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The bile salt deoxycholate induces *Campylobacter jejuni* genetic point mutations that promote increased antibiotic resistance and fitness

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Oxidative damage to DNA is a significant source of mutations in living organisms. While DNA damage must be repaired to maintain the integrity of the genome and cell survival, errors made during DNA repair may contribute to evolution. Previous work has revealed that *Campylobacter jejuni* growth in the presence of bile salt deoxycholate (DOC) causes an increase in reactive oxygen species and the occurrence of 8-oxo-deoxyguanosine (8-oxo-dG) DNA lesions. The fundamental goal of this project was to determine if *C. jejuni* growth in a medium containing DOC contributes to DNA mutations that provide a fitness advantage to the bacterium. Co-culture experiments revealed that *C. jejuni* growth in a DOC-supplemented medium increases the total number of ciprofloxacin-resistant isolates compared to *C. jejuni* grown in the absence of DOC. We recovered two individual isolates grown in a medium with DOC that had a point mutation in the gene encoding the EptC phosphoethanolamine transferase. Transformants harboring the EptC variant protein showed enhanced resistance to the antimicrobial agent polymyxin B and DOC when compared to an *eptC* deletion mutant or the isolate complemented with a wild-type copy of the gene. Finally, we found that the base excision repair (BER), homologous recombination repair (HRR), and nucleotide excision repair (NER) are involved in general oxidative damage repair in *C. jejuni* but that the BER pathway plays the primary role in the repair of the 8-oxo-dG lesion. We postulate that bile salts drive *C. jejuni* mutations (adaptations) and enhance bacterial fitness in animals.

KEYWORDS

sodium deoxycholate, reactive oxygen species, fluoroquinolone resistance, DNA lesions, DNA repair

Introduction

Bacteria encounter bile salts inside the intestines of warm-blooded animals. The concentration of bile salts ranges from 0.2% to 20% in the intestines of humans (Elliot, 1985), 0.7% in the jejunum, and 0.01% in the cecum of chickens (Lin et al., 2003). Bile salts are primarily composed of salts of cholic, deoxycholic, chenodeoxycholic, and lithocholic acids. Deoxycholic acid (DOC), a secondary bile salt, constitutes 15% of the total bile salts. Bacteria have intricate relations with intestinal bile salts. The composition of bile salts is shaped by the metabolic activities of the intestinal anaerobes (Ridlon et al., 2006). In turn, bile salts dictate the composition of intestinal microbiota (Kakiyama et al., 2013) and act as a protective agent against pathogenic microbes due to their antimicrobial activities (Hofmann and Eckmann, 2006; Sannasiddappa et al., 2017). Bile salts disrupt bacterial membranes, denature proteins, and cause DNA lesions in the bacterial genome (Merritt and Donaldson, 2009; Urdaneta and Casadesus, 2017). Intestinal bacteria adapt to the detrimental effect of bile salts through different physiological changes, including the remodeling of the cell envelope and activation of efflux and stress response systems (Hernandez et al., 2012). *Campylobacter jejuni*, a Gram-negative microaerophilic bacterium, is exposed to bile within the gut of vertebrate hosts.

Campylobacter species are the most common culture-proven cause of bacterial gastroenteritis worldwide, accounting for 400–500 million cases of diarrhea each year (Ruiz-Palacios, 2007). In developed countries, infection with certain strains of *C. jejuni* has been correlated with a higher incidence of Guillain-Barré syndrome (GBS), reactive arthritis, and/or post infectious-irritable bowel syndrome (PI-IBS; Spiller et al., 2000; Allos, 2001; Schwerer, 2002; Frenzen, 2008; Sung et al., 2013). In low- and middle-income countries (LMIC), *C. jejuni* infections result in malnourishment and stunted growth (Haque et al., 2019). Alarming reports of antibiotic-resistant *Campylobacter* are increasing (Engberg et al., 2001; Coker et al., 2002; Bae et al., 2005; Gibreel and Taylor, 2006; Ruiz-Palacios, 2007). In a recent report, ~40% of *Campylobacter* isolates recovered from humans were fluoroquinolone (FQ)-resistant (Bravo et al., 2021). FQ-resistant *Campylobacter* spp. have been added to the World Health Organization's (WHO) priority list of antibiotic-resistant bacteria (Tacconelli et al., 2018).

Several mechanisms of resistance to FQs have been reported in Gram-negative bacteria, including mutations in the genes encoding the topoisomerase enzymes [topoisomerase II (DNA gyrase, composed of two subunits of GyrA and two subunits of GyrB) and topoisomerase IV (ParC and ParE)], decreased outer membrane permeability, and export through an active efflux system (Payot et al., 2006). In *Campylobacter* spp., studies have mainly focused on the determination of *gyrA* mutations conferring resistance, as the *parC/parE* appear to be absent in the genomes of sequenced *C. jejuni* isolates (Luo et al., 2003; Luo et al., 2005; Payot et al., 2006). *C. jejuni* isolates resistant to FQs frequently have mutations in DNA gyrase, particularly in the

quinolone resistance determining region (QRDR) of the GyrA. DNA gyrase is responsible for introducing negative supercoils into DNA to relieve the topological stress from transcription and replication complexes (Hawkey, 2003). The Thr-86-Ile mutation is the most common cause of class-wide FQ resistance among *C. jejuni* isolates and confers a high-level FQ resistance (Hakanen et al., 2002; Beckmann et al., 2004). Other less common mutations have been reported in GyrA (i.e., Thr-86-Ala, Thr-86-Lys, Asp-90-Asn, and Asp-90-Tyr; Changkwanyeeun et al., 2016). Poultry has been and continues to be a significant source of FQ-resistant *Campylobacter*, perhaps due to the past practice of using FQs in food animal production. Even though FQs are no longer used in poultry production, FQ-resistant *Campylobacter* organisms have persisted and even increased (Price et al., 2007; Sproston et al., 2018). Previous work has revealed that a single point mutation (i.e., Thr-86-Ile) in *gyrA*, which confers high-level of resistance to FQs, provides *Campylobacter* bacteria with a fitness advantage in which the FQ-resistant isolate outcompetes the FQ-susceptible isolate in chickens (Luo et al., 2005).

The ability of a pathogen to acquire mutations is essential for survival in damaging environments and is closely connected to the mechanisms of prokaryotic DNA repair systems. *C. jejuni* possesses DNA repair systems that function in base excision repair (BER), homologous recombination repair (HRR), and nucleotide excision repair (NER). Repair of double-strand breaks in *C. jejuni* occurs via the AddAB proteins (functionally analogous to the HRR proteins and RecBCD proteins of *Escherichia coli*) working with RecA (Gourley et al., 2017). BER repairs lesions that cause minor distortion in the DNA helix structure, such as those caused by oxidation, deamination, and alkylation (Krokan and Bjørås, 2013). In contrast, NER fixes damage from bulky DNA adducts, such as the pyrimidine dimers caused by UV light (Kisker et al., 2013). It is noteworthy that *C. jejuni* lacks the mutagenic/error-prone DNA polymerases (Pol II, Pol IV, and Pol V) that many bacteria use to adapt to environmental DNA-damaging agents.

C. jejuni passage through poultry and *C. jejuni* infections in humans are known to result in novel genotypic variants characterized by nucleotide polymorphisms and, depending on the strain, genomic rearrangements (Barton et al., 2007; Ridley et al., 2008). However, the mechanism(s) responsible for generating novel *C. jejuni* variants is currently not known. Previously, we reported that the bile salt DOC results in the production of reactive oxygen species (ROS) and causes DNA lesions in *C. jejuni* as determined by the increased production of 8-oxo-deoxyguanosine (8-oxo-dG) (Negretti et al., 2017). These DNA lesions could lead to genotypic variants unless they are repaired by the DNA repair system.

The types of DNA damage caused by ROS are typically repaired by BER, with the NER pathway acting as a secondary defense against oxidative damage (Demple and Harrison, 1994). The first step of the BER process involves DNA glycosylases removing the damaged nitrogenous base while leaving the

sugar-phosphate backbone intact and creating an apurinic/apyrimidinic (AP) site. The 8-oxo-dG in many bacteria is removed by the DNA glycosylase MutM/Fpg (*aka* OGG1 in eukaryotes). If replication of the lesion yields an 8-oxo-dG: adenine base pair, a second DNA glycosylase termed MutY excises the adenine (*aka* hMYH in eukaryotes) to avoid making the mutation permanent in the next round of replication. *C. jejuni* possesses a MutY adenine DNA glycosylase but lacks a MutM/Fpg homolog. A previous study reported that a single nucleotide change in the *mutY* gene (a G → T transversion at position 595) of *C. jejuni* results in an amazing ~100-fold higher frequency of ciprofloxacin resistance than the isogenic wild-type strain (Dai et al., 2015).

We hypothesized that *C. jejuni* growth in the presence of DOC in the gut causes mutations in the genome, some of which confer a fitness advantage in the intestine. The purpose of this study was, in part, to determine if *C. jejuni* growth in a medium containing the ROS-producing bile salt DOC causes DNA damage leading to adaptive mutations that can confer a fitness advantage. We sought to identify mutations, including those in genes and/or gene promoter regions, which could enhance the fitness of *C. jejuni* within an animal host and contribute to human disease. Our findings, together with data where multiple *C. jejuni* PFGE (pulsed-field gel electrophoresis)-type variants have been recovered from chickens inoculated with a single clone, support our hypothesis that stress-induced mutations are an important mechanism to improve fitness and increased virulence in *C. jejuni*.

Materials and methods

Bacterial strains and growth conditions

C. jejuni strain 81–176 was cultured on Mueller-Hinton (MH) agar containing 5% citrated bovine blood (MHB agar, Hardy Diagnostics, Santa Maria, CA) in a microaerobic atmosphere (5% O₂, 10% CO₂, 85% N₂) at 37°C. The *C. jejuni* mutants and complemented isolates were generated as outlined below. When needed, MHB agar plates were supplemented with 8 µg/ml of chloramphenicol or 250 µg/ml of hygromycin. The isolates were passaged every 24–48 h on MHB agar. Sodium deoxycholate (Na-DOC) was used at a concentration of 0.05% unless it is stated otherwise. *E. coli* Stellar™ (Takara Bio Inc., Berkeley, CA) strain was cultured on LB-Miller agar or in LB-Miller broth (Fisher, Hampton, NH) under aerobic conditions at 37°C.

Generation of ciprofloxacin-resistant isolates

C. jejuni were inoculated in MH broth and MH broth supplemented with 0.05% (w/v) Na-DOC at a final OD₅₄₀ of 0.05 and placed on an orbital shaker at 225 rpm under microaerobic conditions at 37°C. The cultures were then passed into new flasks and normalized to the starting OD₅₄₀ of 0.05, and the process was

repeated for a total of 10 days. After 10-days, 10 ml of the cultures were centrifuged, serially diluted with sterile 1x PBS, and spread onto MHB agar plates supplemented with 0.125 µg/ml of ciprofloxacin. Once growth was observed on the MHB-ciprofloxacin plates, individual colonies were selected for PCR amplification and DNA sequencing to determine the mutations of the *gyrA* gene. This 10-day passage was repeated 4 more times for a total of 5 “runs.”

Identifying point mutations in the *gyrA* gene

A fragment of the *gyrA* gene, containing the QRDR, was PCR amplified and sequenced. Individual bacterial colonies were suspended in water and boiled for 5 min. DNA fragments were amplified using CloneAmp™ HiFi PCR Premix (Takara Bio Inc., San Jose, CA) with the following parameters: 98°C for 4 min, 1 cycle; 98°C for 20 s, 55°C for 30 s, and 72°C for 1.5 min, 35 cycles; 72°C for 10 min, 1 cycle. The primers used in the PCR were *gyrA*-F and *gyrA*-R (Supplementary Table S1). PCR products were cleaned up by the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania). The *gyrA* gene sequence from each isolate was analyzed and compared to the sequence of *gyrA* from the 81–176 wild-type strain (ciprofloxacin-sensitive) using SnapGene software to identify DNA mutations at codons 86 and 90, which confer ciprofloxacin resistance to *C. jejuni*.

Antimicrobial susceptibility tests

To determine the 50% inhibitory concentration (IC₅₀) of ciprofloxacin, antimicrobial susceptibility tests were performed using the broth microdilution technique as described previously (Talukdar et al., 2021). The 96-well microtiter plate was incubated in an orbital shaker under microaerobic conditions at 37°C for 48 h. After incubation, the optical density was determined using a 96-well plate reader (BioTek Instruments Inc., Winooski, VT) at 595 nm (OD₅₉₅). The concentrations and absorbance values were graphed on a logarithmic plot using GraphPad Prism (v6.0.h, La Jolla, CA), and the IC₅₀ was calculated from the concentration-effect curve as described previously (Hakeem et al., 2019).

Ciprofloxacin mutation frequency

To determine the mutation frequency of the *gyrA* gene, two cultures of the *C. jejuni* 81–176 wild-type strain were grown; one in a flask containing 50 ml of MH broth and the other in a flask with 50 ml MH broth with 0.05% Na-DOC and normalized to a starting OD₅₄₀ of 0.04–0.05. The serial passage was continued for 5 days. After each day of serial passage, a portion of the Na-DOC-supplemented culture was transferred to a new flask of MH broth to minimize the variability in colony numbers following plating (a portion of the

bacteria lysed in the presence of the detergent, Na-DOC). Following each day of incubation, 10 ml of each culture was centrifuged and resuspended in 1 ml of 1x PBS. Serial 10-fold dilutions were then performed with each sample, and 10 μ l or 100 μ l of each dilution was spread onto MHB agar plates without antibiotics and MHB agar plates with 8 μ g/ml and 10 μ g/ml ciprofloxacin. Once growth on MHB agar and MHB-ciprofloxacin plates could be observed, the colonies growing on each plate for both samples were counted, and the CFU/mL of ciprofloxacin-resistant (mutated) and total *C. jejuni* were determined for each sample. The mutation frequency was calculated by dividing the CFU/ml of *C. jejuni* ciprofloxacin-resistant isolates by the total CFU/ml. The assay was also performed with a *C. jejuni* *mutY* deletion mutant (Δ *mutY*) and complemented isolate.

Passage of *C. jejuni* in MH broth and MH DOC-supplemented medium

C. jejuni were inoculated in MH broth and MH broth supplemented with 0.05% Na-DOC at a final OD₅₄₀ of 0.04 and incubated in a microaerobic chamber with shaking at 37°C. The cultures were passed into new flasks at an OD₅₄₀ of 0.04–0.05 and the process was repeated until the ODs of the cultures in MH broth and MH DOC-supplemented medium were similar (MH-DOC adapted isolates) and the OD₅₄₀ for the DOC-supplemented culture had stabilized (remained constant over several day periods). Thereafter, the cultures were inoculated into new flasks of MH broth and MH broth supplemented with 0.1% (w/v) Na-DOC at an OD₅₄₀ of 0.05, and the process was repeated until the ODs of the cultures in MH broth and MH DOC-supplemented medium became similar (MH-DOC adapted isolates). The passage was repeated one additional time in MH broth and MH broth supplemented with 0.4% (w/v) Na-DOC. After each 24-h incubation period, a sample of the culture was plated on MHB agar plates containing 0.125 μ g/ml of ciprofloxacin. To determine if the isolates had different sensitivities to DOC, individual colonies were selected and grown in MH broth containing 0.1% and 0.4% Na-DOC. The control consisted of the bacteria grown in MH broth without DOC.

Genomic DNA extraction, whole genome sequencing, and analysis

Isolates were subjected to paired-end Illumina sequencing to accurately identify base substitutions, small insertions and deletions (indels), and copy number changes. Briefly, genomic DNA was extracted from *C. jejuni* isolates and Illumina sequencing libraries prepared using the KAPA Low-Throughput Library Preparation Kit with Standard PCR Amplification Module as previously described (Parker et al., 2015). The libraries were sequenced on an Illumina MiSeq instrument using the MiSeq reagent kit v2 (500-cycles; Illumina) following the manufacturer's protocols. Illumina PE reads for each strain were mapped to the appropriate reference *C. jejuni*

genome [*C. jejuni* 81–176 chromosome sequence: (NC_008787)] using Geneious software (v10.2.3; Biomatters, Ltd., Auckland, New Zealand; Kearse et al., 2012) using the parameters of at least 20x coverage and 80% of reads representing the mutation. All identified mutations were confirmed by repeating the analysis using the breseq software (v0.24rc6) package (Deatherage and Barrick, 2014), and later through manual examination. The complete genome sequences of *C. jejuni* isolates were submitted to GenBank under the project PRJNA634604 (accession: SRR22024544–SRR22024548; SRR22024599–SRR22024608).

Generation of *C. jejuni* mutants and complementation constructs

All modified strains were created through homologous recombination using suicide vectors constructed using In-fusion cloning (Takara Bio Inc., Mountain View, CA) and/or standard restriction enzyme-mediated cloning to link the desired DNA fragments. The suicide vectors were generated by the amplification of DNA flanking regions and combined with a PCR-amplified chloramphenicol resistance cassette (SacII) and the pBSK-Kan2 vector backbone (Talukdar et al., 2020). The orientation of the DNA gene flanking fragments was XhoI/SacII upstream and SacII/SacI downstream. The specific suicide vector constructs were electroporated into *C. jejuni* to generate the desired isolates through homologous recombination. Electroporations were performed using a Bio-Rad *E. coli* Pulser and 2 mm gap cuvettes at the 2.5 kV voltage setting. *C. jejuni* mutants (Δ *eptC*, Δ *mutY*, and Δ *uvrC*) were selected on MHB agar supplemented with 8 μ g/ml chloramphenicol. Constructs were verified by restriction digestion and sequencing.

The plasmid vector prRNAHygR was used to construct the complement plasmids (Gourley et al., 2017). Briefly, a fragment of DNA harboring the gene of interest and upstream native promoter region was PCR amplified and cloned into the prRNAHygR vector using XbaI–BamHI sites. The complementation plasmids were electroporated into the *C. jejuni* mutants, and the transformants were selected on MHB agar plates supplemented with 250 μ g/ml hygromycin. Constructs were verified by restriction digestion and PCR using gene-specific primers. A list of the specific primers and constructs is presented in [Supplementary Table S1](#).

EptC phenotypic assays

Motility assays of *C. jejuni* isolates were performed as previously described elsewhere (Malik-Kale et al., 2007; Neal-McKinney and Konkel, 2012). Briefly, overnight grown *C. jejuni* culture was suspended in MH broth at an OD₅₄₀ of 0.1 and 3 μ l of the bacterial suspension was spotted on an MH soft-agar plate (0.4% agar) and incubated for 48 h in microaerophilic condition. The image of the soft agar plate was captured with a GE ImageQuant LAS-4000 mini and the zone of motility was measured. The antimicrobial

susceptibility test with polymyxin B was performed using the broth microdilution technique as described above. To determine the sensitivity of the isolates to DOC, isolates were grown in MH broth and MH broth supplemented with 0.05% Na-DOC for 24 h. Following incubation, the OD₅₄₀ values of each culture were measured in triplicate.

Statistical analysis

All assays were performed in triplicate to ensure reproducibility. Statistical analysis was performed using GraphPad Prism (v9.3.1).

Results

I. Exposure to DOC induces point mutations and ciprofloxacin resistance in *C. jejuni*

We found that growing *C. jejuni* in conditions to mimic the host-gut environment (medium supplemented with 0.05% Na-DOC) resulted in the production of intracellular ROS, which in turn caused DNA damage (Negretti et al., 2017). DNA damage was evident from the increased generation of the mutagenic 8-oxo-dG lesion and DNA breaks. While efficient and accurate repair of DNA damage is crucial to ensure genome stability and cell survival, we hypothesize that culturing *C. jejuni* with 0.05% DOC increases point mutations in the genome. To test this hypothesis, we examined the growth of *C. jejuni* in MH, a nutrient-rich broth, and MH supplemented with 0.05% Na-DOC. Ciprofloxacin resistance was chosen as a marker for DOC-induced mutations, focusing on the established mechanism of GyrA mutations at residue 86 or 90.

IA. *C. jejuni* growth in DOC and selection of ciprofloxacin-resistant isolates

The serial passage of *C. jejuni* in MH and MH-DOC supplemented broths for 10 days resulted in ciprofloxacin-resistant colonies for both conditions. A total of 150 ciprofloxacin-resistant individual colonies (75 ciprofloxacin-resistant *C. jejuni* isolates grown with Na-DOC and 75 isolates grown without Na-DOC) were picked at random. A fragment of the *gyrA* gene was PCR-amplified for each of the isolates, using gene-specific primers, for sequencing to determine if a change had occurred in the QRDR at residue 86 or 90 (Supplementary Figure S1). Two isolates with point mutations in position 86 [ATA₈₆, ACA (Thr) to ATA (Ile) and AAA₈₆, ACA (Thr) to AAA (Lys)] and two in position 90 [TAT₉₀, GAT (Asp) to TAT (Tyr) and AAT₉₀, GAT (Asp) to AAT (Asn)] were selected among the isolates grown in either MH or MH-DOC broths. These point mutations in the *gyrA* gene have been previously reported in *C. jejuni*

(Changkwanyeeun et al., 2016). We observed a higher proportion of isolates with the GAT (Asp) to TAT (Tyr) signature at position 90 when the bacteria were co-cultured with DOC versus bacteria cultured in MH alone in at least two different runs. The increase in recovery of ciprofloxacin-resistant isolates with the G → T transversion suggests that growth in DOC results in the production of intracellular ROS and an increase in the mutagenic 8-oxo-dG lesion.

IB. GyrA variants confer different levels of ciprofloxacin resistance but similar growth rates

Since the DOC induces point mutations in the *gyrA* gene and thus confers ciprofloxacin resistance, we wanted to quantify the effect of each of the point mutations in the *gyrA* gene on ciprofloxacin resistance and bacterial growth. Concentration-effect curves for ciprofloxacin were generated for the *C. jejuni* ciprofloxacin-resistant isolates and the IC₅₀ (50% of inactivated cells) was determined for each of the isolates. Reported are the IC₅₀ values for two ATA₈₆ isolates, two AAA₈₆ isolates, two TAT₉₀ isolates, and two AAT₉₀ isolates. The IC₅₀ was determined for two isolates with each mutation to reduce the chances of selecting an isolate with a second mutation that had occurred elsewhere in the genome that could provide enhanced ciprofloxacin resistance. The IC₅₀ values of two ATA₈₆ isolates were 7.0 μg/ml and 6.7 μg/ml, which were greater than the other isolates (AAA₈₆, TAT₉₀, and AAT₉₀; Figure 1A). The other isolates with the three signature mutations had similar IC₅₀ values; the IC₅₀ of AAA₈₆ isolates was 2.8 μg/ml (Figure 1B), the IC₅₀ of TAT₉₀ isolates were 2.6 μg/ml and 2.7 μg/ml (Figure 1C), and the IC₅₀ of AAT₉₀ isolates were 2.6 μg/ml and 3.1 μg/ml (Figure 1D). Next, the growth of all *C. jejuni* ciprofloxacin-resistant isolates was determined to see whether the differences in ciprofloxacin resistance alter bacterial growth in MH broth. Regardless of the inherent differences in ciprofloxacin resistance, the ciprofloxacin-resistant isolates demonstrated similar growth rates to each other and the wild-type strain in MH broth (Supplementary Figure S2).

IC. Exposure to DOC increases *C. jejuni* ciprofloxacin-resistance mutation frequency and spectra

In this study, we used the *gyrA* gene and ciprofloxacin resistance to determine the mutation frequency of *C. jejuni* grown in the presence and absence of DOC, as a single point mutation (transition and/or transversion) confers ciprofloxacin resistance. Thus, ciprofloxacin resistance is a proxy for genome-wide mutations. To determine if DOC increases the mutation frequency of the *gyrA* gene, *C. jejuni* was grown in a flask containing MH broth and in a flask with MH broth supplemented with 0.05% Na-DOC. Following a 24 h incubation period, the cultures were processed, and

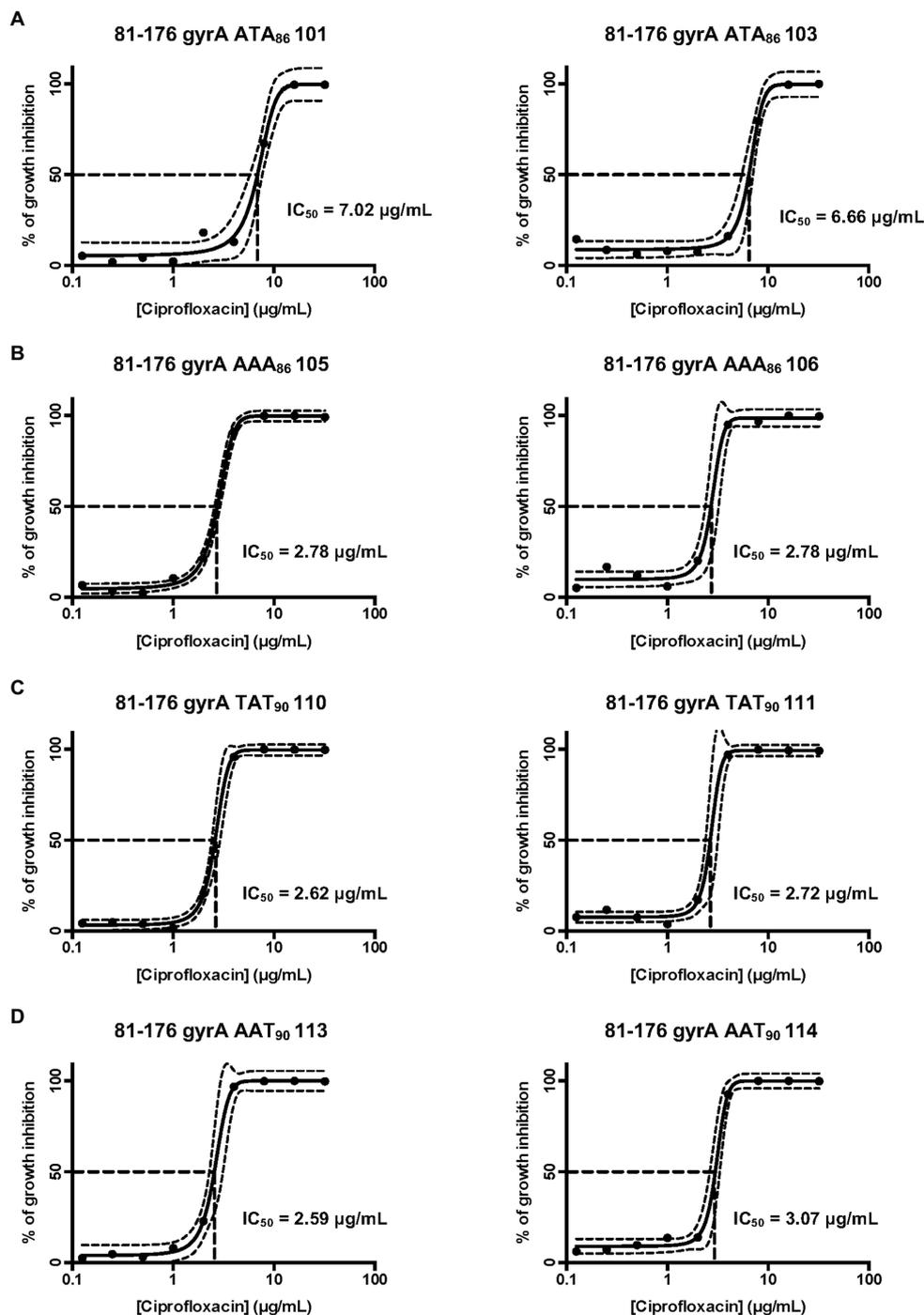


FIGURE 1

Concentration-effect curves for ciprofloxacin on select *C. jejuni* 81–176 isolates. (A) Isolates containing the ACA (Thr) to ATA (Ile) mutation at residue 86; (B) Isolates containing the ACA (Thr) to AAA (Lys) mutation at residue 86; (C) Isolates containing the GAT (Asp) to TAT (Tyr) mutation at residue 90; and (D) Isolates containing the GAT (Asp) to AAT (Asn) at residue 90. The antimicrobial activity of ciprofloxacin was determined by the broth microdilution method, as described in the section “Materials and methods”. Bacterial cultures were treated with different concentrations of ciprofloxacin and incubated for 48h in a microaerobic chamber. The data represent a minimum of three biological replicates. The IC_{50} (50% of inactivated cells) was determined for each isolate. The dashed lines at right angles indicate the ciprofloxacin concentration that is required for a 50% reduction of the cells (IC_{50}). The lines on both sides of the concentration-effect curve represent the 95% confidence intervals.

suspensions were spread onto MHB agar plates and MHB agar plates supplemented with 8 µg/ml and 10 µg/ml of ciprofloxacin. The preliminary assays with MHB agar

supplemented with different concentrations (2, 4, 8, 10, and 12 µg/ml) of ciprofloxacin showed that only ATA_{86} isolates grew well on plates containing 10 µg/ml of ciprofloxacin. The

other ciprofloxacin-resistant isolates either did not grow or grew poorly on plates containing 10 µg/ml of ciprofloxacin. However, all ciprofloxacin-resistant isolates, including the TAT₉₀ isolates grew well on plates containing 8 µg/ml of ciprofloxacin. The ciprofloxacin mutation frequency for the bacteria grown in MH did not significantly change throughout the experiment. In addition, a significant difference was not observed in the mutation frequencies of the bacteria grown in the MH-DOC medium when compared to the MH medium for 1 day when plated on 8 or 10 µg/ml of ciprofloxacin (Figure 2A). However, an increase in the number of *C. jejuni* ciprofloxacin-resistant isolates was observed for the MH-DOC sample plated on 8 and 10 µg/ml of ciprofloxacin versus the MH culture on day 2; a three-fold increase was observed in the frequency of the ciprofloxacin-resistant isolates at 8 µg/ml ($p < 0.001$), and a two-fold increase was observed at 10 µg/ml ($p < 0.01$; Figure 2A). The increase in the number of colonies recovered on 8 µg/ml versus 10 µg/ml of ciprofloxacin, representing an increase in mutation frequencies, likely represented the recovery of isolates with point mutations that do not confer a high level of ciprofloxacin resistance. The increase in mutation frequency observed from day 1 to day 2 with DOC coincided with an increase in bacteria growth in the flask (not shown). Little change was observed in the ciprofloxacin-mutation frequency for the bacteria grown in DOC from day 3 to day 5 (not shown). In summary, *C. jejuni* growth in DOC increased in *gyrA* mutation frequency.

Five *C. jejuni* isolates grown in MH broth and five isolates grown in 0.1% Na-DOC broth for 10 days were selected for whole genome sequencing (WGS) to observe the mutation spectra (Figures 2B,C). In contrast to *C. jejuni* growth in MH, growth in Na-DOC resulted in an increase in DNA transversions versus transitions. The difference in mutation spectra was evident in *gyrA* as well as elsewhere in the genome. A transition is where a purine nucleotide is changed to another purine ($A \leftrightarrow G$), or a pyrimidine nucleotide is changed to another pyrimidine ($C \leftrightarrow T$). A transversion is a point mutation in which a purine (A or G) is changed to a pyrimidine (T or C, one ring) or vice versa. In essence, transitions involve bases of similar shapes, whereas transversions involve bases of different shapes (one ring versus two rings). The types of mutations ($G \rightarrow C$ and $C \rightarrow A$ transversions) identified in the genomes of the *C. jejuni* grown in Na-DOC are consistent with the published data indicating that DOC results in increased levels of ROS and 8-oxo-dG lesions. In summary, *C. jejuni* growth in DOC resulted in a change in the mutation spectra.

II. Exposure to DOC induces mutations that lead to increased fitness/adaptation to DOC

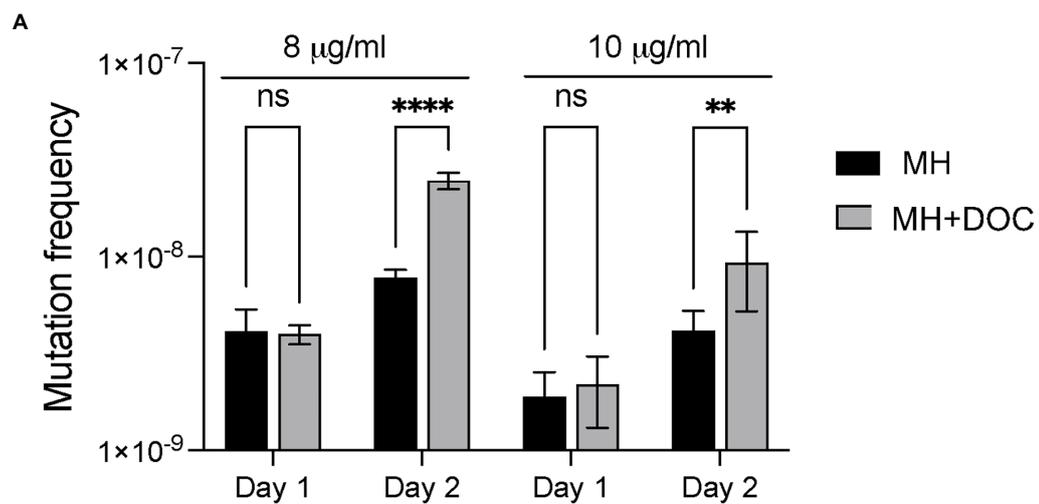
We hypothesize that the increased frequency of the ciprofloxacin-resistant isolates at 8 µg/ml versus 10 µg/ml is

primarily due to an increase in TAT₉₀ isolates, resulting from the $G \rightarrow T$ transversion, as preliminary assays revealed differences in the AAT₉₀ isolates over the course of a 10-day passage in DOC-supplemented media. We further hypothesized that the increased recovery of ciprofloxacin-resistant isolates with $G \rightarrow T$ transversions in the presence of DOC occurs because growth in this medium causes an increase of 8-oxo-dG lesions in the DNA. Based on this hypothesis and our preliminary findings, assays were performed to determine if *C. jejuni* growth in DOC could result in mutation(s) providing a fitness advantage or increased adaptation to DOC.

II. Adaptation of *C. jejuni* to growth in medium with DOC

To determine a specific genomic mutation that occurs upon *C. jejuni* passage in DOC, an experiment was performed whereby the bacteria were subjected to repeated passage in media containing a stepwise increase in the concentration of Na-DOC (from 0.05% \rightarrow 0.1% \rightarrow 0.4%; Figure 3A). A slightly lower bacterial concentration (OD₅₄₀ of 0.04) was used to inoculate the cultures after each day of growth for this experiment than for the ciprofloxacin mutation frequency experiment (described in Results section IC), as we thought this strategy would result in greater pressure for the bacteria to adapt. For the DOC-supplemented culture, the bacteria were grown in MH broth with 0.05% Na-DOC and passaged every day until the OD₅₄₀ for the Na-DOC-supplemented culture was similar to the MH culture over a several-day period (days 16–22; Figure 3B). On day 23, the concentration of Na-DOC in the medium was increased to 0.1%, and the bacterial culture was passaged in 0.1% Na-DOC-supplemented media until the OD₅₄₀ value had increased and was 'similar' to that of the bacteria cultured in MH medium alone at day 34–39 (Figure 3B). At day 40, the concentration of Na-DOC in the medium was increased from 0.1% \rightarrow 0.4%, and the bacterial culture was passaged in 0.4% Na-DOC-supplemented media up to day 50. On days 47–50, the OD₅₄₀ of the MH-DOC culture was similar to the MH culture. Several isolates were picked every few days during the experiment and were frozen. At the conclusion of the experiment, the resistance of selected isolates to DOC was evaluated by measuring the growth (OD₅₄₀) of the bacteria after a 24-h incubation period (Supplementary Figure S3).

Two representative *C. jejuni* isolates (30-1-DOC and 50-2-DOC) that demonstrated increased resistance to Na-DOC after 30 and 50 days of serial passages were selected for WGS, and the data was compared to the sequence of the wild-type parental strain. Illumina sequencing revealed mutations in seven genes in the *C. jejuni* 30-1-DOC isolate and six genes in the *C. jejuni* 50-2-DOC isolate compared to the wild-type isolate (Figure 3C). The *C. jejuni* 30-1-DOC and 50-2-DOC isolates both contained a $C \rightarrow A$ mutation in the *eptC* gene, indicative of a $G \rightarrow T$ transversion on the non-coding strand. Given that both *C. jejuni* 30-1-DOC and 50-2-DOC contained



B

Growth Media	GyrA T86I	GyrA D90N	GyrA D90Y	GyrA D90G
	C>T	G>A	G>T	A>G
MH	3	2	0	0
MH+DOC	1	0	3	1

C

Growth Media	Transition				Transversion					
	G>A	A>G	C>T	T>C	G>C	G>T	A>C	A>T	C>A	T>A
MH	2	0	5	1	0	0	0	0	0	0
MH+DOC	0	1	1	0	0	3	0	0	3	0

FIGURE 2

Culturing *C. jejuni* with deoxycholate (DOC) increases ciprofloxacin mutation frequency and causes an increase in DNA transversions. (A) *C. jejuni* were grown in MH and MH supplemented with sodium deoxycholate (Na-DOC) as outlined in "Materials and methods" and spread onto MHB plates without ciprofloxacin, MHB plates with 8 µg/ml of ciprofloxacin, and MHB plates with 10 µg/ml of ciprofloxacin. The resultant colonies were counted. The mutation frequency was calculated by dividing the CFU of *C. jejuni* ciprofloxacin-resistant by the total CFU. The data on Day 1 indicates the number of bacteria recovered from the bacteria grown in MH broth for one day and in MH broth supplemented with 0.05% Na-DOC for one day. The data are from a single experiment that was representative of three independent assays. Statistical analysis performed: Two-way ANOVA with Sidak's multiple comparison test (** $p < 0.01$; **** $p < 0.001$, ns, non-significant). (B,C) Isolates were recovered after a serial passage in MH and MH supplemented with Na-DOC for 10 days as outlined in "Materials and Methods," and five colonies from each culture were subjected to whole genome sequencing. Culturing *C. jejuni* in MH-DOC supplemented broth versus MH broth resulted in a higher proportion of isolates with DNA transversions at residue 90 of the *gyrA* gene (B). Similarly, WGS revealed more DNA transversions in the isolates recovered from the MH-DOC versus the MH broth (C).

a G → T transversion at residue 60 [CCA (Pro) to CAA (Gln), P360Q mutation] in the *eptC* gene, additional studies were performed to determine if a modification of the EptC native protein would alter an isolate's phenotypic properties. The *eptC* gene encodes for a phosphoethanolamine (pEtN) transferase that modifies a flagellar rod protein, a domain on the lipooligosaccharide (LOS), and several N-linked glycans (Cullen et al., 2013). The *eptC* gene is 1,784 bp in length and encodes a protein of 60 kDa (Supplementary Figure S4).

IIB. Phenotypic characterization of a *C. jejuni eptC* mutant

To explore the relationship between DOC and an *eptC* mutation, an *eptC* deletion mutant ($\Delta eptC$) was generated and the mutant was complemented with a copy of the wild-type gene or a copy of the gene containing the P360Q mutation. The *C. jejuni* wild-type strain and EptC variants were tested for

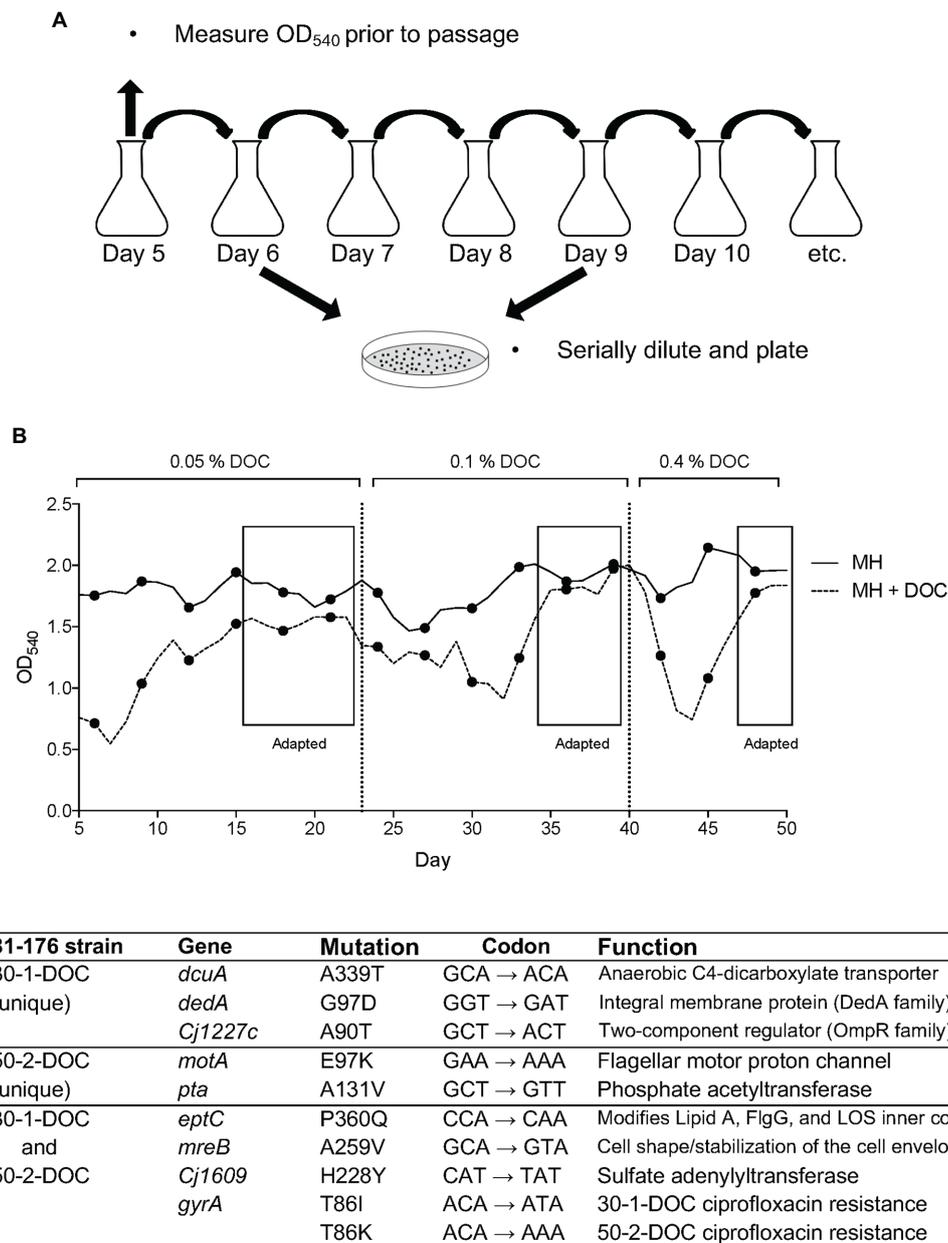


FIGURE 3

Adaptation of *C. jejuni* to growth in medium with deoxycholate (DOC). (A) Method of serial passage used to recover *C. jejuni*-DOC adapted isolates. Serially diluted aliquots of the bacterial growth cultures were spread onto MHB agar plates containing ciprofloxacin. (B) The optical density (OD₅₄₀) values were generated from serially passaged cultures (samples were diluted to be in the linear range of the instrument). (C) Genomic mutations in two *C. jejuni* isolates 30-1-DOC and 50-2-DOC grown in a medium with DOC recovered after 30 and 50 days of passages, respectively.

motility, sensitivity to polymyxin B, and resistance to DOC. Others have reported that a *C. jejuni* Δ *eptC* has reduced motility and increased polymyxin B sensitivity compared to a wild-type isolate (Scott et al., 2012; Fage et al., 2014; Lim and Kim, 2017).

Motility assays revealed that the Δ *eptC* mutant was impaired in motility when compared to the wild-type strain, which is in agreement with previous reports (Cullen et al., 2012; Scott et al.,

2012; Fage et al., 2014). Transformation of the Δ *eptC* mutant with a wild-type copy of the *eptC* gene (*EptC_{WT}*) and the *eptC* gene harboring the CCA to CAA point mutation (P360Q variant, *EptC_{P360Q}*) restored the motility of the isolates to nearly that of the wild-type strain (Supplementary Figure S5).

Polymyxin B is a positively charged lipopeptide that binds to the negatively charged surface of Gram-negative bacteria and disrupts the outer cell membrane. The Δ *eptC* mutant

demonstrated increased sensitivity to polymyxin B when compared to the wild-type strain (Figure 4), which is again consistent with previous studies (Cullen et al., 2012; Scott et al., 2012; Fage et al., 2014). Synthesis of the EptC_{WT} protein in the Δ eptC mutant demonstrated a level of polymyxin B resistance nearly to the same level as the *C. jejuni* wild-type strain. However, unexpectedly, synthesis of the EptC_{P360Q} protein in the Δ eptC mutant demonstrated polymyxin B resistance at a level greater than observed for the wild-type strain (Figure 4).

The growth of the Δ eptC mutant and Δ eptC mutant synthesizing either the EptC_{WT} protein or EptC_{P360Q} variant protein was then examined in a medium supplemented with 0.05% Na-DOC. No significant difference was observed in the growth of the Δ eptC mutant in the DOC-supplemented medium when compared to the wild-type strain (Figure 5). However, an increase was observed in the growth of the Δ eptC transformant synthesizing the EptC_{WT} protein when compared to the wild-type isolate. More interestingly, a significant increase was observed in the growth of the Δ eptC isolate synthesizing the EptC_{P360Q} protein versus the Δ eptC transformant expressing the EptC_{WT} protein. We speculate that the replacement of the Pro, an amino acid that disrupts structures with a hydrophilic residue (Gln) at position 360 of EptC (Figure 6), alters which acceptor molecules that can be modified with pEtN, leading to increased resistance to DOC and polymyxin B. Together, these data indicate that the C \rightarrow A mutation, resulting in a P360Q codon change, enhances the fitness of an isolate to a cationic antimicrobial peptide (polymyxin B) and a secondary bile salt (DOC).

III. MutY repairs G to T mutations caused by DOC

C. jejuni possesses DNA repair systems that function in BER, NER, and HRR but lacks a functional mismatch repair system (Table 1). *C. jejuni* growth in DOC results in increased levels of ROS and 8-oxo-dG. If an 8-oxoG: a base pair is generated during replication, a DNA glycosylase termed MutY excises the adenine to avoid making the mutation permanent in the next round of replication (Banda et al., 2017). We hypothesized that a deletion of the *mutY* gene would increase global genome mutations because non-bulky lesions (repaired by BER) can be bypassed by DNA polymerases. If the hypothesis is correct, we expect to observe an increase in the frequency of ciprofloxacin resistance.

IIIA. A *mutY* deletion results in a hyper-mutation phenotype yielding increased ciprofloxacin resistance

To test the hypothesis that the BER system in *C. jejuni* is involved in counteracting ROS-induced stress, the three DNA repair systems

were targeted by functionally disrupting each pathway. Gene deletions were generated in *addAB* (HRR), *uvrC* (NER), and *mutY* (BER). The growth and the mutation frequency of the Δ uvrC and Δ addAB mutants were observed in MH and MH-DOC supplemented media. The mutants (Δ uvrC and Δ addAB) were grown over a 36-h incubation period and the OD₅₄₀ was measured. The *C. jejuni* wild-type, Δ uvrC and Δ addAB mutants showed impaired growth with MH-DOC compared to MH only (Supplementary Figures S6A–C). To determine the ciprofloxacin mutation frequency, the bacteria were grown in MH and MH-DOC supplemented media and passaged every 24h for a 10-day incubation period. The mutation frequency decreases when either Δ uvrC or Δ addAB mutants are grown with MH-DOC compared to the MH only over 10 days (Supplementary Figures S6E,F), whereas the mutation frequency increased over time for wild-type isolates (Supplementary Figure S6D). Based on these findings, we focused our studies on analyzing the mutations in the *C. jejuni* wild-type strain and Δ mutY mutant in a medium with and without DOC.

Growth of the Δ mutY mutant in MH broth resulted in a 500-fold increase or greater in ciprofloxacin mutation frequency than observed previously with the wild-type strain ($p < 0.001$; Figure 7A). Notable is that the ciprofloxacin mutation frequency observed for the Δ mutY mutant was similar in MH and MH-DOC supplemented media. However, when the Δ mutY mutant was transformed with the wild-type copy of the *mutY* gene, the ciprofloxacin mutation frequency was similar to the wild-type strain (not shown). Based on this finding and the data in Figure 2, we speculate that the growth of *C. jejuni* in DOC-supplemented medium results in levels of ROS that overwhelm the BER system. We concluded that BER plays a significant role in modulating *C. jejuni* mutation programs in response to DOC exposure.

IIIB. A *mutY* deletion results in increased G to T and C to A transversions

The increased mutation frequency of the *C. jejuni* *mutY* deletion mutant prompted an investigation of the types of mutations that occurred in this genetic background versus that of the wild-type strain and *uvrC* deletion mutant. Point mutations [transitions, transversions, and indels (insertions and deletions)] were identified by culturing the bacteria in MH broth and performing WGS. The *C. jejuni* wild-type strain had no G \rightarrow T or C \rightarrow A transversions (Figure 7B). The *C. jejuni* Δ uvrC mutant had one G \rightarrow T and two C \rightarrow A transversions. In contrast, the *C. jejuni* Δ mutY had 26 G \rightarrow T and 18 C \rightarrow A transversions. Overall, the *C. jejuni* wild-type strain and Δ uvrC mutant had fewer transitions and transversions than the Δ mutY mutant. Next, we wanted to determine whether the serial passage of *C. jejuni* wild-type and Δ mutY mutants in MH had any effect on the number of G \rightarrow T and C \rightarrow A transversions. *C. jejuni* wild-type and Δ mutY mutant were grown in MH broth and passaged every 24h for 10 days, and five individual colonies for each strain were randomly selected for sequencing. Consistent with the

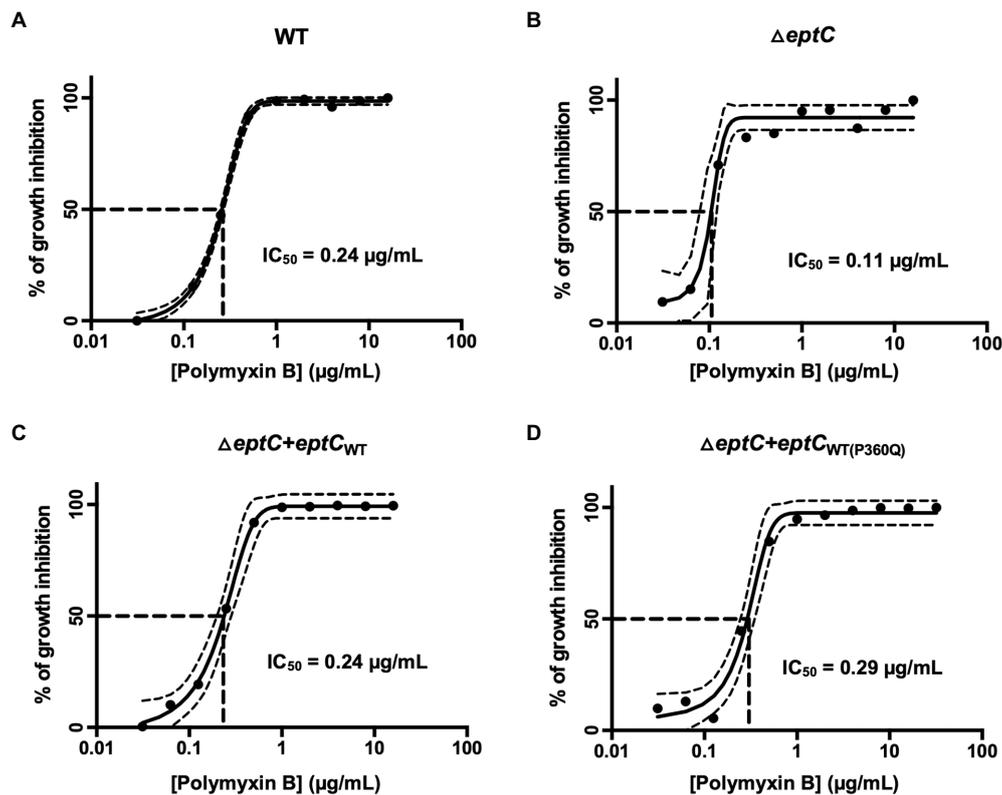


FIGURE 4

A *C. jejuni* Δ *eptC* mutant shows enhanced sensitivity to polymyxin B, whereas a *C. jejuni* variant synthesizing the EptC P360Q protein demonstrates enhanced resistance to polymyxin B. Concentration-effect curves for polymyxin B against a *C. jejuni* (A) wild-type strain, (B) Δ *eptC* deletion mutant, (C) Δ *eptC* deletion mutant transformed with a gene expressing the EptC_{WT} protein (Δ *eptC* + *eptC*_{WT}), and (D) Δ *eptC* deletion mutant transformed with a gene expressing the EptC protein containing the P360Q variant (Δ *eptC* + *eptC*_{WT(P360Q)}). The antimicrobial activity of polymyxin B was determined by the broth microdilution method, as described in the section "Materials and methods". The data represent a minimum of three biological replicates. Dashed lines at right angles indicate the polymyxin B concentration that is required for a 50% reduction of the cells (IC_{50}). The lines on both sides of the concentration-effect curve represent the 95% confidence intervals.

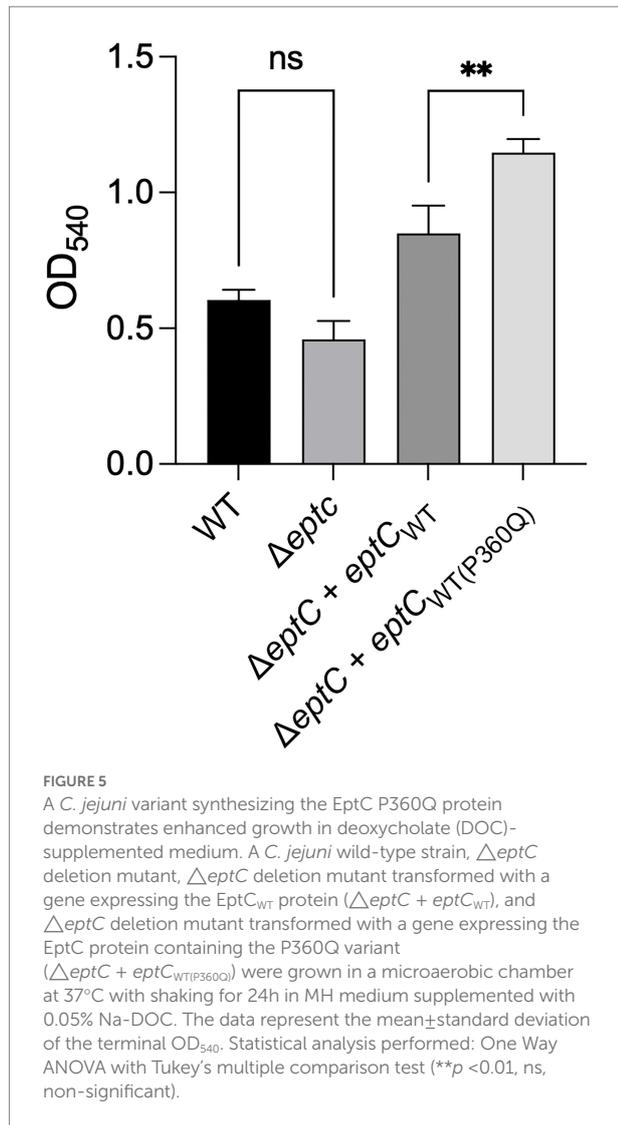
previous finding, there were a higher number of G \rightarrow T and C \rightarrow A transversions in the *C. jejuni* Δ *mutY* mutant compared to the wild-type strain grown in MH (not shown). These findings suggest that the absence of *mutY*, a.k.a. BER system in *C. jejuni*, increases the number of G \rightarrow T and C \rightarrow A transversions, ciprofloxacin resistance isolates, and, potentially, bacterial fitness.

Discussion

Genomic rearrangements have been observed in *C. jejuni* recovered from animals (Mixer et al., 2003; Barton et al., 2007; Ridley et al., 2008). The generation of *C. jejuni* variants *in vivo* is of particular interest because this pathogen lacks the SOS machinery and mutagenic DNA polymerases that *E. coli* utilizes in response to stress. Within the gut of vertebrate hosts, *C. jejuni* is exposed to bile, a protective agent against bacterial colonization. We have shown that *C. jejuni* growth in a medium containing the bile salt DOC results in ROS production (Negretti et al., 2017). Guanine is the most susceptible of the four bases to oxidation, and its oxidation product, 8-oxo-dG, is a common lesion in

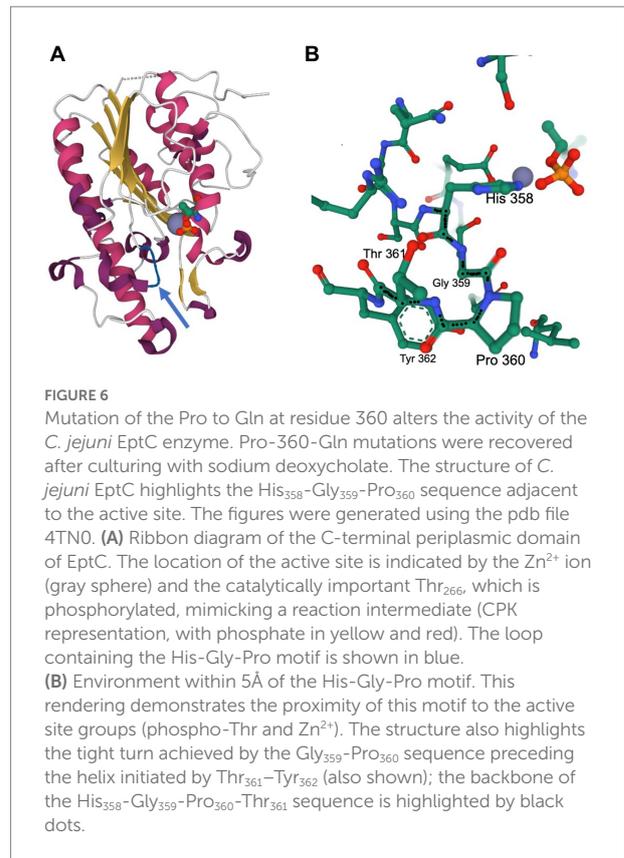
DNA. We hypothesized that the *C. jejuni* DNA repair systems make errors, resulting in mutations (Figure 8). This study aimed to determine if host stressors, such as DOC, can induce genetic mutations and serve as a possible driver of pathogen evolution. We sought to determine if *C. jejuni* growth in a medium containing DOC alters the mutation frequency and has the potential to generate new clonal types with enhanced fitness.

In this study, we initially assessed the mutations in the *gyrA* gene, which confer ciprofloxacin resistance, to determine if *C. jejuni* growth in DOC alters the frequency/type of genomic mutations. The *gyrA* gene represents an ideal target for our study, as it is simple to assess ciprofloxacin resistance and because a variety of point mutations confer antibiotic resistance. We passaged *C. jejuni* in MH medium with and without DOC and readily recovered ciprofloxacin-resistant isolates with four mutations (Thr-86-Ile, Thr-86-Lys, Asp-90-Asn, and Asp-90-Tyr) in the QRDR of GyrA. As predicted from published reports, isolates with the GyrA Thr-86-Ile mutation demonstrated greater resistance to ciprofloxacin than the isolates with other point mutations, as judged by the IC_{50} values (Changkwanyun et al., 2016). Although we selected the *C. jejuni* ciprofloxacin-resistant



isolates cultured in media with and without DOC at random, only isolates with the Asp-90-Tyr (GAT to TAT) signature, a G → T transversion was recovered in greater numbers when the bacteria were cultured with DOC. This result is consistent with the previous findings that *C. jejuni* growth in DOC-supplemented medium results in the production of intracellular ROS and an increase in the mutagenic 8-oxo-dG lesion, thus, a G → T transversion in the DNA (Negretti et al., 2017). WGS of the *C. jejuni* grown in the presence of DOC revealed a greater number of DNA transversions versus transitions, as well as the Asp-90-Tyr (GAT to TAT) mutation, indicating that growth in DOC resulted in a change in the mutation spectra when compared to isolates grown in MH broth. Together, these data show that DOC increases the frequency of *C. jejuni* ciprofloxacin-resistant isolates versus background levels (i.e., *C. jejuni* grown in MH broth) in the absence of antibiotic pressure and shifts the mutations from transitions to transversions.

One question that arises from the apparent increase in *C. jejuni* ciprofloxacin-resistant isolates with the Asp-90-Tyr



signature in our study is why these isolates are not more frequently recovered from poultry. We speculate that the lack of recovery of the Asp-90-Tyr signature in GyrA from poultry is due to the increased virulence potential and fitness of Thr-86-Ile isolates. Some *C. jejuni* strains with the Thr-86-Ile, but not with the Asp-90-Tyr signature, have relaxed DNA supercoiling activity (Changkwanyun et al., 2016; Shortt et al., 2016). DNA gyrase acts by wrapping DNA into a positive supercoil, passing one DNA strand through the other by breakage, and then rejoining the DNA strand (Hawkey, 2003). FQs bind to the complexes between the DNA and gyrase enzyme, and prevent the re-ligation of the broken DNA strands. The residue at the position equivalent to Thr-86 is Ser or Thr in GyrA from several bacterial pathogens, with those containing Thr exhibiting greater resistance to ciprofloxacin (Bax et al., 2010; Blower et al., 2016). This residue forms a conserved hydrogen bond to the carboxylate of ciprofloxacin (Supplementary Figure S7). Replacement of Thr-86 with either Ile or Lys would result in loss of this interaction and additionally may cause steric clashes in the surrounding contacts with ciprofloxacin and the DNA termini. The equivalent residue to Asp-90 is conserved as Asp or Glu. It appears to contribute to the negative charges in the environment around the Mn²⁺ ion chelated by the carboxylate and hydroxyl groups on ciprofloxacin (Supplementary Figure S7). Similarly, a mutation of Asp-90 to either Tyr or Asn would remove the electrostatic interactions formed by Asp or Glu. Overall, the extent and conservation of interactions of the Thr-86 residue are significantly greater than

TABLE 1 List of *C. jejuni* genes that share similarities to DNA repair systems in *Escherichia coli*.

DNA repair system	<i>C. jejuni</i> gene (NCTC 11168 ¹ /81–176 ²)	<i>E. coli</i> gene (K12 ³)	In operon? (Yes/No; position in operon)	% Identity/% Similarity ⁴
<i>Homologous recombination</i>				
RecA	Cj1673c/CJJ81176_RS08060	b2699	Yes (1/2)	57.8%/76.0%
RecB*	Cj1481/CJJ81176_RS07100	b2820	Yes (2/2)	17.1%/32.7%
RecC*	Cj1482/CJJ81176_RS07105	b2822	Yes (1/2)	12.4%/24.8%
RecD*	n/a	b2819	n/a	n/a
RecF	n/a	b3700	n/a	n/a
RecJ	Cj0028/CJJ81176_RS00140	b2892	No	28.3%/49.2%
RecN	Cj0642/CJJ81176_RS03125	b2616	Yes (2/2)	21.8%/42.2%
RecO	Cj0120/CJJ81176_RS00635	b2565	Yes (19/20)?	13.1%/23.9%
RecQ	n/a	b3822	n/a	n/a
RecR	Cj1263/CJJ81176_RS06150	b0472	Yes (3/3)	31.2%/54.1%
SSBP	Cj1071/CJJ81176_RS05200	b4059	Yes (2/3)	33.0%/47.6%
<i>Nucleotide excision repair</i>				
UvrA	Cj0342c/CJJ81176_RS01645	b4058	Yes (1/2)	50.5%/67.6%
UvrB	Cj0680c/CJJ81176_RS03285	b0779	No	50.1%/69.2%
UvrC*	Cj1246c/CJJ81176_RS06065	b1913	Yes (2/3)	29.1%/49.1%
UvrD	Cj1101/CJJ81176_RS05350	b3813	Yes (5/13)	34.5%/53.3%
Mfd	Cj1085c/CJJ81176_RS05270	b1114	Yes (10/15)?	28.2%/46.6%
<i>Base excision repair</i>				
MutY*	Cj1620c/CJJ81176_RS07755	b2961	No	31.8%/50.4%
MutM	n/a	b3635	n/a	n/a
Nei	n/a	b0714	n/a	n/a
Nth	Cj0595c/CJJ81176_RS02900	b1633	Yes (1/3)	38.6%/56.7%
Tag	n/a	b3549	n/a	n/a
AlkA	n/a	b2068	n/a	n/a
Ung	Cj0086c/CJJ81176_RS00465	b2580	No	43.8%/61.7%
XthA/ExoA*	Cj0255c/CJJ81176_RS01235	b1749	No	32.4%/49.2%
Nfo	n/a	b2159	n/a	n/a
Udg	Cj0963/CJJ81176_RS04690		Yes (1/3)	
<i>Mismatch repair</i>				
MutS	Cj1052c/CJJ81176_RS05110	b2733	Yes (2/12)	11.3%/22.0%
MutL	n/a	b4170	n/a	n/a
MutH	n/a	b2831	n/a	n/a
<i>Direct repair</i>				
Ada	n/a	b2213	n/a	n/a
AlkB	n/a	b2212	n/a	n/a
Ogt	n/a	b1335	n/a	n/a
Phr	n/a	b0708	n/a	n/a

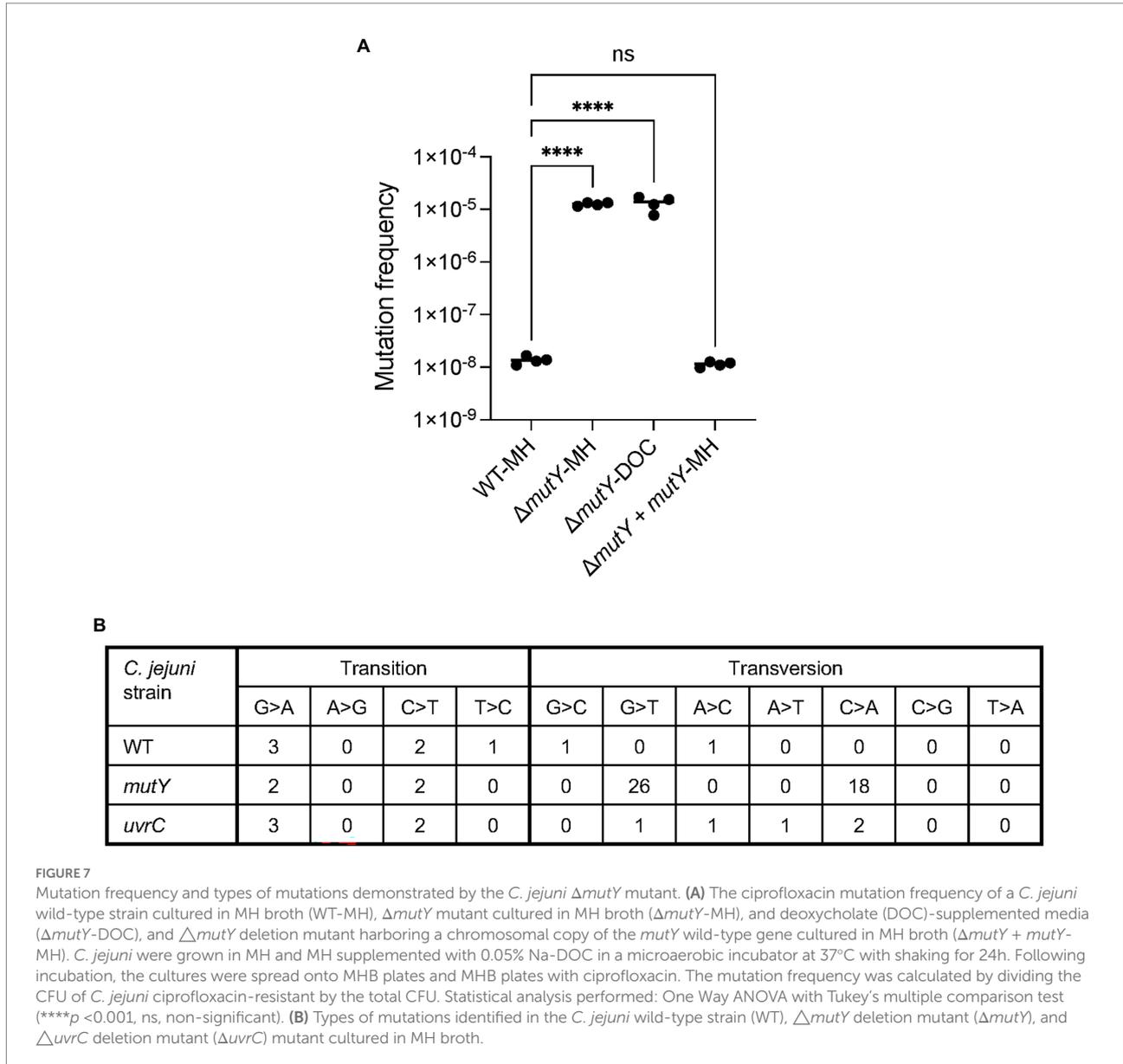
n/a, Not applicable.¹*C. jejuni* NCTC 11168 GenBank accession number: NC_002163.1.

²*C. jejuni* 81–176 GenBank accession number: NC_008787.

³*E. coli* K-12 GenBank accession number: AP009048.

⁴Identity and similarity between *C. jejuni* 81–176 and *E. coli* K-12.

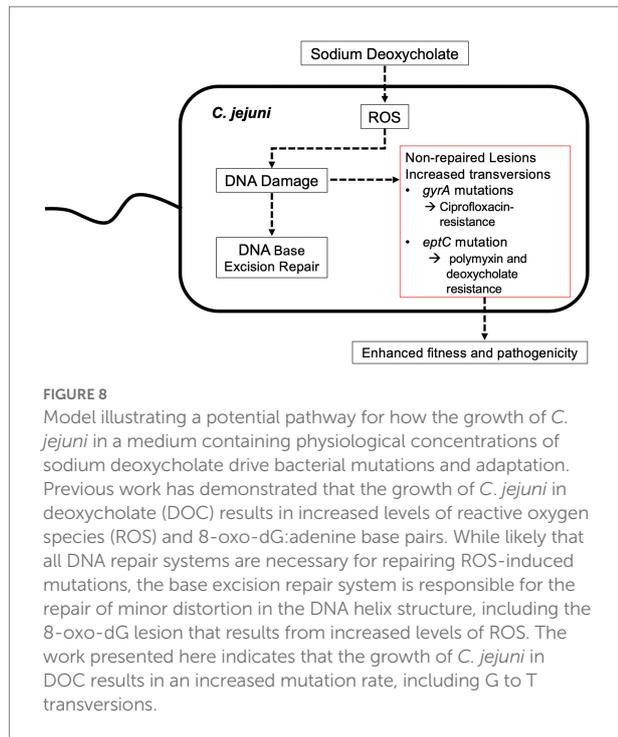
*Genes targeted for mutagenesis (gene deletion).



those of the Asp-90 residue, consistent with the observation that mutation of Thr-86 causes greater resistance to ciprofloxacin, presumably due to weaker binding interactions with the protein: DNA complex. In addition, isolates with the Thr-86-Ile mutation demonstrate an increase in virulence phenotypes, including protein secretion and increased intestinal cell invasion (Scanlan et al., 2017; Whelan et al., 2019). *In vivo* experiments have revealed that a *C. jejuni* Thr-86-Ile isolate has enhanced fitness, as it can outcompete a wild-type isolate in poultry inoculated with a mixture of both isolates, i.e., the Thr-86-Ile isolate dominants and persist in the chickens (Luo et al., 2003). Finally, using the *in vivo* *Galleria mellonella* larvae model of infection, a Thr-86-Ile isolate was found to kill the larvae more quickly than a wild-type isolate (Whelan et al., 2019). It is possible that the low recovery of isolates with the Asp-90-Tyr signature from poultry is due to the low GC

content of *Campylobacter* organisms. *C. jejuni* has mol% (G + C) content of ~30.4%, which might slightly decrease the frequency of guanine oxidation compared with enteric bacteria having mol% (G + C) content of ~50%. However, this does not explain the increased mutation frequency and G → T transversion observed with the *mutY* mutant. Together, we propose that FQ-resistant isolates are prevalent in poultry, the natural reservoir, due to the enhanced fitness of the Thr-86-Ile isolates. We speculate that the increased residency of *C. jejuni* in the intestine of humans, as well as the increased bile concentration in the human intestine compared to poultry, provides an environment where the frequency and spectra of genomic mutations may be increased.

To test if DOC results in mutation(s) that provide enhanced bacterial fitness, *C. jejuni* was continually passaged in DOC-supplemented media and two isolates that



demonstrated enhanced DOC resistance were subjected to WGS. Both isolates demonstrated mutations in several genes, including *eptC*. We focused on the *eptC* mutation in this study because a C → A transversion was observed at position 360 (P360Q variant) and because the protein is involved in host-cell interactions. EptC catalyzes the addition of pEtN to various outer membrane components, including LOS and the flagellar rod protein FlgG. To determine whether the P360Q mutation in the EptC protein conferred a fitness advantage, we first generated a $\Delta eptC$ deletion mutant. In agreement with previous reports, the $\Delta eptC$ showed a defect in motility and increased sensitivity to polymyxin B (Cullen et al., 2013; Lim and Kim, 2017). Complementation of $\Delta eptC$ mutant, by the introduction of a wild-type copy gene driven by its endogenous promoter in the chromosome, restored the isolate's motility and polymyxin B resistance to the level of the wild-type isolate.

Previous work with *Salmonella* spp. has revealed that bile resistance can be acquired through the modification of numerous factors, including lipopolysaccharide, proteins in the cytoplasmic and outer membranes, gene regulatory networks, and DNA repair systems (Hernandez et al., 2012). To determine if the EptC P360Q variant shows evidence of increased virulence potential, a gene encoding the EptC P360Q variant was introduced into the $\Delta eptC$ mutant. Noteworthy is that the EptC residue Pro-360 is central to a conserved sequence motif in this family of bacterial pEtN transferases that includes the essential active site residue His-358 implicated in metal ion binding and a constrained turn that includes the preceding Gly-359. The P360Q mutation does not eliminate EptC activity but appears to allow improved

function, perhaps through broadening the substrate specificity of EptC.

A greater number of DNA transversions versus transitions were observed in the genomic sequences of the *C. jejuni* grown in a medium supplemented with DOC than without DOC. While one would predict that these mutations are repaired by BER pathway (Krokan and Bjørås, 2013), this has not formally been shown in *C. jejuni*. Thus, we analyzed the behavior and genomic background of a $\Delta mutY$ deletion mutant. In brief, we found an increase in G → T and C → A transversions in the genome of the *C. jejuni* $\Delta mutY$ isolate. In contrast, no transversions were observed in the genome of the wild-type isolate, and only a few transversions were observed in the $\Delta uvrC$ mutant. Relevant to this discussion is that the NER pathway serves as a secondary defense against oxidative damage and recombinational repair is also required under certain instances (e.g., single and double-strand breaks; Demple and Harrison, 1994). Finally, both the $\Delta uvrC$ mutant and $\Delta addAB$ mutants demonstrate decreased cell viability and mutation frequency when grown in a medium supplemented with DOC, possibly due to DNA lesions caused by oxidative damage. ROS can stall replicative polymerases during DNA replication, where 8-oxo-dG would allow mutagenic insertion and continued DNA synthesis. Based on these data, we concluded that the BER pathway plays the primary role in the repair of the 8-oxo-dG lesion in *C. jejuni*, but that all three DNA repair systems are involved in general oxidative damage repair.

We present data that demonstrate that the growth of *C. jejuni* in the bile salt DOC, causes DNA mutations that enhance the adaptation of *C. jejuni* to the conditions encountered in a host. While one gene product, EptC, was demonstrated to contribute to the enhanced resistance of polymyxin B (an antimicrobial agent) and DOC resistance, we postulate that the exposure of *C. jejuni* to DOC results in other mutations, which go un-repaired, that may result in either enhanced or reduced fitness of the organism over time. Discovering the single nucleotide polymorphisms (SNPs) that contribute to the pathogenic potential and fitness of *C. jejuni* will require a detailed analysis of mutants generated by stress conditions that mimic the host environment.

Data availability statement

The genome sequences of the *C. jejuni* isolates determined in this study were deposited in GenBank (Project PRJNA634604, accession: SRR22024544, SRR22024545, SRR22024546, SRR22024547, SRR22024548; SRR22024599, SRR22024600, SRR22024601, SRR22024602, SRR22024603, SRR22024604, SRR22024605, SRR22024606, SRR22024607, and SRR22024608).

Author contributions

PT, SR, CP, and MK contributed to the conception and design of the study. PT, TC, SH, KT, SL, TH-W, and MK

performed experiments. PT and MK organized the data and wrote the first draft of the manuscript. PT performed the statistical analysis. LG and CP wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Supplementary material

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