COMPOSITION OF GUT MICROBIOTA AFFECTS *C. JEJUNI*-MEDIATED INFLAMMATION AND AUTOIMMUNITY IN MURINE MODELS

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ABSTRACT

COMPOSITION OF GUT MICROBIOTA AFFECTS *C. JEJUNI*-MEDIATED INFLAMMATION AND AUTOIMMUNITY IN MURINE MODELS

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Campylobacter jejuni is the leading antecedent infection to the acute peripheral neuropathy Guillain-Barre Syndrome (GBS). GBS is debilitating, often causes paralysis, and can require several months or more for recovery. Most concerning is that GBS patients are frequently left with long-term neurologic disabilities. Because a vaccine for *Campylobacter* is lacking there are no viable approaches for preventing this form of GBS. Currently, therapeutic approaches for GBS include plasma exchange and intravenous immunoglobulin but they require specialized equipment, pose significant financial burden, and produce mixed results. These strategies lack a strong rationale because GBS is poorly defined mechanistically. While new working mouse models of GBS may lead to alternative therapies, confirmation of *C. jejuni's* specific role in precipitating GBS and the mechanism(s) through which this occurs remain elusive. Thus far, evidence gathered from murine models demonstrates that multiple factors influence *C. jejuni* pathogenesis, including host genetics and *C. jejuni* genetics, particularly the genetic plasticity of this pathogen. Notably, the gut microbiota can modulate *C. jejuni* colonization- and colitis-resistance; however, its role in modulating *C. jejuni*-triggered autoimmunity remains unknown. The overarching goal of this study is to determine if the composition of gut microbiota affects *C. jejuni*-triggered autoimmunity in murine models. The chapters of this thesis present the following data addressing this goal; mice infected with antimicrobial resistant *Campylobacter* strains from Guillain-Barré syndrome patients produced severe colitis and type 2 autoimmune responses when their microbiota were depleted by antibiotics. Furthermore, we demonstrated that transplanted human fecal microbiota alters the immune response to *Campylobacter jejuni* infection in C57BL/6 mice, potentially increasing the risk of autoimmune sequelae. Finally,

comparative genomic analysis of passaged *C. jejuni* populations revealed genetic variation in multidrug transporter genes *cmeB* and *cmeR* in *Campylobacter jejuni* populations from antibiotic treated mice. CmeR regulates expression of *C. jejuni* cell surface molecules, again potentially impacting the risk of autoimmune sequelae. Taken together, our results demonstrate that composition of gut microbiota is a critical determinant of inflammatory and autoimmune outcomes in *C. jejuni* murine models.

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KEY ABBREVIATIONS

ATP	Adenosine Triphosphate
CPZ	Cefoperazone
DNA	Deoxyribonucleic acid
Hu	Humanized
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Mo	Mouse
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
PMA	Phorbol mysirate acetate
rRNA	Ribosomal Ribonucleic acid

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

GUILLAIN-BARRÉ SYNDROME: A POST-INFECTIOUS DISORDER

General disease characteristics. GBS is the leading cause of polyneuropathy worldwide, excluding locations that have failed to eradicate poliomyelitis (Hughes and Rees 1997). While generally accepted as a disease entity, GBS can manifest in many forms, with a wide range of disease severity, neurological deficits, antecedent infections, electrophysiological responses, and antibody responses (van den Berg, Marrink et al. 1992, Hadden, Karch et al. 2001, Yuki and Hartung 2012, Kuwabara and Yuki 2013, van den Berg, Walgaard et al. 2014). Diagnostic criteria for GBS include acute onset of symptoms, symmetry of symptoms, areflexia, delayed nerve conductivity, and elevated protein levels in cerebral spinal fluid (Asbury and Cornblath 1990). At present, GBS can be divided into at least four distinct subtypes: acute inflammatory demyelinating polyradiculoneuropathy (AIDP) that resembles experimental autoimmune nephritis (EAN), which is a T-cell driven disease. In contrast the two axonal forms, acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN) are known to be antibody mediated. Finally, Miller Fisher Syndrome (MFS) is a rare form of GBS that is not well characterized but often involves areflexia, ataxia, opthalmoplegia, and subclinical motor nerve dysfunction (Hughes and Cornblath 2005).

Associated infecting microorganisms can be identified in approximately fifty-percent of GBS cases, thus it has been deemed a post-infectious disorder [2]. Although, at least sixteen microorganisms have been linked to GBS, most cases have antecedent infections of *Cytomegalovirus*, Epstein-Barr virus, *Mycoplasma pneumoniae*, and *Campylobacter jejuni* (Hughes and Cornblath 2005). Infection with *C. jejuni* infection precedes approximately one-third of GBS cases in which an infectious agent can be identified (Mishu and Blaser 1993, van den Berg, Walgaard et al. 2014), and it is most often associated with the AMAN form.

Acute motor axonal neuropathy. The AMAN form of GBS is likely the result of complement-mediated attack on peripheral nerves following the binding of anti-ganglioside

antibodies elicited by infection. Although mortality is rare with GBS, varying between 3–7%, routine histologic analysis of AMAN patients has been performed and informs model development. Taken together, results of these studies show that the AMAN form of GBS is not associated with significant demyelination or lymphocytic inflammation found with other forms but often results in Wallerian-like degeneration and increased macrophage presence (McKhann, Cornblath et al. 1993, Griffin, Li et al. 1995). Ultrastructural analysis of the dorsal root ganglia and peripheral nerves of 7 patients that died following the onset of neurological symptoms showed the following: immunoglobulins bound to the node of Ranvier, complement deposits on the axolemma, and enhanced macrophage numbers in the axon and paranodal space (Griffin, Li et al. 1996). Furthermore, peripheral nerve dysfunction and paralysis correlated in time with peak presence of anti-ganglioside antibodies (Willison, Jacobs et al. 2016). These results are consistent with the hypothesis that anti-ganglioside antibodies bind to peripheral nerves and activate a complement-mediated immune response that leads to peripheral nerve damage, macrophage scavenging of myelin and axonal surfaces in the peripheral nerves with subsequent loss of nerve conduction velocity (Fig. 1.1). Experimental inoculation of mice with *C. jejuni* strains from patients with GBS evoked anti-ganglioside antibodies; however, whether these antibodies lead to neuropathy was not determined (Malik, Sharma et al. 2014).

CAMPYLOBACTER JEJUNI BIOLOGY

General enteric disease characteristics. *C. jejuni* is not only the leading antecedent infection to GBS but it is also a leading cause of enteric disease in both the developed and developing world (Willison, Jacobs et al. 2016). *C. jejuni* primarily colonizes the gastrointestinal tract and initiates inflammation termed gastroenteritis. Experimental evidence shows that *C. jejuni* gastroenteritis is associated with specific strains of *C. jejuni* (Bell, St Charles et al. 2009, Malik, Sharma et al. 2014) and can be enhanced by serial passage (Bell, St Charles et al. 2009), by

depleting the microbiota with antibiotics (O'Loughlin, Samuelson et al. 2015), or by infecting gnotobiotic animals (Chang and Miller 2006). These outcomes raised the question of whether *C. jejuni*-mediated-autoimmunity would also be enhanced under the environmental conditions employed in these models.

Immune responses to *C. jejuni* infection. *C. jejuni* infection evokes both T helper-1 (T_H1) and T helper-2 (T_H2) cellular responses and in some case anti-ganglioside antibodies (Malik, Sharma et al. 2014). The response to infection is determined by a variety of factors including the *C. jejuni* genetic background. This has been observed in humans where *Campylobacter* initiated autoimmunity results in a wide variety of diseases that includes, but is not limited to, the peripheral neuropathies Guillain-Barré syndrome (GBS) and its variant Miller Fisher syndrome (MFS) that causes descending paralysis (Willison and Veitch 1994). *C. jejuni* strains have been isolated from many patients with AMAN and MFS allowing comparisons to strains from patients with enteritis. GBS has been associated with specific strains of *C. jejuni* that possess lipooligosaccharide (LOS) structures on their outer core that are similar to gangliosides found on peripheral nerves near the node of Ranvier (Wim, Bart et al. 2004). Gangliosides are sialylated glycosphingolipids that are located on cell surfaces throughout the nervous system and play a role in cell-to-cell communication. Variation in ganglioside mimics on *C. jejuni* result from genetic variation in *C. jejuni* LOS loci (Parker, Horn et al. 2005, Parker, Gilbert et al. 2008). Synthesis of ganglioside mimics is dependent upon a variable set of genes and enzymes including glycosyltransferases *cstII* and *cstIII* that attach neuraminic acids to galactose on the outer core.

Experimentally, biochemical analysis of *C. jejuni* 11168 demonstrated that ganglioside mimics or the presence of genes required for their biosynthesis are not the only factors contributing to the autoimmune potential of *C. jejuni* strains (Godschalk, Heikema et al. 2004). Moreover, even strains possessing these mimics do not always elicit significant anti-ganglioside antibody responses as was seen when C57BL/6 IL-$10^{-/-}$ mice were infected with *C. jejuni* strain 11168 (Malik, Sharma et al. 2014). In addition, *C. jejuni's* genome contains hypervariable

nucleotide tracts, some of which are found in genes involved in surface structure biosynthesis; these tracts undergo expansion or contraction during passage in the host (Jerome, Bell et al. 2011) which can affect transcript abundance and gene expression further complicating our understanding of *C. jejuni* pathogenesis. This slip strand mutagenesis was demonstrated to affect genes that encode outer surface structures such as lipo-oligosaccharides (LOS), capsule, and O-linked glycosylation of the flagellum important for pathogenesis (Jerome, Bell et al. 2011).

***Campylobacter*: inflammation and autoimmunity in humans**. The following sections summarize the role of the gut microbiota in mediating colonization and disease in regards to *C. jejuni* and some other well-characterized enteric pathogens. Campylobacteriosis is the disease caused by infection with bacteria from the genus *Campylobacter; C. jejuni* is the most common cause of human *Campylobacter* infection (Cody, McCarthy et al. 2013), followed by *C. coli*. *Campylobacter jejuni* is an important zoonotic pathogen that causes 1.3 million cases of gastroenteritis in the US each year, leading to 13,000 hospitalizations and 120 deaths (Scallan, Griffin et al. 2011). Symptoms of campylobacteriosis often include intestinal inflammation, fever, diarrhea, and abdominal pain (Black, Levine et al. 1988, Young, Davis et al. 2007). These symptoms often resolve 4–7 days after initiation but may last up to 10 days (CDC 2014). Human infection occurs via the oral route and most often results from the consumption of raw or undercooked poultry (Young, Davis et al. 2007). Most avian species are asymptomatic carriers, serving as reservoirs for the *Campylobacter,* and infect other birds through common water and feeding sources (CDC 2014). The National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) reported that almost 50% of raw chicken in stores in the United States was contaminated with *Campylobacter* and that most of these cases may be attributed to contamination during slaughter and subsequent processing. Consumption of raw or unpasteurized milk and untreated water also contributes to *Campylobacter* infection (Young,

Davis et al. 2007). Milk may become contaminated when *Campylobacter* infects the udder or when cow fecal matter containing *Campylobacter* contaminates the milk (CDC 2014).

Although most cases resolve on their own, antimicrobials may be prescribed for *C. jejuni* enteritis when symptoms last longer than 7 days. Azithromycin or ciprofloxacin are most often prescribed to treat *Campylobacter* infection (CDC 2014) but rates of antibiotic resistance in *Campylobacter* species including *C. jejuni* and *C. coli* are high (Engberg, Aarestrup et al. 2001, Gibreel and Taylor 2006). *C. jejuni* was recently designated a serious antimicrobial resistant threat by the Centers for Disease Control and Prevention (CDC 2013). Antibiotic resistant (AR) *C. jejuni* have been suggested to cause more severe infections requiring lengthier hospitalizations when compared to susceptible infections and, thus, represent an important public health concern (Moore, Barton et al. 2006). Death following *Campylobacter* infection is rare, with an incidence of approximately 80 people dying of an estimated total of 1–1.5 million cases of infection per year in the United States (CDC 2014). Death usually occurs in those with underdeveloped or declining immune systems, including children under 2, the elderly, and the immune compromised such as those with acquired immune deficiency syndrome (AIDS) (WHO 2011). In rare instances, infected patients may experience long-term consequences including flare-ups of inflammatory bowel disease (Kim, Hans et al. 2009), irritable bowel syndrome (Qin, Wu et al. 2011), Reiter's arthritis (Garg, Pope et al. 2008), and the acute peripheral neuropathy GBS (Yuki, Taki et al. 1993, Hughes, Hadden et al. 1999).

***Campylobacter*: inflammation and autoimmunity in mouse models**. Many animals have been used to study the effects of *C. jejuni* infection, including mice, rats, rabbits, pigs, chickens and ferrets (Mansfield, Bell et al. 2007). Mice provide many advantages including 1) low cost to maintain, 2) relatively small spaces required for housing, allowing for larger experiments, 3) ease of manipulation, and 4) availability of genetic knockouts. Development of murine *C. jejuni* colonization and colitis models has been greatly advanced by manipulation of host genetics and host microbiota (Chang and Miller 2006, Mansfield, Patterson et al. 2008, Bereswill, Fischer et al.

2011, Malik, Sharma et al. 2014, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015). A summary of these advances is provided in the following sections.

Inflammation is associated with persistent *C. jejuni* colonization. Limited Enteric Flora (LF) C3H Severe Combined Immune Deficient (SCID) mice infected with *C. jejuni* displayed high level *C. jejuni* colonization for up 224 days. In contrast, immune competent congenic LF C3H mice began to clear the bacteria at approximately 28 days (Chang and Miller 2006). LF C3H SCID mice but not LF C3H immune competent mice displayed inflammation of the cecum and the colon (Chang and Miller 2006) suggesting that inflammation may allow *C. jejuni* to persist in the gut. This explanation would be consistent with experimental data from other pathogens including *Salmonella enterica serovar Typhimurium* (Winter, Thiennimitr et al. 2010) and some pathogenic *E. coli* (Horwitz and Silverstein 1980) that have evolved mechanisms to exploit inflammation by utilizing tetrathionate and evading compliment fixation, respectively. In general, two hypotheses exist to explain how pathogens may benefit from inflammation: 1) inflammation alters microbiota structure in a way that frees up nutrients that are exploited by pathogens but not the microbiota (i.e. food hypothesis) and 2) changes in antimicrobial compounds produced by the inflamed tissue may be detrimental to the microbiota but not the pathogen (i.e. differential killing hypothesis) (Stecher and Hardt 2008).

IL-10$^{-/-}$ but not IL-10$^{+/+}$ mice develop colitis after *C. jejuni* infection. Interleukin-10 (IL-10) is a regulatory cytokine produced by T cells, B cells and some monocytes that tends to suppress lymphocyte responses (Couper, Blount et al. 2008). Generally, IL-10 is classified as an anti-inflammatory cytokine that downregulates the host response to invasion by intracellular pathogens by inhibiting several key inflammatory regulators, including major histocompatibility complex II and T-cell co-stimulatory factors B7-1 and B7-2, and expression of interferon (IFNγ) (Moore, Malefyt et al. 2001, Ouyang, Rutz et al. 2011). Congenic C57BL/6 IL-10 deficient mice (C57BL/6 IL-10$^{-/-}$) but not their IL-10$^{+/+}$ counterparts are susceptible to colitis when infected by *C. jejuni* 11168 (Mansfield, Bell et al. 2007). *C. jejuni* 11168 successfully colonized the GI tract of

C57BL/6 wild type and IL-10$^{-/-}$ mice; however, only IL-10$^{-/-}$ mice developed inflammation of the colon and cecum. The cecum was the GI site with the highest level of colonization; and *C. jejuni* was isolated from most GI compartments (i.e. cecum, stomach, colon, and jejunum) or detected by *C. jejuni* specific (*gyr*A) PCR of tissue homogenates. All mice were colonized at comparable levels and colonization was required but not sufficient for GI lesions as only IL-10$^{-/-}$ mice developed disease and lesions (Mansfield, Bell et al. 2007).

Colitis in IL-10$^{-/-}$ mice is *C. jejuni* strain dependent (Bell, Jerome et al. 2013, Malik, Sharma et al. 2014), and genomic composition of the *C. jejuni* strain is an important factor (Bell, Jerome et al. 2013). To date the entire suite of genes required for *C. jejuni* colitis remains unknown; however, comparative genomics of available *C. jejuni* genomes and gene expression analysis of *C. jejuni* strains that caused colitis in C57BL/6 IL-10$^{-/-}$ mice compared to those that did not yielded 201 potential virulence genes, collectively called the *C. jejuni* virulome (Bell, Jerome et al. 2013). In addition, motility is likely a major determinant of *C. jejuni* pathogenesis. *C. jejuni* diminished motility and non-motile mutants colonize at rates 100 to 1000 fold less than the wild-type (Wassenaar, Zeijst et al. 1993) thus variation in motility amongst strains play a role in infection outcomes. Further, an experiment in *C. jejuni* 11168-infected germ-free C57BL/6 mice showed that expression levels of ninety open reading frames (ORFs) were significantly up- or down-regulated in the mouse cecum at least two-fold compared to *in vitro* growth (Bell, Jerome et al. 2013). Genomic content of these ninety *C. jejuni* 11168 ORFs was significantly correlated with the capacity to colonize and cause enteritis in mice. Differences in gene expression levels and patterns are thus an important determinant of pathotype in *C. jejuni* strains in this mouse model.

Antibiotic treated *Campylobacter* mouse models. Results from experimental murine *C. jejuni* inoculation suggest that competition from members of the resident gut microbiota play a role in *C. jejuni* colonization resistance (Chang and Miller 2006, Stahl, Ries et al. 2014, O'Loughlin, Samuelson et al. 2015). Two studies have shown that antibiotic depleted gut microbiota is sufficient to overcome *C. jejuni* colonization resistance in wild-type mice. Consistent

with results in LF C3H mice, antibiotic depletion of gut microbiota with ampicillin enhanced susceptibility to *C. jejuni* colonization and mild enteric disease in CBA/J mice. Mice received two doses of ampicillin at 24 and 48 hours prior to *C. jejuni* infection. 16S rRNA gene amplicon analysis revealed decreased overall diversity in the fecal microbiota of antibiotic treated mice. Notably, antibiotic treated mice that were supplemented with *Enterococcus faecalis or Lactobacillus acidophilus* showed diminished *C. jejuni* loads at 7-days post-inoculation indicating a role for these microorganisms in *C. jejuni* colonization resistance (O'Loughlin, Samuelson et al. 2015).

Similarly, vancomycin treatment enhanced susceptibility to *C. jejuni* colonization and gastrointestinal lesions in C57BL/6 wild-type mice. Mice were pretreated with vancomycin and subsequently infected with *C. jejuni* by oral gavage and maintained for 7-days post-inoculation. In line with previous results in LF microbiota C3H mice (Chang and Miller 2006), enteric disease seen in depleted microbiota wild-type mice was exacerbated in single immunoglobulin interleukin-1 receptor-related protein (SIGIRR) deficient mice. These C57BL/6 Sigrr$^{-/-}$ mice are immune deficient due to this gene knockout, which is a negative regulator of MyD88 signaling. Despite enhanced enteric disease in Siggr$^{-/-}$ mutants, *C. jejuni* pathogen loads were very similar compared to wild-type mice; yet, differences in *C. jejuni* localization in the gut were observed. In wild type mice, *C. jejuni* were primarily found in the lumen and luminal surface of the mucus layer. In stark contrast, *C. jejuni* penetrated the mucus and was often found in the intestinal crypts of Sigrr$^{-/-}$ mice. Further investigation demonstrated that Toll like receptors 2 (TLR2) and 4 (TLR4) were required for enteric disease in Sigrr$^{-/-}$ mice (Stahl, Ries et al. 2014). These results are consistent with several previous reports showing the *C. jejuni* activates TLR2 and TLR4 during inflammatory activation of dendritic cells (Rathinam, Appledorn et al. 2009). Together these studies demonstrate that the gut microbiota plays a significant role in mediating *C. jejuni*-mediated inflammation, which is exacerbated in immunocompromised hosts; however, neither study evaluated autoimmune responses in *C. jejuni* infected mice.

Humanized microbiota *Campylobacter* mouse model. Inoculation of mice with human fecal material has been used to generate humanized microbiota mice (^{Hu}microbiota). In one model, ^{Hu}microbiota mice were generated by using a five-antibiotic cocktail (ampicillin, vancomycin, ciprofloxacin, imipenem, and metronidazole) to deplete the microbiota, followed by inoculation with either murine or human feces. Peroral *C. jejuni* infection of these mice resulted in clearance of *Campylobacter jejuni* in 2 days by murine microbiota mice. In contrast, human microbiota mice remained colonized for 6 weeks and displayed exacerbated T cell, B cell, and pro-inflammatory cytokine responses in the colonic mucosa (Bereswill, Fischer et al. 2011). Notably, murine microbiota controls were also pre-treated with antibiotics thus raising the question of whether this affected their immune responses.

CAMPYLOBACTER JEJUNI WITHIN-HOST ADAPTATION

C. jejuni's genome is not static during *in vivo* passage (Wassenaar, Geilhausen et al. 1998, Nuijten, Berg et al. 2000, de Boer, Wagenaar et al. 2002, Jerome, Bell et al. 2011, Kim, Artymovich et al. 2012, Kivistö, Kovanen et al. 2014). A significant proportion of this genomic variation occurs in virulence associated genes that are involved in the synthesis of antigenic structures including the LOS, flagella, and capsule that are involved in triggering immune responses and potentially aiding in immune evasion (Jerome, Bell et al. 2011, Kivistö, Kovanen et al. 2014). Some genomic variants have direct links to biological outcomes, such as increased motility; thus *C. jejuni* adaptation may influence infection outcomes.

The first evidence for *C. jejuni* adaptation *in vivo* came from variability in pulse-field gel electrophoresis banding patterns following passage in chickens, where analysis of initially clonal isolates of *C. jejuni* revealed multiple banding patterns in recovered isolates, providing evidence that large-scale genomic rearrangements occurred during *in vivo* passage (Wassenaar, Geilhausen et al. 1998). Concurrently, Parkhill *et al.,* (2000) identified hypervariable regions in

the *C. jejuni* 11168 genome consisting of homopolymeric tracts of nucleotides. Since this discovery our laboratory has shown that insertions or deletions in homopolymeric tracts of nucleotides allow *C. jejuni* 11168 to rapidly adapt during passage in mice (Jerome, Bell et al. 2011). *C. jejuni* farm isolates also contained variants in homopolymeric tracts (Kivistö, Kovanen et al. 2014). In both cases, the majority of variants in homopolymeric tracts were found in the LOS, capsular, and flagellar genes. Collectively, these homopolymeric tracts are called contingency loci as they have higher rates of mutation than the rest of the genome. Mutations in contingency loci contribute to phase variation: the ability to turn gene expression on or off (Moxon, Paul et al. 1994); phase variation may directly impact pathogenesis by altering the expression of virulence factors including LOS, capsule, and flagella (Jerome, Bell et al. 2011).

Variation in homopolymeric tract length can result in observable biological outcomes. Notably, passage of *C. jejuni in vivo* led to presence of antigenic ganglioside mimics on the LOS of *C. jejuni* 81-176 that initially lacked any ganglioside mimics (Prendergast, Tribble et al. 2004). Site directed mutagenesis of homopolymeric tracts in the *cgt*A gene (N-acetylgalactosaminyltransferase) in *C. jejuni* 81-176 shifted the ratio of GM2 and GM3 ganglioside mimics and enhanced the invasiveness of the *C. jejuni cgt*A mutant compared to the wild-type strain (Guerry, Szymanski et al. 2002). The host environmental cues that drive evolutionary selection for phase variation are unknown, but it is known that this process allows for rapid adaptation to novel environments, increased diversity, and evasion of the host immune system (van der Woude and Baumler 2004, Jerome, Bell et al. 2011). Slipped strand mutagenesis (Moxon, Paul et al. 1994, Zhou, Aertsen et al. 2014) and the absence of several homologues of *E. coli* DNA repair genes contribute to the high incidence of phase variation in *C. jejuni* (van der Woude and Baumler 2004).

Other mechanisms of *C. jejuni* adaptation. *C. jejuni* has other mechanisms of adaptation to novel environments in addition to variation in homopolymeric tracts. Several recent studies have demonstrated that single nucleotide variants outside of homopolymeric tracts