• Review •

Genomic insights into *Campylobacter jejuni* virulence and population genetics

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Abstract

Campylobacter jejuni has long been recognized as a main food-borne pathogen in many parts of the world. Natural reservoirs include a wide variety of domestic and wild birds and mammals, whose intestines offer a suitable biological niche for the survival and dissemination of the organism. Understanding the genetic basis of the biology and pathogenicity of *C. jejuni* is vital to prevent and control *Campylobacter*-associated infections. The recent progress in sequencing techniques has allowed for a rapid increase in our knowledge of the molecular biology and the genetic structures of *Campylobacter*. Single-molecule realtime (SMRT) sequencing, which goes beyond four-base sequencing, revealed the role of DNA methylation in modulating the biology and virulence of *C. jejuni* at the level of epigenetics. In this review, we will provide an up-to-date review on recent advances in understanding *C. jejuni* genomics, including structural features of genomes, genetic traits of virulence, population genetics, and epigenetics.

Keywords: *Campylobacter jejuni*; Genomics; Virulence factors; Population genetics; Epigenetics

1. Introduction

Campylobacter jejuni is a leading cause of bacterial food-borne gastroenteritis worldwide[1, 2], causing an estimated 400-500 million cases of diarrhea annually[3]. As reported by the Center for Disease Control and Prevention (CDC) FoodNet surveillance program in 2013, *Campylobacter* ranked second (13.82 per 100,000 population), only next to *Salmonella* (15.19 per 100,000 population) among the causes of laboratory-confirmed food-borne illnesses in ten U.S. states covering approximately 15% of the U.S. population[4]. A recent report estimates that *Campylobacter* is not only among the most common causes of domestically acquired food-borne illnesses in humans (over 800,000 cases per year), but also is among the leading causes of hospitalization (over 8,000 annually) in the U.S.[5]. In the European Union, *Campylobacter* is the most commonly reported bacterial gastroenteritis pathogen with an incidence rate of 55.5 per 100,000 population in 2012[6]. In developing countries, *Campylobacter* infections are also an important cause of childhood morbidity by diarrheal illness[7]. Due to the lack of national surveillance programs in developing countries, real incidence of Campylobacteriosis is generally unknown, but case-control community-based studies have provided estimates of 40,000 to 60,000/100,000 for children <5 years of age, significantly higher than the incidence of 300/100,000 for the same age group in developed countries[8]. Most people who become ill with Campylobacteriosis develop a self-limiting diarrhea, cramping, abdominal pain, and fever within two to five days after exposure to the organism[9]. The diarrhea may be bloody and can be accompanied by nausea and vomiting. The illness typically lasts about one week. In persons with compromised immune systems, *Campylobacter* occasionally spreads to the bloodstream and causes a serious life-threatening infection. Some of acute infections can have serious long-term consequences, including the peripheral neuropathies, Guillain–Barré syndrome (GBS) and Miller Fisher syndrome (MFS), and functional bowel diseases, such as irritable bowel syndrome (IBS)[10]. Despite its high importance as a human pathogen, our understanding of the mechanisms of *Campylobacter*-associated diseases is still relatively limited compared with other bacterial pathogens such as *Salmonella* and *E. coli*. The rapid developments in the genomics era in the last two decades have contributed significantly to the increase in our knowledge on the genetic basis of *Campy-*

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lobacter biology. In this review, we summarize the most recent findings and provide an update on different aspects of *C. jejuni* genomics, made by advanced sequencing technologies, including the features of genomes, virulence factors, population genetics, and epigenetics of *C. jejuni*.

2. Genome of *C. jejuni*

The first reference strain NCTC11168 of *C. jejuni* was sequenced in 2000 by the whole-genome shotgun sequencing approach utilizing standard Sanger sequencing technology [11], followed by sequencing of three other reference strains (81116, 81-176, RM1221) within several years [12-14]. However, in the recent several years, with the advent of next-generation sequencing technology, many more *C. jejuni* genomes have been extensively sequenced. Up to the writing of this review, 674 *C. jejuni* genomes, 96 of which are complete, have been sequenced and deposited in GenBank (http://www.ncbi.nlm.nih.gov/genome/genomes/149), and over 7000 draft genomes are available from the PubMLST database (http://pubmlst.org/). The total genome size of *C. jejuni* is relatively small, with a median size of 1.68Mb, and has a relatively low G+C content (median GC%: 30.4) (Figure 1). *C. jejuni* is among the densest bacterial genomes known, with coding sequence in >90% of the genome, encoding a median number of ~1,665 proteins. As with other bacteria, *C. jejuni* has a core genome that is shared by all members of the species, plus an accessory genome composed of partially shared and strain-specific genes. The core genome of *C. jejuni* was estimated to be around 800 genes, while the pan-genomes (e.g., core genome plus all accessory genomes) assessments reach beyond 4,000 genes[15-18]. However, it should be noted that the size of both the core genome and pan-genome has not reached a clear plateau yet. When more diverse genomes are added, even fewer genes would be shared and more diverse accessary genes may be observed, as seen in other bacteria[19]. The genes of the core genome are predicted to be mainly involved in vital bacterial functions such as energy metabolism, cell division, protein and peptide secretion, and synthesis of macromolecules including DNA, RNA, and proteins[20]. However, many core genes remain uncharacterized. The accessory genome consists of plasmids, integrated elements, hypervariable regions, and single or paired variable genes, accounting for at least 21% of the total genes in *C. jejuni* strains[21, 22].

pVir and pTet are the main plasmids present in *C. jejuni* strains[23-25]. The pVir plasmid has been suggested to contain some genes of a potential type IV secretion system, but the contribution of the pVir plasmid to *C. jejuni* virulence is still under debate[26]. The pTet plasmid confers tetracycline resistance and contains genes encoding for a type IV secretion system, which is thought to function in conjugative transfer[27, 28]. A microarray screening for the presence of the pVir and pTetin *C. jejuni* isolates indicated that pTet is more prevalent than pVir[28]. However, both plasmids are not very common in *C. jejuni*, as
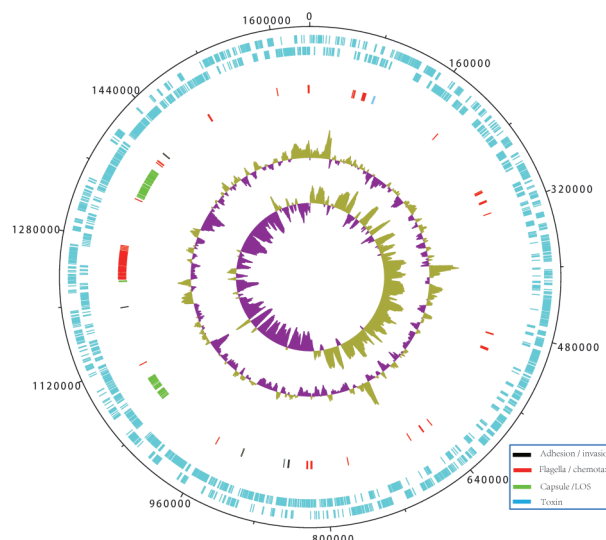

Figure 1. Circular presentation of the genome of *C. jejuni* and the virulence factors, represented by strain *C. jejuni* NCTC11168. The outer circle shows the genome scale; the second and third circles display predicted coding regions on the plus and minus strands, respectively; the fourth circle shows genes related to virulence factors, and the classification of the virulence is shown by different colors as in the box; the fifth circle shows the GC content; the sixth circle depicts the GC skew.

shown by the genomes deposited in GenBank database (pVir 6/674; pTet 6/674). Integrated elements are another contributor to *C. jejuni* accessory genome, which may be introduced by prophages or plasmids. In the reference strain *C. jejuni* RM1221, four integrated elements, CJIE1-CJIE4, have been characterized[22], and recently a fifth integrated element was described in ST-677 clonal complex[29]. Three of these integrated elements carry genes for production of extracellular DNAases, while most of the genes correspond to phage-related and hypothetical proteins. Several variable regions, referred to as hypervariable plasticity regions[20], hypervariable regions[30], and/or regions of divergence[31], were identified in the *C. jejuni* genome. Unlike integrated elements and pathogenicity islands, hypervariable regions in *Campylobacter* do not have a markedly different G+C content to the bulk of the genome and they are not associated with mobile elements important in horizontal DNA transfer[20]. These regions are mainly involved in the biosynthesis of cell surface structures such as flagella, lipooligosaccharide (LOS), and capsular polysaccharide (CPS), as well as restriction-modification systems, and metabolism[20, 21, 30, 32, 33].

3. Virulence factors of *Campylobacter jejuni*

Despite the availability of genomic information of different *C. jejuni* strains from various sources, our understanding of the virulence of *C. jejuni* is still far from complete. Unlike other bacterial pathogens[34], *C. jejuni* does not encode a large number of classical virulence factors. However, several bacterial traits (Figure 1), including the

presence of polysaccharide capsule (CPS) and Lipooligosaccharide (LOS), flagella-driven motility and chemotaxis, colonization of mucus, infection of mucosal cells, and toxin production have been found to be important for *C. jejuni* virulence. A brief description of these factors is provided below.

3.1 Adhesion and invasion factors

Several studies have shown that *C. jejuni* requires adhesion and binding factors to colonize hosts. These experiments have led to the identification of several putative adhesins or binding factors of *C. jejuni*, including the periplasmic binding protein PEB1[35], and several surface exposed proteins such as fibronectin-binding outer membrane protein CadF[36], the lipoprotein JlpA[37], the auto transporter CapA[38], and the major outer membrane protein MOMP[39, 40]. Surface-exposed bacterial molecules directly interact with host cells and play major roles in mediating mucosal adhesion and invasion. PEB1 is a periplasmic protein and crucial for adherence to HeLa cells *in vitro*[41, 42]; however, it is still unclear how periplasmic proteins contribute to host-cell adherence in *C. jejuni in vivo*. Surface-exposed proteins CadF, JlpA, and CapA were demonstrated to be critical in adherence to epithelial cells and colonization in animal models[37, 38, 43, 44]. MOMP is a member of the bacterial porin family and forms voltage-sensitive cation-selective ion channels[45]. In addition to its porin functions, MOMP has also been shown to bind to the surface of human epithelial cells and to the basement membrane protein fibronectin[40, 46]. Our recent findings demonstrated that MOMP is critical for systemic infection and abortion induction(Wu et al, 2016, PNAS, under revision). Cellular invasion is an important pathogenic mechanism for *C. jejuni*. The invasion of epithelial cell *in vivo* results in cellular damage and function loss, which leads to stimulation of host inflammatory responses and diarrhea[47]. *C. jejuni* secretes a protein, CiaB, which is required for the invasion of cultured epithelial cells[48, 49]. Mutants that lack *ciaB* exhibit reduced chick colonization levels [50], implying that cell invasion might be an underappreciated factor in chick colonization.

3.2 Flagella and chemotaxis factors

Flagella and flagellar motility are vital to many aspects of *C. jejuni* pathobiology, including host colonization, host cell invasion, and protein secretion. Non-flagellated mutants were demonstrated to be non-motile and unable to colonize the intestine of experimental animals, indicating that flagella and/or the associated motility is absolutely required for the colonization [51]. Furthermore, aflagellated *C. jejuni* mutants show a significant reduction of internalization by host cells, suggesting the importance of flagella in invasion[47]. Over 40 genes are involved in *C. jejuni* flagella biogenesis and assembly[52]. Structurally, the flagellar filament consists of flagellin subunits FlaA (major subunit) and FlaB (minor subunit)[53]. The two-component signal transduction system, FlgS/FlgR, is central for the regulation of the *Campylobacter* flagellum[52]. Several other genes including *fliA* (sigma28), *rpoN* (sigma54), and the *rpoD* (sigma70) of *C. jejuni* regulate the flagellar biosynthesis and flagellar motility[52, 54]. In addition, two proteins, FlgP and FlgQ, are essential for flagellar motility in *C. jejuni*[55]. Although flagella facilitate *Campylobacter* to move and have a prime importance in chemotactic behavior, signal sensing and transduction are mediated by chemotaxis genes *cheA*, *cheW*, *cheV*, *cheY*, *cheR*, and *cheB*[56]. Signal transduction pathways that regulate motility and chemotaxis of *C. jejuni* still remain open areas for further investigations.

3.3 Capsule and Lipooligosaccharide

Capsular polysaccharide (CPS) and Lipooligosaccharide (LOS) are two predominant cell surface structures important for *C. jejuni* virulence. They are involved in epithelial cell adherence, invasion, and serum resistance [57, 58]. The CPS region in *C.jejuni* is a large gene cluster and composed of three regions: two conserved regions encoding the proteins involved with assembly and transport, which flank the central variable region composed of the genes involved in polysaccharide biosynthesis[59]. The CPS region varies in size from 15 to 34Kb with the central variable region consisting of 11–34 ORFs. Recently, mosaicism in the CPS locus was reported, with the presence of CPS genes elsewhere on the genome of *C. jejuni*, which was thought to add to the antigenic variability of the CPS[60].The LOS clusters in *C. jejuni* strains are also hypervariable. The conserved LOS biosynthesis genes *waaC* and *waaF* were considered the first and last genes of the cluster, respectively[61]. Genes between them are considered as part of the LOS cluster, which are variable from 11 to 30 ORFs[62]. Specific LOS gene clusters containing sialylation genes are associated with the development of GBS in human patients [63]. Structural variation of the CPS and LOS may represent important *C. jejuni* strategies for evading the host immune response, and genetic characterization of *C. jejuni* CPS and LOS genes have suggested multiple mechanisms responsible for such variation[61, 62], including(i) lateral gene transfer, (ii) gene inactivation, duplication, deletion, and fusion, and (iii) phase variable homopolymeric tracts.

3.4 Cytolethal distending toxin

Cytolethal distending toxin (CDT) is the only *Campylobacter* toxin identified so far. It causes diarrhea by interfering with the division and differentiation of cells in intestinal crypts[64, 65]. The toxin activity is encoded by the *cdt* gene cluster, consisting of three adjacent genes: *cdtA*, *cdtB* and *cdtC*[66]. All the three subunits are required for the full toxin activity; CdtB is the active/toxic component of the toxin, while CdtA and CdtC are involved in binding to and internalization into the host cell[67, 68]. CdtA and CdtC interact with CdtB to form a tripartite CDT holotoxin necessary for the delivery of the enzymatically active subunit, CdtB[69]. CdtB is translocated into the host cell cytoplasm and is transported via the Golgi apparatus to the endoplasmic reticulum and from there it finally reaches the nucleus by a retrograde transport mechanism. CdtB shows activity similar to the enzyme deoxyribonuclease (DNaseI)[70] and causes cell cycle arrest in the G2/M transition phase through blocking of CDC2 kinase involved in the entry into mitosis[71].

4. Population genetics of *C. jejuni*

Asa zoonotic pathogen, *C. jejuni* infection in humans has been epidemiologically linked to contact with pets and farm animals and to consumption of contaminated water, milk, and meat (particularly poultry[72]. Reservoirs of *C. jejuni* include a wide variety of birds and mammals, both domesticated and wild, with poultry being the prime source for human infections. Their intestinal mucosa serves as the amplification site of *C. jejuni* and the carriage is usually asymptomatic. The diverse intestinal environment of different hosts represents a variety of niches for *C. jejuni* to adapt. However, questions remain about the genetic basis of host specificity and niche adaptation in *Campylobacter*. Population genetic analyses can be used to correlate the presence of new genetic determinants or changes within existing determinants that enable a given lineage to persist and thrive in a population. *C. jejuni* is composed of a genetically diverse population, represented by the MLST data in the pubMLST database (http://pubmlst.org/campylobacter/) and the large number of accessory genes between strains. The MLST data collected to date show that *C. jejuni* is highly diverse with a total of 3,501 distinct sequence types (STs) (Figure 2) from 31,288 isolates. The STs were clustered into 44 clonal complexes (CC) and 1045 singletons (STs that could not be assigned to any CC). Population genetics analysis using MLST data also indicated that recombination is extensive and the main driver of diversity in *C. jejuni*, generating eight times as much diversity as de novo mutation[73]. The high levels of recombination in *C. jejuni* were demonstrated in several ways[73-75]. First, the tree based on concatenating multiple genetic regions from each strain (MLST data) has little evidence of deep genetic structure that would indicate long periods of independent evolution of different groups. Second, the continued discovery of new genotypes, when the discovery of new alleles has reached an asymptote, suggests that the majority of genetic variation is generated by the reassortment of existing alleles, not the generation of new ones. Finally, *C. jejuni* strains that are distantly related on the global tree often share the same allele at individual MLST loci, which provides strong evidence of genetic exchange. Extensive recombination significantly impacted the population structure and evolution of *C. jejuni*, blurring the boundaries between the different clusters of related genotypes. As a consequence of this, *C. jejuni* strains do not exhibit a highly clonal population structure[76], but are partially clonal[77], and their populations are dominated by clusters of related genotypes which are recognized by MLST as clonal complexes. Clonal complexes reflect the genealogy of *C. jejuni* [74, 78] and have become major units of analysis for *Campylobacter* populations[79]. The 44 clonal complexes of *C. jejuni* populations have little evidence of any phylogenetic relationship among them. Although there are several groups of phylogenetic relationships among some clonal complexes, there is little evidence of a clonal frame link-
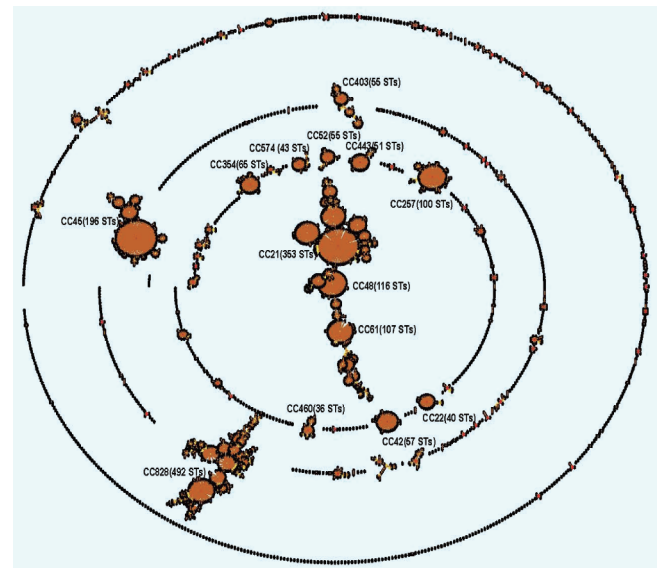

Figure 2. Population snapshot of the 3501 MLST sequence types (STs) listed on the *Campylobacter jejuni* PubMLST database as of May 2016. Black dots represent STs, and lines connect single-locus variants. The snapshot shows all BURST groups (connected STs), singleton STs, ancestral founders (red STs), and subgroup founders (yellow STs). The main clonal complexes (CC) are indicated.

ing all clonal complexes[80].

Genetic analysis using MLST data revealed that *C. jejuni* population contains both specialist lineages and generalist lineages. Specialists are considered host-adapted and strongly associated with certain hosts. For instance, clonal complexes ST-257 and ST-61 are specialists with chicken and ruminants, respectively [81]. Generalists are regularly isolated from multiple animal species. Clonal complexes ST-21 andST-45 are generalists frequently associated with human infections. They are frequently isolated from a wide variety of reservoirs such as chicken, cattle, sheep, wild birds, and starlings [82]. A recently population genetics study further confirmed these clonal complexes as genuine generalist strains based on whole genome sequencing data[83], indicating that they have adapted to transmit between and live within multiple host species. Generalist lifestyle is advantageous for rapid transmission between different hosts. The structuring of the *C. jejuni* population, with many clonal complexes associated with particular host species, highlights the potential role that natural selection also plays in determining the population structure of the species [74].

Although MLST studies provided evidence for the presence of specialists and generalists in *C. jejuni*, the detailed molecular mechanisms for these classifications remain unclear. Recent advances in high-throughput sequencing technologies and the increasing availability of genome sequenced isolate collections provide opportunities for investigating the genetic basis of complex traits. Genome-wide association studies, which have been widely used in human genetics, can identify statistical associations between causal genetic variation and phenotype [84]. The techniques have considerable potential for

enhancing the understanding of how genetic variation in natural bacterial populations may influence their ecology. Sheppard *et al.* investigated the genetic basis of host specificity by analyzing the genome sequences of 192 *C. jejuni* isolates from cattle, chickens, clinical samples, and other sources[85]. The isolates belonged to single-host lineages or the host generalist ST-21 and ST-45 clonal complexes. In the study, a genome-wide association mapping approach was developed, which identified 30-bp DNA sequences (words) associated with colonization of particular species and has the potential to identify host-adapting evolutionary events, including point mutation, homologous recombination, and lateral gene transfer. The method identified the words that are more strongly associated with a particular host than would be expected based on neutral patterns of evolution, given the clonal relationships of the bacteria in the sample and their distribution among hosts. By using the association mapping method, the gain and loss of the *panBCD* genes encoding the vitamin B5 biosynthesis pathway were found to be one factor driving rapid host adaptation of the isolates [83]. Contrastively, another population genetics analysis study did not reveal any lineages closely correlated with any specific clinical presentations in humans for *C. jejuni* [86]. The strains of *C. jejuni* with a specific clinical presentation (i.e., pathovars) usually do not belong to a defined lineage, but are phylogenetically distributed across the species. The virulence genes associated with the disease causation were frequently recombined between lineages, which might have blurred a potential genotype-phenotype correlation in the *C. jejuni* population [87]. However, the only exception to this overall observation is our recent work that has demonstrated the predominance of an emergent *C. jejuni* clone (ST8) in the etiology of sheep abortions in the United States [88-90].

5. Epigenetics in *C. jejuni*

Methylation of DNA widely occurs in bacteria and is the only known mechanism by which prokaryotes might achieve epigenetic inheritance. Base methylation can modulate the interaction of DNA-binding proteins with their cognate sites, and controls chromosome replication, correction of DNA mismatches, cell cycle-coupled transcription, and formation of epigenetic lineages by phase variation [91-93]. DNA methylation in bacteria is controlled by restriction-modification(R-M) and 'solitary' DNA methyltransferases. R-M systems are generally believed to have an 'immune' function, protecting cells from invading foreign DNA. R-M systems in general are made up of methyltransferases (MTases) and restriction endonucleases that recognize a target sequence (motif) and catalyze specific base methylation on the motif or cleave the sequence, respectively [94]. In addition, 'solitary' MTases, which occur in the genome without an associated restriction enzyme (RE), have been found to play important regulatory roles in global gene expression and other biological processes[95, 96]. Furthermore, the ability of such MTases to target their recognition motifs

for methylation often depends on competitive binding at the target site between several DNA binding proteins[95]. These epigenetic regulators of gene expression, including both MTases and competing DNA-binding proteins, are a source of phase variation that increases the robustness of the population and provides opportunities to modulate transcription in response to changing environmental conditions [97].

In *C. jejuni,* the first description of an R-M system was in the first sequenced genome of *C. jejuni* strain NCTC11168[11]. As of recently, four types of R-M systems (Type I through IV, including orphan MTases) have been identified in various *C. jejuni* strains [98-101]. These R-M system types are based on the composition of the protein complex, sequence specificities, cleavage position, cofactor requirements, symmetry of the motif on the double-stranded DNA, and regulation of their expression[102, 103]. Information about these R-M systems and their components are all available on a comprehensive web-based database named Restriction Enzyme dataBASE (REBASE: http://rebase.neb.com/rebase/rebase.html)[104]. The R-M systems found in *C. jejuni* display strain-specific differences. For example, NCTC11168 contain fewer R-M systems than *C. jejuni* ATCC 43431 [105]. Secondly, 81116, 81-176 and several other *C. jejuni* strains possess highly divergent or lack the R-M systems that are found in NCTC11168 [106]. Several studies also observed different G+C content in the R-M genes compared to the rest of the genome, which suggest that *C. jejuni* may have acquired these systems through horizontal gene transfer (HGT) events [105, 106].

In recent years, *C. jejuni* methylation studies have greatly advanced since the advent of next-generation sequencing technologies like Pacific Biosciences' Single Molecule Real-Time (SMRT) [107-110] and Illumina's DNA sequencing systems [99, 100]. Edmonds *et al.* described adenine base methylation in *C. jejuni* [111]. More recent studies confirmed the same observation (i.e., adenine methylation) in strains NCTC11168, 81-176, and IA3902, and specifically found N6-methyladenine (m6A) base modifications that were catalyzed by N-6 adenine MTases [107, 109, 112]. Another type of base methylation, N4-methylcytosine (m4C), was also identified in *C. jejuni* F38011 isolate and postulated to be catalyzed by N-4 cytosine MTase [108]. The authors found m4C motifs in NCTC11168, 81-176 and RM1221, but remarked that the cytosine methylation observation will need to be confirmed.

Comprehensive methylome (i.e, methylation on the whole genome scale) profiles of *C. jejuni* have been relatively few. However, of the few, two studies showed that the motifs and associated MTases of several commonly studied *C. jejuni* strains [112] and *C. jejuni* F38011 isolate [108] were similar or even shared across these different strains. The most comprehensive comparison of *C. jejuni* methylome profiles to date involved strains IA3902, NCTC11168 and 81-176 [109]. Bioinformatic analysis generated methylation distribution plots that showed hypo- and hypermethylated regions of the genome. IA3902

belongs to an emergent hypervirulent clone of *C. jejuni,* and is associated with systemic infection and abortion induction in ruminant animals [88]. The locations of the hypomethylated regions in IA3902 were clearly distinct from its gastroenteric counterparts 11168 and 81-176. In addition, the genes found at these hypomethylated areas were extraordinarily similar between NCTC11168 and 81-176, while IA3902 had a completely different and much more diverse set of genes. Hypermethylated regions varied between the three strains and the genes found in these areas were even less similar among the genomes. The findings from this study led to the postulation that restriction and modification activities may play a stronger role in expression of IA3902 genes more so than NCTC11168 and 81-176. In addition, such activities might also correlate with the hyper virulence and abortion-causing phenotype of IA3902.

A recent review article pointed out that *Campylobacter* have an abnormally higher number of R-M systems compared to other organisms in the 1.5-2Mbp genome size class[94]. As some of these R-M systems seem to have been acquired through HGT, and with *Campylobacter* being naturally competent, it is no surprise that *Campylobacter* possess a higher number of R-M systems in their genome. Characterization of these R-M systems, along with their respective MTases, may reveal their unique roles in phenotypic expression of pathogenesis-associated factors in *C. jejuni*. This may be a reason for why *Campylobacter* maintain so many R-M systems in their genome.

One role that MTases play is in the form of a phase varion, which is a novel mechanism to generate phase variation and consists of a set of genes regulated by phase variable MTases[107]. It has been postulated that these phase varions and phase variable R-M systems may be an evolutionary response to pressures of the environment and serve as an adaptive strategy. The first studies to suggest the role of R-M systems in *C. jejuni* pathogenesis advocated a greater functional role of R-M proteins in host colonization and survival in the environment [101, 113]. When characterizing Type I R-M systems in 73 *C. jejuni* strains, Miller *et al*. found that the length of the polyG tract in the Type I R-M specificity subunit gene varied among several *C. jejuni* strains [101]. This length depended on the strain and that some strains carried multiple types of G-tract populations. The authors suggested R-M genes that carry these homopolymeric tracts are subject to slipped strand mispairing which then introduce gene variations that may be beneficial for their survival in the host environment.

More recent studies have identified several phase variable MTases in *C. jejuni*, but the mechanisms and roles of these MTases in the pathobiology of *C. jejuni* have yet to be defined. In NCTC11168, *cj0031* was characterized as a phase variable MTase, containing a phase variable polyG tract and postulated to behave like a phase varion [114, 115]. A majority of *Campylobacter* cells with this MTase in the phase ON state from chicken and mice infection studies suggests the importance of this MTase

in *C. jejuni* colonization [114, 116]. However, methylome profiling of the *cj0031* deletion mutant provided no clear picture to correlate the distribution of *cj0031* methylation sites with gene expression profiles or reductions in adherence, invasion, and biofilm formation that were observed in the mutant [107].

Subsequent studies found two other MTase homologues with *cj0031*, but all displayed drastic differences in gene structure. *cjsa_rs00180*(formerly known as *cjsa_0032*) from IA3902 has the exact same gene sequence as *cj0031*, but contained no homopolymeric tract, leaving this gene and the resulting MTase in a constitutive phase ON state. Though the mutation had no effect on growth, motility or mucin penetration, it is hypothesized that mutation of this gene would affect colonization, which remains to be tested [117]. *cjh00185* was identified from a clinical isolate *C. jejuni* F38011 strain and shares homology with *cj0031*but contains a shorter polyG tract. However, the main difference between this MTase gene and *cj0031* is the presence of a frame shift mutation that splits *cjh00185* into two potential protein-encoding open reading frames. The authors suggested that this may or may not be a new class of split DNA MTases consisting of 2 or 3 subunit enzymes required for full activity [118]. It is speculated that these unique changes are a function of the physiology of the organism and may have ecological benefits for adaptation and survival in the host intestinal environment [117].

Several studies have also found phenotypic evidence suggesting *C. jejuni* MTases' epigenetic regulatory role in phenotypes critical to pathogenesis of this organism. For example, epigenetic regulation of motility and adhesion was suggested in three studies after changes in these two phenotypes were observed in several different MTase mutants. In 81-176, the mutation of the *cj1461*M-Tase caused a defective flagellar structure that may be responsible for the mutant's hyper-adherence and severe invasion defect [119]. *cj0588* in 81-176 is an RNA MTase involved in 23S rRNA methylation, and the mutant strain of this gene displayed loss of motility and reduced adhesion to Caco-2 cells [120]. In 81116, a *cj0588* mutant also showed reduced adherence and invasion to Caco-2 cells [121].

Changes in antimicrobial resistance phenotypes of *C. jejuni* in association with R-M systems were found in two studies. In the first study involving *cj0588*rRNAMTase mentioned earlier, it was shown that increased resistance to the antimycobacterial drug capreomycin was observed in the MTase mutant *C. jejuni* strain [120]. A second study characterized the *erm*(B) rRNA MTase gene, which confers resistance to macrolides and found in multiple *Campylobacter* species including *C. jejuni*[122]. Though at this stage the rate of *C. jejuni* carrying *erm*(B) is relatively low, it is a major concern because *erm*(B) confers high-level resistance to macrolide antibiotics (important for clinical treatment of campylobacteriosis), is associated multidrug resistance genomic islands, and can be readily transferred between strains via natural transformation [123].

Growth in laboratory media, on the other hand, does not seem to be affected by the lack of a functional MTase

in three *C. jejuni* strains that were studied. Growth was comparable between wildtype and their respective MTase mutants in three different studies: IA3902 (*cjsa_rs00180*mutant), F38011 (*cjh00185* mutant), and 81-176 and 81116 (*cj0588* mutant) [108, 117, 120, 121]. Even with the addition of deoxycholate, growth rate and survival over time did not change in the *cjh00185* mutant compared to wildtype F38011[118].

Just as phenotypic characterization of individual MTases and their R-M systems on *C. jejuni* pathobiology are beginning to emerge, so too are the tools used to analyze the genetic impact of methylation on *C. jejuni* genomes. The study of methylation patterns using current SMRT sequencing methods rely on a population-level consensus, which lack the resolution to analyze epigenetic heterogeneity within a sample. This past year, a powerful new tool, single-molecule modification analysis of long reads (SMALR), provided the framework to analyze methylation at the single-molecule resolution [110]. This method is amenable to modifications to accommodate changing sequencing techniques, has diverse applications beyond study of a single culture, and can be used for sequences with low coverage and analyze methylation patterns in a mixed population of bacteria. With this new tool, future studies will likely reveal a more detailed picture of methylation and its role in the adaptability, biology and disease pathogenicity of *C. jejuni*.

6. Conclusion

The rapid development of genomic techniques in recent years provides unprecedented opportunities for us to understand the genetic basis of biology and pathogenicity of *Campylobacter* at the level of both single strain and population. Although *C. jejuni* does not encode a large number of classical virulence factors, genome sequencing coupled with gene mutation experiments has revealed the genetic basis of several genetic factors associated with the pathogenesis of *C. jejuni* in colonization, adherence and invasion, and immune evasion[124, 125].Additionally, recent studies also revealed that DNA methylation (bacterial epigenetics) modulated the virulence and pathogenesis of *Campylobacter*[119, 121], but the underlying molecular mechanisms remain unknown. As a bacterial pathogen implicated in multiple clinical diseases and adapted to a wide range of hosts, *C. jejuni* displays high population diversities in both phenotypes and genotypes.

Population genetics analysis using MLST data identified both specialists and generalists in *C. jejuni* population. Whole genome based population genetics analyses discovered *panBCD* genes driving rapid host adaptation of some *C. jejuni* isolates.

Currently, there are a large number of complete and nearly complete genome sequences for various *Campylobacter* species and strains available in public databases. The new generation of methylation determination tool (SMALR) will also likely foster the generation of more data on methylation and epigenetics. How to make sense of these BIG data is the next biggest challenge. The omics approaches, in most cases, can only unravel correlation and association, which is not sufficient for determining the causative effect for a disease phenotype. Thus, omics approaches should be augmented and empowered by other approaches in future research efforts. By combining genomics with other tools, we will be able to precisely elucidate the genetic basis of *C. jejuni* host adaptation and pathogenesis. A good example is the recent identification of a few specific SNPs in MOMP that are responsible for the hypervirulence (abortion induction) of *C. jejuni* clone SA (Wu *et al*, 2016, PNAS, under revision). In this particular case, the novel strategy involved natural transformation between two genetically similar but phenotypically different (abortifacient and non-abortifacient) strains, positive selection in an animal model for the transformants that gained virulence, and subsequent whole-genome sequence analysis of the transformants responsible for the disease phenotype. Application of this strategy allowed us, in one experiment, to identify the specific target mutations in a single gene out of more than 8,000 SNPs spanning the entire genomes of the two closely related strains. This example illustrates the power of and need for creative integration of omics approaches with traditional methodologies as well as model systems. With these innovations in mind, it is entirely possible that we will be able to discover novel targets for effective prevention and control of *C. jejuni* infections in both animals and humans.

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