

GENOME EVOLUTION OF CAMPYLOBACTER JEJUNI DURING EXPERIMENTAL
ADAPTATION

By

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ABSTRACT

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Campylobacter jejuni is a leading cause of foodborne bacterial enteritis in humans. An important reservoir for *C. jejuni* is in chickens, but it has been shown to colonize a large host range. Passage through a mouse model of campylobacteriosis resulted in a hypervirulent phenotype in mice for *C. jejuni* strain NCTC11168. After analyzing the wild-type and mouse-adapted variants by phenotype assays, expression microarray, pulse-field gel electrophoresis and whole genome sequencing we discovered that the genetic changes in the mouse-adapted variant were confined to thirteen hypermutable regions of DNA in contingency loci. We also show that specific contingency loci changes occurred in parallel during mouse infection when reisolates from multiple mice were analyzed. Furthermore, a mathematical model that considers contingency loci mutation rates and patterns does not explain the observed changes. Taken together, this is the first experimental evidence that contingency loci play a role in the rapid genetic adaptation of *C. jejuni* to a host, which results in increased virulence.

In contrast to the observed virulence increase by serial host passage, we showed that *C. jejuni* rapidly loses an essential host colonization determinant during adaptive laboratory evolution. Passage in broth culture selected for flagellar motility deficient *C. jejuni* cells in parallel for five independently evolved lines. Moreover, the loss of motility occurred by two genetic mechanisms: reversible and irreversible. Reversible loss of

motility occurred early during broth adaptation, followed by irreversible motility loss in the majority of cells by the end of the experiment. Whole genome sequencing implicated diverse mutation events that resulted in the loss of gene expression necessary for flagellar biosynthesis. Furthermore, reversible mutations in homopolymeric DNA tracts of adenine/thymine residues, and irreversible types of mutation such as gene deletion, were discovered in the broth-evolved populations. In all evolved lines, an alternative sigma factor necessary for flagellar structural gene expression was removed from the genome.

Overall, this dissertation contains the first accounts of *C. jejuni* experimental evolution. The results provide insight into the biological importance of reversible mutations in homopolymeric DNA tracts, and provide a basis for future studies of *C. jejuni* evolvability.

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CHAPTER 1

Introduction

CAMPYLOBACTER JEJUNI

Campylobacter jejuni was described as early as 1886 but was not recognized as an important gastrointestinal pathogen until the 1970s, when *Campylobacter* was isolated from the blood and feces of a human patient with diarrhea (20, 36, 45). It is now known to be a spiral or curved-rod shaped, flagellated, ϵ -proteobacterium (Figure 1.1) that is often considered commensal of avian species, and an enteric pathogen of humans and other mammals. *C. jejuni* can colonize a diverse host range, but colonized chicken flocks seem to be the most important reservoir of *C. jejuni* for the infection of humans (198). The genome sequencing of a human clinical strain of *C. jejuni* in 2000 revealed a small genome (~1.6 Mbp) that was low in G+C content (~30%), contained hypervariable DNA elements, and lacked homologs to many previously identified virulence factors (148).

C. jejuni infection is one of the most prevalent foodborne causes of human bacterial enteritis in industrialized nations (5, 165), and costs the United States economy approximately four billion dollars each year (192). Despite this, the molecular mechanisms of *C. jejuni* virulence are not well understood. Most research attempting to identify and describe genes important for *C. jejuni* virulence has relied on mammalian cell lines and chicken models of colonization (198). In chicken models, *C. jejuni* rarely induces gastrointestinal inflammation as is seen during human infection (198). However, studies using cell lines and chickens have identified a number of factors that may be involved in *C. jejuni* pathogenesis through their roles in surface structure biosynthesis,

motility, and toxin production. The importance of these factors for *C. jejuni* virulence in a small animal mammalian model, where an intense inflammatory response is initiated, has only rarely been studied (53, 60, 66, 138).

There is little agreement among researcher concerning the importance of various *Campylobacter jejuni* virulence factors. This is in part due to the lack of a small animal model of disease until recently (122), which would allow rigorous testing of Falkow's molecular Koch's postulates for microbial pathogenecity factors (46). Recently, a literature search implicated approximately 300 genes as being involved directly or indirectly in *C. jejuni* virulence (J.A. Bell, J.P. Jerome, *et al.*, submitted manuscript). This suggests that just over 18% of all predicted protein products in the genome of *Campylobacter jejuni* are involved in virulence. However, many putative virulence genes are not present in all clinical *C. jejuni* isolates from patients with campylobacteriosis. It appears that many of these implicated genes are necessary for virulence in some strains but not sufficient for *C. jejuni* virulence. Also, the large number of identified factors may be reflective of the lifestyle of *C. jejuni*, which only involves replication in a host. In addition, the potential for false positive results when assessing *C. jejuni* virulence is high due to potential for confounding random mutations as us shown in this dissertation, and suggested by others (11). Unfortunately this is rarely discussed and false positive results may be present in the literature due to the often-accepted practice to not complement targeted deletions in *C. jejuni* research. This section reviews what is widely accepted concerning the ability of *C. jejuni* to colonize and elicit an inflammatory response within a host.

The present knowledge of *C. jejuni* virulence provides an insufficient picture of *C. jejuni* pathogenesis in the human host. What is known is that *C. jejuni* can survive passage through the stomach to colonize the lower gastrointestinal tract, including the ileum, colon, and cecum. *C. jejuni* is able to chemotax toward mucus (87), and pass through the mucus layer to adhere to the apical side of the epithelium, and invade epithelial cells. The route of invasion and its role in *C. jejuni* pathogenesis is still vague. Uptake by M cells followed by basolateral invasion, a paracellular entry, or apical invasion routes have all been demonstrated (86, 131, 178, 182). No matter which route(s) is most relevant *in vivo*, the interaction with and breach of the epithelium elicits a strong inflammatory response involving infiltration of mononuclear cells, along with the release of pro-inflammatory cytokines (42, 122, 153). This inflammatory response may result in tissue damage and pathology that may manifest clinically as watery to bloody diarrhea, and occasional fever, vomiting, and headache before clearance of the bacteria.

Based on work that has been done using mammalian cell culture and *in vivo* infections, certain factors likely play a role in *C. jejuni* virulence. Cell surface structures including lipooligosaccharide (LOS) are thought to be important for *in vivo* colonization, survival, and immune system reactivity (31, 117, 198). Although the mechanistic role for LOS *in vivo* has not been well studied, purified *C. jejuni* LOS is capable of stimulating cytokine production in human dendritic cells (85), and LOS core truncations affect *C. jejuni* mouse colonization (137). Also, modifications of the LOS mimic human gangliosides and are likely the cause of the post-*C. jejuni* infection autoimmune neuropathies, Guillain-Barré and Miller Fisher syndromes (58, 151). In support of this

suggestion, specific LOS structures that mimic human gangliosides are capable of eliciting an immune response in mice that includes production of specific subclasses of anti-ganglioside antibodies (149). The LOS structure of *C. jejuni* has also been shown to mimic multiple other mammalian glycan structures including P blood group antigens (84), but the biological importance of this is unknown. The importance of molecular mimicry in *C. jejuni* pathogenesis certainly deserves more attention in future work, since molecular mimicry is a common strategy employed by infectious agents to manipulate and/or avoid their host's immune response (44, 69).

Capsular polysaccharide is an important surface structure for immune system avoidance, and thus virulence, in many bacterial pathogens (159), and *C. jejuni* has been shown to express a capsule (102). In *C. jejuni* the genes that define the carbohydrate moieties of the capsule are highly variable between strains, and many are subject to phase variation (99). The capsule has been shown to be important for *in vivo* survival; it was shown that a capsule deficient mutant was attenuated in a ferret diarrheal disease model (7). However, capsule deficiency seems to play no role in the survival of *C. jejuni* during its commensal colonization in the chicken gastrointestinal tract (6). It is possible that capsule is only important during infection of a mammalian host, but more data is needed to support this. Also, considering the role of capsule as a virulence determinant in *C. jejuni*, workers have sought to develop a capsular polysaccharide conjugate vaccine against *C. jejuni* diarrheal disease (130). However, caution must be taken when developing a vaccine against *C. jejuni* capsular polysaccharides that are known to be highly variable between strains, and even within a given strain via phase variation.

The foremost tenet in *C. jejuni* pathogenesis research is that the ability to colonize any host depends on another surface structure: flagella (15, 72, 139). There is direct evidence that non-flagellated, non-motile mutants are colonization deficient in chickens, piglets, mice and humans (15, 37, 72, 96, 121). The formation of functional flagella is a complex process involving an estimated ninety genes (including chemotaxis related genes) (73). The flagellum is known to be heavily glycosylated, which has been shown to be important for flagellar biosynthesis, and thus motility (66, 120). Because of their role in flagellar motility many of these genes are essential for host colonization. Motility is thought to be important during chemotaxis through the gastrointestinal lumen toward the mucus and underlying epithelial cell layer. Also, non-motile *C. jejuni* mutants are generally defective in epithelial cell interaction in culture (63, 185, 196). A complete flagellar apparatus appears to be required for secretion of the Campylobacter invasion antigens (Cia) that are necessary for maximal invasion of cultured epithelial cells (106, 107). This has lead to speculation that the flagellar export apparatus may act as type III secretion system to deliver effector molecules to the host. In some bacterial pathogens, delivery of flagellin through a T3SS acts as a trigger for inflammation by activation of the inflammasome by intracellular NOD-like receptors (NLRs) (105), but this has not been studied for *C. jejuni*. Currently, there is still uncertainty in the field concerning the importance of flagellar functions aside from motility in *C. jejuni* pathogenesis.

Since it is indispensable for host colonization, it is interesting to note that flagellar motility is a phase variable phenotype in some clinical strains of *C. jejuni* (100). It seems counterproductive for bacteria that only replicate naturally within a host to variably express an essential colonization factor. However, avoidance of bacteriophage that bind

flagellar structures may drive the random, variable expression of flagella and flagellar modifications (30). The genes that are apparently responsible for motility phase variation are the motility accessory factor, *maf1* (100), and the *flgS* and *flgR* genes of the FlgSR two-component regulatory system (74). The *maf1* open-reading frame contains a hypervariable homopolymeric guanine tract and is located in the flagellar glycosylation locus, but its function remains unknown. If *maf1* does indeed contribute to flagellar modification as suggested by genomic location, it may be a promising target for vaccine development or phage therapy, considering its importance for flagellar motility.

Beyond the importance of surface structures for *C. jejuni* virulence in mammalian hosts, one known toxin is produced—a cytolethal distending toxin (CDT) encoded by three genes, *cdtA*, *cdtB* and *cdtC* (150). This toxin has been suggested to play a role in cell cycle arrest and intestinal epithelial cell apoptosis, and the release of the pro-inflammatory cytokine interleukin-8 (53, 79, 80). CDT may also be involved in persistent infection of mice through immune system avoidance (53). Furthermore, it has been shown that neutralizing antibodies to CDT are formed during human infection (3). Recently it was shown that functional CDT is released from *C. jejuni* in outer membrane vesicles (115). This may be the main route of CDT secretion given the lack of other virulence related secretion systems. Still, CDT does not seem to be necessary for virulence in all *C. jejuni* strains considering it is missing from some clinical isolates (3).

Aside from the intensively studied factors described above, the *C. jejuni* genome contains potential virulence genes identified based on similarity to other known virulence factors (148). These include an integral membrane protein with hemolysin domain (Cj0183), a hemolytic cytotoxin (Cj0588), and a phospholipase A (putatively

pldA) (148). There are also a number of factors that have been shown to be important for epithelial cell adherence *in vitro*. These include a fibronectin binding protein (CadF), a lipoprotein that may be involved in initiating a pro-inflammatory response (JlpA), and a periplasmic protein (Peb1). However, the role of these factors in colonization and lesion production in an *in vivo* mammalian system has not been adequately defined. Direct study of all of these factors in a small animal model could shed light on their currently putative roles.

Overall, knowledge of *C. jejuni* pathogenesis is dominated by research of surface structures, and in particular the carbohydrate modified LOS, capsule, and the flagella (67). This is reasonable considering these structures are able to directly interact with host cells, are important for adherence to epithelial cells, contribute to development of post-infection autoimmune disease, and are necessary for flagellar motility. These structures were likely evolved for functions in avian species—considered the natural host, and main reservoir of *C. jejuni*—but they also happen to be important for pathogenesis in other hosts. It may be that *C. jejuni* infection of humans is opportunistic since infection of humans is not likely to lead to transmission of the bacteria—impeding the evolution of human specific adaptations by *C. jejuni*. This is supported by the lack of known effectors and toxins in *C. jejuni* that have arisen during co-evolution of well-characterized pathogens and their human hosts. Therefore, understanding the basic biology of *C. jejuni*, including how it replicates within a host, and how this contributes to pathogenesis in other novel hosts, such as a small animal model or humans, may be most appropriate.

WITHIN-HOST EXPERIMENTAL EVOLUTION OF BACTERIAL PATHOGENS

The evolution of virulence in bacteria is an extremely complex process that is difficult to study. Sex, kin selection, and the cost to bacterial fitness associated with host death have been cited as factors affecting the evolution of bacterial virulence in a host (18, 19, 126). Much of the work done to study the evolution of bacterial virulence has been theoretical or comparative, rather than via experimental laboratory evolution. To study the evolution of virulence experimentally, researchers have performed *in vivo* serial passage experiments. This is when a bacterial strain is inoculated in a host, allowed to grow, and then isolated for re-inoculation into a new host (41). Thus, the experimenter has the ability to monitor the evolution of the bacteria isolated from each passage, and study the effect of pathogen evolution on the host.

Multiple *in vivo* passages of a bacterial strain often select for increased virulence (41). This has been observed for bacterial pathogens including *Helicobacter pylori* (16), *Salmonella typhimurium* (41) and *Campylobacter jejuni* (13). When serially passaged in animal models, these pathogens cause more severe disease than the ancestral strain from which they were derived. Other studies show that the fitness of the pathogen in the host increases during serial passage experiments (41, 57, 140). It therefore seems as if there is the relation that as the bacterial fitness increases, host fitness during infection decreases (41, 169). However, in natural systems there may be a trade-off for bacteria between increased fitness, and the consequences of disease in the host (i.e. it may be more difficult for host to host transmission if the original host dies) (18). This trade-off

does not play a role during serial passage experiments since the pathogen is artificially passaged from host to host.

Ebert offers three non-mutually exclusive hypotheses to explain the increase in virulence during serial passage (41). First, the artificial transmission of the bacteria to a new host relieves any cost associated with killing the host. If the host is killed in a natural system it may inhibit transmission, so that it may be beneficial for a strain to become less virulent. Second, the evolution of the host may be stifled since the experimental hosts are most often genetically similar lab animals. This allows the bacteria to adapt to a single host environment during serial passage and precludes any effects of an evolutionary arms race. Finally, costs associated with survival during transmission are reduced by the artificial serial passages. This may neglect the potentially important between-host survival of horizontally transmitted pathogenic bacteria. Giraud *et al.* and Nilsson *et al.* discovered that mutations that cause auxotrophy accumulate during serial passage experiments (57, 140). It could be reasoned that the ability to catabolize essential compounds is necessary for pathogen survival outside the host during transmission, but that during the artificial inoculations between metabolite rich environments there is no need for the ability to grow in minimal medium environments. It has been suggested that the increase in auxotrophy may be a trade-off for the fitness increase during a serial passage experiment (57).

Other serial passage experiments have attempted to understand the bacterial fitness costs and benefits of mutation rates within a host, without observation of host disease. Still, based on the conclusion that bacterial fitness and virulence increase concomitantly, certain hypotheses can be made about bacterial mutation rate and

virulence. Nilsson and colleagues performed serial passage experiments in a mouse model with the pathogenic bacteria *Salmonella typhimurium* LT2 (140). Inoculation size was varied and two strains were used: a wild type and a strain deficient in DNA repair with a high mutation rate (mutator). After evolution in 18 separate lineages for 8-10 passages, the evolved strains were competed against the unevolved parental strain *in vivo*. All 18 lineages showed an increase in mouse-specific fitness relative to the parental strain that was greater for larger initial population (inoculation) sizes.

Furthermore, the mutator lineages had an increased adaptive mutation rate relative to the wild type strains. This experiment showed that a pathogen's fitness may increase in the niche provided by a specific host. Also, increased mutation rates may lead to a faster increase in fixation of beneficial mutations. Presumably, these results are observed since the bacterial strain is far from an adaptive peak when first inoculated into the host. Higher mutation rates and larger population sizes are predicted to increase the chance that beneficial mutations will arise and become fixed. What the authors did not discuss was the effect of the bacterial fitness increase on the mouse host. From the studies, it could be hypothesized that a mutator population, or a larger transmitted population, leads to higher virulence in a new host (140). However, to my knowledge this has not been tested.

The experimental evolution of bacterial strains toward increased virulence in animal models has been lightly researched. The effect of bacterial mutation rates and population size on bacterial fitness has been assessed (140), but serial passage experiments generally neglect important selective pressures that occur during the transmission of bacteria between hosts. It is possible to design experiments to study a

more natural transmission of bacteria simply by forcing contact between infected, and a non-infected, naïve hosts. I would predict that bacterial virulence would not increase as rapidly and to the same extent as seen in artificial passage experiments if there is a true trade-off associated with growth in the host and survival in the environment.

Potentially useful information could be gained from a comparative study of a pathogenic bacterial strain that is only slightly virulent, and the same strain after evolution in the host during serial passage. This type of experiment may be considered a forward genetic screen to identify important factors for fitness and/or virulence in the animal model used. These comparisons may also lead to conclusions about the evolution of bacterial adaptation to a host environment.

WITHIN-HOST EXPERIMENTAL EVOLUTION OF *CAMPYLOBACTER JEJUNI*

Aside from humans and birds *C. jejuni* can colonize a broad range of animal hosts. Some researchers have argued that natural *C. jejuni* populations have host-specific genotypic and phenotypic adaptations indicative of a long-term association with specific host species (47, 125), but others have suggested *C. jejuni* genotypic and phenotypic patterns are characteristic of an opportunistic, or generalist, lifestyle (64). However, irrespective of the long-term evolutionary history of *C. jejuni*, direct comparisons of ancestral and host-adapted *C. jejuni* populations suggest an ability to quickly adapt through heritable changes (13, 15, 21, 94, 162).

Heritable adaptation is a stochastic process since it is dependent on the generation of random mutations for adaptive change to occur by natural selection (134). This is distinct from deterministic adaptation that is prescriptive through gene regulation in response to environmental cues (135). Describing the prescriptive responses of pathogenic bacteria by classical gene regulation has been a main focus of infectious disease microbiology (38, 127), and for *C. jejuni* (56). However, recent work with *C. jejuni* has also highlighted the ability to adapt to an environment by mutation (11, 93, 103, 166), often in homopolymeric DNA—the simplest form of simple sequence DNA repeats (SSRs), which will be discussed in the following section.

Researchers have noted that specific *C. jejuni* phenotypes are selected by passage through chickens (94, 162), mice (13), rabbits (21), and humans (15). Most often, *C. jejuni* motility increases during host passage, and along with the motility increase, an increased ability to colonize a host has been documented (15, 94). As

described above, *C. jejuni* motility is essential for efficient host colonization. The genetic basis of the motility increase is unknown, but hypermutable homopolymeric DNA within some motility accessory factor (*maf*) genes may play a role (101). Also, whether standing phenotypic variation in motility, as has been described (100), is selected during passage, or whether the increased motility phenotype arises by mutation during passage is also unknown. Although not tested, it is possible that selection for motility also explains the observation that chicken passage generates a *C. jejuni* variant with approximately 1000-fold lower infectious dose to yield sustained colonization (158). That is, if only a fraction of *C. jejuni* cells are motile before passage then a higher dose will be needed for colonization compared to the passaged *C. jejuni* population that is mostly, or completely, made up of motile cells.

Other phenotypic changes have been documented in *C. jejuni* as a result of host passage. Antigenic variation in lipooligosaccharide (LOS) modifications occurs in *C. jejuni* during infection of humans (151). Prendergast *et al.* noted that *C. jejuni* strain 81-176 inoculated into human volunteers initially lacked ganglioside mimics in the LOS that are linked to the development of the post-infection neuropathy, Guillain-Barré syndrome (GBS). However, *C. jejuni* that had been recovered from volunteers had undergone antigenic variation to express multiple ganglioside mimics. It was previously shown that strain 81-176 was capable of producing various potential ganglioside mimics since some genes responsible for LOS modification were variably expressed due to the presence of mutable homopolymeric DNA within their open reading frames (68). Though not directly tested it seems likely that the variation in LOS antigenicity that occurred during human infection was driven by mutations in homopolymeric DNA within

LOS biosynthesis genes. Future work should focus on understanding why certain ganglioside mimicking LOS structures appear to have been selected by passage through humans, as this may have implications for the pathogenesis of *C. jejuni*-induced GBS.

Finally, our laboratory showed that serial passage through mice leads to an increased virulence phenotype of *C. jejuni* in a C57BL/6 IL-10^{-/-} mouse model of campylobacteriosis (13). Passaged strains included NCTC11168 (ATCC 700819), which was isolated in the 1970s, sequenced in 2000, and is widely used in *Campylobacter* research (148), along with four recent clinical isolates of *C. jejuni* from patients with gastroenteritis: D0835, D2586, D2600 and NW. After three rounds of passage there was an increase in fecal *C. jejuni* populations (NW, D2600, and 11168), and an increase in the number of mice harboring *C. jejuni* in the jejunum (D2600, D0835, and 11168). Survivorship of infected mice was decreased by passage, and there was an increase in the fraction of mice exhibiting pathological changes, and histopathological changes in the gut (13). Collectively these results suggest that passaged *C. jejuni* had increased fitness (based on colonization levels) and increased virulence (based on host damage) as a result of serial passage, which is consistent with numerous other pathogen serial passage experiments (41). However, this study did not address phenotypic or genetic changes occurring in *C. jejuni* as a result of passage that accompanied the increases in fitness and virulence.

Only a few studies have identified the genetic changes that occur during *C. jejuni* host passage. However, with the relative ease and low cost of new sequencing technologies, using host passage as a forward genetic screen will likely become more

common. This should lead to more analysis of the genetic basis of adaptation during host passage that is contributing to the phenotypic changes described above. Until the work completed during this dissertation, no study had completed a comprehensive analysis of the genomic basis of *C. jejuni* adaptation that occurs during host passage. Nevertheless, as might be expected considering the phenotypic changes described, the *C. jejuni* genome is not static *in vivo* as shown by pulse field gel electrophoresis (PFGE) (32, 166, 186), evidence for intergenomic DNA transfer (32), and targeted re-sequencing of chicken passaged isolates (11, 103, 193).

Multiple studies have shown *C. jejuni* is undergoing rapid intragenomic recombination during chicken colonization (32, 166, 186), and in some cases the functional consequences of DNA rearrangements have been reported (166). Scott *et al.* showed that a rearrangement of the *C. jejuni* genome occurred during chicken colonization between prophage DNA sequences. This mutation, involving approximately one-third of the genome, was associated with the avoidance of phage predation and the generation of infectious bacteriophage (167). Interestingly, although the rearranged *C. jejuni* was more resistant to phage attack, it had a decreased ability to colonize chickens, suggesting a trade-off between phage resistance and the ability to colonize the host. Nuijten *et al.* also documented functional DNA rearrangements that occurred during chicken colonization (145). This group showed that deletions in *flaA*, which is sufficient for flagellar motility, could be repaired through rearrangements with the *flaB* duplication located immediately downstream during chicken colonization. As discussed above, the importance of flagellar motility for *C. jejuni* colonization is undisputed, so there is a great selective pressure to regain motility during chicken colonization. The

restoration of a functional *flaA* likely represents one mechanism for DNA mutation repair in *C. jejuni* via intragenomic recombination.

These studies have shown that *C. jejuni* is constantly mutating via intragenomic recombination during chicken commensalism to generate genomic diversity for rapid genetic adaptation. Alternatively, intragenomic recombination may play a role in the stability of functional DNA sequences in the genome, since *C. jejuni* lacks some DNA mutation repair genes present in other bacteria. Further work should elucidate the biological significance of the extensive *C. jejuni* intragenomic recombination that occurs during chicken commensalism, and in the diversity of hosts in which *C. jejuni* can colonize.

Other studies have further examined the potential for *C. jejuni* to generate genetic diversity during chicken commensalism. It has been known for some time that *C. jejuni* is naturally transformable (183), but the importance of this phenotype during chicken colonization had not been assessed. De Boer *et al.* created two *C. jejuni* strains that were distinguishable by different antibiotic resistance genes, but otherwise isogenic (32). When these strains were used to co-colonize chickens, strains were recovered after colonization that contained both antibiotic resistance genes. From this work the authors concluded that *C. jejuni* interstrain recombination occurred *in vivo*, and should be considered another potential source of genomic diversity. However, multiple researchers have noted that *C. jejuni* natural transformation is preferential to DNA from genetically similar *C. jejuni* strains. That is, natural transformation is most efficient between homologous DNA, and not very efficient when incorporating DNA from other species into *C. jejuni* (183). In this work by de Boer *et al.*, the only difference between

the genomes of the experimental *C. jejuni* “strains” was in a single antibiotic resistance gene (32), and the rate of recombination between more distant *C. jejuni* strains, or other *Campylobacter* species was not tested.

Finally, Wilson and colleagues demonstrated that during chicken colonization, mutations accumulated in the homopolymeric tract of the lipooligosaccharide modifying contingency gene, *Cj1139c* (152, 193). The diversity that was generated at this locus during chicken colonization was diminished during subsequent mouse passage. This finding led to their conclusion that the chicken reservoir may also serve as a reservoir for contingency loci mutations that are important for colonization of different hosts. What was not tested though, was whether chicken colonization led to mutations in all contingency loci, or only those that were not subject to purifying selection for a single allele. Also, it was not tested if diversity in contingency loci mutation is generated whenever a population of *C. jejuni* is grown, or whether the chicken gastrointestinal environment is specifically contributing to the generation of contingency loci mutational diversity. In either case, it is likely that mutations in contingency loci are always present in a population of *C. jejuni* within a flock of chickens, considering the estimated size of the *Campylobacter* population on a large chicken farm (184).

EVOLVABILITY BY HOMOPOLYMERIC DNA TRACTS IN *CAMPYLOBACTER JEJUNI*

Bacteria adapt to changing environments through two major mechanisms: a change in gene expression or a change in genotype. Expression may be regulated at multiple levels including transcription, translation, and post-translation and has been the main focus of molecular microbiology studies for three decades (38). Expression regulation to alter phenotype (a prescriptive response) occurs *after* the environment changes and *in response* to specific environmental cues. A “sense and respond” approach to expression change has evolved into elaborate functional response systems in bacteria that are necessary for replication in different environments, but there are definite drawbacks to this strategy. First, the environment may change too rapidly for a bacteria to mount an appropriate regulatory response. This may be especially true for bacterial pathogens that are actively under attack from a host’s immune system. Secondly, a programmed adaptive response is the product of selection on an evolutionary time scale, so that bacteria will only be able to respond to a limited set of re-occurring environmental changes.

In contrast to prescriptive gene expression changes, adaptive genotypic changes must occur *before* the environment changes, and offer *no response* by gene regulation. Instead, mutations generate stochastic diversity within a population of otherwise related cells, and as a result of environmental change, selection may favor certain cells within the population of variants. Often, bacterial pathogens have mechanisms for generating mutational diversity in defined genes to stochastically alter expression at a high rate.

These genes have been termed contingency genes in bacteria (134). This leads to diversity of genotypes/phenotypes within a population of cells derived from a single ancestral cell, and may be considered a bet-hedging strategy, or contingency plan. That is, some members of the population may be more fit than others in future environments, but the mutations that occur have little or no effect on fitness in the present environment.

Multiple human and animal bacterial pathogens have a genetic-based mechanism for the stochastic generation of mutations in defined loci. These include *Bordetella pertussis*, *Neisseria* spp., *Helicobacter* spp., *Haemophilus influenzae*, *Yersinia pestis*, and *Campylobacter* species (59, 95, 148, 160, 172, 179). Most often the increased mutation rate is driven by the presence of simple sequence DNA repeats (SSRs) in the promoter or open reading frame of a contingency gene (135). Repeats consist of iterations of DNA sequence, where the repeat unit may be one or more nucleotides in length. SSRs are thought to be prone to slipped-strand mispairing during genome replication which leads to a high rate of insertion or deletion of the repeat unit (113). These mutations may affect RNA polymerase binding to the promoter region of a gene, as is the case in the intergenic region between the *H. influenzae* fimbriae genes, *hifA* and *hifB*₁, where insertion and deletion of TA repeats changes the distance between the -35 and -10 RNA polymerase binding sites leading to more or less transcriptional starts (179). Alternatively, SSRs present within the open reading frame of a gene lead to the introduction or loss of premature translational stop codons. An example of this is the reversible expression of the adhesin/antigen gene, *opa*, in *Neisseria gonorrhoeae* driven by loss or gain of the pentameric repeat unit, 5'-CTCTT-3' (175). No matter the

location of the SSR in a contingency gene, the effect is the same: high frequency, stochastic, reversible, heritable, genotypic switching that rapidly generates genotypic and phenotypic diversity in an otherwise clonal bacterial population.

Mutability of *Campylobacter jejuni* SSRs was first discovered when Parkhill and colleagues sequenced the genome of strain NCTC11168 in 2000 (148). This genome sequence was published under a title that highlighted the presence of hypervariable tracts of homopolymeric DNA (148). The authors defined these tracts as variable since traditional Sanger genome sequencing could not resolve their length to one specific value—indicative of a high rate of insertion and deletion (indel) mutations. Most of the mutations described by Parkhill *et al.* occurred in 8–12 base tracts of homopolymeric cytosine/guanine DNA (polyC/G tracts), but one variable homopolymeric adenine/thymine (polyA/T) tract was also reported. Subsequent analyses by whole genome sequencing (WGS) (52, 93) and direct length analysis of PCR products (103, 187), have confirmed that polyC/G tracts are unstable due to indel mutations in *C. jejuni*. In addition, researchers have described phase variation in *C. jejuni* flagellar biosynthesis genes through reversible mutations in polyA/T tracts (72, 75). These findings show that homopolymeric DNA in *C. jejuni* is unstable, and may be a source for rapid genetic adaptation.

Homopolymeric DNA repeats in *C. jejuni* likely mutate by slipped strand mispairing of the repeat units during replication, such as other SSRs (113, 135). When a gene is affected by a homopolymeric DNA tract, base insertion and deletion mutations dictate the status of gene expression. Therefore, SSR-containing genes are subject to high rate, stochastic, heritable switching of gene expression, and are considered to be

in an “ON” or “OFF” state based on the length of the SSR. Genes associated with hypermutable SSRs are called contingency genes because of their high rate of mutation relative to the rest of the genome (134, 135). In the *C. jejuni* genome, contingency genes are defined by the presence of a polyC/G tract due to the instability of polyC/G DNA in the genome. As will be discussed below, nearly all *C. jejuni* genes contain a polyA/T tract. Although it has been shown that polyA/T tracts facilitate genotypic and phenotypic switching in *C. jejuni* (72), the frequency of switching must be much lower than polyC/G DNA in most polyA/T tracts. Therefore, genes containing a polyA/T tract are not considered contingency genes, but are genetically reversible by slipstrand mutation.

For this review we focus on the literature concerning the ability of *C. jejuni* to adapt from phenotypic variation generated via mutations in defined DNA sequences: homopolymeric DNA tracts. We detail the current state of knowledge concerning the mutability of homopolymeric DNA in *C. jejuni*, and at times draw comparisons to relevant features of SSRs found in other pathogenic bacteria. Finally, we speculate on the implications of homopolymeric DNA tract mutability for *C. jejuni* evolutionary biology and research.

Multiple researchers have observed mutations in *C. jejuni* polyC/G tracts after streaking an agar plate for DNA isolation from a single colony (52, 148, 187). These mutations are the result of a high mutation rate in polyC/G tracts, and possibly adaptive evolution during *in vitro* culture (163). When first observed, the rate of indel mutation in polyC/G tracts was assumed to be high (148), but had not been determined experimentally. Subsequently, Bayliss and colleagues determined the rate of switching

in *C. jejuni* polyC/G tracts to be in the range of 10^{-2} to 10^{-4} mutations per division (11).

They also noted that this rate varies with the length of the tract such that longer tracts have a higher switching rate. SSR switching rates comparable to those of *C. jejuni* polyC/G tracts have been observed for long tetranucleotide repeats (18-37 repeat units) in *Haemophilus influenzae* (33), but not for relatively short homopolymeric DNA tracts. Intriguingly, in *Neisseria meningitidis*, the rate of polyC/G switching is around 10^{-5} , but when components of the mismatch repair system (MMR) are inactive, the switching rate increases to frequencies comparable to those observed for *C. jejuni* polyC/G DNA (123, 155). Considering these findings, it is most likely that the high polyC/G-mediated switching rate in *C. jejuni* is largely due to an inactive MMR system (54).

Observations of polyC/G mutations suggest that currently undefined molecular mechanisms affect contingency loci mutability in the *C. jejuni* genome. First, polyC/G tract lengths are mostly between 8 and 12 bases in *C. jejuni*. Maintenance of tract length in this range likely results from selection for switching rate, since longer tracts are more unstable, and shorter tracts might not switch enough to be advantageous in changing environments. A bias for insertion mutations when the tract is short (8 or 9 bases), versus a bias for deletion mutations when the tract is long (10 or 11 bases), has also been described (11). This bias for insertion or deletion mutations based on tract length may result in the maintenance of tract length, but how this bias is achieved is unknown.

Another feature of *C. jejuni* polyC/G DNA tracts is that most are present within the open reading frame of a contingency gene. For instance, of the 29 polyC/G tracts in

C. jejuni strain NCTC11168, 26 are found in the open reading frame of a contingency gene. Indel mutations within the coding sequence result in the gene being turned ON or OFF at the level of translation, and not transcription, which likely leads to the complete loss of function since a full-length protein is not synthesized. This is in contrast to SSR mutations that modulate the level of transcription of a functional protein, such as SSRs in the promoter regions of some fimbrial contingency genes in *Haemophilus influenzae* (179) and *Bordetella pertussis* (191). It is possible that the bias toward polyC/G DNA occurring within the ORF is simply a constraint due to the organization of the *C. jejuni* genome, which encodes very little intergenic space (148).

An additional feature of polyC/G tracts in *C. jejuni* is a strong bias for the homopolymeric guanine bases of polyC/G tracts to be located on the coding DNA strand (11). In NCTC11168 there are 29 contingency loci, and for 28 of these, polyG is on the coding strand and polyC is on the template strand. The effect of having guanine instead of cytosine tracts in the transcript is unknown. Presumably, this bias would not affect mutation rates since a coding strand bias is not predicted to alter the frequency of slipped strand mispairing during replication. Also, polyG tracts are distributed on both DNA strands of the genome so a difference during replication of the leading versus lagging strands appears irrelevant. Moreover, the one contingency locus in NCTC11168 with a transcribed polyC tract (*Cj0685* or *cipA*) does not appear to have altered mutability (11, 93). Therefore, the bias for coding strand polyG tracts is likely the result of an unknown function at the level of transcription. It is known that in some contexts, polyG DNA, and presumably RNA, has altered secondary structure (128) that may

result in altered function, but this remains untested for *C. jejuni* contingency loci transcripts.

Finally, work has shown evidence for transcript termination, or decreased transcript stability, when a polyC/G mutation within the open reading frame introduces a premature translational stop codon (93). A similar link between transcription and translation has been shown in a phase variable type III restriction-modification system in *Helicobacter pylori* (34). Due to this link, phase variable mutations that disrupt the translation of a contingency locus, may also result in a polar affect on the transcription of the contingency gene, and downstream genes. Low transcript abundance when a contingency gene is OFF at the level of translation might be advantageous since expression of non-functional peptides is both energetically costly, and potentially cytotoxic. Interestingly, there is evidence that transcript termination affects mutation patterns in homopolymeric DNA in a phase variable capsule locus of *Neisseria meningitides* (109). In this case, low transcript abundance in OFF mutants, as observed for *C. jejuni*, is the result of Rho-dependent transcript termination, which leads to an increased OFF to ON switching rate. This phenomenon has not been studied in *C. jejuni*, but is thought to be the result of transcription-coupled DNA repair enabled by the product of the *mfd* gene (164, 168). Increased Mfd expression is known to increase the frequency by which antibiotic resistance mutations occur in *C. jejuni* (70), but the effect of Mfd on contingency gene switching has not been tested.

PolyC/G tract length distributions of contingency loci may change as a result of host passage (11, 93, 103, 193). Genome sequencing of mouse passaged *C. jejuni*, and targeted contingency loci analysis of chicken passaged *C. jejuni* have discovered

changes in the frequency of ON and OFF phases in contingency loci (93, 103, 193). In a mouse model of campylobacteriosis the contingency loci changes that occur during infection are associated with increased virulence, as passage of *C. jejuni* in the model results in increased inflammation of the gastrointestinal (GI) tract (13). Furthermore, changes in *C. jejuni* contingency loci that occur during chicken passage are associated with the successful colonization of mice (103), even when the mice are kept on a high fat diet that has been shown to be less permissive to *C. jejuni* colonization (13). This suggests that the microevolution of contingency loci in the avian intestine—an important reservoir of *C. jejuni*—may promote subsequent infection of different hosts, such as humans.

The observed changes in polyC/G length distributions during animal infection suggest that specific phase variants have differential fitness *in vivo*. However, these changes may also result from genetic drift due to polyC/G mutation rates and patterns, bottlenecks of *C. jejuni* cells during initial colonization or by active immune system clearance, epistasis in a genome that can encode millions of genotypes through contingency loci switches, and/or genetic hitchhiking. In chickens and mice, the changes in contingency loci that occur during gut passage are not explained by mathematical models of polyC/G genetic drift (11). Also, in a mouse serial passage experiment, hitchhiking does not seem to drive the observed contingency loci changes, since the differences in polyC/G tract distributions between ancestral and mouse-adapted *C. jejuni* were the only genetic changes detectable by WGS, expression microarray, and pulse-field gel electrophoresis (93). However, some combination of these effects may contribute to the variability in contingency loci changes sometimes

observed between replicate experimental animals (11, 103). The combined use of next-generation sequencing technologies, mathematical models that incorporate bottleneck effects, and targeted analysis of contingency loci dynamics during controlled evolution experiments, should be able to elucidate the evolutionary forces driving the observed contingency loci changes during host infection.

Despite experimental evidence that polyC/G mutability is important for the *in vivo* lifestyle of *C. jejuni*, the molecular functions of most contingency loci are unknown. Based on genome sequences and structural analyses, specific glycan structures of the lipooligosaccharide (LOS), glycosylated flagella, and capsule are variably expressed in *C. jejuni* due to polyC/G DNA (117, 148). All of these structures are important for *C. jejuni* pathogenesis (7, 66, 137), but the purpose of their phase variable glycan moieties, and their influence on disease outcome, is not well understood. It is known that *C. jejuni* flagellin must be glycosylated for flagellar filament polymerization (61, 120), and the *maf* contingency loci involved in flagellar glycosylation (*maf1* and *maf7*) may affect motility (100). However, the biological significance of reversible flagella expression is confounding considering the importance of flagellar motility for *C. jejuni* colonization of a host—the only known place of replication. Still, it is possible that the reversible expression of flagellar motility, or flagellar glycans, may be a mechanism for phage avoidance, since phage are known to attach to phase variable surface structures (30).

Similarly, the reversible expression of specific glycan residues on the capsule via contingency loci mutation appears to be a mechanism for phage avoidance. Recent work has shown that loss of an O-methyl phosphoramidate capsule modification based

on a polyC/G mutation, results in resistance to the lytic bacteriophage, F336 (171). In this work the authors also noted that F336 could not promote lysis of *C. jejuni* in liquid culture—consistent with the omnipresence of polyC/G mutations in a populations of *C. jejuni* cells, which in this case influences reversible phage resistance. This result suggests that selective pressure from virulent phage may have played an underappreciated role in the history of contingency loci evolution in *C. jejuni*.

Aside from phage avoidance, it is possible that phase variable carbohydrates contribute to immune system avoidance, or modulation, which is beneficial for *C. jejuni* survival in the host. In fact, some carbohydrate structures on the *C. jejuni* LOS mimic human gangliosides, and other mammalian glycans (58, 68, 84, 116, 151), and polyC/G DNA within LOS biosynthetic genes affects this mimicry (68, 84, 116). The benefit for *C. jejuni* to express human glycan mimics is unknown, but multiple pathogens have evolved mechanisms to interfere with the host's immune response through molecular mimicry (104). Mimicking host structures may make it difficult for the host to discriminate *C. jejuni* from itself, and encoding multiple molecular mimics through contingency loci variation might allow *C. jejuni* to persistently evade the host's immune system. Alternatively, it is possible that variable glycan structures have differential fitness during infection of distinct hosts.

Mimicry through LOS contingency loci may also affect disease outcome in humans infected with *C. jejuni* infected, because mimicry of human ganglioside, GM1, which is variable due to polyC/G mutation (116), appears to play a significant role in the development of Guillain-Barré syndrome (199). Moreover, phase variation of LOS structure has been observed during *C. jejuni* infection of human volunteers (151).

The suite of polyC/G tract containing contingency loci varies between *C. jejuni* strains. This is not surprising considering most contingency loci are located in regions of the genome that are genetically divergent for different strains (40). Of 14 genomes deposited to NCBI, the number of polyC/G tracts over 7 bases in length ranges from 11 in strain M1, to 29 in strain NCTC11168 (ATCC700819). Strains that cluster by gene content also appear have a similar combination of contingency loci (JP Jerome, unpublished data). Interestingly, there are examples of strain-to-strain sequence variations in polyC/G tracts between homologous loci that are predicted to alter mutability. For example, homologs in different strains may have an interrupted polyC/G tract by the insertion of a non-C/G base, or a shortened homopolymeric tract length to below 7 bases. It may be hypothesized that selection for, or against, mutability during the recent evolutionary history of a given *C. jejuni* strain has resulted in these changes, but extensive phylogenetic and comparative genomic analyses considering contingency loci have yet to be performed.

Whole genome sequencing of *C. jejuni* reveals less indel mutations in polyA/T tracts in comparison to the polyC/G mutations of contingency loci (148). Nonetheless, there are numerous accounts of indel mutations in polyA/T DNA in the *C. jejuni* genome (72, 75, 90, 93, 190). These observations are anecdotal evidence that the rate of indel mutation in polyA/T DNA is somewhere between the relatively low frequency of point mutations (54, 71), and the high frequency of polyC/G indel mutations, but the exact rate of polyA/T tract mutation has not been determined experimentally.

The difference in mutational diversity in polyA/T tracts and polyC/G tracts of the same length within a population of *C. jejuni* cells distinguishes these two types of

homopolymeric tracts, but the molecular mechanism for this difference in mutability is unknown. Also, the distribution of polyA/T tracts on the genome of *C. jejuni* suggests they are an incidental source of mutational diversity. This is because polyA/T tracts are present in nearly every predicted *C. jejuni* open reading frame, including those of essential genes in which phenotypic switching would be strongly selected against. Because of their distribution and presumably lower mutation rate, these tracts do not define contingency loci in the *C. jejuni* genome, as do polyC/G DNA tracts. However, we speculate that since indel mutations will most often lead to the loss of gene function, a slightly increased polyA/T mutation rate in the *C. jejuni* genome might allow efficient and rapid exploration of a novel fitness landscape through reversible inactivation of gene functions. This appears to be the case when flagellar motility is selected against during experimental laboratory adaptation of *C. jejuni* (Chapter 3).

Multiple researchers have observed the loss of flagellar motility by indel mutations in polyA/T DNA tracts. Interruptions by indel mutation in the protein coding sequence of flagellar biosynthetic genes, *flgS*, *flgR*, *flhA*, *flhB*, *fliP*, *pflA*, and *fliR*, have been reported (72, 75). Loss of many of these proteins disrupts the flagellar biosynthesis transcriptional cascade, resulting in an aflagellated phenotype (76). The biological significance of reversible flagella expression is unknown. As described above, polyA/T DNA is ubiquitous in the *C. jejuni* genome, so why do we observe so many polyA/T indel mutations in flagellar genes? It does not appear that there is an increased occurrence of polyA/T tracts in flagellar genes (JP Jerome, unpublished data), and in any case, a single mutable tract is all that is necessary to reversibly inactivate a gene. It may be possible that the sequence context of polyA/T DNA affects mutability, so that

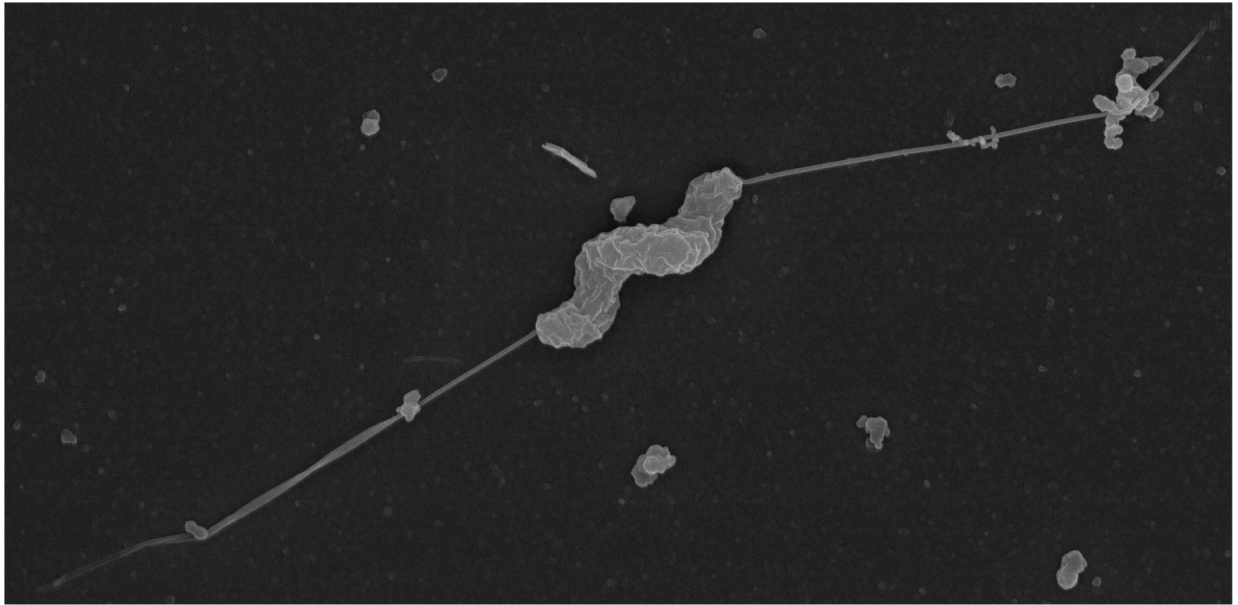
only certain tracts, such as those in flagellar biosynthesis genes in which mutations have been observed, have increased mutation rates. However, experimental evidence shows that loss of flagellar biosynthesis increases *C. jejuni* fitness in laboratory culture (21). This finding, and the ease with which motility deficient mutants are observed in the laboratory, suggests that lab-based observations of motility phase variation by polyA/T mutation are biased. Therefore, it is likely that polyA/T indel mutations occur across the entire genome in a replicating population of *C. jejuni*, but are not observed unless they are beneficial and rise in frequency, such as is the case for flagellar mutants in lab culture (Chapter 3).

Work with reversible motility mutants has shown that indel mutations in polyA/T tracts are phase variable in *C. jejuni* (72, 75). Motility can be restored in non-motile mutants containing an ORF-disrupting polyA/T tract indel by selection in the laboratory, or during host colonization, that results in the restoration of the ORF (75). When analyzing motile revertants from an ancestral strain containing mutations in relatively long *flgS* polyA/T tracts (6 or 7 bases), Hendrixson observed restoration of the functional *flgS* allele through true phase variation in the affected long polyA/T tracts (75). However, restoration of *flgS* from ancestral strains with a deletion mutation in a short polyA/T tract (4 bases), or deletion of one unit of a pentanucleotide duplication, resulted in *flgS* restoration by various mutational events unrelated to polyA/T tract mutability. Thus true phase variation was observed during restoration from long polyA/T tract lesions, but not from short tract, or heteropolymeric, DNA lesions. This is consistent with the following conclusions: polyA/T DNA mutations in *C. jejuni* are reversible; they occur at a higher rate than other mutation events; and that the rate of

polyA/T mutation is affected by tract length, as for other SSRs including *C. jejuni* polyC/G tracts (11, 33, 156).

Diversity in contingency loci expression through polyC/G mutation in an otherwise clonal *Campylobacter jejuni* population appears to be the rule, not the exception. Multiple researchers have shown that *C. jejuni* cells harvested from an agar plate are diverse in genotype and phenotype, and although there are changes in the frequency of contingency loci genotypes, diversity is not lost during infection of chickens or mice (93, 103, 193), or by extended culture in the laboratory (JP Jerome, unpublished data). Since relatively large numbers of *C. jejuni* colonize and replicate within the ceca of chickens (12, 77), or mice (Chapter 3), the high switching rates of polyC/G tracts are expected to generate genetic diversity in contingency loci during host colonization. Moreover, this diversity creates the potential for individual *C. jejuni* cells to express distinct sets of proteins relative to their immediate ancestors. Therefore, when unpredictable environmental stresses occur, subpopulations of cells with higher fitness may already be present in the *C. jejuni* population, and emerge when under selective pressure.

Figure 1.1 *Campylobacter jejuni* NCTC11168 (ATCC 700819) cell. Scanning electron micrograph showing the spiral shape and polar flagella of *C. jejuni*. Image taken at the Center for Advanced Microscopy at Michigan State University, by J.P. Jerome.



CHAPTER 2

Standing genetic variation in contingency loci drives the rapid adaptation of *Campylobacter jejuni* to a novel host

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ABSTRACT

The genome of the food-borne pathogen *Campylobacter jejuni* contains multiple highly mutable sites, or contingency loci. It has been suggested that standing variation at these loci is a mechanism for rapid adaptation to a novel environment, but this phenomenon has not been shown experimentally. In previous work we showed that the virulence of *C. jejuni* NCTC11168 increased after serial passage through a C57BL/6 IL-10^{-/-} mouse model of campylobacteriosis. Here we sought to determine the genetic basis of this adaptation during passage. Re-sequencing of the 1.64Mb genome to 200-500X coverage allowed us to define variation in 23 contingency loci to an unprecedented depth both before and after *in vivo* adaptation. Mutations in the mouse-adapted *C. jejuni* were largely restricted to the homopolymeric tracts of thirteen contingency loci. These changes cause significant alterations in open reading frames of genes in surface structure biosynthesis loci and in genes with only putative functions. Several loci with open reading frame changes also had altered transcript abundance. The increase in specific phases of contingency loci during *in vivo* passage of *C. jejuni*, coupled with the observed virulence increase and the lack of other types of genetic changes, is the first experimental evidence that these variable regions play a significant role in *C. jejuni* adaptation and virulence in a novel host.

INTRODUCTION

Campylobacter jejuni is a leading cause of bacterial gastroenteritis in humans and is a common antecedent infection to the acute flaccid paralysis, Guillain-Barré syndrome (20, 181, 184). It is considered a commensal of avian species, and most human infections likely arise from contact with raw chicken meat (88). However, *C. jejuni* has a large natural host range including cows, pigs, dogs, cats, migratory birds, and insects (4, 24, 51, 132, 146). Despite the ability to colonize a diverse range of hosts there has been a lack of small animal models for study of human disease, and little understanding of the molecular basis of *C. jejuni* virulence.

When the genome of *C. jejuni* NCTC11168 was sequenced in 2000 it was found to contain multiple hypervariable regions, or contingency loci, that could not be resolved to a consensus sequence (148). Contingency loci are important for virulence properties in other pathogenic bacteria, including *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *Helicobacter pylori* (95, 172, 179). In *C. jejuni* these regions consist of homopolymeric tracts of nucleotides that are prone to slipped-strand mispairing during replication (113). This leads to a high rate of indel mutation that can change the open reading frame of a gene. These mutations are a mechanism for phase variation, as they often control ON/OFF phenotypic switches. Contingency loci are thought to be a bet-hedging strategy in case of exposure to novel situations, since cells derived from a single ancestral cell can have numerous heritable phenotypes (135). To test whether these genes are important for rapid adaptation in *C. jejuni*, Wassenaar *et al.* attempted to determine patterns of variation in six contingency loci during experimental adaptation (187). In response to heat and cold stress, passage through tissue culture cells, and

passage through the chicken gut, the distribution of variation across the six sites under investigation was stable; providing no evidence for the role of contingency genes in adaptation to a novel environment. However, it is possible that contingency genes not investigated had mutated or that the conditions tested were not selective for specific contingency gene phases.

Serial passage experiments are a method to increase pathogen virulence by adaptation to a specific host (41). This experimental evolution involves host infection, followed by re-isolation of the pathogen, and then inoculation of the re-isolated pathogen into a new host. Serial passage of viral, bacterial, and eukaryotic pathogens almost exclusively results in increased fitness, virulence, and growth rate in the host, and decreased virulence to a former host (41, 169). Comparative sequence analysis of the adaptation that occurs in such experiments has only been performed for viruses. For example, Brown *et al.* re-sequenced the viral genome of influenza A after experimentally increasing virulence in the mouse lung (17). In this case, serial passage followed by genome re-sequencing led to the discovery of multiple novel virulence modulators. Studies like this show that serial passage leading to increased virulence followed by the analysis of genetic mutations can be used as a forward genetic screen for virulence factors.

In vivo serial passage of *C. jejuni* alters virulence. Jones *et al.* showed that passage of a poorly motile variant of *C. jejuni* through chickens resulted in restored motility and increased ability to colonize and persist in the avian gastrointestinal tract (94). A reduction in the minimum infectious dose of *C. jejuni* has also been reported as a result of passage through chickens (158). Prior work described by Bell *et al.* showed

that when *C. jejuni* was serially passaged through the cecum of C57BL/6 IL-10^{-/-} mice, the virulence of mouse-adapted bacteria increased (13). Higher measures of pathology that included the presence of blood in the cecal lumen, decreased timing of the onset of disease, and higher fecal and jejunal *C. jejuni* population sizes indicated that the mouse-adapted bacterial population was more virulent than the ancestral *C. jejuni*. Increased virulence following serial passage was observed for three of five *C. jejuni* strains tested, and higher virulence could often be observed in bacteria re-isolated after a single 35-day *in vivo* passage. These results suggest that adaptation occurs rapidly during the course of *C. jejuni* infection.

Our objective in this study was to test the hypothesis that specific genetic changes in *C. jejuni* control adaptation to a novel host. The capacity to increase virulence by *in vivo* passage is well documented, and uncovering mutations on the genome-scale is now feasible using new sequencing technologies (10, 78, 170, 180). We used Illumina sequencing to re-sequence the genome of *C. jejuni* NCTC11168 before (referred to as wild-type) and after mouse serial passage (referred to as mouse-adapted), as well as expression microarrays, pulse-field gel electrophoresis, and phenotypic assays to determine the basis of *C. jejuni* host adaptation during passage. We found that variation present in contingency loci in the ancestral inoculum is driving the adaptation of *C. jejuni* during *in vivo* serial passage. Deep re-sequencing showed that the frequencies of specific phases at contingency loci changed during passage. Furthermore, several contingency loci with sequence changes also had altered transcript abundance based on microarray and real-time qRT-PCR. Many contingency genes that mutated during passage are putatively involved in surface carbohydrate

biosynthesis, and as expected, phenotypic assays that are affected by surface structure changes in *C. jejuni* revealed differences between the mouse-adapted and wild-type variants.

RESULTS

Deep re-sequencing of contingency loci variation

We suspected that variations in the 8-13 base homopolymeric tracts of contingency loci would play a role in *C. jejuni* adaptation during serial passage. However, the utility of next-generation sequencing technologies in evaluating variations in contingency loci had not been reported. We chose to re-sequence the NCTC11168 genome using Illumina short-read technology for two reasons. First, this platform provides very high coverage, which is necessary to observe frequencies of indels in homopolymeric tracts. Second, in contrast to pyrosequencing, Illumina sequencing is not known to suffer from a higher than average error rate for indels in homopolymeric DNA. One channel of an Illumina Genome Analyzer II flow cell was used per variant to generate sequence coverage of approximately 200-500X across the genome. Manual curation of reads mapping to homopolymeric tracts yielded an average coverage of 107X per tract, with a range of 42-252X, for reads that unambiguously defined the number of bases within the homopolymeric tract. Additionally, the estimated error rate for indel mutations in this data set was lower than the estimated base substitution error rate (Figure 2.1), as has been shown by others (39), suggesting that indel mutation analysis is more robust to sequencing errors than SNP discovery. When we characterized homopolymeric tracts by colony PCR and Sanger sequencing, the results were consistent with the Illumina short read sequence data, further verifying the use of this technology for re-sequencing the variable tracts of contingency loci in *C. jejuni*.

Variations in contingency genes after *in vivo* passage

Twenty-three variable homopolymeric tracts, and nine potentially variable tracts, were reported when the NCTC11168 genome was sequenced in 2000 (148). In our re-sequencing analysis 28 homopolymeric tracts were variable. We found no indel mutations for *Cj1367c*, containing a reportedly variable two base guanine tract, and *Cj1677*, with a 7 base thymine tract. *Cj0628* contains two adjacent homopolymeric tracts, and both were variable. The homopolymeric thymine tract in *Cj0628* was the only variable thymine tract found, despite the presence of numerous long thymine tracts in the AT-rich *C. jejuni* genome. Because sequences surrounding six tracts (*Cj1305c*, *Cj1310c*, *Cj1318*, *Cj1335*, *Cj1421c*, and *Cj1422c*) match multiple sites in the genome, short sequence reads could not be unambiguously mapped to these loci. These tracts were defined by colony PCR and Sanger sequencing as described in the Methods section. Every homopolymeric tract containing at least 8 guanines or cytosines in the NCTC11168 genome was variable with the exception of the 9 base tract in *Cj1310c*. However, this tract only had coverage of 10X by colony PCR and Sanger sequencing. Variations in the entire suite of NCTC11168 contingency loci before and after mouse-adaptation are shown in Figure 2.2.

For several contingency loci a specific phase increased in frequency during passage. As an example, in *Cj1296*, 3.6% of sequence reads had 10 guanine residues before passage, and after passage 75.5% of reads showed 10 guanines. Insertion of a guanine at this site creates a fusion of open reading frames *Cj1296* and *Cj1297*. For other contingency loci such as *Cj1139c* and *Cj1420c* the distribution of indel variants was stable through passage. Overall, chi-square tests of association revealed significant differences at thirteen homopolymeric tracts as a result of mouse adaptation

(Table 2.1). All of these differences are associated with open reading frame changes, except for the tract at *Cj0565*, which is upstream of the potential ORF. Both positive and negative in-frame ORF fold changes can be seen in Table 2.1. Therefore, the homopolymeric tract length corresponding to the in-frame ORF was enriched due to mouse adaptation for some contingency loci (*Cj0031*, *Cj0046*, *Cj0676*, *Cj1295*, *Cj1296/Cj1297*, *Cj1325*, and *Cj1429c*), while for others there appears to be selection for an ORF with a premature stop codon.

Five significant changes in contingency genes were in the flagellar glycosylation locus, and there was a single change in each of the lipooligosaccharide (*Cj1145c*) and capsule biosynthesis (*Cj1429c*) loci. Several contingency genes annotated as pseudogenes (*Cj0046*, *Cj0565*, *Cj0676*) and a conserved hypothetical protein (*Cj0170*) also changed significantly. Altered open reading frames of contingency genes annotated as a restriction/modification enzyme (*Cj0031*) and an iron-binding protein (*Cj0045c*) were also enriched after passage.

Parallel changes observed in contingency loci during mouse infection

The results presented thus far compared two time points during the serial passage experiment: unpassaged (wild-type) and mouse passaged (mouse-adapted) *C. jejuni*. From this we showed significant changes in the frequency of the ON/OFF status of 13 contingency loci, and the absence of other DNA mutation (93). As described by Bell *et al.* (13), the mouse-adapted variant we analyzed was passaged through 3 sets (3 passages) of 5 mice each, and the re-isolates were pooled from each mouse to make the inoculum for each subsequent passage.

To determine whether the same genes changed significantly in each individual mouse during infection, we first analyzed 4 contingency loci (*Cj0170*, *Cj1429*, *Cj1295*, and *Cj1296*) by direct PCR analysis of *C. jejuni* populations from 4 infected mice (Figure 2.5). For each gene, there was a statistically significant shift in the distribution of tract lengths for at least 3 of the 4 mice (Table 2.8). For *Cj0170*, *Cj1429*, *Cj1295*, and *Cj1296*, the length of the tract that increased in frequency relative to the inoculum was 9, 10, 9, and 10, respectively, which corresponds to the OFF phase for *Cj0170*, and the ON phase for *Cj1429*, *Cj1295*, and *Cj1296*. The ON versus OFF phases that increased in frequency by this analysis were the same phases that were enriched after 3 serial passages (93).

The tract length distribution was not significantly altered compared to the inoculum for genes *Cj1295*, *Cj1296*, and *Cj1429* in 1 of the 4 mice. However, the mouse for which a shift in frequency did not occur was different for each gene. For example, the frequency of tract lengths present in the *C. jejuni* population infecting Mouse 1166 was not significantly different from the inoculum for gene *Cj1296*. However, the population in Mouse 1166 was significantly different from the inoculum for loci *Cj0170*, *Cj1295*, and *Cj1429*. Analogous comparisons can be made for other genes, in 1 of the 4 mice analyzed.

Overall, this analysis indicated that there were parallel changes in the analyzed contingency loci in 4 independently evolving *in vivo* populations. However, there was also variation in each population such that the distribution of lengths in a given gene never shifted to the same degree in all mice.

Significant shifts in genotype of *in vivo* *C. jejuni* populations relative to the inoculum

Contingency loci genotypes present in a given *C. jejuni* population were estimated from the genome sequence reads by converting the ON/OFF status of six loci (*Cj1326*, *Cj0031*, *Cj1139*, *Cj0685*, *Cj0045* and *capA* (*Cj0685c*)) to a binary format (1 for ON phenotypic phase; 0 for OFF phenotypic phase) (11). The resulting distributions of genotypes are plotted in Figure 2.6. There was a significant difference in the distribution of genotypes present for 3 of the 4 *in vivo* derived *C. jejuni* populations compared to the inoculum by Kolmogorov-Smirnov test (K-S test) for differences in genotype distributions ($P < 0.005$ for Mice 1141, 1166, and 1233) (Figure 2.6). The genotype distribution from reisolated *C. jejuni* colonizing Mouse 1067 was not significantly different from the inoculum ($P = 0.929$, by the K-S test) considering these 6 genes. The significant difference in genotypes observed for Mice 1141, 1166 and 1233 was mainly driven by increases in the genotypes 1-1-1-0-0-0 and 0-1-1-0-0-0 that were present at low frequency in the inoculum (2.5% and 3.7%, respectively), but rose to high frequency in parallel for 3 of the 4 mice (10.9%, 13.3%, and 49.6% for 1-1-1-0-0-0; 49.0%, 62.7%, and 25.6% for 0-1-1-0-0-0, in Mice 1141, 1166, and 1233, respectively). These genotypes resulted from an increase in the observed frequency of the ON state for genes *Cj1325*, *Cj0031* and *Cj1139* in mouse infecting *C. jejuni* populations.

Concomitant to the parallel frequency spike of specific genotypes due to mouse passage was a decrease in the frequency of multiple genotypes present in the inoculum. The inoculum was predicted to contain 14 genotypes with a frequency greater than 2%. However, after mouse passage there were 11, 7, 5, and 7 genotypes

predicted to occur at a frequency of 2% or more in the populations derived from Mice 1067, 1141, 1166, and 1233, respectively. This suggests a general trend toward the loss of genotypic diversity during mouse passage when considering these six genes.

Modeling of contingency loci mutability does not result in the genotypes observed during mouse infection

We have shown that homopolymeric tract lengths, and concurrently, estimated contingency loci genotypes, change in frequency during mouse infection. The observed changes generally occurred in parallel for 3 of 4 independently derived populations (independent replicates of mice). This suggests that selective pressure for specific contingency loci phases is occurring *in vivo*. However, multiple evolutionary forces may affect homopolymeric DNA tract length changes in *C. jejuni* contingency loci during mouse infection.

Even in the absence of selective pressure on contingency gene products, frequency shifts in ON/OFF status may occur due to the high switching rates and mutational patterns that have been described for *C. jejuni* polyC/G DNA (11). In order to determine whether mutation rates and patterns alone may account for the contingency gene changes we observed during mouse serial passage, we estimated the genotypes predicted by a published stochastic mathematical model of contingency loci mutations (11). Genotypes observed from the 3 mice with significantly different genotype distributions relative to unpassaged *C. jejuni*, were significantly different from the distributions predicted by genetic drift of the inoculum by this model, no matter the number of divisions input into the model ($P < 0.01$, K-S test) (Figure 2.6).

Since the difference in genotypes observed in the mouse-derived *C. jejuni* populations appeared to be driven mainly by the increased frequency of the ON phase for genes *Cj1325*, *Cj0031* and *Cj1139*, we increased the mutation rate input into the model by ten times for these genes. Still, the model predicted genotypes did not reproduce the experimentally observed distributions in the 3 genotypically altered mouse-adapted populations. Taken together, the stochastic drift model driven by the mutability of homopolymeric DNA in contingency loci could not produce the observed changes resulting from mouse passage. This strongly suggests that other evolutionary forces, such as selective pressure on contingency loci phase, are driving the observed genotypic changes during mouse colonization.

Mutations outside of contingency genes

Single-nucleotide polymorphisms (SNPs) along with duplications, insertions, and other polymorphisms (DIPs) have been reported as the dominant genetic basis of adaptation in evolution experiments with microbes (10, 17, 180). We analyzed the Illumina short reads for the presence of SNPs and DIPs. There was no evidence of DIPs, and only one SNP was found to change in frequency by more than 20% during passage. This is a nonsynonymous mutation that changes a valine to alanine in *pbpC* (V30A), and was confirmed by Sanger sequencing. This mutation arose during passage and increased in frequency to approximately 62% after three *in vivo* passages. Six other SNPs were predicted to have changed in frequency during passage by at least 5%, but less than 16% (Table 2.2). The absence of fixed SNPs and DIPs during passage was not a result of the sequencing methods or analysis, as multiple fixed SNPs and DIPs

were found in the genome of the NCTC11168 (ATCC 700819) culture we used compared to the NCTC11168 that was sequenced in 2000 (Table 2.3). Differences in phenotype between NCTC11168 isolates have been described (55), so genetic differences in our stock culture were not unexpected, and will be informative for future work.

Absence of large genomic rearrangements

Researchers have described large intra-genomic inversions in *C. jejuni* during avian colonization (157, 186) and in particular, in response to bacteriophage predation (166). Since certain large genomic rearrangements are difficult or impossible to discover using short read re-sequencing technology, we performed pulse-field gel electrophoresis (PFGE) on the wild-type and mouse-adapted *C. jejuni*. PFGE showed no evidence of genome rearrangements in mouse-adapted *C. jejuni* (Figure 2.3).

***In vitro* gene expression: wild-type versus mouse-adapted variants**

Expression microarrays were used to determine whether adaptation to increased virulence had a basis in transcriptional regulation. From direct comparisons of RNA isolated during growth *in vitro*, nine ORFs were found to have significantly altered expression in the mouse-adapted bacteria relative to wild-type (Table 2.4). Of these, the five ORFs with the highest fold changes in expression were associated with contingency loci (*Cj0170*, *Cj0565*, *Cj1295*, *Cj1296*, and *Cj1297*). As shown above, these loci also had significant changes in homopolymeric tract length. The homopolymeric tract is located immediately upstream of the ORF predicted for *Cj0565*, but the tract is within

the predicted ORFs of *Cj0170*, *Cj1295*, and *Cj1296*. Alteration in transcript abundance was verified for four of these ORFs by real-time qRT-PCR (Table 2.4). Changes observed by microarray could not be verified for *Cj1190c* and *Cj1381*, which are not contingency loci. Notably, the five contingency loci with altered expression after three passages in mice also showed significant changes by microarray in *C. jejuni* isolated after a single passage (data not shown).

***In vivo* gene expression: wild-type versus mouse-adapted variants**

Since many *C. jejuni* genes are specifically regulated *in vivo* (177), we sought to compare transcript abundance of wild-type and mouse-adapted bacteria during mouse infection. To avoid isolation of RNA from commensal microflora, C57BL/6 IL-10^{-/-} germ-free mice were infected with wild-type or mouse-adapted *C. jejuni*, and a protocol to isolate intact, bacterial RNA, free of eukaryotic-RNA contamination, from the cecum was developed (Methods). Four germ-free mice were inoculated with each variant and infection was allowed to proceed for four days. In this short infection period, both variants caused only mild pathology which is consistent with the results of acute *C. jejuni* infection of specific-pathogen-free mice (122), and germ-free mice (118). At necropsy all mice were colonized only with *C. jejuni*. RNA was obtained from cecal samples and used to directly compare wild-type and mouse-adapted gene expression during *in vivo* growth. This comparison yielded nine significant expression differences between variants (Table 2.5). Contingency loci *Cj0170*, *Cj0565*, and *Cj1297* were among those with significant transcript abundance changes at $p < 0.05$, while *Cj1296* was significant at $p < 0.10$ by microarray. As described above, these contingency genes

also had altered expression *in vitro* and the transcript abundance differences were confirmed by real-time qRT-PCR. However, gene expression differences could not be verified by real-time qRT-PCR for *Cj1506c*, *Cj1523c*, *Cj0144*, and *frdC*, none of which are contingency loci.

***In vitro* phenotypes of *C. jejuni* variants**

The majority of contingency genes are coincident on the genome with surface carbohydrate biosynthetic loci (148), and seven of the thirteen significant contingency gene changes in mouse-adapted bacteria were in these loci. Genes in the flagellar glycosylation locus are known to affect motility and autoagglutination (66), and lipooligosaccharide and capsular biosynthesis genes are known to affect the interaction of *C. jejuni* with epithelial cells in culture (7, 68). We investigated possible changes in these phenotypes in mouse-adapted *C. jejuni*.

It has been reported that non-motile and non-autoagglutinating *C. jejuni* mutants are attenuated (129), and we hypothesized that these phenotypes may have changed during passage in mice. Spreading on soft agar plates was equal in the wild-type and mouse-adapted variants, indicating that both *C. jejuni* were fully motile (Figure 2.4a). Also, darting motility was seen in both variants when observed in wet mounts by bright field microscopy. However, the ability to autoagglutinate was slightly decreased in mouse-adapted cells (Figure 2.4b). Although the change was small, it was statistically significant and reproducible.

Adherence to, and invasion of, epithelial cells is likely important for *C. jejuni* pathogenesis. Thus, we hypothesized that adherence and invasion would be increased

in *C. jejuni* after mouse adaptation. Young adult mouse colon (YAMC) epithelial cells were used to compare the ability of *C. jejuni* variants to infect epithelial cells in culture. Both variants were able to adhere to, and invade, YAMC cells. However, wild-type *C. jejuni* associated with YAMC epithelial cells approximately 5 times more than mouse-adapted bacteria (Table 2.6). Invasion of wild-type *C. jejuni* into YAMC cells was also higher than the mouse-adapted variant by approximately 10 times. Invasion was assayed using *C. jejuni* that had been passaged 1 or 3 times. The data presented in Table 2.6 are from *C. jejuni* passaged a single time, indicating that this change occurred rapidly. The adherence and invasion results from this *in vitro* study would suggest the mouse-adapted variant is less virulent. However, as described in the Introduction, the mouse-adapted *C. jejuni* was more virulent than wild-type *in vivo* in C57BL/6 IL-10^{-/-} mice (13).

DISCUSSION

Bacteria generally respond to their surroundings by changes in gene activity through regulation at the level of transcription, translation, and/or posttranslation. However, genotypic variation in a closely related population of bacterial cells provides an alternative method for immediate adaptation to novel environmental conditions. Pathogenic bacteria are faced with novel, changing circumstances during the course of infection as they pass through different host microenvironments, and are met with an active immune response. It has been argued that passage through dynamic environments between and within hosts selects for highly mutable loci, or contingency genes (108, 134). Here we show that all homopolymeric guanine tracts over seven bases long in the *C. jejuni* genome have variable length, and that this variation provides the genetic basis for adaptation during serial passage in a mouse model of campylobacteriosis. Since serial passage also leads to higher bacterial virulence in the model (13), mutability in contingency loci should be considered an integral part of *C. jejuni* pathogenesis.

We present multiple lines of evidence to support the conclusion that adaptive evolution is occurring by selection for existing contingency loci variants during serial passage. First, the frequencies of specific phases change significantly during *in vivo* passage. No open reading frame is completely fixed at any locus, but this is expected due to the high slipped-strand mutation rate reported for these sites (148, 187). However, some contingency genes, namely *Cj0045c*, *Cj0170*, *Cj0565*, *Cj1145c*, *Cj1295*, and *Cj1429c*, appear to be under stabilizing selection for a single phase after passage,

with only small populations of one base insertion and deletion mutants. This suggests that one phase is beneficial, but due to the stochasticity of slipped-strand mutation, a background population of variants is always present. As described in the Methods section, the mouse-adapted *C. jejuni* is actually a pool of re-isolated cells from five infected mice. Therefore, we can assume that the dominant phase in our re-sequence analysis was overrepresented in multiple mice from the previous passage, providing strong evidence of selection for the phase in question. Re-sequencing of re-isolated *C. jejuni* from individual mice in the future could provide insight into how animal-to-animal variation affects *C. jejuni in vivo* adaptation.

Differential transcript abundance of contingency genes due to serial passage provides more evidence for the importance of homopolymeric variation in *C. jejuni* adaptation. Based on *in vitro* and *in vivo* transcript comparisons, the only confirmed differences between wild-type and mouse-adapted variants were in ORFs associated with contingency genes. Additionally, all of the ORFs with differential expression also had altered phase frequencies, strengthening evidence that these gene phases are being selected *in vivo*. Interestingly, transcript abundance is decreased for *Cj0170*, which is mainly in a truncated, likely “OFF,” frameshifted phase after passage, while transcript abundance is increased for *Cj1295* and *Cj1296/7*, which are in extended, likely “ON,” in-frame phases after passage. It is possible that ribosome-coated, in-frame contingency gene transcripts have greater stability *in vivo*, and thus a longer half-life (35). Also, rho-dependent transcript termination may occur during ribosome pausing when a premature stop codon is reached for frameshifted contingency genes (109). Alternatively, it is possible that one or more contingency genes may be directly

influencing transcription of other contingency loci. In what has been termed the “phasevarion,” phase variable DNA methylation by a type III restriction-modification enzyme has been shown to control transcription of a set of genes (173, 174). Some contingency genes are controlled under the phasevarion in *Haemophilus influenzae*, but many non-contingency genes are also part of this regulon (174). It may be that the observed increase in the extended form of *Cj0031*, encoding a putative type IIS restriction-modification enzyme, is driving transcriptional changes in a fraction of the cell population in mouse-adapted *C. jejuni*. The mechanism of differential transcript abundance in contingency genes is under investigation in our laboratory.

Finally, our conclusion that contingency genes provide the genetic basis for adaptive microevolution during serial passage is supported by the lack of other genetic changes in the genome of mouse-adapted *C. jejuni*. No SNP or DIP reached fixation after three rounds of serial passage in the mouse model, and there were no large rearrangements detectable by PFGE. The SNPs that were detectable below the level of fixation, such as the one that arose in *pbpC*, may be beneficial mutations, but are not likely driving the increased virulence that is observed. If these SNPs were the cause of increased virulence, we would expect them to become fixed after multiple rounds of passage. Also, increased virulence after a single passage is consistent with our conclusion that beneficial mutations at contingency loci are present in the original inoculum. Still, the SNPs described may be beneficial mutations that we could expect to become fixed in the population after more rounds of passage. Such a complete genome analysis is only possible through re-sequencing, and in particular, through the next-generation sequencing technologies that make comparative genome sequencing

feasible. Traditional sequencing and genome assembly, or next-generation sequencing to low coverage, would not have detected any difference between wild-type and mouse-adapted *C. jejuni* due to insufficient coverage across homopolymeric tracts.

Although we have implicated thirteen contingency loci in host adaptation and enhanced virulence, the functions during pathogenesis of these altered loci are not well established. Three significant changes were in putative pseudogenes, but for *Cj0046* and *Cj0676*, passage results in an extended ORF. It is possible that this extension results in some gene product activity. Of the thirteen gene changes, only *Cj1295* has been functionally characterized (81). The *Cj1295* gene product has been associated with a pseudaminic acid modification that is part of the *C. jejuni* O-linked flagellar glycan profile, but a role in virulence or host adaptation has not been previously described. In addition to *Cj1295*, four other putative flagellar glycosylation genes were significantly altered in mouse-adapted *C. jejuni*, suggesting flagellar modifications are important for host adaptation and enhanced virulence. Apart from flagellar glycosylation changes, putative carbohydrate-modifying contingency genes in the capsule locus and lipooligosaccharide (LOS) locus also changed significantly. Because flagella and surface structures are known stimulators of innate and adaptive immunity, we speculate that these changes may decrease recognition by host cell surface and cytoplasmic receptors allowing expansion of the *C. jejuni* population. In particular, the large decrease (-7.6 fold) of in-frame *Cj1145c*, a homolog to an LOS glycosyltransferase in *C. jejuni* LIO87 (83), may be due to immune system recognition and clearance of bacterial cells expressing the in-frame, active protein product of *Cj1145c*.

A surprising finding in this study was that the mouse-adapted bacteria appeared to be less virulent by *in vitro* assays. Mouse-adapted *C. jejuni* was less able to autoagglutinate than wild-type, but this was a very small difference. When Guerry *et al.*, correlated autoagglutination and virulence, they compared strains that showed large differences in the ability to autoagglutinate (66). The small difference observed in this work is likely related to subtle alterations in surface carbohydrate modifications that are affected by the phase variable changes we observed by re-sequencing. The decreased ability of mouse-adapted *C. jejuni* to adhere and invade in cultured cells was unexpected since the ability to interact with gastrointestinal tract epithelial cells *in vivo* must be important for *C. jejuni* host colonization (197). However, discordance between *in vitro* adherence and invasion results and *in vivo* virulence measures has been described before (110). Also, screens using epithelial cell culture models have been unable to discover significant invasion factors, outside of genes affecting motility. Furthermore, it is known that different cell lines often produce mixed results (143). It is likely that the inability to mimic complex host physiologic factors in the limited environment of the culture dish makes *in vitro* models inappropriate for studying some aspects of the molecular basis of *C. jejuni* pathogenesis.

Based on homopolymeric hypervariation, Parkhill *et al.* suggested that *C. jejuni* has quasispecies properties similar to RNA viruses such as human immunodeficiency and hepatitis C virus (82, 124, 148). Our analysis shows that *C. jejuni* exists as a population of genotypes within a host, which is a main property of quasispecies. However, another feature of the quasispecies concept is that selection is acting on the population and not individual variants. From our data it can be seen that some

contingency loci phases sweep to near fixation during passage, while others are stable as a mixed population. If selection was acting on individuals within the population, all contingency genes should be nearly fixed to one phase since they would have been selected, or hitchhiking, in the most fit genome. In our analysis it appears that selection at one locus does not have any affect on the distribution of mutations at other loci. However, this may be a consequence of the high mutation rate at homopolymeric tracts leading to the re-diversification of tract length distributions at contingency loci under no selective pressure. Also, it is possible that multiple *in vivo* microenvironments, or a variable host immune response, exert dynamic selective pressures on variants within the population to maintain genome diversity in the *in vivo* population. Based on our data and the work of Parkhill *et al.* (148), researchers should consider *C. jejuni* as a population of genotypes and phenotypes, and not a clonal isolate.

Generation of genetic diversity during *in vivo* growth has been described in *C. jejuni* (157, 166, 186, 193), but these studies mainly focused on *C. jejuni* infection of chickens. In regards to homopolymeric tract variation, Wilson *et al.* suggested that generation of genetic diversity occurs in specific, permissive environments such as the avian intestine where *C. jejuni* is a commensal organism (193). Generation of diversity in avian species may be advantageous for transmission to novel hosts, such as different mammalian species, where diversity is lost due to stabilizing selection. In our re-sequencing study, some contingency genes had a specific phase enriched after passage, concomitant with a loss of diversity. However, in this experiment contingency gene phases are never lost from the population and sixteen genes maintained a stable distribution of variants after passage through mice. Also, the generation of

homopolymeric diversity implies there are no restrictions on the mutations that arise and are maintained in these tracts. However, as suggested by Wassenaar *et al.*, some tract length frequency distributions do not appear to be stochastically maintained since the frequency of one frameshifted length is favored (187). If a purely stochastic, slipped-strand mutational process was driving this distribution we would expect to see no bias between the insertion and deletion frameshift mutations since they both result in the same prematurely stopped protein product. As an example, for *Cj0685c*, only two homopolymeric tract lengths are observed in our analysis (in-frame and one base deletion frameshift), and this bi-allelic distribution is indistinguishable before and after mouse-adaptation. Here we show that a subset of contingency loci had changes in the frequency of homopolymeric tract lengths due to mouse passage, and we suggest that similar changes would occur in some contingency loci during avian passage.

The genotypic analysis we performed was based upon work by Bayliss *et al.* (11). Their work showed that the contingency loci genotypes derived from six genes were significantly altered during chicken passage for *C. jejuni* strains NCTC11168 and 81-176, and that laboratory culture did not result in significant genotypic changes. Also, it was shown that mutability (rates and patterns) of homopolymeric DNA in contingency loci alone can result in significant changes in genotype in a replicating *C. jejuni* population. In our work, mutability alone does not explain the genotypic changes in contingency loci that we observed as a result of mouse infection. Also, parallel changes in specific contingency loci, and in contingency loci genotype, were observed when multiple mice were analyzed. This strongly suggests that selection is acting on contingency loci during mouse infection, as has been suggested to occur during chicken

infection (11). However, our observed changes might be the result of a cellular bottleneck during initial colonization of *C. jejuni* upon mouse inoculation, or due to genetic hitchhiking of specific contingency locus homopolymeric tract lengths and genotypes, in a genome with an unrelated beneficial mutation. A random bottleneck effect would not result in the observed parallelism in our data considering multiple mice. Also, no mutations outside of contingency were discovered by our extensive genetic analysis. Therefore, selection on contingency loci is likely the main driver of the observed contingency loci changes. Although, we cannot with the data presented distinguish the contingency genes that are more or less important in the mouse model, since multiple changes were observed. Also, considering the number of mutable loci in the genome, hitchhiking of one contingency locus phase on other more important contingency loci changes, along with possible epistatic effects, will make determining the importance of specific contingency loci *in vivo* a difficult task.

As noted by Moxon *et al.*, contingency gene switching is combinatorial (135). Therefore, the 29 variable regions described in *C. jejuni* may produce at least 2^{29} , or ~536 million genotypes, and a potentially equal number of phenotypes that provide a substrate for rapid adaptation. In this work we show that standing genetic variation in these genes is capable of driving the rapid adaptation of *C. jejuni in vivo*. Aside from contingency gene variations, the mouse-adapted variant has a nearly identical genome to wild-type, but with significantly different virulence properties. Therefore, it is necessary to consider the potential effects of contingency gene variability during *C. jejuni* human clinical trials, molecular epidemiology studies, and when developing successful vaccines and therapies against *Campylobacter jejuni*. Additionally,

contingency loci affecting surface structures could be particularly significant from a clinical perspective when considering that outer membrane changes in *C. jejuni* have the potential to initiate autoimmune disease in the host, such as Guillain-Barré syndrome.

METHODS

Ethics statement

All experiments involving mice were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were reviewed and approved by the University of Michigan Committee on Use and Care of Animals (Application Number: 09116).

Bacteria

C. jejuni NCTC11168 was obtained from the American Type Culture Collection (ATCC 700819), and was streaked to generate the wild-type inoculum for serial passage. The mouse-adapted variant was generated in the serial passage experiments described Bell *et al* (13). Briefly, 5 mice were colonized with wild-type NCTC11168 for 35 days or until clinical signs necessitated early euthanasia. At necropsy, cecal tissue was streaked on selective media to re-isolate *C. jejuni* from all 5 mice. Re-isolations were pooled, frozen, and used to generate the inoculum for the next passage. The mouse-adapted variant that was re-sequenced in this study was the inoculum that was used to infect the final round of mice and had been passaged three times. Variants were generally streak-plate cultured at 37°C on Bolton or Blood agar in vented GasPak jars in a microaerobic environment generated by atmosphere evacuation, followed by equilibration with a gas mixture of 80% N₂, 10% CO₂, and 10% H₂. At most, one subculture was performed prior to genotypic or phenotypic analysis.

Illumina sequencing and SNP analysis

The wild-type and mouse-adapted inocula were streaked onto Tryptic Soy Agar with 5% defibrinated sheep's blood (TSAB) and grown for 48 hours as described above. The entirety of the growth on streaked plates was harvested into phosphate buffered saline and pelleted before DNA extraction using the Qiagen DNeasy blood and tissue kit according to the manufacturer's instructions. Sequencing was carried out at the Michigan State University Research Technology Support Facility (MSU RTSF). DNA was prepared for sequencing using the Illumina Genomic DNA Sample Prep Kit following the manufacturer's instructions. The flow cell was generated via the Single Read Cluster Generation Kit (v. 2) and sequencing carried out on an Illumina GAIIx using the Sequencing Reagent Kit (v. 3). Data was collected with Sequencer Control Software v2.6 and base calling was performed with Real Time Analysis v1.6.

Analysis of SNP mutations in mixed bacterial genotypes was as described by Barrick *et al.* (9, 10). Briefly, reads were mapped to the reference genome (GenBank: AL111168.1) using SSAHA2 (141). The frequencies of all possible base mismatches and single-base indels at read bases with a given quality score were counted at reference genome positions with only uniquely aligned reads to create an empirical error model. Then, the probability of observing the aligned bases and their quality scores at every position with only uniquely aligned reads in the reference genome was calculated according to two models. The first model assumed that the entire population had only one base at this position and that all disagreements were therefore due to sequencing errors. A polymorphism was predicted when the second model that allowed

the population to be an arbitrary mixture of the two most frequent bases at this position explained the data better by a likelihood ratio test ($E\text{-value} \leq 0.01$).

Because this procedure alone has a high false-positive rate due to biases in sequencing errors that are not accounted for by base quality scores, polymorphism predictions had to pass three further tests. First, the distribution of reads with each of the two bases between the two possible genome strands had to be similar by Fisher's exact test ($P\text{-value} \geq 0.01$). Second, the qualities of bases supporting the polymorphism had to not be biased toward lower scores compared to those supporting the reference base by a Kolmogorov-Smirnov test ($P\text{-value} \geq 0.05$). Finally, only polymorphisms where both bases are predicted to be present at frequencies $\geq 5\%$ in the population are reported.

Illumina read data has been deposited into the NCBI short-read archive (SRA023661.1).

Contingency gene variation analysis from Illumina read data

Reads were mapped onto the *C. jejuni* NCTC11168 reference genome (GenBank: AL111168.1) using ZOOM v1.5 Next Generation Sequencing Software (114). Parameters were set to allow at most 2 mismatches per read with at least 8 high quality bases, and an indel length of at most 3 bases. Therefore, if the reference homopolymeric tract contained 9 bases, reads with between 6 and 12 bases in the tract could be mapped. After mapping, manual curation of indel variation in known and suspected variable regions was performed. Reads were considered informative if they satisfied two conditions. First, the read had to extend across the homopolymeric tract

with a consistently high quality score throughout. Second, there had to be at least one high quality base before or after the tract that matched the reference sequence and signaled the end of the homopolymeric tract. Changes in frequencies of observed homopolymeric tract lengths were considered significant at P-value < 0.01 from chi-square tests of association. It was recognized that tracts with different numbers of bases in a given gene could result in the same open reading frame. When this happened, tract lengths that gave the same ORF were grouped for statistical analysis. Where possible, the longest ORF based on the homopolymeric tract lengths observed was considered to be the full-length, in-frame form of the contingency locus. Then, a fold change of the in-frame ORF was calculated by comparing the frequency that the in-frame ORF was observed in the mouse-adapted population relative to the unpassaged wild-type population.

Contingency gene variation analysis by direct PCR length determination

The distributions of homopolymer lengths present in a *C. jejuni* population for a specific contingency locus were estimated by a fragment length (or GeneScan) analysis, essentially as described previously (11, 103, 187). Briefly, primers were designed to amplify between 70 and 150 bases of DNA that contained the homopolymeric tract within a contingency gene. One of the primers was labeled with 6-FAM fluorescent dye (Integrated DNA Technologies, Coralville, Iowa). PCR was performed using PfuTurbo DNA polymerase (Stratagene, La Jolla, California) according to the manufacturer's instructions. Products were cleaned using the Qiaquick PCR clean-up kit (Qiagen) according to the manufacturer's instructions, and approximately 4-

10 ng of product was analyzed on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California) at the MSU RTSF. Peak data generated was analyzed using Peak Scanner Software v1.0 (Applied Biosystems) to determine the lengths of PCR products (peak size) that corresponded to the lengths of the homopolymeric tract plus or minus any base insertion(s) or deletion(s), and the frequency that each length was observed (peak area). This method was consistent with the results of length distributions from the Illumina read data (data not shown).

Genotype analysis

C. jejuni was reisolated from the cecum of 4 mice that had been infected with the wild-type variant for 35 days in a previously published experiment (13). Sequencing was performed as described above at the Michigan State University Research Technology Support Facility (MSU RTSF) with few modifications. Briefly, the Illumina Genome Analyzer IIx was used according to the manufacturer's instruction. Libraries of each sample were prepared with the Illumina TruSeq kit, and samples were given unique multiplex ID tags and pooled for sequencing. Read files were deposited in the Sequence Read Archive at NCBI (Submission Accession Number SRA049039).

All homopolymer repeats with eight or more bases in the reference genome (GenBank: AL111168.1) were analyzed. For each repeat, we counted the number of bases in the homopolymer tract in each read that perfectly matched at least the five adjacent bases on each side of the repeat. Reads were locally re-aligned for this test relative to their original alignments by SSAHA2.

Read counts were used to determine the frequency of the ON and OFF phases of a contingency gene based on the number of homopolymeric cytosine or guanine residues that corresponded to the full-length ORF (ON), or a prematurely truncated ORF (OFF). The ON and OFF frequencies were then used to estimate the frequency by which a specific genotype (presented in a binary format where 1 refers to the ON phase, and 0 refers to the OFF phase) would be expected in the population considering 6 genes previously shown by Bayliss *et al.* to mutate approximately independently (11). For example, if the same number of ON and OFF reads were observed for each of the 6 genes analyzed, then the expected frequency for all 64 possible genotypes would be equal, 0.5^6 or 1.5625%. The six contingency genes analyzed here were: *Cj1326*, *Cj0031*, *Cj1139*, *Cj0685*, *Cj0045* and *capA* (*Cj0685c*), and genotype distributions were generated for the wild-type inoculum, and 4 independently derived (4 replicate mice) mouse passaged populations of *C. jejuni*.

A stochastic mutation model (11) was used to determine the effect of homopolymeric DNA mutability (drift), on the experimentally observed genotype distributions. This model considers the switching rates and patterns of mutations in polyC/G DNA tracts to determine the effects on genotype distributions in the absence of other microevolutionary forces (e.g. selection, bottlenecks). The parameters input to the model were the estimated ON-to-OFF and OFF-to-ON rates from Bayliss *et al.* for the 6 genes considered (*Cj1326*: 10.3×10^{-4} and 17.9×10^{-4} ; *Cj0031*: 10.3×10^{-4} and 17.9×10^{-4} ; *Cj1139*: 6.9×10^{-4} and 2.1×10^{-4} ; *Cj0685*: 2.1×10^{-4} and 6.9×10^{-4} ; *Cj0045*: 38.8×10^{-4} and 3.7×10^{-4} ; *capA*: 38.8×10^{-4} and 3.7×10^{-4} , for ON-to-OFF and OFF-to-ON, respectively,

for each gene) (11), and the distribution of genotypes in the unpassaged inoculum based on our observed ON/OFF frequencies for these 6 genes. Since we could only estimate the number of *C. jejuni* divisions that occurred during 35 days of mouse colonization, we ran the model to include a range of divisions (70, 140, 280, 420, 560, 1000, 2000, and 3000) to predict genotype distributions for comparison to distributions observed by genome sequencing. By 3000 divisions the model predicted genotypes were approaching equilibrium (estimated to occur at ~6000 divisions).

Genotype distributions were deemed significantly different by Kolmogorov-Smirnov (K-S) test when the P-value was found to be less than 0.01.

Colony PCR and Sanger sequencing

A small amount of intact *C. jejuni* cells from a single colony on a streak plate were toothpicked into PCR master mix. Denaturation/lysis at 95 °C, 10 minutes was done before initiation of thermocycling. PCR products were purified using the Qiaquick PCR clean-up kit (Qiagen) according to the manufacturer's instructions, and DNA sequencing was performed at the MSU RTSF.

Pulsed field gel electrophoresis

C. jejuni cells were harvested from Bolton agar plates, and PFGE was performed on SmaI-digested genomic DNA of each variant as described by Ribot *et al.* (154), using the Chef-DR™ system (Bio-Rad).

Germ-free mouse experimental design for gene expression comparisons

C57BL/6 IL-10^{-/-} mice were raised in the germ-free colony at the University of Michigan (Ann Arbor). Mice were housed in soft-sided bubble isolators, and fed autoclaved water and laboratory chow. At six weeks of age, male mice were inoculated with approximately 1×10^{10} CFU of either wild-type or mouse-adapted *C. jejuni*.

Preparation of the inoculum is described in Mansfield *et al.* (122). All mice infected with a given variant were housed together in an autoclaved, polycarbonate filter-top cage inside a laminar flow hood. Infection was allowed to proceed for 96 hours before euthanasia. Mice were humanely sacrificed by giving an overdose of an inhalant anesthetic according to AVMA guidelines (1). The cecum was immediately extracted, submerged in RNAlater (Ambion) and the cecal contents were squeezed out using a sterile cell lifter (Corning). Samples were stored in 50ml conical tubes in RNAlater on ice for approximately 2.5 hours until they were stored at -80°C until RNA extraction.

RNAlater has been used successfully with *C. jejuni* (176), and in our hands these storage conditions did not alter *C. jejuni* NCTC11168 gene expression in control microarray experiments.

***In vivo* RNA isolation**

This protocol was modified from Zoetendal *et al.* (200). Briefly, cecal contents in RNAlater were thawed on ice, and an equal volume of PBS plus a 1:100 volume of phenol (Invitrogen) was added directly to each tube. Tubes were vortexed before slow-speed centrifugation, supernatant extraction, and then high-speed centrifugation. This

procedure resulted in pellets that consisted of only *C. jejuni* cells, and small cecal content particles. RNA was then extracted using TRIzol[®] (Invitrogen) according to the manufacturer's instructions. To remove proteins, fat, polysaccharides, proteoglycans, and insoluble materials, centrifugation after homogenization, and precipitation using a high salt solution and isopropanol was performed as described in the TRIzol[®] reagent protocol. Samples were treated for vigorous DNA contamination with the TURBO DNA-free[™] kit (Ambion) and then precipitated overnight with a 1:10 volume of 3M sodium acetate, pH 5.5, and 3 volumes of 100% ethanol.

***In vitro* RNA isolation**

Frozen inocula were streaked onto Bolton agar plates and harvested into sterile tryptic soy broth with 15% glycerol to OD₆₀₀ 0.3, before being aliquoted and stored at -80°C. Ten milliliters Bolton broth in 25 cm² vented tissue culture flasks were inoculated with 100µl of thawed aliquot. Cells were grown at 37°C with 80 r.p.m. agitation inside a microaerobic GasPak jar. After 18 hours, a 1:10 volume of phenol "stop solution" (14) was added to the culture before RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The optional on-column DNase step was performed, and after elution samples were precipitated overnight with a 1:10 volume of 3M sodium acetate, pH 5.5, and 3 volumes of 100% ethanol. Samples were then treated using the TURBO DNA-free[™] kit.

Microarrays and analysis

The above methods produced intact RNA as verified by analysis on the Agilent BioAnalyzer 2100, and visualization on non-denaturing TAE agarose gels. During real-time qRT-PCR, residual DNA could not be detected in control wells before 32 cycles, further verifying the lack of genomic DNA in RNA samples. RNA was reverse transcribed overnight in the presence of aminoallyl-dUTP (Ambion) using SuperScript III (Invitrogen), and cDNA was labeled with Cy3 or Cy5 dye (GE Healthcare). Whole-ORF arrays containing ~99% of *C. jejuni* NCTC11168 ORFs were hybridized overnight in a rotating 54 °C oven using microarray hybridization chambers and backing slides from Agilent. Array details can be found at the NCBI GEO (GPL8707 and GPL8954). Scanning and fluorescence data generation was done using GenePix scanner and software. LimmaGUI was used for global loess normalization of fluorescence intensities, and to determine significant expression differences (188). Results were considered significant at $p < 0.05$ after applying a false discovery rate control. Reports from microarray experiments are deposited in the NCBI GEO (GSM437708-437714 and GSM587271-587274) and conform to MIAME guidelines.

Real-time quantitative reverse transcription PCR (qRT-PCR)

Primers for real-time qRT-PCR were designed using web-based Primer3 (161) to amplify 80-120bp of template (Table 2.7). Reactions were carried out in 96-well PCR plates using a Bio-Rad iQ5 iCycler and iScript One-Step RT PCR Kit with SYBR Green (BioRad). Threshold cycle number was determined for four biological replicates from

each variant. Relative expression was normalized to the 16S rRNA threshold cycle and analyzed by the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (119).

Cell culture

Young Adult Mouse Colon (YAMC) epithelial cells are described in Whitehead et al. (189) and were obtained with permission from Dr. Robert Whitehead (Ludwig Institute for Cancer Research, Melbourne, Australia). YAMC cells proliferate at 33°C in the presence of IFN-γ due to IFN-γ inducible expression of the temperature sensitive SV40 large T antigen. Cells were propagated in 5% CO₂ at 33°C in RPMI medium 1640 with L-glutamine and 25mM HEPES (Invitrogen), supplemented with 5% fetal bovine serum, ITS[®] (BD Biosciences; insulin 6.25 µg/ml, transferrin 6.25 µg/ml and selenous acid 6.25 ng/ml), 5 IU/ml of murine IFN-γ, and 100000 IU/l penicillin and 100 mg/l streptomycin (permissive conditions). Before *C. jejuni* infection, cells were moved to 5% CO₂, 37°C and incubated without antibiotics, ITS[®], or IFN-γ (non-permissive conditions). It has been reported that these cells behave similarly to normal colonic epithelial cells at the non-permissive temperature in that they are contact inhibited, produce brush border enzymes, and undergo apoptosis as they reach maximal confluence (48, 189).

Association and invasion assays

Approximately 1.5×10^5 YAMC cells were transferred to each well of a 24 well tissue culture plate and incubated to ~80% confluence for 48 hours. YAMC cells were moved to 37°C infection conditions 18 hours prior to addition of *C. jejuni*. For association assays, *C. jejuni* was added to an MOI of 100:1 and allowed to colonize for 1 hour before 3 warm PBS washes, lysis with 0.1% Triton X-100, and enumeration by serial dilution. The number of cells associated includes both adhered and invaded *C. jejuni*. Invasion assays were performed with *C. jejuni* MOI 100:1, and following a two hour incubation, cells were washed with PBS and incubated for one hour with 250 µg/ml gentamicin to kill extracellular bacteria. YAMC cells were then washed again, lysed, and released bacteria were enumerated on agar plates. Results presented above are mean CFU ± standard deviation from triplicate wells during a single experiment. The association and invasion assays were performed twice to ensure reproducibility.

Autoagglutination assays

Assays for autoagglutination were performed as described in Misawa and Blaser (129). Briefly, *C. jejuni* variants grown on Bolton agar were suspended in 3-5 ml PBS, pH 7.2 in 5 ml polystyrene tubes (BD Falcon) to an equal OD₆₀₀. Suspensions were allowed to incubate at 37°C for 24 hours before reading the OD₆₀₀ of the top 1 ml. The ability to autoagglutinate is reflected in the decrease in OD₆₀₀ reading from the original suspension. Samples were processed in triplicate or quadruplicate and the assay was repeated 3 separate times at different starting OD₆₀₀ readings. Results presented

above were from the assay with starting OD₆₀₀ 1.0. All 3 independent assays showed the same statistically significant trend (i.e. the mouse-adapted bacteria having a higher final OD₆₀₀ measure), as presented in Figure 2.4b above.

Motility assay

C. jejuni variants were grown on Bolton agar plates for 24-48 hours, before being harvested and resuspended in TSB to an equal OD₆₀₀ of 0.2-0.3. Five microliter aliquots were spotted onto a Bolton agar plate containing 0.4% agar. Cells were incubated at 37°C, 10% CO₂ for 48 hours before being photographed to observe the diameter of spreading.

Statistics

If data from wild-type and mouse-adapted variants did not have significantly different variance by F-test, then comparisons were considered significant when $p < 0.05$ by Student's t-test. Other statistical tests used are as described for specific assays.

Acknowledgments

We would like to thank Dr. Kathryn Eaton and Sara Poe for assistance with the germ-free mice.

Table 2.1 Significant open-reading frame changes due to serial passage.

Gene	Putative Function	Variations Detected	In-frame ORF tract length ^a	Enriched ORF	Percent with enriched ORF		Fold change of in-frame ORF ^c
					Wild- type	Mouse- adapted	
<i>Cj0031</i>	Restriction/Modification	G(8-11)	G(9)	In-frame	24.0	50.5	2.1
<i>Cj0045c</i>	Iron-binding protein	C(9-13)	C(11)	Frameshifted	74.0	93.5	-4.0
<i>Cj0046</i>	Pseudogene	G(8-13)	G(9)	In-frame	13.2	68.7	5.2
<i>Cj0170</i>	Conserved hypothetical	G(8-10)	G(8)	Frameshifted	54.6	88.0	-3.8

Table 2.1 (cont'd)

<i>Cj0565</i>	Pseudogene	G(9-11)	None apparent	G(10) ^b	52.3	72.8	
<i>Cj0676</i>	Pseudogene	G(8-11)	G(10)	In-frame	15.1	38.7	2.6
<i>Cj1145c</i>	Glycosyltransferase	C(8-11)	C(10)	Frameshifted	65.6	95.5	-7.6
<i>Cj1295</i>	Aminopeptidase	G(6-11)	G(9)	In-frame	54.2	86.8	1.6
<i>Cj1296/Cj1297</i>	Aminoglycoside N3'- acetyltransferase	G(8-10)	G(10)	In-frame	3.6	75.5	20.9
<i>Cj1306c</i>	Unknown	C(8-11)	C(9)	Frameshifted	25.0	50.0	-1.5
<i>Cj1325</i>	Acetimidino-N- methyltransferase	G(8-11)	G(9)	In-frame	32.2	61.9	1.9
<i>Cj1342c</i>	Unknown	C(8-11)	C(9)	Frameshifted	19.9	56.3	-1.8

Table 2.1 (cont'd)

<i>Cj1429c</i>	Unknown	C(9-13)	C(10)	In-frame	28.9	86.1	3.0
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^a The “In-frame ORF tract length” was defined as the most frequently observed homopolymeric tract length that generated the longest potential open-reading frame. All tract lengths that introduced a premature stop codon were considered frameshifted.

^b A tract with length 10 increases in frequency during passage, but no ORF change could be detected since the tract is upstream of the predicted coding region.

^c The tract lengths observed for *Cj0046*, *Cj1295*, and *Cj1429c* each contained a single read that differed from the “In-frame ORF tract length” by 3 guanines (or cytosines), and these were considered in-frame since they do not introduce a premature stop codon. A positive fold change indicates an increase of the in-frame ORF in mouse-adapted *C. jejuni*, and a negative fold change indicates a decrease.

Table 2.2 SNP changes during passage: All detected SNPs that changed frequency in the population by 5% or more are shown. Verification of SNPs was through traditional sequencing of PCR products to observe a mixed peak at the relevant nucleotide position.

Gene	Position	Mutation	% wild-type reads with mutation	% mouse-adapted reads with mutation	PCR, Sanger sequence verification
<i>pbpC</i>	611,386	T→C	0%	62.1%	yes
<i>porA</i>	1,189,659	A→G	82.4%	100%	yes
<i>cstA/prsA</i>	854,539	G→A	0%	16.0%	yes
<i>Cj0019c</i>	24,605	C→G	0%	6.8%	not tested
<i>Cj0590</i>	550,127	G→A	5.4%	0%	not tested
<i>cipA</i>	638,796	A→G	13.7%	0%	not tested
<i>Cj1564</i>	1,492,193	C→A	0%	4.9%	not tested

Table 2.3 Consensus mutations in our NCTC11168 (ATCC 700819) culture: These mutations were found through mapping to the reference genome sequence (GenBank AL111168.1), and analysis as described in the Methods.

Consensus changes in contingency loci are not shown here, but are pictured in Figure 2.2.

Gene	Mutation start	Mutation end	Reference sequence	Change	Type	Codon change	Amino acid change
<i>Cj0184c</i>	180710	180711	AC	deleted	frameshift	GTA→ATA	V→I
<i>mreB</i>	253191	253191	A	G	substitution	GAT→GGT	D→G
<i>cheA</i>	262345	262345	A	G	substitution	ATA→ACA	I→T
<i>Cj0431</i>	393542	393542	T	A	substitution	TAA→AAA	*→K
<i>Cj0455c</i>	420550	420550	A	G	substitution	TAA→CAA	*→Q
<i>Cj0807</i>	760188	760188	A	G	substitution	AAA→GAA	K→E
<i>porA</i>	1189659	1189659	A	G	substitution	GAA→GGA	E→G
<i>gapA/nadD</i>	1338390	1338390	.	A	noncoding	N/A	N/A
<i>Cj1470c</i>	1404347	1404347		T inserted	frameshift	ATG→TAT	M→Y

* indicates a translational stop codon.

Table 2.4 Genes differentially expressed in the mouse-adapted variant during *in vitro* growth.

Gene	Contingency locus	P-value	Fold Change	
			Microarray	qRT-PCR
<i>Cj1297</i>	Yes	0.004	2.5	3.5
<i>Cj1296</i>	Yes	0.004	2.1	2.6
<i>Cj1295</i>	Yes	0.131	1.6	2.5
<i>Cj0565</i>	Yes	0.015	-2.1	NT ^a
<i>Cj0170</i>	Yes	0.015	-1.8	-18.9
<i>Cj0299</i>	No	0.015	1.8	NT
<i>Cj1190c</i>	No	0.016	-1.7	NV ^b
<i>Cj1021c</i>	No	0.036	1.6	NT
<i>Cj1381</i>	No	0.039	1.6	NV

^a NT – not tested

^b NV – tested, but not verified

Table 2.5 Genes differentially expressed in the mouse-adapted variant during *in vivo* growth.

Gene	Contingency locus	P-value	Fold Change	
			Microarray	qRT-PCR
<i>Cj1523c</i>	No	<0.001	2.1	NV ^b
<i>frdC</i>	No	<0.001	1.8	NV
<i>Cj1297</i>	Yes	<0.001	1.7	2.6
<i>Cj0565</i>	Yes	<0.001	-1.6	-2.0
<i>rpmB</i>	No	0.003	-1.5	NV
<i>Cj1506c</i>	No	<0.001	-1.4	NV
<i>Cj0170</i>	Yes	0.027	-1.4	-3.0
<i>Cj0704</i>	No	0.027	-1.4	NT ^a
<i>Cj0144</i>	No	0.046	-1.4	NT

^a NT – not tested

^b NV – tested, but not verified

Table 2.6 Epithelial cell interaction of *C. jejuni* variants.

Variant	Colony Forming Units	
	Associated	Internalized
Wild-type	$(1.79 \pm 0.24) \times 10^5$	$(5.53 \pm 0.83) \times 10^4$
Mouse-adapted ^a	$(3.25 \pm 0.16) \times 10^4$	$(4.87 \pm 2.19) \times 10^3$
Mouse-adapted efficiency ^b	0.18 ± 0.03	0.09 ± 0.04

^a Mouse-adapted bacteria in this assay had been passaged through mice a single time.

^b Fraction of mouse-adapted CFU relative to wild-type.

Table 2.7 Primers used for real-time PCR.

Gene	Primer 1	Primer 2	Product size (bp)
<i>16S rRNA</i>	gcgcaacccacgtatttagt	ataagggccatgatgacttga	106
<i>Cj0144</i>	ctcaaccaaattggcgaactt	cagctttccataagtccaaaat	81
<i>Cj0170</i>	gcaacatgatgagcgataa	tcctttacttcacgcaagct	81
<i>Cj1295</i>	aatgggtggactcaaagca	ccaagattgggttcgcataca	93
<i>Cj1296</i>	ggcaaggaaggcactcttct	gcactcccactttgccttta	97
<i>Cj1297</i>	agggctttgtgattgacgagcta	gccgcacgatttcatttatct	117
<i>Cj1190c</i>	cggttgtggctgatgaagtt	acttgctcactgccactttgga	124
<i>Cj1381</i>	cttgaccaatgcaactttca	tgactgggattttgaaaggaa	112
<i>Cj1523c</i>	accatcttcatcacgccatcga	tgactgttgctttcttgctctt	104
<i>frdC</i>	tcgttatggagggttcaagc	gacatatcgccgctgatttta	107
<i>Cj1506c</i>	atcgctgagcaaacgaatct	ttccgcaagctgtcttacct	114
<i>Cj0565</i>	cggcaaattgcaacacttaca	aggctgggctagctaaatcca	102

Table 2.8 Significant ORF changes during *C. jejuni* infection of multiple mice.

Gene	Mouse	Fold Change of in-frame ORF ^a	P-value ^b
<i>Cj0170</i>	1166	In-frame ORF not observed	0.001
	1141	-5.3	0.04
	1233	In-frame ORF not observed	0.0001
	1067	-19.1	0.0007
<i>Cj1429</i>	1166	10.2	<0.0001
	1141	2.5	NS
	1233	7.4	<0.0001
	1067	6.2	<0.0001
<i>Cj1295</i>	1166	1.8	<0.0001
	1141	1.8	<0.0001
	1233	1.3	NS
	1067	1.8	<0.0001
<i>Cj1296</i>	1166	1.2	NS
	1141	39.6	<0.0001
	1233	12.8	0.0002
	1067	32.4	<0.0001

^a Fold change description in Table 2.1.

^b P-values from Chi-square test of association using Illumina read count data as described in the Methods.

Figure 2.1. Estimated indel error rates. Representative graph of estimated error rates for different base substitution and indel mutations by quality score. This graph is from the re-sequencing data for wild-type when the reference base is adenine, but in all estimations, indel error rates fall below base substitution error rates. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis (or dissertation).

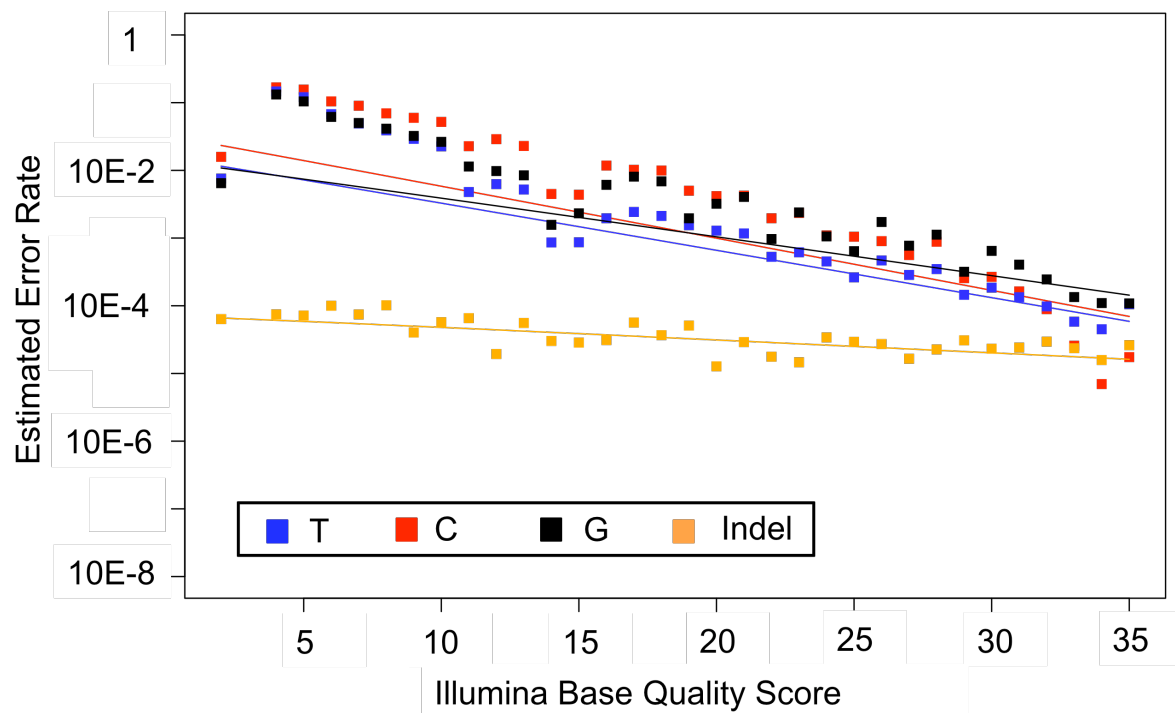


Figure 2.2. Microevolution of contingency loci. For each contingency gene, the percentage of sequence reads with a particular base count in the homopolymeric tract is graphed. The distribution of homopolymeric variations before (wild-type *C. jejuni* NCTC11168), and after (mouse-adapted *C. jejuni* NCTC11168) passage, for each variable gene in the genome is shown. *Cj0628* contains 2 variable tracts, but is not pictured. The distribution of variation at this tract was stable through passage. * $p \leq 0.01$ and ** $p \leq 0.001$ by chi-square test of distribution for open reading frame variations. Green bracket: Lipooligosaccharide biosynthesis locus; Blue bracket: Flagellar glycosylation locus; Reb bracket: Capsular biosynthesis locus.

Figure 2.2 (cont'd)

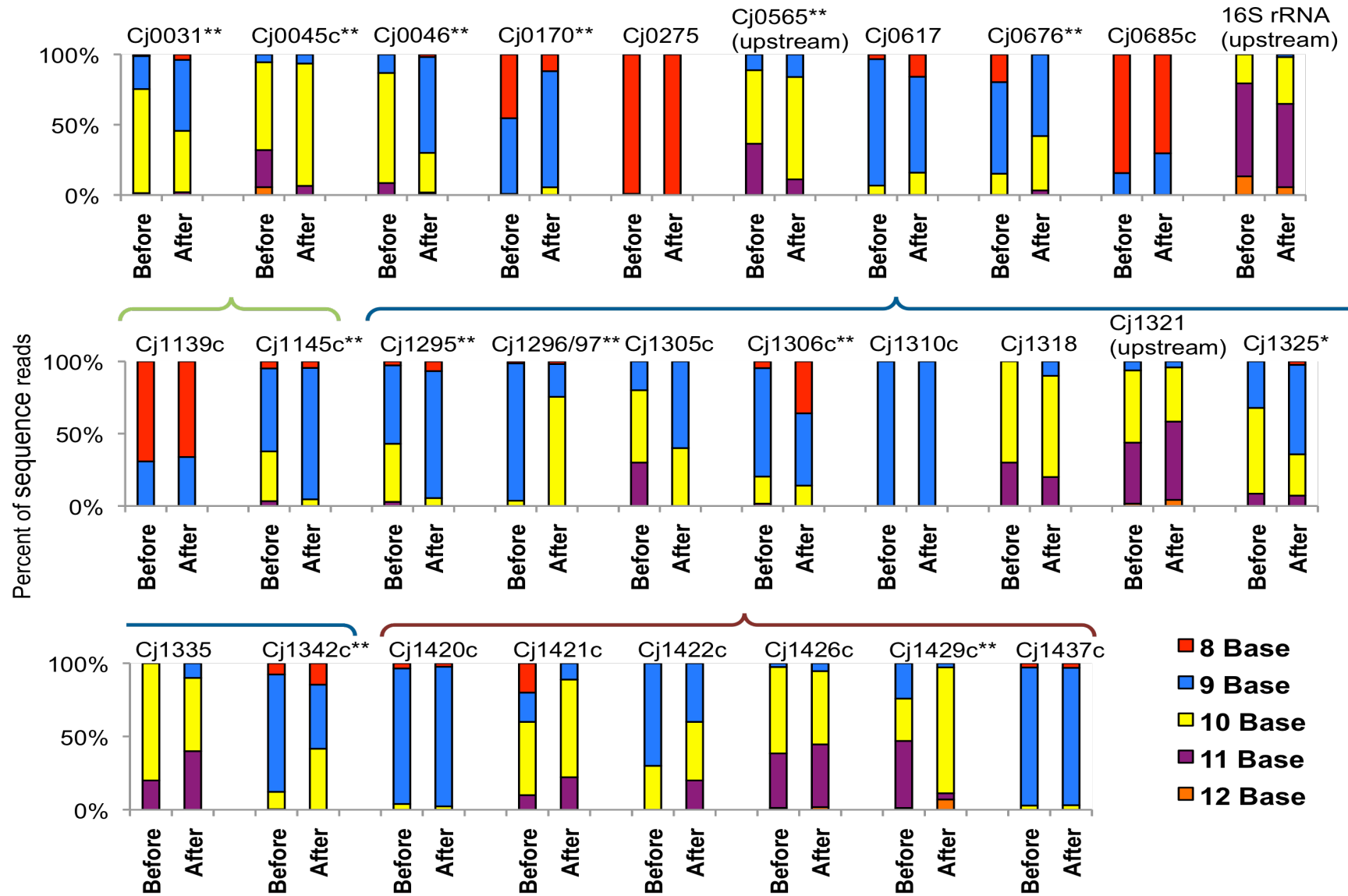


Figure 2.3 Absence of large genomic changes during passage. Left: Pulse field gel electrophoresis image to compare wild-type and mouse-adapted bacteria. Right: The same gel with an image filter as an attempt to detect rare bands.

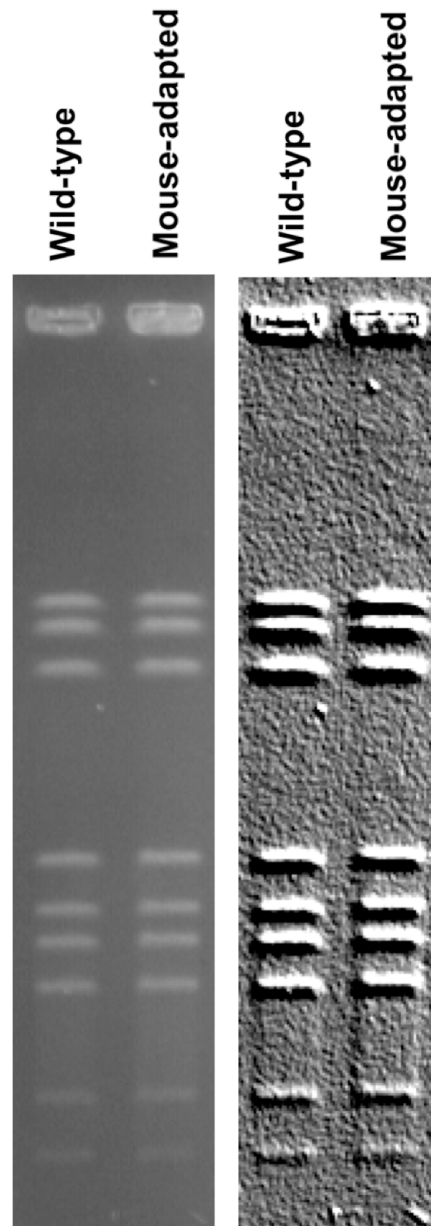


Figure 2.4 *In vitro* phenotypes of mouse-adapted *C. jejuni*. (A) Soft agar plates allow motile *C. jejuni* to spread from the center of inoculation. The ability to spread is based on flagellar motility. The top right spot is the wild-type variant and shown going clockwise are mouse-adapted variants passaged one, two, or three times through mice. All have spread an equal amount after 48 hours. (B) A higher final OD₆₀₀ for the mouse-adapted variant indicates a decreased ability to autoagglutinate. Standard error bars are shown.

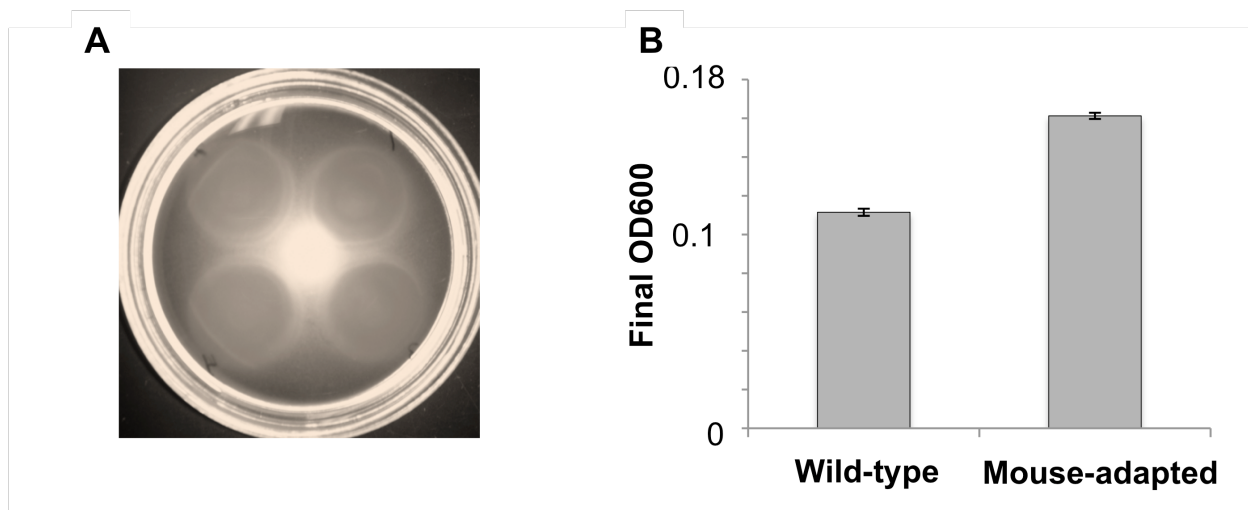


Figure 2.5 Microevolution of contingency in multiple mice by direct PCR analysis.

The distribution of homopolymeric DNA tract lengths were determined for four populations of *C. jejuni* infecting independent replicate mice by a genescan method of PCR product lengths (Methods). This analysis was performed for 4 genes that changed significantly during serial passage: (A) *Cj0170*; (B) *Cj1429*; (C) *Cj1295*; (D) *Cj1296/7*. For statistical significance of changes occurring in these populations refer to Table 2.8. “Before” refers to the inoculum before ever being passaged through the mouse model. For reference, the results from Illumina sequence read analysis of serially passaged, mouse-adapted *C. jejuni* is also shown (labeled “3 passages”).

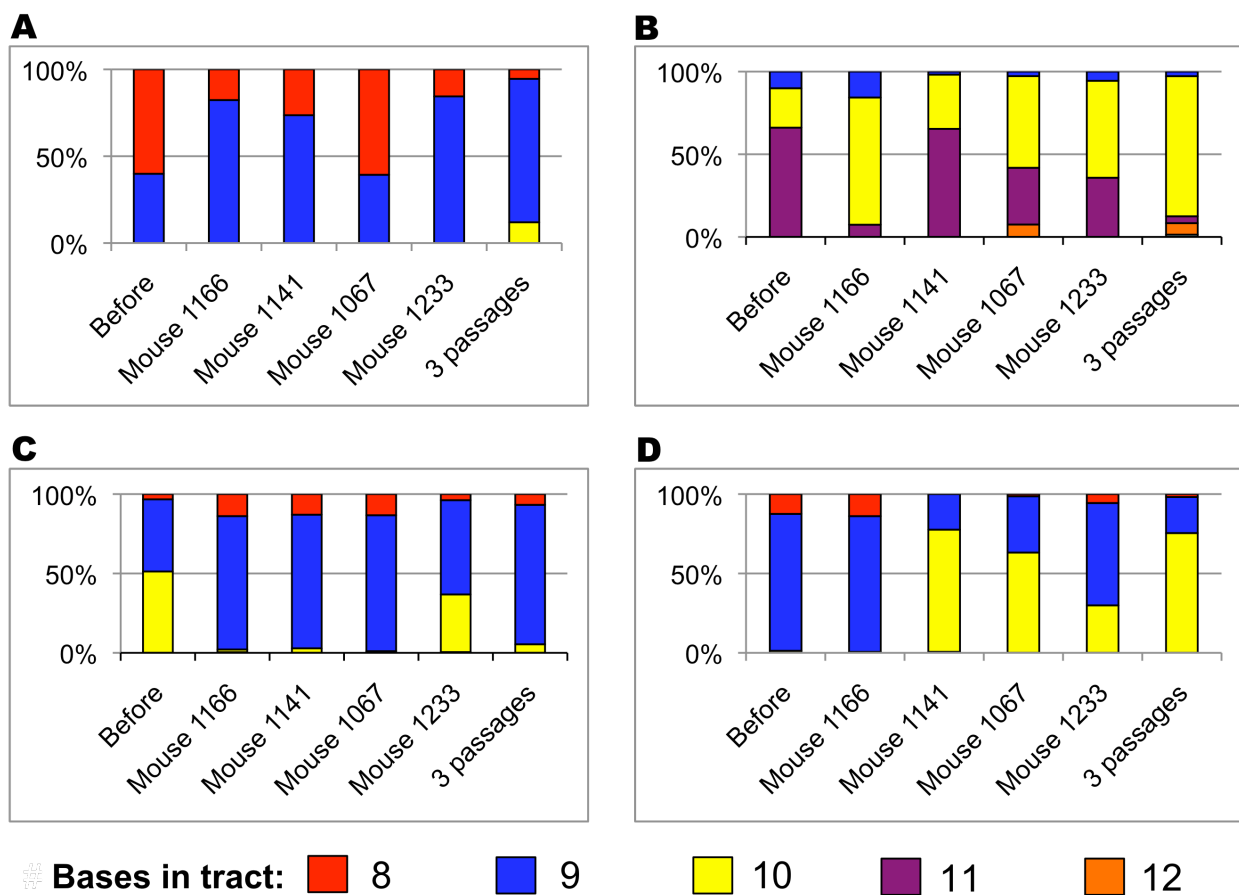
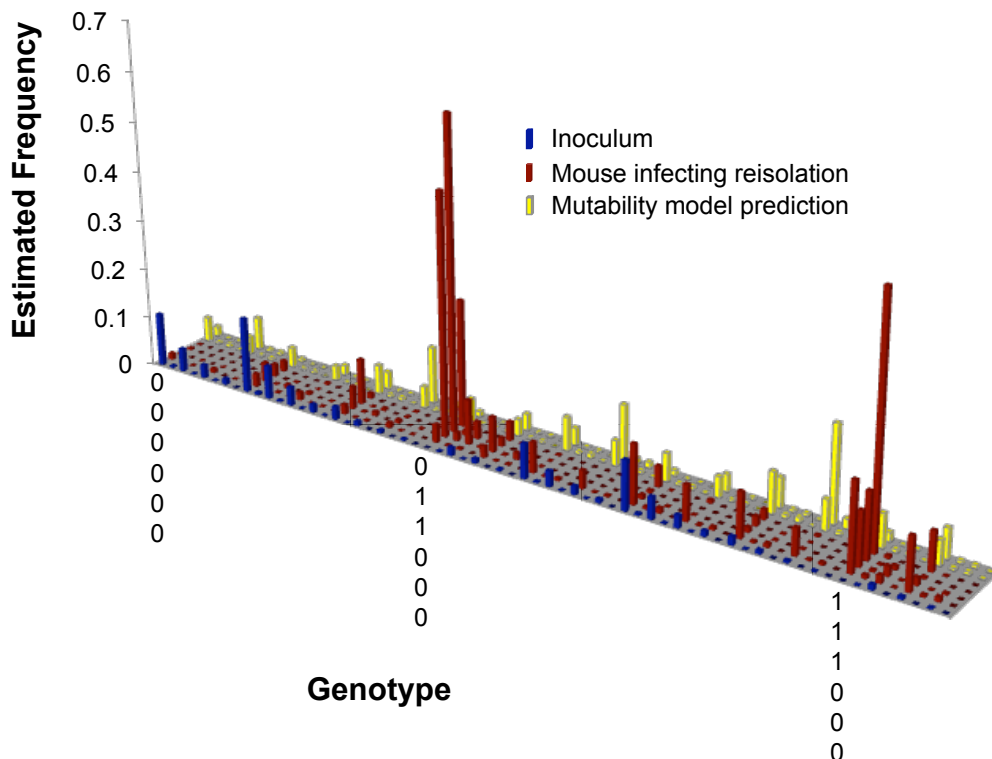


Figure 2.6 Changes in the distribution of genotypes observed during mouse infection, or by a model of contingency loci mutability. Distributions of genotypes were derived as in the methods according the frequency that ON and OFF phases were observed for 6 contingency loci. The gene order of the binary genotype code is: *Cj1326*, *Cj0031*, *Cj1139*, *Cj0685*, *Cj0045* and *capA* (*Cj0685c*). A 0 signifies the “OFF” phase, and a 1 signifies the “ON” phase for a contingency gene. The genotype distributions isolated from 4 mice are pictured in the following order going back in the plane of the chart: 1067, 1141, 1166, and 1233. The model predicted genotypes at generation 560, and at the stationary distribution (furthest back) are also shown.



CHAPTER 3

**Reversible motility mutations and parallel σ^{54} loss during experimental evolution
of *Campylobacter jejuni***

ABSTRACT

Evolution experiments in the laboratory have focused heavily on model organisms, often to the exclusion of clinically relevant pathogens. *Campylobacter jejuni* is a foodborne bacterial pathogen that only replicates naturally within a host. When we experimentally evolved *C. jejuni* to rich broth medium, we observed the loss of flagellar motility—an essential function for efficient host colonization. At early time points during broth adaptation the motility defect was often reversible by selection for motility in semi-solid media, but after 35 days of laboratory culture, motility was lost irreversibly in the majority of cells in 5 independently evolved populations. Genome re-sequencing revealed numerous disruptive mutations to genes in the *C. jejuni* flagellar transcriptional cascade, including genes known to affect expression of the σ^{54} (RpoN) regulon and chromosomal deletion of *rpoN* in all evolved lines. Furthermore, we demonstrate that a phase variable (reversible) motility mutant is deficient for colonization in a C57BL/6 IL-10^{-/-} mouse disease model, despite the reversibility of the motility defect. This reversible motility mutant contained an adenine deletion within a homopolymeric tract of DNA resulting in truncation of the flagellar biosynthesis gene, *fliR*. Re-insertion of an adenine residue by selection for motility on semi-solid agar partially restored motility and the ability to colonize mice. Our work shows that a pathogenic *C. jejuni* strain is quickly attenuated by experimental laboratory evolution, and provides insight into the role of genomic instability for *C. jejuni* evolvability. Based on this data we suggest a potential stepwise mechanism of *C. jejuni* genetic adaptation to a novel environment by high-rate,

reversible mutations for rapid phenotype selection, followed by irreversible mutations during prolonged selective pressure.

INTRODUCTION

The natural reservoir of *Campylobacter jejuni* is in avian species, but it is known to colonize numerous other animals, including humans (20, 198). In birds, *C. jejuni* infection rarely results in disease and colonization is stable over long time periods (198). Infection in humans often results in gastroenteritis and is generally self-limiting, but chronic autoimmune diseases such as inflammatory bowel disease (62), and long-term neurological sequelae such as Guillain-Barré syndrome may occur (20). In the United States *C. jejuni* is currently estimated to be the third leading cause of human infections, and the second leading cause of hospitalizations, due to a foodborne bacterial pathogen (165).

Relatively little is known about the molecular factors essential for *C. jejuni* pathogenesis, but it is widely accepted that flagellar motility is necessary for efficient colonization of an animal host (15, 94, 121, 139). Motility is likely needed for navigation through the gastrointestinal lumen and mucus layer to enable *C. jejuni* to colonize and invade the underlying epithelial cell monolayer. Additionally, non-motile mutants are often attenuated in their interaction with epithelial cells in culture (63, 185) and incapable of secreting the Cia proteins necessary for maximal invasion of epithelial cells (107).

Construction of a functional flagellum is a complex process involving numerous genes and an elaborate transcriptional cascade. Current models of *C. jejuni* flagellar biosynthesis show that formation of a type III secretion system (T3SS) influences the activity of the FlgSR two-component signal transduction pathway to stimulate

expression of the alternative sigma factor, σ^{54} (RpoN) (56). The σ^{54} regulon is almost exclusively composed of flagellar structural genes in *C. jejuni*, and expression of these genes generates essential components of flagella, including the basal body, hook and minor flagellin subunits (25). Researchers have also shown that *C. jejuni* flagellin must be extensively glycosylated for filament polymerization, and therefore some genes involved in flagellar glycosylation are necessary for motility (61). Finally, a number of other gene products have been implicated in flagellar motility, but their molecular roles in the process are unknown. These include a predicted transmembrane protein, Cj0390 (26, 30); the GTPase, FlhF, that contributes to the expression of σ^{54} (8); and a protein that is potentially secreted out of the cytoplasm, PflA (196).

Researchers have also described reversible, or phase variable, expression of flagellar motility in *C. jejuni* (21, 72, 75, 100, 144). It has been noted that the rate of motility phase ON to OFF is significantly higher than OFF to ON during *in vitro* culture (21). Moreover, mutants defective in flagellar biosynthesis have been reported to have an increased growth rate in culture (194). In contrast, motile phase ON mutants seem to have higher fitness *in vivo* since non-motile cells are completely lost during gut passage when ON and OFF spontaneous mutants are co-inoculated into rabbits (21), humans (15) or mice (Jerome JP, unpublished data). Furthermore, phase OFF non-motile isolates are reported to have low efficiencies for colonizing chickens and infant mice (22, 55, 75, 139). *C. jejuni* may also adapt in the gastrointestinal tract of chickens during the course of infection from low to high levels of colonization (23, 94), and the increased colonization phenotype is associated with increased motility (94).

In most work concerning the reversible motility phenotype in *C. jejuni* the genetic basis of the motility deficiency has not been investigated. However, Hendrixson has described phase variable expression through homopolymeric adenine (and/or thymine) tract slipstrand mutation of both the histidine kinase and response regulator of the FlgSR two-component system in *C. jejuni* (72, 75). Spontaneous mutations in *flgS* also occurred by nucleotide deletions within a short heteropolymeric repeat (75). When motile revertants from the frameshifted *flgS* mutants were selected *in vitro*, intragenic indels ranging from 1 to 368 nucleotides were discovered that resulted in restoration of a functional open reading frame (75). Conversely, during chicken colonization an *flgS* mutant reverted to the motile phenotype through an extragenic single nucleotide substitution in *flgR* that suppressed the *flgS* mutant phenotype. Genetically reversible motility expression may also be affected by slipstrand mutations in the motility accessory factor genes, *maf1* and *maf7*, that contain hypervariable homopolymeric tracts of guanine residues (100). Also, in the closely related species *Campylobacter coli*, reversible flagellar expression has been linked to slipstrand mutation in a homopolymeric tract of thymine residues in the *flhA* gene that is part of the flagellar T3SS (147). It is known that *C. jejuni* lacks many DNA repair systems (98, 148), and taken together with the findings described here, researchers anecdotally suggest that the *C. jejuni* genome is highly plastic.

To begin to define how the *C. jejuni* genome can adapt to different environments we have taken a simple approach: evolve *C. jejuni* experimentally to a given environment, and then sequence the genomes of the evolved and ancestral variants. Using this approach, we previously showed that serial passage of *C. jejuni* NCTC11168

through a mouse model of campylobacteriosis resulted in significant changes in allele frequency in hypermutable homopolymeric guanine and cytosine tracts termed contingency loci (93). In the present study we assessed the genetic basis of *C. jejuni* NCTC11168 adaptation during experimental evolution in a more controlled environment—namely, rich broth media in the laboratory. We hypothesized that relieving the selective pressure of the host environment would result in the loss of functions required for *C. jejuni* growth *in vivo*. We also predicted that the observed rate and genetic patterns of laboratory adaptation would lead to a better understanding of *C. jejuni* evolvability.

This work shows that experimental laboratory adaptation of *C. jejuni* results in the rapid loss of flagellar motility, which is an important colonization determinant of this foodborne pathogen. We further characterized the motility loss as reversible or irreversible based on the ability or inability to restore motility by selecting for motile cells in a semi-solid agar environment. By defining the genetic reversibility of the motility deficiencies over the time course of the experimental evolution, we found that subpopulations with reversible motility loss appeared early, before subpopulations with irreversible motility deficiencies. Sequencing the ancestral and evolved populations defined multiple mutations that are known to disrupt flagellar motility. Many of these mutations are predicted to disrupt a protein coding sequence by a phase variable (reversible) mechanism driven by slipstrand mutations in homopolymeric DNA. However, the evolved lines also contained mutations in essential flagellar motility genes that are not genetically reversible, such as base substitutions that introduced stop codons, and large genome deletions. Finally, one reversibly non-motile isolate was

characterized further and found to be deficient for mouse colonization in the C57BL/6 IL-10^{-/-} mouse model of campylobacteriosis. This variant had a low rate of reversion to the motile phenotype *in vivo* despite maintaining the ability to rapidly evolve restored motility in the laboratory. The genotypic and phenotypic patterns of adaptive evolution defined in this work will likely apply to other highly mutable bacteria such as pathogenic *Helicobacter* species.

RESULTS

Motility is lost during evolution in broth

Five independent populations of *C. jejuni* were maintained in broth media for 35 days by transferring a 1:100 culture volume to fresh broth every 24 hours. From wet-mount microscopic observations used to monitor the evolving cultures, it appeared that a fraction of cells in each population were losing motility over time. To further observe the motility phenotypes of individual cells in each evolving population, we used a semi-solid agar pour-plate technique (21) on cultures saved over the course of the evolution experiment. In this assay, dividing cells spread outward toward more nutrients and form a diffuse circle of growth if they are motile (Figure 3.2a, top right), while non-motile cells form a small, dense, orange colony in the medium (Figure 3.2a, top left). The ancestral inocula used to seed each evolved line had been passaged through C57BL/6 IL-10^{-/-} mice (13), and only motile colony forming units (CFUs) were observed by the pour plate assay (N=203).

After 3 days of culture, or approximately 12 divisions, non-motile cells could be observed in the population (data not shown), and by day 5 there was a significant loss of motile cells in all 5 independent populations ($P < 0.05$, Fisher's Exact test) (Figure 3.1a). After 35 days 100% of observed colonies were non-spreading in 4 populations (N=81, 23, 40, and 37 for Populations 2, 3, 4, and 5, respectively). One population still contained motile CFUs after 35 days in culture, but they were present in low frequency in the population (N=61 with 8 motile CFUs). A loss of motility was also evident when archived cultures were re-grown and spotted onto semi-solid agar as a population of

cells at various time points (Figure 3.1b). However, it should be noted that when approximately 4×10^6 cells from the day 35 stock cultures were incubated for extended periods (96 hours) on semi-solid agar, *C. jejuni* cells could be observed spreading into the media. Therefore, although motility was lost completely in 4 populations as assayed by pour-plate (Figure 3.1b), subpopulations of cells that were non-motile, but had the ability to re-evolve functional flagella were still present after 35 days.

Higher frequency of reversibly non-motile cells at early time points during experimental evolution

During the pour-plate assays described above we noticed that many CFUs in the populations at day 5 were able to re-evolve motility during extended incubation in the semi-solid agar environment. The restoration of motility from initially non-motile mutants in semi-solid agar during long incubations has been shown before (72, 100). Therefore, extended incubation of the pour-plates allowed us to further define non-motile cells as having reversible or irreversible phenotypes (Figure 3.2a).

As shown in Figure 3.1a above, there was a significant increase in non-spreading CFUs between the time of inoculation and day 5 in all lines. Initially it was observed that of all non-motile CFUs at day 5 (N=108 across all five lines), 23 spread after extended incubation, meaning that they were reversibly non-motile. At day 35 nearly all CFUs were non-motile in each population, with little reversion to a motile phenotype after 72 hours. The proportion of reversibly non-motile CFUs was significantly greater at day 5 than day 35 for Populations 1, 2 and 4 by Fisher's Exact test ($P=0.05$, <0.0001 , and 0.0006 , respectively). However, highly motile CFUs in the day 5 populations rapidly

spread through the entire plate making it difficult to accurately observe motility reversion during incubations longer than 72 hours.

To avoid a takeover of the pour-plates by highly motile CFUs, we repeated the assay using day 10 and day 35 cultures: time points when few motile CFUs remained in each population. Nearly all observed CFUs at both times were non-motile, but a significantly higher frequency of reversibly non-motile CFUs was observed at day 10 versus day 35 for all 5 independent populations ($P \leq 0.01$, Fisher's exact test) (Figure 3.2B). The frequency of reversibly non-motile CFUs at day 10 was 3.6-, 2.3-, 6.8-, 22.9-, and 2.9-fold higher than after 35 days for Populations 1 through 5, respectively. The decrease in reversibly non-motile CFUs between day 10 and day 35 indicated that reversible motility mutations appeared rapidly during laboratory evolution, but were eventually outcompeted by *C. jejuni* cells that were irreversibly deficient for motility.

Multiple flagellar motility mutations including parallel *rpoN* deletions were detected in evolved populations

In an effort to understand the genetic basis of adaptation, we re-sequenced whole population samples of each *C. jejuni* line before and after 35 days of broth culture. Consistent with a loss of the motility phenotype, we discovered multiple mutations in the evolved lines that disrupt the open reading frame (ORF) of known flagellar biosynthesis genes and genes previously shown to be involved in flagellar motility (Figure 3.3). Affected genes included *flgS*, *flgR*, *flhA*, *flhB*, *fliP*, *fliF*, *motA*, *Cj0390*, *pflA*, and *rpoN*. The estimated frequencies of many of the flagellar gene mutations were low in the population by day 35 (Table 3.1). However, none of these

mutations were detectable in the ancestral inocula, and a subset of these predictions was confirmed by independent methods (Table 3.1). It should be noted that synonymous and nonsynonymous single nucleotide polymorphisms (SNPs) were also detected at low frequency in many of these genes after broth adaptation, but without testing the phenotypic effect of these alleles there is no clear evidence for whether they alter motility. Unless a SNP introduced a premature stop codon it is not listed in Table 3.1, but presumably some nonsynonymous SNPs in the evolved populations might also disrupt gene function.

We found ORF-disrupting mutations in the flagellar secretion apparatus genes *flhA*, *flhB*, and *fliP*, and the two-component signaling system encoded by *flgRS*. Functional protein products of these genes are necessary for expression of the σ^{54} regulon (76, 97). In addition, all 5 evolved populations contained a fraction of cells that had lost some portion of the σ^{54} -encoding gene, *rpoN* (Table 3.1). Deletions that included part of *rpoN* ranged in size from 21 to 8448 bases. One evolved population (Population 4) contained an *rpoN* mutation that approached fixation during broth evolution. This was an approximately 8.5 kilobase deletion that removed the majority of *rpoN* along with the downstream genes *dcuB*, *Cj0672*, *kdpB*, *kdpC* and 330 amino acids from the N-terminus of *kdpD*. Consistent with the irreversible motility loss described above, deletion of *rpoN* from the *C. jejuni* genome results in the loss of expression of flagellar biosynthesis genes (25, 76), and a non-flagellated, non-motile phenotype (92).

To assay for a growth advantage under the experimental evolution conditions, we revived frozen day 1 and day 35 samples from Population 4 to estimate the number of

doublings per 24-hour growth period. On average, day 35 samples doubled significantly more (4.1 times), than day 1 samples (3.7 times) ($P = 0.007$). This suggested the deletion of *rpoN* resulted in a higher cell density after 24 hours of growth when serial transfer to new media occurred during the evolution experiment. The parallel loss of σ^{54} expression through mutations disrupting the flagellar transcriptional cascade or actual deletion of *rpoN* in all 5 lines, along with the evidence of a growth advantage, supports the conclusion that motility loss through erosion of the σ^{54} regulon is favorable under the experimental evolution conditions.

ORF-disrupting reversible and irreversible types of mutations were detected

Since we observed phenotypically reversible and irreversible motility phenotypes during the pour-plate assay, we hypothesized that some of the ORF-disrupting mutations discovered might be subject to phase variable expression. The disruptive types of mutations we found included single base insertion and deletion mutations—often in homopolymeric tracts of DNA; expansion of a TA dinucleotide repeat; large DNA deletions; and SNPs that introduced premature stop codons. Of these mutation types, insertions and deletions in homopolymeric DNA are considered highly mutable by slipstrand mispairing (113), and are the only documented source of phase variable gene expression in *C. jejuni*. Mutations in long tracts (7–8 base) of homopolymeric DNA were found in *flgS*, *flgR*, *flhB*, and *fliP* genes, while mutations in short homopolymeric tracts (3–5 base) that may be reversible, but presumably have a lower mutation rate, were found in *flgS*, *flgR*, *flhA*, *rpoN*, *pflA*, and *motA* genes. All of the homopolymeric tract indel mutations discovered were in adenine or thymine tracts, except for the mutation in

motA that occurred within a 5 base guanine tract and was present in all 5 evolved lines. It should be noted that some of these mutations occurred in multiple lines or have been described in past literature (Table 3.1), strongly supporting the conclusion that these are defined sites of increased mutability and sources of phase variation based on the instability of homopolymeric DNA in *C. jejuni*.

Isolation of a spontaneous motility mutant and experimental selection of a motile revertant

Our evolution experiment shows that motility deficient cells are rapidly selected in *C. jejuni* during laboratory culture, and we predicted that this might lead to spontaneous motility-deficient mutants in stock cultures. Indeed, when 20 individual colonies from a wild-type NCTC11168 culture were spotted onto semi-solid agar we observed subclonal motility phenotype variation. Specifically, 14 of 20 colonies showed little to no spreading during semi-solid agar culture indicating that this NCTC11168 stock was mostly made up of non-motile cells.

From our stock culture a non-spreading mutant was isolated and stored frozen. Twenty colonies from this putatively non-motile stock were assayed for spreading on semi-solid agar, and zero colonies spread by flagellar motility from the point of inoculation. However, when these non-motile cells were left on the semi-solid agar for an extended incubation, offshoots of presumed motile *C. jejuni* cells arose (Figure 3.4a), indicating that the motility loss was phase variable. Cells from one of these offshoots were isolated and stored. When colonies from this variant were assayed for motility in semi-solid agar, all 20 colonies were relatively uniform in spreading phenotype, spread

significantly more than the nonspreading control ($P < 0.0001$) by approximately 3-fold, but spread on average approximately 70% as much as the wild-type culture. Based on the phenotypes described we refer to the original mixed-motility stock culture as 11168wt, the non-motile isolate as 11168mot⁻, and the motile revertant as 11168mot⁺.

By genome sequencing we discovered that the motility defect of 11168mot⁻ was due to a single nucleotide deletion in an eight base homopolymeric thymine tract within the open-reading frame of *fliR*, a gene that codes for a structural protein of the flagellar T3SS in *C. jejuni* (56). This mutation shifts the *fliR* open-reading frame to introduce a stop codon after 39 amino acids; a significant truncation considering the full-length ORF is predicted to be 255 amino acids. No mutations in *fliR* were detected during broth experimental evolution, but as is the case with *flhA*, *flhB*, *flgS*, *flgR* and *fliP*, *fliR* disruption results in repression of the σ^{54} regulon (76). Consistent with our motility phenotype observations, this deletion was present in approximately 70% of the reads in 11168wt culture and 100% of reads for 11168mot⁻ (Figure 3.4b). Selection for motility on semi-solid agar resulted in the re-insertion of an adenine residue in this tract to restore the *fliR* ORF, and only the allele that generated the full-length ORF was present in 11168mot⁺ (Figure 3.4b). The presence of motile and non-motile cells within a stock culture of *C. jejuni* as a consequence of a reversible mutation to disrupt σ^{54} expression is entirely consistent with our finding that motility is lost by mutations affecting σ^{54} expression during laboratory evolution as described above.

Finally, it must be noted that genome sequencing revealed subtle genetic differences between 11168wt, 11168mot⁻, and 11168mot⁺. We found that 11168mot⁻

and 11168mot⁺ contained a nonsynonymous single nucleotide polymorphism (E179K) in the non-heme iron protein, *rrc* (*Cj0012c*), which was not detectable in 11168wt. Also, there were significant differences in the frequency of alleles present in some contingency loci caused by homopolymeric guanine tract hypervariability (data not shown).

11168mot⁻ is deficient for mouse colonization and the *in vitro* revertant, 11168mot⁺, is partially restored for mouse colonization

After defining the genotype and reversible motility phenotype of 11168wt, 11168mot⁻, and 11168mot⁺ we sought to assess the ability of these variants to colonize the C57BL/6 IL-10^{-/-} mouse model of campylobacteriosis (122). As can be seen in Figure 3.5, 11168wt was being shed in feces at a significantly higher level than the 11168mot⁻ variant at day 1 and day 8 post-inoculation for both independent experiments ($P < 0.05$ by ANOVA). *In vitro* reversion of 11168mot⁻ back to the motile phase did not fully complement the mouse colonization defect of 11168mot⁻. The 11168mot⁻ strain colonized 2 mice and the motile revertant (11168mot⁺) colonized 3 mice when inoculated concurrently. When all mice were considered, the level of 11168mot⁺ colonization was not statistically different than 11168mot⁻. However, the average level of 11168mot⁺ colonization from day 8 feces and day 10 cecal tissue (1.0×10^6 CFU/gram feces; 6.7×10^8 CFU/gram tissue) was significantly greater than 11168mot⁻ (1.8×10^3 CFU/gram feces; 7.8×10^6 CFU/gram tissue), if mice that were not detectably colonized were excluded from the statistical analysis ($P < 0.01$, ANOVA).

Intriguingly, although not statistically significant, the mice that were colonized with 11168mot+ actually had higher average loads of *C. jejuni* from fecal and cecal samples than the mice colonized with 11168wt (2.3×10^5 CFU/gram feces; 2.8×10^8 CFU/gram tissue). This indicated that although 11168mot- initially colonized some mice, it was never able to reach loads as high as 11168wt or 11168mot+ by 10 days post-inoculation.

Two mice were colonized with 11168mot- despite the initial motility defect of this variant. When reisolated *C. jejuni* from the cecal tissue of these mice were assayed by pour-plate, all detectable CFUs were highly motile, indicating that 11168mot- had reverted to a motile form *in vivo*.

DISCUSSION

Genome studies suggest *Campylobacter jejuni* may be particularly evolvable due to a lack of mutational repair pathways (98), few instances of genetic redundancy (148), and the presence of hypermutable homopolymeric DNA (93, 148). Researchers have described large intragenomic rearrangements (166), horizontal genetic exchange (32), homopolymeric adenine and thymine tract indel mutations (72), and allele frequency changes in contingency loci (11, 93, 193) during *in vivo* growth of *C. jejuni*. These studies show that *C. jejuni* has the ability to evolve in real-time during host infection. In this work we sought to understand how *C. jejuni* evolves outside of the host during continuous culture in laboratory media.

We discovered that in 5 independently evolving populations motility was quickly lost through parallel erosion of the σ^{54} regulon. Since motility is an important colonization determinant, this result is consistent with anecdotal evidence suggesting that multiple passages of *C. jejuni* in the laboratory leads to attenuation in animal models (Mansfield LS, unpublished data). Spontaneous loss of motility has been observed in *Campylobacter* (21), and in cases where the genetic basis of motility loss was determined, ORF-disrupting indel mutations in *flgR*, *flgS* and *flhA* were discovered (72, 75, 147). Consistent with our data these mutations were found in stocks that had been cultured in the laboratory, and they disrupt downstream expression of σ^{54} (76). In addition, Gaynor *et al.* described extensive virulence differences in the original clinical isolate of strain NCTC11168 (11168-O) and the genome sequenced variant (11168-GS)

that included a severe motility defect in 11168-GS (55). Considering the pleiotropic effects of flagellar motility loss on virulence traits (65), and our finding that motility deficiency is selected during laboratory culture, it is possible that the attenuated 11168-GS isolate from Gaynor's study had acquired a motility-disrupting mutation during routine culture. This would be analogous to the 11168mot- variant (*fliR* mutation) described in this work, or the *flgR*, *flgS*, or *flhA* mutants described previously (72, 75, 147).

Although the parallel loss of a sigma factor encoding gene has not been reported, loss of sigma factor function by point mutation and small indels during experimental laboratory adaptation of *E. coli* have been observed (28, 142). It has been proposed that mutations affecting regulatory hubs such as sigma factors result in large expression changes within the cell and can therefore yield a large fitness benefit. In some cases, these mutations of large effect have been shown to increase fitness during experimental evolution, and have occurred in parallel (27, 29). As an example, adaptive evolution experiments using *E. coli* have shown that mutations in *rpoS*, the alternative sigma factor necessary for mounting a stress response, are frequently observed in parallel (50, 142). It is hypothesized that an altered balance between self-preservation and nutritional-competence (SPANC) may be achieved by functional changes to an alternative sigma factor such as RpoS to relieve competition for RNA polymerase to bind with the housekeeping sigma factor (σ^{70}) (49).

In *C. jejuni*, σ^{54} reportedly contributes to a motility phenotype (92), and resistance to some stresses (89). These are two phenotypes not necessary in our

experimental evolution conditions, and the biosynthesis of flagella is presumably energetically costly for *C. jejuni*. The energy cost associated with flagella biosynthesis is implicated in the loss of flagellar gene expression in laboratory evolved *E. coli* (29, 43). It is likely that the σ^{54} loss we observed promotes faster *C. jejuni* growth through decreased expression of multiple unnecessary flagellar proteins, and decreased competition for RNA polymerase binding with the housekeeping sigma factor, σ^{70} .

In this work we report mutations in genes that affect downstream expression of σ^{54} through known regulatory networks (*flhA*, *flhB*, *flgS*, *flgR* and *fliP*), and also in the σ^{54} -encoding gene, *rpoN*. Multiple large DNA deletions that removed the majority of the *rpoN* reading frame were detected. In some of the deletion events, genes downstream of *rpoN* were also lost from the genome. Loss of downstream genes was likely collateral to the removal of *rpoN*, considering our evidence that σ^{54} loss was the beneficial mutation, and that a number of the downstream ORFs are pseudogenes. These large DNA deletions are genetically irreversible. On the contrary, other mutations we detected were in tracts of homopolymeric DNA, which are likely subject to a higher mutation rate and are reversible by slipstrand mispairing mutations in *C. jejuni* (148). One notable homopolymeric tract mutation occurred within a 5 base guanine tract within a gene encoding the flagellar motor protein, *motA*, and was predicted in every population. This mutation is intriguing considering the hypervariability that occurs in longer (≥ 8 base) homopolymeric guanine tracts in *C. jejuni* (93, 148). Presumably homopolymeric guanine tract mutations occur at a lower rate for shorter tracts, but considering this

mutation occurred in all lines suggests that ORF-disrupting indel mutations in short guanine or cytosine tracts are a biologically relevant source for *C. jejuni* genomic diversity. Finally, we show evidence for multiple previously undescribed mutations in long and/or short homopolymeric adenine and thymine tracts in *flgS*, *flgR*, *flhA*, *rpoN*, and *pflA* that are expected to result in reversible motility loss. Homopolymeric adenine and thymine tracts are ubiquitous in the AT-rich *C. jejuni* genome, and our data is consistent with other work suggesting these are a substantial source of genomic diversity (72), and therefore contribute to evolvability.

The biological significance of reversible motility loss through homopolymeric adenine tract mutations for *C. jejuni* pathogenesis has not been determined. The reversibly non-motile *C. jejuni* isolate (11168mot-) in this work was most often cleared from the mouse gastrointestinal (GI) tract before it could establish infection in the C57BL/6 IL-10^{-/-} mouse model, or else colonized to significantly lower levels than wild-type (11168wt) and the *in vitro* revertant (11168mot+). Although the mutation in 11168mot- was readily reversible on semi-solid agar through slipped strand mutation in a homopolymeric adenine tract, *in vivo* restoration of motility occurred at a low rate such that we only detected *C. jejuni* in 2 of 10 11168mot- inoculated mice. From this we conclude that reversible motility loss is highly detrimental for *C. jejuni* fitness in this non-avian animal model of infection. In contrast, Hendrixson showed that a reversibly non-motile isolate was able to consistently colonize the chicken gastrointestinal (GI) tract, but only to low loads unless reversion to the motile phenotype was selected *in vivo* (72). It is possible that the avian intestine, where *C. jejuni* is considered a commensal organism (198), is more permissive to colonization by motility defective cells than the

mouse GI tract, providing a reservoir for the expansion of mutational diversity, and increased colonization capacity by selection for motility. Within an avian host, *C. jejuni* may also be more exposed to attack by phage that attaches to *C. jejuni* flagella (30), such that reversible flagella expression could act as a mechanism for phage avoidance. Nevertheless, based on our data and given the importance of flagellar motility for colonization in this pathogen that only replicates naturally within a host, it may be wise to consider this alternative hypothesis: reversible motility expression is not necessary for *C. jejuni* pathogenesis, and is simply a consequence of high evolvability and selective pressure against flagella production during laboratory culture.

We also report here that the restoration of motility *in vitro* did not completely restore the degree of spreading on semi-solid agar, or the ability to colonize mice. The incomplete complementation of the phenotype, despite complementation of the *fliR* frameshift mutation, suggests that subtle genomic differences in 11168mot⁻ also contributed to the motility and colonization deficiency. Indeed, we discovered a nonsynonymous single base substitution in *rrc* that was present in the motility mutant (11168mot⁻) and the revertant (11168mot⁺), but not wild-type (11168wt). In this case, the modified amino acid is not conserved or part of any known functional motif in homologous proteins from other bacterial species (195), but the phenotypic effect of this change, if any, is unknown. We also could observe differences in homopolymeric guanine tract lengths in contingency loci between 11168wt, 11168mot⁻, and 11168mot⁺, and these genes have been shown to be important for *C. jejuni* pathogenicity in this model (93). Genomic differences in these variants derived from the same stock through minimal laboratory culture, are likely inevitable as a consequence of the hypermutability

of the *C. jejuni* genome, are only detectable when the entire genome is re-sequenced, and may contribute to false positive and negative results when analyzing cloned *C. jejuni* strains.

In order to persist, organisms must maintain a balance between genome stability (genome robustness), and the ability to adapt by natural selection (genome evolvability) (111). Mutations are necessary for evolvability. The rapid accumulation of reversible mutations in *C. jejuni* likely increases evolvability, and in contrast to irreversible mutations, may actually maintain genomic robustness since some cells within a population are always one high-rate mutation away from restoration of the ancestral phenotype. Here we observed reversible and irreversible loss-of-function mutations, and the pattern of evolution was characterized by reversible loss of function, before an irreversible loss of function. This suggests that one mechanism used by *C. jejuni* to attain high evolvability without sacrificing robustness is through high-rate reversible mutations that allow a rapid exploration of the fitness landscape, before total function loss during prolonged selective pressure. This fast and reversible pattern of adaptive evolution may contribute to the ability of *C. jejuni* to colonize a diverse host range, and might also be relevant for other highly mutable bacteria such as pathogenic *Helicobacter* species.

METHODS

Ethics Statement

All mouse experiments were performed according to recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were also reviewed and approved by the Michigan State University Institutional Animal Use and Care Committee (approvals 04/07-030-00 and 06/09-092-00).

Bacteria

Campylobacter jejuni NCTC11168 was used for the evolution experiments described in this study. The frozen stock used was originally obtained from the American Type Culture Collection (ATCC 700819) and had been passaged on agar plates fewer than 4 times before being stored frozen at -80°C in 15% glycerol/tryptic soy broth (TSB).

Unless otherwise noted, *C. jejuni* cultures were grown from freezer stocks by re-streaking onto Bolton (Oxoid) agar plates and incubation for 48–72 hours at 37°C in gas exchange jars in a microaerobic environment generated by atmosphere evacuation, and equilibration with a gas mixture of 80% N_2 , 10% CO_2 , and 10% H_2 .

Strain 11168mot⁻ was isolated as a single colony from an NCTC11168 freezer stock, and was subsequently found to be deficient in spreading by the semi-solid agar motility assay described below. To generate 11168mot⁺, cells from a primary streak of 11168mot⁻ were used to seed the center of a semi-solid Bolton agar plate. Plates were

incubated and monitored at 48, 72 and 96 hours. After 96 hours, a sterile inoculating loop was used to touch the outermost ring of spreading cells (black arrow in Figure 3.4a) before streaking onto a new Bolton agar plate. The new plate was incubated for 48 hours before cells were harvested and stored as a freezer stock. We then analyzed this culture by the semi-solid agar motility assay to confirm an increased spreading phenotype, and dubbed it 11168mot+.

Experimental evolution in broth medium

From a previously published experiment from our lab (13), we had saved host-adapted *C. jejuni* NCTC11168 cultures that had been passaged through the C57BL/6 IL-10^{-/-} mouse model. Mouse passage was found to alter only the frequency of alleles present in contingency loci in these populations (93). We grew *C. jejuni* from cultures reisolated from 5 individual mice that had been through two passages of mouse infection. These 5 non-clonal lines were used to seed five 25 cm² flasks containing 10 ml autoclaved/filter sterilized Bolton Broth with approximately 1x10⁸ cells per flask. Flasks were placed in a gas exchange jar in a microaerobic environment generated by atmosphere evacuation and equilibration with a gas mixture of 80% N₂, 10% CO₂, and 10% H₂. The jar was then incubated at 37^o C with 200 r.p.m. agitation in a tabletop shaker. Every 24 hours 100 ul of culture was transferred to a flask containing 9.9 ml fresh media (1:100 dilution), as has been done in other evolution experiments (112).

The 5 lines were kept as independently evolving populations for 35 days and cultures were saved at days 1–5, 10, 15, 25, and 35 at -80°C in 15% glycerol/TSB.

To estimate the cell density achieved, and the number of doublings, during 24-hours of culture, samples from day 1 and day 35 freezer stocks were first grown on Bolton agar plates, re-suspended to equal OD₆₀₀ densities of approximately 0.3, and grown under the experimental evolution conditions for approximately 24-hours. For each time point, one hundred microliters of the 24-hour culture was used to seed each of three replicate flasks containing 9.9 ml of fresh media. Serial dilution and plating of the inocula and test cultures after 24 hours of growth under the experimental evolution conditions was performed. The difference in the number of doublings at each time point were considered significant by a P-value less than 0.05 by Student's t-test.

Semi-solid agar motility assays

To determine the motility of an entire *C. jejuni* population within a freezer culture, *C. jejuni* cells were streaked onto Bolton agar plates and grown for 48 hours. The flat end of an autoclaved wooden applicator (Puritan[®], Guilford, Maine) was then pressed onto *C. jejuni* cells in the primary streak and transferred to semi-solid (0.4% agar) Bolton agar plates and incubated at 37°C , 10% CO₂. The degree of spreading was monitored at 24 and 48 hours. Images were recorded after 48 hours for analysis. For 11168wt, 11168mot⁻, and 11168mot⁺ we used the pictures to measure the amount of spread using ImageJ software (2). Since spreading occurred concentrically during

growth up to 48 hours we used ImageJ to measure the distance from the point of inoculation to the edge of the spread (the radius). This distance was normalized to the spreading distance of the positive control (11168wt from primary streak) on each plate. The normalized data from 11168mot- and 11168mot+ were compared to each other by a two-sample t-test, or to a hypothetical mean of 1 (the normalized distance of the positive control) by single sample t-test and were considered significant at $P < 0.0001$.

Finally, for strains 11168wt, 11168mot-, and 11168mot+ we spotted 20 single colonies onto semi-solid agar plates. Two colonies per variant were assayed for their ability to spread on the medium per semi-solid agar plate. Primary streaks of 11168wt and 11168mot- variant were also inoculated on each plate as positive and negative spread plate controls respectively. A colony was considered motile if the degree (radius) of spreading at 48 hours was greater than the non-spreading 11168mot- population that grew on the semi-solid agar, but did not spread outward by flagellar motility.

Pour-plate motility assays

To observe motility phenotypes of each evolved line during the time course of the broth evolution experiment we performed pour-plate assays as described by Caldwell *et al.* (21), with a few modifications. For our assays we used Bolton medium with 0.33% agar cooled to 42°C. *C. jejuni* cells were then added to the liquid agar solution and allowed to solidify and dry for 2–3 hours at room temperature. We aimed to dilute each sample to achieve a plate count of approximately 10-100 CFUs per plate, with 2 or 3 plates cultured per sample. These plates were incubated at 37°C in a 10% CO₂, non-humidified incubator for 48 hours or less to differentiate motile and non-motile CFUs. All

non-motile CFUs were counted and marked on the plate, before extended incubation for a further 24–72 hours to determine the number of initially non-motile CFUs that could revert to the motile form. Motile CFUs never formed a dense colony of growth, but rather a large, diffuse zone of spreading in the pour-plate by 48 hours, while non-motile CFUs formed a dense, orange colony within the media by 48 hours. Reversibly non-motile colonies were characterized by the appearance of a diffuse spreading originating from the dense, orange colony after extended incubation. CFUs of the three mutually exclusive phenotypes: motile, reversibly non-motile and irreversibly non-motile, were counted. As expected, all CFUs in the 11168mot- population were reversibly non-motile by this assay. Examples of each CFU type are shown in Figure 3.2a.

Fisher's exact test was used to determine statistically significant differences in the frequency of motile and non-motile CFUs between days 0 and 5 or days 0 and 35. This test was also used to show significant frequency differences of reversibly and irreversibly non-motile CFUs between days 5 and 35 or 10 and 35. P-values less than 0.05 were considered significant.

Genome sequencing and analysis

Samples to be sequenced were re-streaked from frozen cultures onto tryptic soy agar supplemented with 5% defibrinated sheep's blood. The entirety of the streak plate was resuspended in tryptic soy broth and pelleted before DNA extraction using the QIAGEN DNeasy Blood and Tissue kit per the manufacturer's instructions. Sequencing was performed at the Michigan State University Research Technology Support Facility (MSU RTSF) on an Illumina Genome Analyzer IIx according to the manufacturer's

instruction. Briefly, libraries of each sample were prepared with the Illumina TruSeq kit. Twelve samples were given unique multiplex ID tags (barcodes) and pooled for sequencing in one lane. Reads that passed filtering were sorted according to their respective barcode sequence for analysis. Read files were deposited at NCBI in the Sequence Read Archive (Submission Accession Number SRA049039; Study Accession Number SRP011023).

The *breseq* pipeline was used to predict consensus mutations present in all individuals in a population and mutations present in only a certain percentage of individuals in a sample. The *breseq* pipeline is described in documentation included with the freely available source code (<http://barricklab.org/breseq>). The *C. jejuni* NCTC11168 genome sequence (RefSeq:NC_002163.1) was used as the reference for mapping reads. Consensus mutation predictions were essentially as described previously (10). They included predictions of new junctions from split-read matches, which can define the endpoints of deletions or locations of new mobile element insertions, and predictions of large deletions from regions lacking read coverage.

Point mutations present at intermediate frequencies in the population were predicted according to a procedure described previously (9) but with relaxed parameters that required only 90% of the length of a read to match the reference genome for inclusion in the analysis. Percentages of large deletions, such as those reported for *rpoN* in each population, were estimated by counting reads supporting the unique sequence junction formed by the deletion versus the number of reads supporting the mutually exclusive junction in the ancestral genome sequence indicating no deletion, as in a study of mobile-element insertions in mixed *E. coli* populations (136). Overall read

coverage across the *rpoN* gene region was checked to be sure it was consistent with deletion frequencies predicted by this procedure.

A subset of mutations predicted from the *breseq* pipeline were assayed with an alternative method to validate their presence in the population. To assay for large genome deletions of the *rpoN* gene region, the Roche Expand Long Template PCR system and/or the Invitrogen Platinum[®] *Taq* DNA Polymerase PCR system was used according to the manufacturer's instructions to amplify across the putatively missing regions of DNA. The presence of PCR products associated with deletions (or lack of deletions) predicted by *breseq* was visualized by agarose (0.8%) gel electrophoresis. DNA from the ancestral *C. jejuni* populations was used as a negative control.

To confirm small insertion or deletion mutations predicted to be present at relatively low frequency in the evolved populations, the Invitrogen Platinum[®] *Taq* DNA Polymerase PCR system followed by Sanger sequencing at the MSURTSF was performed. From the sequencing chromatograms, a minor set of shifted peaks after the predicted indel that corresponded to the length of the indel was considered positive confirmation of the *breseq* prediction. DNA from the ancestral *C. jejuni* populations was used as a negative control.

Mouse infection experimental design

All mouse breeding, husbandry, randomization to different treatments, inoculation and other procedures are described in detail by Mansfield *et al.* (122). Briefly, C57BL/6 IL-10^{-/-} mice were conventionally reared in a specific pathogen free environment at

Michigan State University. Prior to inoculation mice were transferred to the University Research Containment Facility, separated into individual sterile filter-top cages, and fed a 12% fat diet (Harlan Teklad diet 7904) with sterile water *ad libitum*.

Inocula were prepared and delivered orally to 8-10 week old mice as described in Mansfield *et al.* (122). Equal amounts of *C. jejuni* 11168wt, 11168mot-, or 11168mot+ (approximately 1×10^8 CFU) were inoculated into 5 mice per variant. For the first experiment (Experiment 1) only 11168wt and 11168mot- were compared in 5 mice each. Subsequently all three strains were screened concurrently (Experiment 2) in 5 mice each. Serial plating and counting of CFUs in cultures before and after inoculation confirmed that viability was not altered during the inoculation process. After inoculation, mice were checked each day for the presence of clinical signs indicative of enteritis. At 10 (Experiment 2) or 11 (Experiment 1) days post inoculation, mice were humanely euthanized by CO₂ overdose and necropsied.

***C. jejuni* colonization in mouse fecal and cecal samples**

Feces were collected from each mouse on day 1 and day 8 post-inoculation. A fecal pellet was collected into 1.5 ml microcentrifuge tubes and kept on ice. Four hundred microliters of TSB/15% glycerol was added to fecal pellets before they were homogenized using a wooden applicator (Puritan[®]) and stored at -80°C . Serial dilution and plating of fecal slurries was performed to determine colonization level. Five dilutions were plated per sample onto Bolton agar supplemented with the antibiotics cefoperazone (25 µg/ml), vancomycin (10 µg/ml), and amphotericin B (2 µg/ml) and

incubated at 37°C in a gas exchange jar containing 80% N₂, 10% CO₂, and 10% H₂ for 72 hours. One hundred microliters of the slurry was also placed into a pre-weighed microcentrifuge tube and dried for 72 hours at 70°C. The weight of the dried fecal slurry was measured and used to normalize the amount of feces plated between samples. Results are presented as CFU per gram dried feces. Differences in means between groups were deemed statistically significant at P<0.05 using one-way analysis of variance (ANOVA) with statistically different means separated by the Tukey HSD test. Data from Experiments 1 and 2 was kept separate for statistical analyses.

During necropsy the cecum was extracted and placed into a petri plate containing approximately 20 ml phosphate buffered saline. Contents in the cecum were lightly scraped away from the tissue using a scalpel and forceps. A piece of cecal tissue was saved on dry ice, and then approximately 10 millimeters of cecum that included the cecal apex was placed into a microcentrifuge tube containing 400 µl sterile TSB. Tissue was ground using a cordless pellet pestle motor (Kontes) with an autoclaved microcentrifuge pestle. Grinding was performed to yield a homogenous TSB/tissue slurry, and only a small piece of white connective tissue remained. Samples were then stored on ice until CFU per gram cecal slurry was determined by serial plating as described for fecal samples.

After CFU counts were made from fecal or cecal samples, re-isolated *C. jejuni* samples were saved in TSB/15% glycerol and stored at -80°C.

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Table 3.1 Mutations predicted to disrupt flagellar motility in evolved populations.

Gene	Genome Position	Mutation ^a	Estimated frequency ^b	Sequence repeat length ^c	Population ^d
<i>flgS</i>	743561	+A ^e	2.8, 2.8%	7	2, 5
	743832	+T	6.2, 1.5%	5	<u>2</u> , 4
	743842	E162*	2.6%		1
	743936	+T	3.1%		5
<i>flgR</i>	955527	ΔA	6.1%	4	<u>5</u>
	956188	+T ^f	2.5, 4.0%	8	2, 3
	956457	ΔT	2.5%	8	1
<i>flhA</i>	818832	+A	3.0%	3	2
	819015	+TA	2.3%	2	2
	819272	+G	1.9%		3
	819285	Q157*	3.6%		2
	819550	+T	5.1%		1
	819366	Δ399 bp	10.6%		1
	818865	Δ12 bp	1.1%		2
	818853	Δ225 bp	1.3%		3

Table 3.1 (cont'd)

<i>rpoN</i>	624667	ΔGA	11.4%		5
	624837	AAAG → TCAGGA	2.6%		5
	624428	Δ3490 bp	3.4%		<u>1</u>
	625292	Δ993 bp	19.8%		<u>2</u>
	624741	Δ5143 bp	11.6%		2
	624194	Δ5809 bp	57.9%		<u>3</u>
	625117	Δ3579 bp	19.4%		3
	625149	Δ414 bp	15.4%		3
	624211	Δ8448 bp	100%		4
	624832	Δ1181 bp	30.3%		<u>5</u>
	624622	Δ3463 bp	2.9%		<u>5</u>
	624842	Δ21 bp	3.3%		5
<i>fliP</i>	768476	+A	1.8%	7	5
<i>motA</i>	305107	ΔC	29.2, 3.3, 27.9, 5.7, 5.7%	5	1, 2, 3, 4, 5
<i>pflA</i>	1494929	+A	10.5%	3	1
<i>fliF</i>	289652	Δ96 bp	15.5%		1

Table 3.1 (cont'd)

<i>Cj0390</i>	355937	ΔA	15.8%	3	5
	356475	ΔC	1.6%		1
	357769	Δ9	30.6%, 23.2%, 18.4%, 39.6%, 21.5%		<u>1</u> , 2, 3, 4, 5

^a + indicates an insertion of the following base(s); Δ indicates a deletion of the following

base(s), or for longer deletions Δ is followed by the number of bases deleted; *

symbolizes a stop codon.

^b When the same mutation was found in multiple populations, the estimated frequencies observed for each population are listed in numerical order.

^c When a mutation occurred in a homopolymeric tract or sequence repeat of two or more units, the number of repeat units observed in the ancestral genome at the site is listed here. These mutations likely occurred by a slipstrand mechanism and are potentially reversible. Nothing is listed in this column if the mutation did not occur in a DNA sequence repeat.

^d Population numbers that are underlined identify mutations that were assayed and confirmed by an alternative method to the Illumina data as described in the Materials and Methods.

^e This mutation has been reported previously by Hendrixson (75).

Table 3.1 (cont'd)

^f This mutation is in a phase variable region identified by Hendrixson (72), but mutation in this particular thymine tract was not previously reported.

Figure 3.1 Motility loss during adaptive laboratory evolution. (A) Fractions of motile cells in each population over the time course of the evolution experiment. * $p < 0.05$ by Fisher's exact test for difference from day 0 between motile and non-motile CFUs for all 5 independent populations. (B) Semi-solid agar spreading phenotypes of a representative population over the time course of experimental evolution. The degree of spreading is representative of flagellar motility.

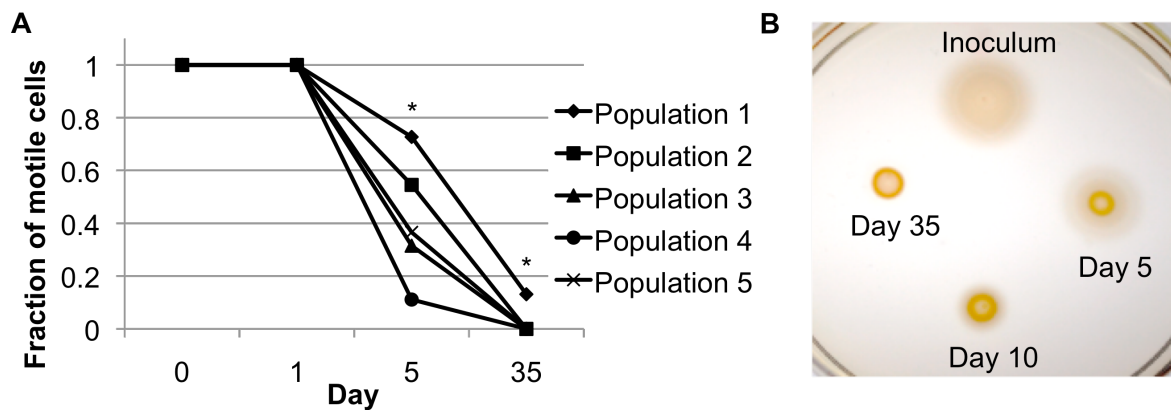


Figure 3.2 Reversible versus irreversible motility during adaptive laboratory

evolution. (A) Phenotypes of motile, irreversibly non-motile, and reversibly non-motile CFUs in the pour-plate assay. From top left clockwise: Irreversibly non-motile CFU, extended incubation (120 hours); motile CFU, standard assay incubation time, (48 hours); reversibly non-motile CFU, extended incubation (72 hours); reversibly non-motile CFU, extended incubation (120 hours). (B) The percentage of reversibly non-motile, irreversibly non-motile, and motile CFUs at day 10 and day 35 were plotted for each population. * $p < 0.01$ and ** $p < 0.00015$ by Fisher's exact test for difference between reversibly and irreversibly non-motile CFUs at day 10 or day 35.

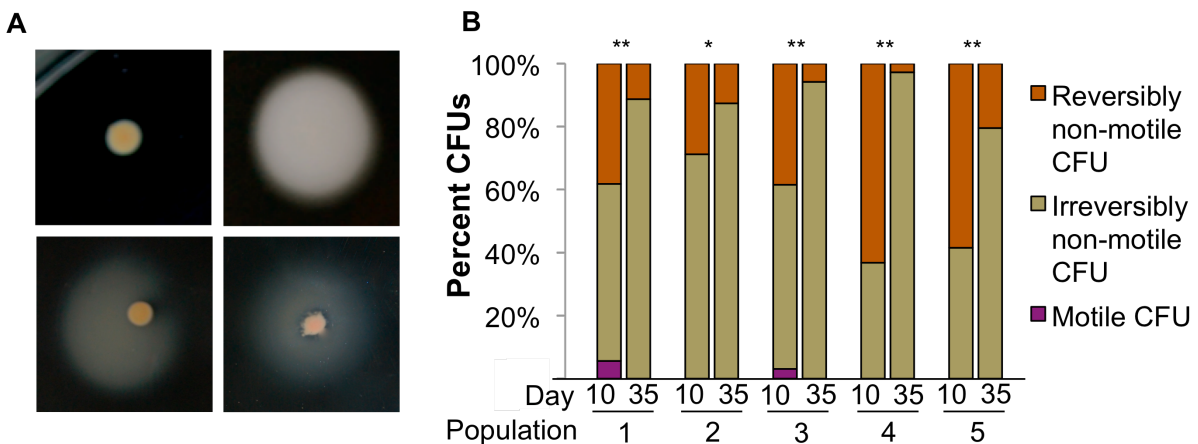


Figure 3.3 Genomic changes in evolved populations that are predicted to disrupt motility. The ancestral, mouse passaged inocula are represented by the inner black circle. Experimentally evolved lines are pictured as green circles with the innermost circle representative of Population 1 and the outermost circle representative of Population 5. Different types of mutations are indicated on each genome according to the key. Some lines were predicted to have multiple subpopulations of *rpoN* genome deletions with varying lengths, but this is not represented here: see Table 3.1 for details. Underlined gene names indicate genes that are predicted to decrease expression of σ^{54} and the genes controlled by this sigma factor.

Figure 3 (cont'd)

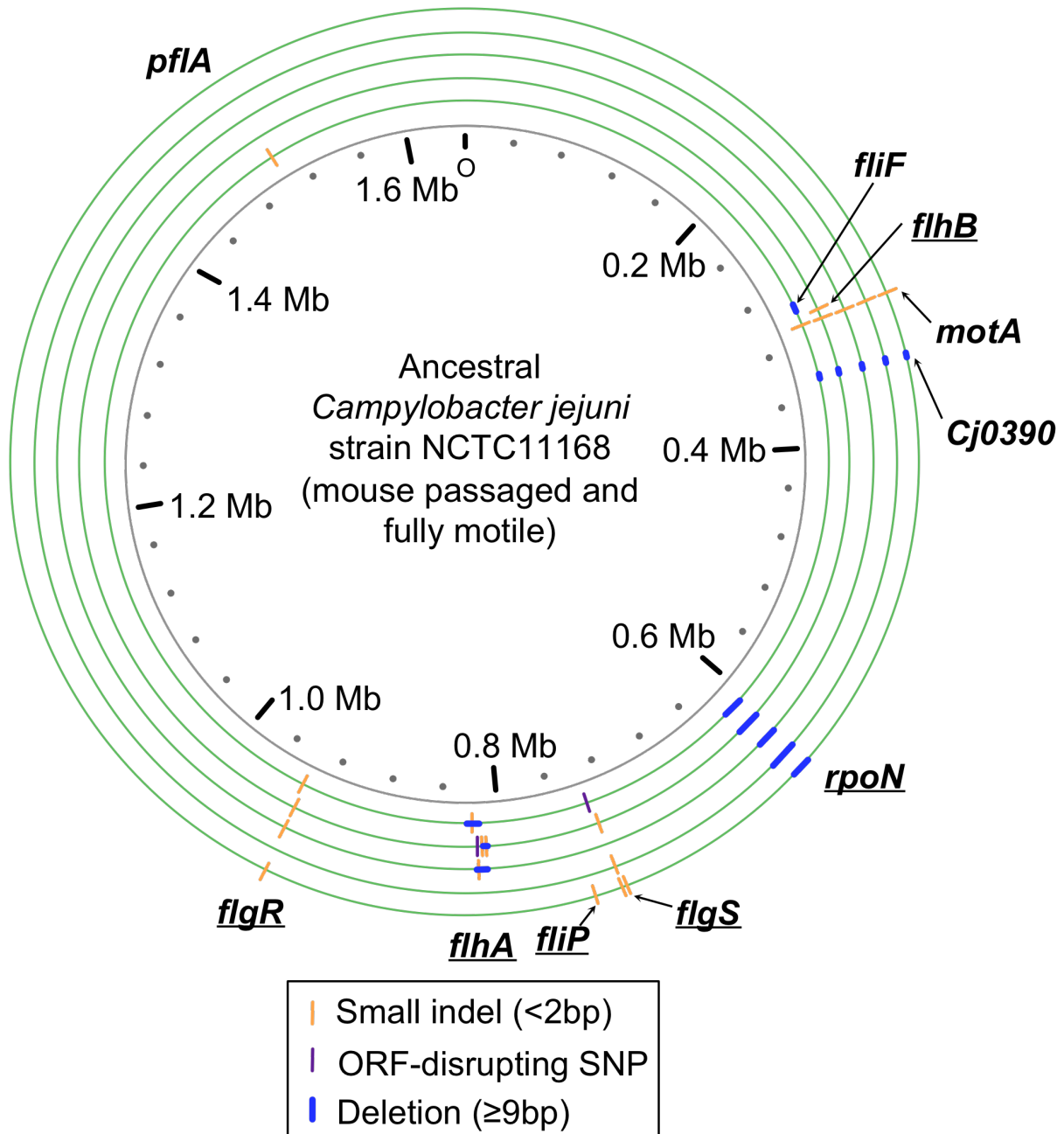


Figure 3.4 Phenotype and genotype data for 11168wt, 11168mot- and 11168mot+.

(A) Spreading phenotypes of the 3 variants after incubation on semi-solid agar. Plates were imaged after 48 hours for each variant. Also pictured is the 11168mot- variant after extended incubation (96 hours) to show the selection for motile revertants. The two images of 11168mot- are from the same plate incubated for different times and the black arrow indicates the location where cells were isolated to generate 11168mot+. Variants and incubation times are listed on the figure. (B) The predicted frequency of full-length FliR open-reading frame alleles present in each variant by the Illumina sequence data is plotted. This frequency is based on the number of sequence reads with an 8 base homopolymeric adenine tract (full-length ORF), or 7 base tract (truncated ORF by frameshift mutation). The observed frequency of motile CFUs for each population is listed above each bar showing the tight association of the population level motility phenotype to *fliR* genotype.

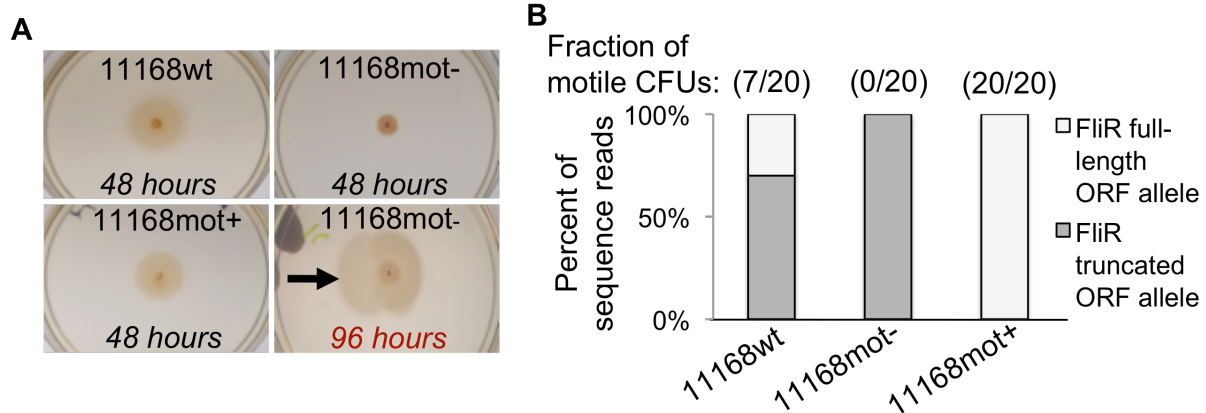
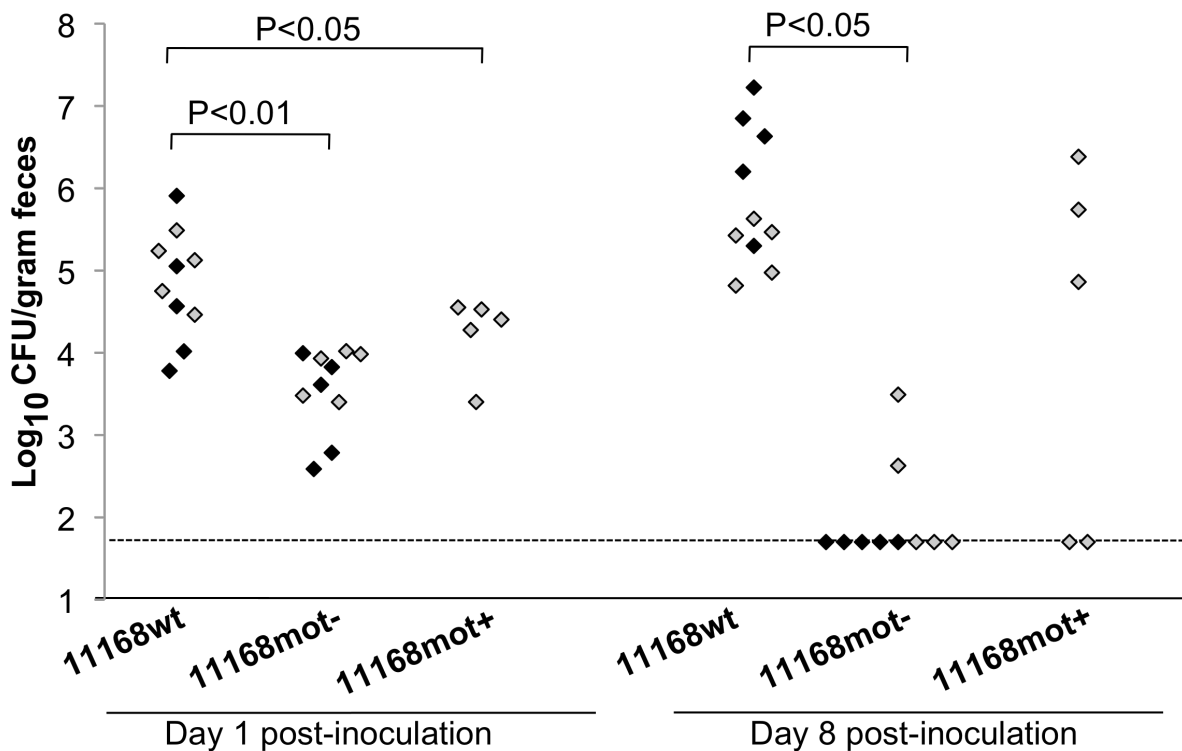


Figure 3.5 Mouse colonization of 11168wt, 11168mot-, and 11168mot+. The *C. jejuni* load in each mouse was estimated from fecal samples collected at day 1 and day 8 post-inoculation, and plotted here as $\log_{10}$ CFU per gram dried feces. Black or grey diamonds distinguish mice that were sampled in separate experiments (Experiment 1 is in black; Experiment 2 is in grey). The dashed line indicates the limit of detection (1.69 $\log_{10}$ CFU per gram dried feces) by culture, and mice in which no *C. jejuni* were detected are plotted on this line. P-values are shown above groups with means deemed significant by ANOVA from Experiment 2 data, and more detailed statistical analyses are described in the text.



CHAPTER 4

Conclusion

SUMMARY

Campylobacter jejuni is a prevalent foodborne pathogen of humans. Despite this, little is known about the molecular factors that are necessary for *C. jejuni* pathogenesis. This is partly due to the lack of a small animal model of *C. jejuni* colonization and enteritis until recently (122). Also, the evolutionary history of *C. jejuni* is not likely to have yielded specific adaptations to human infection. This is because human-to-human transmission does not seem to occur, making human infection an evolutionary impasse. Therefore, the hunt for conserved human-specific virulence effectors may be in vain. Still, *C. jejuni* colonizes and causes disease in humans, so there must be factors that are necessary for this ability.

One potential virulence trait of *C. jejuni* that has been alluded to, but not directly studied until this work, is evolvability. The first genome sequence of *C. jejuni* showed the genome contained multiple hypermutable DNA sequences, and lacked some DNA repair systems (148), which lead to the speculation that the genome might be particularly prone to certain mutations, and as a consequence have high evolvability. The ability to rapidly evolve in a novel environment might partly explain the diverse range of hosts in which a single *C. jejuni* strain (like NCTC11168) can colonize and replicate.

Prior experiments in our laboratory and others, along with anecdotal evidence, suggested that *C. jejuni* rapidly adapted genetically during host infection, or during laboratory culture. Host-adaptation of *C. jejuni* seemed to result in increased ability to colonize and/or cause gastrointestinal disease, and conversely, laboratory adaptation

was suggested to lead to attenuation. These findings led us to hypothesize that specific mutations occurred in the *C. jejuni* genome during mouse serial passage, and/or experimental laboratory adaptation, that were the basis of the observed phenotypic changes. To test this we used a simple procedure that is analogous to a forward genetic screen: 1) experimentally evolve *C. jejuni* by mouse, or laboratory, passage; 2) define phenotypic changes in the evolved variants relative to the ancestral *C. jejuni*; 3) use Illumina technology to re-sequence *C. jejuni* populations pre- and post-experimental evolution to determine genetic differences; 4) verify genetic changes. This experimental pipeline was used in Chapters 2 and 3 of this dissertation to show that reversible mutations in homopolymeric cytosine/guanine (polyC/G), or adenine/thymine (polyA/T) DNA tracts, as well as large genome deletions, play a significant role in the rapid genetic adaptation of *C. jejuni* during mouse infection, and/or during laboratory culture.

Chapter 2 summary: Contingency loci are important for *C. jejuni* genetic adaptation in mice

Serial host passage of viral, bacterial, or eukaryotic parasites almost exclusively leads to increased virulence and fitness within the host, and decreased virulence to an alternate host (41). By serial passage experiments using a mouse model of campylobacteriosis, a detectable increase in virulence of some *C. jejuni* strains was observed (13). One of these strains (NCTC11168 or ATCC700819) was analyzed further. Whole genome sequencing indicated that the genome of unpassaged (wild-type) NCTC11168, which was less virulent, and passaged (mouse-adapted) NCTC11168, which was more virulent, were nearly identical except by insertion and

deletion mutations in the homopolymeric cytosine and guanine (polyC/G) tracts of contingency loci.

PolyC/G DNA has been shown to be hypermutable in *C. jejuni* (11, 148, 187), and mediates the stochastic, reversible switching of contingency genes between a functional (ON) phase, and a non-functional (OFF) phase. Contingency loci are the product of adaptive evolution for mutability (133), and are considered a bet-hedging strategy since they generate a population of genotypes from a single ancestral cell. The different genotypes, in an otherwise clonal population, may have different fitness in dynamic environmental conditions. During mouse serial passage we showed that a large increase in virulence occurred after only a single 35-day mouse infection experiment (13). The finding that only hypermutable contingency loci contained the only mutations observed (93) is consistent with such a rapid heritable phenotypic change, since contingency loci mutations are ubiquitous in almost any given population of *C. jejuni* cells. In other words, the mutations that appear to have been selected during mouse infection did not arise during infection—they were already present in the inoculum. This is necessary if contingency loci are to serve as an effective bet-hedging strategy. Overall, this work, and that of others (11, 103, 187, 193) shows that genetic diversity in contingency loci due to polyC/G mutability in *C. jejuni* should be considered a constant feature of this pathogen.

Whole genome sequence analysis, expression microarray of *C. jejuni* growing *in vivo* and *in vitro*, pulse field gel electrophoresis, and targeted gene sequencing showed that the only significant difference between the mouse evolved and ancestral populations was in contingency loci. Since the virulence phenotype of these variants

was significantly altered, this strongly suggested that the observed contingency loci changes were responsible for increased fitness and virulence of the mouse-adapted variant. The key words in the previous sentence are “strongly suggested.” This is because further proof that these contingency loci were driving the observed phenotypic change would involve satisfying Falkow’s molecular Koch’s postulates (46). However, many of these postulates are difficult to satisfy when a pathogen genome is not static, as is the case of the *C. jejuni* genome that actually exists as a population of genotypes. Also, since 13 contingency genes were significantly changed during passage, it is possible that some combination of those 13 changes would be necessary to reproduce the evolved population phenotype. Despite the imminent pitfalls, we removed a contingency locus (*Cj1296/7*) that appeared to be under selection for the ON phase from the ancestral genome and assessed the virulence phenotype: we found no significant difference between wild-type and knockout (data not shown). In any case, what we really wanted to know was not which specific contingency loci were important in the mouse model, but whether contingency loci mutability in general was necessary for genetic adaptation to increased virulence. To determine the importance of contingency loci mutability for *C. jejuni* pathogenesis gene we would need a method to remove, and then reintroduce, the mutability of *C. jejuni* polyC/G DNA. Unfortunately too little is currently known about contingency loci mutability in *C. jejuni* to alter this property in *C. jejuni* (However, see Future Directions for ideas on how to do this).

Since we did not have the tools to alter contingency loci mutability in *C. jejuni* we determined whether the contingency loci changes we observed could be explained by stochastic genetic drift, and whether certain changes occurred in parallel in multiple

mice. The result: a model of drift did not reproduce the observed changes during mouse infection, and certain loci did change to the same phase in parallel when multiple mice were examined. Still, there was some variability between mice in the contingency loci that increased in frequency during infection. Overall, Chapter 2 of this thesis shows that some contingency loci phases increased in frequency in parallel during mouse passage, that mutations were not detected outside of contingency loci by extensive genetic analyses, and that mutability of polyC/G DNA in and of itself does not explain these observed contingency loci changes.

Chapter 3 summary: Reversible and irreversible mutations occur during selection against flagellar motility in laboratory culture.

In contrast to the relatively subtle changes that occurred in contingency loci during mouse serial passage, multiple diverse mutation events were observed to occur during laboratory passage. When we adapted 5 independent populations of *C. jejuni* to rich broth media by serial transfer every 24 hours, one of the main phenotypic changes that occurred was the loss of flagellar motility. Cells that had lost motility were seen as early as day 3 during this 35-day experiment. Although, 35 days was the same number of days that *C. jejuni* colonized mice in the serial passage experiment for which only contingency loci mutations were observed, by laboratory adaptation we observed mutations outside of contingency loci that included base substitutions, indels in short and long polyA/T tracts, indels in heteropolymeric DNA, parallel deletion in a short (5 base) polyC/G tract, and the parallel loss of an alternative sigma factor in the 5 independently evolved lines by multiple distinct genomic deletions.

The 5 evolved lines in this experiment were seeded from *C. jejuni* that had been passaged through the mouse model, and contained only motile colony forming units. This indicated that motility is selected during *in vivo* passage, as has been shown for *C. jejuni* before (15, 21, 72, 94). Concurrently, when a population of *C. jejuni* that contains both motile and non-motile CFUs (11168wt), is inoculated into the C57BL/6 IL-10^{-/-} mouse model, only highly motile CFUs are reisolated from the mouse cecum. We also showed that a *C. jejuni* strain containing a mutation that disrupted motility, but is phase variable, is inoculated into this model, the only mice that are detectably colonized contained only motile revertants. Taken together, motility is essential for efficient *C. jejuni* colonization in this model.

In contrast, motility is not essential for growth in laboratory culture, and based on our data, the production of flagella is actually detrimental to fitness in this environment. Before this work, there was evidence buried in the literature to suggest motility may be selected against *in vitro*: there is data presented by Wosten *et al.* that shows an *rpoN* deletion mutant had a higher growth rate in culture relative to wild-type (194), and when exploring the rate of reversible motility switching in *C. jejuni*, Caldwell *et al.* reported an approximately 10000-fold higher rate of ON-OFF switching relative to OFF-ON switching of flagellar expression *in vitro* (21). Although neither work suggested flagellar motility deficiency increased fitness during laboratory growth, from our work it is clear that these observations are due to selection against flagellar biosynthesis during *C. jejuni* culture. Furthermore, numerous reports in the literature describe spontaneous motility deficient *C. jejuni* isolates, which has apparently lead to significant differences in the phenotype of *C. jejuni* strain NCTC11168 in different laboratories (55). Perhaps the

results presented here will shed light on this issue, and why there is often conflicting results concerning the ability of specific *C. jejuni* strains to colonize cell culture, and animal, models.

Experimental evolution in the laboratory showed that flagellar motility was lost in two stages. Initially, motility was lost in a significant proportion of the population, but the phenotype was reversible if cells were switched to an environment that selected for motility. This indicated that reversible types of mutation occurred early during laboratory adaptation, and in support of this, numerous indel mutations were discovered in homopolymeric DNA tracts that are known to be subject to phase variation in *C. jejuni*. However, prolonged selection against motility eventually lead to the irreversible loss of flagella production in all 5 independently evolved lines. Although each population had a diversity of mutations that were predicted to irreversibly disrupt motility, the dominant mutation event in 4 of 5 populations was the genomic deletion of alternative sigma factor, σ^{54} . This sigma factor is necessary for expression of multiple flagellar structural genes in *C. jejuni* (25, 76).

Finally, based on this work we suggest that *C. jejuni*'s propensity for reversible mutations through homopolymeric DNA increases robustness and evolvability of the genome. Evolvability is increased since indel mutations in homopolymeric DNA occur at a high rate in *C. jejuni*, and often effect significant functional change through frameshift mutation. An increase in mutation rate, and consequently, evolvability, is usually accompanied by a decrease in genome robustness (i.e. the ability to maintain genomic function). However, homopolymeric mutation, which seems to accumulate frequently in *C. jejuni*, is reversible. Therefore, robustness of genome function is actually partially

maintained in this system since reversion to the ancestral phenotype is accomplished by a single, high-rate mutation. Whether the reversible expression of *C. jejuni* genes by homopolymeric DNA mutations outside of contingency loci has been selected during its evolutionary history is unknown, but based on our work, it is important for rapid genetic adaptation.

FUTURE DIRECTIONS

Are *C. jejuni* contingency loci necessary for host immune system evasion?

The role of contingency loci in *C. jejuni* biology is unknown. Although we have presented compelling evidence that the ON/OFF frequencies of multiple contingency loci change during mouse passage, and others have described similar changes during chicken passage (11, 99, 187), we still do not know why hypermutability in these specific loci has arisen during the course of *C. jejuni* evolutionary history. Based on the genomic locations, and *in silico* predicted functions, of most contingency loci in *C. jejuni*, surface carbohydrate structures are most affected by contingency loci mutability.

Did mutability via contingency loci evolve as a mechanism for host immune system evasion? *C. jejuni* only replicates naturally within a host. To remain within a host to replicate *C. jejuni* must evade clearance by the immune system. Therefore, the ability to present variable sets of antigens on the cell surface via contingency loci mutations might be advantageous. If the immune system recognizes specific *C. jejuni* surface structures that are phase variable, then subpopulations of cells with mutations that turn these structures OFF may go unrecognized.

It may be possible to screen for contingency loci that are important for immune system evasion in mice by studying the populations of *C. jejuni* cells present during the time course of infection in two mouse strains: wild-type and immune system deficient (Recombination activating gene(s) (RAG)-deficient). For example, if a specific contingency locus was responsible for expression of an antigen that is recognized by the immune system, then it would be expected that *C. jejuni* cells with that gene in the

OFF phase would be selected during infection and increase in frequency during the wild-type host's immune response. However, the same pattern would not occur in mice deficient in immune system activity. Contingency genes that appear to be important could be analyzed further in these mouse models by targeted mutations that disrupt mutability of the gene in question.

Defining the evolutionary forces that drive contingency loci mutation patterns during host infection.

We showed here that contingency loci phases, and the genotypes they generate, change during host passage. Furthermore, using a model of contingency loci mutability, we have shown that the rates and patterns of mutation in the homopolymeric DNA tracts of *C. jejuni* contingency do not result in our observed changes. Also, a bottleneck effect during initial colonization whereby only a relatively small number of *C. jejuni* cells successfully establish colonization would not result in the parallel changes we saw when multiple mice are analyzed. A bottleneck of cells could result in large shifts in the contingency loci mutations observed in re-isolated populations, but the effect would be random, such that the same changes would not be predicted to occur in multiple mice.

However, it is possible that many of the observed contingency loci mutations observed are hitchhiking on other beneficial mutations. We could not detect mutations in mouse-adapted populations that were outside of contingency loci, but our analyses are only as good as the current state of technology. As more advanced sequencing techniques are realized, currently undetectable mutations might be found, even though it is unlikely.

Still, many of the contingency loci changes observed could be hitchhiking on one, or a few, other contingency loci mutations that are under selective pressure in the mice. We observed 13 significant changes during mouse passage, but we do not know their relative contributions to *in vivo* fitness. It might be possible to determine the fitness effect of removing each of these genes, or their ability to mutate, from the genome and analyzing fitness through *in vivo* competition experiments with wild-type. However, it is impossible to control for any beneficial or deleterious mutations that will be present in contingency loci in the genomic background of each of these mutants. Furthermore, it may be the case that multiple contingency loci changes are necessary to exact any observable fitness effect. Essentially, it would be very difficult to accurately assess which specific loci are most important *in vivo* by analyzing “defined” mutants, unless the introduced contingency loci mutation resulted in a large fitness change.

The effects of natural selection could be added to the currently available contingency loci mutation model (11) to predict which forces (or combinations thereof) are most likely to result in the observed contingency loci changes. If a successful model that incorporated these evolutionary forces was available it may also be possible to determine which loci contribute most to fitness in a given environment. Currently, the contingency loci genotype model relies on inputted ON-OFF and OFF-ON mutation rates that were determined experimentally for 2 genes. If these rates were defined for each contingency locus in the genome, then microevolution due to drift would be better defined, and selection might be estimated for each given contingency locus. For example, the model could vary the ON-OFF and OFF-ON rates to estimate the rates for each locus that yield the experimentally observed genotypes. The difference between

the known switching rates, and the rate that is necessary to achieve the experimentally derived pattern, could be a proxy measure for selection. That is, the rates do not actually vary, but the effect of selection will drive the observed increase or decrease of the ON or OFF phase, and this could be modeled and estimated as an increase in ON-OFF or OFF-ON switching for each locus.

What are the molecular drivers of homopolymeric DNA mutability in *C. jejuni*?

Trans-acting factors are known to affect the rate of mutation in simple sequence DNA repeats in other bacterial pathogens with contingency loci. For example, disruption of the mismatch repair (MMR) genes, *mutS* and *mutL*, and the nucleotide excision repair gene, *uvrD*, in *Neisseria meningitidis* results in a greatly increased rate of mutation in homopolymeric cytosine/guanine tracts (123, 155). *C. jejuni* lacks a functional MMR system, but appears to have other functional DNA repair systems including nucleotide excision repair, recombinational repair, and base excision repair (54). Genes involved in these repair systems could be inactivated, and the rate of switching in contingency loci could then be determined in various DNA repair deficient backgrounds. The switching rate may be determined by engineering reporter genes that are reversibly expressed based on the length of a homopolymeric tract, or through other functional assays that measure a reversible phenotype driven by mutation in a specific contingency locus. These methods have been used when studying *C. jejuni* contingency loci before (11, 116).

As noted in the Introduction, another trans-acting factor that may modulate switching rates in *C. jejuni* is *mfd*. Mfd is linked to transcription-coupled DNA repair

(168), and in *N. meningitidis* it may play a role in the distinct rates of ON-OFF versus OFF-ON switching in a polyC/G mutation driven contingency locus (109). As described by Han *et al.*, overexpression of *mfd* increases the rate of spontaneous mutation in *C. jejuni* (70).

Finally, given the similarity between the polyC/G tract switching rates of wild-type *C. jejuni* that lacks MMR, and the rate observed in *N. meningitidis* when the MMR system is removed (described in the Introduction), it may be hypothesized that a functional MMR system in *C. jejuni* would decrease the rate of polyC/G switching. The effect of removing MMR in *N. meningitidis* destabilizes polyC/G tracts by 100-fold or more, so the effect of adding MMR back into the genome of *C. jejuni* might greatly decrease the rate of contingency locus switching. Since the *C. jejuni* genome does not appear to encode the majority of known MMR genes, an entire functional MMR system would need to be added, which may prove to be difficult. However, if MMR were successfully reintroduced into *C. jejuni*, and polyC/G DNA was stabilized, it would be possible to associate the loss of contingency locus mutability with an increased or decreased ability to colonize an animal host.

Does the rate of homopolymeric DNA switching vary in different *Campylobacters*?

Hundreds of *Campylobacter* genomes from multiple sources have been sequenced by Illumina technology and deposited to NCBI, and/or EMBL-EBI. Based on what is known, and described in this work, each genome should have diversity in the tract lengths of multiple contingency loci. These lengths may be observed from the

Illumina read data by mapping onto a reference *C. jejuni* genome (93). Do all *C. jejuni* strains have hypermutable polyC/G DNA? What about related species such as *C. upsaliensis* that is known to contain many more, and much longer, polyC/G tracts (52)?

From publicly available Illumina data it may be possible to estimate the switching rate based on the observed tract length diversity, and using the model of genotype mutability described by Bayliss *et al.* (11). As noted, mutation rates are input into this model. However, it should be possible to reverse the model, such that an estimated starting genotype (from a single colony) and the observed genotype after DNA preparation/sequencing are input to estimate switching rates for each contingency locus in question. To do this it would be necessary to estimate the number of generations that occurred during the DNA preparation (approximately 30-50 perhaps (163)), and we need to assume that mutations are not being selected during DNA preparation. Also, it would be necessary to identify the contingency genes that are conserved in most *Campylobacter* genomes to for this analysis. This procedure might identify large differences in switching rates, and thus mutability, in different *Campylobacters* that could be verified with traditional switching rate calculations, and further targeted analysis.

Can “evolvability factors” be screened for in *C. jejuni*?

Chapter 3 showed that motility is lost through multiple mutation events in parallel when *C. jejuni* is experimentally evolved in broth medium. There was a significant loss of motility observed after 5 days of serially transfer in broth, but many of the cells in day 5 populations had a reversible motility deficient phenotype. This finding could be used to

develop a relatively high-throughput screen for factors that contribute to evolvability through reversible mutations in homopolymeric DNA.

C. jejuni strains with defined mutations (such as the reintroduction of MMR), or transposon libraries of random *C. jejuni* mutants, could be experimentally evolved in broth medium in microtiter plates to screen for mutants that do not rapidly evolve the loss of flagellar motility—a phenotype that is easily tractable. It would be hypothesized that a beneficial mutation (in this case, the loss of flagellar motility) in a strain that is less evolvable due to genome stabilization would not rapidly lose flagellar motility during broth experimental evolution.

Does contingency loci mutability contribute to *in vivo* fitness?

Along with this work, others have also shown that mutations in *C. jejuni* contingency loci appear to be under selection during host infection. To determine whether mutability is important for within-host fitness it would be necessary to compete two *C. jejuni* strains within an animal model: mutable wild-type versus a less mutable strain. The problem is obtaining a “less mutable” strain. A number of trans-acting factors may play a role in mutability as described above, but mutants deficient in these factors have not been assayed for contingency locus mutability in *C. jejuni*. An alternative approach is to “lock” all contingency loci, or some subset, into the ON and/or OFF phase. Since there are at least 10 contingency loci per *C. jejuni* genome, this would necessitate a high efficiency recombineering, or a multiplex automated genome engineering (MAGE) technique (91) to alter multiple polyC/G tracts to remove their

ability to mutate rapidly by slipstrand DNA mutation. These techniques are not currently in use for *C. jejuni*.

REFERENCES

REFERENCES

1. 2001. 2000 Report of the AVMA Panel on Euthanasia. J Am Vet Med Assoc **218**:669-96.
2. **Abramoff, M. D., P. J. Magalhaes, and S. J. Ram.** 2004. Image Processing with ImageJ. Biohotonics International **11**:36-42.
3. **Abuoun, M., G. Manning, S. A. Cawthraw, A. Ridley, I. H. Ahmed, T. M. Wassenaar, and D. G. Newell.** 2005. Cytolethal distending toxin (CDT)-negative *Campylobacter jejuni* strains and anti-CDT neutralizing antibodies are induced during human infection but not during colonization in chickens. Infection and Immunity **73**:3053-62.
4. **Acke, E., K. McGill, A. Lawlor, B. R. Jones, S. Fanning, and P. Whyte.** 2010. Genetic diversity among *Campylobacter jejuni* isolates from pets in Ireland. Vet Rec **166**:102-6.
5. **Adak, G. K., S. M. Meakins, H. Yip, B. A. Lopman, and S. J. O'Brien.** 2005. Disease risks from foods, England and Wales, 1996-2000. Emerg Infect Dis **11**:365-72.
6. **Bachtiar, B. M., P. J. Coloe, and B. N. Fry.** 2007. Knockout mutagenesis of the *kpsE* gene of *Campylobacter jejuni* 81116 and its involvement in bacterium-host interactions. FEMS Immunol Med Microbiol **49**:149-54.
7. **Bacon, D. J., C. M. Szymanski, D. H. Burr, R. P. Silver, R. A. Alm, and P. Guerry.** 2001. A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. Molecular Microbiology **40**:769-77.
8. **Balaban, M., S. N. Joslin, and D. R. Hendrixson.** 2009. FlhF and its GTPase activity are required for distinct processes in flagellar gene regulation and biosynthesis in *Campylobacter jejuni*. Journal of Bacteriology **191**:6602-11.
9. **Barrick, J. E., and R. E. Lenski.** 2009. Genome-wide Mutational Diversity in an Evolving Population of *Escherichia coli*. Cold Spring Harb Symp Quant Biol.

10. **Barrick, J. E., D. S. Yu, S. H. Yoon, H. Jeong, T. K. Oh, D. Schneider, R. E. Lenski, and J. F. Kim.** 2009. Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* **461**:1243-7.
11. **Bayliss, C. D., F. A. Bidmos, A. Anjum, V. T. Manchev, R. L. Richards, J. P. Grossier, K. G. Wooldridge, J. M. Ketley, P. A. Barrow, M. A. Jones, and M. V. Tretyakov.** 2012. Phase variable genes of *Campylobacter jejuni* exhibit high mutation rates and specific mutational patterns but mutability is not the major determinant of population structure during host colonization. *Nucleic Acids Res.*
12. **Beery, J. T., M. B. Hugdahl, and M. P. Doyle.** 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl Environ Microbiol* **54**:2365-70.
13. **Bell, J. A., J. L. St Charles, A. J. Murphy, V. A. Rathinam, A. E. Plovanich-Jones, E. L. Stanley, J. E. Wolf, J. R. Gettings, T. S. Whittam, and L. S. Mansfield.** 2009. Multiple factors interact to produce responses resembling spectrum of human disease in *Campylobacter jejuni* infected C57BL/6 IL-10-/- mice. *BMC Microbiol* **9**:57.
14. **Bhagwat, A. A., R. P. Phadke, D. Wheeler, S. Kalantre, M. Gudipati, and M. Bhagwat.** 2003. Computational methods and evaluation of RNA stabilization reagents for genome-wide expression studies. *J Microbiol Methods* **55**:399-409.
15. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis* **157**:472-9.
16. **Bleich, A., I. Kohn, S. Glage, W. Beil, S. Wagner, and M. Mahler.** 2005. Multiple in vivo passages enhance the ability of a clinical *Helicobacter pylori* isolate to colonize the stomach of Mongolian gerbils and to induce gastritis. *Lab Anim* **39**:221-9.
17. **Brown, E. G., H. Liu, L. C. Kit, S. Baird, and M. Nesrallah.** 2001. Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: identification of functional themes. *Proc Natl Acad Sci U S A* **98**:6883-8.
18. **Brown, N. F., M. E. Wickham, B. K. Coombes, and B. B. Finlay.** 2006. Crossing the line: selection and evolution of virulence traits. *Plos Pathogens* **2**:e42.

19. **Buckling, A., and M. A. Brockhurst.** 2008. Kin selection and the evolution of virulence. *Heredity* **100**:484-8.
20. **Butzler, J. P.** 2004. *Campylobacter*, from obscurity to celebrity. *Clin Microbiol Infect* **10**:868-76.
21. **Caldwell, M. B., P. Guerry, E. C. Lee, J. P. Burans, and R. I. Walker.** 1985. Reversible expression of flagella in *Campylobacter jejuni*. *Infection and Immunity* **50**:941-3.
22. **Carrillo, C. D., E. Taboada, J. H. Nash, P. Lanthier, J. Kelly, P. C. Lau, R. Verhulp, O. Mykytczuk, J. Sy, W. A. Findlay, K. Amoako, S. Gomis, P. Willson, J. W. Austin, A. Potter, L. Babiuk, B. Allan, and C. M. Szymanski.** 2004. Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals coordinate regulation of motility and virulence by *flhA*. *Journal of Biological Chemistry* **279**:20327-38.
23. **Cawthraw, S. A., T. M. Wassenaar, R. Ayling, and D. G. Newell.** 1996. Increased colonization potential of *Campylobacter jejuni* strain 81116 after passage through chickens and its implication on the rate of transmission within flocks. *Epidemiol Infect* **117**:213-5.
24. **Chatre, P., M. Haenni, D. Meunier, M. A. Botrel, D. Calavas, and J. Y. Madec.** 2010. Prevalence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from cattle between 2002 and 2006 in France. *J Food Prot* **73**:825-31.
25. **Chaudhuri, R. R., L. Yu, A. Kanji, T. T. Perkins, P. P. Gardner, J. Choudhary, D. J. Maskell, and A. J. Grant.** 2011. Quantitative RNA-seq analysis of the *Campylobacter jejuni* transcriptome. *Microbiology* **157**:2922-32.
26. **Colegio, O. R., T. J. t. Griffin, N. D. Grindley, and J. E. Galan.** 2001. In vitro transposition system for efficient generation of random mutants of *Campylobacter jejuni*. *Journal of Bacteriology* **183**:2384-8.
27. **Conrad, T. M., M. Frazier, A. R. Joyce, B. K. Cho, E. M. Knight, N. E. Lewis, R. Landick, and B. O. Palsson.** 2010. RNA polymerase mutants found through adaptive evolution reprogram *Escherichia coli* for optimal growth in minimal media. *Proc Natl Acad Sci U S A* **107**:20500-5.

28. **Conrad, T. M., N. E. Lewis, and B. O. Palsson.** 2011. Microbial laboratory evolution in the era of genome-scale science. *Mol Syst Biol* **7**:509.
29. **Cooper, T. F., D. E. Rozen, and R. E. Lenski.** 2003. Parallel changes in gene expression after 20,000 generations of evolution in *Escherichiacoli*. *Proc Natl Acad Sci U S A* **100**:1072-7.
30. **Coward, C., A. J. Grant, C. Swift, J. Philp, R. Towler, M. Heydarian, J. A. Frost, and D. J. Maskell.** 2006. Phase-variable surface structures are required for infection of *Campylobacter jejuni* by bacteriophages. *Appl Environ Microbiol* **72**:4638-47.
31. **Dasti, J. I., A. M. Tareen, R. Lugert, A. E. Zautner, and U. Gross.** 2009. *Campylobacter jejuni*: A brief overview on pathogenicity-associated factors and disease-mediating mechanisms. *Int J Med Microbiol*.
32. **de Boer, P., J. A. Wagenaar, R. P. Achterberg, J. P. M. van Putten, L. M. Schouls, and B. Duim.** 2002. Generation of *Campylobacter jejuni* genetic diversity in vivo. *Molecular Microbiology* **44**:351-359.
33. **De Bolle, X., C. D. Bayliss, D. Field, T. van de Ven, N. J. Saunders, D. W. Hood, and E. R. Moxon.** 2000. The length of a tetranucleotide repeat tract in *Haemophilus influenzae* determines the phase variation rate of a gene with homology to type III DNA methyltransferases. *Molecular Microbiology* **35**:211-22.
34. **de Vries, N., D. Duinsbergen, E. J. Kuipers, R. G. Pot, P. Wiesenekker, C. W. Penn, A. H. van Vliet, C. M. Vandenbroucke-Grauls, and J. G. Kusters.** 2002. Transcriptional phase variation of a type III restriction-modification system in *Helicobacter pylori*. *Journal of Bacteriology* **184**:6615-23.
35. **Deana, A., and J. G. Belasco.** 2005. Lost in translation: the influence of ribosomes on bacterial mRNA decay. *Genes Dev* **19**:2526-33.
36. **Dekeyser, P., M. Gossuin-Detrain, J. P. Butzler, and J. Sternon.** 1972. Acute enteritis due to related vibrio: first positive stool cultures. *J Infect Dis* **125**:390-2.
37. **Diker, K. S., G. Hascelik, and S. Diker.** 1992. Colonization of infant mice with flagellar variants of *Campylobacter jejuni*. *Acta Microbiol Hung* **39**:133-6.

38. **DiRita, V. J., and J. J. Mekalanos.** 1989. Genetic regulation of bacterial virulence. *Annu Rev Genet* **23**:455-82.
39. **Dohm, J. C., C. Lottaz, T. Borodina, and H. Himmelbauer.** 2008. Substantial biases in ultra-short read data sets from high-throughput DNA sequencing. *Nucleic Acids Res* **36**:e105.
40. **Dorrell, N., J. A. Mangan, K. G. Laing, J. Hinds, D. Linton, H. Al-Ghusein, B. G. Barrell, J. Parkhill, N. G. Stoker, A. V. Karlyshev, P. D. Butcher, and B. W. Wren.** 2001. Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. *Genome Research* **11**:1706-1715.
41. **Ebert, D.** 1998. Experimental evolution of parasites. *Science* **282**:1432-5.
42. **Edwards, L. A., K. Nistala, D. C. Mills, H. N. Stephenson, M. Zilbauer, B. W. Wren, N. Dorrell, K. J. Lindley, L. R. Wedderburn, and M. Bajaj-Elliott.** 2010. Delineation of the innate and adaptive T-cell immune outcome in the human host in response to *Campylobacter jejuni* infection. *PLoS One* **5**:e15398.
43. **Edwards, R. J., R. E. Sockett, and J. F. Brookfield.** 2002. A simple method for genome-wide screening for advantageous insertions of mobile DNAs in *Escherichia coli*. *Curr Biol* **12**:863-7.
44. **Elde, N. C., and H. S. Malik.** 2009. The evolutionary conundrum of pathogen mimicry. *Nat Rev Microbiol* **7**:787-97.
45. **Escherich, T.** 1886. Beitrage zur Kenntniss der Darmbakterien. III. Ueber das Vorkommen von Vibrionen im Darmcanal und den Stuhlgangen der Sauglinge. (Articles adding to the knowledge of intestinal bacteria. III. On the existence of vibrios in the intestines and feces of babies.). *Münchener Med Wochenschrift* **33**:815-817.
46. **Falkow, S.** 1988. Molecular Koch's postulates applied to microbial pathogenicity. *Rev Infect Dis* **10 Suppl 2**:S274-6.
47. **Falush, D.** 2009. Toward the use of genomics to study microevolutionary change in bacteria. *PLoS Genet* **5**:e1000627.

48. **Fenton, J. I., and N. G. Hord.** 2006. Stage matters: choosing relevant model systems to address hypotheses in diet and cancer chemoprevention research. *Carcinogenesis* **27**:893-902.
49. **Ferenci, T.** 2005. Maintaining a healthy SPANC balance through regulatory and mutational adaptation. *Molecular Microbiology* **57**:1-8.
50. **Ferenci, T.** 2008. The spread of a beneficial mutation in experimental bacterial populations: the influence of the environment and genotype on the fixation of *rpoS* mutations. *Heredity (Edinb)* **100**:446-52.
51. **Forster, M., K. Sievert, S. Messler, S. Klimpel, and K. Pfeffer.** 2009. Comprehensive study on the occurrence and distribution of pathogenic microorganisms carried by synanthropic flies caught at different rural locations in Germany. *J Med Entomol* **46**:1164-6.
52. **Fouts, D. E., E. F. Mongodin, R. E. Mandrell, W. G. Miller, D. A. Rasko, J. Ravel, L. M. Brinkac, R. T. DeBoy, C. T. Parker, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, S. A. Sullivan, J. U. Shetty, M. A. Ayodeji, A. Shvartsbeyn, M. C. Schatz, J. H. Badger, C. M. Fraser, and K. E. Nelson.** 2005. Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. *Plos Biology* **3**:72-85.
53. **Fox, J. G., A. B. Rogers, M. T. Whary, Z. Ge, N. S. Taylor, S. Xu, B. H. Horwitz, and S. E. Erdman.** 2004. Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Campylobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. *Infection and Immunity* **72**:1116-25.
54. **Gaasbeek, E. J., F. J. van der Wal, J. P. van Putten, P. de Boer, L. van der Graaf-van Bloois, A. G. de Boer, B. J. Vermaning, and J. A. Wagenaar.** 2009. Functional characterization of excision repair and RecA-dependent recombinational DNA repair in *Campylobacter jejuni*. *Journal of Bacteriology* **191**:3785-93.
55. **Gaynor, E. C., S. Cawthraw, G. Manning, J. K. MacKichan, S. Falkow, and D. G. Newell.** 2004. The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. *Journal of Bacteriology* **186**:503-17.

56. **Gilbreath, J. J., W. L. Cody, D. S. Merrell, and D. R. Hendrixson.** 2011. Change is good: variations in common biological mechanisms in the epsilonproteobacterial genera *Campylobacter* and *Helicobacter*. *Microbiol Mol Biol Rev* **75**:84-132.
57. **Giraud, A., I. Matic, O. Tenaillon, A. Clara, M. Radman, M. Fons, and F. Taddei.** 2001. Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *Science* **291**:2606-8.
58. **Godschalk, P. C., M. L. Kuijf, J. Li, F. St Michael, C. W. Ang, B. C. Jacobs, M. F. Karwaski, D. Brochu, A. Moterassed, H. P. Endtz, A. van Belkum, and M. Gilbert.** 2007. Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barre and Miller Fisher syndromes. *Infection and Immunity* **75**:1245-54.
59. **Gogol, E. B., C. A. Cummings, R. C. Burns, and D. A. Relman.** 2007. Phase variation and microevolution at homopolymeric tracts in *Bordetella pertussis*. *Bmc Genomics* **8**:122.
60. **Goon, S., C. P. Ewing, M. Lorenzo, D. Pattarini, G. Majam, and P. Guerry.** 2006. A sigma(28)-regulated nonflagella gene contributes to virulence of *Campylobacter jejuni* 81-176. *Infection and Immunity* **74**:769-772.
61. **Goon, S., J. F. Kelly, S. M. Logan, C. P. Ewing, and P. Guerry.** 2003. Pseudaminic acid, the major modification on *Campylobacter* flagellin, is synthesized via the Cj1293 gene. *Molecular Microbiology* **50**:659-671.
62. **Gradel, K. O., H. L. Nielsen, H. C. Schonheyder, T. Ejlersen, B. Kristensen, and H. Nielsen.** 2009. Increased short- and long-term risk of inflammatory bowel disease after salmonella or campylobacter gastroenteritis. *Gastroenterology* **137**:495-501.
63. **Grant, C. C., M. E. Konkel, W. Cieplak, Jr., and L. S. Tompkins.** 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. *Infection and Immunity* **61**:1764-71.
64. **Gripp, E., D. Hlahla, X. Didelot, F. Kops, S. Maurischat, K. Tedin, T. Alter, L. Ellerbroek, K. Schreiber, D. Schomburg, T. Janssen, P. Bartholomaeus, D. Hofreuter, S. Woltemate, M. Uhr, B. Brenneke, P. Gruening, G. Gerlach, L. Wieler, S. Suerbaum, and C. Josenhans.** 2011. Closely related *Campylobacter*

- jejuni strains from different sources reveal a generalist rather than a specialist lifestyle. *Bmc Genomics* **12**:584.
65. **Guerry, P.** 2007. Campylobacter flagella: not just for motility. *Trends in Microbiology* **15**:456-461.
 66. **Guerry, P., C. P. Ewing, M. Schirm, M. Lorenzo, J. Kelly, D. Pattarini, G. Majam, P. Thibault, and S. Logan.** 2006. Changes in flagellin glycosylation affect Campylobacter autoagglutination and virulence. *Molecular Microbiology* **60**:299-311.
 67. **Guerry, P., and C. M. Szymanski.** 2008. Campylobacter sugars sticking out. *Trends in Microbiology* **16**:428-435.
 68. **Guerry, P., C. M. Szymanski, M. M. Prendergast, T. E. Hickey, C. P. Ewing, D. L. Pattarini, and A. P. Moran.** 2002. Phase variation of Campylobacter jejuni 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro. *Infection and Immunity* **70**:787-793.
 69. **Hajishengallis, G., and J. D. Lambris.** 2011. Microbial manipulation of receptor crosstalk in innate immunity. *Nat Rev Immunol* **11**:187-200.
 70. **Han, J., O. Sahin, Y. W. Barton, and Q. Zhang.** 2008. Key role of Mfd in the development of fluoroquinolone resistance in Campylobacter jejuni. *Plos Pathogens* **4**:e1000083.
 71. **Hanninen, M. L., and M. Hannula.** 2007. Spontaneous mutation frequency and emergence of ciprofloxacin resistance in Campylobacter jejuni and Campylobacter coli. *J Antimicrob Chemother* **60**:1251-7.
 72. **Hendrixson, D. R.** 2006. A phase-variable mechanism controlling the Campylobacter jejuni FlgR response regulator influences commensalism. *Molecular Microbiology* **61**:1646-59.
 73. **Hendrixson, D. R.** 2008. Regulation of Flagellar Gene Expression and Assembly, p. 548-549. *In* a. M. J. B. I. Nachamkin; C. M. Szymanski (ed.), *Campylobacter*, 3rd ed. ASM Press, Washington, DC.

74. **Hendrixson, D. R.** 2008. Restoration of flagellar biosynthesis by varied mutational events in *Campylobacter jejuni*. *Molecular Microbiology* **70**:519-536.
75. **Hendrixson, D. R.** 2008. Restoration of flagellar biosynthesis by varied mutational events in *Campylobacter jejuni*. *Molecular Microbiology* **70**:519-36.
76. **Hendrixson, D. R., and V. J. DiRita.** 2003. Transcription of sigma54-dependent but not sigma28-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus. *Molecular Microbiology* **50**:687-702.
77. **Hermans, D., K. Van Deun, A. Martel, F. Van Immerseel, W. Messens, M. Heyndrickx, F. Haesebrouck, and F. Pasmans.** 2011. Colonization factors of *Campylobacter jejuni* in the chicken gut. *Vet Res* **42**:82.
78. **Herring, C. D., A. Raghunathan, C. Honisch, T. Patel, M. K. Applebee, A. R. Joyce, T. J. Albert, F. R. Blattner, D. van den Boom, C. R. Cantor, and B. O. Palsson.** 2006. Comparative genome sequencing of *Escherichia coli* allows observation of bacterial evolution on a laboratory timescale. *Nat Genet* **38**:1406-12.
79. **Hickey, T. E., G. Majam, and P. Guerry.** 2005. Intracellular survival of *Campylobacter jejuni* in human monocytic cells and induction of apoptotic death by cytolethal distending toxin. *Infection and Immunity* **73**:5194-7.
80. **Hickey, T. E., A. L. McVeigh, D. A. Scott, R. E. Michielutti, A. Bixby, S. A. Carroll, A. L. Bourgeois, and P. Guerry.** 2000. *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. *Infection and Immunity* **68**:6535-41.
81. **Hitchen, P., J. Brzostek, M. Panico, J. A. Butler, H. R. Morris, A. Dell, and D. Linton.** 2010. Modification of the *Campylobacter jejuni* flagellin glycan by the product of the Cj1295 homopolymeric-tract-containing gene. *Microbiology* **156**:1953-62.
82. **Holmes, E. C.** 2010. The RNA virus quasispecies: fact or fiction? *J Mol Biol* **400**:271-3.
83. **Houliston, R. S., S. Bernatchez, M. F. Karwaski, R. E. Mandrell, H. C. Jarrell, W. W. Wakarchuk, and M. Gilbert.** 2009. Complete chemoenzymatic synthesis

of the Forssman antigen using novel glycosyltransferases identified in *Campylobacter jejuni* and *Pasteurella multocida*. *Glycobiology* **19**:153-9.

84. **Houliston, R. S., E. Vinogradov, M. Dzieciatkowska, J. Li, F. St Michael, M. F. Karwaski, D. Brochu, H. C. Jarrell, C. T. Parker, N. Yuki, R. E. Mandrell, and M. Gilbert.** 2011. Lipooligosaccharide of *Campylobacter jejuni*: similarity with multiple types of mammalian glycans beyond gangliosides. *Journal of Biological Chemistry* **286**:12361-70.
85. **Hu, L., M. D. Bray, M. Osorio, and D. J. Kopecko.** 2006. *Campylobacter jejuni* induces maturation and cytokine production in human dendritic cells. *Infection and Immunity* **74**:2697-705.
86. **Hu, L., B. D. Tall, S. K. Curtis, and D. J. Kopecko.** 2008. Enhanced microscopic definition of *Campylobacter jejuni* 81-176 adherence to, invasion of, translocation across, and exocytosis from polarized human intestinal Caco-2 cells. *Infection and Immunity* **76**:5294-304.
87. **Hugdahl, M. B., J. T. Beery, and M. P. Doyle.** 1988. Chemotactic behavior of *Campylobacter jejuni*. *Infection and Immunity* **56**:1560-6.
88. **Humphrey, T., S. O'Brien, and M. Madsen.** 2007. *Campylobacters* as zoonotic pathogens: a food production perspective. *Int J Food Microbiol* **117**:237-57.
89. **Hwang, S., B. Jeon, J. Yun, and S. Ryu.** 2011. Roles of RpoN in the resistance of *Campylobacter jejuni* under various stress conditions. *BMC Microbiol* **11**:207.
90. **Hyytiäinen, H., and M. L. Hanninen.** 2012. Quality control strain *Campylobacter jejuni* ATCC 33560 contains a frameshift mutation in the CmeR regulator. *Antimicrob Agents Chemother* **56**:1148.
91. **Isaacs, F. J., P. A. Carr, H. H. Wang, M. J. Lajoie, B. Sterling, L. Kraal, A. C. Tolonen, T. A. Gianoulis, D. B. Goodman, N. B. Reppas, C. J. Emig, D. Bang, S. J. Hwang, M. C. Jewett, J. M. Jacobson, and G. M. Church.** 2011. Precise manipulation of chromosomes in vivo enables genome-wide codon replacement. *Science* **333**:348-53.
92. **Jagannathan, A., C. Constantinidou, and C. W. Penn.** 2001. Roles of rpoN, fliA, and flgR in expression of flagella in *Campylobacter jejuni*. *Journal of Bacteriology* **183**:2937-42.

93. **Jerome, J. P., J. A. Bell, A. E. Plovanich-Jones, J. E. Barrick, C. T. Brown, and L. S. Mansfield.** 2011. Standing genetic variation in contingency loci drives the rapid adaptation of *Campylobacter jejuni* to a novel host. *PLoS One* **6**:e16399.
94. **Jones, M. A., K. L. Marston, C. A. Woodall, D. J. Maskell, D. Linton, A. V. Karlyshev, N. Dorrell, B. W. Wren, and P. A. Barrow.** 2004. Adaptation of *Campylobacter jejuni* NCTC11168 to high-level colonization of the avian gastrointestinal tract. *Infection and Immunity* **72**:3769-76.
95. **Josenhans, C., K. A. Eaton, T. Thevenot, and S. Suerbaum.** 2000. Switching of flagellar motility in *Helicobacter pylori* by reversible length variation of a short homopolymeric sequence repeat in *fliP*, a gene encoding a basal body protein. *Infection and Immunity* **68**:4598-603.
96. **Josenhans, C., and S. Suerbaum.** 2002. The role of motility as a virulence factor in bacteria. *Int J Med Microbiol* **291**:605-14.
97. **Joslin, S. N., and D. R. Hendrixson.** 2009. Activation of the *Campylobacter jejuni* FlgSR two-component system is linked to the flagellar export apparatus. *Journal of Bacteriology* **191**:2656-67.
98. **Kang, J., and M. J. Blaser.** 2006. Bacterial populations as perfect gases: genomic integrity and diversification tensions in *Helicobacter pylori*. **4**:826-836.
99. **Karlyshev, A. V., O. L. Champion, C. Churcher, J. R. Brisson, H. C. Jarrell, M. Gilbert, D. Brochu, F. St Michael, J. J. Li, W. W. Wakarchuk, I. Goodhead, M. Sanders, K. Stevens, B. White, J. Parkhill, B. W. Wren, and C. M. Szymanski.** 2005. Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. *Molecular Microbiology* **55**:90-103.
100. **Karlyshev, A. V., D. Linton, N. A. Gregson, and B. W. Wren.** 2002. A novel paralogous gene family involved in phase-variable flagella-mediated motility in *Campylobacter jejuni*. *Microbiology-Sgm* **148**:473-480.
101. **Karlyshev, A. V., D. Linton, N. A. Gregson, and B. W. Wren.** 2002. A novel paralogous gene family involved in phase-variable flagella-mediated motility in *Campylobacter jejuni*. *Microbiology* **148**:473-80.

102. **Karlyshev, A. V., M. V. McCrossan, and B. W. Wren.** 2001. Demonstration of polysaccharide capsule in *Campylobacter jejuni* using electron microscopy. *Infection and Immunity* **69**:5921-4.
103. **Kim, J. S., K. A. Artymovich, D. F. Hall, E. J. Smith, R. Fulton, J. Bell, L. Dybas, L. S. Mansfield, R. Tempelman, D. Wilson, and J. E. Linz.** 2012. Passage of *Campylobacter jejuni* through the chicken reservoir or mice promotes phase variation in contingency genes Cj0045 and Cj0170 that strongly associates with colonization and disease in a mouse model. *Microbiology*.
104. **Knodler, L. A., J. Celli, and B. B. Finlay.** 2001. Pathogenic trickery: deception of host cell processes. *Nat Rev Mol Cell Biol* **2**:578-88.
105. **Koizumi, Y., C. Toma, N. Higa, T. Nohara, N. Nakasone, and T. Suzuki.** 2012. Inflammasome activation via intracellular NLRs triggered by bacterial infection. *Cell Microbiol* **14**:149-54.
106. **Konkel, M., B. Kim, V. Rivera-Amill, and S. Garvis.** 1999. Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. *Mol Microbiol* **32**:691 - 701.
107. **Konkel, M. E., J. D. Klena, V. Rivera-Amill, M. R. Monteville, D. Biswas, B. Raphael, and J. Mickelson.** 2004. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *Journal of Bacteriology* **186**:3296-303.
108. **Kussell, E., and S. Leibler.** 2005. Phenotypic diversity, population growth, and information in fluctuating environments. *Science* **309**:2075-8.
109. **Lavitola, A., C. Bucci, P. Salvatore, G. Maresca, C. B. Bruni, and P. Alifano.** 1999. Intracistronic transcription termination in polysialyltransferase gene (*siaD*) affects phase variation in *Neisseria meningitidis*. *Molecular Microbiology* **33**:119-27.
110. **Law, B. F., S. M. Adriance, and L. A. Joens.** 2009. Comparison of in vitro virulence factors of *Campylobacter jejuni* to in vivo lesion production. *Foodborne Pathog Dis* **6**:377-85.
111. **Lenski, R. E., J. E. Barrick, and C. Ofria.** 2006. Balancing robustness and evolvability. *Plos Biology* **4**:2190-2192.

112. **Lenski, R. E., and M. Travisano.** 1994. Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc Natl Acad Sci U S A* **91**:6808-14.
113. **Levinson, G., and G. A. Gutman.** 1987. Slipped-Strand Mispairing - a Major Mechanism for DNA-Sequence Evolution. *Molecular Biology and Evolution* **4**:203-221.
114. **Lin, H., Z. Zhang, M. Q. Zhang, B. Ma, and M. Li.** 2008. ZOOM! Zillions of oligos mapped. *Bioinformatics* **24**:2431-7.
115. **Lindmark, B., P. K. Rompikuntal, K. Vaitkevicius, T. Song, Y. Mizunoe, B. E. Uhlin, P. Guerry, and S. N. Wai.** 2009. Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from *Campylobacter jejuni*. *BMC Microbiol* **9**:220.
116. **Linton, D., M. Gilbert, P. G. Hitchen, A. Dell, H. R. Morris, W. W. Wakarchuk, N. A. Gregson, and B. W. Wren.** 2000. Phase variation of a beta-1,3 galactosyltransferase involved in generation of the ganglioside GM1-like lipooligosaccharide of *Campylobacter jejuni*. *Molecular Microbiology* **37**:501-14.
117. **Linton, D., A. V. Karlyshev, and B. W. Wren.** 2001. Deciphering *Campylobacter jejuni* cell surface interactions from the genome sequence. *Curr Opin Microbiol* **4**:35-40.
118. **Lippert, E., T. Karrasch, X. Sun, B. Allard, H. H. Herfarth, D. Threadgill, and C. Jobin.** 2009. Gnotobiotic IL-10; NF-kappaB mice develop rapid and severe colitis following *Campylobacter jejuni* infection. *PLoS One* **4**:e7413.
119. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods* **25**:402-8.
120. **Logan, S. M.** 2006. Flagellar glycosylation - a new component of the motility repertoire? *Microbiology-Sgm* **152**:1249-1262.
121. **Malik-Kale, P., B. H. Raphael, C. T. Parker, L. A. Joens, J. D. Klena, B. Quinones, A. M. Keech, and M. E. Konkel.** 2007. Characterization of genetically matched isolates of *Campylobacter jejuni* reveals that mutations in

genes involved in flagellar biosynthesis alter the organism's virulence potential. *Appl Environ Microbiol* **73**:3123-36.

122. **Mansfield, L. S., J. A. Bell, D. L. Wilson, A. J. Murphy, H. M. Elsheikha, V. A. Rathinam, B. R. Fierro, J. E. Linz, and V. B. Young.** 2007. C57BL/6 and congenic interleukin-10-deficient mice can serve as models of *Campylobacter jejuni* colonization and enteritis. *Infection and Immunity* **75**:1099-115.
123. **Martin, P., L. Sun, D. W. Hood, and E. R. Moxon.** 2004. Involvement of genes of genome maintenance in the regulation of phase variation frequencies in *Neisseria meningitidis*. *Microbiology* **150**:3001-12.
124. **Mas, A., C. Lopez-Galindez, I. Cacho, J. Gomez, and M. A. Martinez.** 2010. Unfinished stories on viral quasispecies and Darwinian views of evolution. *J Mol Biol* **397**:865-77.
125. **McCarthy, N. D., F. M. Colles, K. E. Dingle, M. C. Bagnall, G. Manning, M. C. Maiden, and D. Falush.** 2007. Host-associated genetic import in *Campylobacter jejuni*. *Emerg Infect Dis* **13**:267-72.
126. **Michod, R. E., H. Bernstein, and A. M. Nedelcu.** 2008. Adaptive value of sex in microbial pathogens. *Infect Genet Evol* **8**:267-85.
127. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science* **243**:916-22.
128. **Milton, D. L., M. L. Casper, N. M. Wills, and R. F. Gesteland.** 1990. Guanine tracts enhance sequence directed DNA bends. *Nucleic Acids Res* **18**:817-20.
129. **Misawa, N., and M. J. Blaser.** 2000. Detection and characterization of autoagglutination activity by *Campylobacter jejuni*. *Infection and Immunity* **68**:6168-75.
130. **Monteiro, M. A., S. Baqar, E. R. Hall, Y. H. Chen, C. K. Porter, D. E. Bentzel, L. Applebee, and P. Guerry.** 2009. Capsule polysaccharide conjugate vaccine against diarrheal disease caused by *Campylobacter jejuni*. *Infection and Immunity* **77**:1128-36.

131. **Monteville, M. R., and M. E. Konkel.** 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. *Infection and Immunity* **70**:6665-71.
132. **Moore, J. E., J. Lanser, M. Heuzenroeder, R. M. Ratcliff, B. C. Millar, and R. H. Madden.** 2002. Molecular diversity of *Campylobacter coli* and *C. jejuni* isolated from pigs at slaughter by *flaA*-RFLP analysis and ribotyping. *J Vet Med B Infect Dis Vet Public Health* **49**:388-93.
133. **Moxon, E. R., R. E. Lenski, and P. B. Rainey.** 1998. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Perspect Biol Med* **42**:154-5.
134. **Moxon, E. R., P. B. Rainey, M. A. Nowak, and R. E. Lenski.** 1994. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr Biol* **4**:24-33.
135. **Moxon, R., C. Bayliss, and D. Hood.** 2006. Bacterial contingency loci: the role of simple sequence DNA repeats in bacterial adaptation. *Annu Rev Genet* **40**:307-33.
136. **Nahku, R., K. Peebo, K. Valgepea, J. E. Barrick, K. Adamberg, and R. Vilu.** 2011. Stock culture heterogeneity rather than new mutational variation complicates short-term cell physiology studies of *Escherichia coli* K-12 MG1655 in continuous culture. *Microbiology* **157**:2604-10.
137. **Naito, M., E. Fridrich, J. A. Fields, M. Pryjma, J. Li, A. Cameron, M. Gilbert, S. A. Thompson, and E. C. Gaynor.** 2010. Effects of sequential *Campylobacter jejuni* 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. *Journal of Bacteriology* **192**:2182-92.
138. **Nemelka, K. W., A. W. Brown, S. M. Wallace, E. Jones, L. V. Asher, D. Pattarini, L. Applebee, T. C. Gilliland, Jr., P. Guerry, and S. Baqar.** 2009. Immune response to and histopathology of *Campylobacter jejuni* infection in ferrets (*Mustela putorius furo*). *Comp Med* **59**:363-71.
139. **Newell, D. G., H. McBride, and J. M. Dolby.** 1985. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. *J Hyg (Lond)* **95**:217-27.

140. **Nilsson, A. I., E. Kugelberg, O. G. Berg, and D. I. Andersson.** 2004. Experimental adaptation of *Salmonella typhimurium* to mice. *Genetics* **168**:1119-30.
141. **Ning, Z., A. J. Cox, and J. C. Mullikin.** 2001. SSAHA: a fast search method for large DNA databases. *Genome Research* **11**:1725-9.
142. **Notley-McRobb, L., T. King, and T. Ferenci.** 2002. *rpoS* mutations and loss of general stress resistance in *Escherichia coli* populations as a consequence of conflict between competing stress responses. *Journal of Bacteriology* **184**:806-11.
143. **Novik, V., D. Hofreuter, and J. E. Galan.** 2010. Identification of *Campylobacter jejuni* genes involved in its interaction with epithelial cells. *Infection and Immunity* **78**:3540-53.
144. **Nuijten, P. J., N. M. Bleumink-Pluym, W. Gaastra, and B. A. van der Zeijst.** 1989. Flagellin expression in *Campylobacter jejuni* is regulated at the transcriptional level. *Infection and Immunity* **57**:1084-8.
145. **Nuijten, P. J., A. J. van den Berg, I. Formentini, B. A. van der Zeijst, and A. A. Jacobs.** 2000. DNA rearrangements in the flagellin locus of an *flaA* mutant of *Campylobacter jejuni* during colonization of chicken ceca. *Infection and Immunity* **68**:7137-40.
146. **Pacha, R. E., G. W. Clark, E. A. Williams, and A. M. Carter.** 1988. Migratory birds of central Washington as reservoirs of *Campylobacter jejuni*. *Can J Microbiol* **34**:80-2.
147. **Park, S. F., D. Purdy, and S. Leach.** 2000. Localized reversible frameshift mutation in the *flhA* gene confers phase variability to flagellin gene expression in *Campylobacter coli*. *Journal of Bacteriology* **182**:207-10.
148. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. M. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**:665-668.

149. **Perera, V. N., I. Nachamkin, H. Ung, J. H. Patterson, M. J. McConville, P. J. Coloe, and B. N. Fry.** 2007. Molecular mimicry in *Campylobacter jejuni*: role of the lipo-oligosaccharide core oligosaccharide in inducing anti-ganglioside antibodies. *FEMS Immunol Med Microbiol* **50**:27-36.
150. **Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russell, A. N. Erdem, and H. Zeytin.** 1996. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* gene. *Infection and Immunity* **64**:2070-8.
151. **Prendergast, M. M., D. R. Tribble, S. Baqar, D. A. Scott, J. A. Ferris, R. I. Walker, and A. P. Moran.** 2004. In vivo phase variation and serologic response to lipooligosaccharide of *Campylobacter jejuni* in experimental human infection. *Infection and Immunity* **72**:916-22.
152. **Raamsdonk, L. M., B. Teusink, D. Broadhurst, N. Zhang, A. Hayes, M. C. Walsh, J. A. Berden, K. M. Brindle, D. B. Kell, J. J. Rowland, H. V. Westerhoff, K. van Dam, and S. G. Oliver.** 2001. A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. *Proc Natl Acad Sci U S A* **98**:45-50.
153. **Rathinam, V. A., K. A. Hoag, and L. S. Mansfield.** 2008. Dendritic cells from C57BL/6 mice undergo activation and induce Th1-effector cell responses against *Campylobacter jejuni*. *Microbes Infect* **10**:1316-24.
154. **Ribot, E. M., C. Fitzgerald, K. Kubota, B. Swaminathan, and T. J. Barrett.** 2001. Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. *J Clin Microbiol* **39**:1889-94.
155. **Richardson, A. R., and I. Stojiljkovic.** 2001. Mismatch repair and the regulation of phase variation in *Neisseria meningitidis*. *Molecular Microbiology* **40**:645-55.
156. **Richardson, A. R., Z. Yu, T. Popovic, and I. Stojiljkovic.** 2002. Mutator clones of *Neisseria meningitidis* in epidemic serogroup A disease. *Proc Natl Acad Sci U S A* **99**:6103-7.
157. **Ridley, A. M., M. J. Toszeghy, S. A. Cawthraw, T. M. Wassenaar, and D. G. Newell.** 2008. Genetic instability is associated with changes in the colonization potential of *Campylobacter jejuni* in the avian intestine. *Journal of Applied Microbiology* **105**:95-104.

158. **Ringoir, D. D., and V. Korolik.** 2003. Colonisation phenotype and colonisation potential differences in *Campylobacter jejuni* strains in chickens before and after passage in vivo. *Vet Microbiol* **92**:225-35.
159. **Roberts, I. S.** 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annual Review of Microbiology* **50**:285-315.
160. **Rosqvist, R., M. Skurnik, and H. Wolf-Watz.** 1988. Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. *Nature* **334**:522-4.
161. **Rozen, S., and H. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**:365-86.
162. **Sang, F., S. Shane, K. Yogasundram, H. Hagstad, and M. Kearney.** 1989. Enhancement of *Campylobacter jejuni* virulence by serial passage in chicks. *Avian Dis* **33**:425 - 430.
163. **Saunders, N. J., A. C. Jeffries, D. W. Hood, and E. R. Moxon.** 2000. High rates of phase variation in *Campylobacter jejuni*? *Molecular Microbiology* **36**:1504.
164. **Savery, N.** 2011. Prioritizing the repair of DNA damage that is encountered by RNA polymerase. *Transcription* **2**:168-172.
165. **Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin.** 2011. Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis* **17**:7-15.
166. **Scott, A. E., A. R. Timms, P. L. Connerton, C. L. Carrillo, K. A. Radzum, and I. F. Connerton.** 2007. Genome dynamics of *Campylobacter jejuni* in response to bacteriophage predation. *Plos Pathogens* **3**:1142-1151.
167. **Scott, A. E., A. R. Timms, P. L. Connerton, C. Loc Carrillo, K. Adzfa Radzum, and I. F. Connerton.** 2007. Genome dynamics of *Campylobacter jejuni* in response to bacteriophage predation. *Plos Pathogens* **3**:e119.
168. **Selby, C. P., and A. Sancar.** 1993. Molecular mechanism of transcription-repair coupling. *Science* **260**:53-8.

169. **Slev, P. R., and W. K. Potts.** 2002. Disease consequences of pathogen adaptation. *Curr Opin Immunol* **14**:609-14.
170. **Smith, E. E., D. G. Buckley, Z. Wu, C. Saenphimmachak, L. R. Hoffman, D. A. D'Argenio, S. I. Miller, B. W. Ramsey, D. P. Speert, S. M. Moskowitz, J. L. Burns, R. Kaul, and M. V. Olson.** 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A* **103**:8487-92.
171. **Sorensen, M. C., L. B. van Alphen, A. Harboe, J. Li, B. B. Christensen, C. M. Szymanski, and L. Brondsted.** 2011. Bacteriophage F336 recognizes the capsular phosphoramidate modification of *Campylobacter jejuni* NCTC11168. *Journal of Bacteriology* **193**:6742-9.
172. **Srikhanta, Y. N., S. J. Dowideit, J. L. Edwards, M. L. Falsetta, H. J. Wu, O. B. Harrison, K. L. Fox, K. L. Seib, T. L. Maguire, A. H. Wang, M. C. Maiden, S. M. Grimmond, M. A. Apicella, and M. P. Jennings.** 2009. Phasevarions mediate random switching of gene expression in pathogenic *Neisseria*. *Plos Pathogens* **5**:e1000400.
173. **Srikhanta, Y. N., K. L. Fox, and M. P. Jennings.** 2010. The phasevarion: phase variation of type III DNA methyltransferases controls coordinated switching in multiple genes. *Nat Rev Microbiol* **8**:196-206.
174. **Srikhanta, Y. N., T. L. Maguire, K. J. Stacey, S. M. Grimmond, and M. P. Jennings.** 2005. The phasevarion: a genetic system controlling coordinated, random switching of expression of multiple genes. *Proc Natl Acad Sci U S A* **102**:5547-51.
175. **Stern, A., M. Brown, P. Nickel, and T. F. Meyer.** 1986. Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. *Cell* **47**:61-71.
176. **Stintzi, A.** 2003. Gene expression profile of *Campylobacter jejuni* in response to growth temperature variation. *Journal of Bacteriology* **185**:2009-16.
177. **Stintzi, A., D. Marlow, K. Palyada, H. Naikare, R. Panciera, L. Whitworth, and C. Clarke.** 2005. Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. *Infection and Immunity* **73**:1797-1810.

178. **van Alphen, L. B., N. M. Bleumink-Pluym, K. D. Rochat, B. W. van Balkom, M. M. Wosten, and J. P. van Putten.** 2008. Active migration into the subcellular space precedes *Campylobacter jejuni* invasion of epithelial cells. *Cell Microbiol* **10**:53-66.
179. **van Ham, S. M., L. van Alphen, F. R. Mooi, and J. P. van Putten.** 1993. Phase variation of *H. influenzae* fimbriae: transcriptional control of two divergent genes through a variable combined promoter region. *Cell* **73**:1187-96.
180. **Velicer, G. J., G. Raddatz, H. Keller, S. Deiss, C. Lanz, I. Dinkelacker, and S. C. Schuster.** 2006. Comprehensive mutation identification in an evolved bacterial cooperator and its cheating ancestor. *Proc Natl Acad Sci U S A* **103**:8107-12.
181. **Vucic, S., M. C. Kiernan, and D. R. Cornblath.** 2009. Guillain-Barre syndrome: an update. *J Clin Neurosci* **16**:733-41.
182. **Walker, R. I., E. A. Schmauder-Chock, J. L. Parker, and D. Burr.** 1988. Selective association and transport of *Campylobacter jejuni* through M cells of rabbit Peyer's patches. *Can J Microbiol* **34**:1142-7.
183. **Wang, Y., and D. E. Taylor.** 1990. Natural transformation in *Campylobacter* species. *Journal of Bacteriology* **172**:949-55.
184. **Wassenaar, T. M.** 2011. Following an imaginary *Campylobacter* population from farm to fork and beyond: a bacterial perspective. *Lett Appl Microbiol* **53**:253-63.
185. **Wassenaar, T. M., N. M. Bleumink-Pluym, and B. A. van der Zeijst.** 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. *EMBO J* **10**:2055-61.
186. **Wassenaar, T. M., B. Geilhausen, and D. G. Newell.** 1998. Evidence of genomic instability in *Campylobacter jejuni* isolated from poultry. *Appl Environ Microbiol* **64**:1816-21.
187. **Wassenaar, T. M., J. A. Wagenaar, A. Rigter, C. Fearnley, D. G. Newell, and B. Duim.** 2002. Homonucleotide stretches in chromosomal DNA of *Campylobacter jejuni* display high frequency polymorphism as detected by direct PCR analysis. *FEMS Microbiol Lett* **212**:77-85.

188. **Wettenhall, J. M., and G. K. Smyth.** 2004. limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics* **20**:3705-6.
189. **Whitehead, R. H., P. E. Vaneeden, M. D. Noble, P. Ataliotis, and P. S. Jat.** 1993. Establishment of Conditionally Immortalized Epithelial-Cell Lines from Both Colon and Small-Intestine of Adult H-2kb-Tsa58 Transgenic Mice. *Proceedings of the National Academy of Sciences of the United States of America* **90**:587-591.
190. **Wiesner, R. S., D. R. Hendrixson, and V. J. DiRita.** 2003. Natural transformation of *Campylobacter jejuni* requires components of a type II secretion system. *Journal of Bacteriology* **185**:5408-18.
191. **Willems, R., A. Paul, H. G. van der Heide, A. R. ter Avest, and F. R. Mooi.** 1990. Fimbrial phase variation in *Bordetella pertussis*: a novel mechanism for transcriptional regulation. *EMBO J* **9**:2803-9.
192. **Wilson, D. J., E. Gabriel, A. J. Leatherbarrow, J. Cheesbrough, S. Gee, E. Bolton, A. Fox, P. Fearnhead, C. A. Hart, and P. J. Diggle.** 2008. Tracing the source of campylobacteriosis. *PLoS Genet* **4**:e1000203.
193. **Wilson, D. L., V. A. Rathinam, W. Qi, L. M. Wick, J. Landgraf, J. A. Bell, A. Plovanich-Jones, J. Parrish, R. L. Finley, L. S. Mansfield, and J. E. Linz.** 2010. Genetic diversity in *Campylobacter jejuni* is associated with differential colonization of broiler chickens and C57BL/6J IL10-deficient mice. *Microbiology* **156**:2046-57.
194. **Wosten, M. M., J. A. Wagenaar, and J. P. van Putten.** 2004. The FlgS/FlgR two-component signal transduction system regulates the fla regulon in *Campylobacter jejuni*. *Journal of Biological Chemistry* **279**:16214-22.
195. **Yamasaki, M., S. Igimi, Y. Katayama, S. Yamamoto, and F. Amano.** 2004. Identification of an oxidative stress-sensitive protein from *Campylobacter jejuni*, homologous to rubredoxin oxidoreductase/rubrerhythrin. *FEMS Microbiol Lett* **235**:57-63.
196. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry.** 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. *Molecular Microbiology* **14**:883-93.

197. **Young , K., L. Davis, and V. Dirita.** 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev Microbiol* **5**:665 - 679.
198. **Young, K. T., L. M. Davis, and V. J. DiRita.** 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. **5**:665-679.
199. **Yuki, N., K. Susuki, M. Koga, Y. Nishimoto, M. Odaka, K. Hirata, K. Taguchi, T. Miyatake, K. Furukawa, T. Kobata, and M. Yamada.** 2004. Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain-Barre syndrome. *Proc Natl Acad Sci U S A* **101**:11404-9.
200. **Zoetendal, E. G., C. C. Booijink, E. S. Klaassens, H. G. Heilig, M. Kleerebezem, H. Smidt, and W. M. de Vos.** 2006. Isolation of RNA from bacterial samples of the human gastrointestinal tract. *Nat Protoc* **1**:954-9.