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A single nucleotide change in *mutY* increases the emergence of antibiotic-resistant *Campylobacter jejuni* mutants

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Objectives: Mutator strains play an important role in the emergence of antibiotic-resistant bacteria. *Campylobacter jejuni* is a leading cause of foodborne illnesses worldwide and is increasingly resistant to clinically important antibiotics. The objective of this study was to identify the genetic basis that contributes to a mutator phenotype in *Campylobacter* and determine the role of this phenotype in the development of antibiotic resistance.

Methods: A *C. jejuni* isolate (named CMT) showing a mutator phenotype was subjected to WGS analysis. Comparative genomics, site-specific reversion and mutation, and gene knockout were conducted to prove the mutator effect was caused by a single nucleotide change in the *mutY* gene of *C. jejuni*.

Results: The *C. jejuni* CMT isolate showed ~100-fold higher mutation frequency to ciprofloxacin than the WT strain. Under selection by ciprofloxacin, fluoroquinolone-resistant mutants emerged readily from the CMT isolate. WGS identified a single nucleotide change (G595→T) in the *mutY* gene of the CMT isolate. Further experiments using defined mutant constructs proved its specific role in elevating mutation frequencies. The *mutY* point mutation also led to an ~700-fold increase in the emergence of ampicillin-resistant mutants, indicating its broader impact on antibiotic resistance. Structural modelling suggested the G595→T mutation probably affects the catalytic domain of MutY and consequently abolishes the anti-mutator function of this DNA repair protein.

Conclusions: The G595→T mutation in *mutY* abolishes its anti-mutator function and confers a mutator phenotype in *Campylobacter*, promoting the emergence of antibiotic-resistant *Campylobacter*.

Introduction

Campylobacter jejuni, a Gram-negative microaerophilic bacterium, is one of the most prevalent bacterial foodborne pathogens causing gastroenteritis in humans.[1] Infection with *C. jejuni* is also associated with the development of a paralysing neuropathy, the Guillain–Barré syndrome.[2] The main sources of human *Campylobacter* infections are poultry meat, water and milk contaminated by *Campylobacter*.[3,4]

Fluoroquinolone (FQ) antimicrobials are often prescribed for clinical treatment of diarrhoea caused by enteric bacterial pathogens, including *Campylobacter*.[5,6] However, *Campylobacter* resistance to FQ antimicrobials is increasing,[7–9] and some countries have even reported 98%–100% FQ resistance (FQ^R) rates among *Campylobacter* isolates originating from broiler chickens.[10,11] In a recent report from the US CDC,[12] drug-resistant *Campylobacter* is listed as a serious threat to public health.

The main targets of FQs in bacteria are DNA gyrase (GyrA) and/or topoisomerase IV.[13] However, *Campylobacter* species lack topoisomerase IV, and point mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene confer resistance to FQ antimicrobials. For example, the Thr-86→Ile amino acid modification in GyrA is frequently associated with high-level resistance to FQs,[14,15] while other amino acid substitutions in the QRDR region are linked to low to intermediate levels of resistance.[16] In addition to the point mutations, the chromosomally encoded and constitutively expressed multidrug efflux pump CmeABC is also a key player in FQ^R. This was shown by gene-specific inactivation of the *cmeABC* operon, which led to significant reductions in FQ MICs even in mutant strains that harboured resistance-conferring mutations in GyrA.[17–21] Plasmid-mediated FQ^R genes, such as *qnr*, *aac(6′)-Ibcr* and *qepA*, which have been discovered in other Gram-negative bacteria, have not been reported in *Campylobacter*.[22] Thus, GyrA mutations and CmeABC are synergistically responsible for conferring FQ^R in *Campylobacter*.

In addition to contributing to FQ^R, point mutations mediate resistance to other antimicrobials in *Campylobacter*, such as macrolides and β-lactams. Macrolide resistance is mainly

mediated by a single nucleotide transition (A→G) or transversion (A→C) at position 2058 or 2059 (*Escherichia coli* coordinates) of 23S RNA.[8,23] Recently, a G→T transversion in the promoter region of bla_{OXA-61}, which encodes the sole β-lactamase gene in *C. jejuni*, has been linked to high-level ampicillin resistance (Amp^R) by restoring the expression of bla_{OXA-61}.[24]

Bacterial strains exhibiting elevated mutation frequencies are mutators, and the mutator phenotype facilitates bacterial adaptation to environmental changes.[25] Mutators also play an important role in the emergence of antibiotic resistance in the clinical setting.[26] Mutators have also been reported among natural isolates of different bacterial species, including *E. coli*, *Salmonella enterica*,[27] *Helicobacter pylori*[28] and *Streptococcus pneumoniae*.[29] The majority of naturally occurring mutators are related to defects in the methyl-directed mismatch repair (MMR) system and the nucleotide excision repair (NER) system, such as mutations in *mutS*, *mutH*, *mutL* and *uvrD*.[26] In *E. coli*, the MMR system, including MutS, repairs replication errors that arise from misincorporations (mismatches) and strand slippage (frameshift errors). In addition, the MMR system inhibits recombination between homologous sequences.[30] Epsilonproteobacteria, including *Campylobacter* and *Helicobacter*, do not have most of the MMR genes, except for a *mutS* homologue in both species. However, inactivation of *mutS* in *H. pylori* did not confer a mutator phenotype and there is no evidence that MutS mutations are present in naturally occurring mutators of *H. pylori*.[28] Gaasbeek *et al.*[31] characterized several putative DNA repair genes in *Campylobacter* and found that none of the investigated genes (including *recA*, *uvrA*, *uvrC*, *mutS* and *ung*) altered the spontaneous FQ^R mutation frequency. However, mutation of *ung* was found to be associated with increased mutation frequency in *H. pylori*.[32] A previous study documented high variability (up to 700-fold) in FQ^R mutation frequencies among *C. jejuni* and *Campylobacter coli* strains,[16] suggesting mutator strains may exist among *Campylobacter* isolates. However, the genetic basis conferring the mutator status in *Campylobacter* is still unknown.

In this study, we identified a variant (named CMT in this study) of *C. jejuni* NCTC 11168,[33] which demonstrated a spontaneous FQ^R mutation frequency >100-fold higher than that of 11168 and other *C. jejuni* isolates. This finding prompted us to examine the mutator phenotype by utilizing WGS, comparative genomics analysis and other genetic methods. A single nucleotide change (G595→T transversion) was identified in the putative base excision repair (BER) gene *mutY* and was found to be responsible for the elevated frequency of mutation to FQ^R resistance in *Campylobacter*. Additionally, the G595→T mutation in *mutY* also significantly increases the emergence of spontaneous Amp^R mutants in *C. jejuni*. To our knowledge, this is the first identified genetic factor conferring the naturally occurring mutator phenotype in *Campylobacter*, promoting the emergence of antibiotic resistance mutants.

Materials and methods

Bacterial strains and growth conditions

The CMT isolate is a natural variant of *C. jejuni* 11168 identified in our laboratory. All of the *C. jejuni* strains used in this study are listed in Table 1. *C. jejuni* was cultured using Mueller–Hinton (MH) broth or agar (Difco) at 42°C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2). *E. coli* was grown on LB agar or in LB broth at 37°C for 24 h under aerobic conditions. When needed, culture media were supplemented with ciprofloxacin (1 mg/L), ampicillin (100 mg/L), kanamycin (30 mg/L) or chloramphenicol (10 mg/L).

Ciprofloxacin susceptibility testing

The susceptibilities of *C. jejuni* strains to ciprofloxacin (Fisher Bioreagents) were determined by a standard microtitre broth dilution method with an inoculum of 10^6 cfu/mL as described previously.[19] MICs were determined as the lowest concentration of ciprofloxacin showing complete inhibition of bacterial growth after 24 or 48 h of incubation at 42°C.

C. jejuni survival assay under ciprofloxacin treatment in vitro

In order to test the ability of *C. jejuni* cells to generate FQ^R mutants under FQ selective pressure, different strains were tested for their survival under treatment with a lethal concentration of ciprofloxacin. The assay was carried out in 96-well plates. Overnight cultures on MH agar plates were resuspended in fresh MH broth and adjusted to an inoculum of 10^6 cfu/mL. Ciprofloxacin was added at a final concentration of 1 mg/L (~10–20×MIC). Aliquots of 200 μL inoculum (~10^5 cfu) with ciprofloxacin were added to each well. The plates were then incubated at 42°C for 48 h in the incubator under microaerobic conditions. Triplicate samples were taken at 0, 6, 12, 24, 36 and 48 h for viable counts by dropping 10 μL of different dilutions onto non-selective MH agar plates. Then, 50 colonies (if any) were randomly picked from the counting plates at each timepoint, and streaked onto MH plates containing 1 mg/L ciprofloxacin and checked for growth after overnight incubation to confirm the susceptibility of *C. jejuni* cells in the culture to ciprofloxacin at designated

Table 1. *C. jejuni* strains used in this study

Bacterial strain	Genotype or phenotype	Source
NCTC 11168	*C. jejuni* isolate	33
W7	*C. jejuni* isolate	42
IA3902	*C. jejuni* isolate	41
CMT	a naturally occurring variant of *C. jejuni* NCTC 11168	this study
CMT_{W199G}::Km	CMT derivative; *cj1621*::Km^R, 199W→G reversion in MutY	this study
CMT::Km	CMT derivative; *cj1621*::Km^R	this study
11168_{G199W}::Km	NCTC 11168 derivative; *cj1621*::Km^R, 199G→W mutation in MutY	this study
11168::Km	NCTC 11168 derivative; *cj1621*::Km^R	this study
11168 mutY::cat	NCTC 11168 derivative; *mutY*::*cat* insertional mutation	this study
CMT mutY::cat	CMT derivative; *mutY*::*cat* insertional mutation	this study

timepoints. To ensure that the inoculum was free of pre-existing resistant mutants, three 200 μL precultures were plated on selection plates. The test results were used only if no FQ^R mutants were present in these 200 μL precultures.

Assay for spontaneous FQ^R and Amp^R mutation frequencies

To determine spontaneous mutation frequencies for FQ^R or Amp^R in *Campylobacter*, the methods of Bjorkholm *et al.*[28] and Hanninen *et al.*[16] with minor modifications were applied. Briefly, for each *C. jejuni* isolate 30 μL (~10^5 cfu) overnight cultures were distributed into 20 tubes containing 3 mL of MH broth. Cultures were shaken for 24 h at 42°C. After incubation, colony counts (cfu) of evolved mutants in each tube were determined by spreading 1.5 mL on MH agar plates containing 1 mg/L ciprofloxacin or 100 mg/L ampicillin (~10×MIC). The number of total viable bacteria was determined from three tubes by dropping 10 μL of 10^{-4}, 10^{-5} and 10^{-6} dilutions onto non-selective MH agar plates. After 2 days of incubation in a microaerobic atmosphere at 42°C, colonies were counted. The frequency of resistant mutants was expressed as the mean number of resistant colonies divided by the mean of the total number of viable cells. The mutation frequencies were calculated from the mean of 20 cultures for the respective isolate. To ensure that the 30 μL of bacteria used to inoculate the 3 mL sample cultures was free of pre-existing resistant mutants, three 30 μL precultures were plated on selection plates. The sample cultures were used to measure the mutation frequency only if no mutants were present in these 30 μL precultures. Nine or 10 colonies growing on selective MH plates from the spontaneous FQ^R or Amp^R mutation frequency test were randomly picked to sequence the QRDR in the *gyrA* gene and the promoter region of the bla_{OXA-61} gene, respectively (primers are listed in Table 2). Meanwhile, these colonies were sub-cultured and tested for their susceptibility to ciprofloxacin and ampicillin, respectively, as described above.

WGS of *C. jejuni* CMT

Briefly, genomic DNA of *C. jejuni* NCTC 11168 and its CMT variant were prepared from bacteria grown on MH agar medium using a Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. An Illumina Genome Analyzer was used to sequence the genome of the CMT isolate according to the manufacturer's instructions, with read lengths of 150 bp. The generated reads were assembled *de novo* into contigs using the Velvet (v 1.2.10) and VelvetOptimiser (v 2.2.5).[34]

Comparative genomics analysis

The contigs were aligned against the reference genome *C. jejuni* NCTC 11168[33] by using Mauve (v 2.3.1).[35,36]

Site-specific reversion (W199→G)/mutation (G199→W) of MutY in *C. jejuni* isolates

In order to investigate the role of the single nucleotide change (G→T) of *mutY* in promoting the emergence of FQ^R mutants in *C. jejuni*, this mutation in the CMT isolate was reverted to the WT sequence using a method previously reported, with some modifications.[20,37] Cj1621 and *mutY* (*cj1620c*) are tandemly positioned on the chromosome of *C. jejuni*, but transcribed in opposite directions. Cj1621 encodes a possible periplasmic protein with unknown function. A Km^R marker was inserted in the *cj1621* gene upstream of *mutY* to facilitate the reversion of the specific mutation in *mutY* by homologous recombination. Briefly, a 896 bp fragment containing 702 bp of the cj1621 encoding sequence with 194 bp immediately downstream of the gene, and another 712 bp fragment containing the 5′ region of *cj1621*, the intergenic region and the 5′ *mutY* sequence up to the mutation site, were amplified by PCR using *C. jejuni* 11168 DNA as the template and primers listed in Table 2. These two PCR fragments were then linked with the Km^R marker by overlap PCR using the listed primers. The overlap PCR product was purified and used to naturally transform *C. jejuni* CMT. Transformants were screened on MH agar plates containing 30 mg/L

Table 2. PCR primers used in this study

Primer	Sequence	PCR products
mutY-FFm	CAAAGCCCaTATATCAAGCAAA	712 bp fragment containing *mutY*(T595) for MutY mutation
mutY-FF	CAAAGCCCcTATATCAAGCAAA	712 bp fragment containing *mutY*(G595) for MutY reversion
mutY-FR	**CATTCTATAGATATATTGATAAGCG**AAAAACCTACACCTAAACCTT	with mutY-FFm/mutY-FF for amplifying the 712 bp fragment for MutY mutation/reversion
aphA3-F	CGCTTATCAATATATCTATAGAATG	*aph* gene
aphA3-R	GATAATGCTAAGACAATCACTAAA	
mutY-RF	**TTTAGTGATTGTCTTAGCATTATC**TTTGTTGCAGGAGTAGTTTTTA	896 bp fragment for MutY mutation/reversion
mutY-RR	TTTTAAGTCCCTTGTGTCTACC	
mutY-5F	TCGGGTTTTAGCGTATTGCT	*mutY*-5′ end
mutY-5R	CGGGGTACCAGCCCCAAACTTATCCACG	
cat-F	CGGTGGTACCTGGAGCGGACAACGAGTAAA	*cat gene*
cat-R	CGCGGATCCTCAGTGCGACAAACTGGGATT	
mutY-3F	CGCGGATCCATTTGCGATACAGAAAAGCCAA	*mutY*-3′ end
mutY-3R	GCTGTTTTTGGAGGATCTGC	
GyrAF1	CAACTGGTTCTAGCCTTTTG	*gyrA* QRDR region
GyrAR1	AATTTCACTCATAGCCTCACG	
p0299-F	TCTCATTTTGCATACCTCAA	bla_{OXA-61} promoter region
p0299-R	CTCCATAGCCCTTGAAAAGT	

Lowercase letters represent the T595 or G595 nucleotide in the *mutY* gene.
The nucleotides in bold represent sequences from the *aphA3* gene designed for overlap PCR.
The underlined nucleotides represent the restriction sites added to the primers (KpnI and BamHI).

kanamycin and confirmed by chromosomal DNA amplification of the gene flanking the insertion site. This resulted in CMT_{W199G}::Km with reversion (W199→G) in MutY through homologous recombination, which was confirmed by DNA sequencing. Another construct, CMT::Km, containing only a Km^R cassette in *cj1621* of the CMT isolate (without the reversion) served as a control to demonstrate that neither the presence nor the location of the Km^R cassette in *cj1621* had any effect on the emergence of FQ^R mutants.

A similar protocol was utilized to construct 11168_{G199W}::Km, with a point mutation (G199→W) in MutY of *C. jejuni* 11168 in order to confirm its mutator effect in *C. jejuni* cells. Another construct, 11168::Km, containing only a Km^R cassette in *cj1621* of the 11168 isolate, served as a control as described above.

Construction of an insertional MutY mutant in C. jejuni isolates

Briefly, primers *mutY*-5F and *mutY*-5R were used to amplify the 5′ part of *mutY* and its upstream region (*mutY*-5′ fragment), while primers *mutY*-3F and *mutY*-3R were used to amplify the 3′ part of *mutY* and its downstream region (*mutY*-3′ fragment). Primer pair *cat*-F/*cat*-R was used to amplify the *cat* gene from the pRY112 plasmid, encoding chloramphenicol resistance, using Phusion High-Fidelity DNA Polymerase (New England Biolabs, USA). After KpnI and BamHI digestion, the *mutY*-5′, *cat* and *mutY*-3′ PCR fragments were ligated by T4 DNA ligase (New England Biolabs, USA) and amplified utilizing *mutY*-5F and *mutY*-3R primers, resulting in the construction of a *mutY*-5′-*cat*-*mutY*-3′ PCR product. The purified *mutY*-5′-*cat*-*mutY*-3′ product was then electroporated into *C. jejuni* competent cells. Transformants were selected on MH agar plates containing 10 mg/L chloramphenicol. The insertion of the resistance marker into the *mutY* gene was confirmed by PCR using primers *mutY*-5F and *mutY*-3R.

Structural modelling of C. jejuni MutY

The crystal structure of MutY in epsilonproteobacteria is still unknown. To shed light on how the G199→W mutation might affect the function of MutY in *C. jejuni*, the MutY structure was modelled according to the crystallized MutY structures in other bacterial species. The modelling was conducted and completed using the SWISS-MODEL web site (http://swissmodel.expasy.org/). Briefly, the amino acid sequence of *C. jejuni* MutY was searched with BLAST[38] against the primary amino acid sequences deposited in the SWISS-MODEL template library (SMTL, last update 3 December 2014; last included PDB release 28 November 2014). For each identified template, the template's quality was predicted from features of the target template alignment. The templates with the highest quality were then selected for model building. Structural models were built using the target template alignment with Promod-II[39] and MODELLER.[40] The modelled structure of MutY of *C. jejuni* was then animated and visualized by the PyMOL Molecular Graphics System, Version 1.7.4 (Schrödinger, LLC).

Results

Abnormal ciprofloxacin susceptibility pattern of C. jejuni isolate CMT

During regular MIC testing, it was observed that the MIC of ciprofloxacin in isolate CMT varied with the duration of incubation. At 24 h of incubation, the CMT isolate showed an MIC of 0.06 mg/L, similar to that of other three *C. jejuni* strains, NCTC 11168, IA3902[41] and W7[42] (Figure 1). However, after 48 h of incubation growth was observed in wells with ciprofloxacin concentrations as high as 2 mg/L for the CMT isolate (Figure 1). This abnormal pattern did not occur with other tested strains. This observation was reproducible in multiple experiments. The wells that did not show visible growth until after 48 h of incubation were passaged onto MH plates with 1 mg/L ciprofloxacin. All these passaged cultures from CMT grew on the selective MH plates after overnight incubation, while the passaged cultures from 11168 did not show any growth. These observations suggested that FQ^R mutants might have developed from the CMT isolate during the MIC test.

CMT rapidly accumulates FQ^R mutants under ciprofloxacin treatment in vitro

To further evaluate the ability of isolate CMT to generate FQ^R mutants under ciprofloxacin treatment, *C. jejuni* CMT and 11168 were compared for growth in MH broth with addition of 1 mg/L ciprofloxacin (Figure 2a). The cfu number of the initial inoculum in each well was adjusted to that normally used for the broth

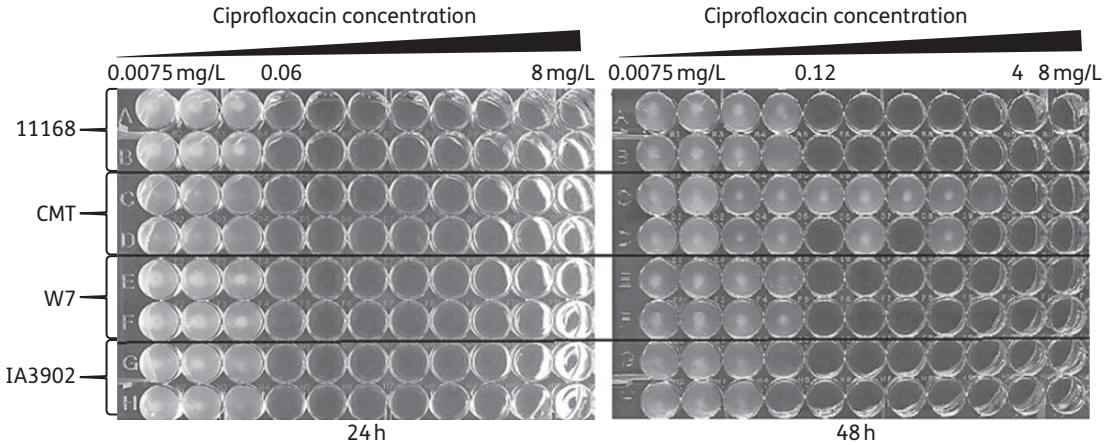

Figure 1. Ciprofloxacin susceptibility test for *C. jejuni* 11168, CMT, W7 and IA3902 utilizing a broth microdilution method in a 96-well plate. The concentration range of ciprofloxacin used for this assay was from 0.0075 to 8 mg/L. Each of the isolates was assayed in duplicate. Pictures were taken of the same plate at 24 and 48 h of incubation.

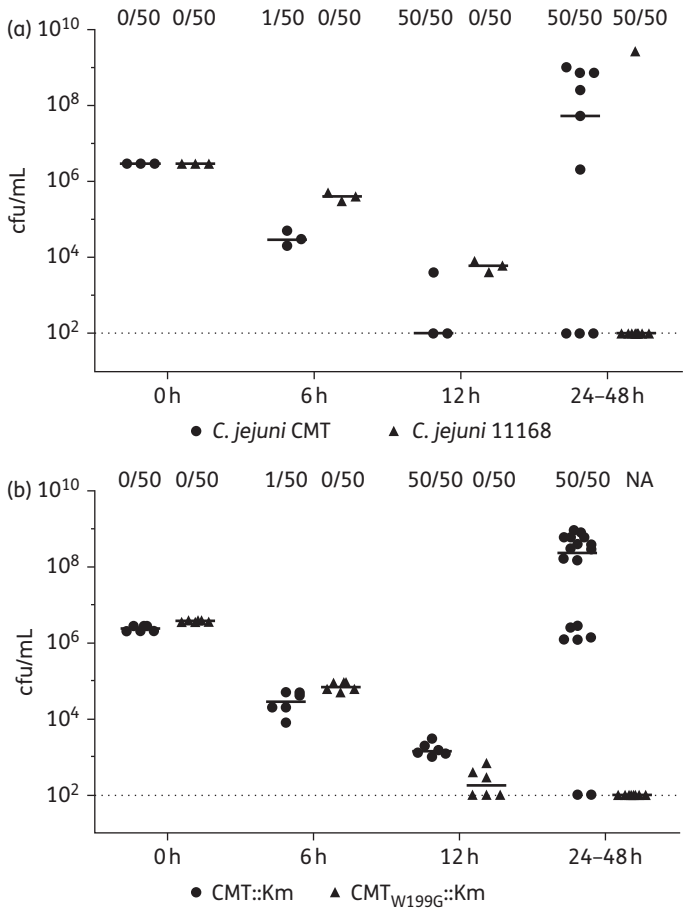

Figure 2. Growth of *C. jejuni* under ciprofloxacin (1 mg/L) treatment. (a) Comparison of *C. jejuni* CMT (filled circles) with NCTC 11168 (filled triangles). (b) Comparison of *C. jejuni* CMT$_{W199G}$::Km (filled circles) with CMT::Km (filled triangles). For both panels, at each timepoint the number of FQ^R mutants among 50 randomly picked colonies from antibiotic-free enumerating plates is shown at the top. The broken line in both panels represents the detection limit of the plating method. NA, no colonies available.

microdilution test (~10^5 cfu/well). The bacterial counts for both CMT and 11168 started to decrease steadily after the addition of ciprofloxacin. Interestingly, CMT experienced greater growth reductions than 11168 during the first 12 h of treatment. However, a drastic increase in cfu/mL was observed with the CMT cultures after 24 h of incubation. Six of the nine cultures of CMT yielded $>10^6$ cfu/mL bacteria after 24 h of treatment (Figure 2a). In contrast, eight of the nine cultures of 11168 did not show bacterial growth. For each strain, up to 50 colonies from each timepoint were randomly picked from the enumerating plates (no antibiotics) and streaked onto MH plates with 1 mg/L ciprofloxacin. None of the colonies from 11168 collected at 0–36 h timepoints grew on the selective MH plates, but all 50 colonies from the single 11168 culture showing growth at 48 h grew on the ciprofloxacin plate. For the CMT cultures, no colonies from the 0 h cultures grew on the selective MH plate. One in 50 colonies from 6 h cultures grew on the MH plate with ciprofloxacin. Notably, all of the colonies from the CMT cultures at each of the 12, 24, 36 and 48 h timepoints grew on ciprofloxacin plates, which was distinct from that of 11168 cultures. These results suggest that the deferred growth in the CMT cultures after 24 h of incubation was not due to the efficacy loss of ciprofloxacin in the medium, but was mainly attributable to the growth of FQ^R mutants in these cultures. It is interesting to note that the percentage of FQ^R mutants increased rapidly in the CMT cultures during the first 12 h of incubation, which subsequently allowed further accumulation of FQ^R mutants.

CMT has notably elevated spontaneous FQ^R mutation frequency

CMT and the other three *C. jejuni* isolates, 11168, IA3902 and W7, were tested for spontaneous FQ^R mutation frequency in MH broth under conventional conditions without adding any antibiotics. This assay was performed in triplicate. *C. jejuni* 11168, IA3902 and W7 had similar spontaneous FQ^R mutation frequencies (10^{-9}). The average FQ^R mutation frequency for the CMT isolate was 1.2×10^{-7}, ~2 logs higher than that of the other three *C. jejuni* isolates (Figure 3a).

Several FQ^R colonies growing on selective plates were picked for strains 11168 and CMT and subsequently sequenced for the QRDR region in the *gyrA* gene and tested for susceptibility to ciprofloxacin (Table 3). For strain 11168, characteristic mutations had occurred either as a single substitution consisting of C257→T or G268→A transition, which leads to a Thr-86→Ile or Asp-90→Asn amino acid change in FQ^R mutants, with MIC values of 8–16 mg/L. However, all colonies sequenced for the CMT strain carried a C257→A or G268→T transversion in the QRDR region, which resulted in a Thr-86→Lys or Asp-90→Tyr amino acid change. Antibiotic susceptibility tests showed that all FQ^R mutants carrying Thr-86→Lys or Asp-90→Tyr showed an MIC of 4 mg/L for ciprofloxacin. This result revealed that FQ^R mutants generated from the CMT strain had distinct *gyrA* mutation patterns compared with that of 11168 or other FQ^R mutant *C. jejuni* isolates that were also sequenced for QRDR regions in the *gyrA* gene.[16] Together, spontaneous FQ^R mutation tests and the ciprofloxacin treatment assay indicate that that the CMT strain generates FQ^R mutants much more frequently than does the 11168 isolate.

Genome sequencing and comparative genomics analysis

To fully investigate the genetic basis of the high-frequency emergence of FQ^R mutants in the CMT isolate, this isolate was subjected to WGS analysis. *De novo* assembled contigs were aligned against the NCTC 11168 genome. Few artificial gaps were observed due to the presence of repetitive sequences in the genome, which makes the software unable to align some tiny contigs appropriately (data not shown). Excluding the gaps, multiple single nucleotide changes, frame shifts (due to single nucleotide deletion or insertion) and previously reported homopolymeric nucleotide stretches were observed in the CMT genome compared with the genome of 11168. As shown in Table S1 (available as Supplementary data at *JAC* Online), most of the mutations occurred in genes not related to DNA repair and thus were not expected to affect spontaneous mutation. However, there was a G→T transversion in *cj1620*, which leads to a G199→W amino acid change in the coding sequence. *cj1620* is an orthologue

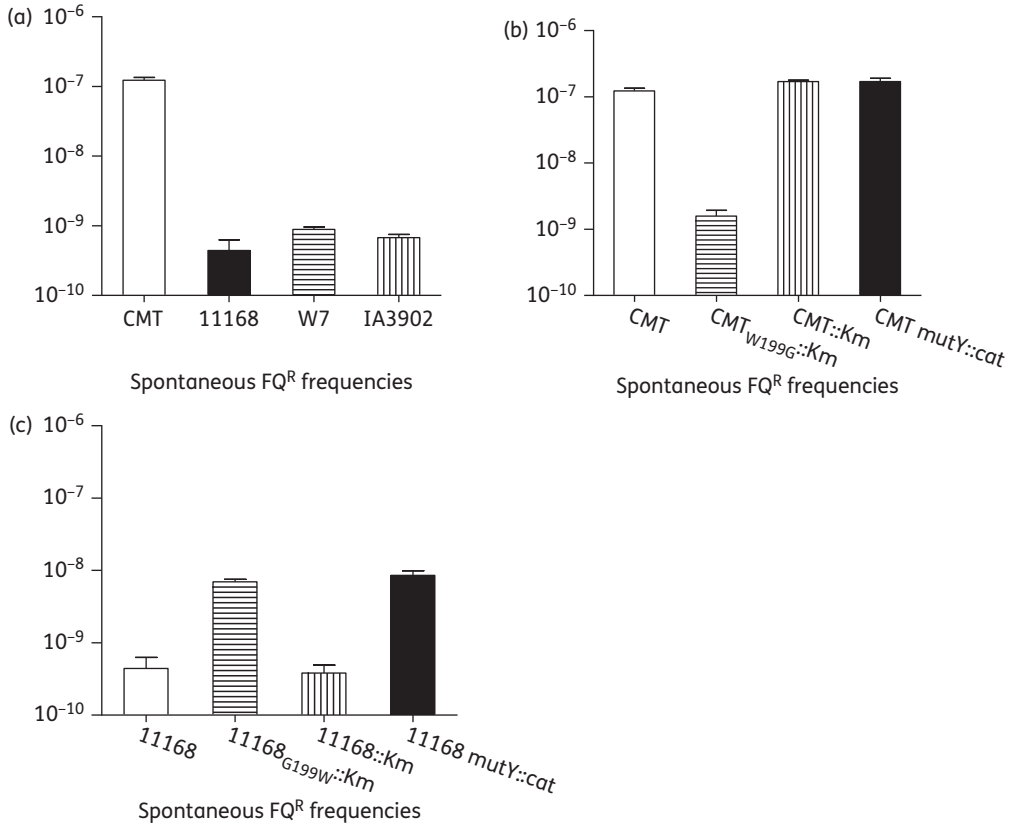

Figure 3. Spontaneous FQ^R mutation frequencies of various *C. jejuni* isolates. (a) Elevated mutation frequency in *C. jejuni* CMT compared with strains 11168, W7 and IA3902. (b) Reduced mutation frequency in the revertant of MutY of the CMT isolate. (c) Mutation of *mutY* increases spontaneous FQ^R frequencies in *C. jejuni* 11168. Each bar represents the mean value of triplicate experiments.

Table 3. Differences in the GyrA mutations of FQ^R mutants from different strains

		No. of isolates with different mutations in GyrA				
C. jejuni isolate	No. of total isolates (colonies)	Asp-90→Asn (G268→A)	Thr-86→Ile (C257→T)	Asp-90→Tyr (G268→T)	Thr-86→Lys (C257→A)	MIC of ciprofloxacin (mg/L)
CMT	9	0	0	8	1	4
CMT_{W199G}::Km	10	9	1	0	0	8–16
NCTC 11168	10	6	4	0	0	8–16

of DNA repair gene *mutY*, encoding a putative adenine glycosylase that has the ability to remove adenines from adenine/7,8-dihydro-8-oxoguanine (8-oxoG) mismatches in *E. coli*, which is specific for the repair of the G→T or C→A transversion.[43] In *C. jejuni* 11168, the *mutY* gene encodes a protein of 339 amino acids. At the amino acid level, the MutY products of *C. jejuni* 11168 and *Escherichia coli* K-12 MG1655 share 35% identity. The G199 is well conserved in MutY proteins among the 10 bacteria species that were analysed in this study (Figure S1), suggesting that it might be important for the function of MutY. Thus we hypothesized that the single point mutation in *mutY* was related to the increased spontaneous mutation frequencies in the CMT isolate.

Reversion of the G199→W mutation in MutY significantly decreases the spontaneous FQ^R mutation frequency of the CMT isolate

To define the role of the G→T transversion in *mutY* in affecting the spontaneous mutation rate in *C. jejuni*, the G199→W mutation in the CMT isolate was reverted by homologous recombination, creating construct CMT_{W199G}::Km, in which the reversion was accompanied by insertion of a kanamycin resistance cassette in the adjacent gene *cj1621*. Another construct, CMT::Km, which only contained the kanamycin resistance cassette in *cj1621*, was also made as a control. As shown in Figure 3(b), the CMT::Km isolate did not show any change in spontaneous FQ^R mutation

Table 4. Spontaneous Amp^R mutation frequencies and the point mutation in the bla_{OXA-61} promoter region in ampicillin-resistant *C. jejuni* mutants

C. jejuni isolate	Spontaneous Amp^R mutation frequencies	No. in (total no.) picked ampicillin-resistant colonies with the G→T mutation in bla_{OXA-61} promoter region	Ampicillin MIC (mg/L) before mutation	Ampicillin MIC (mg/L) after G→T mutation
CMT::Km	3.31E−07	7 (7)	16	256
CMT_{W199G}::Km	4.26E−10	8 (8)	16	256

frequency ($\sim 1.7\times10^{-7}$) compared with that of the CMT isolate ($\sim 1.2\times10^{-7}$), while CMT_{W199G}::Km showed a drastic decrease in spontaneous FQ^R mutation frequency ($\sim 1.6\times10^{-9}$). Sequence analysis of the QRDR region in the *gyrA* gene of FQ^R mutants from the CMT_{W199G}::Km isolate revealed that the mutation patterns had changed to C257→T or G268→A transition in FQ^R mutants, instead of the C257→A or G268→T transversion in FQ^R mutants from the CMT isolate (Table 3). Together, these results indicated that the G199→W mutation was responsible for the increased spontaneous FQ^R mutation frequency in the CMT isolate, and reversion of this mutation recovered the function of MutY in repairing G:C→T:A transversion in *C. jejuni* cells, substantially decreasing the emergence of FQ^R mutants in *C. jejuni*.

The CMT::Km and CMT_{W199G}::Km constructs were further compared for growth in MH broth in the presence of 1 mg/L ciprofloxacin. This assay was conducted twice under the same conditions; the results of the two independent experiments were combined and are shown in Figure 2(b). Similar to the results for CMT and 11168 isolates, the cfu/mL of both CMT::Km and CMT_{W199G}::Km decreased steadily in the first 12 h of incubation. As expected, significant increases in cfu/mL were observed in the CMT::Km cultures after 24 h of incubation. Sixteen of the 18 cultures of CMT::Km yielded $>10^6$ cfu/mL bacteria after 24 h of incubation. In contrast, none of the 18 cultures of CMT_{W199G}::Km showed bacterial growth after 24 h, which indicated that the W199→G reversion in *mutY* prevented the emergence of FQ^R mutants in the CMT isolate under the experimental conditions used in this study. For each isolate, up to 50 colonies were randomly picked for each strain and at each timepoint from the non-antibiotic plates, and streaked onto MH plates with 1 mg/L ciprofloxacin. None of the colonies from CMT_{W199G}::Km cultures at any timepoints grew on the selective MH plates. For CMT::Km, no colonies from the 0 h cultures grew on the selective MH plate, while one of the 50 colonies from 6 h cultures grew on the ciprofloxacin-containing plates. Similar to the results of CMT cultures, all 50 colonies from CMT::Km cultures at each of the 12, 24, 36 and 48 h timepoints grew on the selective MH plates. These results from the selective plates confirmed that the regrowth after 12 h of treatment was due to emergence of FQ^R mutants and that the W199→G reversion in MutY prevented the emergence of the mutants.

The G199→W mutation in MutY abolishes the anti-mutator function of MutY in Campylobacter

To verify the mutator effect of the G199→W mutation in MutY, the point mutation was introduced into *C. jejuni* 11168 by homologous recombination. A MutY insertional mutant of 11168 was also constructed to confirm the anti-mutator role of MutY in *Campylobacter*. As shown in Figure 3(c), the G199→W mutation in MutY of 11168 significantly increased the spontaneous FQ^R mutation frequency compared with that of the 11168 isolate. The same increased frequency was also observed in the *mutY* insertional mutant. As a control, the Km^R insertion in the *cj1621* gene alone did not change the mutation frequency of 11168. These results indicated that the G199→W mutation had the same effect on mutation frequencies as the *mutY* insertional mutation, suggesting that this mutation significantly compromised the anti-mutator effect of MutY in *C. jejuni*. Additionally, an isogenic MutY insertional mutant was also made in strain CMT and compared with the CMT isolate for spontaneous FQ^R mutation frequencies. No difference in the FQ^R mutation frequency was observed between CMT and its isogenic *mutY* insertional mutant (Figure 3b). Together, these results indicate that the G199→W mutation abolishes the anti-mutator function of MutY, which leads to the elevated frequency of emergence of FQ^R mutants in *C. jejuni*.

The G199→W mutation in MutY also significantly increases the spontaneous Amp^R mutation frequency in C. jejuni

To investigate whether the G199→W mutation in MutY has a general effect on the emergence of antimicrobial-resistant mutants in *C. jejuni*, the CMT::Km and CMT_{W199G}::Km constructs were compared for the spontaneous Amp^R mutation frequency. As shown in Table 4, there was a ~700-fold difference in mutation frequency between the CMT::Km isolate and the CMT_{W199G}::Km revertant, in which the G199→W mutation was reverted, Sequencing of the spontaneous Amp^R mutants randomly picked from both the CMT::Km and the CMT_{W199G}::Km background revealed the same G→T transversion in the −10 region of the bla_{OXA-61} promoter. This G→T transversion was previously shown to restore the TATA box, leading to expression of β-lactamase bla_{OXA-61} and consequently high-level resistance to ampicillin in *C. jejuni*.[24] These results indicate that the G199W mutation in MutY also results in a significant increase in the emergence of Amp^R mutants, due to the defect of the MutY mutant in repairing G:C→T:A transversions in the *C. jejuni* genome.

The G199→W mutation probably affects the catalytic domain of MutY in C. jejuni

Structural modelling of *C. jejuni* using the published MutY structure from *Geobacillus stearothermophilus*[44] suggests that G199 is located in helix 10 (Figure S2) and is close to the catalytic domain (the six-helix barrel module and the [4Fe–4S] cluster), which is vital for MutY catalytic enzyme activity. In the MutY of the CMT isolate a hydrophobic amino acid, tryptophan (W), with an extra indole ring

replaced the simple amino acid glycine (Figure S2). This modification may affect the function of the catalytic domain and thus interfere with the removal of adenine by the MutY catalytic domain.

Discussion

Antibiotic resistance in *C. jejuni* is an increasing problem worldwide and is considered a serious threat to public health in the USA.[12] Both acquisition of antimicrobial resistance genes and mutations contribute to the prevalence of antibiotic-resistant *Campylobacter*. Multiple antimicrobial resistance genes have been found in *Campylobacter*, such as the *tet*(O) gene for tetracycline resistance, bla_{OXA-61} for β-lactam resistance, *erm*(B) for macrolide resistance and several aminoglycoside resistance genes.[14,45,46] However, mutation is the most common mechanism of resistance in *Campylobacter* for several classes of clinically important antimicrobial agents, such as FQs[45,47] and macrolides.[15] Except for Mfd, which was previously reported to promote mutation in *Campylobacter*,[48] little is known about genetic factors that affect spontaneous mutation frequencies in this organism. In this study, the single G595→T transversion in *mutY* of *C. jejuni* was found to promote spontaneous FQ^R and Amp^R mutation frequencies. This mutation abolishes the anti-mutator function of MutY and leads to significantly increased emergence of FQ^R and Amp^R mutants in *Campylobacter*. To our knowledge, genetic factors contributing to the naturally occurring mutator *Campylobacter* have not been reported and the findings in this study represent a previously undescribed genetic mechanism that is involved in hypermutation for antibiotic resistance in this important foodborne pathogen.

The G595→T mutation in *mutY* was initially identified by WGS analysis. Although the mutator isolate CMT shows significantly elevated spontaneous mutation frequencies compared with the WT 11168 isolate, there were few ambiguous sites in the alignment of the genome sequences of CMT and 11168 genomes (Table S1). This is due to the fact that the assembled whole-genome sequence was actually a consensus sequence from multiple reads in a mixed population. In the mutator CMT strain, although the mutation frequency is elevated, the vast majority of DNA alleles are still WT. Without a selection step to enrich the mutations, these mutations are only a tiny fraction and massively outnumbered by the WT sequences in the population, Thus, the high mutation frequency in CMT cannot be revealed by WGS alone.

To prove that the G199W change in MutY was linked to elevated mutation frequencies, the mutation was reverted at its original location utilizing homologous recombination. Significant decreases in spontaneous FQ^R and Amp^R mutation frequencies were observed in the resulting revertants (Figure 3 and Table 4). Additionally, the same strategy was utilized to make the site-specific G199→W change in MutY of *C. jejuni* 11168, which showed an effect on the spontaneous FQ^R mutation frequency similar to that of the MutY insertional mutant. Furthermore, insertional inactivation of the *mutY* gene in the CMT isolate did not further increase its mutation frequency, suggesting that G199W alone is sufficient to abolish the anti-mutator function of MutY. Together, these results establish that the naturally occurring mutation in *mutY* confers a mutator phenotype in *Campylobacter*.

The MutY protein in *C. jejuni* is specific for the repair of the G→T or C→A transversion, which is similar to that observed in *E. coli*.[43] The G199→W mutation resulted in loss of the repair function and consequently an elevated mutation rate with the G→T or C→A transversion. This was shown by the findings that all of the FQ^R mutants of the CMT isolate carried a C257→A or G268→T transversion in the QRDR region of GyrA (Table 3) and also all of the Amp^R mutants of the CMT isolate carried a G→T transversion in the bla_{OXA-61} promoter region (Table 4). We also tested the mutation rate for macrolides, and no apparent difference was observed in the frequency of erythromycin resistance between WT 11168 and the CMT isolates (data not shown). This can be explained by the fact that macrolide resistance is mediated by an A→G transition or A→C transversion in the ribosomal RNA of *C. jejuni*,[8,23] and MutY is not expected to repair these types of mutations in *C. jejuni*. Thus, loss of MutY function selectively affects antibiotic resistance mechanisms that are mediated by G:C→T:A transversions in target genes.

In this study, all of the FQ^R mutants from the CMT isolate showed intermediate-level FQ^R (MIC = 4 mg/L). When the concentration of ciprofloxacin was increased to 4 mg/L in the mutant-enumerating plates, no obvious difference was observed in the spontaneous FQ^R mutation frequency between NCTC 11168 and the CMT isolates (data not shown), consistent with the MIC results and suggesting that 4 mg/L prevented the emergence of the FQ^R mutants carrying a C257→A or G268→T transversion. Blondeau *et al*.[49] introduced the concept of mutant prevention concentration (MPC), which represents a threshold above which the selective proliferation of resistant mutants is expected to occur only rarely. In this study, increasing the ciprofloxacin concentration from 1 to 4 mg/L significantly reduced the spontaneous FQ^R mutation frequency of the CMT isolate. Thus, understanding MPC and managing the doses of FQs may significantly reduce the development of resistance in *Campylobacter*.

The crystal structures of MutY from *E. coli* and another thermophilic bacterium (Geo), *Bacillus stearothermophilus*, were resolved,[44,50] revealing the basis for recognizing lesions in the A·oxoG pair and for catalysing removal of the adenine base. However, the function of G199 in MutY was not defined in these crystallization studies. To shed light on how the G199→W mutation might affect the function of MutY, we conducted modelling of the *C. jejuni* MutY structure (Figure S2), which suggested that G199 is located next to the MutY catalytic domain and that the G199→W mutation may interfere with the function of this vital domain. However, this finding from structural modelling remains to be confirmed experimentally in future studies.

The growth rates of CMT, its revertant and *C. jejuni* 11168 appeared to be similar, as measured by their growth curves in MH broth and the colony sizes on MH plates (data not shown), which suggests that there is no obvious growth defect related to the G199W mutation in MutY of *Campylobacter*. However, it is still unknown whether there is a long-term fitness cost of the G→T transversion in *mutY* for *Campylobacter*. Although many mutations are deleterious to organisms, there are multiple reports of naturally occurring pathogenic organisms that exhibit permanent mutator phenotypes due to defects in the DNA repair system.[28,29,51–53] Thus, bacterial organisms have great potential to adapt to stress by fixing novel mutations that increase fitness.[54] There are only limited numbers of *C. jejuni* MutY sequences available in the NCBI database. However, examination of the 35 published *C. jejuni* genomes indeed revealed a number of nucleotide sequence polymorphisms of MutY (Figure S3), some of which are

also located near the functional domains of the MutY protein. Whether these mutations in MutY also contribute to a mutator phenotype in *Campylobacter* remain to be determined.

To our best knowledge, this is the first identification of a genetic factor conferring a natural mutator phenotype in *Campylobacter*. This mutator phenotype is due to a single amino acid change (G199W) in MutY, which results in loss of the repairing function. Antibiotic resistance associated with a G→T or C→A transversion is influenced by the G199W mutation in MutY. Thus, the mutator phenotype conferred by the *mutY* mutation may help *Campylobacter* adapt to antibiotic stresses. Mutators are a risk factor for clinical treatment of bacterial infections as they tend to promote the selection of mutants with resistance to antibiotics.[26] Thus, identification of mutator strains and understanding their associated mechanisms may help to guide the clinical use of antibiotics and treatment regimens, reducing the development of antibiotic resistance.

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Transparency declarations

None to declare.

Disclaimer

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Supplementary data

Table S1 and Figures S1 to S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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