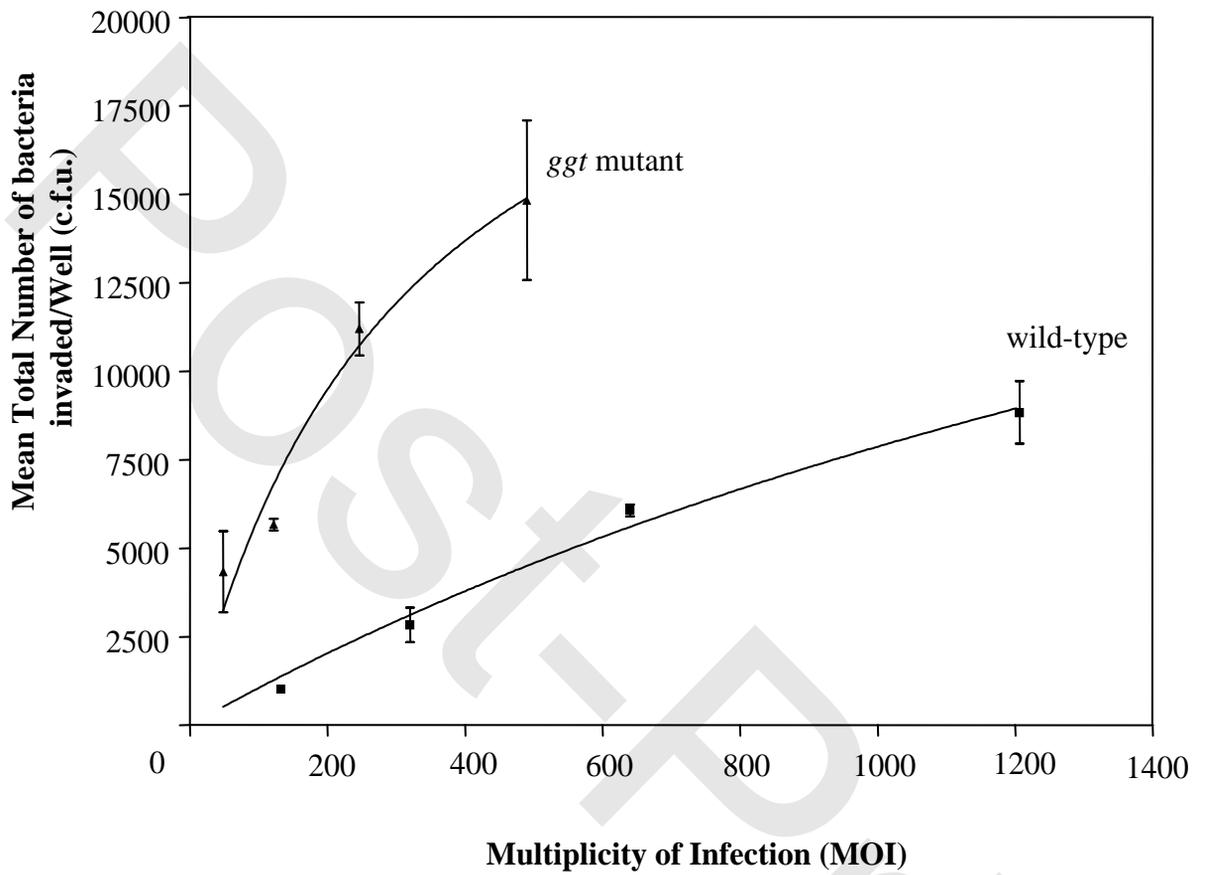


Fig. 3

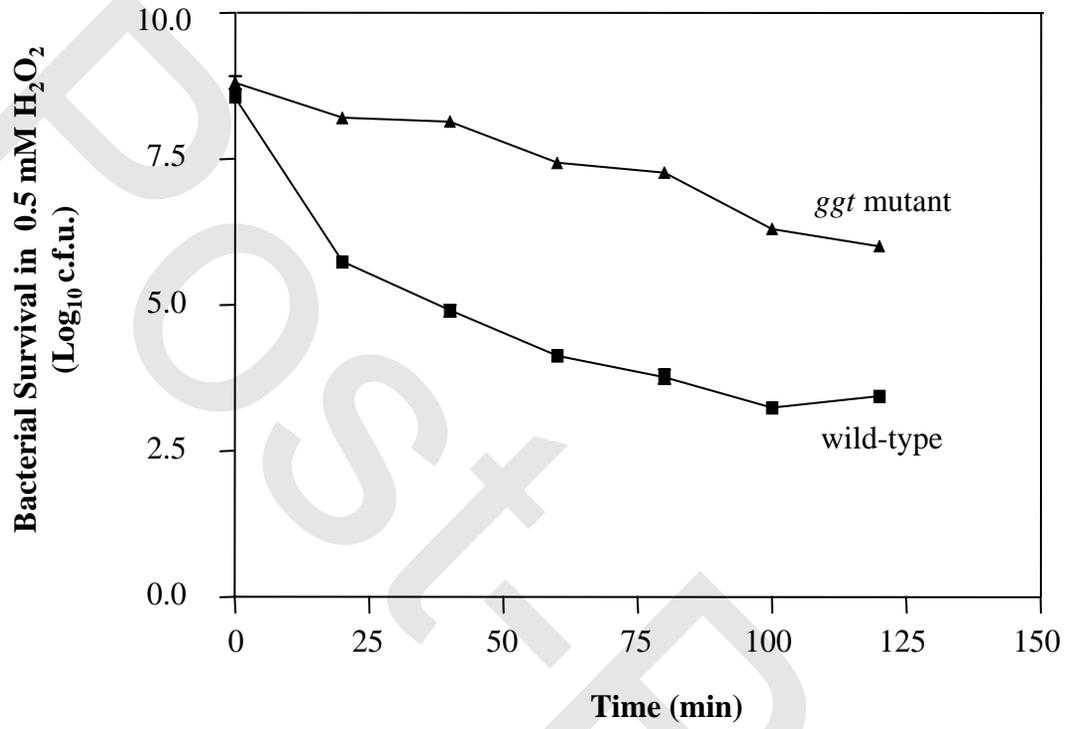


Fig. 4

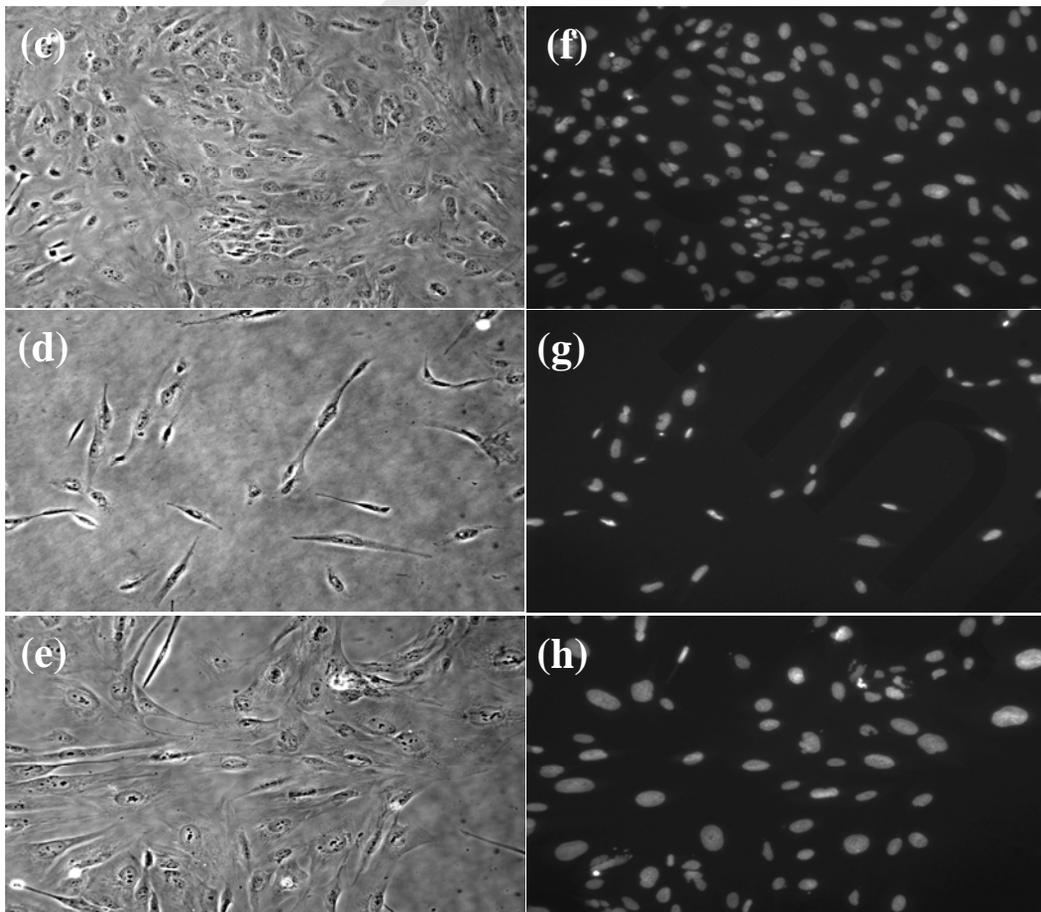
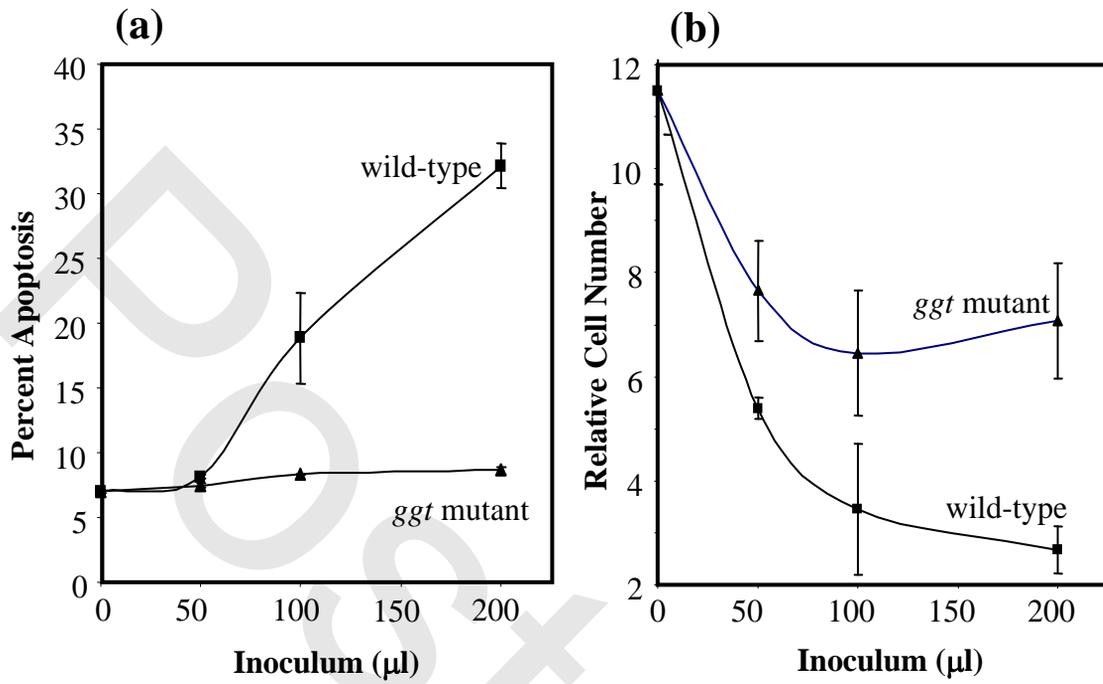


Fig. 5

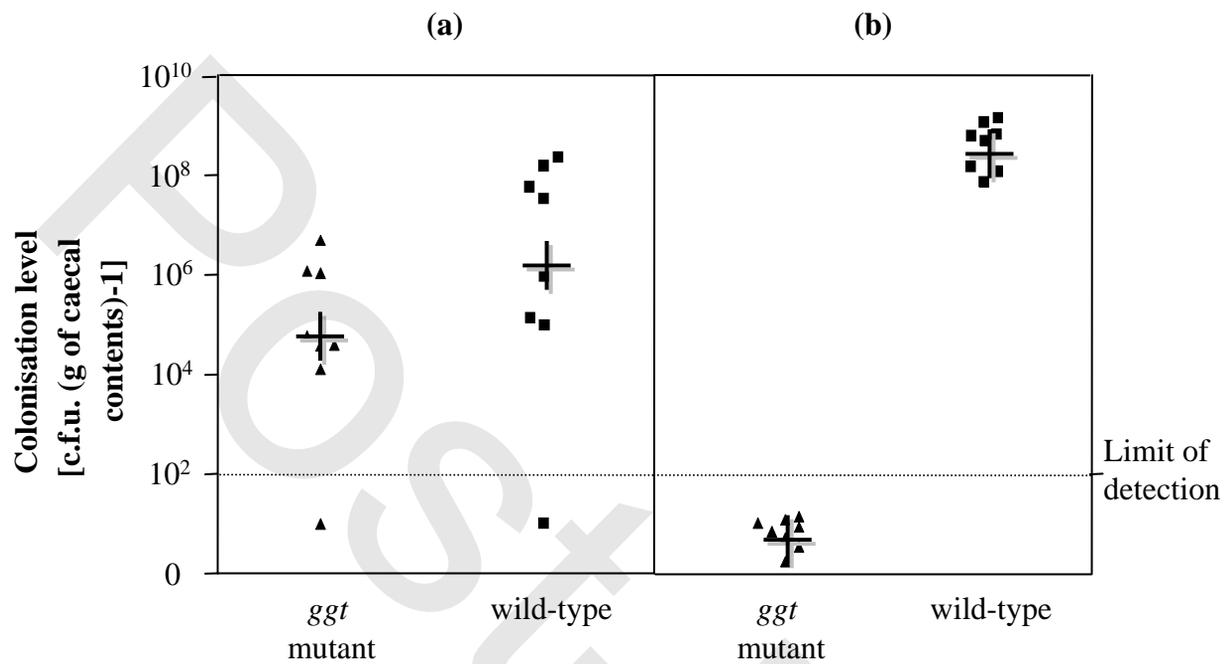


Table 1

(a) Standard and recombinant vectors used in this study. (b) Primers used in this study (T_m, 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	<i>C. coli</i> Kan cassette in pUC19	van Vliet <i>et al.</i> , 1998
	pCR-Script-ggt	ggt in cloning vector, Amp	This study
	pIHA-ggt	ggt suicide vector, Amp, Kan	This study

(b)	Oligo	T _m	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 °C	21.40 U/ml/mg	12.49 U/ml/mg
37 °C	22.95 U/ml/mg	12.12 U/ml/mg
42 °C	25.63 U/ml/mg	13.76 U/ml/mg
47 °C	22.25 U/ml/mg	12.01 U/ml/mg

The sensitivity of the assay is 0.001 Δ Abs/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.

<i>C. jejuni</i> 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%)