



UNIVERSITY OF
LEICESTER

**Investigation of the Influence of Phase Variation on the
Biological Phenotypes and Immunity to the Flagella in
*Campylobacter jejuni***

Thesis submitted for the degree of Doctor of Philosophy
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Abstract

Campylobacter jejuni is the leading cause of foodborne bacterial gastroenteritis worldwide. Chickens are considered the main source of human infection. Flagellar-mediated motility plays a central role in commensal and pathogenic behaviours of *C. jejuni*. The FlaA protein, a major subunit of the flagellar filament, is decorated with variable glycans that are synthesised or attached by glycosylation enzymes. Some of these glycosylation enzymes are encoded by phase-variable genes that may have an influence on motility. The decoration of the flagella with sugar moieties is also likely to influence immune recognition. In this study, phase variation (PV) and whole genome sequence analyses of multiple motile and hypermotile variants of the *C. jejuni* 11168Ca strain showed that the formation of hyper-motility is associated with mutations in functional domains of the *cbrR* gene rather than the reversible expression of PV genes. This study also revealed the slow elicitation of *C. jejuni*-specific antibody responses following challenge of chickens against glycosylated FlaA (gFlaA) in contrast to fast responses to recombinant non-glycosylated FlaA (rFlaA) in birds after two weeks of colonisation. However, after 6 weeks of colonisation, all birds generated FlaA-specific antibodies against both gFlaA and rFlaA protein as detected in western blots. Similar reactivity for the flagella proteins in a range of flagellin glycosyltransferase mutants (non-PV gene; *maf2* and PV genes *Cj1295* and *Cj1310c*) was also observed. Finally, this study showed little to no influence of PV genes on motility and aggregation and strong association with colonisation via being expressed in a high proportion of colonies. In conclusion, this study showed a potential role for glycosylated PV genes in delaying *C. jejuni*-specific FlaA antibody responses as a mechanism for long-term colonization in chickens.

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Abbreviations

::	Insertional mutant
Amp	Ampicillin
APS	Ammonium persulphate
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine Serum Albumin,
C°	Degrees Celsius
cat	Chloramphenicol
CDT	Cytolethal distending toxin
cfu	Colony forming unit
CJIEs	<i>Campylobacter jejuni</i> -integrated elements
Cm	Centimetres
CV	Column volume
ddH ₂ O	Double distilled H ₂ O
dH ₂ O	Distilled H ₂ O
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
DUF	Domain of Unknown Function
EDTA	Ethylenediaminetetraacetic acid
g	Gram
GST	Glutathione S-transferase
His6	Hexahistidine tag
IL-10	Interleukin-10
INDELS	Insertion-deletion polymorphisms
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan	Kanamycin
Kb	Kilobase
kDa	Kilodalton
L	Litre
LB	Luria-Bertani Agar
LOS	Lipooligosaccharides
M	Molar
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation-Time Of Flight
mg	Milligram
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
Min	Minute
ml	Millilitre
mM	Millimolar

MOMP	Major outer membrane protein
NCTC	National Collection of Type Cultures
ng	Nanogram
Ni ²⁺	Nickel ion
nm	Nanometre
OD	Optical density
ORF	Open reading frame
PAGE	Poly-acrylamide gel electrophoresis
PBP	Periplasmic binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNACL	Protein and Nucleic Acid Chemistry Laboratory
PV	Phase variation
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
SDS	Sodium dodecyl sulphate
Sec	Second
Ser	Serine
SNP	Single nucleotide polymorphisms
SOE	Splicing overlap extension
spp.	Species
TAE	Tris base, acetic acid and EDTA containing buffer
TBS	Tris-Buffered Saline
TCR	Two-component regulatory
TEMED	N,N,N',N' - tetramethylethylenediamine
Thr	Threonine
Tris-HCl	Tris hydrochloride
U	Unit
UV	Ultraviolet
V	Volt
v/v	Volume/volume
VAIN	Variable atmosphere incubator
VNC	Viable-non-culturable
w/v	Weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactosidase
Xg	Times gravity
Δ	Deletion mutant
μ g	Microgram
μ l	Micro litre

μl	Microlitre
μM	Micromolar

Chapter 1: General introduction

1.1. Overview

Bacteria have to face a wide range of challenges, which has led to various processes and mechanisms evolving in bacteria in order to overcome these challenges. Phase variation is a process, which involves high frequency, stochastic, reversible, and heritable changes in phenotype. Phase variation occurs due to mechanisms that generate heterogeneity in the population and hence give the bacterium the ability to adapt to environmental fluctuations at the population level. Several bacterial species contain mechanisms that generate phase variation including *Campylobacter jejuni*, which is a leading cause of foodborne disease in humans. Phase variation impacts a variety of important bacterial features, including motility and immune evasion, that can have an influence on virulence. Understanding the contributions of phase variation to specific phenotypes is important for designing strategies for controlling spread of pathogenic bacteria.

Phase variation in *C. jejuni* is associated with poly G/C tracts that mediate a high frequency of insertion/deletion mutations due to the potential for slipped-strand mis-pairing during DNA replication. The genes known to contribute to the glyco-modification of *Campylobacter* flagellin are phase variable genes and are clustered in a single locus, the flagellar glycosylation locus. Thus, variations in motility, the ability to evade immune responses of host cells/systems against the flagellin and other alterations in bacterial behaviour can be correlated to phase variation. This thesis will focus on chicken immune responses to the phase-variable glycosylation of FlaA, a major subunit of *Campylobacter* flagellin and how this PV-glycosylation contributes to motility and autoagglutination behaviours of *C. jejuni* 11168Ca.

1.2. Phase variation

Pathogenic and commensal bacteria face many challenges in order to maintain their fitness. These challenges emerge from their exposure to fluctuations in the external environment and to a range of selective pressures such as the immune response of the host (Moxon *et al.*, 1994). Several reports highlight the importance of phase variation as a mechanism that could facilitate bacterial survival in the face of variability in the external environment and that enable adaptation to the new environmental conditions due to the selective replication of one variant of a clonal population (Woude and Bäumler, 2004; Moxon *et al.*, 2006; Wisniewski-Dyé and Vial, 2008). Phase variation can be described as reversible, heritable and high frequency switching between “all-or-none” (on/off) expression of contingency genes of bacterial cell surface structures or phenotypic states of bacterial cells (Woude and Bäumler, 2004). The term contingency loci refers to a specific subset of genes that are the target of increased diversity of mutational and combinational processes associated with localised hyper-mutation (Bayliss, 2009). The majority of contingency loci are characterized by the presence of simple sequence repeats (SSR) within the open reading frame of genes (Figure 1.1) (Bayliss *et al.*, 2001). The SSRs location may undergo slippage during replications by the replicative DNA polymerase that results in either expansion or contraction in the numbers of tandem repeat units. When the repeat unit is not a multiple of three, this results in a frame shift which may either truncate an open reading frame (ORF), if resulting in a premature stop codon, or combine with a second ORF by escaping an impending stop signal (van Ham *et al.*, 1993). Also, simple sequence repeats could be located in the promoter region, which leads to changes in the level of expression of a gene with a tendency to switch between three levels of expression (high, intermediate, and low). Variations in expression can also arise when multiple initiation codons are present. For example, The transcription of *porA* gene can be affected by changes in a repeat tract (poly-G) found in the promoter of *porA*, the alterations in the repeat lengths affects spacing between -10 and -35 promoter elements thereby causing changes in gene expression (Ende *et al.*, 2000).

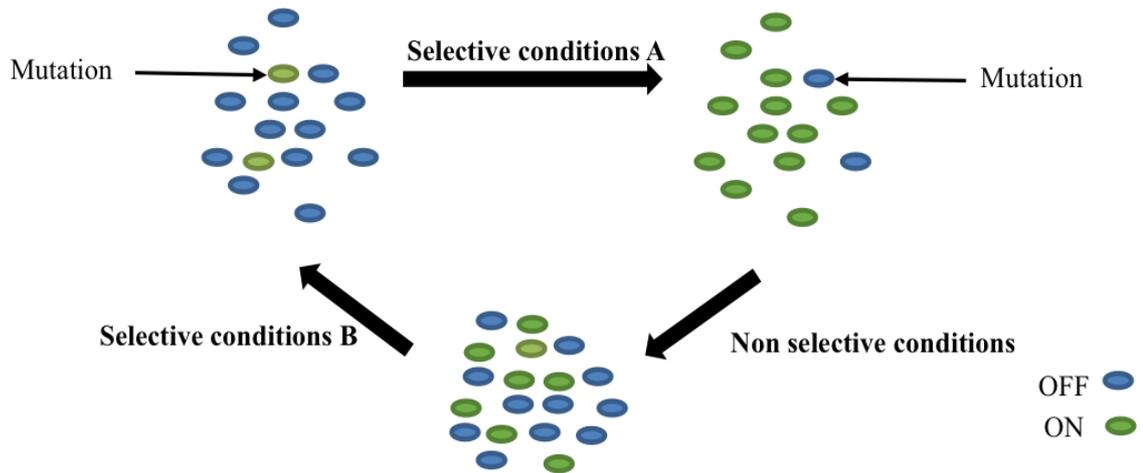


Figure 1.1. Schematic representation of phase variation.

Phase variation starts with mutational processes that occur at contingency loci in order to acquire small numbers of ON variants among an OFF population. Under selective condition A, the proportion of the ON variants increases to yield a majority ON population with a small number of OFF variants. Alternatively, under the selective condition B, the majority of the population is OFF variants. The same pathway is followed for ON to OFF phase variation, i.e. reversal to the previous state.

1.3. Molecular mechanisms of phase variation

1.3.1. Short sequence repeats and slipped-strand mispairing

Slipped-strand mispairing is achieved through changes in simple sequence repeat tracts (Bayliss, 2009). The stability of these repeats fluctuates during DNA replication resulting in the misalignment of the template and copy strands, referred to as slipped-strand mispairing, which leads to the addition or removal of one or more repetitive sequence units with high frequency (Henderson *et al.*, 1999; Levinson and Gutman, 1987). This misalignment causes the appearance of a kink in either the template or copy strand that effectively hides a single repeat unit from the replication machinery. If the production of a kink occurs on the copy strand, then there is expansion in the repeat tract length by addition of a repeat unit. Alternatively, if this kink occurs on the template strand there is contraction of a repeat tract length by deletion of a repeat unit (Moxon *et al.*, 2006). This is illustrated in Figure 1.2. The mutations generated by the slipped strand mispairing mechanism only have an influence in the newly copied DNA and hence are passed onto only one of the two daughter cells. Changes in the number of repeat units are reversible and can produce changes in phenotype by altering gene expression at the level of either transcription or translation (Woude and Bäumlner, 2004).

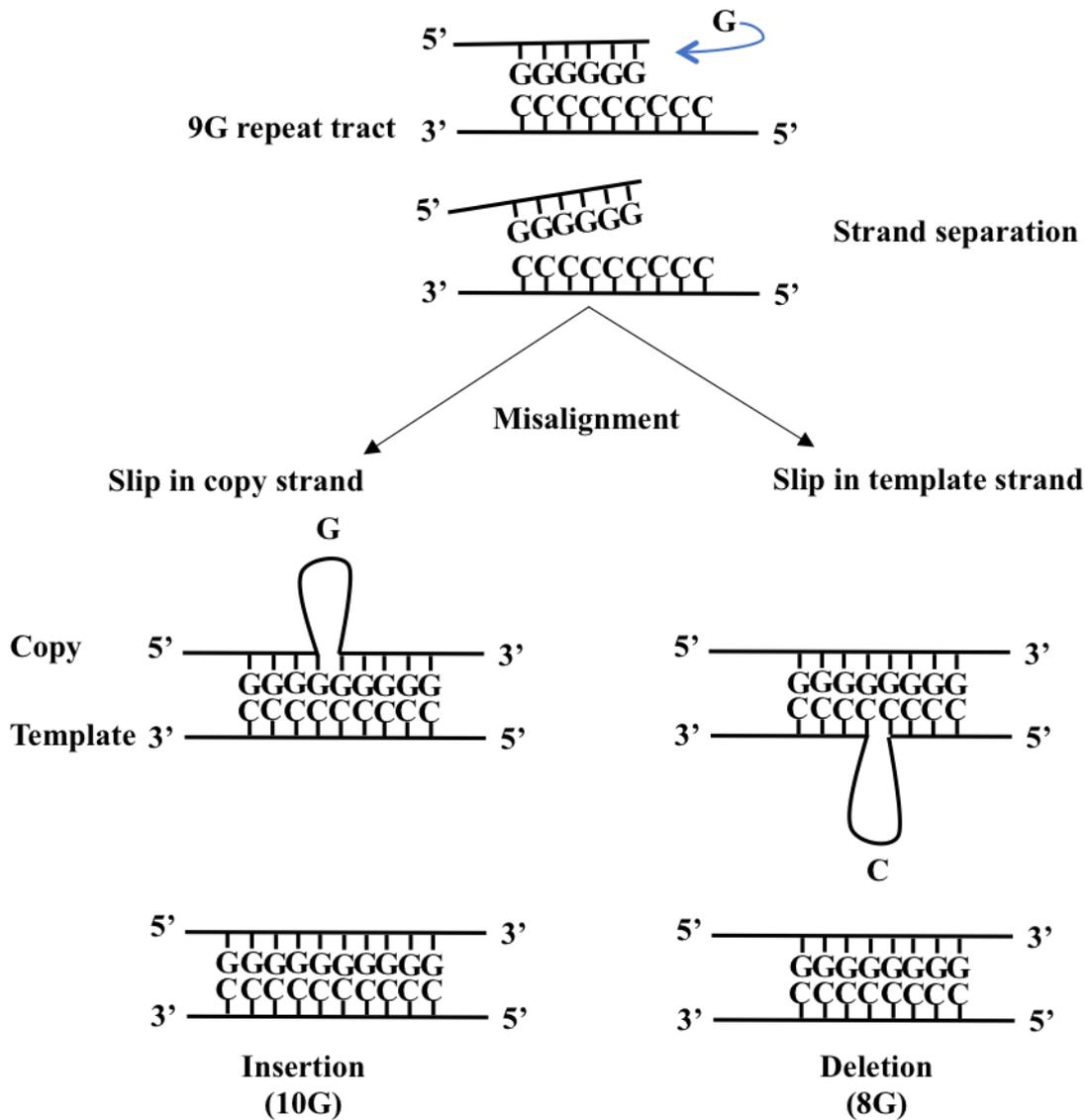


Figure 1.2. Slipped strand mispairing can cause phase variation by producing an insertion or deletion of a repeat unit.

This diagram explains likely slipped strand-mispairing events. During DNA synthesis, the newly synthesised strand separates from the template. This is followed by re-annealing between both strands, template and copy, during which a misalignment can take a place. A slip in the template strand (shown on right) causes a kink to appear with displacement of one repeat unit in the newly synthesised strand, the consequence is deletion of a repeat in the copy strand (8G). Conversely, if the slippage event occurs in the copy strand (shown on left), this leads to the production of a kink in this strand and hence addition of a repeat to the new synthesised DNA (10G).

1.3.2. Homologous recombination

Homologous recombination involves numerous distinct recombination pathways composing of proteins essential for the repair and maintenance processes of the cell. Homologous recombination usually occurs along regions of homology greater than 50bp. This process requires the existence of two copies of a gene or two segments of DNA with a homologous sequence (Woude and Bäumlner, 2004).

In some cases this recombination mechanism occurs between alleles of a gene in a unidirectional exchange of DNA (Woude and Bäumlner, 2004). A good example of this type of unidirectional homologous recombination as a mechanism of phase variation is for the *pilE* locus of *Neisseria gonorrhoeae*, which can mediate antigenic variation of the PilE protein (Figure 1.3). This variation derives from the insertion of *pilS* sequences into the expression locus *pilE*, which is facilitated in part by the multifunctional recombinase, RecA. Occasionally, these insertions contain a premature stop codon or result in deletion of *pilE* and hence ON/OFF switches in pilin expression or an irreversible OFF phenotype for gonococcal pili can be generated as a result of these recombination events (Manning *et al.*, 1991; Segal *et al.*, 1985).

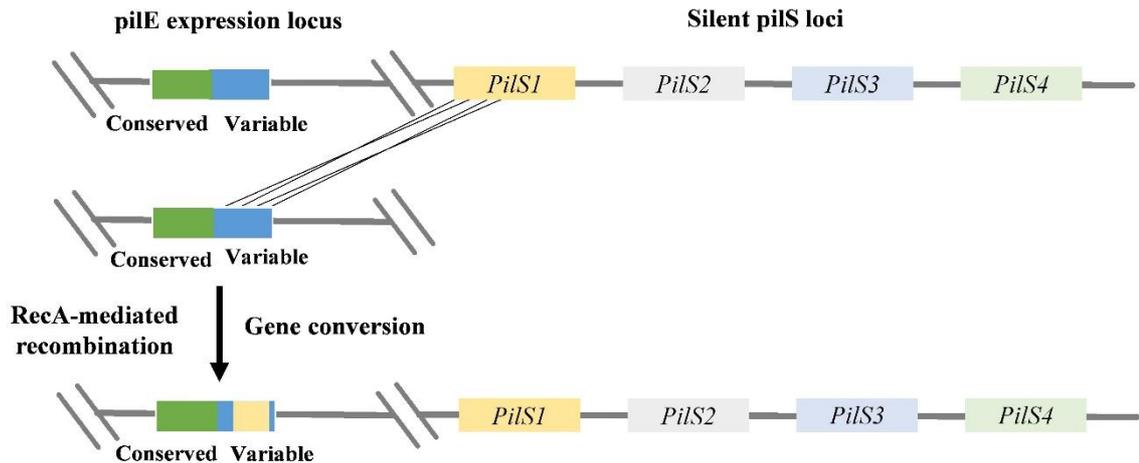


Figure 1.3. Homologous recombination between the silent *pilS* loci and the single *pilE* expression locus.

There is one functionally active locus for the *pilE* in *N. gonorrhoeae* and one to six copies of transcriptionally inactive *pilS* alleles, which differ in sequence to the *pilE* gene and each other. These *pilS* loci are promoterless, containing the 5' end of the gene and variable sequences. The *pilE* gene contains conserved and variable regions resulting from the donation of a stretch of nucleotides from the silent partial *pilS* genes and consequently the sequence of this gene is altered resulting in antigenic variation (Davidsen and Tønjum, 2006).

1.3.3. Site-specific recombination

Site-specific recombination is another mechanism of phase variation that involves non-homologous recombination events in which a particular enzyme recognises and cuts a specific sequence comprising ~30 bps and an inverted repeat (Hallet and Sherratt, 1997). The presence of, at least, two copies of the target sequences, is necessary for recombination and phase variation occurs when these sequences flank a promoter region or part of the open reading frame of a gene. In this scenario, the gene expression or the product changes as a result of alternation in the direction of the DNA segment, which is located between the target sequences (Bayliss, 2009). Site-specific recombination within the locus encoding a surface structure can provide simple switching between ON and OFF states of divergent gene alleles (Bayliss, 2009; Dybvig, 1993; Henderson *et al.*, 1999) (Figure 1.4). The best characterised example of variation achieved by site-

specific DNA inversions is the phase-variable expression of type 1 fimbriae, which are important surface structures for adhesion in *Escherichia coli*. The expression of the *fim* locus is controlled by inversion of a 314bp DNA sequence comprising the promoter upstream of the *fimA* gene (encoding the major fimbrial structural protein). The inversion is mediated by two recombinases, *fimB* and *fimE*, placed upstream of *fimA* with phase variation being caused by a change in the orientation of the inversion fragment. FimB protein has the ability to invert the switch in the ON-to-OFF and OFF-to-ON directions with similar efficiencies, whereas FimE inverts it preferentially in the ON-to-OFF direction (Bryan *et al.*, 2006; McCusker *et al.*, 2008).

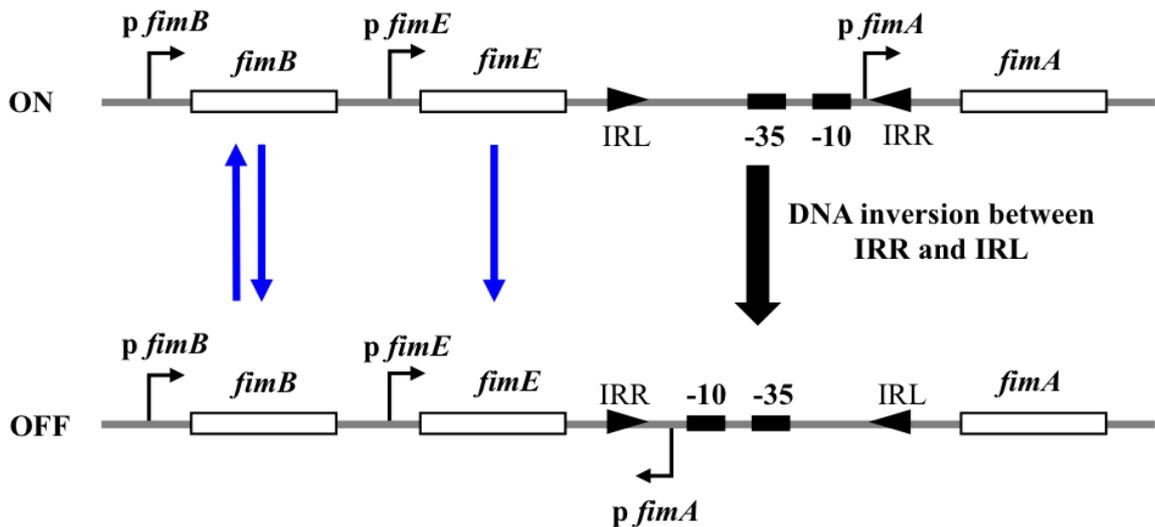


Figure 1.4. Site-specific recombination mechanism.

In *E. coli*, the expression of type fimbriae I mediates phase variation through site-specific inversion to regulate the expression of *fimA*, the major subunit of the pili. The orientation of the *FimA* promoter (indicated as *p fimA* and mRNA indicated as arrow) can be altered by two site-specific recombinases, FimB (its promoter indicated as *p fimB*) and FimE (its promoter indicated as *p fimE*), by inverting the IRR and IRL (IRR is inverted repeat right and IRL is inverted repeat left) and subsequently the transcription of *fimA* will turn either to on (top) or off (bottom). The FimE recombinase mediates only one direction expression from on to off, while FimB can facilitate the inversion in both directions (as shown by blue arrows). -35 and -10 refers to promoter elements where sigma factor binds to and hence recruits RNA polymerase, and transcription begins. The diagram is adapted from van den Broek et al. (2005).

1.3.4. Methylation

DNA sequence-specific methylases can interact with DNA binding proteins to produce reversible changes in promoter expression, leading to the process of phase variation. Thus, switches in gene expression result from the variations in methylation, which are correlated with changes in the binding of transcription factors (Bayliss, 2009). At the level of transcription initiation, there is a complex interaction between promoter-binding of protein regulators and methylation by DNA adenine methyltransferase (Dam), whose activities mediate the phase variable expression of certain bacterial genes (Haagmans and van der Woude, 2000). One example is *ag43*, which encodes the outer membrane protein Ag43

in *E.coli* and is regulated by Dam-dependent phase variation (Wallecha *et al.*, 2002). The DNA- methylating activity of Dam and the global regulator OxyR, oxidative stress response protein, are required for phase variation of Ag43 (Haagmans and van der Woude, 2000). In the OFF state, OxyR binds to the regulatory region containing unmethylated GATC recognition sequences of Dam in the *ag43* promoter, therefore preventing the binding of Dam to these GATC sequence. Conversely, the activation of *ag43* expression is achieved by methylation of the three GATC sequences by Dam which inhibits OxyR binding and permits binding of the RNA polymerase resulting in the ON phase (Figure 1.5) (Wallecha *et al.*, 2002).

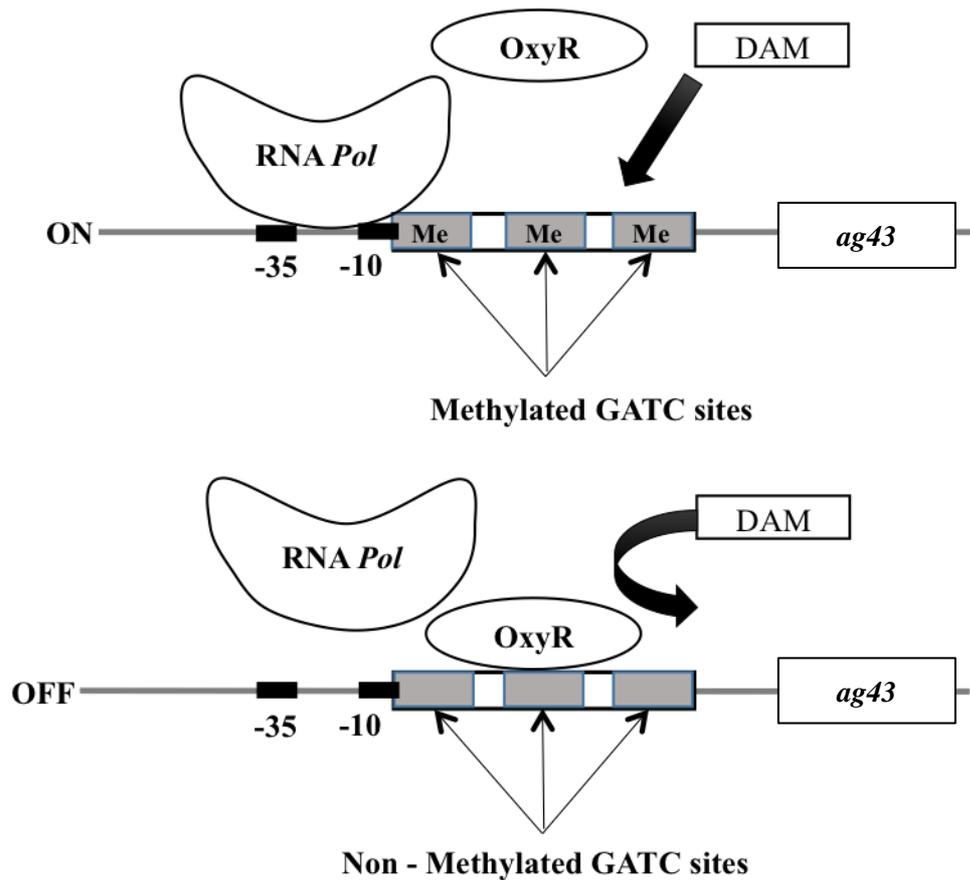


Figure 1.5. Methylation mechanism mediated phase variation of the *ag43* expression.

The methylation of GATC sites by DNA adenine methyltransferase prevents binding of OxyR and leads to production of the ON phase (top). The OFF phase is obtained when methylation of the GATC target sites is inhibited by binding of OxyR to these sequences, which also blocks RNA polymerase from initiating transcription from the *ag43* promoter (bottom).

1.4. A brief history of *Campylobacter* spp.

Whilst certain *Campylobacter* species were identified as a pathogenic bacteria for humans 1972 by Dekeyser and Butzler, they were possibly discovered in 1886 by German bacteriologist Theodor Escherich, who described spiral shaped bacteria, isolated from the colons of infants with an enteric disease called “cholera infantum (Snelling *et al.*, 2005; Silva *et al.*, 2011; King and Adams, 2008). Escherich attempted to cultivate this bacterium on solid media but, unfortunately, his effort was unsuccessful. Interestingly, despite the increasing incidence of diarrhoeal cases due to this spiral shaped bacterium, he thought this bacterium was not a leading cause of disease. As these reports were written and published in the German language, they were broadly unknown for many years until the Third International *Campylobacter* Workshop held in Ottawa in 1985 when Kits stated Escherich's findings (Butzler, 2004).

For many years later, *Campylobacter* was isolated frequently from animals and therefore veterinarians considered it as a veterinary disease. In 1909 McFadyean and Stockman, two veterinary surgeons, isolated an unknown bacterium from aborted foetuses of ewes which they described as a vibrio-like organism (Butzler, 2004). Afterwards, a spiral bacterium was isolated from aborted bovine foetuses by Smith in 1919 in USA and this bacterium was identical to the vibrio-like bacterium of McFadyean and Stockman. Following this, the name of ‘*Vibrio fetus*’ was proposed for this organism by Smith and Taylor. Later, in 1931, a bacterium isolated from calves with ‘winter dysentery’ was characterised as *Vibrio jejuni* and Doyle in 1944 associated a similar organism related with swine dysentery. After that, the role of *Vibrio fetus* in cows was reported by Stegnga and Terpstra in 1949.

While research was establishing the roles of *Campylobacters* as pathogenic bacteria, the first occurrence of human *Campylobacter* infections was reported in 1938. This occurred in Illinois when there was a milk-borne outbreak of enteritis that affected 355 prisoners. A microscopic examination was positive for ‘*V. jejuni*’ in 10% of the cases, while faecal cultures tested negative in 20% of those infected. However, organisms like ‘*V. jejuni*’ were grown in liquid cultures of the blood of 3.5% of victims. In 1947, the first human infection was described when three pregnant women were admitted to hospital with a fever of unknown source,

Vinzent and colleagues identified *V. fetus* from their blood (Butzler, 2004). Two of them aborted after four weeks in the hospital and examination of the placenta detected large inflammatory and necrotic areas. In 1957, *Vibrio* was temporarily described as 'related *Vibrio*' by King. She observed that the characterisation of this organism was similar to the Vinzent description, but with different biochemical and antigenic features. The development of filtration techniques led to the isolation of *Campylobacter* from faeces and was achieved by Dekeyser and Butzler with his team in 1972. This new technique involved a filter with a pore size of 0.65 μm , stool suspensions were passed through the filter and then the filtrate was cultured on selective media as this pore size allowed passage of *Campylobacter* and retention of other bacteria in the filter. Following this, in 1977, the selective media technique was developed by Skirrow, and this led to another revolution in *Campylobacter* investigation when it was combined with increasingly sophisticated equipment and technique for generating microaerobic conditions. Finally, *Campylobacter jejuni* was identified in the mid-1980 (Butzler, 2004).

In summary, key findings on this bacterium were made by Escherich, King and Butzler; Escherich defined the shape of *Campylobacter* as a spiral bacterium; King pointed out that this bacterium was not uncommon; and Butzler successfully developed a selective method for culturing *Campylobacter*. During the period of time from 1963 to 1972, reports about the occurrence of *Campylobacter* were few compared to the later decades, probably due to blood samples being utilised for diagnosis and because of the lack of selective methods for *Campylobacter* isolation. These key advances led to a major improvement in isolation and diagnosis of *Campylobacter* infections so that nowadays this bacterium is considered a major pathogenic bacterium and major cause of disease in humans.

1.5. General features of *Campylobacter jejuni*

Campylobacter jejuni is categorised as a member of the family *Campylobacteraceae*, which belongs to the delta-epsilon group of proteobacteria. It is a Gram-negative flagellated spirally curved rod-like bacterium (Yeh *et al.*, 2014) of approximately 1.5-6.0 μm long and 0.2-0.5 μm wide (Figure 1.6). *C. jejuni* requires an atmosphere of 3-15% O_2 , 3-10% CO_2 for growth (Ketley and Konkel, 2005) and is considered as a moderate thermophilic microorganism, which grows in the range from 30 to 42°C (Ursing *et al.*, 1994). The ability of this microorganism to grow under microaerobic conditions is related to its occurrence as a commensal in the intestines of many animals, in particular poultry, with growth at 42°C being indicative of a specific adaptation to survival in the avian gut (Park, 2002). In addition, *C. jejuni* can generate coccoid cell structures termed a viable but non-culturable (VBNC) state. *C. jejuni* is thought to convert to a VBNC state as a result of exposure to various stressors, such as starvation, low temperature, and low pH (Trevors, 2011).

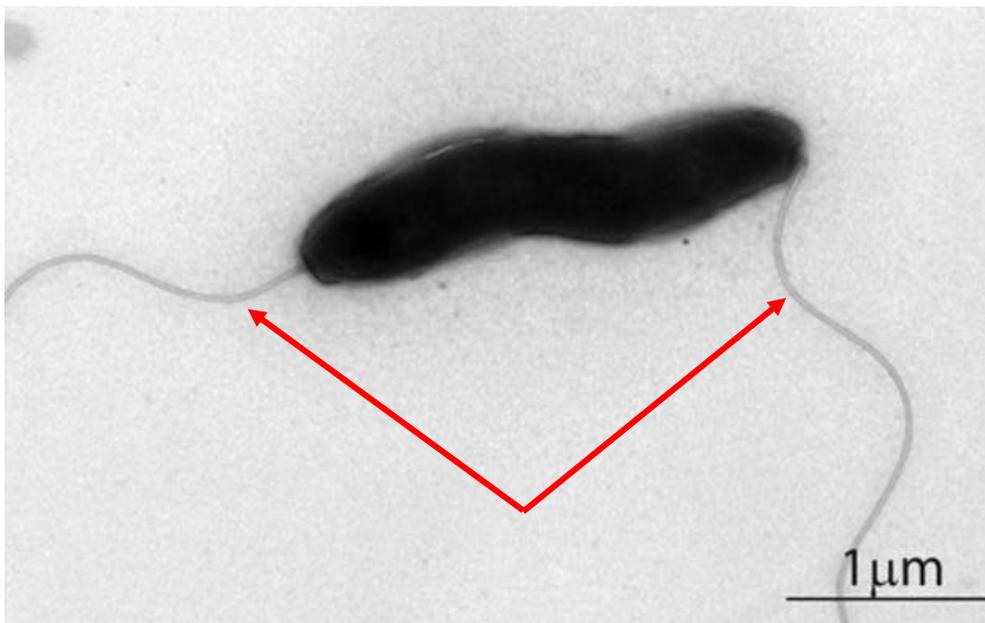


Figure 1.6. Transmission electron microscopy of the *C. jejuni*.

This figure shows curved s shaped of *C.jejuni* with a single polar flagellum at both ends (red arrows) (Gao *et al.*, 2014).

1.6. Genomic analysis of *C. jejuni* NCTC 11168

C. jejuni NCTC11168 was selected for sequencing as it is genetically tractable and widely available. This strain was isolated in 1977 from a patient with severe gastroenteritis (Skirrow, 1977). Key features identified in the original annotation of the *C. jejuni* NCTC11168 genome were the presence of three loci containing multiple genes involved in synthesis or modification of CPS (Capsular Polysaccharides), flagella and LOS (Lipooligosaccharides). The complete sequence of the genome of *C. jejuni* strain NCTC 11168 revealed a circular chromosome of 1,641,481 base pairs (30.6% G+C) in length that was expected to encode 1,643 open reading frame (ORF)s, with no plasmids (Parkhill *et al.*, 2000).

Other strains have been fully genome sequenced and all have a similar length to this initial genome sequence (Pearson *et al.*, 2007; Zhang *et al.*, 2010; Shyaka *et al.*, 2015). These sequences include *C. jejuni* strain RM1221, isolated in 2000 from a chicken carcass in the USA (Miller *et al.*, 2000), and *C. jejuni* strain 81-176, which is utilised widely in research and is considered as a highly pathogenic strain (Hofreuter *et al.*, 2006) as it was recovered from a patient during a milk-associated outbreak of campylobacteriosis in the USA (Hofreuter *et al.*, 2006). Another genetically interesting *C. jejuni* strain 81116 was isolated from a case of campylobacteriosis associated with a human waterborne outbreak (Pearson *et al.*, 2007) while CG8486 was isolated from a patient with inflammatory diarrhoea in Thailand (Poly *et al.*, 2007). Finally a number of different *Campylobacter* species have been sequenced and annotated including multiple strains of *C. lari*, *C. upsaliensis* and *C. coli* (Fouts *et al.*, 2005).

Interestingly, the genome of *C. jejuni* NCTC 11168 contains multiple hypervariable sequences. These are short homopolymeric tracts of nucleotides usually present in genes encoding enzymes with functions in the biosynthesis or modification of surface structures, or in closely linked genes of unknown function. There was also a significant absence of insertion sequences or phage-associated sequences within the genome. A number of known and potential pathogenic factors were identified including genes encoding for a cytolethal distending toxin (CDT), haemolysin-like toxins, putative type II export and chemotaxis genes (Parkhill *et al.*, 2000; Fouts *et al.*, 2005). Since the publication of this work,

extensive studies have revealed the genetic loci that code for important surface structures on *C. jejuni* strains; the surface polysaccharide region (*Cj1413c – Cj1448c*), which has been renamed as the capsule locus (Karlyshev *et al.*, 2002; Karlyshev *et al.*, 2005); flagella modification locus (*Cj1293 – Cj1342c*), which has been confirmed as an O-linked glycosylation pathway and is responsible for glycosylation of the flagellin structural proteins FlaA and FlaB (Thibault *et al.*, 2001; Szymanski *et al.*, 2003; Liu and Ochman, 2007; Karlyshev *et al.*, 2002); the LOS locus (*Cj1131c - Cj1152c*), which has been investigated and shown to be highly variable (Guerry *et al.*, 2000; Linton *et al.*, 2000). In addition, an N-linked glycosylation pathway (*Cj1119c – Cj1130c*) was identified and extensively characterized (Linton *et al.*, 2005; Szymanski *et al.*, 2003; Kelly *et al.*, 2006).

Despite the high degree of homology that comparative genome analyses have shown for different *C. jejuni* strains, differences have been identified in hypervariable regions, strain-specific regions and genomic islands. For example, *C. jejuni* strain RM1221 identified four large genomic elements, *Campylobacter jejuni*-integrated elements (CJIEs), that were absent from *C. jejuni* strain NCTC 11168 (Parker *et al.*, 2006). In terms of the differences in hypervariable regions, Gilbert *et al.* (2002) have compared the lipo-oligosaccharide (LOS) biosynthesis loci from 11 *C. jejuni* strains and found that these strains express a total of 8 different ganglioside mimics in their LOS outer cores associated with structural variation in LOS. Functional genomics (whole-genome-based transcriptomics and proteomics) was applied also to achieve a comprehensive understanding of *Campylobacter* biology (Wu *et al.*, 2013). The application of this approach displays that changes in global gene expression can change the pathogenic potential of *C. jejuni* isolates and explains the important phenotypic differences between genetically close *C. jejuni* isolates (Gaynor *et al.*, 2004; Malik-Kale *et al.*, 2007; Seal *et al.*, 2007).

1.7. Epidemiology of *C. jejuni*

Campylobacter is the main cause of foodborne diarrhoeal disease in the world with 550 million cases being reported every year of which 220 million occur in children under 5 years of age (WHO, 2018). <http://www.who.int/mediacentre/factsheets/fs255/en/.in>. *Campylobacter* infections in children less than 5 years old occur mainly in developing countries, and may lead to the development of immunity with increasing age resulting in *Campylobacter* infections being more frequently asymptomatic (Kaakoush *et al.*, 2015; Ketley, 1997). In industrialized countries, a similar boosting of immunity against *Campylobacter* has been observed among poultry breeders and people who drink unpasteurized milk (Moore *et al.*, 2005). The main agents of campylobacteriosis in humans are *C. jejuni* and *C. coli*, although other *Campylobacter* species have been reported to cause disease in humans. Many reports have shown that *C. jejuni* causes around 90% of campylobacteriosis cases whereas approximately 10% are caused by *C. coli* and very low numbers by other non-*jejuni/coli* strains (Bronowski *et al.*, 2014; Friedman *et al.*, 2000; Gillespie *et al.*, 2002; Snelling *et al.*, 2005).

The numbers of *Campylobacter* enteritis cases are frequently reported in America, Europe and Australia, while in Africa, Asia and the Middle East, *Campylobacter* infection is considered to be endemic even though epidemiological data is incomplete. In these developing countries, there is a direct connection between poor hygiene and human and animals contacts on the hyperendemicity of *C. jejuni* (Ketley, 1997). Furthermore, the occurrence of campylobacteriosis and the number of reported cases are significantly different between countries or areas in the same country (Figure 1.7) (Kaakoush *et al.*, 2015).

The number of reported cases of campylobacter infections steadily increased in 1998 up to a peak of 58,059 cases in England and Wales. Cases have subsequently decreased slowly possibly due to the introduction of measures for control of infections and contamination in animals, specifically poultry (Cabiscol *et al.*, 2000). The Public Health England (PHE) annual data report on *Campylobacter* infection in England and Wales for January 2017 has shown that 51831 cases were confirmed in the laboratory in 2007, whereas the number of

laboratory reports in 2016 was 52381 cases (Figure 1.8) (*Campylobacter* data 2006 to 2015, 2017). Recorded reports about the actual number of *Campylobacter* enteritis cases are considered to be less than the true number of cases because many campylobacteriosis cases do not require medical assistance (Friedman *et al.*, 2000).

In 2013, campylobacteriosis in the European Union (EU) was reported to occur at a rate of 64.8 per 100,000 populations. While in the United States of America it was estimated that *C. jejuni*, and *C. coli* cause 1.3 million cases of illness each year (Fitzgerald *et al.*, 2016). According to the Centers for Disease Control and Prevention (CDC), the occurrence of *Campylobacter* infections increased by 13% in 2014 in comparison with 2006-2008 (CDC, 2018) <https://www.cdc.gov/campylobacter/technical.html>. Outbreaks of campylobacteriosis are rare and sporadic (i.e. single individual) (Black *et al.*, 1988). Foodborne disease outbreaks are defined by CDC as a sudden increase in the incidence of cases in a specific region or within a specific group of individuals through a specific period of time, usually with a common cause. It has been found that males are more susceptible to campylobacteriosis than females and *C. jejuni* infection is often seasonal (Friedman *et al.*, 2000). The number of outbreaks of campylobacteriosis have been linked to the type of event and environmental source of infection. Poultry products or water are the most common reported sources of outbreaks caused by *Campylobacter*. For example, in England and Wales, 143 outbreaks were recorded between 1992 and 2009 of which 114 cases were due to consumption of contaminated food or water, while 2 related to animal contact and 22 to an unknown source of infection. Unfortunately, data on campylobacteriosis outbreaks from developing countries are not available (Kaakoush *et al.*, 2015).

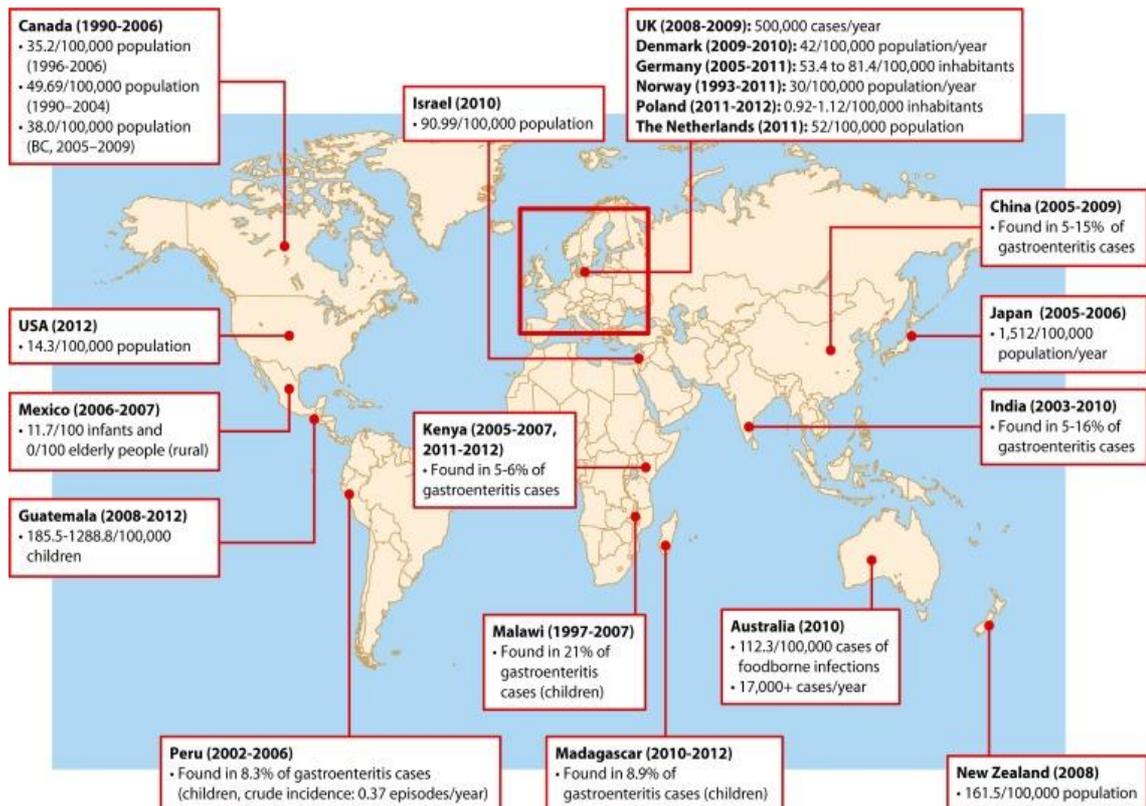


Figure 1.7. Occurrence and spread of campylobacter infection (*C. jejuni/C. coli*).

The latest information on the global epidemiology of campylobacteriosis from the literature is shown by (Kaakoush *et al.*, 2015).

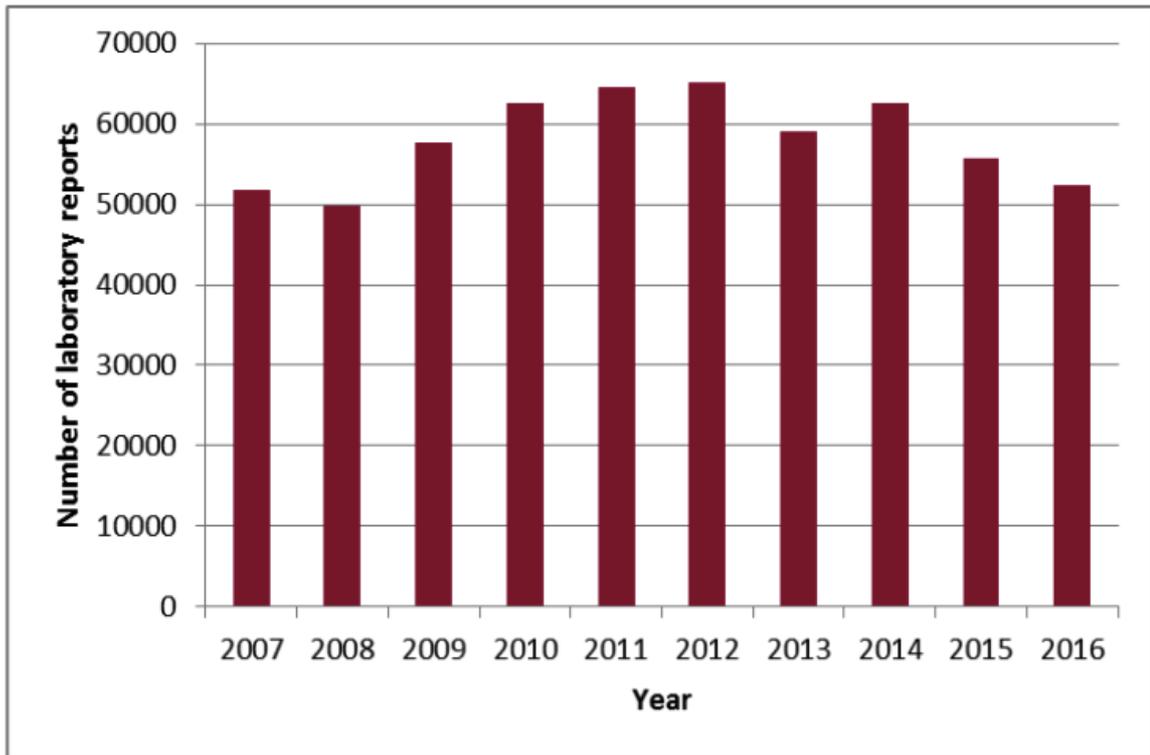


Figure 1.8. Annual report from Public Health England.

This figure illustrates that the number of confirmed cases of *Campylobacter* infection increased in England and Wales between 2007 and 2016.

1.8. *C. jejuni* infections

1.8.1. Reservoirs of *C. jejuni*

Both wild and domesticated animals are the main reservoirs of *C. jejuni* as these microorganisms are part of the normal intestinal microbiota in the gastrointestinal tract of animals (Blaser, 1997; Bronowski *et al.*, 2014; Bull *et al.*, 2006; Ketley and Konkel, 2005; Sopwith *et al.*, 2008; Whiley *et al.*, 2013). However, Humphrey *et al.* (2014) have found that *C. jejuni* can cause disease in chickens as there was a strong inflammatory response to *C. jejuni* infection that may lead to diarrhoea when chicken breeds are growing rapidly in an intensive production system.

Poultry and poultry products are sources of 50–70 % human *Campylobacter* infection (Hermans, Van Deun, Martel, *et al.*, 2011), including chickens, cattle and sheep. Additionally, it has been found that pigs are the main source of *C. coli* (Alter *et al.*, 2005; Jensen *et al.*, 2006; Oporto *et al.*, 2007). Furthermore, *C. jejuni* have been isolated from wild ducks (Colles *et al.*, 2011), monkeys and marine mammals (Foster *et al.*, 2004; Kalashnikova *et al.*, 2002). Interestingly, flies have been associated as an important vector to transmit *Campylobacter* in the broiler house and in kitchens (Ekdahl *et al.*, 2005; Hald *et al.*, 2004). However, the relative contributions of each of these animal reservoirs to *Campylobacter* infection in humans is unclear (Oporto *et al.*, 2007). Potential sources of human infection by *C. jejuni* are shown in Figure 1.9.

Another factor attributed to *Campylobacter* cross-contamination is environmental conditions (Whiley *et al.*, 2013). A number of researchers have isolated *Campylobacter* from water and soil, and this is a clear indicator of the ability of *Campylobacter* to adapt for survival in the farm environment (Bull *et al.*, 2006; Ellis-Iversen *et al.*, 2012; Peyrat *et al.*, 2008). Surface water can become contaminated in a variety of ways, including faeces of wild and domestic animals, effluent sewage, unpasteurised milk. Also, unwashed fruit and vegetables have been associated with human infection as important environmental reservoirs (Whiley *et al.*, 2013). Overall the commonest environmental reservoir is contaminated drinking water, which has been linked with spread of *Campylobacter* colonisation in herds and has an essential role in the distribution of *Campylobacter* (Abe *et al.*, 2008; Whiley *et al.*, 2013).

Contamination of these environmental sources with *C.jejuni* suggests that this species has survival mechanisms that enable adaptation to and survival in extreme conditions (Murphy *et al.*, 2006). Identification of these survival mechanisms may help with development of intervention strategies and lead to improved control of the prevalence of *Campylobacter* infections.

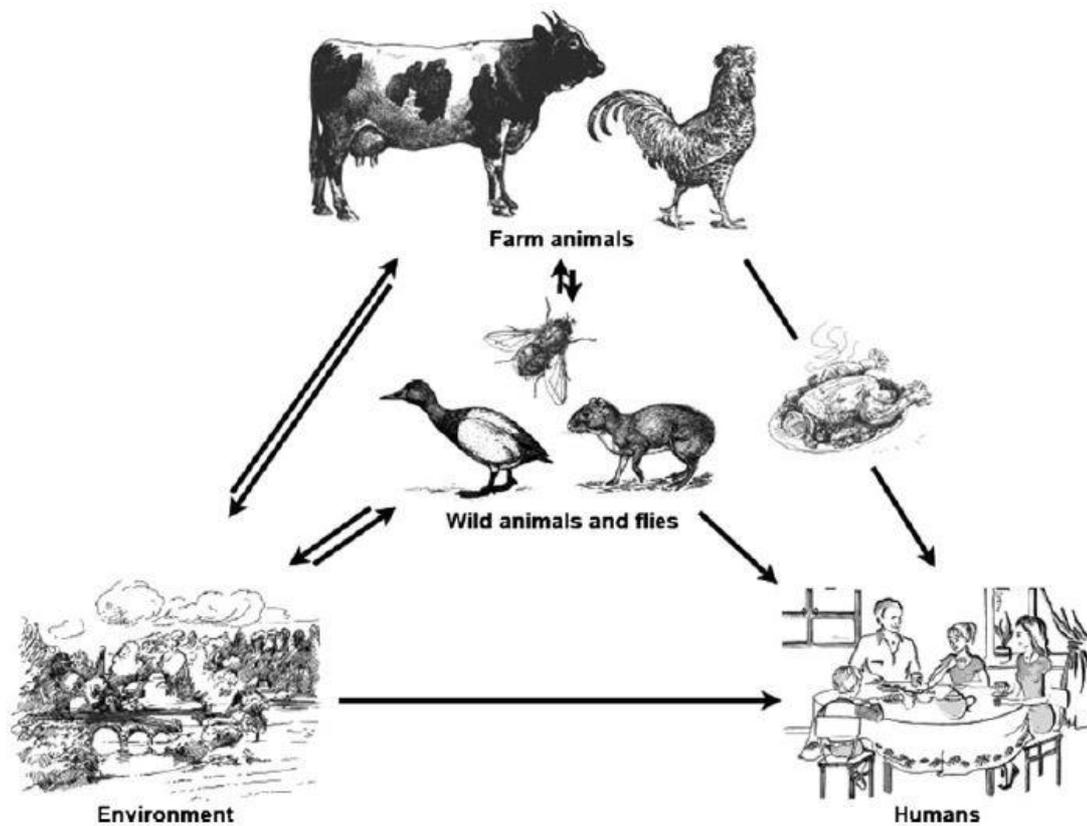


Figure 1.9. Illustration of potential sources of *C. jejuni* leading to campylobacteriosis infections.

The important sources of *Campylobacter* infection in humans are wild and domestic animals. In addition, environmental sources are linked to the transmission of *C. jejuni* to humans through contaminated drinking water or consumption of contaminated animal products (Bronowski *et al.*, 2014).

1.8.2. Clinical manifestations and complications arising from *C. jejuni* infection

The oral ingestion of as few as 500-800 *C. jejuni* can cause infection of humans (Black *et al.*, 1988). Usually, the incubation period after oral ingestion of *C. jejuni* is 24-72 h; however, it could take one week or more in cases of infection with a lower inoculum. The main initial symptom of infection is cramping abdominal pain, which can be heavy in some cases, and is difficult to distinguish from appendicitis. Diarrhoea associated with *C. jejuni* can vary in individuals from mild to bloody (Blaser, 1997). Additionally, other clinical symptoms include fever, vomiting and headaches (Allos, 2001). It is likely that the virulence of the infecting strain determines disease outcome, however the host response and host immune status are also important (Ketley, 1997).

Campylobacteriosis is self-limiting reaching its peak and continuing at least for two days, followed by a gradual resolution in the week after (Blaser, 1997). It is very rare that *C. jejuni* enteritis is correlated with death with severe cases mainly occurring in developing countries when treatment is absent (Silva *et al.*, 2011). Post-infection complications associated with *Campylobacter* infection include acquired immune-mediated neuropathies such as Guillain-Barré Syndrome (GBS), the most severe and well-studied, or Miller Fischer Syndrome (MFS) (Nachamkin *et al.*, 1998; Salloway *et al.*, 1996). The development of GBS results from molecular mimicry between *C. jejuni* ganglioside-like LOS epitopes and human neuronal gangliosides; thereby the host generates anti-*Campylobacter* antibodies that recognise, and cross react with host gangliosides in the peripheral nerves. This molecular mimicry is thought to lead to inflammation, tissue damage, and neuromuscular paralysis that can be severe and occasionally life-threatening (Linton *et al.*, 2005; Poly and Guerry, 2008; van den Berg *et al.*, 2014; Young *et al.*, 2007).

1.8.3. Treatment of *C. jejuni* infection

Normally the *C. jejuni* infection is a self-limiting disease and the only required therapies for the majority of patients are the compensation of lost fluids and electrolytes. A requirement for antibiotic therapy is associated with cases of prolonged illness and for patients who are suffering from acute enteritis disease. Additionally, HIV-positive or immunocompromised individuals should receive antibiotic treatment (Butzler, 2004). The most commonly prescribed antibiotics are macrolides such as erythromycin, clarithromycin and azithromycin and fluoroquinolone antibiotics, such as ciprofloxacin (Allos, 2001). However, there is increasing resistance of *Campylobacter* to both choices of antibiotics, potentially due to the utilisation of antibiotic as food additives in poultry farming to prevent and control infection and promote growth rate (Snelling *et al.*, 2005).

1.9. Pathogenesis

Despite the recognition of *C. jejuni* as an important human pathogen for more than 30 years, the molecular pathogenesis and virulence of its infections is not well understood (Bolton, 2015). *C. jejuni* enters into the host through either contaminated food or water, as a result the organism has to confront several innate immune defence mechanisms such as gastric acid in the stomach. The virulence mechanisms of *C. jejuni* are up-regulated during colonization of the host intestine (Hermans, Van Deun, Martel, *et al.*, 2011) and lead to colonisation of mucosal surfaces, adherence to and invasion of the underlying intestinal epithelial cells, cell death, tissue inflammation and the subsequent diarrhoea due to the damage to the absorptive epithelium (Everest *et al.*, 1992). In chickens, *C. jejuni* can colonise the gastrointestinal tract in very high numbers (up to 10^{10} CFU per gram of infected intestine) (Young *et al.*, 2007). Colonisation starts with penetration of the mucus layer and is followed by adherence to the epithelial cells of the distal ileum, and finally relocation to the colon, which is the target niche for colonisation (Neal-McKinney and Konkel, 2012; Poly and Guerry, 2008; Stef *et al.*, 2013).

1.9.1. Colonisation virulence factors of *C. jejuni*

C. jejuni host colonisation is a multifactorial process. Several regulatory systems in *C. jejuni* enable this microorganism to react to the hostile environment of the host and may contribute to colonisation, these systems include two-component regulatory (TCR) systems (Brás *et al.*, 1999). The ability of *C. jejuni* to penetrate the mucus layer is enabled by its spiral shape and motility by means polar flagellum at one or both pole cells (Young *et al.*, 2007).

The main virulence factors for *C. jejuni* to colonise the intestinal tract are chemotaxis and motility, mutant strains of *C. jejuni* lacking chemotaxis and motility genes are severely compromised in colonisation and pathogenicity (Hermans, Van Deun, Martel, *et al.*, 2011; Van Vliet and Ketley, 2001). *C. jejuni* has also a number of virulence mechanisms which help these bacteria to establish colonisation in the host intestine and evade the host immune defence mechanisms. These potential virulence factors include: iron acquisition, motility, chemotaxis, bacterial surface proteins that mediate adhesion to and invasion of

hos cells, capsule formation, lipooligosaccharide, secretion of toxins, and biofilm formation. The most extensively studied of the *C. jejuni* virulence factors will be discussed below.

1.9.1.1. Iron acquisition

Campylobacteriosis is characterized by diarrhoea ranging from mild to bloody stools in humans. The ability of *C.jejuni* to establish successful infections in the human intestine and to colonise the gastrointestinal tract of chicks requires iron. Iron is an important nutrient for all living organisms including microorganisms and is a cofactor for many enzymes that are associated with essential cellular processes such as the iron sulphur proteins, which participate in electron transport, anaerobic respiration, amino acid metabolism and energy metabolism (van Vliet *et al.*, 2002). The requirement level of iron for the growth of bacteria is between 10^{-7} and 10^{-5} M whereas the concentration of freely available iron in the mammalian body is only around 10^{-18} M (Andrews *et al.*, 2003). This is because host fluids contain heme or iron-binding glycoproteins, such as transferrin in serum and lactoferrin in mucosal secretions that bind to and sequester the extracellular iron (Palyada *et al.*, 2004; Debruyne *et al.*, 2008).

In order to overcome this iron limitation some *C.jejuni* strains utilise low molecular mass complexes that have high affinity for iron called siderophores (Miller *et al.*, 2009). These compounds have high affinities for iron and can compete with the iron-binding proteins of the host to bind free iron (Field *et al.*, 1986). Exogenous siderophores used by *C. jejuni* include enterochelin, ferrichrome (Baig *et al.*, 1986) and rhodotorulic acid (Miller *et al.*, 2009). As siderophores are too large to be transferred through the bacterial outer membrane barrier, there are specific receptors in the *C.jejuni* membrane that facilitate entry. One of these receptors is CfrA, which is a Ferric-enterobactin (FeEnt) outer membrane receptor, and is responsible for iron acquisition (Palyada *et al.*, 2004). Additionally, these receptors require the inner membrane ABC transport system CeuBCDE to be transferred to the cytosol (Konkel *et al.*, 2001). In addition to the siderophore systems, genomic analysis of *C. jejuni* NCTC 11168 has revealed other iron acquisition systems such as the haemin/haemoglobin uptake system, which is encoded by *chuABCD* (Ridley *et al.*, 2006).

A critical aspect of iron uptake is regulation and homeostasis. Maintaining and equilibrating the level of iron inside the cells is crucial as excess amounts of iron can couple with oxygen to produce harmful reactive oxygen species (ROS). Therefore, during iron- starvation genes controlling iron uptake must be switched on to permit sufficient iron-uptake for survival while these genes should be suppressed when the amount of iron reaches a high level to avoid the production of damaging ROS (Miller *et al.*, 2009). The ferric uptake regulator, Fur (Cj0400), and PerR (peroxide stress regulator; Cj0322) are two regulatory proteins, which are responsible for maintaining iron homeostasis in *C. jejuni* (van Vliet *et al.*, 1999).

The regulation of intracellular iron levels and iron uptake are essential for *C.jejuni* colonisation of chickens (Hermans, Van Deun, Martel, *et al.*, 2011). It has been reported that the inactivation of *fur* and other important genes in iron transport such as *cfrA* and *ceuE* compromises the ability of *C.jejuni* to colonise chickens (Woodall *et al.*, 2005). In addition, there was a significant 50-fold reduction in the ability of a *Cj0178* mutant *C. jejuni* strain to colonise the gastrointestinal tract of chicks (Palyada *et al.*, 2004). The *Cj0178* gene was identified as being important for iron uptake from Transferrin and Lactoferrin (Miller *et al.*, 2009). These findings clearly show that iron uptake systems play an essential role in survival and host colonisation of *C.jejuni*.

1.9.1.2. Flagella

Many bacterial species achieve motility by means of flagella. Motility is beneficial for pathogenic bacteria as it allows migration towards favourable conditions (Terashima *et al.*, 2008). A flagellum is an important virulence determinant in *C.jejuni* enabling motility and intestinal colonisation (Hendrixson, 2006a; Hendrixson and DiRita, 2004a). Key functions of the flagellum are to enable the bacterium to penetrate mucus, to avoid being washed out of the intestine via the motion of peristalsis and to escape from the intestine lumen and penetrate underlying intestinal epithelial cells (Van Vliet and Ketley, 2001). Additionally the flagella apparatus enables secretion of important virulence factors in order to adhere to and invade surface epithelial cells (Stef *et al.*, 2013).

1.9.1.2.1. Biosynthesis of flagella

Approximately 47 genes are involved in *C. jejuni* flagellar biosynthesis (Parkhill *et al.*, 2000) with a complex regulatory system that controls the biosynthesis of this organelle (Hendrixson, 2008). The *C.jejuni* flagellum is a long, spiral shaped hair-like structure that protrudes from the surface of the cell. A flagellum consists of three distinct structural subunits comprising of the basal body, located within the inner membrane ring, the rigid filament and related ring structures, and the extracellular hook structure (Lertsethtakarn *et al.*, 2011) (Figure 1.10). The essential components of the extracellular filament are FlaA, an important flagellin protein product of *flaA* and FlaB a lesser protein product of *flaB* both of which are post-translationally modified by O-linked glycosylation (Neal-McKinney *et al.*, 2010; Guerry, 2007). This modification is required for flagellar assembly and is, therefore, important for motility, virulence and epithelial cell adherence and invasion (Lertsethtakarn *et al.*, 2011).

The expression of flagellar genes is regulated by RpoN (σ^{54}), FliA (σ^{28}) and the two-component regulatory system FlgSR (Hendrixson, 2008). The sensor kinase FlgS and the response regulator FlgR are the dual elements of a mechanism that modulate σ^{54} -dependent transcription (Hendrixson, 2006a). The expression of *flaB*, the hook and basal-body genes are regulated by RpoN, whereas FliA regulates expression of *flaA* (Balaban *et al.*, 2009). It has been revealed that insertional activation of RpoN and FliA generate a non-flagellated cell (Jagannathan *et al.*, 2001). Other studies have shown that FlhF is required for flagella development and σ^{54} -dependent flagella expression (Balaban *et al.*, 2009). In addition, FlgM can inhibit the activity of σ^{28} until the flagella secretory system has formed. Once the secretory system is formed, σ^{28} can initiate the expression of genes such as *flaA* encoding the major flagellin due to the transportation of FlgM out of the cytoplasm (Hendrixson and DiRita, 2004a).

The early understanding of flagella biosynthesis and regulation was developed from mutagenesis studies on *Salmonella* and *Vibrio* species (Hughes *et al.*, 1993; Klose and Mekalanos, 1998; Karlinsey *et al.*, 2000). Despite the identification of many orthologues in *C.jejuni*, significant differences are observed between the flagella biosynthesis mechanisms of these microorganisms such as *E.coli* and *Salmonella*. In addition, *Campylobacter* species have their flagella genes

distributed throughout the genome at 32 different loci and not organized into one operon (Fouts *et al.*, 2005; Chilcott and Hughes, 2000; Liu and Ochman, 2007; Parkhill *et al.*, 2000) whereas the flagella genes of other bacteria are clustered together into operons with a single promoter controlling expression. Another major difference is that a master regulator controls flagella formation in other motile bacteria; FlhDC in *Salmonella* species, FlrA for *Vibrio* species and FleQ for *Pseudomonas* species (Klose and Mekalanos, 1998; Arora *et al.*, 1997; Karlinsey *et al.*, 2000; Kutsukake *et al.*, 1990). These simple systems contrast with the complex *C. jejuni* regulatory network described above.

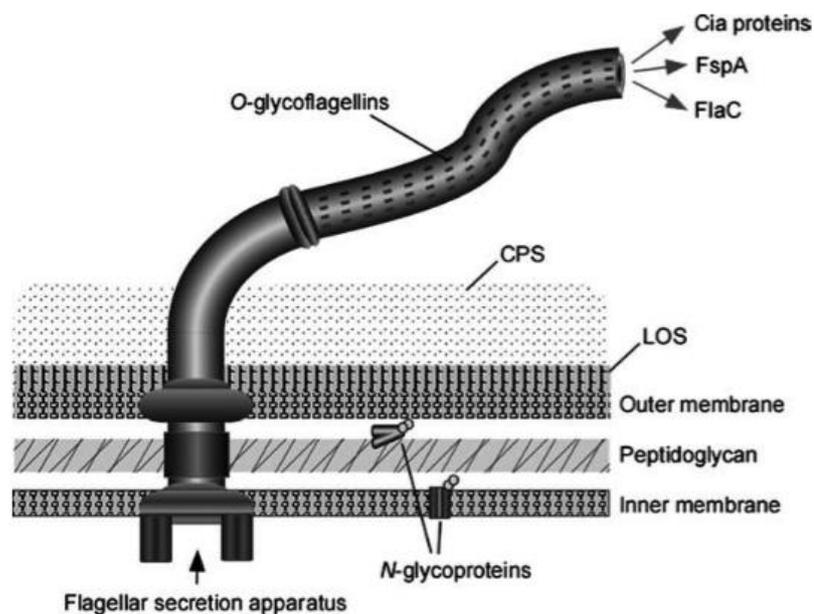


Figure 1.10. Structure of the flagellum of *C. jejuni*.

Three distinct elements are involved in the formation of the *C. jejuni* flagellum. The three elements are: basal body (MS and C-ring) attached with rod, PL ring, hook and filament. The essential components of the extracellular filament (FlaA and FlaB) proteins are modified by O-linked glycosylation. The outer membrane is decorated with variable lipooligosaccharide (LOS) and polysaccharide capsule (CPS). N-glycoproteins are found in the inner membrane and periplasm of the organism. In addition to flagellin proteins the flagellum also secretes FlaC, FspA and Cia proteins. This modified picture was taken from van Putten *et al.* (2009).

1.9.1.2.2. Flagella and Motility

Several studies have been performed to characterise the roles of *flaA* and *flaB* genes. It has been shown that the only requirement for motility is *flaA* gene. Mutagenesis of these genes has shown that *flaA* mutants produce a truncated flagellum, whilst *flaB* mutants do not assemble flagella (Wassenaar *et al.*, 1991; Matz *et al.*, 2002). A clear demonstration that pathogenesis requires motility was shown by studies, where a combination of phase variants, positive and negative for motility, were ingested by volunteer subjects; motile variants alone were subsequently isolated from the subjects' stool (Black *et al.*, 1988; Guerry, 2007). The importance of flagellin in *C.jejuni* in protective immunity was demonstrated when a recombinant protein containing part of the FlaA flagellin of *C. coli* was attached to the maltose binding protein (MBP) of *E. coli* to generate an effective subunit vaccine against *C. jejuni* (Lee *et al.*, 1999). The flagellum in *C. jejuni* is a target to host immune responses, indicating that it has a high immunogenicity. Avoidance of these responses is thought to be achieved by antigenic variation through DNA rearrangements within the flagellin locus and slipped-strand mispairing mechanisms in flagellin glycosylation genes (Karlyshev *et al.*, 2002; Nuijten *et al.*, 2000). Some studies have indicated that the *C. jejuni* flagellin is a target for phase variation and antigenic variation (Caldwell *et al.*, 1985; Harris *et al.*, 1987)

Flagellum based motility has a significant role for adherence to and invasion of intestinal epithelial cells, (Grant *et al.*, 1993; Wassenaar *et al.*, 1991; Yao *et al.*, 1994). Strains with mutations in *flaA* produce aflagellate and nonmotile variants resulting in loss of the ability to adhere to and invade human embryonic intestinal (INT407) cells (Wassenaar *et al.*, 1991; Yao *et al.*, 1994). Adherence of these mutants can be partially restored by centrifugation of mutant bacterial cells onto host cells (Wassenaar *et al.*, 1991). While nonflagellated mutants could not restore its ability to invade INT407, thus this is suggested that flagellum has an additional role in invasion (Hu and Kopecko, 2005). Another study carried out by Yao *et al.* (1994) showed that a paralysed flagella mutant (termed *pflA*, encodes a predicted protein of 788 amino acid residues and a molecular weight of 90,977 with no significant homology to known proteins) of *C. jejuni* strain 81-176 was able to adhere to but not invade INT407 cells. These studies indicated that the

flagellum is not only necessary for reaching the epithelial cells but is also important for subsequent adherence and invasion. The aflagellate (*flaA*⁻) and *pflaA* mutants were also used to investigate the colonisation of the intestinal tracts of suckling mice and three day old chicks. The motile strain, which had a shorter flagellar filament than that of the wild type strain had colonised the intestinal tract of suckling mice only when mice were challenged with a large inoculum. Also only the fully motile, wild type strain colonised the chick ceca. Both studies concluded that motility and an intact flagellum were important for colonisation (Morooka *et al.*, 1985; Nachamkin *et al.*, 1993).

The *C. jejuni* flagella apparatus also promotes virulence through export of Cia proteins (Campylobacter invasion antigens), which are synthesised via *C. jejuni* during co-culture with epithelial cells to be secreted (Poly and Guerry, 2008). These proteins play a role in both commensal colonization of poultry and *C. jejuni* invasion of human epithelial cells (Konkel *et al.*, 1999; Fernando *et al.*, 2008). Such insertional mutagenesis of the gene (*ciaB*) encoding a 73-kDa secreted protein (CiaB) resulted in a significant reduction in the number of *C. jejuni* cells internalised compared to a *C. jejuni* wild type isolate (Konkel *et al.*, 2004). Flagellar apparatus is required for secretion due to the absence of classical type III secretion system from *C. jejuni* (Konkel *et al.*, 2004). It has been found that mutations that disrupted filament assembly were found to be nonmotile (Mot⁻) and did not secrete Cia proteins (S⁻), a *flaA* (*flaB*⁺) filament mutant was found to be nonmotile but Cia protein secretion competent (Mot⁻, S⁺). The bacterial flagellum also has the capacity to modulate the formation of microcolonies through an autoagglutination mechanism (Guerry *et al.*, 2006) as shown by mutation of one of these *maf* genes in NCTC 11168 (*maf5* or *Cj1337*) resulted in a non-motile phenotype and failed to autoagglutinate (Butler, 2009). Microcolonies are likely to be essential for adhesion to host surfaces. Additionally, the flagellum seems to be essential for biofilm formation in which in *C. jejuni* biofilms, flagella may improve or facilitate initial attachment or biofilm structuring but are not essential for this process (Reuter *et al.*, 2010) and also flagella may have a role in avoidance of the innate immune response (Andersen-Nissen *et al.*, 2005).

1.9.1.2.3. Flagella and chemotaxis

A key element in flagella motility is chemotaxis, which is used by motile bacteria in order to direct their movements in the direction of more favourable conditions via sensing their chemical environment. This mechanism is employed by many pathogenic bacteria to invade their hosts (Hamer *et al.*, 2010). Similar to motility, chemotaxis is fundamental for virulence in bacteria, for instance, a chemotactic mutant of the medically important bacterium *Helicobacter pylori* lost its ability to colonise mucosa and was not able to cause gastric cancer (Porter *et al.*, 2008). Recently, chemotaxis has been found to contribute positively to the potential of *C. jejuni* strains to competitively colonise the chicken gastrointestinal tract (Thibodeau *et al.*, 2015). Extracellular signals, often sugars or amino acids, are sensed by chemoreceptors called methyl-accepting chemotaxis proteins (MCPs), which typically contain a periplasmic domain that binds to the signal. There are around 10 chemoreceptor proteins that are referred to as Transducer Like Proteins (Tlps) associated with signal transduction chemotaxis pathway in *C. jejuni*. The fundamental components of this pathway includes the essential two-component regulatory system histidine kinase (CheA) and response regulator (CheY) (Chang and Miller, 2006; Hendrixson and DiRita, 2004b) and coupling proteins, CheV and CheW, which show different affinities to various chemoreceptors (Alexander *et al.*, 2010). CheW is thought to attach with the signal domains of Tlps to form a Tlp-CheW-CheA ternary complex (Chandrashekar *et al.*, 2017), hence CheA catalyses its own auto-phosphorylation, then transfer of the phosphoryl group to the response regulator CheY (Young *et al.*, 2007). Phosphorylated CheY interacts with the FliM of the flagella motor to initiate movement (Yao *et al.*, 1997). Jama (2015) showed that Cj0700 can promote dephosphorylation of CheY and hence is likely to be a CheZ orthologue, a phosphatase which is dephosphorylates CheY in *E.coli*. In addition, the N-terminal region of CheV has a “CheW-like” domain and a carboxyl terminal CheY-like domain, so has been hypothesised to act as a phosphate sink for the chemotaxis signal-transduction machinery (Young *et al.*, 2007) (Figure 1.11). According to Hartley-Tassell *et al.*, (2010), the movement of pathogenic bacteria towards or away from a chemical gradient is attributable to the initiation of a molecular signal transduction cascade associated with the flagellar motor, producing an alteration in the direction of flagellum rotation. The bipolar flagella

phenotype of *C. jejuni* confers darting motility (Balaban and Hendrixson, 2011) to bacterium which is likely depending on the flagellar rotation either to confer run and reverse bias, as in *Pseudomonas aeruginosa*, or switching between run and stop motion as in *Rhodobacter sphaeroides* for chemo-attractant or repellent conditions respectively (Eisenbach, 2011). *C. jejuni* is mainly a commensal of the intestinal tract of many species of birds, in particular the mucus-filled crypts of the caeca; thereby this organism uses chemotaxis in order to direct movement towards these sites for colonisation. The main chemotactic agents in the colonisation process are mucins and glycoproteins, i.e., the primary constituent of mucus (Bolton, 2015). *C. jejuni* has chemotactic response towards L-fucose and L-serine, constituents of mucin whereas bile acids are repellent for *C. jejuni* (Hugdahl *et al.*, 1988). Mutagenesis studies have already been created in the *che* genes. The *cheY* gene is involved *C.jejuni* pathogenesis since a *cheY* mutant has been shown to be defective in colonisation of the intestine in chickens (Hendrixson, 2006a). In contrast, *cheY* mutants have been found to be hyperinvasive and also showed increasing adherence and invasion by threefold compared to the wild-type strain (Golden and Acheson, 2002; Yao *et al.*, 1994). Another study has shown that *cheA* mutants in *C. jejuni* were unable to colonise mice and were shown to lose invasiveness when trying to invade intestinal epithelial cells (Hendrixson and DiRita, 2004b; Chang and Miller, 2006).

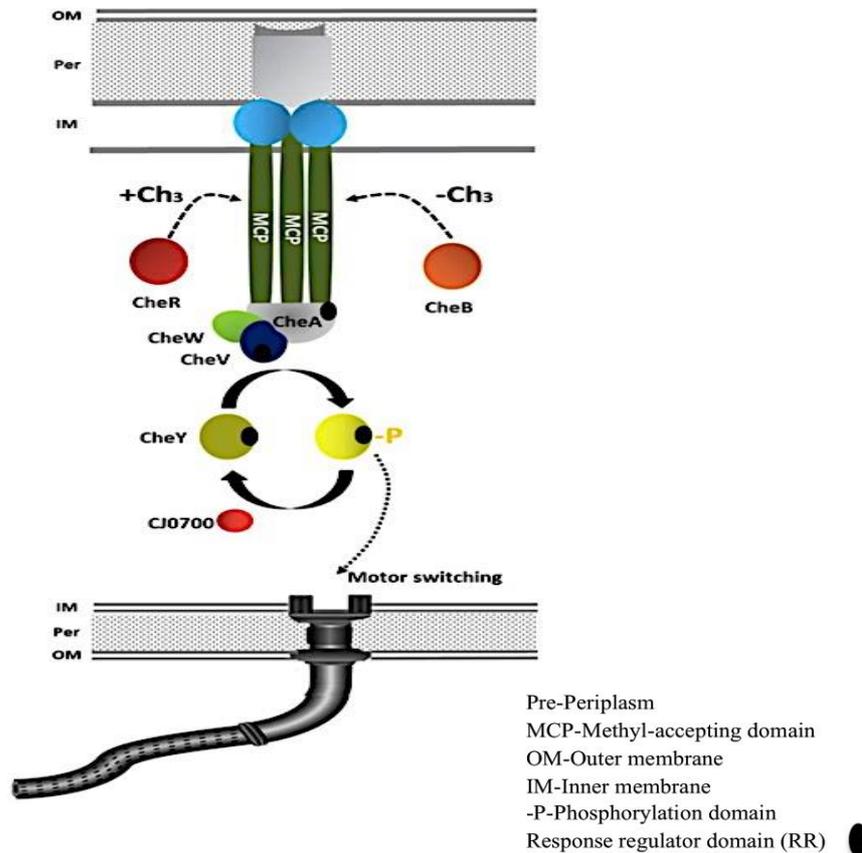


Figure 1.11. *C. jejuni* chemotaxis pathway.

Chemotactic signals are detected by dedicated groups of transmembrane chemoreceptors (MCPs). Upon receiving the stimulus, the N-signal domain of the membrane spanning protein, a histidine kinase CheA, activates the C-terminal sensor kinase causing autophosphorylation of conserved histidine residue in the sensor kinase domain. Autophosphorylated CheA-P phosphorylates CheY via transferring the phosphate (P) to conserved aspartic acid residue in N-terminal receiver domain of CheY. Phosphorylated CheY binds to flagellar motor and stimulates a switch in the direction of rotation from anticlockwise to clockwise (Raphael *et al.*, 2005). Cj0700 (a CheZ-like protein) dephosphorylates CheY-P (Jama, 2015). Methylation/demethylation of methyl accepting chemotactic domains in the MCPs regulates the whole process via two enzymes, a methyltransferase CheR to activate CheA by adding methyl group (Ch₃) and methylesterase CheB to deactivate CheA by removing methyl group. The presence of response regulator (RR) domain in CheV could inhibit the activity of Methylesterase CheB (missing a response regulator domain). The image adapted from (Elgamoudi, 2016).

1.9.1.3. Adhesion

Adhesion is an essential virulence factor for most bacterial pathogens in terms of establishing infection and inducing disease (Konkel *et al.*, 2004). The commensal colonisation in poultry and other animals or pathogenic colonisation in humans are linked with establishing *C. jejuni* cells themselves within the mucus layer of the intestinal epithelium (Byrne *et al.*, 2007; Ketley and Konkel, 2005).

The interaction between *Campylobacter* and host cells is a difficult process including interaction between different surface structures of the bacterial cells and specific host cells receptors. There are various molecules that have been demonstrated to have a role in adherence, involving the Lipooligosaccharides (LOS), the flagellum and the major outer membrane protein (Konkel *et al.*, 2004). Peb1, Peb4, CapA, CadF, JlpA, FlpA, and major outer membrane protein (MOMP) are well-studied protein adhesion determinants (Flanagan *et al.*, 2009; Goulhen *et al.*, 2004; Konkel *et al.*, 2010). For example, it has been suggested that CadF protein supports *C. jejuni* binding to fibronectin (Fn) on the host epithelium, the mutation in *cadF* resulted in 50% decrease in adhesion to human cells compared with the wild-type (Konkel *et al.*, 2004), suggesting, the importance of CadF protein as an adhesion factor.

1.9.1.4. Invasion

Another important virulence factor in *C.jejuni* is the invasion of host cells (Byrne *et al.*, 2007; Hermans, Van Deun, Messens, *et al.*, 2011). The potential approach of *C.jejuni* to invade the intestinal epithelial cells starts after colonisation of the human intestinal mucus layer in which *C. jejuni* cells may modify the signal transduction pathways of its host in order to be able, then, to enter and passage the epithelial monolayer (Backert *et al.*, 2013; Konkel *et al.*, 2004). The flagellum is deemed essential for *C. jejuni* to invade and penetrate the human intestinal, causing host responses that link to the inflammatory enteritis characteristics of *C. jejuni* diarrhoeal disease (Barrero-Tobon and Hendrixson, 2014).

A number of bacterial proteins have been associated with invasion. For maximal invasion of host epithelial cells, *C. jejuni* must secrete proteins from the flagellar type III secretion system (T3SS) (Christensen *et al.*, 2009). *C. jejuni* was seen to produce at least 14 additional proteins following the incubation of *C.jejuni* with

INT407 cells compared to bacteria cultured without cells (Konkel and Cieplak, 1992). Later, Konkel *et al.* (1993) have been identified nine new proteins from media-grown bacteria, one of these proteins has found to be necessary for the secretion of the other eight and termed CiaB (Campylobacter invasion antigen B) (Konkel *et al.*, 1999). The consideration of CiaB proteins as an important factor in *C. jejuni* pathogenesis has been shown through the mutagenesis studies with *ciaB* gene, which is deficient in Cia protein secretion. In piglets inoculated with *C. jejuni ciaB* mutant developed diarrhoea after three days post inoculation whilst all piglets inoculated with a *C. jejuni* wild type isolate developed diarrhoea within 24 h (Christensen *et al.*, 2009).

1.9.1.5. Toxin production

The production of cytolethal distending toxin (CDT) is existence in many pathogenic Gram-negative bacteria and considered as virulence factor (Zilbauer *et al.*, 2008). However, cytolethal distending toxin (CDT) has been only confirmed genetically in *C. jejuni* (Young *et al.*, 2007). Sequencing analysis has provided an insight into the *cdt* gene cluster in *C. jejuni* which comprises of three adjacent genes, termed *cdtA*, *cdtB* and *cdtC* (Pickett *et al.*, 1996). CdtA and CdtC are required to bind to the susceptible cells and hence delivering CdtB into target cells while CdtB subunit is responsible for toxin activity due to its ability to break the host DNA double-strand by producing DNase I-like activity (Ge *et al.*, 2008). This toxin induces the distention and death cells as a consequence of the blocking of cell division through arresting cells in the G2/M phase of the cell cycle (Zilbauer *et al.*, 2008). CDT is expressed during colonisation and causes distension of the epithelial cells, which manifests in bloody diarrhoea (Dasti *et al.*, 2010). A correlation between CDT and *C. jejuni* pathogenicity has been shown in a rat model incubated with a mutated *C. jejuni* 81-176 that does not express functional *cdtB*, due to an insertion of the kanamycin gene (CDT-). Therefore, diarrhoea and severe inflammation were observed in the entire gastro-intestinal tract due to CDT-induced damage to the epithelial barrier (Jain *et al.*, 2008; Pokkunuri *et al.*, 2012). Therefore, it is clear that the notion of bacterial toxins play a role is consistent with the presence of a transient watery diarrhoea that progresses to a bloody diarrhoea in *C. jejuni* enteritis (Dasti *et al.*, 2010).

However, CDT has not been shown to play a role in colonisation of chickens (Hu and Kopecko, 2008).

1.9.1.6. Lipooligosaccharide

Gram-negative bacteria have an outer membrane comprising of lipopolysaccharide (LPS) or LOS, which are variable structures with a key role in virulence (Moran *et al.*, 1996). LPS is composed of an O-polysaccharide chain, core oligosaccharide and a lipid A component in the outer membrane, whereas LOS lacks an O-polysaccharide repeating structure. Nevertheless, the structural variation in the outer core of LOS is comparable with that of LPS (Gilbert *et al.*, 2008).

An early observation was that variation in the LOS structures of *C.jejuni* was associated with differences in *C. jejuni* serotypes. Later genomic and mass spectrometry studies showed that there was a huge amount of variation in the LOS locus and LOS structure between *Campylobacter* strains and species. The variability of *C. jejuni* LOS is correlated with the existence of a number of genetic mechanisms, which influence glycosyltransferase activity and hence vary the LOS outer core structures. These mechanisms include; i) phase variation due to homopolymeric tracts; ii) gene inactivation due to the deletion or insertion of single or multiple bases, but without phase variation; iii) substitution of amino acid leading to an inactive variant of the glycosyltransferase and, iv) single or multiple mutations resulting in glycosyltransferases with different acceptor specificities. The high diversity of LOS structures produced by these genetic mechanisms allows *C. jejuni* to change the structure of these cell-surface carbohydrates (Gilbert *et al.*, 2008).

A key feature of *C. jejuni* is molecular mimicry between lipooligosaccharides (LOS) and gangliosides in peripheral nerves (Godschalk *et al.*, 2007). The presence of sialic acid-containing epitopes in the LOS core structure are responsible for the ganglioside mimicry and may lead to the development of GBS (Godschalk *et al.*, 2007) (section 1.8.2). These sialylated LOS epitopes may be a feature exploited by *C. jejuni* to avoid the immune response of the host, thus the loss of sialic acid from the LOS of one *C. jejuni* strain resulted in increased immunogenicity and increased susceptibility to being killed by normal human

serum (Guerry *et al.*, 2000). Five LOS locus classes - A, B, C, M and R - encode genes responsible for the production of sialylated LOS while D, F and G lack a *cst* gene encoding a sialic acid transferase (Parker *et al.*, 2008). These LOS structures include terminal tetra- and pentasaccharide moieties identical to those of GM1a and GD1a gangliosides (Aspinall *et al.*, 1994; Moran and O'Malley, 1995).

It is clear that the genetic variation leads to the diversity of LOS structures and hence raises an important feature for *C.jejuni* to colonise a wide range of intestinal niches, which is a natural environment for *C. jejuni*. Additionally, structural variation of LOS acquires *C.jejuni* the ability to survive in different non-intestinal niches (Karlyshev *et al.*, 2005).

1.10. Immune response to *C. jejuni* infection

1.10.1. Human Immune Response

The innate immune response is important for the activation of the subsequent adaptive immune response (Boyd *et al.*, 2005). Innate immune responses are initiated by contact between host pattern recognition receptors (PRRs) located on the surface of intestinal epithelial cells and pathogen components termed pathogen-associated molecular patterns (PAMPs). Host signal transduction pathways are triggered by this recognition leads to secretion of proinflammatory molecules (Eckmann and Kagnoff, 2005). The most widely studied PRRs are the Toll-like receptors (TLRs) family, which contain a highly conserved group of molecules. TLRs have a significant role in pathogen detection and regulation of immune responses during infections. One of these TLRs is toll like receptor 5 (TLR5), which is located on the apical surface of intestinal epithelial cells and recognises conserved epitopes within some bacterial flagellins. This recognition leads to the initiation of signalling and expression of interleukin-8 (IL-8). The release of IL-8 and other proinflammatory molecules leads to recruitment of specific phagocytes such as macrophages, dendritic cells (DC), monocytes and neutrophils, which then interact with and clear *C. jejuni* cells (Figure 1.12) (Fleckenstein and Kopecko, 2001; Young *et al.*, 2007). This interaction leads to stimulation of a large innate pro-inflammatory response via the activation of nuclear transcription factor kappaB (NF- κ B) with release of multiple cytokines and tumour necrosis factor alpha (TNF- α). There is also activation of T helper cells type 1 (T_H-1), which helps to elicit a specific adaptive cell-mediated immune response (Young *et al.*, 2007).

DCs have essential roles in both the innate and adaptive immune response to microbial pathogens as they are major antigen-presenting cells and commonly present in tissues within or adjacent to the intestinal mucosa (Medzhitov and Janeway, 1997). The determination of the type of T-cell mediated immune response to an invader is regulated by DCs, this includes initiating the primary immune response and allow formation of immunological memory (Palucka and Banchereau, 2002). Rapid internalisation of *C. jejuni* into human monocyte-derived dendritic cells (DCs) occurs over a 2 hours period following exposure but after longer times of incubation (24 hr or 48 hr) the number of viable intracellular

bacteria is reduced (Hu *et al.*, 2006). Further, maturation of the DCs is induced by both live and heat-killed *C. jejuni* as indicated by up-regulation of cell surface marker proteins, secretion of interleukins and activation of NF- κ B. An adaptive immune response has been demonstrated against *C. jejuni* during infections in humans (Young *et al.*, 2007) with specific serum immunoglobulins being found in 80–90% of patients with culturable *C. jejuni*. The levels of immunoglobulin (Ig) A reach a peak 7 to 10 days after the start of symptoms and then decrease rapidly. IgG reaches a peak after 3-4 weeks and persists at high levels for long periods of time. IgM is found on mucosal surfaces, secreted through an active secretion mechanism, and its levels remain high for longer than IgA but shorter than IgG. Levels of antibodies (IgA, IgG and IgM) were detected in infected children younger than 6 months of age (Blaser, 1997; Cawthraw *et al.*, 2002; Strid *et al.*, 2001).

The elicitation of protective immunity against *Campylobacter* requires only a few protein antigens (Newell and Nachamkin, 1992). However, studies have demonstrated that serum antibodies obtained from individuals persistently exposed to *C. jejuni* reacted with a wide range of antigens (Cawthraw *et al.*, 2000). Antibodies present in human sera recognise several bacterial components including flagella, the major outer-membrane protein (MOMP), other outer membrane proteins and LOS (Guerry *et al.*, 2000). The absence of *N*-acetylneuraminic acid (NeuNAc) from the LOS core reduces its immunogenicity. Additionally, pooled anti-sera from infected patients was found to contain antibodies against CDT that neutralised the action of this toxin (Young *et al.*, 2007).

1.10.2. Chicken Immune Response

C.jejuni is often considered to be a commensal organism for chickens through its ability to be tolerated by the chicken immune system (Hermans *et al.*, 2012a). Conversely, Humphrey *et al.*, (2014) reported that some chicken breeds that have a strong inflammatory response to *C.jejuni* that leads to diarrhoea. Thus, it is unclear if unknown factors either inhibit the immune response or direct it towards tolerance.

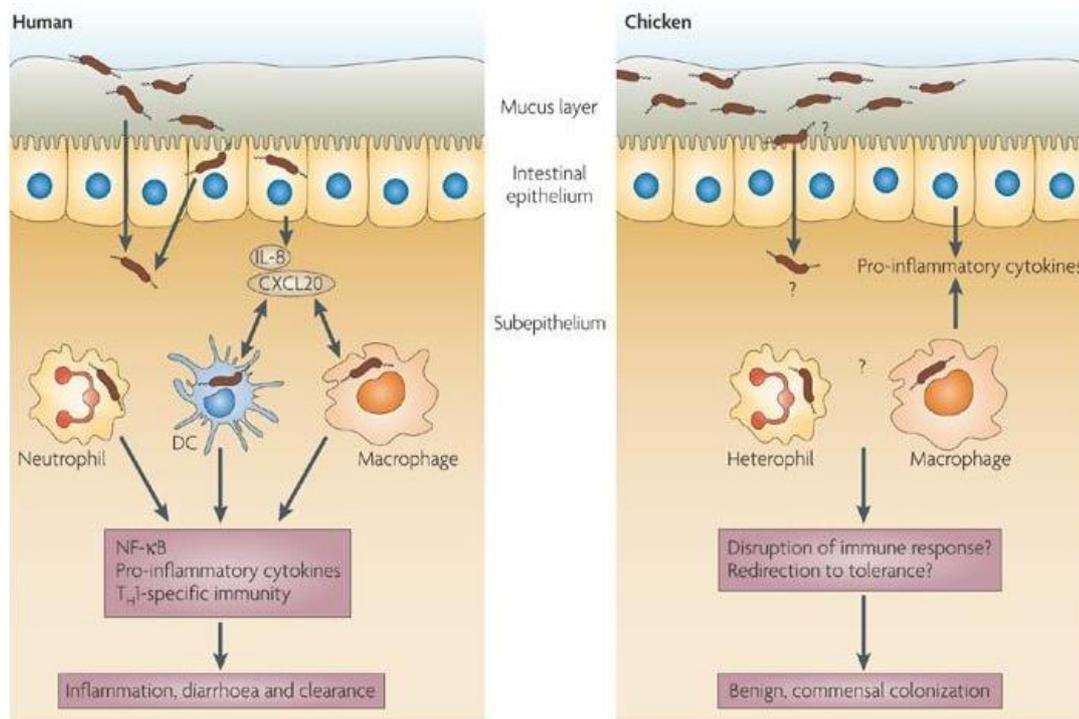
On the first day of hatching, the guts of chicks do not contain a microbial flora while the chicks also have an immature mucosal immune system. After 3 to 7 days, an increase in caecal pro-inflammatory chemokine and cytokine expression is observed as a result of exposure to feed and microflora (Bar-Shira and Friedman, 2006). The potential for infection is reduced by these intestinal innate immune responses, which limits invasion by both commensal and pathogenic bacteria. This innate response involves several tissues, cell types (such as epithelial cells, monocytes /macrophages, dendritic cells, natural killer cells and neutrophils), germline-encoded molecules such as chemo and cytokines and mucous secretions. Another mechanism of innate defence is antimicrobial proteins, which are effective at killing a wide variety of bacteria. A key example is the defensins, which are cationic proteins with the ability to cause cell lysis and comprise of three subfamilies α -, β - and θ -defensins. To date, 13 avian β -defensins, also called gallinacins or Gal have been described (Brisbin *et al.*, 2008).

The ability of *C. jejuni* to stimulate an innate immune response in chickens has been shown *in vitro* using both epithelial and macrophage cell lines with these infections resulting in production of IL-1 β , IL-6 and inducible nitric oxide (NO) synthase (Figure 1.12) (Young *et al.*, 2007). An activation of Toll-like receptors is an important step in the initiation of innate immune responses to many bacteria infections in the gastrointestinal tract (Linde *et al.*, 2008). This activation results in enhancement of expression of effector molecules, such as proinflammatory cytokines. The interaction between pathogen recognition receptors (PRR) expressed by innate defence cells with their respective ligands leads to generation of a cytokine microenvironment. This affects the type of immune response generated. However, the switching from innate to adaptive immunity in chickens is not fully understood and not all PRR and cytokines have been completely identified (Brisbin *et al.*, 2008). Several chicken TLRs have been shown to recognise specific *C. jejuni* ligands, including the chicken cell-surface-expressed chTLR2 and the chicken TLR4/myeloid differentiation protein-2 (chTLR4/chMD-2) complex (Hermans *et al.*, 2012). Activation of chTLR2, chTLR4 and chTLR21 leads to the secretion of inflammatory cytokines and chemokines through myeloid differentiation primary response gene 88 (MyD88)–dependent activation of nuclear transcription factor kappaB (NF- κ B). Production of inducible

nitric oxide synthase-mediated NO from chicken monocytes may also be triggered by chTLR4 and chTLR21 ligands (Hermans *et al.*, 2012). Finally, TLR5 specifically senses and responds to conserved regions in bacterial flagellins. However, TLR5 signalling does not play an essential role in the chick immune response against *C. jejuni*, due to the lack of recognition sites for this PRR in the flagellin of *C. jejuni* and hence an inability to stimulate the receptor (de Zoete *et al.*, 2010).

An alternative protection for chicks is maternal antibodies. These antibodies have been detected in the first two weeks after hatching and may protect the new-born chicks against *C. jejuni* colonisation while the innate and adaptive immune system is undergoing further development (Cawthraw *et al.*, 1994). Sahin *et al.* (2003) have proven that partial protection against colonization in young chickens following exposure to both homologous and heterologous *C. jejuni* strains could be mediated by anti-*C. jejuni* maternal antibodies. It has also been shown by immunoblotting that maternally derived antibodies react strongly with outer membrane surface components including flagellin, LOS, and MOMP of *C. jejuni* (Cawthraw *et al.*, 1994; Sahin *et al.*, 2001; Shoaf-Sweeney *et al.*, 2008).

Overall, there seems to be some evidence to indicate that cytokine responses are elicited in the guts of chickens following infection with *C. jejuni* and that are specific antigens such as cell-surface carbohydrate (LPS or LOS) and flagella trigger antibody responses (Lacharme-Lora *et al.*, 2017).



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Figure 1.12. Innate immune response to *C. jejuni* in human and chicken.

C. jejuni invades the mucous layer in the human intestine and then adheres to, and is internalised by, the intestinal epithelial cells, leading to the production of interleukin (IL-8). The recruitment of dendritic cells (DC), macrophages and neutrophils is induced by the secretion of IL-8. These interactions generate an enormous pro-inflammatory response that is correlated with the increases in cytokines. Similarly, the primary niche of *C. jejuni* is the mucosal layer of the chicken intestines with *in vitro* evidence indicating that *C. jejuni* infection stimulates the production of IL-1 β , IL-6 and intracellular nitric oxide synthase from chicken epithelial cells and macrophages. However, the subsequent host response does not typically lead to inflammatory diarrhoea in chickens (Young *et al.*, 2007). Question marks refer to parts that require more investigations to fill the gaps in the knowledge regarding *Campylobacter*–chicken interactions.

1.11. Protein glycosylation in *C. jejuni*

Protein glycosylation is the covalent attachment of sugar moieties to an amino acid linkage via a post-translational protein modification process. The process of attachment is accomplished by glycosyltransferases. This process starts when glycosyltransferases acquire energy through use of activated sugar or lipid-phospho sugar donors to direct the synthesis of glycosidic bonds that covalently attach the glycan to a protein (Merino and Tomás, 2014). The transformation of glycans into a protein receptor can be achieved by two different mechanisms; the glycan chains can be constructed on a lipid carrier and transferred *en bloc* onto target proteins by an oligosaccharyltransferase (OTase)-dependent mechanism, or sugars can be linked consecutively onto carrier proteins by an OTase-independent mechanism (Schirm *et al.*, 2003). Glycosylation modifications contribute to protein, and specifically flagellin, diversity in a number of gram-negative pathogenic bacteria, including *Pseudomonas aeruginosa*, *Helicobacter pylori* and *C. jejuni*. These glycosylation modifications are predicted to affect the immunogenicity of bacterial cells, their interactions with eukaryotic cells and to circumvent host immune responses (Howard *et al.*, 2009a).

The genome sequencing of *C. jejuni* strain NCTC 11168 provided several notable findings with regard to glycosylation and lead to further analyses that provided important information about the the O-linked glycosylation systems that modifies the flagellum and illustrated the existence of a novel N-linked glycosylation pathway (Karlyshev *et al.*, 2005).

1.11.1. *N*-glycosylation of *C.jejuni*

The *N*-linked protein glycosylation locus contains a cluster of genes in a locus with a size of 17 kilobase pairs (kbp) and are called the *pgl* genes for protein glycosylation. *Pgl* genes encode enzymes facilitating glycosylation of several proteins. The glycans are covalently linked with proteins via asparagine residues in the motif Asn-Xaa-Ser/Thr (Merino and Tomás, 2014). PglB is the putative oligosaccharyltransferase, considered to be the essential product in *N*-linked glycosylation and with a probable responsibility for linking glycan to the asparagine (Szymanski *et al.*, 2003). Mass spectrometry and NMR spectroscopy have been applied to determine the structure of the *N*-glycan which consists of a heptasaccharide GalNAc- α 1,4-GalNAc- α 1,4-(Glc β 1,3)-GalNAc- α 1,4-GalNAc- α 1,4-GalNAc- α 1,3-Bac- β 1,N-Asn, where Bac is bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucose) (Knauer and Lehle, 1999). It has been proposed the process for synthesising *N*-linked glycoproteins starts with the conversion of UDP-HexNAc to bacillosamine through serial alteration by PglF (dehydratase), PglE (aminotransferase) and PglD (acetyltransferase) in the cytoplasm. PglC then transfers the bacillosamine residue to a lipid carrier. Diacetamido-trideoxy hexose (DATDH), a sugar related to bacillosamine but with an unknown hexose character is attached to the α -1,3-linked galactose component by PglA, an orthologue of an *N. meningitidis* gene. It has thus been proposed that PglA connects the α -1,3-linked *N*-acetylglucosamine (GalNAc) structure to bacillosamine in *C. jejuni*. PglH, an α -1,4-GalNAc transferase, along with its homologous family member PglJ, is thought to function in attaching the next four α -1,4-linked GalNAc moieties. The branching glucose component is most likely added by a third glycosyltransferase, PglI (Figure 1.13). The ATP-binding cassette (ABC) transporter orthologue, WlaB, has been suggested to 'flip' the assembled heptasaccharide through the inner membrane and into the adjacent periplasm. A similar mechanism exists in eukaryotes, whereby the assembled sugars are 'flipped' into the endoplasmic reticulum (ER) (Karlyshev *et al.*, 2005).

The processes of glycosylation occurs in the periplasm and more than 30 proteins may be modified by this locus (Young *et al.*, 2002). The majority of these proteins seem to be either surface exposed or excreted. The *pgl* genes are highly conserved between strains of *C. jejuni* and *C. coli*, unlike the genes

involved in the glycosylation of *C. jejuni* flagellin (Szymanski *et al.*, 2003). This suggests that generation of antigenic variation is not the principal role of the *pgl* system, even though the majority of proteins decorated by this system are surface-exposed or secreted. Another possibility is that this system plays an essential role in terms of offering protection against extracellular proteases.

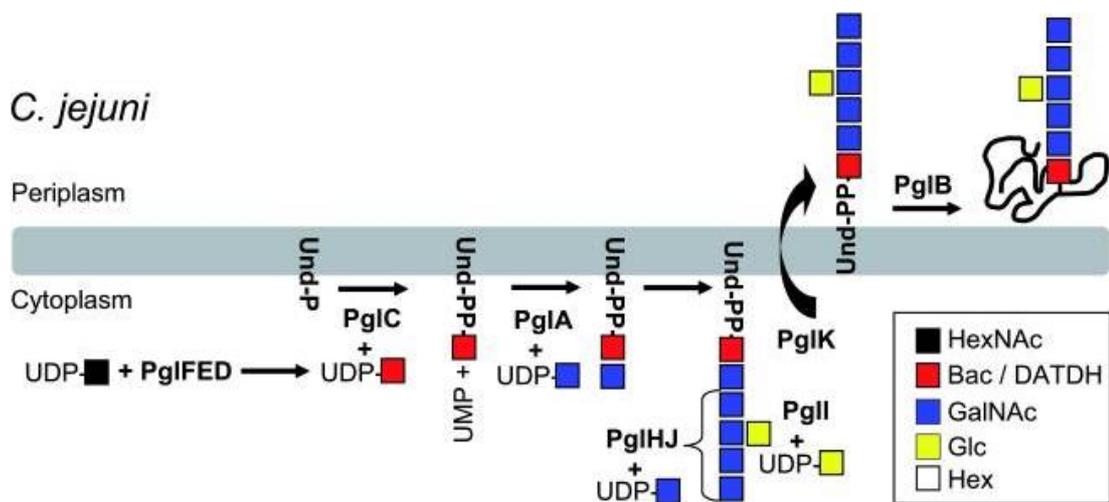


Figure 1.13. Diagrammatic representation of the current model of the *N*-linked glycosylation pathway in *C. jejuni*.

Cluster of genes named *pgl* (protein glycosylation locus) encodes the *N*-glycosylation machinery and includes at least 30 different proteins. The consecutive action of PglF, PglE and PglD catalyse the biosynthesis of UDP-bacillosamine from UDP-*N*-acetyl glucosamine (*N*-GlcNAc). PglC transfers bacillosamine to undecaprenyl pyrophosphate (UPP) and then the action of PglA, PglJ and PglH GalNAc transferases extend it with five *N*-acetylgalactosamine (GalNAc) residues. After that, glucose is added on a β 1, 3-linkage by PglI, which is a branching enzyme. Finally, PglK flips the assembled heptasaccharide into the periplasmic space, which is then transferred *en bloc* to the asparagine residue of the acceptor polypeptide in the periplasm by an *N*-OTase named PglB (Merino and Tomás, 2014). The figure adapted from (Aas *et al.*, 2007). *N*-acetylhexoseamine (HexNAc); Bacillosamine (Bac) 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH); *N*-Acetylgalactosamine (GalNAc); D-Glucose (Glc); Hexose (Hex).

1.11.2. Structural analysis of the O-linked flagellin glycosylation system in *Campylobacter* spp.

Logan et al. (1989) reported that the *C. coli* VC167 flagellin was posttranslationally modified on serine residues in the central region of the amino acid chain. Subsequent studies by Doig et al. (1996) showed the sensitivity of *C. jejuni* flagellins to periodate oxidation and recognition by a sialic-acid-specific lectin, suggestive of an O-linked flagellin glycosylation process and indicative of modification with sialic-acid (Neu5Ac). Subsequently, whole flagellins were isolated from three *C. jejuni* strains (81-176, NCTC 11168 and OH4384) and one *C. coli* strain (VC167 T2) and analysed for their sugar content, these experiments demonstrated that the flagellins are modified, mainly with pseudaminic acid (Pse), which is structurally related to sialic acid (Thibault *et al.*, 2001). The predominant O-glycans are derivatives of pseudaminic acid (PseAc, where Ac represents an acetamido group) and an acetamidino form of legionaminic acid (LegAm, where Am represents acetamidino) (Logan *et al.*, 2008). The process by which O-linked glycosylation of flagellin arises is not fully understood, unlike the N-linked mechanism. The attachment site is not linked to a consensus amino acid; however, the majority of modified residues are located in a narrow hydrophobic section of the central core structure. The linkage is exclusively on Ser/Thr residues as is the case with eukaryotic O-linkage (Szymanski *et al.*, 2003).

Thibault et al. (2001) investigated the flagellin-associated glycan structures from *C. jejuni* strain 81-176 and found that to be modified with pseudaminic acid (PseAc) on 19 Ser (serine) / Thr (threonine) residues (Figure 1.14), these modifications increased the molecular mass of the flagellin protein by approximately 10% (6000Da). Although, PseAc has been identified as the main glycosyl component, other modifications, which are structurally similar to PseAc, are present, including acetamidino pseudaminic acid (PseAm), an O-acetyl derivative of PseAc (PseOAc) and a dihydroxy propionyl derivative, (PsePr) (Thibault *et al.*, 2001). Both PseAc and PseAm were identified in *C. coli* VC167 flagellin whereas PseOAc and PsePr were not found. McNally et al. (2007) revealed that both Pse5Ac7Am and Leg5Am7Ac have the same mass with an N-methylacetimidoyl form, MeLegAm, also existent on *C. coli* VC167 flagellins.

In terms of NCTC11168, the types of modification present in the flagellin was initially analysed by Logan et al. (2009). The glycans in NCTC11168 decorate a specific number of amino acid residues in the flagellin (Ser /Thr residues), however it is unclear whether the presence of particular types of O-glycan is permanent or whether there is variation within the population of molecules in the nature of the glycan or the extent of glycosylation (Zampronio *et al.*, 2011). Serine residues were found to comprise about 11% of residues in the flagellin of this strain whilst threonine comprised approximately 6%, however the glycosylation is not present on many of these motifs. The mechanism for selection of specific residues to be targets for glycosylation is still unidentified, suggesting that this process either is tightly regulated or stochastic in nature (Ulasi *et al.*, 2015).

MGFRINTNVAALNAKANS^SDLNAK^SLDASLSRL^{SS}GLRINSAADDASGM^AIAD^SLR^SQANT 60
 LGQAI^SNGNDALGILQ^TADKAMDEQLKILD^TIK^TKATQAAQDGQ^SLK^TRTMLQADINKLM 120
 EELDNIAN^TTSFNGKQL^LSGNFTN^QEFQIGASS^NQTVKATIGAT^QSSKIGVTRFETGA^QS 180
 FTSGVVGL^TIKNYNGIEDFKFDNVVI^STSVGTGLGALAE^EINKSADKTGVRATYDVK^TTTG 240
 VYAIKEG^TTSQEFAINGV^TIGKIEYKDG^DNG^SLI^SAINAVK^DTTGVQASKDENGKLV^LT 300
 SADGRGIK^ITGDIGVGS^GILANQKENYGR^LSLVKNDGRDINI^SGTNL^SAIGMG^TTD^MIS^Q 360
 SSV^SLR^SES^KGQ^ISATNADAMGF^NSYKGGGK^FV^FQNV^SSI^SAFM^SAQ^GG^SFR^GSG^SGF^SVG 420
 SGKNL^SVGL^SQGIQ^IISAA^SMSNTYV^VAG^SGF^SSG^SGN^SQFAALKTTAANTTDETAGV 480
 T^SLKGAMAVMDIAETAITNLDQIRADIG^SIQN^QV^TSTINNI^TVTQVNVKAAESQIRDVDF 540
 ASESANY^SKANILAQ^SGSYAMAQAN^{SS}QQNVLRLLQ 576

Figure 1.14. Modification sites with glycans on *C. jejuni* 81-176 flagellin

Serine and threonine residues modification with pseudaminic acid derived glycosyl groups are indicated in red and other non-modified residues (Ser/Thr) are indicated in yellow (Thibault *et al.*, 2001).

1.11.3. Genetic structure of the O-linked flagellar glycosylation locus in *C.jejuni*

The flagellar glycosylation locus was identified following sequencing of the *C. jejuni* strain NCTC11168 genome; this locus was estimated to contain over 47 genes including genes encoding the flagellin structural proteins FlaA and FlaB (Parkhill *et al.*, 2000). This locus exhibits considerable variation between strains with a size of 20 kbp in *C. jejuni* 81-176 versus 50kbp in both *C. jejuni* strains NCTC 11168 and RM1221 (Fouts *et al.*, 2005; Parkhill *et al.*, 2000). A feature of these loci is the presence of highly similar orthologous genes including the *maf* (motility-accessory factor) and 0627 gene families. The *maf* gene family was directly linked to motility and includes *maf1* (*Cj1318*), *maf2* (*Cj1333*), *maf3* (*Cj1334*), *maf4* (*Cj1335 /Cj1336*), *maf5* (*Cj1337*), *maf6* (*Cj1340c*) and *maf7* (*Cj1341c*) (Karlyshev *et al.*, 2002).

Genetic studies have linked flagellin genes to host colonisation. A comparative phylogenomic study has characterised a set of five genes (*Cj1321* to *Cj1326*) as a livestock clade (Champion *et al.*, 2005) with *Cj1324* partially increasing the ability of *C. jejuni* to colonise chickens. While the *Cj1324* (*ptmG*) mutants were motile and had full-length flagella, their hydrophobicity was decreased and there was a reduction in their ability to autoagglutinate and form biofilms compared with the wild type. Analysis of *Cj1324* mutant flagellin revealed the absence of specific legionaminic acid derivatives from flagellin. Hence, the specificity of these glycans indicates their importance in *Campylobacter* strain isolated from chickens, showing a strong evidence in the involvement of flagellin glycosylation in colonisation and explaining the preference of certain *Campylobacter* isolate to potentially colonise chickens than others (Howard *et al.*, 2009a).

Genetic analysis has identified 23 genes as being involved in PseAc and PseAm biosynthesis in *C. jejuni* 81-176, which are characterised as the *pse* family (Figure 1.15). Genes encoding the enzymatic machinery for conversion of UDP-*N*-acetylglucosamine to pseudaminic acid and related derivatives are *pseB/Cj1293*, *pseC/Cj1294*, *pseH/Cj1313*, *pseG/Cj1312*, *pseI/Cj1317* and *pseF/Cj1311* (Guerry *et al.*, 2006; Thibault *et al.*, 2001; McNally *et al.*, 2007). The *pse* pathway appears to be conserved among *Campylobacter* members including in *C. coli* (Logan *et al.*, 2002). However, *pseA* is a pseudogene in *C. coli* VC167

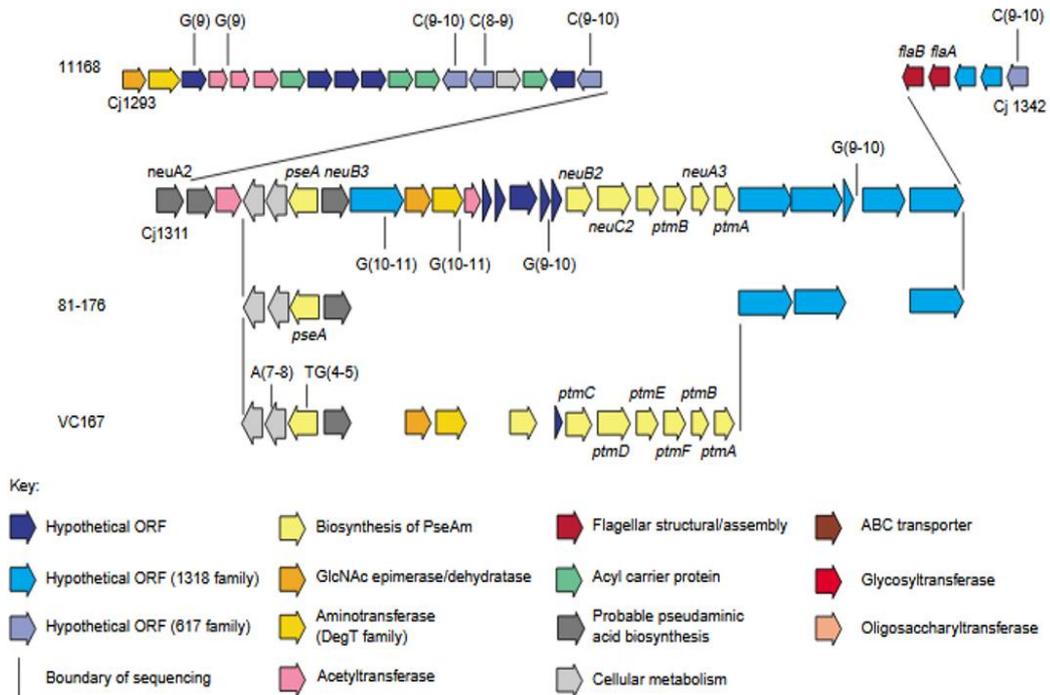
whereas in *C. jejuni* 81-176 this gene is involved in synthesis of PseAm directly from PseAc (Logan *et al.*, 2009). Additionally legionaminic acid and its derivatives are synthesised in *C. coli* VC167 by a separate pathway encoded by the posttranslational modification (*ptm*) genes (Figure 1.15) (Logan *et al.*, 2009; McNally *et al.*, 2007). Contrastingly, both the *pse* and *ptm* pathways are present in the flagellar glycosylation locus of *C. jejuni* NCTC11168 (Figure 1.15) (Guerry *et al.*, 2006; Thibault *et al.*, 2001; McNally *et al.*, 2007). These results indicate that *C. jejuni* NCTC11168 flagellins are likely to be glycosylated with glycans found in both *C. jejuni* 81-176 and *C. coli* VC167 strains (Logan *et al.*, 2009). This high complexity of the glycosylation locus in *C. jejuni* NCTC 11168 may lead to high diversity in the flagella modifications including distinct pathways such as the addition of di-*O*-methylglyceric derivatives to both PseAc and PseAm (Logan *et al.*, 2009).

A key feature of the NCTC 11168 genome was the presence of 29 phase variable genes with poly-G tracts. Eight of these phase variable genes have been putatively linked to flagellar glycosylation; three are in the *maf* family while the remainders are of the 617 family. The *maf* genes encompass *Cj1318* (*maf1*) and *Cj1335* (*maf4*) with 98% amino acid sequence similarity and *Cj1342c* (*maf7*) that is more distantly related. Differences in motility are known to arise from slipped-strand mispairing in the G-tracts of some *maf* family genes (Karlyshev *et al.*, 2002). Van Alphen *et al.* (2008) investigated the functions of the *maf4* gene in *C. jejuni* strain 108 and showed that it contributed to changes in flagellin glycosylation and bacterial agglutination behaviour suggesting that this modification provides the basis for the change in motility. Three other phase variable genes in the flagella locus are *Cj1342c* (*maf7*), *Cj1321* and *Cj1325*. The location of the repeat tract in *Cj1321* gene is 46bp upstream, which may influence binding of transcription factors and hence lead to variations in transcriptional expression. *Cj1321* is a homologous to amino transferase in other species, whereas *Cj1325* is a putative methyltransferase (Aidley, 2017). As the experimental investigation revealed that the importance of *Cj1324* in chicken colonization is likely related to *Cj1321* and *Cj1325* that may influence the behaviour of *C. jejuni* via structural changes in the flagellar glycoproteins.

Cj1295 is another phase variable genes, which is identified as a flagellar glycosyltransferase. The *Cj1295* gene product attaches a di-O- methylglyceroyl-modified derivative of pseudaminic acid to FlaA as shown by Hitchen et al. (2010). Enrichment of the 9G-ON variants of *Cj1295* was detected following serial passage of *C. jejuni* NCTC11168 through a mouse model by Jerome et al. (2011). These authors also found enrichment of the ON variants in the adjacent PV locus *Cj1296* another putative flagellar glycosylation gene.

Finally, *Cj1305c*, *Cj1306c* and *Cj1310*, located in the flagellin glycosylation locus, are predicted to be phase-variable flagellin glycosyltransferases. However, the exact functions of these genes are not known although *Cj1306c* exhibited significant enrichment for the ON state after serial passage through a mouse model (Jerome *et al.*, 2011).

Together, the flagellin glycosylation locus consists of eight genes containing homopolymeric tracts of nucleotides that are prone to slipped-strand mispairing during replication. Thus, it is possible that structural variation in the flagellar glycosylation due to PV may contribute to immune escape or immune modulation.



1.11.4. Glycosylated Flagellin in *C. jejuni*

There are two flagellin proteins; FlaA is the major component comprising 90% of the total structure; and FlaB. FlaA consists of 572 amino acid residues with an approximate size of 59 kDa, however SDS-PAGE analysis indicates an apparent Mw about one-tenth greater than this, probably as a result of the modification with numerous glycan residues (Ulasi *et al.*, 2015). The *flaA* and *flaB* genes share 93% homology, with the majority of the amino acid differences found in the amino and carboxy termini. The *flaA* and *flaB* genes are chromosomally adjacent to each other, separated by 163 bp, and are each 1731 bp in length (Wassenaar *et al.*, 1991). The alternative sigma factors σ^{28} and σ^{54} , comprise a two-part regulatory system including the sensor kinase FlgS and the σ^{54} -response regulator FlgR, in addition to the flagellar export apparatus, and are thought to tightly regulate flagellar biogenesis in *C. jejuni* (Hendrixson, 2006a). The sequences of the flagellar filament genes of *C. jejuni* (FlaA and FlaB) and *Salmonella* (FliC) are similar, which led to the assumption that *C. jejuni* flagella are composed of 11 protofilaments. However, Galkin *et al.* (2008) used electron microscopy to show that these filaments actually have seven protofilaments. Flagellin proteins have a highly conserved amino acid sequence at the *N*- and *C*-terminal regions, which are also known as D0 and D1 domains, and the central hypervariable region forms the outside surface-exposed domains (D2 and D3) in the assembled filament. The sites of *O*-glycosylation are localised to the D2 and D3 domains (Merino and Tomás, 2014).

Assembly of the flagella encompasses multiple steps. The *N*-terminal sequence of the unfolded flagellin monomers is recognised and drives export through the basal body structure to the tip. Polymerisation is promoted by a capping protein, which maintains flagellin at a high level of polymerisation to avoid diffusion into the media (Macnab, 2004). Interaction between the *N*- and *C*-terminal sequences drag the following monomer into the growing flagellum (Evans *et al.*, 2013). Prevention of this interaction in the cytoplasm is achieved by the cytosolic chaperone FliS which binds to a *C*-terminal 40 amino acid region of the flagellin monomers (Merino and Tomás, 2014).

The flagellin assembly process requires O-linked glycosylation as inhibition of glycosylation leads to an inability to assemble the flagella and generate non-motile variants. The glycan modifications of the monomers also have an effect on the stability of flagellin. Therefore, the levels of flagellin in mutant cell lysates are less compared to the wild type due to the disruption of PseAc biosynthesis in *C. jejuni* (Goon *et al.*, 2003). Glycosylation occurs either in the cytoplasm, potentially near the flagella machinery, or in the basal body compartment (Logan *et al.*, 2008). Data on the occurrence of glycosylation close to the basal body and before exporting the flagellin can be found in *C. jejuni* 81–176 as some enzymatic constituents of the O-linked flagellin glycosylation apparatus have been localised at the end of the cell alongside the flagella biogenesis region. Additionally, the molecular weight of flagellin in flagellar mutants of *C. jejuni* 81–176 at multiple levels of the regulatory hierarchy is equivalent with the mass of glycosylated flagellin in the wild type (Ewing *et al.*, 2009). Flagellin glycosylation in *C. jejuni* begins with sequential transfer of nucleotide-activated sugars to the serine or threonine residues in the flagellin subunits (Figure 1.16) (Nothaft and Szymanski, 2010). Overall, these data indicate that unmodified monomers will not be transferred via the export apparatus, whereas modified flagellin monomers should be recognised by the export apparatus before export.

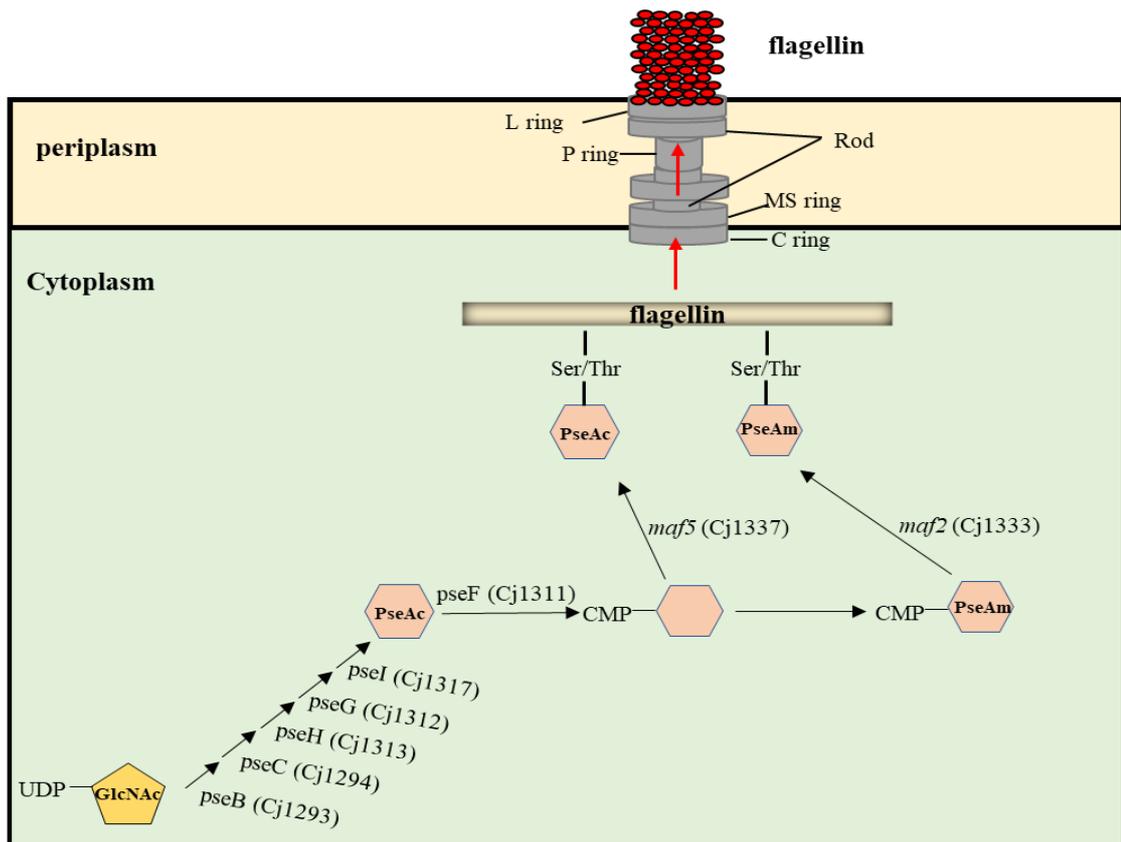


Figure 1.16 Schematic representation of the hypothetical model for O-linked flagellar glycosylation.

The pathway occurs in the cytoplasmic inner membrane in which initiates from UDP-GlcNAc and develops to UDP-2 acetamido-2,6-dideoxy- β -L-arabino-hexos-4-ulose through PseB. PseC further converts this to UDP-4-amino-4,6-dideoxy- β -L-AltNAc. PseH converts this to UDP-2,4-diacetamido-2,3,6-trideoxy- β -L-altropyranose. PseG performs as a sugar hydrolase leading to the release of 2,4-diacetamido-2,4,6-trideoxy-L-altropyranose. This is then converted by Psel to the nine-carbon structure Pse5Ac7Ac (pseudaminic acid) PseF allows CMP activation of Pse5Ac7Ac. Sequentially, CMP-activated sugars are relocated to the Ser or Thr residues in the flagellin monomers by specific glycosyltransferases which are encoded by one of *maf* genes *maf 5* or *maf2*. Image obtained from Merino and Tomás, (2014). Pse, 5,7-diamino-3,5,7,9 tetra-deoxynon-2-ulosonic acid (Pse5Ac7Ac); PseAm, 5-acetamidino analogue of Pse (Pse5Am7Ac); UDP, uridine diphosphate; CMP, cytidine monophosphate.

1.12. Biological role of O-linked glycosylation in *Campylobacter*

The use of mutants that have reductions in the glycosylation of flagellin but that can still export flagellum monomers has assisted analysis of the functional roles of specific glycans. These studies have demonstrated that adherence to and invasion of human intestinal epithelial cells requires PseAm and this factor is a determinant of virulence in a ferret model of pathogenesis (Guerry *et al.*, 2006; Guerry, 2007; Guerry and Szymanski, 2008). Similarly, the loss of production of LegAm derivatives leads to reductions in colonisation of chickens compared to that of the parent strain (Howard *et al.*, 2009b). These results indicate that heterogeneity in the glycans on *C. jejuni* flagellin is required for optimal interaction with various host surfaces (Thibault *et al.*, 2001). The potential for generating a wide diversity of flagellin glycoproteins suggests a mechanism for immune system or bacteriophage evasion by antigenic diversity; this is supported by the fact that the flagellin is both surface exposed and an immunodominant protein.

Autoagglutination is another identified phenotype influenced by glycosylation modification. Consequently, the loss of PseAm or PseAc affects autoagglutination, however there is no effect on the motility, suggesting that glycans interact with other flagellar glycans on adjacent bacteria (Guerry *et al.*, 2006). Thus, it is likely that the ability of *Campylobacter* to form microcolonies and biofilms is affected by autoagglutination, which is an important factor in host colonisation (Howard *et al.*, 2009). Further, specific glycan may mediate filament-filament interactions affect autoagglutination behaviour of *C. jejuni* while other are essential for within filament interactions through influencing structural subunit-subunit interaction (Ewing *et al.*, 2009).

1.13. Phase variable loci of NCTC 11168

The NCTC 11168 strain of *C. jejuni* was the first strain of this species for which a closed, whole genome sequence was obtained (Parkhill *et al.*, 2000; Gundogdu *et al.*, 2007). This sequence revealed the presence of 29 potentially variable poly-G tracts with 27 out of these tracts being located within or close to coding regions. Three of these loci contained pseudogenes, however, the majority were associated with genes involved in modification of surface structures (i.e. capsular polysaccharide (CPS), LOS and flagella modification). A few of these poly-G tracts were located in genes encoding surface proteins or restriction-modification enzymes (Lango-Scholey *et al.*, 2016). Table 1.1 lists all 29 tracts in NCTC11168 strain and shows the distribution of these phase variable genes among the loci that encode *C.jejuni* surface structures.

Table 1.1. Phase variable loci of strain NCTC 11168.

This table presents all 29 poly-G/C tracts of length seven or more, which have been identified in the NCTC 11168 strain. The name of the ORF where the tract is located or the nearest closet ORF when the tract is not within a reading frame. Names shown in brackets refer to an alternative name of the same locus. The repeat number for an ON length is shown, these numbers are putative and are predicted depending on the length that gives the longest reading frame. ON lengths shown between brackets refer to intergenic tracts and tracts positioned in pseudogenes (chosen for the length that results from the longest run before a stop codon). Table adapted from (Aidley, 2017). ¹Not included in 28-locus fragment analysis assay (see methodology section). *c* indicates that the coding sequence of the gene is located on the complementary strand.

PV locus	ON tract	Function
<i>Cj0031</i>	G9	Type IIG R/M system
<i>Cj0045c</i>	G11	Iron uptake system
<i>Cj0046</i>	(G11)	Pseudogene (sodium sulfate transmembrane transport)
<i>Cj0170</i>	G8	Influences motility
<i>Cj0275 (ClpX)</i>	G8	Protein degradation pathway
<i>Cj0565</i> (upstream)	(G10)	Pseudogene
<i>Cj0617</i>	G10	Flagellar modifying transferase (617 family)
<i>Cj0628 (CapA)</i>	G9	Cell adhesion (Lipoprotein autotransporter)
<i>Cj0676</i>	G9	Pseudogene (potassium transport ATPase)
<i>Cj0685c (CipA)</i>	G9	Cell invasion protein
<i>Cj0742</i> (downstream)	N/A1	Pseudogene (outer membrane protein)
<i>Cj1139c</i>	G8	LOS-modifying β -1,3 galactosyltransferase
<i>Cj1144c</i>	G10	LOS-modifying
<i>Cj1295</i>	G9	Flagellar modifying glycosyltransferase

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PV locus	ON tract	Function
<i>Cj1296</i>	G10	Flagellar modifying
<i>Cj1305c</i>	G9	Flagellar modifying (617 family)
<i>Cj1306c</i>	G9	Flagellar modifying (617 family)
<i>Cj1310c</i>	G9	Flagellar modifying (617 family)
<i>Cj1318 (maf1)</i>	G8	Motility accessory factor, flagellar glycosylation
<i>Cj1321</i> (upstream)	G10	Flagellar modifying
<i>Cj1325</i>	G10	Flagellar modifying methyltransferase
<i>Cj1335 (maf4)</i>	G8	Motility accessory factor, flagellar glycosylation
<i>Cj1342 (maf7)</i>	G9	Motility accessory factor
<i>Cj1420c</i>	G9	Capsular methyltransferase
<i>Cj1421c</i>	G9	CPS MeOPN transferase
<i>Cj1422c</i>	G9	CPS MeOPN transferase
<i>Cj1426c</i>	G10	CPS methyltransferase
<i>Cj1429c</i>	G10	Capsular modification
<i>Cj1437c</i>	G9	Capsular modification

1.14. Aims of the project

Flagella-mediated motility is an important factor for *C. jejuni* to move into favoured colonization site. Therefore, the starting hypothesis for this thesis is that the variation in motility leading to formation of the hypermotility phenomena are due to reversible switching of 28-phase variable genes. Additionally, there are several phase-variable genes located in the flagellar glycosylation locus that encode enzymes involved in the modification of the flagella sub units (FlaA and FlaB) by attachment of sugar moieties. Previous studies have shown that flagellar filament proteins, in particularly FlaA, are highly immunogenic (Grant *et al.*, 1993; Nachamkin *et al.*, 1993; Widders *et al.*, 1998; Wassenaar *et al.*, 1993; Sahin *et al.*, 2003; Newell and Fearnley, 2003; Neal-McKinney *et al.*, 2014; Boyd *et al.*, 2005; Hendrixson and DiRita, 2004; Hermans *et al.*, 2011; Paul *et al.*, 2014; Shoaf-Sweeney *et al.*, 2008; Smith *et al.*, 2008) and that natural anti-flagella immune responses may play a role in protection against colonization. This suggest that the flagellum is the major target for an adaptive immune response against this pathogen, hence the second hypothesis is that phase variable changes in the glycan structure of the filament of the flagella affect immune recognition.

These hypotheses were investigated through fur objectives:-

- Determine the PV states of hyper-motile variants recovered from *C. jejuni* 11168Ca by using the GeneScan method.
- Investigate if glycosylation makes a difference in the reactivity of antibodies against the flagella of *C. jejuni* during the colonisation of chickens
- Test the immunogenicity of flagellin proteins from isogenic strains of *C. jejuni*, which have been mutated in selected flagellin-glycosylation genes to understand whether there are antibodies against different glycans and if differences in reactivity are controlled by phase variable genes.
- Determine the influence of selected phase variable genes on motility and aggregation.

Chapter 2: Materials and Methods

2.1. Bacterial culture, growth condition, sterilisation and storage

Mueller-Hinton Agar (MHA, Oxoid, and Basingstoke, UK) was frequently used for growth of *C. jejuni* as a solid medium and Mueller-Hinton Broth (MHB, Oxoid) was used to grow *C. jejuni* in liquid cultures. These media were prepared according to manufacturer's instructions and sterilised by autoclaving (121°C, 15 minutes and 15 psi of pressure). The media was cooled to 55°C before any antibiotics could be added to the media. Once cooled, agar was poured into Petri dishes (Sterilin). Plates were stored at 4°C for up to two weeks.

C. jejuni strains (Table 2.2) were grown at 42°C under microaerophilic environment of 85% N₂, 10% CO₂ and 5% O₂ in a VAIN cabinet (Variable Atmosphere Incubator; Don Whitley Scientific Ltd, Shipley, UK) on Mueller-Hinton Agar (MHA) or Mueller-Hinton Broth (MHB) with shaking at 500 rpm (Vibrax VXR, IKA, Germany). These media were frequently supplemented with vancomycin and trimethoprim as standard antibiotics to minimize contamination. For mutant selection, chloramphenicol and kanamycin were added to the growth media. All antibiotics were prepared as concentrated stock, filter-sterilised using a 0.22µm pore diameter filter sterilisation membrane (Gelman Sciences) with 25mm syringe filter membrane (PALL Life Sciences) and stored at 4°C in the dark until used. All antibiotics were purchased from Sigma. Final concentrations in media are mentioned in the table (Table 2.1). *C. jejuni* cells were recovered from glycerol stock and incubated for three days, then sub-cultured by swabbing onto fresh MHA plates. After five days of incubation, colonies were normally visible and hence, they were ready to be utilised in experiments.

E. coli strains DH5α and BL21 (DE3) were obtained from laboratory stocks (Laboratory 121, Department of Genetics and Genome Biology, University of Leicester). *E. coli* cells were normally grown on agar plates (LA; Luria-Bertani Agar, Oxoid) or in liquid media (LB; Luria-Bertani Broth, Oxoid) with shaking (250 rpm) at 37°C under aerobic conditions. LA and LB media were supplemented with the appropriate antibiotics required for selection in order to maintain presence of any plasmids present in strains (Table 2.1).

For long-term storage, *C. jejuni* and *E. coli* stains were preserved in MHB and LB, respectively, containing 25% (v/v) glycerol at -80°C as a cryo-preserved. To prepare the glycerol stock, *C. jejuni* bacteria were grown on MHA plates supplemented with vancomycin and trimethoprim and then incubated at 42°C for 1-3 days in the Variable Atmosphere Incubator (VAIN). *E. coli* cells were grown either on LA plates or in 5ml of LB overnight at 37°C with shaking at 250rpm. Cell growth was then re-suspended in 2ml of MHB or LB for *C. jejuni* and *E. coli*, respectively, and the cells suspension from plates was transferred to 1.5ml micro-centrifuge tubes. Then after, cells pelleted at 1100 X g in an Eppendorf MiniSpin plus centrifuge machine for 5 minutes at room temperature. Pelleted cells had been re-suspended with 1ml MHB or LB for *C. jejuni* and *E. coli* respectively and aliquots of 500µl cells suspension was transferred into cryo-tubes in which equal volumes of 50% (v/v) glycerol was added, snap frozen on dried ice and stored at -80°C. For sub-culturing glycerol stocks, frozen cell stocks were put on dry-ice and then cells were scrapped off from freezing vials with stick end of sterilised swab before being spread on to the solid media, and then incubated under suitable growth conditions with appropriate antibiotics.

Table 2.1. Antibiotic preparation and concentrations.

Name	Stock Concentration	Diluted with	Final Concentration	
			<i>C. jejuni</i>	<i>E. coli</i>
Vancomycin	10 mg/ml	ddH ₂ O	10 µg/ml	
Trimethoprim	5 mg/ml	50% EtOH	5 µg/ml	
Kanamycin	50 mg/ml	ddH ₂ O	50 µg/ml	50 µg/ml
Chloramphenicol	20 mg/ml	50% EtOH	10 µg/ml	20 µg/ml
Ampicillin	100 mg/ml	ddH ₂ O		100 µg/ml

Table 2.2. Bacterial strains and plasmids used in this research

Bacterial strains	Description	Source
<i>Escherichia coli</i> BL21 (DE3)	Overexpression of recombinant protein His6	Lab strain
<i>E. coli</i> DH5αE	Cloning host strain	Lab strain
<i>Campylobacter jejuni</i> 11168 Ca	Chicken adapted hypermotile derivative <i>C. jejuni</i> NCTC 11168-wild type	Dr. Richard Haigh, University of Leicester
<i>C. jejuni</i> 11168H	A hypermotile derivative of <i>C. jejuni</i> NCTC 11168-wild type	Lab strain
<i>C. jejuni</i> Δ <i>Cj1310c</i>	NCTC 11168 Δ <i>Cj1310::Kan</i> (<i>Kan^R</i>) <i>Kan</i> replaces <i>Cj1310</i> deletion	This study
<i>C. jejuni</i> Comp-Δ <i>Cj1310c</i>	NCTC 11168 Δ <i>Cj1310::kan</i> (<i>Kan^R</i>):: <i>Cj0046::Cj1310</i> (<i>Cm^R</i>)-Fixed ON	This study
<i>C. jejuni</i> Δ <i>flaA</i> Ca	NCTC 11168 Δ <i>flaA::kan</i> / natural transformation	This study
<i>C. jejuni</i> Δ <i>flaA</i> H	NCTC 11168 Δ <i>flaA::kan</i>	Dr. Dennis Linton, University of Manchester
NCTC 11168 <i>maf2::kan^R</i>	Knockout mutant / <i>maf2::kan^R</i>	Dr. Dennis Linton, University of Manchester
NCTC 11168 Δ <i>maf5::kan^R</i>	Deletion mutant/Δ <i>maf5::kan^R</i>	Dr. Dennis Linton, University of Manchester
NCTC 11168 <i>Cj1295::aphA</i>	Knockout mutant/ <i>Cj1295::aphA</i>	Dr. Dennis Linton, University of Manchester
NCTC 11168 Comp <i>Cj1295</i>	NCTC 11168 <i>Cj1295::aphA::Cj0223::Cj1295</i> (<i>Cm^R</i>)-Fixed ON	Dr. Dennis Linton, University of Manchester

Plasmids	Description	Source
pLEICS-01	Expression vector, Amp ^R , N-His6, TEV cleavage site-1	(PROTEX, University of Leicester)
pLEICS-01-KH <i>flaA</i>	<i>flaA</i> gene cloned into N-terminal of pLEICS01 plasmid multi-cloning site, Amp ^R	This study
pC46 <i>fdxA</i>	Complementation plasmid contains <i>Cj0046</i> flanks, <i>fdxA</i> constitutive promoter (medium expression), Cm ^R	(Reuter and Vliet, 2013)
pKM46	Complementation plasmid containing <i>Cj0046</i> flanks and Km ^R	Dr.Amit Kumar verma
pAV35	Source of chloramphenicol resistance cassette	Van Vliet et al., 1998
pGEM-T Easy	High copy number cloning vector, Amp ^R , allows blue/white screening	Promega
pCΔ <i>Cj1295/Cj1296</i>	Upstream flanking of <i>Cj1295</i> gene and downstream flanking of <i>Cj1296</i> gene cloned into pGEM-T Easy, Cm ^R	This study
pKMLFlank <i>Cj1310</i>	Plasmid contains left flank of <i>Cj1310c</i> gene and Km ^R	This study
pKMΔ <i>Cj1310c</i>	Plasmid contains left flanking region of <i>Cj1310</i> and right flanking region of <i>Cj1310</i> gene derived from pKM46 plasmid, Km ^R	This study
pC46 <i>fdxA-Cj1310</i>	Plasmid contains <i>Cj1310</i> gene cloned into pC46 <i>fdxA</i> vector, Cm ^R	This study
pC46 <i>fdxA-SDMCj1310</i>	Plasmid derived from pC46 <i>fdxA-Cj1310</i> containing fixed 8G repeat tract generated by Site directed mutagenesis, Cm ^R	This study

Amp^R, Ampicillin resistant and Cm^R, chloramphenicol resistant, which is conferred by the *cat* (chloramphenicol acetyl transferase) cassette, Km^R: Kanamycin resistant.

2.2. Extraction of chromosomal DNA

The modified method of Chen and Kuo (1993) was used to prepare the chromosomal DNA of *C. jejuni*. Briefly, colonies of *C. jejuni* were recovered from confluent MHA plates into 2 ml of MHB. The cell suspension was transferred into a micro-centrifuge tube and centrifuged at 11300 X *g* for 3 min. Pelleted cells were re-suspended in 600 µl of buffer 1 (40 mM Tris-acetate pH 7.8, 20 mM sodium acetate, 1mM EDTA, 1% SDS). Then, 200µl of 25mM NaCl was added to the lysate and mixed by shaking. Proteins and cell debris were pelleted by centrifugation at 11300 X *g* for 15 min. The supernatant was transferred into a micro-centrifuge tube and 600µl of chloroform/iso-amyl alcohol (24:1, v/v) was added and mixed by inverting the tube approximately 100 times. The upper layer was transferred into a new micro-centrifuge tube. This was repeated twice. The separated top layer was added to an equal volume of 100% ethanol and mixed by inversion before centrifugation at 11300 X *g* for 5 min. Finally, the supernatant was mixed with 800µl of 70 % ethanol and centrifuged at 11300 X *g* for 2 min. The dried pellet was re-suspended in H₂O. Concentration and yield of genomic DNA samples was estimated by agarose gel electrophoresis via comparing the sample DNA intensity to that of a DNA quantitation standard (Hyper I ladder) or using a Nanodrop 2000c spectrophotometer (Thermo Scientific).

2.3. Extraction of plasmid DNA

For plasmid extraction, *E. coli* was grown in LB overnight at 37°C with suitable antibiotic supplementation. The cell suspension was used to extract plasmid DNA with small size plasmid/DNA preparation kit (Omega, Bio-Tek. Inc, USA) as per manufacturer's instructions. The concentration of extracted plasmid DNA was quantified either by comparison to a DNA standard following agarose gel electrophoresis or by Nano Drop 2000C (Thermo Scientific, UK). Purified plasmids were stored at -20°C for future use.

2.4. DNA analysis methods

2.4.1. Polymerase chain reactions (PCR)

2.4.1.1. Primer design

Primers were designed using Clone Manager (version 9) (Scientific and Educational Software, 2012) and SnapGene 1.1.3 software. The designed primers were synthesised by Sigma-Aldrich. Stock and working primer solutions were prepared in distilled water. In addition, necessary primers also contained restriction sites at the 5' end to facilitate subsequent cloning of amplified fragments. Template of sequences of *flaA* and other genes in *C. jejuni* for primer use in primer design were acquired from NCBI database (<https://www.ncbi.nlm.nih.gov>). All primers used in this study are listed in Table 2.3.

Table 2.3. Primers used in this research. Restriction enzyme sites are underlined.

Primer name	Sequence (5' -> 3') Restriction sites are underlined	Note
KO1310_LF lankR (<i>Sac</i> II)	GAT <u>CCGCGGG</u> CTTTAAATCT TTTTGATTTAATCTTGA	Amplification of left flanking region of <i>Cj1310c</i>
KO1310_LF lankF (<i>Bss</i> HII)	GGAT <u>GCGCGCT</u> GACAGCAT ATTTACATATAGGAAC	Amplification of left flanking region of <i>Cj1310c</i>
KO1310_RF lankF2 (<i>Xba</i> I)	GCGTCTAGATTAAGCCTTTT ATTGTATTTTAAAGAC	Amplification of right flanking region of <i>Cj1310c</i>
KO1310_RF lankR2 (<i>Nde</i> I)	CTGGC <u>CATATG</u> CGCTTCTAAA AAGCACTTTCATGATTTAC	Amplification of right flanking region of <i>Cj1310c</i>
Comp1310F 2 (<i>Bsm</i> BI)	GGCCGTCTCGATCCACCAC GAGCAGGGATTATGCAAAG ATTTTTCATA	For cloning <i>Cj1310</i> into pC46fdxA plasmid
Comp1310 R2 (<i>Bsm</i> BI)	TGAGC <u>GTCTCT</u> ATTAGTAGT TCCTATATGTAAATATGCTG T	For cloning <i>Cj1310</i> into pC46fdxA plasmid
FlaA-F	TACTTCCAATCCATGGGATT TCGTATTAACAC	Cloning primer to amplify <i>flaA</i> gene and clone it into the expression vector
FlaA-R	TATCCACCTTTACTGCTACT GTAGTAATCTTAAAC	Cloning primer to amplify <i>flaA</i> gene and clone it into the expression vector
FlaA_int-F	GCA GGA GCT ACT TCA GAT AC	Primer design to sequence the internal region of <i>flaA</i> gene
FlaA_int-R	CCT GCG CTA CTC ATA TAG GC	Primer design to sequence the internal region of <i>flaA</i> gene
T7 promoter	TAATACGACTCACTATAGGG	Vector-specific primer-used to amplify/sequencing
pLEICS-01- Seq-R	ATTAACATTAGTGGTGGTGG T	Vector-specific primer-used to amplify/sequencing
CATinvR	GCGGTCCTGAACTCTTCAT GTC	For sequencing pCfdxA- <i>Cj1310</i> plasmid
Cj0046F	CTCTCTCCGCTAGAAATTAA ATCC	For sequencing pCfdxA- <i>Cj1310</i> plasmid
1310SDM-F	AAAATTTATGGAGGGGGGT CTTATGGAGG	Site directed mutagenesis of <i>Cj1310</i> gene
1310SDM- R2	CTCCATAAGA CCT CCC CCC ATAAATT	Site directed mutagenesis of <i>Cj1310</i> gene

newCj0046e ndF	GCTATGTTTATTACAGCTAC AGC	For sequencing pCfdxA- SDM1310 plasmid
Cj1310- fwd2	GAACAAATTATTCTCTTAT AG	For sequencing pCfdxA- SDM1310 plasmid
Cj1310-rev	AATCACACCATCCACATACC	For sequencing pCfdxA- SDM1310 plasmid
flank0046se q-F	GTGTAGGGTTTTTTTTGTAA AATTAG	For sequencing C. jejuni Comp-Δ <i>Cj1310c</i> strain
flank0046se q-R	AACATGAGGGCTATATGCA CTATACC	For sequencing C. jejuni Comp-Δ <i>Cj1310c</i> strain
Cj1310F-out	TTTCTTCTTGCCATAAAAAG CAAAGAAT	For sequencing C. jejuni Δ <i>Cj1310</i> strain
Cj1310R- out	TCGCGTTTAATGTGTCCAAA GCCTATTT	For sequencing C. jejuni Δ <i>Cj1310</i> strain
STMinvKan F	CTGGGGATCAAGCCTGATT G	For sequencing pKMLFlank <i>Cj1310</i> plasmid
kno1295up_ F	GACTTTCTAGAGAGAGTGAA AATATAGGAGCTATTAGCG	Splicing overlap extension PCR- <i>Cj1295/Cj1296</i> -Up flanking region
Ko1295/96S OE-R3	ACTTGCTGAATAAATTA TTTTCAAGAATGCTAAATAA AGTATC	Splicing overlap extension PCR- <i>Cj1295/Cj1296</i> -Up flanking region
Ko1295/96S OE-F3	ATCCACTTTTCAATCGTGTA TATGAAATTTTAACTAGGGA	Splicing overlap extension PCR- <i>Cj1295/Cj1296</i> -Down flanking region
kno1296do_ R	CACAAAGCTTCCCAAACAG CTTGTTGTATC	Splicing overlap extension PCR- <i>Cj1295/Cj1296</i> -Down flanking region
Cat-pro- SOE-F	GATTGAAAAGTGGATAGATT TATGAT	Amplification of chloramphenicol cassette for Splicing overlap extension PCR
Cat-SOE-R	ATTTATTCAGCAAGTCTTGT AATTCATC	Amplification of chloramphenicol cassette for splicing overlap extension PCR
CATinvF	GGAATGTCCGCAAAGCCTA ATCC	For sequencing chloramphenicol cassette
CATinvR	GCGGTCCTGAACTCTTCAT GTC	For sequencing chloramphenicol cassette
pUCF	GCCAGGGTTTTCCAGTCA CGA	For sequencing pΔ <i>Cj1295/Cj1296</i>
pUCR	GAGCGGATAACAATTTCA CAGG	For sequencing pΔ <i>Cj1295/Cj1296</i>
F-kan	ATGGCTAAAATGAGAATATC	For sequencing Kanamycin cassette
R-kan	CGGAATTCTAGGTAATAAAA CAATTCAT	For sequencing Kanamycin cassette

2.4.1.2. PCR reaction mix

For PCR reactions greater than 1 kb in length, high fidelity Phusion DNA polymerase (New England, Bio labs, UK) was used according to the instructions provided by manufacturer. Phusion DNA polymerase was used in order to reduce the incorporation error rate of PCR when cloning genes from *C. jejuni* and *E. coli* strains. However, for PCR reactions where the amplicon was less than 1 kb in length or those testing primer specificity and screening for recombinant clones, Taq DNA polymerase (New England, Biolabs® inc., UK) was used. Normally, 20µl and 50µl reaction volumes were used with a concentration of 1x reaction buffer comprising 1.5mM MgCl₂; 10pmol dNTP; 10pmol primers; 1-30ng chromosomal DNA or 1-20ng plasmid DNA. For every PCR reaction and dilutions as well, autoclaved ddH₂O was utilised. DNA amplification was carried out in a thermal cycler (Eppendorf, Scientific Support Inc., UK) using the cycle programs shown in Table 2.4. The extension time and annealing temperature was varied according to the primers used Table 2.3. A gradient PCR was performed to determine the best conditions and concentrations of components to amplify the gene, and then the best result of this PCR was considered as the standard condition for the next PCR targeting this gene.

Table 2.4. Conditions of PCR reactions.

Steps	Temperature	Time	Number of cycles
Initial denaturation	94 °C	2 minutes	1X
Denaturation	94 °C	15 seconds	30X
Annealing *	55.70 °C	30 seconds	
Extension **	72 °C	Phusion: 30 seconds/ 1000 bp Kapa: 60 seconds/ 1000 bp	
Final extension	72 °C	5 minutes	1X

*Primer annealing temperature is calculated as 5-10°C lower than your primer without the restriction enzyme added.**Extension time is calculated as approximately 1kb PCR product size per minute.

2.4.1.3. Splicing overlap extension (SOE) PCR for constructing a *Cj1295* and *Cj1296* double mutant

The SOE PCR protocol was used to perform the splice PCR to splice the multiple fragments of DNA for cloning experiment (Choi and Schweizer, 2005). Furthermore, multiple separated fragments containing 5' prime end overlapping homologies to the other fragments to be spliced were generated by using a Q5 High-Fidelity DNA Polymerase kit (NEB). Additionally, PCR generated amplicon were mixed in an equimolar ratio in a single PCR reaction (Table 2.5) and the generation of multiple independent PCR fragments including 5' end overlapping. The PCR reaction was run in the primary thermocycle without primers. Initially with fourteen cycles, the overlapping regions of the fragments should anneal to each other and create single spliced fragment. Additionally, the primers that bind 5' to 3' end of the fusion product were then added to amplify the new spliced fragment and those conditions were mentioned in the table 2.6.

Table 2.5. SOE PCR reaction mixture setup

Reagents in Master mixture (Final concentration)	Volume (μ l) for 1 reaction
5X Q5 Reaction Buffer (1X)	Volume (μ l) for 1 reaction
10 μ M dNTPs (0.2mM each) (200 μ M)	10 μ l
Splicing fragment 1 (50ng)	1 μ l
Splicing fragment 2 (50ng)	As required
Splicing fragment 3 (50ng)	As required
10 μ M Forward primer (0.5 μ M) (added after 14th cycle)	As required
10 μ M Reverse primer (0.5 μ M) (added after 14th cycle)	2.5 μ l
Q5 High-Fidelity DNA Polymerase (0.02 U/ μ l)	2.5 μ l
Nuclease-Free Water	0.5 μ l
	Final volume 50 μ l

Table 2.6. SOE PCR thermocycling condition

Steps	Number of cycles	Temperature	Time
Polymerase activation	1	98°C	2 min
Primary thermocycle without primers			
Denaturation	14	98°C	10 sec
Annealing		2°C below the lowest T _m of overlapping region	30 sec
Extension		72°C	20 - 30 sec/kb of DNA
Cycling after adding primers			
Initial denaturation	1	98°C	30 sec
Denaturation	20-50	98°C	10 sec
Annealing		2°C below the lowest T _m of primers	30 sec
Extension		72°C	20 - 30 sec/kb of DNA
Final extension	1	72°C	2 min
Final hold	1	15°C	∞

2.4.1.4. Colony PCR

To verify the presence of a correct recombinant plasmid, colony PCR was performed. Individual colonies were picked by toothpick and re-suspended in 100µl of distilled water in a 500µl PCR micro-centrifuge tube (Eppendorf AG, Germany). The same toothpick was used to streak the individual clones onto selective agar and the plate was incubated at 37°C to provide a stock of each clone. The cell suspension was heated at 98°C for 5 minutes then cells were centrifuged at 1100 X g for 3 min. Finally, the PCR reaction was performed according to the conditions outlined in 2.4.1.2 and shown in table 2.4.

2.4.1.5. PCR products purification

PCR products were purified using the E.Z.N.A cycle pure kit from (Omega, Bio-Tak, Inc, USA) as per manufacturer's instructions.

2.4.2. Restriction enzyme digestion of DNA

Appropriate restriction endonucleases were used for sub-cloning and plasmid analysis. All restriction enzymes and buffers were purchased from NEB (New England Biolabs Ltd, Hitchin, UK) and used as per the manufacturer's recommendations. Usually, 1µg of DNA was digested with appropriate enzymes and compatible buffers in a 50µl total volume and incubated at 37°C in a water bath for 1-3 hours or overnight.

For cloning purposes, the vector was treated with Shrimp Alkaline Phosphatase (SAP, Roche Diagnostics, Germany) to remove 5' phosphate with the purpose of preventing self-ligation of the vector alone. Dephosphorylation reactions were carried out in a 50µl total volume and incubated for 1 hour at 37°C in accordance to manufacturer's guidelines. After that, the reaction was purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

2.4.2.1. Ethanol precipitation

Ethanol precipitation of DNA was used to desalt, concentrate or change buffering conditions of purified DNA fragments. Briefly, DNA was precipitated by the sequential addition of 1/10th to reaction volume of 3M sodium acetate, 2.5 volumes of ice-cold 100% ethanol and 10mM of tRNA. The mixture was incubated on ice for 5 minutes then precipitated nucleic acid was pelleted at 1300 X g for 10 minutes. Finally, the DNA pellet was washed in 70% (v/v) ethanol (800µl), re-centrifuged at 1300 X g for 2 minutes. Subsequently, ethanol was aspirated, and the DNA pellet was dried at room temperature. DNA was again re-suspended with sterile dH₂O and stored at -20°C until used for further analysis.

2.4.3. Gel-electrophoresis for DNA visualisation

Amplified DNA samples were examined on the 1% agarose gel (1 X TAE buffer (40mM Tris acetate, 1mM EDTA, pH 8.2). Ethidium bromide (Etbr) approximately 2µl per 100ml agarose gel was added, to enable the visualisation of DNA fragment bands on exposure of the gel under the ultraviolet light. Additionally, DNA fragment size were measured with a DNA Hyper Ladder 1kb (Bioline

Reagents Ltd) which was used along with the PCR samples on agarose gel. The gel was usually run at 100 volts for 45 minutes.

2.4.3.1. Gel Purification of DNA fragments

DNA fragments were purified from agarose gels using the ZymoClean™ Gel DNA Recovery Kit (Cambridge Bioscience, UK) following the manufacturer's instructions. Gel-purified DNA were eluted in 10µl to 50µl of pure distilled water.

2.4.4. DNA ligation

To clone a piece of DNA, both insert, and vector were digested with the same restriction enzymes then ligated together. Appropriate amounts of vector and insert DNA were added in the ratio 1:3 and the reactions were assembled in final volume of 20µl. Gel electrophoresis was used to estimate the concentrations of restriction-digested vector and insert DNA fragments as outlined in section 2.4.3. Typically, 50ng of vector and 150ng of insert DNA were used in the total volume of 20µl reaction mixture. The 20µl reaction volume containing 1µl T4 DNA ligase (5-10 units for 1µg of DNA), which enhances the formation of phosphodiester bonds between the 5' phosphate and 3' hydroxyl groups in DNA, 2µl 10 x ligase buffer (New England Biolabs Ltd, Hitchin, UK), vector DNA, insert DNA and sterile distilled water. The reaction was incubated overnight at 16°C. A control reaction, where the insert DNA was substituted for an equal volume of sterile distilled water, was used to assess the amount of vector self-ligation. The ligation product was cleaned with either the QIA quick PCR purification kit (Qiagen) or using ethanol precipitation protocol for downstream applications.

2.4.5. TA-cloning

Amplicons generated from the high-fidelity PCRs were blunt-ended as the Phusion polymerase does not incorporate an extra adenine residue at the 3' end of amplicons, unlike *Taq* polymerase. Therefore, amplicons from these PCRs were A-tailed prior to cloning into the pGEM-T® Easy vector. Completed PCR reactions were purified and eluted in 10µl of distilled water. From this 10 ul of purified PCR amplicon, 7µl were A-tailed in a reaction mixture containing 1 ul 10X Kapa buffer, 1 ul 2mM dATP and 1 ul Kapa *Taq* polymerase. The A-tailed process

was completed by incubating the mixture at 70°C for 30 minutes. A-tailed amplicons were subsequently TA-cloned by using the manufacture's protocol (Promega).

2.4.6. DNA sequencing

DNA fragments were sequenced by using a BigDye® Terminator v3.1 cycle sequencing kit (ABI Applied Biosystems®) provided by the University of Leicester's Protein and Nucleic Acids Laboratory (PNAACL). Each sequencing reaction mixture was composed of 1µl template DNA, 1µl of the primer, 4µl of BigDye v3.1 mixture (0.5µl BigDye v3.1, 0.8µl 5 X sequencing buffer, 2.7µl distilled water) and 4µl distilled water to give a total 10µl reaction mixture. Cycling conditions were as follows: an initial 5 minutes 96°C denaturing step before 29 cycles of a 10 second denaturing step at 96°C, 10 second for annealing step at 50°C, and 4 minutes extension step at 60°C. Subsequently, the reaction mixture was purified by adding 2.2% SDS into reaction mixture and placed in a thermal cycler at 98°C for 5 minutes followed by 25°C for 10 minutes. Finally, PCR products from the sequencing reaction were purified by using Performa® DTR gel filtration cartridges (EdgeBio) in accordance with the manufacturer's instructions in order to remove any unincorporated dye from the sequencing reaction. The products were analysed by using an automated sequencer, ABI 3730 DNA sequencer (ABI, Applied Biosystems) at PNAACL.

2.4.7. 28-locus-CJ11168 PV-analysis assay

Fragment analysis was used to detect the state of 28 phase variable loci of NCTC11168 by using methods described in Lango-Scholey *et al*, 2016. This assay was performed by PCR amplification of gene fragments that contain the target SSRs with fluorescently-labelled primers. These tags could be detected by capillary electrophoresis which records the specific size of each amplified fragment and therefore the length of repeats in the SSR to be determined.

The design of primer pairs was done with considering these primers to be very specific to amplify different fragments either by size of fragment or by DNA ladder type thus, that 28 loci could be measured in a single capillary electrophoresis assay.

Analysis was carried out in 96 well PCR plates. Individual colonies were resuspended in 100µl of purified dH₂O, heated at 98°C for 5 to 8 minutes and then centrifuged at 768 X g for 4 minutes to pellet cell debris. Clear supernatants were transferred into labeled tubes for further analysis. PCR amplification was performed (Table 2.7) with total six sets of multiplexed primers (Listed in Appendix A). For PCR master mix, 2µl of each PCR reaction was mixed with Taq polymerase; MgCl₂ buffer was added as required to elevate the higher productivity of Taq polymerase. Subsequently, the mixture was heated to 72°C for 45 minutes to allow the attachment of the untemplated A-residues by the Taq polymerase to complete phase. After that, from that mixture only 0.5µl mixture was added to 9.25µl of formamide and 0.25µl of GeneScan™ LIZ-500 or LIZ-600 size standard. The mixture was then transferred to PNACL for capillary electrophoresis using Applied Biosystems 3730 Genetic Analyser.

Table 2.7. PCR conditions for 28-locus-CJ11168 PV-analysis assay

Temperature	Time	Number of cycles
94°C	5 minutes	1x
98°C	30 seconds	30x
50°C	30 seconds	
72°C	60 seconds	

The raw data was analysed using the Peak Scanner™ software to produce a full list of peaks present in the electrophoresis data. PSA analysis (Aidley, 2017) was subsequently used to calculate fragment lengths by comparison to a sample of known tract lengths (as confirmed by sequencing) and scored as ON and OFF based on whether the tract length produces the longest possible reading frame. In the case of four tracts which are either intergenic or located in pseudogenes, an arbitrary ON length was chosen (Table 1.1).

2.5. Preparation of competent cells and transformation of plasmid DNA

In *E.coli* bacteria, transformation was performed by heat shock (CaCl₂) method. *C. jejuni* was transformed by electroporation or taking advantage of its natural competence by natural transformation.

2.5.1. Preparation of Chemically-competent *E. coli* cells.

In this method *E. coli* competent cells were prepared by using calcium chloride. A 50µl aliquot of an overnight culture of *E. coli* cells was inoculated into 5ml LB media and incubated at 37°C until the OD_{600nm} reached 0.5 – 0.6. Those cells were then centrifuged at 3000 X *g* for 10 minutes at 4°C, and the cell pellet resuspended in 1ml 0.1M CaCl₂ and kept on ice for 1 hour before the final wash. Finally, resuspension was done in 1ml of ice cold sterile CaCl₂ (100mM) in 15% glycerol w/v. The cell suspension was dispensed into 100µl aliquots, flash-frozen in dry ice and stored at -80°C until further experimental analysis.

2.5.2. Preparation of electrocompetent *C. jejuni*

For the preparation of the electrocompetent *C. jejuni*, cell pellets were gently resuspended in 1ml of filter-sterilised ice-cold wash buffer (272mM sucrose and 15% v/v glycerol) and centrifuged at 13,000 rpm for 2 minutes at 4°C. This step was repeated thrice before resuspension in 1ml ice-cold wash buffer, distributed into 100µl aliquots, flash-frozen in dry ice and stored at -80°C.

2.5.2.1. Wash buffer preparation

To prepare 10ml stock of washing buffer, the following components were added; 0.931g sucrose, 1.5ml glycerol and 8.5ml, sterile dH₂O. Final mixture was sterilised by using 0.2µm-membrane filter (Acrodisc PALL life science, Portsmouth, UK) into a clean universal tube.

2.5.3. Transformation of *E. coli* cells by heat shock

Chemically-prepared 100µl aliquot of competent *E. coli* were taken from -80°C storage and kept on ice for 20 minutes until thawed. After that, 5µl of plasmid

DNA were mixed with 100µl of competent *E. coli* cells on ice for 30 minutes. The mixture was then heat shocked at exactly 42°C for 40 seconds and transferred onto ice for another 10 minutes. The cell mixture was mixed with 950µl of LB media which were then incubated aerobically at 37°C for 1 hour. Transformed cells were spread on LA media plates with appropriate antibiotic selection and incubated overnight at 37°C incubator for further growth. Positive control for the transformations was the high copy-number plasmid, pUC19, while negative control was competent cells without addition of plasmid DNA, used to assess the antibiotic sensitivity of the cells if spread onto supplemented plates and to assess the viability of the cells following the procedure, when spread onto plain LA plate. The ligation control was also transformed to assess the amount of the circular vector DNA existing due to re-ligation of the vector occurring in the reaction or failure to achieve complete digestion.

2.5.4. Transformation of *C. jejuni* by electroporation

For transformation of *C. jejuni*, cells kept on ice were mixed with 5µl of plasmid DNA and the mixture was transferred into pre-chilled electroporation cuvettes. The mixture was pulsed once using GenePulser electroporator (Bio-Red) set as 2.5KV, 200Ω, and 25µF. The optimum time constant (resistance-capacitance time constant) was 4.6(ms) units. Finally, cells were transferred from the cuvette with 1000µl of MHB and spread on non-selective MHA plates and incubated overnight at 42°C, micro-aerobically in the VAIN to allow for expression of antibiotic resistance genes during overnight growth. Cells were subsequently harvested from non-selective plates with 1ml of MHB and spread on MHA plates containing appropriate selection of antibiotics and incubated in the VAIN at 42°C for 3 – 5 days.

2.5.5. Natural transformation

C. jejuni cells grown on MHA plates were recovered in 2ml MHB and OD_{600nm} were adjusted to ~ 0.5 by using a spectrophotometer. A cell suspension of 500µl was then transferred into 1.5ml sterilized Eppendorf tube containing 1ml MHA and incubated for 3 hours. Subsequently, 5µl of DNA was added and incubated

statically O/N at 42°C temperature. Bacteria were removed from the surface of MHA and plated on MHA antibiotic selection plates at dilutions (1/10 and 9/10).

2.6. Protein analysis methods

2.6.1. Cell lysate preparation

Bacterial cells of the *C. jejuni* were grown on MHA media as stated in section 2.1. Bacterial cells were re-suspended in PBS and then concentration of the cells was adjusted to an optical density ($OD_{600} = 0.5$). Cell pellets were harvested by centrifugation at 13000 rpm for 5 minutes. Subsequently, pellets were re-suspended in 100µl of 2X SDS loading buffer (Laemmli buffer) (10% SDS, 1M Tris pH 6.8, 1% Bromophenol blue, 2.5ml glycerol, 200mM DTT and make up to 10ml with dH₂O) further process by heat treatment (98°C, 5 minutes) and spin for 2 minutes at 13,000 rpm. However, when SDS-PAGE was not conducted immediately, the cell lysate was stored at -20°C.

For purified proteins, certain concentration of protein were mixed with 2X SDS loading buffer, heat treated at 98°C for 5 minutes.

2.6.2. *C. jejuni* outer membrane fraction preparation

The FlaA protein from *C. jejuni* was prepared by the Sarkosyl method. The extraction of outer membrane *C. jejuni* cells was performed at 4°C or on ice (this method is similar to van Vliet et al. (1998)). *C. jejuni* strains were recovered from -80°C freezer stocks onto MHA-TV plates (MHA supplemented by Trimethoprim and Vancomycin) and grown for 2-3 days in the VAIN at 42°C and used to inoculate 5 ml of MHB medium. The cultures were incubated overnight (with shaking at 500rpm), and $OD_{600} = 0.3$ was used to equalise the cultures containing approximately (6×10^7 cfu/ml). Cells were harvested by centrifugation at 4000 rpm for 15 minutes and re-suspended in 1.0ml envelope buffer (EB; 10mM Tris-HCl, pH 7.5), then transferred to Eppendorf tubes (250µl/tube). Cells were frozen at -80°C for at least 30 minutes, defrosted, and lysed by sonication (10 minutes total, with 15 seconds on/off). The non-lysed cells were removed by centrifugation, the supernatants were transferred to 2 ml clear Beckman tubes and crude total membranes pelleted by ultracentrifugation at 50,000 g for 10

minutes at 4°C in the Beckman TL-100 ultracentrifuge. Supernatants were discarded and the pellets (total membranes) were re-suspended in 1.0ml of EB containing 0.6% (w/v) sodium sarkosyl (N-lauryl-sarcosine; EBS) and incubated at 4°C for 30 minutes. Sarkosyl-insoluble fractions were pelleted by ultracentrifugation (as above), the supernatants were discarded and the pellets re-extracted in 1.0ml of EBS for a further 30 minutes at 4°C. Finally, a second ultracentrifugation step (as above) the supernatants were discarded and the pellets (outer membrane) were re-suspended in 100µl EB and 100µl 2X SDS-PAGE loading buffer for SDS-PAGE and Western blot and stored at -20°C.

2.6.3. SDS polyacrylamide gel electrophoresis (SDS-PAGE) and staining

Cell lysates and protein samples were analysed on 10% SDS-PAGE gels as described by Laemmli (1970). The separating gel was prepared with the following components: 2.7ml buffer A (750 mM Tris, 0.2% SDS, pH 8.8), 1.83ml UltraPure Protogel® (Geneflow), 2.3ml distilled water, 190µl 1% APS and 15µl TEMED (Sigma-Aldrich®). After that, 1ml of isopropyl alcohol was added to the gel to remove the air bubbles and to enhance the straightness of the gel. Once the bottom separating gel had solidified, the stacking gel, which is composed of: 1ml buffer B (250mM Tris, 0.2% SDS, pH 6.8), 330µl UltraPure Protogel® (Geneflow), 1.9ml distilled water, 50µl 1% APS and 4µl TEMED (Sigma- Aldrich®), was poured and the comb was instantly inserted into the gel. When the polymerisation of stacking gel was completed, the gel casting unit was placed in the electrophoresis tank containing 1 X SDS-PAGE running buffer (25mM Tris- HCl, 192mM Glycine, 1 % (w/v) SDS), and the comb was removed. The pre-stained protein marker (PageRuler Prestained Protein Ladder size range 10 to 170kDa, Thermo Scientific) was loaded, followed by loading an equal volume of samples into wells of the stacking gel. Electrophoresis was performed at constant voltage of 80 Volts for 3 hours.

Following electrophoresis, gels were fixed and stained in Coomassie Brilliant Blue dye solution (10% glacial acetic acid, 45% methanol and 0.25% w/v Brilliant Blue R (Sigma-Aldrich)) for 2 hours to overnight at room temperature with shaking (40 rpm). After that, de-staining solution (7.5% glacial acetic acid, 20% methanol)

was used to show the protein positions on the SDS-PAGE gel.

2.6.4. Western blotting

For immunoblotting of proteins, electrophoresed samples were transferred from SDS-PAGE gels onto pre-activated polyvinylidene fluoride membrane (PVDF) (Millipore, UK) (pre-activated with methanol). Transfer was performed in a tank (Geneflow, UK) containing ice-cold transfer buffer (25mM Tris pH ~8.3, 192mM glycine, 20% methanol) at constant voltage of 100 Volts for 1 hour before membranes were blocked overnight at 4°C with blocking buffer containing 5% skimmed milk in TBS-T (20mM Tris, 150mM NaCl, 0.05% Tween 20, pH 7.6), then gently rinsed in ddH₂O. Membranes were incubated with an appropriate concentration of primary antibodies in blocking buffer for 1 hour at room temperature with gentle shaking. Unbound primary antibodies were removed by washing membranes with 50ml of 1 X TBS-T washing buffer, followed by washing the membranes three times for 5 minutes, twice for 15 minutes and then a final three times for 5 minutes each, each wash using 20ml of TBS-T. Above all incubation and washing steps were performed with gentle agitation in shaker. Membranes were then incubated in an appropriate amount of Horseradish peroxidase conjugated antibodies (secondary antibodies) prepared in blocking buffer, for 1 hour at room temperature. Unbound secondary antibodies were removed as previously described with wash buffer before signals were developed with an EZ-ECL Chemiluminescence kit for HRP (Geneflow). The image was captured using Fuji medical X-ray film (Fujifilm, UK), with different exposure time. Blots were quantified with ImageJ software (<https://imagej.net/Welcome>).

2.6.5. Expression and purification of recombinant FlaA and generation of anti-FlaA antibodies

Amplification of FlaA from NCTC11168 *C. jejuni* strain was performed using primers listed in Table 2.3. A cloning service provided by the PROTEX laboratory (PROTEX, University of Leicester) was employed to clone the open reading frame (ORF) of *flaA* into pLEICS-01 (Table 2.1) in which *flaA* was fused at the N-terminal to 6xhistidine residues. The pLEICS-01::*flaA* construct (pLEICS-01-KH*flaA*) was confirmed by sequencing the insert using vector-specific primers

and DNA sequencing of the insert (Table 2.3) and also using restriction enzymes (Section 2.4.2).

In order to express recombinant FlaA protein, clones were transformed into an *E. coli* expression strain BL21 and grown overnight in 10ml of Luria broth (LB) supplemented with ampicillin (100µg/ml) at conditions outlined in 2.1. Overnight cell cultures were added into 100ml of LB-ampicillin (100µg/ml) broth (dilution of 1/100) and grown until cell density an OD₆₀₀ of ~0.5 was reached. Isopropyl-beta-D-thiogalactopyranoside (IPTG) at a final concentration of 1mM was added to the cell suspension in order to induce FlaA protein expression. Culture mixture was incubated at 30°C and 37°C for 5 hours with shaking. During induction of the expression of FlaA protein, 500µl of the culture was taken at hourly intervals (including pre-induction) and boilates prepared for analysis on SDS-PAGE and detection by Western blot as described in sections 2.6.3 – 2.6.4. The cell pellet was stored at 4°C or at -80°C depending on the required storage period.

Recombinant FlaA protein was purified from *E. coli* BL21 (DE3) cells under denaturing condition. Cells were harvested from 1000ml inducible culture by centrifugation at 3200 X g at 4°C for 20 minutes. Then, pellets were re-suspended in appropriate volumes of lysis buffer (50mM Tris-HCL (pH=7.4), 15mM NaCl) containing protease inhibitor tablets (Roche); 1 tablet for 50ml of lysis buffer according to the manufacturer's instructions and lysozyme (350µg/ml) (Sigma, UK). The mixture was vigorously mixed by vortexing then incubated at 37°C for 30 minutes with rocking. The cells were homogenised by using a Bioruptor sonicator (Diagenode, Belgium) in ice-cold conditions with 30 seconds on/off for 30 minutes, and subsequently derbies cells (inclusion bodies) were isolated by centrifugation at 13000xg for 30 minutes to separate soluble and insoluble fractions then washed three times in 5ml of washing buffer (50mM Tris-HCL (pH=7.4), 100mM NaCl, 2M urea 0.5% Triton X-100) at 12000 xg for 30 minutes. The pellet was re-suspended in solubilisation buffer with 8M urea and 250mM NaCl (50mM Tris-HCL (pH=7.4), 250mM NaCl, 20mM imidazole, 8M urea) and incubated at room temperature for 1 hour/ON with stirring (10-30mg wet weight/ml) to allow for complete solubilisation. After that the solubilised proteins was centrifuged at 12000 xg for 30 minutes. The Histidine-tagged FlaA protein was purified from contaminant *E. coli* proteins by using HisTrap FF column with Ni²⁺ Sepharose packed (GE Healthcare Life sciences) resins, which has affinity

for His-tagged fusion protein. Briefly, the column was washed thoroughly with 25ml of ddH₂O to remove residual alcohol and after that the equilibration of 5ml resin was achieved with 25ml binding buffer (50mM Tris-HCl pH=7.4, 250mM NaCl, 20mM Imidazole) before adding proteins. Solubilised FlaA fusion protein was run through the column which was then washed with a set of buffers with decreasing urea concentration: 8M, 4M, 2M, 1M, 0.5M, 0.25M, 0.13M, 0.06M urea solution in 20mM Tris, 250mM NaCl, 20mM imidazole pH 7.4 (5ml of each buffer was used, 40ml in total). The final washing step was performed with 5ml of 20mM Tris, 250mM NaCl, and 20mM imidazole pH 7.4. His-tagged proteins were eluted from the nickel column with 5ml of 20mM Tris, 250mM NaCl, 300mM imidazole, pH=7.4. After 5 days of room temperature incubation in refolding buffer (20mM Tris-HCL (pH=8), 500mM NaCl, 1% Zwittergent 3-14). The protein was then dialysed against three changes of 1L BPS using Slide-A-Lyzer G2 Dialysis Cassette (MWCO = 20kDa) (Thermo scientific) and concentrated using Amicon Ultra-15 Centrifuge Filter Units (MWCO = 30kDa) (Millipore, UK). Pure rFlaA protein was quantified by SDS-PAGE against dilutions of a BSA standard and concentration was measured (0.49mg/ml) by Quickstart Bradford kit (Biorad Laboratories Inc.), as per the manufacturer's instructions.

The polyclonal antiserum reactive against *C. jejuni* FlaA was generated by Eurogentec (<http://www.eurogentec.com/eu-home.html>) using Speedy 28-Day programmer. Two rabbits were immunised four times with 100µg/ml of recombinant FlaA from *C. jejuni* 11168.

2.7. Biological assays

2.7.1. Growth assay

Frozen *C. jejuni* strains were thawed on dry ice from -80°C freezer and then streak-out with loop under the flow hood onto MHA-TV plates, then incubated at 42°C overnight for the further growth in VAIN. Next day, bacterial colonies were swabbed into new fresh MHA-TV plates, which contains appropriate antibiotics selections and then re-incubated at 42°C overnight for the further growth in VAIN. Positive colonies were selected for inoculation in 5ml MHB with TV starter culture in universal sterile bottles, then the culture was transferred for incubation in shaker at 50 rpm overnight in the VAIN. The optical density (600nm) of each

starter culture was measured in spectrophotometer then culture diluted further accordingly in fresh MHB-TV media for final volume of culture to give OD₆₀₀ of 0.05 (about 5 X 10⁷ CFU/ml).

Multiple 200µl aliquots of diluted cultures were inoculated into individual wells of 96-well microtiter plate. A total of four technical replicates and two MHB blanks per strains were prepared. Microtiter plates containing samples that sealed with a gas permeable pre-pierced seal. However, seal was carefully positioned towards the pierced slits and away from the center of the walls to avoid the interference with the absorbance measurements. Microtiter plates were incubated in the FLUOstar Omega at 42°C with atmospheric control unit set that independently control the O₂ and CO₂ levels. After that, absorbance at 60nm was measured (20 flashes/cycle) for total 40 cycles at least 30 minutes with continuous shaking at 500 rpm between measurements. Cell growth were measured and calculated using the mean from three independent experiments and plotted + / - one standard deviation from the mean.

2.7.2. Motility assay

Motile and hyper motile variants of 11168Ca cells were produced by sub-culturing on semi-solid (0.4%) agar plates. The low concentration of the agar allowed bacterial cells to expand in size and create visible halo within the agar. Briefly, cell suspensions of *C. jejuni* culture were carried out by overnight incubation at 42°C at 500 rpm in shaker. *C. jejuni* culture was diluted with MHB to an absorbance of 0.3 at OD₆₀₀ (6 X 10⁷ cfu/ml). After overnight incubation next day, 10µl aliquots were spotted into the center of the swarm plates (0.4% MHA-TV) and again incubated at 42°C under microaerophilic conditions for 3 days without any inversion.

Motility was then scored visually based on the colonies of bacterial strains extension on the agar from the point of inoculation. The expansion of the halo was examined by measuring the respective diameter from 24 hours to 96 hours intervals. Photos were taken following incubation and the size of the swarming halos expansion in diameter were recorded and calculated under an indirect light source by using a Syngene-Gene Genius unit (Syngene, Cambridge, UK) in dark

room. Additionally, each experiments were performed for three times respectively.

2.7.3. Autoagglutination assay

Bacterial cells were suspended in PBS, adjusted to an OD₆₀₀ of 0.5 and 2ml of suspensions were dispensed into 10ml glass test tubes (Fisher Scientific, UK) and kept undistributed at RT for 24 hours. After that, mutant cells were capable for AGG fell at the bottom of the tube, and leaving a clear supernatant. The top layer of the suspension culture was removed by 1ml and then measured the OD₆₀₀ to quantify the degree of AGG.

2.8. Illumina genome sequencing and bioinformatics analysis

2.8.1. Illumina genome sequencing

Genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions and quantified with Quanti-iT PicoGreen (Thermo-Fisher Scientific). Purified DNA of minimum concentration 30ng/μl resuspended in dH₂O was sent to Oxford Genomics Centre for Illumina genome sequencing workflow. Library for Illumina sequencing were prepared by using NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs) as follows: initially, the amount of around 300ng genomic DNA was sheared using Episonic Multifunctional Bioprocessor. Fragmented DNA were repaired, bead purified (Ampure paramagnetic beads; Beckman Coulter Inc) and A-tailed. Adaptors were ligated and Illumina-specific sequencing indexes were introduced to samples via PCR. Indexed DNA samples were purified for final time before quantification proceeded with Quanti-iT PicoGreen, and pooled at equimolar amounts. Pooled samples were then run on a sequencer (Illumina HiSeq 4000) for paired-end 150-bp sequencing.

2.8.2. Illumina sequence data analysis

Bioinformatics analysis had been carried-out under supervision Dr. Roxana Zamudio. Special computational teaching and research environment (SPECTRE)

workshops, which are high performance cluster hosted by University of Leicester were all bioinformatics analysis works done. First, raw sequence reads quality checked by using FastQC <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>. Following by read trimming and filtering using Trimmomatic (v0-36) <http://www.usadellab.org/cms/?page=trimmomatic> (Trimmomatic: a flexible trimmer for Illumina sequence data). This program filtered-out poor quality reads and end-trimmed the remaining reads to remove poor quality bases and barcodes. Trimmed reads were then assembled into contigs and scaffolds with the SPAdes assembler (v 3.9.0) (SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing) <http://bioinf.spbau.ru/en/spades>. SPAdes was run with the recommended parameters (-k 21, 33, 55, 77--careful). These assemblies were evaluated with QUAST tool (QUAST: quality assessment tool for genome assemblies) <http://bioinf.spbau.ru/quast>. Trimmed and filtered FastQ files were mapped to the reference genome *C. jejuni* NCTC 11168 (GenBank accession no AL111168.1) using Snippy v2.5/BWA-MEM v0.7.12 <https://github.com/tseemann/snippy>. Variants consisting of single base substitutions, insertions and deletions were detected by using Snippy v2.5/Freebayes v0.9.21-7 <https://github.com/tseemann/snippy> with a minimum mapping coverage threshold of 10 and a base-call stringency of 90%.

2.9. Bioinformatics tools

The following bioinformatics tools were used in this study

- 1- *C. jejuni* genome sequence data was obtained from NCBI database (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and also the analysis of sequences was performed by the Blast programmes on the NCBI website and by Clone Manager Professional Suite (Sci Ed Central, version 9.0, copyright 1994-2005, Scientific and Educational Software).
- 2- Plasmid maps and genomes were visualized and constructed using Clone Manager (version 9) (Scientific and Educational Software, 2012) and SnapGene 1.1.3.
- 3- The sequence trace files were viewed using finch TV version 1.4.0 (Geospiza Research team).

4- An amino acid alignments between two proteins was done by using the Pairwise Sequence Alignment (EMBOSS,Needle) https://www.ebi.ac.uk/Tools/psa/emboss_needle/ and also clustal omega online programme from the website: <http://www.ebi.ac.uk/Tools/msa/clustalo/> EMBOSS Sixpack tool was used at https://www.ebi.ac.uk/Tools/st/emboss_sixpack/ to display DNA sequences with 6-frame translation.

5- The TOPCONS web server <http://topcons.cbr.su.se/pred/> was used to examine, if *Cj1310* has signal peptide and transmembrane structure.

6- To identify functional domains and motifs in protein sequences used Interproscan server www.ebi.ac.uk/interpro/ and for the SMART database was used to identify conserved domains <https://smart.emble.de>.

2.10. Statistical analysis

Statistical analyses and graphs were performed using GraphPad Prism, version 7.00 (for Windows; GraphPad Software) or Microsoft Excel 2010 (version 12.0.6024.5000, Microsoft Corporation). Data were expressed as mean \pm SD. Motility test results were analysed by one-way ANOVA followed by Sidak's multiple comparisons test. Auto-agglutination test results were also analysed by one-way ANOVA followed by Tukey's multiple comparisons test. A paired student's *t*-test was performed to determine whether the data was statistically significant. Statistical significance was indicated by asterisks (*) using the following scale: ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Chapter 3: Analysis of motile variants to determine the genetic basis for differences in motility

3.1. Introduction

The genome of *C. jejuni* NCTC 11168 is subject to considerable genetic variation, and this variation may have an important role in the survival of the species within host organisms and the environment. The sequencing of this strain by Parkhill et al (2000) led to the identification of 29 potentially variable poly-G tracts of which 27 out of are located within or close to coding sequences and the other two are found associated with pseudogenes. Genes containing these simple sequence repeats are mostly clustered in three loci involved in the alteration of capsular polysaccharide, lipo-oligosaccharide (LOS) and flagella structures (Lango-Scholey *et al.*, 2016) (Table1.1). These genes undergo phase variation via slipped-strand mispairing during replication, which can result in insertions and deletions (i.e. indels) of repeats causing frame shift mutations (Thomas *et al.*, 2014). Phase variation, which is identified as the reversible switching of gene expression between an "on" and "off" state is a major mechanism of *C. jejuni* that gives rise to genetic heterogeneity in its population (Woude and Bäumlner, 2004; Bayliss, 2009). Gaasbeek et al. (2009) suggested that the frequent occurrence of phase variation maybe due to the absence of a functional mismatch repair system, which can result in more frequent indels in repetitive tracts.

Flagella-mediated motility plays a fundamental role as a primary requirement for commensal and pathogenic behaviours of *C. jejuni* (Lertsethtakarn *et al.*, 2011). Motility is necessary for *C.jejuni* in order to be able to travel through and colonise the highly viscous mucus layer covering the surface of epithelial cells (Szymanski *et al.*, 1995). Moreover, the flagellum filament acts as an adhesion element to promote the binding of bacterial cells to host epithelial cells(Backert and Hofreuter, 2013). In a similar manner, *C. jejuni* motility is important in order to achieve maximal invasion of human intestinal epithelial cells *in vitro* (Wassenaar *et al.*, 1991). Therefore, functional flagella in *C.jejuni* are required in several steps of the infection process.

C. jejuni is motile through the action of a polar flagella at one or both ends of the bacterial cells which is comprised of a membrane-embedded basal body, a hook structure and a long helical filament (Parker *et al.*, 2014). The filament is

composed of repeating subunits known as flagellins, which consist of the major flagellin subunit FlaA and the minor subunit FlaB (de Vries *et al.*, 2015). The genes encoding the flagellin proteins FlaA and FlaB are the only flagellar structural genes included in the flagellin glycosylation cluster (Karlyshev *et al.*, 2002). The role of FlaA and FlaB in the biosynthesis of fully functional flagella in *C. jejuni* has been investigated (Guerry *et al.*, 1991). A mutagenesis study revealed that an isogenic *C. jejuni flaA* mutant had a truncated flagellum and lost its ability to colonise the host (Wassenaar *et al.*, 1991). While the *flaB* gene has been found to be not essential for motility (Josenhans *et al.*, 1999), its product was reported to play an important role in defence of *C. jejuni* against bacteriophage infection (Lis and Connerton, 2016).

The *C. jejuni* flagellar filaments are posttranslationally modified by O-linked glycans which is an important step in the assembly of the flagellum and hence motility (Doig *et al.*, 1996; Thibault *et al.*, 2001). Additionally, O-linked glycans attached to *C. jejuni* flagellins can contain more than one type of sugar, including Pse, legionaminic acid and related derivatives of nonulosonate (Thibault *et al.*, 2001). The high variability of O-linked glycans in *C. jejuni* flagella are correlated with phase variable genes involved in the Pse biosynthesis pathways (L. B. van Alphen *et al.*, 2008; Hitchen *et al.*, 2010) (Table 1.1). As motility in this microorganism is known to frequently switch between non-motile, motile and hyper-motile states (van Alphen *et al.*, 2008), it seems likely that genetic diversity in the flagellar glycosylation locus leads to variation in decoration of the flagellar filament with glycans and hence causes these frequent alternations in motility.

Phase variable genes involved in the modifications of flagellar filaments including *maf* genes (*maf1* and *maf4*) are generating considerable interest in terms of their contributions to the motility alternations in *C. jejuni*. In 2000, Karlyshev *et al.* found that inactivation of *maf5* in a hyper-motile variant of *C. jejuni* NCTC11168 (H11168 Δ *maf5*) generated non-motile variants. This mutagenesis study revealed the importance of the Maf5 protein in flagellar biosynthesis. However, prolonged incubation of H11168 Δ *maf5* resulted in the formation of motile cells due to the phase variation of *maf1* gene via slipped-strand mispairing which partially restored the motility of the *maf5* mutant. Later in 2008, van Alphen *et al.* were investigating only the function of the potentially phase-variable *maf4* gene in *C. jejuni* strain 108 as *maf1* (which is identical to *maf4*) is absent in this strain. Van

Alphen et al. (2008) found that *maf4* is not essential for motility of *C. jejuni*. However, the function of *maf4* changes one of the glycan modifications of the flagellin and increased bacterial autoagglutination. It is likely that the reversible expression of *maf4* changes the population behaviour of *C. jejuni* and may lead to motility variations, which helps *C. jejuni* adapt in various environments. Finally, Hitchen et al. (2010) found that *Cj1295* is responsible for structural changes in the flagellin glycoprotein but does not change the autoagglutination of *C. jejuni* NCTC 11168.

The motility of the original sequenced strain *C. jejuni* NCTC11168 was noted to be significantly lower than that of fresh clinical isolates. Other researchers had noted that there was variation in motility ranging from non-motile to hyper-motile for variants of wild-type parental strain NCTC11168 (Karlyshev *et al.*, 2002). Therefore, this study was hypothesised that the formation of hyper-motile cells arose due to the presence of phase variable expression of homopolymeric G/C tract containing genes, as identified by Parkhill et al. (2000), that are predicted to encode enzymes involved in modification of surface structures.

The aim of this chapter is to understand the genetic basis behind the formation of outgrowths of hyper-motile cells from a low motility inoculum in motility plates of reduced agar concentration (0.4 % agar). The starting variant was *C. jejuni* NCTC11168 Ca, which is a chicken-adapted hypermotile variant with multiple phase variable genes (Table1.1). Multiple, independent motile and hyper-motile variants were obtained from the 11168Ca strain and examined in the motility assay, and also by gene scan analysis and whole genome sequencing.

3.2. Variable motility in single-colony isolates of *C. jejuni* 11168 Ca.

The importance of changes in the motility of *C. jejuni* NCTC11168 have been indicated by the ability of the hyper motile isolate 11168H to colonize the chicken intestine at a high level (Karlyshev *et al.*, 2002). The swarming motility of bacterial cells in semisolid agar media can be measured by the Swarm Assay (Niu *et al.*, 2005). Thus, the Swarm Assay was performed in order to isolate hypermotile variants *in vitro* from 11168Ca. Primary screening was carried out with three single colonies which were reisolated from 11168Ca and designated as A, B, and C. The three isolates were grown in MHB media and inoculated into the swarm plates as described (section 2.7.2). After 72 hours incubation under microaerobic conditions, the diameters of halo, representing the distance moved by respective isolates in soft agar, were measured. The parental isolates (A, B and C) were significantly more motile than the 11168Ca $\Delta flaA$ mutant (Figure 3.1) The mean diameters of the motility zones of A, B and C variants ranged between 40 and 45 mm; the mutant showed significantly less motility (12 mm, $P < 0.0001$). Therefore, it is suggested that the three (A, B, C) selected single colonies were motile (M) as compared to the mutant. Furthermore, during this experiment sweeps of hyper-motile cells (HM) were collected with a sterile loop from the outer edges of halo growth rings for further screening (Figure 3.1). These potential hyper-motile variants were visualised on the edges of the halo growth after 48 and 72 hours incubation and then isolated. These sweeps of isolates were labeled as A2, B2, C2, A3, B3 and C3 respectively. Figure 3.2 shows the formation of the hyper-motile phenotype on a swarm assay plate in comparison with the morphology of motile cells.

A comparison of the motility of the HM and M variants was performed using the swarm assay. After 48 hours of incubation, halo areas were measured. The mean diameters of the halo growth rings of the HM variants were between 45 and 70 mm while, the mean diameters of growth zones of M variants were between 25 mm and 29 mm, respectively. The HM variants showed significantly greater movement from the centre of the plate compared to M variants ($P < 0.001$) (Figure 3.3).

Based on the above information, this study was initially classified the motility of the 11168 Ca strain into two categories motile and hyper motile variants depending on their phenotype on the semi-agar plates (Figure 3.2). A comparison of the diameters of the growth zones of M and HM indicated that hyper-motile variants had a 15 mm halo after 1 day of incubation that expanded to cover a 100 mm petri dish after three days of incubation, whereas the diameter of swarm growth of motile variants after 72 hours was between 40mm and 50mm (Figure 3.4). After 48 hours, the hyper-motile variants with the lowest halo ring size of ≤ 40 mm were still significantly better at spreading than the motile variants. Therefore, we speculated that HM outgrowths consistently swarm more than motile variants with a threshold value for HM defined as 40 mm. To validate this observation, further isolations of motile and hyper-motile cells were picked from the edges of swarming colonies on motility plates of M and HM variants at two different times of incubation (24 and 48 hours). These new patches of M and HM colonies were isolated from M variants (A, B and C) and HM variants (A2, B2, C2 A3, B3 and C3) (approximately 41 variants in total as shown in figure 3.5) and assessed in the motility assay. The diameters of swarming rings for motile variants were compared to the lower diameter of halo growth for hyper-motile variant B.2.3. These results supported the observation that hyper-motile cells had a minimum diameter for the growth ring of ≤ 40 and had diameters that were significantly higher than motile cells. As showing in appendix B, there is a significant difference ($p < 0.001$) between the lower diameter of halo growth for hyper-motile variant B.2.3 (41mm) and the maximal diameter of ring size for motile variant B.1.1 (29.75mm).

There was also interesting observation that hyper-motile derivatives of *C. jejuni* 11168 Ca maintained their hyper-motile phenotype when they were moved to new plates suggesting a genetic basis for this phenotype. This observation formed the basis for further genetic analyses. In order to perform these phenotypic and genetic analyses, all putative variants with motile or hyper-motile phenotypes were picked from the edge of the halo growth and directly cultivated on a fresh MH agar plate for three days at 42 °C in a microaerobic atmosphere. Subsequently, cells scraped from these MH agar plates were assayed in terms for their motility, for the PV states of 28 polyG tracts (section 2.4.7) and finally a glycerol stock (50 % v/v glycerol) was prepared for subsequent tests.

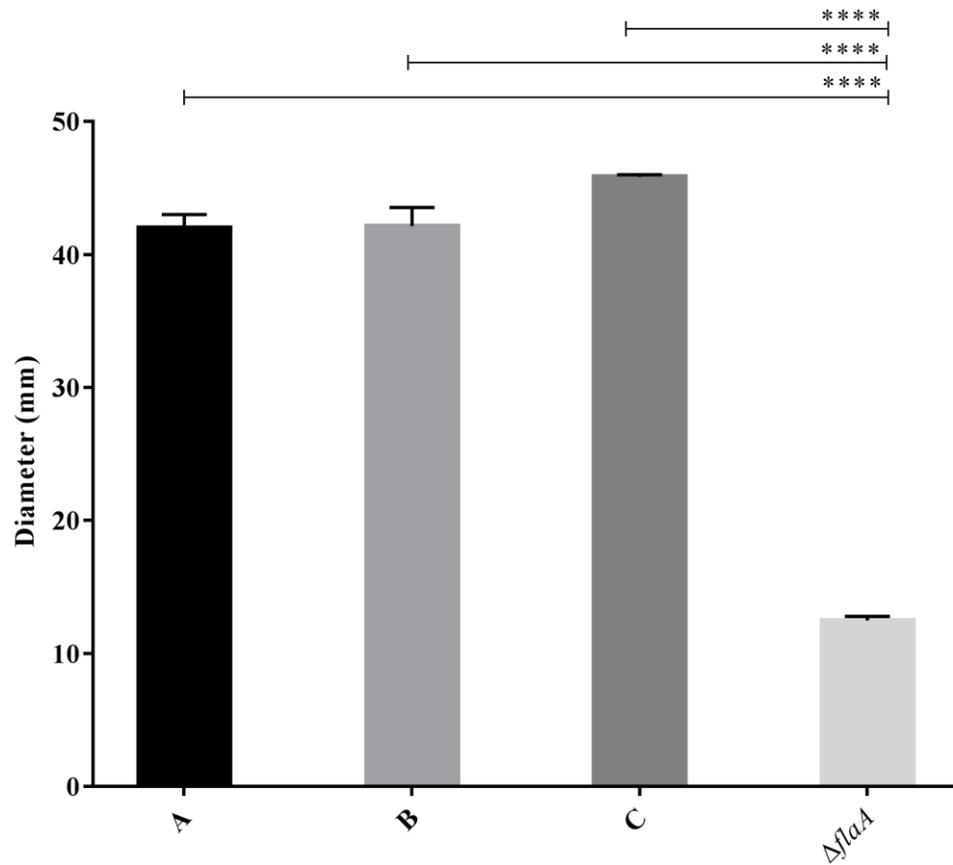


Figure 3.1. Comparison of the difference in swarm diameters between three selected colonies A, B and C from *C. jejuni* 11168 Ca with 11168 Ca Δ *flaA* mutants. Swarming motility was analysed on MH plates with 0.4 % (w/v) agar. All swarm diameters were measured following 72 hours incubation under microaerobic conditions. Values are averages of data from three independent experiments and there were 3 replicates per experiment. The standard deviations (S.D) are represented by error bars. Student t-test ****significant difference ($P < 0.0001$).

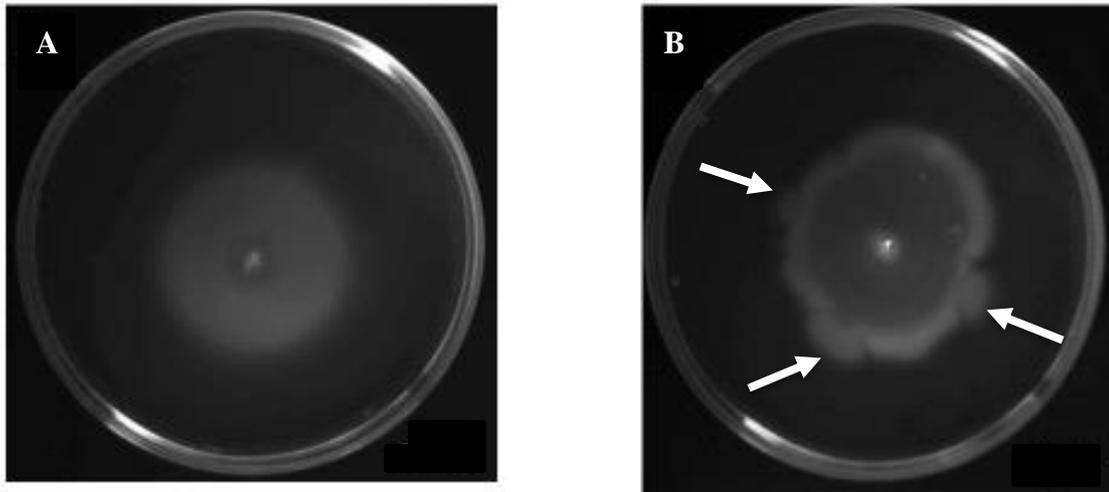


Figure 3.2. Representative images showing the formation of the hyper-motile phenotype in comparison with the motile phenotype of *C.jejuni* 11168Ca on semi-solid media.

A) Swarm plate showing normal motility of *C. jejuni* cells spreading beyond the site of inoculation. B) Hyper-motility of 11168Ca strain on soft agar plate with evidence of rapidly migrating zones of bacteria moving away from the initial zone of inoculation faster than the majority of cells. Arrows point to areas in which hyper-motile cells have been picked. A suspension of cells at an $OD_{600} \approx 0.5$ were stabbed into the centre of a 0.4% (w/v) MHA plate and grown for 48 hours at 42 °C in a microaerobic atmosphere.

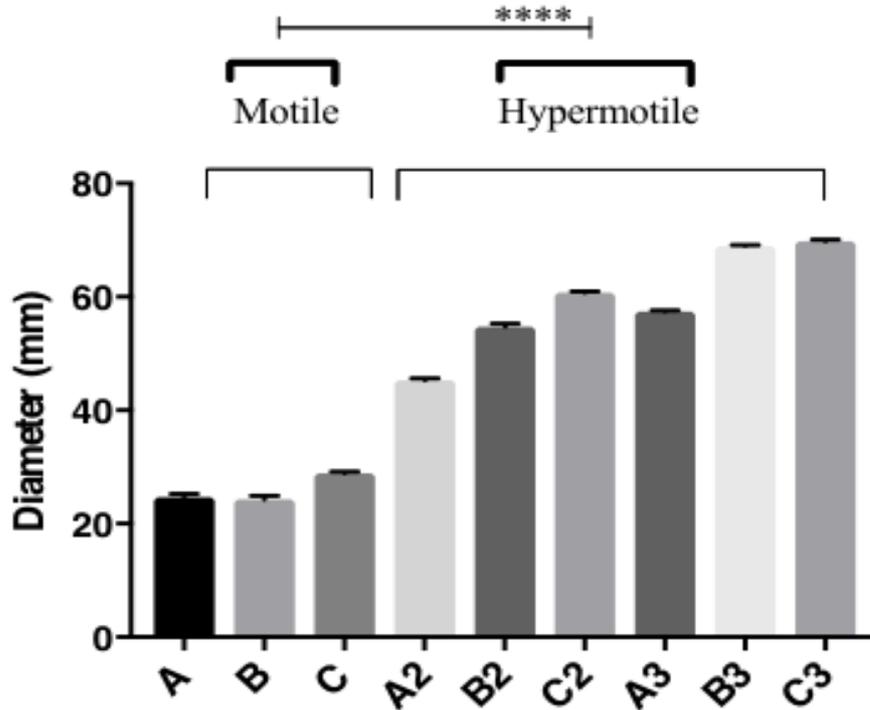


Figure 3.3. Diameters of the motility zones of the *C. jejuni* 11168 Ca isolates, motile isolates (A, B and C) and hyper-motile isolates (A2, B2, C2 A3, B3 and C3).

Swarm assays were performed on 0.4% MH agar plates inoculated with the indicated variants and incubated at 42 °C under microaerophilic conditions for 48 hours. Values are averages of data from three independent experiments and there were 3 replicates per experiment. The standard deviations (S.D) are represented by error bars. Student t-test ****significant difference ($P < 0.0001$).

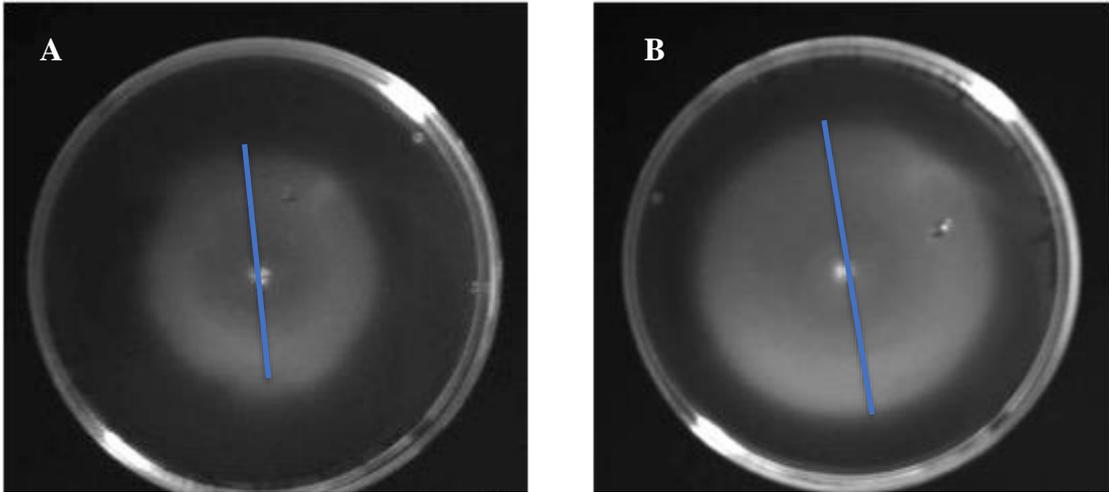


Figure 3.4. Confirmation of hypermotility phenotypes in 11168 Ca strain.

Stationary phase cultures of (A) a motile (represent variant A in Figure 3.5) and (B) hyper-motile (represent variant A2 in Figure 3.5) variants were normalized by OD_{600} and were stabbed onto a 0.4% (w/v) MHA plate and grown for 48 hours at 42 °C in a microaerobic atmosphere. Blue lines show the diameters of the bacterial spread on the swarm plates.

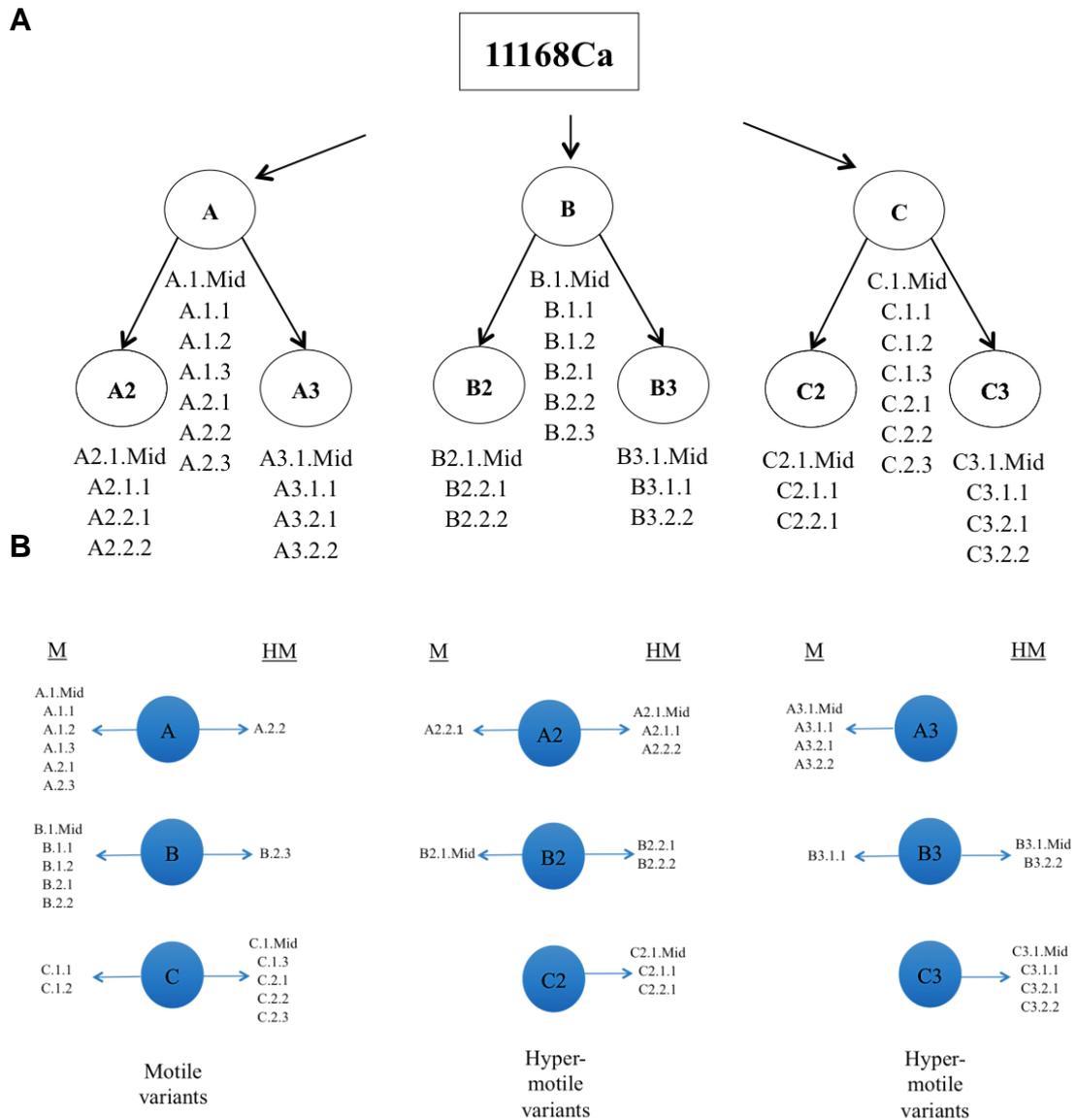


Figure 3.5. Schematic representation of parental motile strains (A, B and C), hypermotile strains (A2, B2, C2, A3, B3 and C3) and their M and HM derivatives as used for the motility analysis.

This diagram shows the track that has been followed to isolate M and HM variants. (A) Three original colonies A, B and C were picked from *C. jejuni* 11168Ca strain. The A2, B2, C2, A3, B3 and C3 were isolated from the edges of halo growth of these colonies after two days (2 refers to the date of the isolation) and three days (3 refers to the date of the isolation). Further variants were isolated from these 9 variants. (B) These variants were classified into M and HM relative to their morphology on the swarm plates.

3.3. Analysis of 28 PV genes in motile and hyper-motile variants using GeneScan and PSAnalyse.

Phase variation is a potential determinant of the variation in motility. As the sequence of *C.jejuni* NCTC11168 demonstrated the presence of SSRs in a number of genes involved in expression of surface molecules (Table 1.1) this work was performed to determine if there is a difference in the expression of phase variable genes between motile and hyper-motile variants of this strain and, if so, which combination of PV genes results in maximum motility.

A combination of GeneScan™ and PSAnalyse was utilised to determine the expression states of the 28 phase variable tracts. The analysis of these samples was carried out once at the time of collection of each M and HM variants. In order to reduce the required number of PCRs to encompass all 28 of the polyG/polyC tracts present in *C. jejuni* NCTC11168, a high throughput method was employed (Lango-Scholey *et al.*, 2016). This method consists of three steps; (1) amplification of the 28 PV loci in five multiplex PCR reactions; (2) analysis of fragment length; (3) use of PSAnalyse for the automated calling of repeat numbers and determination of ON/OFF states for each gene. A control sample (conA) of PCR products derived from a single colony of *C.jejuni* NCTC11168 was included on each plate of tested samples. This control had previously been subject to GeneScan and Sanger sequencing in order to assign and verify the tract lengths of each PV gene.

The PSAnalyse script uses a control data file, referred to as peaksets that contains the tract lengths, which correspond to the ON expression states. The potential of switching by three nucleotides to the next available ON state was observed in some variants and hence further columns were added to the peakset files for the other expression states. PSAnalyse has a default setting for the 'scan width' of 3.5 bp as this default leads to collection of all peaks that are located within the range of the expected size for a desired peak (± 3.5 bp) and subsequently the script selects the highest peak as the target peak. Another parameter is the minimum peak height, which had a default setting of 1,000. This setting excludes spurious peaks that result from unbound primers and inefficient PCR reactions that occur at much lower sizes than the target peaks. Further, slippage during PCR amplification and the presence of variation within a colony

can lead to the appearance of normally smaller “side” peaks positioned 1bp above and below the main peak, PSAnalyse recognises these side peaks and compares their area to the main peak. Therefore, the ratio of the main peak area to the side peak areas was set to 1.5. Practically, the migration of PCR products leads to the difference between the peak positions in base pairs as result of errors in tract length size. Therefore, the maximum acceptable error of observed peak sizes from expected peak sizes was set to ± 0.33 bp.

The tract length of each PV gene was converted into a ‘0’ or ‘1’ (0=‘OFF’; 1=‘ON’) indicating the expression status of each gene and generating 1,456 values from the whole populations (i.e. a sweep) of the motile and hyper-motile isolates. The average scores for the ON state for each gene in both M and HM variants were compared (19 samples for M and 32 for HM). As shown in Figure 3.6, the most surprising observation is that seven PV genes, including *Cj1295* (G8), *Cj1296* (G9), *Cj1318* (G9 and G10), *Cj0045* (G10), *Cj0171* (G9 and G10), *Cj0676* (G8 and G9) and *Cj0685* (G8), were stable in the OFF state in both screened samples (M and HM variants). This observation suggests that the OFF state of these genes was consistent between motile and hyper-motile isolates and hence there is no correlation between motility and the expression of these genes. However, it is possible that the presence of SNP in these genes (or some of them) would change the outcome of the tract length due to a change in reading frame 5’ to the tract and hence altering the OFF state to repeat number association in these genes.

A second set of genes, *Cj1321*, *Cj1139*, *Cj0046*, *Cj0275* and *Cj0565*, exhibited a mixed population of ON and OFF expression states but with a high proportion of ON expression states for the majority of M and HM populations. It could be that expression of these genes may have an association with motility but not with the variability in motility. It is also possible that the combination of ON status in *Cj1321*, *Cj1139*, *Cj0046*, *Cj0275* and *Cj0565* genes and OFF states in *Cj1295*, *Cj1296*, *Cj1318*, *Cj0045*, *Cj0171*, *Cj0676* and *Cj0685* genes may be linked to motility.

For the remaining 11 genes (*capA*, *Cj0031*, *Cj0617*, *Cj1305*, *Cj1306*, *Cj1420*, *Cj1421*, *Cj1422*, *Cj1426*, *Cj1429*, *Cj1437*), there were mixed populations between ON and OFF expression states in both M and HM variants. However, a

Chi-square test did not show any significant differences in the average score of the ON state between M and HM variants.

Taken together, the data indicated that the phase variable expression of 28 PV genes was not responsible for the formation of hyper-motility in the 11168Ca strain and that therefore another source of variation may be responsible for the this phenomena.

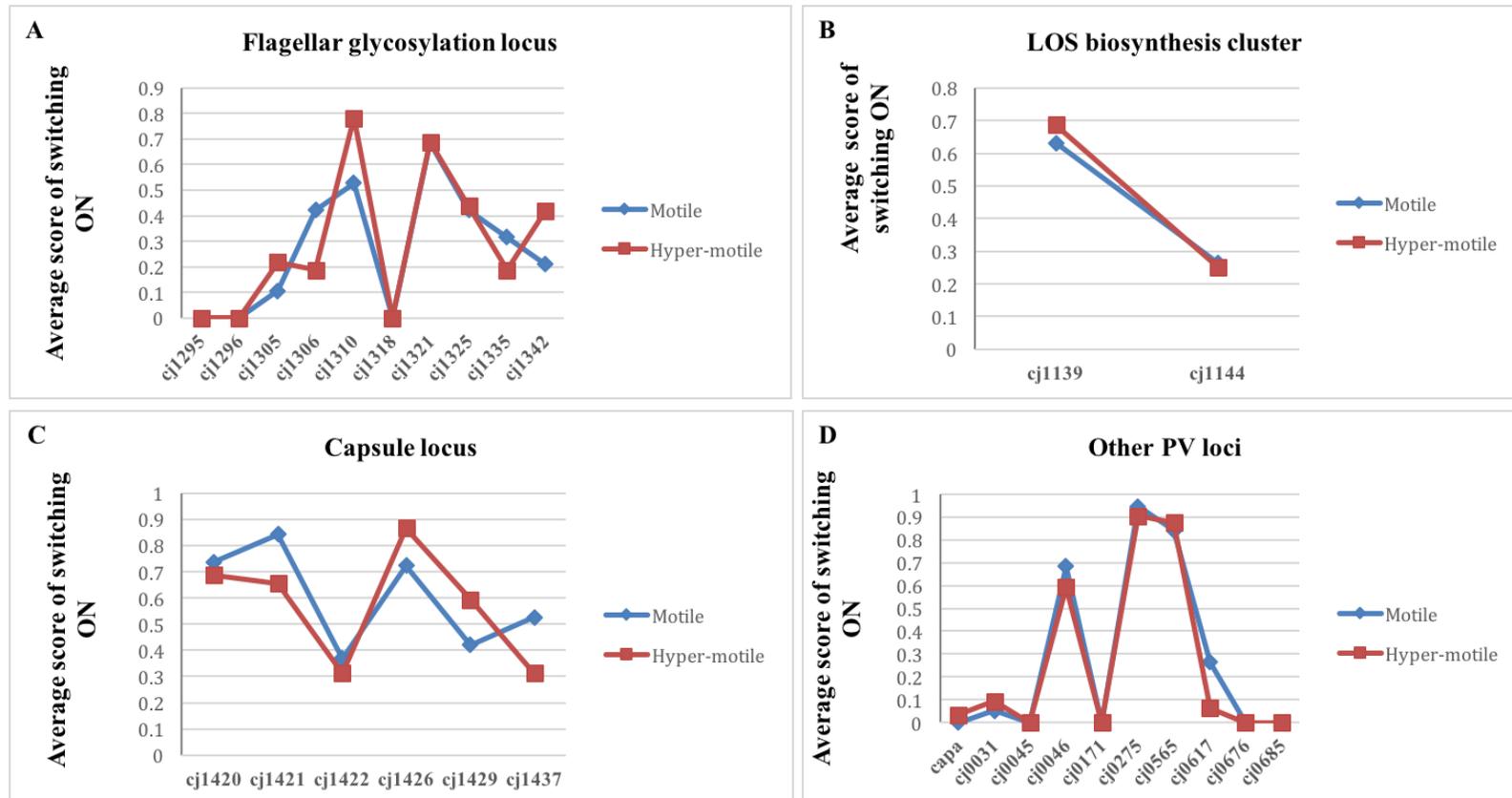


Figure 3.6. Phase states of 28 variable poly-G tracts in motile and hyper-motile variants.

This Figure shows the changes in proportion of each poly-G tract in the ON state for PV genes in (A) flagellar glycosylation locus; (B) LOS locus; (C) Capsule locus; (D) different locations throughout the genome. The tract lengths of the 28 PV genes for a population was determined by GeneScan™ and scoring using PSAnalyse for a total DNA extract of motile and hyper-motile populations. The average score of ON for each gene in either type of motility was plotted (Blue diamonds indicate motile variants; Red squares indicate hyper-motile variants) by dividing populations with an ON repeat number (ON refers as 1 in the score input file generated from PSAnalyse script) by the total number of populations.

3.4. Identification of genetic differences between putative motile and hyper-motile strains using Next Generation Sequencing (NGS)

The reproducibility of the hyper-motile phenotype when assessed through the swarm assay (section 3.2) and the absence of a correlation between the reversible expression states of PV genes and fluctuations in motility (section 3.3) have indicated a stable, genetic basis of the formation of hyper-motility in *C.jejuni*. To test this hypothesis Illumina whole genome sequencing was employed and whole genome comparisons were implemented between M and HM isolates in order to identify any potential mutations (SNPs/ INDELS) associated with the hyper-motility phenotype.

Twenty-eight M and HM variants were selected for Illumina next generation sequencing (NGS). These variants are shown in Table 3.1 and labeled as V with a given number from 1 to 28 while the parental 11168Ca strain was designated as V.29 in order to easily distinguish each isolate through the bioinformatics analysis. Total chromosomal DNAs were extracted and analysed for concentration and purity (section 2.8.1). These samples were then shipped to Oxford Genomics Centre, UK for library preparation and paired end Illumina NGS on an Illumina HiSeq 4000 machine. FastQ files were quality checked and trimmed. Trimmed FastaQ files were *de novo* assembled with SPAdes and assessed with QUAST.

SNPs and INDELS in M and HM genomes were called relative to the reference sequence *C. jejuni* NCTC 11168 (GenBank accession no AL111168.1) using Snippy. A total of 16 SNPs and 11 INDELS differences were present in the 28 genomes. Seven SNPs from the reference were found in every sample (Table 3.2). The motility of the reference strain was found to be substantially less motile compared to fresh clinical isolates. However, derivatives of the wild-type parent strain (reference) ranging from non-motile to hyper-motile were recovered (Karlyshev *et al.*, 2002). Additionally, parental 11168Ca strain that used to isolate M and HM variants is considered as hyper-motile strain. Therefore, these seven SNPs were presumed to be either mutations that may lead to increased motility

in M and HM isolates compared to the reference genome or errors in the reference assembly.

The analysis of genotypic variants associated with phenotypic changes focused on the remaining base substitutions, deletions and insertions that were either unique to individual samples or common between some samples but not all. These remaining SNPs were only found in 6 motile variants and 3 hyper-motile variants (Table 3.3), while INDELs were identified in 9 motile variants and 13 hyper-motile variants (Table 3.4).

The first interesting observation to emerge from the comparison of motile and hyper-motile variants with the reference was that two M (M-V.3 and M-V.12) and three HM (HM-V.6, HM-V.18 and HM-V.26) isolates had the same SNP in the *pta* gene (Cj0688) (Figure 3.7). This gene encodes a phosphotransacetylase enzyme containing an N-terminal phosphate acetyltransferase and phosphate butyryltransferase domain, which is involved in the acetogenesis pathway of *C. jejuni*. In brief, Pta enzyme converts acetyl-CoA to acetyl-phosphate, resulting in release of CoA for reuse. This is followed by production of acetate from acetyl-phosphate through the action of acetate kinase [AckA (Cj0689)], which results in acetate excretion and build up in the surrounding medium. However, the AckA–Pta pathway is reversible to allow for the uptake of acetate into the cell when other sources of nutrients become scarcer (Wright *et al.*, 2009). This mechanism may help *C. jejuni* to identify the optimal niches for growth to colonise the avian intestinal tract and probably infection of human hosts leading to diarrheal disease through an impact on both metabolic and signal transduction pathways in a bacterial cell.

The location of the SNP in *pta* was found in the C-terminal domain and altered a G to an A resulting in a non-synonymous amino acid change from serine to asparagine. This location suggests that this SNP has no influence on the function of the Pta protein (Figure 3.9A). Importantly, as shown in Figure 3.5A, there was a correlation between these variants in which M-V.12 (C.1.1), HM-V.6 (C2), HM-V.18 (C3.1.1) and HM-V.26 (C2.2.1) variants were all derived from the M-V.3 (C) variant, which was characterized as motile (Figure 3.3 and 3.5). Thus, the co-inheritance of this variation in *pta* from the original motile variant M-V.3 (C) to some of its derivatives, suggests that M-V.3(C) contained a mixture of cells with

and without the *pta* SNP so that isolates C.1.1, C2, C3.1.1 and C2.2.1 had this SNP and the other isolates did not. Overall, this finding indicates that this SNP within *pta* gene does not influence the variability in *C. jejuni* motility.

Another similar observation was that the two hyper-motile variants, HM-V.4 (A2) and HM-V.13 (A2.1.1), and their parental variant M-V.1 (A) (Figure 3.5) had seven identical SNPs (Table 3.3 and Figure 3.7) and five identical INDELS (Table 3.4 and Figure 3.8). One of these SNPs was in the active domain of *pta* gene and resulted in a change of the amino acid from alanine to glutamate (Figure 3.9B), indicating a potential change in Pta protein function in this group of motile variants.

Another single point mutation altered A to G in the glycosyl transferases domain of (*Cj0685c*) *cipA*, (a putative *Campylobacter* invasion protein), leading to a non-synonymous change of amino acid from Valine to Alanine. This gene was previously annotated as a glycosyl transferase due to the presence of an unknown functional domain (DUF2972) that was also found in two genes involved in capsule biosynthesis (*Cj1421c* and *Cj1422c*) (Parkhill *et al.*, 2000). *cipA* shares 24.3% and 22.0% identity in this domain with the *Cj1421c* and *Cj1422c* gene products, respectively (Figure 3.10). The specific and ubiquitous nature of this domain suggests a common biological role (Prakash *et al.*, 2011). Recently, Crofts *et al.* (2018) have suggested that *cipA* may facilitate invasion and persistence in the human intestinal epithelial cells via flagella modification. The SNP in *cipA* was predicted to cause a single Isoleucine to Threonine amino acid substitution in the DUF2972 domain. The occurrence of this single point mutation within the open reading frame of *cipA* in particular in the glycosyl transferases domain may cause a change in the function of CipA protein.

Despite the importance of both proteins (Pta and CipA) for *C. jejuni* growth and adaptation, they do not contribute to the motility of *C. jejuni* (Raphael *et al.*, 2005; Joshua *et al.*, 2006). In summary, there was co-inheritance of seven SNPs and five INDELS from the parental A variant to two (A2 and A2.1.1 variants) but not all progeny suggesting that the parental culture was a mix of two variants.

Finally, the most striking result to emerge from the genome sequencing approach is the presence of one SNP (Figure 3.7) and four INDELS (Figure 3.8) mutations

that were unique to hyper-motility isolates and within the *Campylobacter* bile resistance regulator (*cbrR* coding region Cj0643) gene. This gene is part of a multidrug efflux pump pathway and encodes the *Campylobacter* bile resistance orphan response regulator protein (Hermans, Van Deun, Martel, *et al.*, 2011). The *cbrR* gene was first described by Raphael *et al.* (2005) as encoding a 414-amino-acid protein with a calculated molecular mass of 47-kDa and having two tandem N-terminal response regulator (RR) domains that have the aspartyl residue required for phosphorylation and a C-terminal GGDEF domain (Figure 3.11A). The alignment of CbrR receiver domains with *E. coli* CheY shows that the N-terminal receiver domain I of CbrR contain a high number of residues that are similar with CheY residues (Figure 3.11B). While only two residues in CheY are conserved in receiver domain II of CbrR (Figure 3.11C), both receiver domains contain D57 (relative to CheY residue numbering), which is the likely site of phosphorylation (Raphael *et al.*, 2005). The GGDEF domain has been reported to be a diguanylate cyclase containing a putative nucleotide-binding loop (Pei and Grishin, 2001). Many bacterial-signaling proteins such as two proteins in *Acetobacter xylinum* contain a GGDEF motif, including a diguanylate cyclase and phosphodiesterase A and both proteins contributed to the regulation of cellulose biosynthesis (Tal *et al.*, 1998). PleD is another similar protein in *Caulobacter crescentus* that has a 22% amino acid identity with CbrR and contains two tandem N-terminal RR domains and a C-terminal GGDEF motif. This protein is essential for the onset of motility prior to cell division (Hecht and Newton, 1995; Aldridge *et al.*, 2003).

The precise location of aforementioned mutations in HM variants within CbrR domains was determined (Table 3.5). In A2.2.2 (HM-V.24), there was a non-synonymous change from Isoleucine to Asparagine. This amino acid substitution is located in receiver domain I, which includes a phosphoacceptor site that is phosphorylated by histidine kinase homologues. The deletion of an A within receiver domain I was observed in three HM variants C2, C2.1.1 and C2.2.1. C2.1.1 and C2.2.1 were picked from the C2 swarm plate after 24hr and 48hr, respectively. Similarly, the HM variants C3.1.1 and C3.2.1 were collected from the swarm plate of HM variant C3 (after incubation of 24hr and 48hr, respectively) with all of these variants having a deletion of a G within receiver domain II. These results indicate the possibility of a correlation of this genotype with the hyper-

motility phenotype. For H.M variant B3 and the two HM variants A3 and A3.1.1, there was a deletion of GC-dinucleotide and a G located in GGDEF domain, respectively. Therefore, the inactivation of the GGDEF domain may correlate with hyper motility in order to facilitate host colonisation,

Overall, genotypic data retrieved from Illumina genome sequence analysis has mainly indicated that mutations (SNPs and INDELS) in motile and hyper-motile variants are due to co-inheritance from the original isolate or its derivatives. As HM variants on swarm plates of motile variants were collected using a swabbing technique and were not purified through single colonies, these variants may have mixed populations of genotypes and phenotypes (M and HM). Importantly, the existence of genotypic variations of *cbrR* only in HM isolates suggests that mutations (SNPs and INDELS) in this gene can have a significant impact on the formation of hyper-motile cells. However, additional research would need to be conducted to be able to confirm the potential phenotypic consequences of these mutations.

Table 3.1. Illustrates M and HM variants that were Illumina sequenced

Motile isolates	Hyper-motile isolates
A (V.1)	A2 (V.4)
B (V.2)	B2 (V.5)
C (V.3)	C2 (V.6)
A.2.1 (V.19)	A3 (V.7)
A.1.1 (V.10)	B3 (V.8)
A2.2.1 (V.23)	C3 (V.9)
A.2.3 (V.20)	A2.1.1 (V.13)
B.1.1 (V.11)	C2.1.1 (V.15)
B3.1.1 (V.17)	A3.1.1 (V.16)
B.2.1 (V.21)	C3.1.1 (V.18)
C.1.1 (V.12)	B.2.3 (V.22)
	A2.2.2 (V.24)
	B2.2.1 (V.25)
	C2.2.1 (V.26)
	A3.2.1 (V.27)
	B3.2.2 (V.14)
	C3.2.1 (V.28)
	11168Ca (V.29)

Table 3.2. Position of SNP differences between the published *C. jejuni* NCTC 11168 (GenBank accession no AL111168.1) and all motile and hyper-motile variants subjected for sequence.

Position	Type	Reference	Substitution	Locus tag	Gene	Product
192288	SNP	T	C	<i>Cj0196c</i>	<i>purF</i>	amidophosphoribosyltransferase
253500	SNP	C	T	<i>Cj0276</i>	<i>mreB</i>	rod shape-determining protein MreB
262345	SNP	A	G	<i>Cj0284c</i>	<i>cheA</i>	chemotaxis histidine kinase
393542	SNP	T	A	<i>Cj0431</i>		ATP/GTP-binding protein
420550	SNP	A	G			
760188	SNP	A	G	<i>Cj0807</i>		oxidoreductase
1019088	SNP	C	T	<i>Cj1087c</i>		peptidase

Table 3.3. Position of SNP differences between the published *C. jejuni* NCTC 11168 (GenBank accession no AL111168.1) and individual M and HM samples.

H.motile; Hyper-motile

Position	Type	Reference	Substitution	Locus tag	Gene	Product	Variant name	Type of motility
253191	SNP	A	G	<i>Cj0276</i>	<i>mreB</i>	Rod shape-determining protein MreB	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
393542	SNP	T	A	<i>Cj0431</i>		ATP/GTP-binding protein	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
437316	SNP	C	G	<i>Cj0475</i>	<i>rplA</i>	50S ribosomal protein L1	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
638796	SNP	A	G	<i>Cj0685_c</i>	<i>cipA</i>	Invasion protein CipA	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
643379	SNP	C	A	<i>Cj0688</i>	<i>pta</i>	Phosphate acetyltransferase	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
1426404	SNP	C	T	<i>Cj1491_c</i>		Two-component regulator	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
1526302	SNP	C	T	<i>Cj1597</i>	<i>hisG</i>	ATP phosphoribosyltransferase	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
642122	SNP	G	A	<i>Cj0688</i>	<i>pta</i>	Phosphate acetyltransferase	C (V.3)/ C.1.1 (V.12)	Motile
							C2 (V.6)/ C3.1.1 (V.18)/ C2.2.1 (V.26)	H-motile
604879	SNP	T	A	<i>Cj0643</i>	<i>cbrR</i>	Two-component response regulator	A2.2.2 (V.24)	H-motile

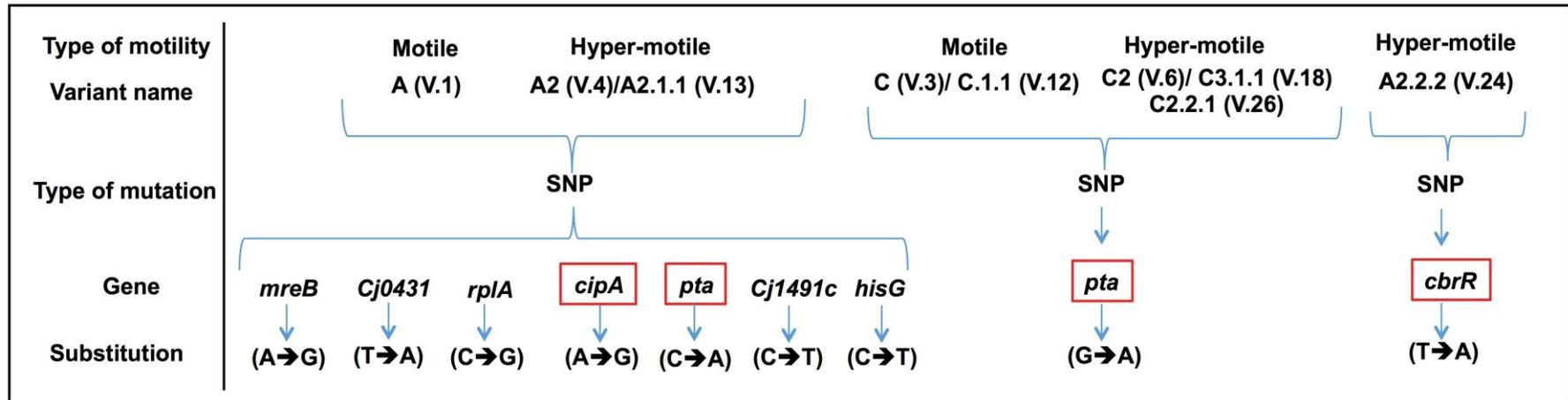


Figure 3.7. Schematic diagram of the location of single nucleotide polymorphisms (SNPs) identified in genes within individual M and HM samples in comparison to the published *C. jejuni* NCTC 11168 (GenBank accession no AL111168.1).

Three groups of SNPs were identified in two groups of mixed motile and hyper-motile variants and one group of hyper-motile variant. *cipA*, *pta* and *cbrR* genes, which are indicated by red open rectangle, were assumed to play an important role in motility.

Table 3.4. Location of INDELS differences between the published *C. jejuni* NCTC 11168 (GenBank accession no AL111168.1) and individual M and HM samples.

*** The expression state of the phase variable genes (*wlaN* and *cipA*) was OFF within variants that contained INDELS mutation of these two genes in comparison with the reference strain. H-motile;Hyper-motile**

Position	Type	Reference	Substitution	Locus tag	Gene	Product	Variant name	Type of motility
87370	INS	GAA	GAAA	Cj0076c	<i>lctP</i>	L-lactate permease	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
98004	INS	CTTTAA	CTTTATTTA A	Cj0088	<i>dcuA</i>	Anaerobic C4-dicarboxylate transporter	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	Hyper-motile
180705	DEL	TACACAC T	TACACT	Cj0184c		Serine/threonine protein phosphatase	A (V.1))	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
181488	DEL	ATTTTTTT TA	ATTTTTTTA	Cj0184c		Serine/threonine protein phosphatase	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
1074192	INS	ACCCCCC CCA	ACCCCCC CCA	Cj1139c	<i>wlaN</i> - OFF*	Beta-1,3 galactosyltransferase	A (V.1)	Motile
							A2 (V.4)/ A2.1.1 (V.13)	H-motile
639006	DEL	TGGGGG GGGT	TGGGGGGG GT	Cj0685c	<i>cipA</i> - OFF*	Invasion protein CipA	B (V.2) /C (V.3)/ B.1.1 (V.11)/ C.1.1 (V.12)/ B3.1.1 (V.17)/ A.2.3 (V.20)/ B.2.1 (V.21)/ A2.2.1 (V.23)	Motile
							B2 (V.5)/ C2 (V.6)/ A3 (V.7)/ B3 (V.8)/ C3 (V.9)/ B3.2.2 (V.14)/ C2.1.1 (V.15)/ C3.1.1 (V.18)	H-motile

Continued from previous page...

Position	Type	Reference	Substitution	Locus tag	Gene	Product	Variant name	Type of motility
605228	DEL	TAAAAAA AT	TAAAAAAT	Cj0643	<i>cbrR</i>	Two-component response regulator	C2 (V.6)/ C2.1.1 (V.15)/ C2.2.1 (V.26)	H-motile
605813	DEL	AGA	AA	Cj0643	<i>cbrR</i>	Two-component response regulator	A3 (V.7) A3.1.1 (V.16)	H-motile
605897	DEL	GGCT	GT	Cj0643	<i>cbrR</i>	Two-component response regulator	B3 (V.8)	H-motile
605384	DEL	AGA	AA	Cj0643	<i>cbrR</i>	Two-component response regulator	C3 (V.9)/ C3.1.1 (V.18)/ C3.2.1 (V.28)	H-motile
261177	INS	ACCA	ACCCCA	Cj0284 c	<i>cheA</i>	chemotaxis histidine kinase	A.2.3 (V.20)	Motile

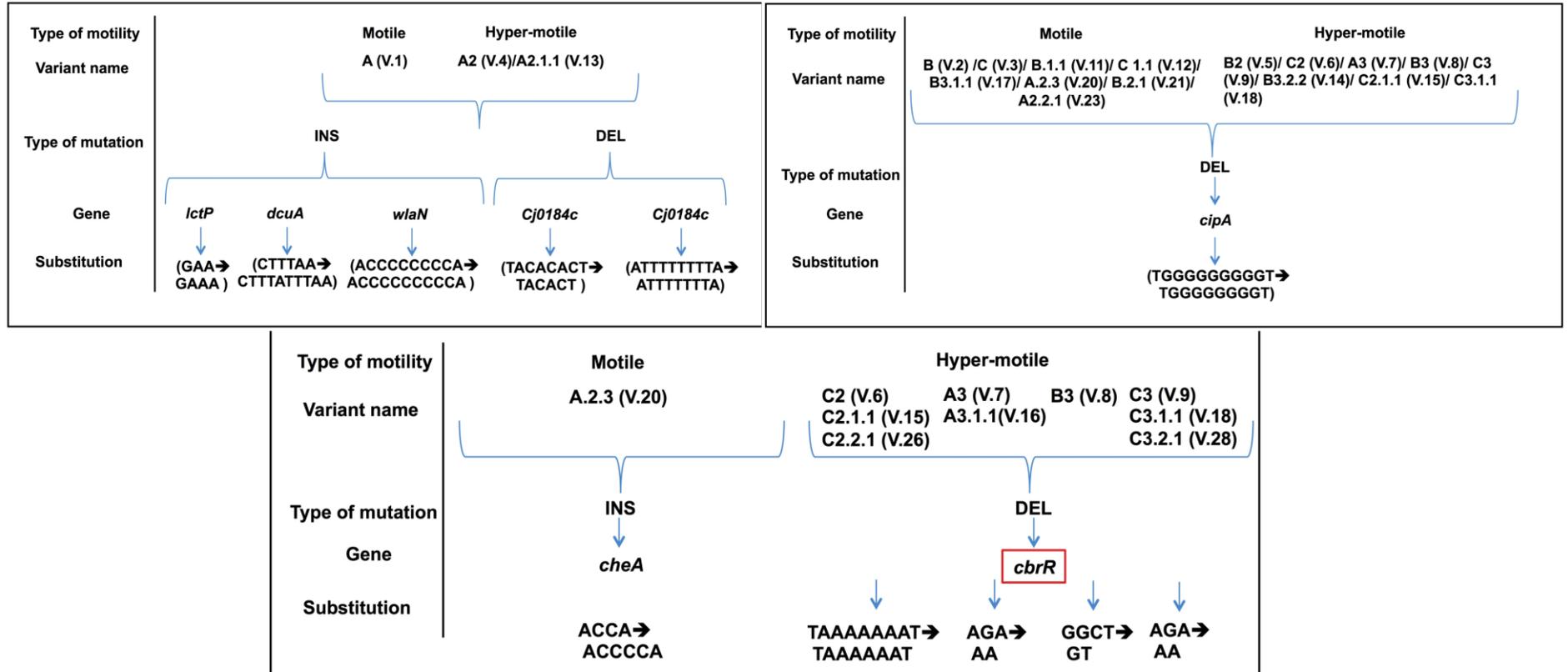


Figure 3.8. Schematic diagram of the location of insertion and/or deletion of nucleotides (INDELs) identified in genes within individual M and HM samples in comparison to the published *C. jejuni* NCTC 11168 (GenBank accession no AL111168.1).

INDELs mutations were found in two groups of mixed motile and hyper-motile variants, one group of motile variant and one group of hyper-motile variant. *cbrR* gene, which is indicated by a red open rectangle, may have an essential role in increased motility due to the unique presence of deletion mutations in hyper-motile variants within this gene.

Table 3.5. Domain location of SNPs and INDELS within *cbrR* gene associated with only H.M variants.

H.M Variant	Position	Type	Reference	Substitution	CbrR Domain
V.24 (A2.2.2)	604879	SNP	T	A	Receiver domain I
V.6 (C2)	605228	Deletion	A	-	Receiver domain I
V.15 (C2.1.1)	605228	Deletion	A	-	Receiver domain I
V.26 (C2.2.1)	605228	Deletion	A	-	Receiver domain I
V.7 (A3)	605813	Deletion	G	-	GGDEF
V.16 (A3.1.1)	605813	Deletion	G	-	GGDEF
V.8 (B3)	605897	Deletion	GC	-	GGDEF
V.9 (C3)	605384	Deletion	G	-	Receiver domain II
V.18 (C3.1.1)	605384	Deletion	G	-	Receiver domain II
V.28 (C3.2.1)	605384	Deletion	G	-	Receiver domain II

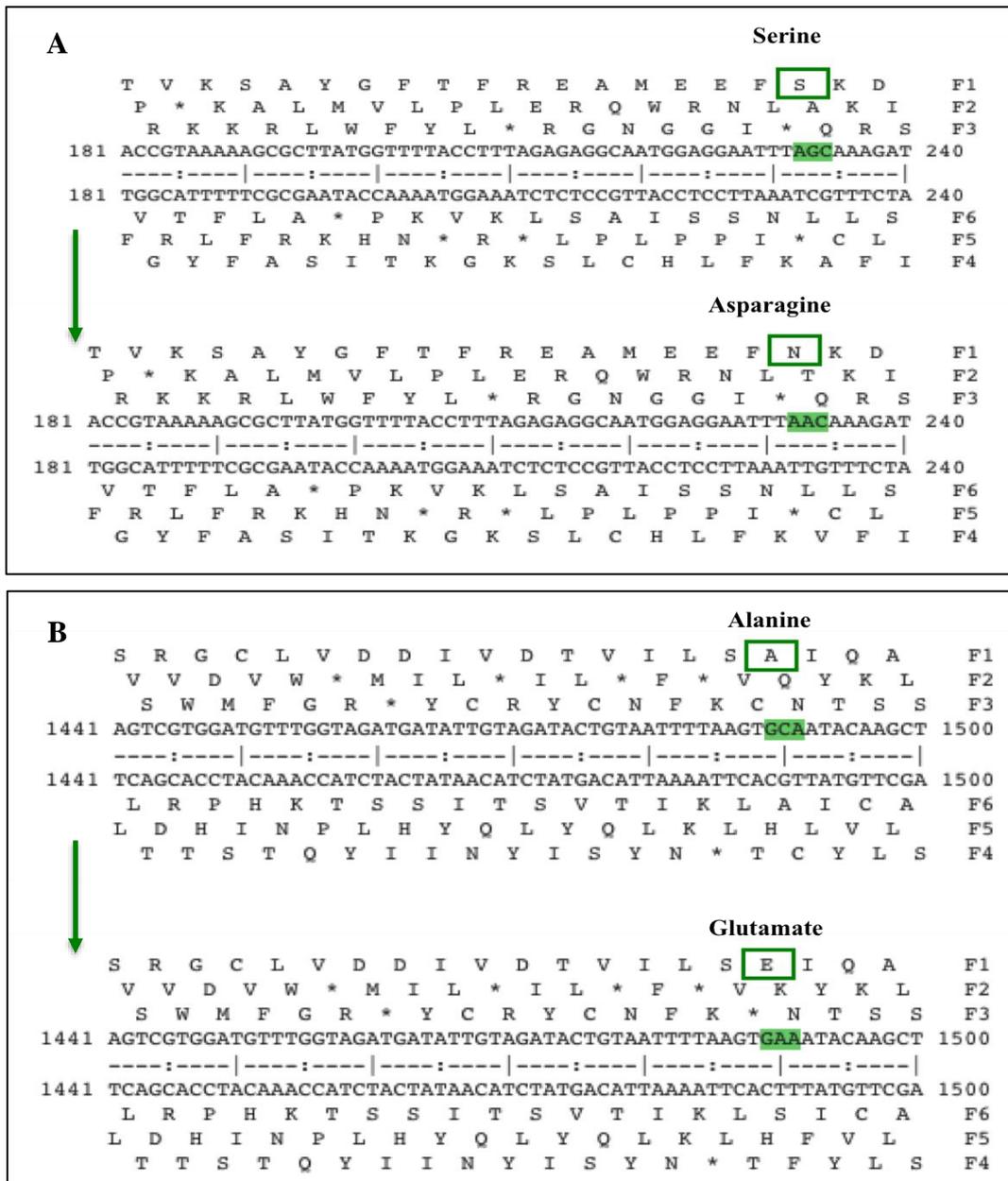


Figure 3.9. Amino acid changes in the amino acid sequences of the *pta* gene as result of substitutions of single nucleotides.

Conversion of a DNA sequence of *pta* gene into an amino acid sequence. The open rectangle indicates amino acid change from Serine to Asparagine (A) in motile and hyper-motile variants and from Alanine to Glutamate (B) in motile variants introduced by the SNP. The highlighted nucleotide represents the location of the SNP. EMBOSS Sixpack tool was used at https://www.ebi.ac.uk/Tools/st/emboss_sixpack/ to display DNA sequences with 6-frame translation and ORFs and identify amino acid change.

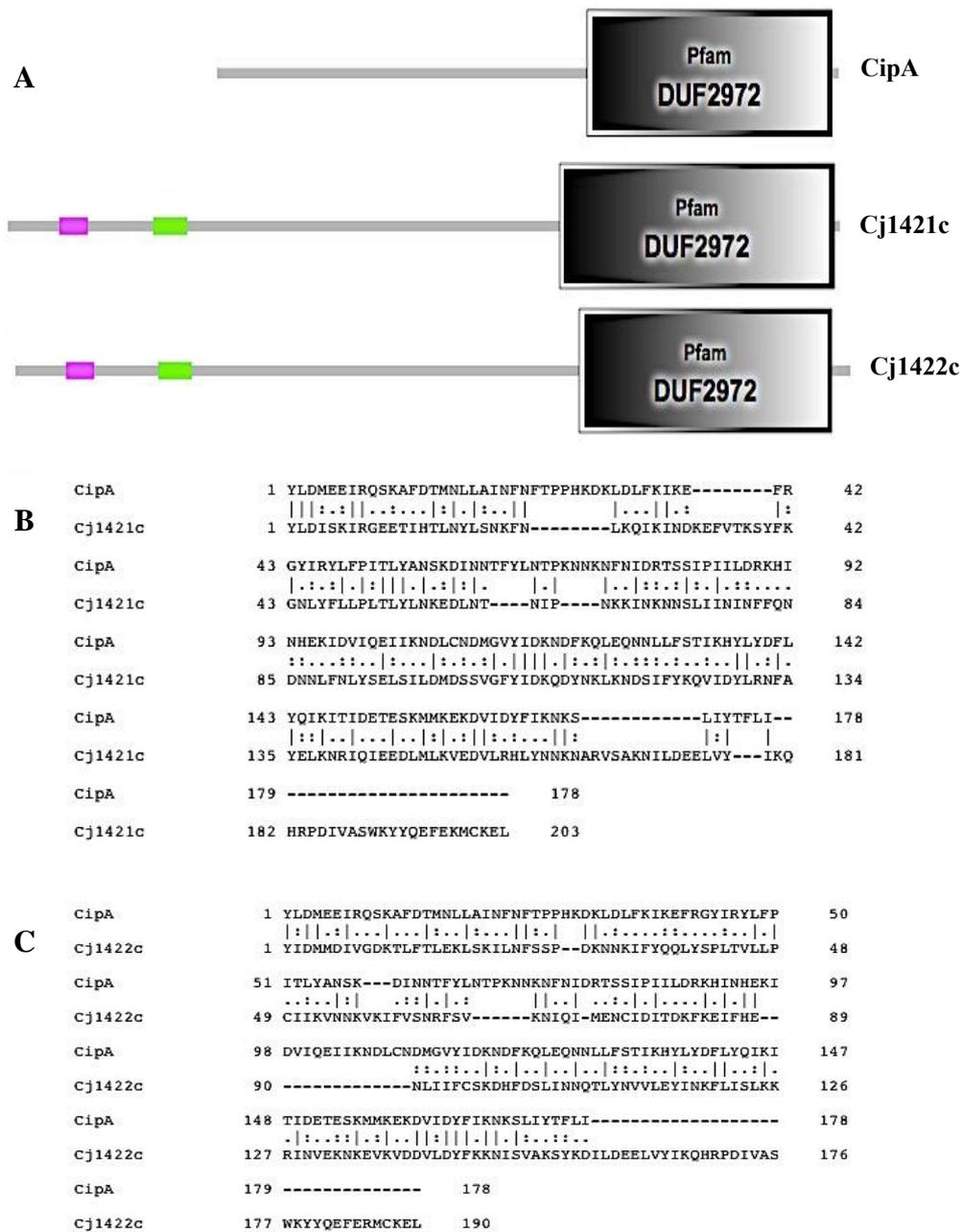


Figure 3.10. The DUF (Domain of Unknown Function) 2972 domain structure of *C. jejuni* 11168 *CipA*, *Cj1421c* and *Cj1422c*.

(A) The DUF 2972 domain structure of the *CipA*, *Cj1421c* and *Cj1422c* proteins was obtained using from the SMART database (<https://smart.embl.de>) and aligned. The alignment of DUF 2972 domain sequence of *CipA* protein with *Cj1421c* (B); and *Cj1422c* (C). Bars, dots, and hyphens indicate identical amino acids and gaps, respectively. Numbers at the right side are the positions on the related protein sequence. EMBOSS Needle from the European Bioinformatics Institute was used to align protein sequences (http://www.ebi.ac.uk/Tools/psa/emboss_needle).

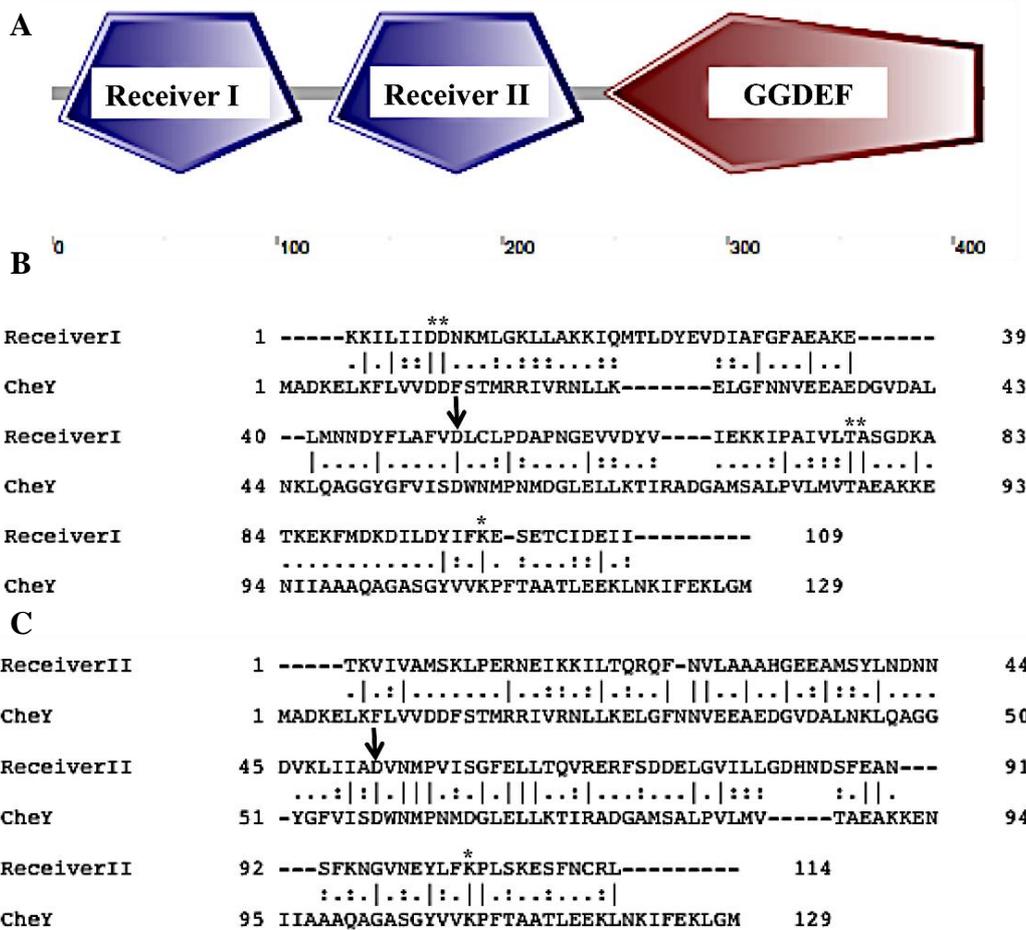


Figure 3.11. Presentation of domains in the CbrR protein.

(A) Domain structures of *C. jejuni* 11168 CbrR protein was predicted using SMART PROTEIN online sequence analysis (<https://smart.embl.de>). Number of amino acids is indicated underneath the protein. Purple = two receiver domains; red = GGDEF. (B) Amino acid sequence alignment of receiver domain I with *E. coli* CheY. (C) Sequence alignment of receiver domain II with *E. coli* CheY. Identical amino acid residues are indicated by vertical bars. Highly conserved residues are indicated by stars. Arrows indicate the location of aspartyl residue (D57), which is required for phosphorylation of response regulators. Alignment was done using EMBOSS Needle pairwise sequence alignment from the European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

3.5. Discussion

During motility experiments with *C. jejuni* 11168Ca, hyper-motility zones were detected on the edges of halo growth at random locations (Figure 3.2). The mechanism leading to production of this phenotype is unknown. Hyper-motility phenotypes have not been well studied in *C. jejuni* but two reports have described hyper-motility as a phenotype in *C. jejuni* (Karlyshev *et al.*, 2002; Du *et al.*, 2016) while a limited number of other reports have described hypermotility in several bacteria including *Proteus mirabilis* (Belas *et al.*, 1998) *Pseudomonas aeruginosa* (Waite *et al.*, 2012) (Waite *et al.*, 2012) and *Salmonella enterica* serotype Typhimurium (Bogomolnaya *et al.*, 2014).

In this chapter, the objective was to determine the genetic basis that leads to generation of hyper-motile derivatives of *C. jejuni* 11168Ca selected *in vitro*. Three single motile colonies derived from *C. jejuni* 11168Ca were used to isolate A2, B2, C2, A3, B3 and C3 hyper motile variants from swarm plates at different time points. PV has been previously implicated in variations in motility and hence the PV states of all the known phase-variable genes was examined. The analysis of the expression states of the 28 phase variable genes in these M and HM did not show a specific pattern of expression in PV genes emerging in hyper-motile variants. This was surprising because many of these genes are involved in modification of the flagella (Guerry *et al.*, 1996; Guerry *et al.*, 2006; Hitchen *et al.*, 2010; Logan *et al.*, 2009; Hitchen *et al.*, 2010). Specific findings regarding these PV genes are discussed in the following paragraphs.

The results of the Gene-Scan analysis show that the expression of *Cj1321*, *Cj1139c*, *Cj0046*, *Cj0275* and *Cj0565* genes were ON in both motile and hypermotile variants. Of these genes, *Cj1321* gene, which is involved in the O-glycan modification of *C. jejuni* flagellin, was in an ON state in 68.42% of M and 68.75% of HM variants (Figure 3.6). This stability of the ON state in both variants (M and HM) indicates the absence of an effect on variations in motility. This finding is in agreement with Howard *et al.* (2009) who has shown that gene-specific disruption of *Cj1321*, *Cj1322*, *Cj1323*, *Cj1324*, or *Cj1325/6* or the removal of the entire locus (*Cj1321* to *Cj1325/6*) did not affect motility.

The expression state of both *Cj1295*, which has been identified as a flagellar glycosyltransferase for attachment of a di-O-methylglyceroyl-modified version of

pseudaminic acid to FlaA protein (Hitchen *et al.*, 2010), and the adjacent PV locus *Cj1296* were in an OFF state in all variants (M and HM). Both ON and OFF expression variants of *Cj1295* of *C. jejuni* NCTC11168, as well as a NCTC11168Δ*Cj1295* mutant were previously shown to be fully motile (Guerry *et al.*, 2006; Hitchen *et al.*, 2010; Baldvinsson *et al.*, 2014), indicating that the OFF state of *Cj1295* is unlikely to be associated with variations in motility and hence to increases in motility.

Cj1318 is another homopolymeric-tract-containing gene (*maf1*) and one of the *maf* family members in *C. jejuni*. In NCTC 11168 strain, *maf1* and *maf4* have 98% amino acid sequence similarity to each other and both genes contain poly G tracts which mediate phase variation (Karlyshev *et al.*, 2002) The expression state of *Cj1318* was OFF in both analysed phenotypes (M and HM), suggesting the absence of a potential association between the *Cj1318* OFF state and the motility phenotypes of 11168Ca. This finding is in agreement with Karlyshev *et al.* (2002) who sequenced variants of the NCTC11168 strain with motility phenotypes ranging from almost non-motile to hyper-motile and found that the *Cj1318* (*maf1*) and *Cj1335* (*maf4*) genes were out of frame in all variants. In contrast, gene-scan analysis of *Cj1335* in the M and HM variants (Figure 3.6) showed that a notable proportion (31.5%) of motile isolates contained an 11G ON variant of *Cj1335* while only 18.75% of hyper-motile variants had this gene in the ON state. However, there was not a statistically significant difference between the motility phenotypes of ON and OFF variants of *Cj1335*. It could be that the expression of *Cj1335* may influence the glycosylation status of flagellin and alters the population behaviour of *C. jejuni*. This finding is in agreement with van Alphen *et al.* (2008) who showed that the putative phase-variable *Cj1335* gene of *C. jejuni* strain 108 was involved in glycan modification of the flagellin leading to the enhancement of bacterial autoagglutination as compared with a *Cj1335*-negative strain. Another study has shown that homologs of *Cj1318* (*maf1*) and *Cj1335* (*maf4*) genes are found in strain 81-176 (*maf2* (*Cj1333*) and *maf3* (*Cj1334*)) and are also involved in modifications of the glycoforms of the flagella but again mutations in these genes were not found to affect the motility (Guerry *et al.*, 2006).

Overall it seems likely that the occurrence of phase variation in genes containing homopolymeric stretches of nucleotides, including genes involved in the flagellin modification, does not correlate with the formation of hyper-motile cells. However, further research would need to be conducted with single colonies derived from M and HM variants by GeneScan assay to be able to determine potential associated of PV genes with a variable motility phenotypes. In addition to consider the combinatorial effects in which the state of one gene can affect the impact of another.

In the absence of an effect of PV, NGS was performed to determine if other types of genetic variation influenced motility. A recent study by de Vries et al (2015) used the NGS approach in order to determine the molecular basis of the variation in motility resulting from the deletion of the flagellin B gene (*flaB*) in *C. jejuni* M1 mutants clones. The analysis of severely attenuated and intermediate motility *flaB* mutant clones revealed second-site mutations a TA-dinucleotide deletion in *fliW* and an A deletion in *flgD*, respectively (de Vries *et al.*, 2015).

The genomic analysis of SNPs and INDELS revealed that two SNPs were identified in the *pta* (*Cj0688*) gene. The first single point mutation of *pta* was found within two M (M-V.3 and M-V.12) and three HM (HM-V.6, HM-V.18 and HM-V.26) variants. The location of this SNP was not in the conserved domain of Pta protein, indicating the absence of a biological role of this protein in *C. jejuni* associated with motility. In the same way, the second SNP of *pta* also occurred within M and HM variants (M-V.1, HM-V.4 and HM-V.13) but in the functional N-terminal domain. However, genotype variations of *pta* were not associated with the variability in motility phenotypes. This finding is supported by a review of the literature that did not find any association between *Cj0688* and motility. Previous studies have shown that the formation of aggregate biofilms, which describes unattached bacteria connected to each other via an extracellular polymeric matrix (EPM), was severely reduced in a mutant with insertional inactivation of the phosphate acetyltransferase (*Cj0688*) (Joshua *et al.*, 2006). Therefore, it could be assumed that this gene may have an inhibitory affect on other genes that have a direct influence on variations in motility.

In addition, there was a SNP located in the DUF2972 domain of *cipA* (cell invasion protein A) within three variants (M-V.1, HM-V.4 and HM-V.13) that could have caused a possible change of CipA protein function and may have led to the variation in motility. Several studies have reported that this gene is involved in invasion of host cells *in vitro* (Javed *et al.*, 2010; Poli *et al.*, 2012; Crofts *et al.*, 2018). A previous study showed that the *cipA* mutant of *C.jejuni* 11168 strain was more motile and better able to autoagglutinate than the wild-type, suggesting that a loss of this gene does not result in reduced motility or autoagglutination (Javed *et al.*, 2010). A recent study has shown a role for this gene *in vivo* via its association with cellular invasion and persistence in humans and then found that the *cipA* gene is involved in modifications of the flagella (Crofts *et al.*, 2018). The importance of the CipA protein appears through switches in expression into the ON state in every relapse infection of human volunteers, despite its being in an OFF state in the inoculum. A possible explanation of the presence of the SNP in the glycosyl transferase domain in CipA may be to affect the modification of the flagella with a consequent affect on motility.

Interestingly, mutations in *cbrR* were identified and only found within hyper-motile variants. Several studies have shown that the ability of *C. jejuni* to sense and respond to the presence of bile is required for chicken colonisation. This gene was shown to provide resistance to sodium deoxycholate; mutants were highly sensitive and the complemented mutant had restored resistance. *cbrR* mutants were also less able to colonise in the chick model (Lin *et al.*, 2003; Raphael *et al.*, 2005). A mutagenesis study has shown that a mutant with a deletion of *cbrR* in *C. jejuni* displayed motility similar to that of the *C. jejuni* F38011 wild-type strain (Raphael *et al.*, 2005). The occurrence of these mutations was distributed through conserved domains (domain receiver I, domain receiver II and GGDEF domain) within HM variants and potentially altered the function of the CbrR protein. It is possible that this genotypic variation of *cbrR* may have increased the motility. A future study could focus on complementation of each SNP to observe if there is an obvious phenotypic difference in motility.

Mixtures of M and HM variants that had the same mutations were observed as outgrowths of 11168Ca strain in these experiments. It is important to bear in mind

the possible bias in classification of motility of this and other studies. The motility phenotypes depends on the phenotype of growth in swarm assays and on the strain or variant used to examine this phenomenon. Starting from 11168Ca, which is considered as a hyper-motile strain, may have biased detection to only hyper-motile cells. Karlyshev et al (2002) used a different 11168 variant and observed that the probability of emergence of spontaneous clones with altered motility had frequencies of approximately 1×10^{-3} , which is suggestive of PV (Karlyshev *et al.*, 2002). This suggests that differences between motility experiments are highly influenced by the starting strain. These differences may explain why other genes that could be responsible for promoting hypermotility were not detected. This includes the mutant strain of *Cj0371*, a virulence-associated gene of *C.jejuni*, which displayed hypermotility, enhanced chemotaxis, and enhanced growth kinetics (Du *et al.*, 2016) and the frequent mutations in the regulators of flagellin expression (i.e. FlgR, FlgS, and MotA (Hendrixson, 2006b; Mohawk *et al.*, 2014). Another source of variation that was not examined was the influence of DNA supercoiling in regulating motility in *C. jejuni* (Shortt *et al.*, 2016). Supercoiling can be influenced by environmental factors and these were not investigated in this study.

In conclusion, the importance of changes in motility of *C. jejuni* serves as an indication of its adaptation capabilities. Therefore, I attempted in this chapter to determine the genetic basis of generation of the hypermotile phenomena in the chicken adapted strain (11168 Ca), which was isolated after serial passage in chickens (Prof Ketley, personal communication). Initially, the association between the phase variable genes that are involved in the decoration of the *C.jejuni* flagella with glycans and the stochastic formation of hypermotility was speculated. Analysis of 28-PV genes in *C. jejuni* 11168Ca strain was performed but did not detect any association between the specific patterns of these genes expressions with hyper-motility. However, as these genes are involved in the modifications of the flagella, it is possible to conclude that the level and diversity of glycosylation may contribute to variations in motility. Also, while the biological role of flagellin glycosylation is unknown, it is possible to hypothesise that these modifications could be utilised by bacteria to adapt to different environments via increasing motility in response to environmental signals. Then, screening the potential mutations occurs in other genes that would influence motility was

achieved via using genome sequence and genotypic and phenotypic characterization. Surprisingly, the one common dominator was the *cbrR* gene, which exhibited allelic variation in only hyper-motility variants. However, experimental work is necessary to confirm that the genotypic variation of *cbrR* is responsible for the observed hypermotility cells.

Chapter 4: Investigation of immune responses of infected chickens against rFlaA and native FlaA protein of *C. jejuni*

4.1. Introduction

C. jejuni is the most common bacterial infections of humans, often a foodborne illness in many developed countries. Infection with this microorganism occurs primarily through the chicken host, which is a natural host for *Campylobacter* species, and the caeca of chicken is colonised with *C.jejuni* present at densities of 10^8 CFU per gram of ceacal contents or higher (Cawthraw *et al.*, 1994; Sahin *et al.*, 2003a; Knudsen *et al.*, 2006; Huang *et al.*, 2010). Therefore, several strategies have been intensively investigated to prevent contamination of human food supplies via reducing abundance and occurrence of *Campylobacter* in poultry flocks (Lin, 2009; Hermans *et al.*, 2011). It is estimated that a 30% reduction in the occurrence of human campylobacteriosis could be resulted from a 2-log reduction of *C. jejuni* on chicken carcasses (Rosenquist *et al.*, 2003).

Several studies have demonstrated that anti-*Campylobacter* antibodies reduce colonisation of *C. jejuni* in the broiler chicken gastrointestinal tract (Sahin *et al.*, 2003; Cawthraw and Newell, 2010; Layton *et al.*, 2011). Therefore, the identification of essential proteins that promote the colonization of chickens, which would then be ideal vaccine candidates if found to be immunogenic, is ongoing to combat *C. jejuni* in poultry (Neal-McKinney *et al.*, 2014). Flagellar proteins have been shown to be required for motility, colonization, and protein secretion (Wassenaar *et al.*, 1991; Grant *et al.*, 1993; Konkel *et al.*, 2004; Guerry, 2007). Hence, these antigens could be exploited as vaccine candidates to reduce the colonisation of chicken by *C. jejuni*. FlaA protein, which is a major subunit of the flagellar filament, is required for chicken colonisation (Myszewski and Stern, 1990; Nachamkin *et al.*, 1993), and considered to be the most immunodominant antigen for *C. jejuni* (Cawthraw *et al.*, 1994; Nachamkin *et al.*, 1994; Lee *et al.*, 1999; Cawthraw *et al.*, 2000; de Zoete *et al.*, 2010; Radomska *et al.*, 2016). Furthermore, chicken antibody studies revealed that flagellin proteins, particularly FlaA protein, were predominantly recognised by maternal antibodies (Sahin *et al.*, 2001; Sahin *et al.*, 2003; Cawthraw and Newell, 2010) and also reacted with

chicken sera containing anti-*Campylobacter* specific antibodies (Cawthraw *et al.*, 1994; Widders *et al.*, 1998; Radomska *et al.*, 2016).

C. jejuni flagellin subunits (FlaA and FlaB) of the flagellar filament are exclusively modified with as many as 19 O-linked glycans that can increase the weight of flagellin by 10% (Thibault *et al.*, 2001). Enzymes that are involved in the biosynthesis of flagellar filament glycans and their glycosyltransferases are encoded by genes that are located adjacent to the flagellin structural genes in one of the more hypervariable regions of the *Campylobacter* genome, the flagellar glycosylation locus (Cj1293 – Cj1342c) (Taboada *et al.*, 2004; Dorrell *et al.*, 2001; Hofreuter *et al.*, 2006; Quiñones *et al.*, 2008) in which these genes synthesise both pseudaminic and legionaminic acids and their derivatives in a phase-variable manner (Karlyshev *et al.*, 2002a). The process of flagellar glycosylation seems to be required for the formation and function of an intact flagellar filament, which is important in colonization, as the mutation in genes involved in the glycosylation pathway of flagellar filament results in non-motile cells (McNally *et al.*, 2006). Moreover, *C. jejuni* utilizes glycoform variations on the flagellin protein to generate antigenic diversity and hence immune evasion for this surface-exposed and immunodominant protein (Szymanski and Wren, 2005). Therefore, it is clear that changes of the glycans decorating the surface of the flagella filament may help *C.jejuni* to evade the chicken immune system.

Despite intensive research into the chicken immune responses to *C.jejuni* over the past few years, there is still a poor understanding of the mechanism by which *C.jejuni* is able to evade the chicken immune system to colonise the gastrointestinal tract. One possibility is that the glycosylation of flagella is a mechanism for the evasion of the immune system. Therefore, it could be that there is a specific anti-*Campylobacter* antibody response against modified flagellin protein. However, no study has yet been performed to compare the immunogenicities of glycosylated and non-glycosylated forms of the flagella protein upon infecting chickens with *C. jejuni*. Consequently, the present study hypothesised that phase variable changes in the glycan structure of the filament (FlaA) of the flagella affects immune recognition. To investigate this hypothesis, western blotting was performed to detect if there are any differences between the reactivities of native glycosylated FlaA protein [from *C. jejuni* whole-cell lysates of two variants (chicken adapted Ca, hyper-motile H)] and recombinant non-

glycosylated FlaA protein with longitudinal sera from infected chickens with either 11168Ca or 11168H (Table 2.1).

4.2. The source of serum samples used to determine the antigenicity of glycosylated FlaA protein

The chicken sera were derived from a study by Bayliss et al. (in preparation) This study used *Campylobacter*-free commercial broilers. Briefly, a total of 60 birds were divided into 5 groups. The first two groups (I and II) were challenged with 1×10^9 colony-forming units (CFU) of the *C. jejuni* Ca (chicken adapted) variant orally at 14 days of age, whereas the second two groups (III and IV) received *C. jejuni* H (hyper-motile) variant also by oral gavage and with the same dose. It should be noted that these two variants are different from each other in terms of their phase state of 28 variable poly-G tracts profile (Bayliss, personal communication). The last group (V) was challenged with sterile phosphate-buffered saline (PBS) (control group). One day (D-1) before challenge, sera (collected by wing bleeds) and fecal swabs were obtained from all birds. At 14 days post-challenge (D+14), six birds from each group of twelve birds were culled, sera were collected by heart-puncture and caecal contents were plated on selective media to detect *C. jejuni*. These birds were B2, B3, B4, B7, B9 and B10-group I, B14, B19, B20, B21, B22 and B24-group II, B25, B26, B27, B30, B33 and B35-group III, B39, B43, B44, B45, B47 and B48-group IV and B49, B52, B54, B57, B59 and B60 from control group. While the remaining six birds from each group were checked by fecal swab and sera obtained by wing bleed. At 28 days post-challenge (D+28), the collection of sera and detection of *C. jejuni* cells were done in the same way as D-1. Finally, at day 52 post-challenge (D+52), all remaining birds were culled, therefore sera were collected by heart-puncture and caecal contents were recovered and plated (Bayliss *et al.*, in preparation). These sera were designated as following D-1 *C. jejuni* sera, D+14 *C. jejuni* sera, D+28 *C. jejuni* sera and D+52 *C. jejuni* sera. Figure 4.1 shows a flowchart displaying the time line and the source of collection sera and *C.jejuni* colonies.

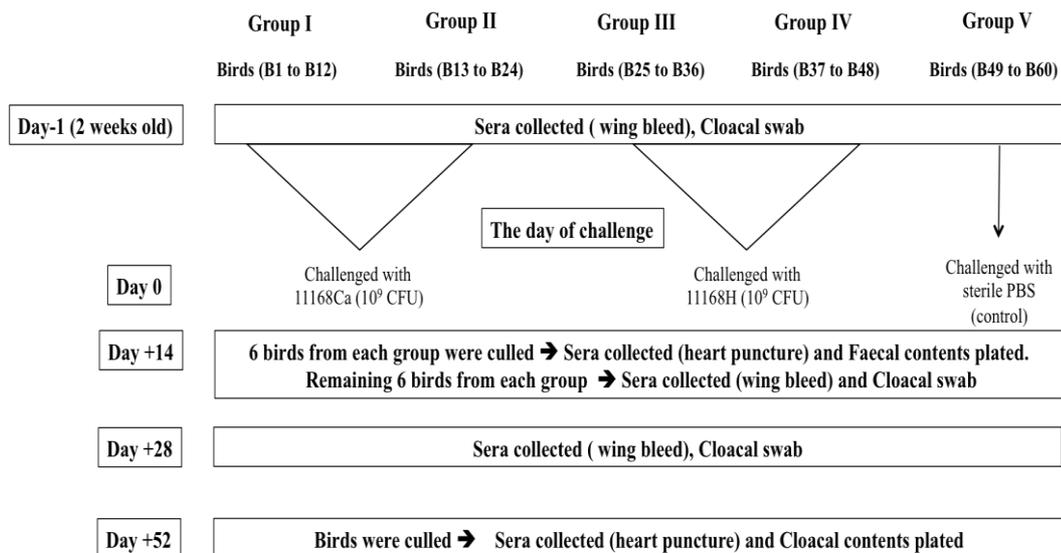


Figure 4.1. Flowchart showing source and selection of sera for assessment of immunogenicity of native glycosylated and recombinant non-glycosylated FlaA.

10^9 CFU of *C. jejuni* strains 11168 Chicken-adapted (Richard Haigh, University of Leicester) and 11168 hypermotile (H; Karlyshev *et al.*, 2002) were used to challenge 4 groups of 2-week old birds by oral gavage. Each group contained 12 birds: Groups I (B1–B12) and II (B13 – B24) were challenged with 11168Ca while Groups III (B25 – B36) and IV (B37 – B48) were challenged with 11168 H. A fifth group (Group V; B49 – B60) remained unchallenged and received sterile PBS buffer only. Sera were collected at four timepoints: days -1 (before challenge), 14, 28 and 52 (post-challenge) by wing bleed or heart puncture. Sera selected for western blot analysis were from: B6 (Ca Group I); B16, B17, B18 and B23 (Ca Group II); B28, B29, B31 and B34 (H Group III); and B40 and B42 (H Group IV).

4.3. Constructing His6-tagged *flaA* expression plasmid

The cloning of the *flaA* gene was done by employing a service at the Protein Expression laboratory (PROTEX) at the Department of Biochemistry, University of Leicester. The *flaA* gene was amplified from genomic DNA of *C. jejuni* NCTC11168Ca using primers (FlaA-F and FlaA-R) (Table 2.3) designed in accordance with guidelines provided by PROTEX in which 12 nucleotides corresponding to the vector sequence at the 5'-end (5'- TACTTCCAATCC -3') and 15 nucleotides at 3'-end (5'- TATCCACCTTTACTG -3') were included in the forward and reverse primers, respectively, so that the *flaA* gene could be inserted into a pLEICS01 expression vector (Table 2.2) The pLEICS01 vector was selected for cloning of the *flaA* gene as it allows for tagging recombinant protein with a 6x-His at the N-terminus.

Initially, a PCR product with the predicted fragment size of 1719bp fragment long was amplified from chromosomal DNA of *C. jejuni* NCTC11168Ca using FlaA-F and FlaA-R primers. After that PCR products were cleaned with a PCR purification kit (section 2.4.1.5) and sequenced using FlaA-F, FlaA-R, FlaA_int-F and FlaA_int-R primers in order to confirm that the fragment sequence was correct (section 2.4.6). Subsequently, the FlaA PCR product was given to PROTEX to construct the *flaA* (pLEICS-01::*flaA*) recombinant plasmid.

The *flaA* allele was cloned into the pLEICS-01 expression vector by Dr. X. Yang (PROTEX, department, University of Leicester) in which this plasmid will fuse the FlaA protein with the His-tag at the N-terminus. Three pLEICS-01::*flaA* constructs were obtained from PROTEX. Insertion of *flaA* into putative pLEICS-01::*flaA* was confirmed by PCR using FlaA-F and FlaA-R primers and again this was confirmed to be error-free by sequencing.

For expression, pLEICS-01::*flaA* was transformed into competent cells of the *E. coli* BL21 expression strain via heat shock (section 2.5.3). The *flaA* gene was amplified from pLEICS-01::*flaA* in *E. coli* BL21 (DE3) transformants by colony PCR using FlaA-F and FlaA-R in comparison to PCR amplified from chromosomal DNA. The PCR product of *flaA* amplified from a pLEICES-01::*flaA* and *flaA* amplified from chromosomal DNA showed the same molecular size (Figure 4.2). The construct was also verified by digestion with the *HindIII*, which showed the presence of 681bp and 216bp *flaA* fragments with the 6435bp

pLEICS-01 fragment (Figure 4.2, lane 5) as predicted by using the Clone Manager software. Also, the appearance of a 970bp fragment arises from the treatment of pLEICS-01::*flaA* construct with *Xba*I, which is located downstream of the T7 promoter (Figure 4.3A), and *Hind*III, which is positioned within the *flaA* gene, indicates that the fragment is in the correct orientation (Figure 4.3, lane 4). In addition, more restriction enzymes were used to confirm the insertion of *flaA* gene into pLEICS-01 vector as shown in Figure 4.3 in which the sizes of resultant fragments were as predicted by Clone Manager software. Finally, the sequence of putative pLEICS-01::*flaA* construct using FlaA-F, FlaA-R, FlaA_int-F, FlaA_int-R, T7 promoter and pLEICS-01-Seq-R primers (Table 2.3) was proved to be correct and was designated pLEICS-01-KH*flaA* after sequence conformation (Figure 4.4).

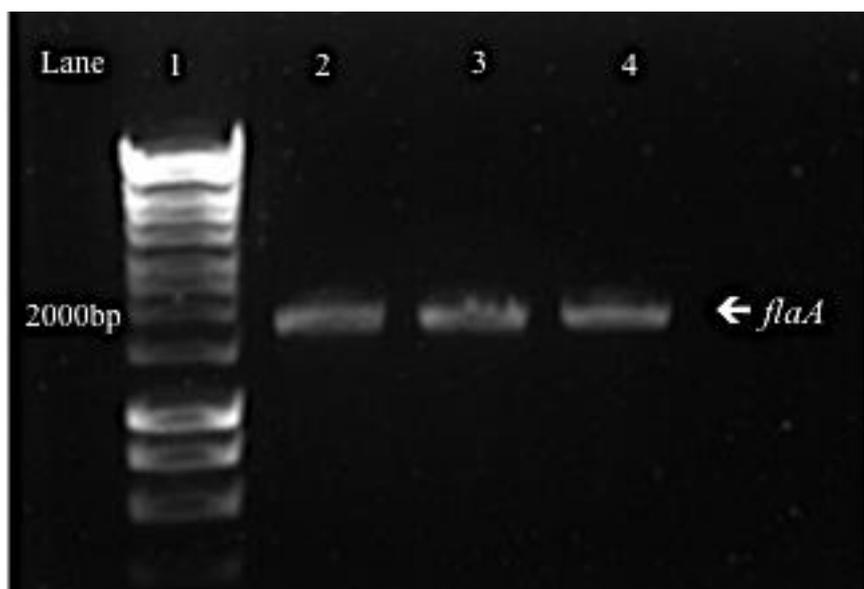


Figure 4.2. Verification of the presence of *flaA* from pLEICS-01::*flaA* constructed plasmid in competent cells of the *E. coli* BL21.

The *flaA* gene was amplified from pLEICS-01::*flaA* plasmid using FlaA-F and FlaA-R primers. Lane 1, hyper-ladder I marker. Lanes 2,3, amplification of *flaA* gene (1719bp) from pLEICS01::*flaA* plasmid extracted from three random selected of *E. coli* BL21 clones; lane 4, *flaA* gene (1719bp) from chromosomal DNA of *C. jejuni* as positive control.

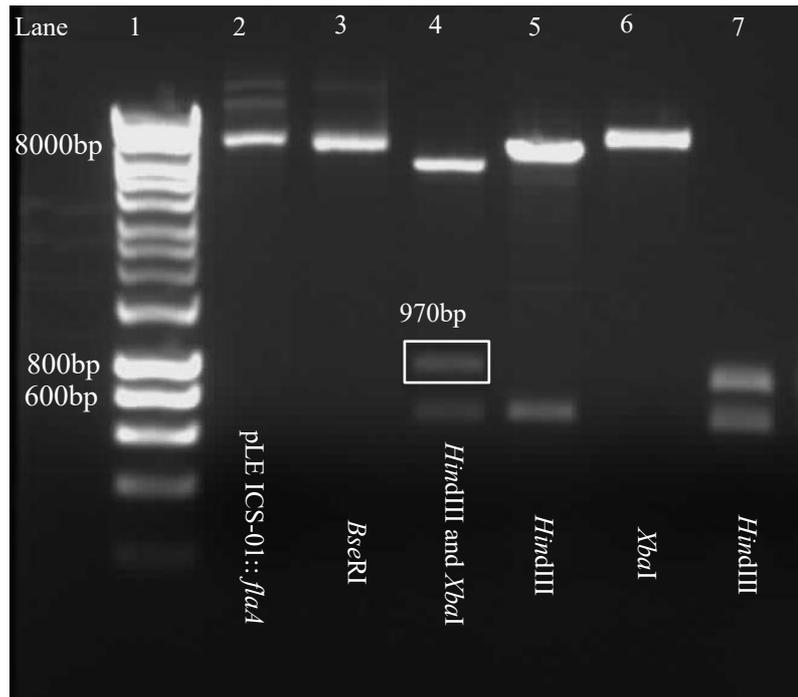


Figure 4.3. Verification of the pLEICS-01-*flaA* by restriction digestion.

The presence of the target gene (*flaA*) in the putative pLEICS-01::*flaA* constructed plasmid was verified by digestion with *Hind*III. Lane 1, Hyper-ladder I marker. Lane 2, uncut pLEICS-01::*flaA* constructed plasmid. Lane 3, pLEICS-01::*flaA* constructed plasmid digested with *Bse*RI, which is located in a multiple cloning site of pLEICS-01, and therefore pLEICS-01::*flaA* has not been cut with this restriction enzyme. Lane 4, three fragments with 5465bp, 970bp, which is confirmed the orientation of the insert, as indicate by open white box and 681 with pLEICS-01::*flaA* constructed plasmid digested with *Hind*III and *Xba*I. Lane 5, the digestion of constructed plasmid with *Hind*III resulted in three fragments with expected sizes of 6435bp, 681bp and 216bp, which had low intensity. Lane 6, recombinant plasmid with *Xba*I. Lane 7, three fragments with 863bp, 640bp and 216bp (low intensity) of digested *flaA* PCR product with *Hind*III.

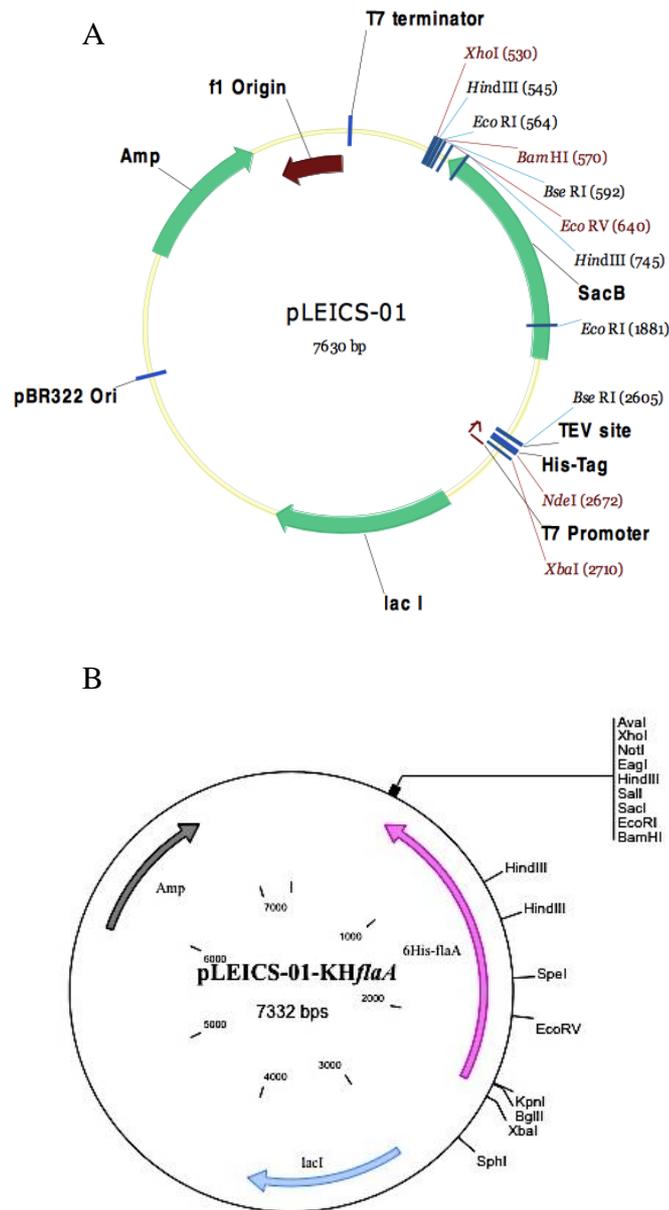


Figure 4.4. Schematic map of the *flaA* gene fused into His-tagged plasmid.

The pLEICS-01-KH*flaA* plasmid (B) was derived from pLEICS-01 plasmid (A) and carries 6xHistidine residues for purifying FlaA fusion protein (pink), *lacI* gene (green, left and blue right) and ampicillin resistance (grey).

4.4. Optimization of His6-tagged *flaA* expression and purification

The purification method and concentration of the inducer were optimised in order to achieve efficient purification and expression of FlaA protein. Expression of pLEICS-01-KH*flaA* was tested using different IPTG concentrations at 30°C and 37°C, as outlined in section 2.6.5. Visualisation of 6His-tagged FlaA protein was achieved using 10% SDS-PAGE gel electrophoresis combined with either staining of gels with Commassie Brilliant Blue stain or probing of Western blots with an anti-HIS-HRP antibody (dilution 1:20000, Fisher Scientific, UK) or D+52 *C. jejuni* serum that were taken after 52 days of colonisation with *C. jejuni* (dilution 1:100, Bird36). The recombinant 6His-tagged FlaA protein has a predicted molecular weight of 59 kDa (kilodalton).

Initially, overnight cultures of *E. coli* BL21 cells were diluted 1/100 with Luria broth and grown shaking at 37°C until the cell concentration reached to 0.5 OD at A₆₀₀. Then cells were induced with 0.5 mM or 0.8 mM concentrations of IPTG and incubated at both temperatures of 30°C or 37°C for 4 hours. Recombinant FlaA protein was produced at low levels, and hence could not be detected in a Western blot with the anti HIS-HRP antisera (data not shown). Due to low level expression, the concentration of IPTG was increased to 1mM and cells were incubated at 30°C or 37°C for 5 hours taken at hourly intervals. In this experiment, the optimum expression was found to be 2 hours post-induction with 1 mM IPTG at 37°C (Figure 4.5A, lane 2, right). For further confirmation and to see if the expressed FlaA protein migrated at its predicted molecular weight, Western blot analysis with anti-HIS-HRP antibody (dilution 1:20000) was performed and found to recognise a protein of the expected molecular weight for the FlaA fusion protein when expressed in induced cells lysates with a 1 mM concentration of IPTG for 3 hours at 30°C as shown in figure 4.5C (left) and for 2 hours at 37 °C (Figure 4.5C, lane 2, right). Another Western blot analysis showed that the D+52 *C. jejuni* serum, from B36, also reacted with the putative FlaA fusion protein (Figure 4.5B). As 6His-FlaA was optimally produced using 1 mM of IPTG at 37°C for 2 hours, this IPTG and temperature condition was chosen for use in subsequent expression of *flaA*.

After the expression conditions for FlaA were optimised, different purification conditions and methods were also tried (data not shown). These experiments established that FlaA purification was optimal with a binding buffer containing 20 mM Tris, 250 mM NaCl, 20 mM imidazole adjusted to pH 7.4 in the presence of 8M urea using affinity chromatography (HiTrap chelating nickel-affinity column) as detailed in section 2.6.5. As seen in figure 4.6B (lane1), one specific purified protein band corresponding to the expected molecular weight of 59kDa for recombinant FlaA was observed. Purified FlaA protein at the predicted molecular weight was confirmed using anti-HIS-HRP antibody (Figure 4.6C). The protein was subsequently subjected to MALDI-TOF mass spectrometry, which also confirmed its identity (performed by The Protein Nucleic Acid Chemistry Laboratory, UK). In addition, the amount and the concentration of purified FlaA protein was enough for generation of anti-FlaA antibodies according to the company recommendations (Eurogentec, Belgium) that to be used in the planned assays.

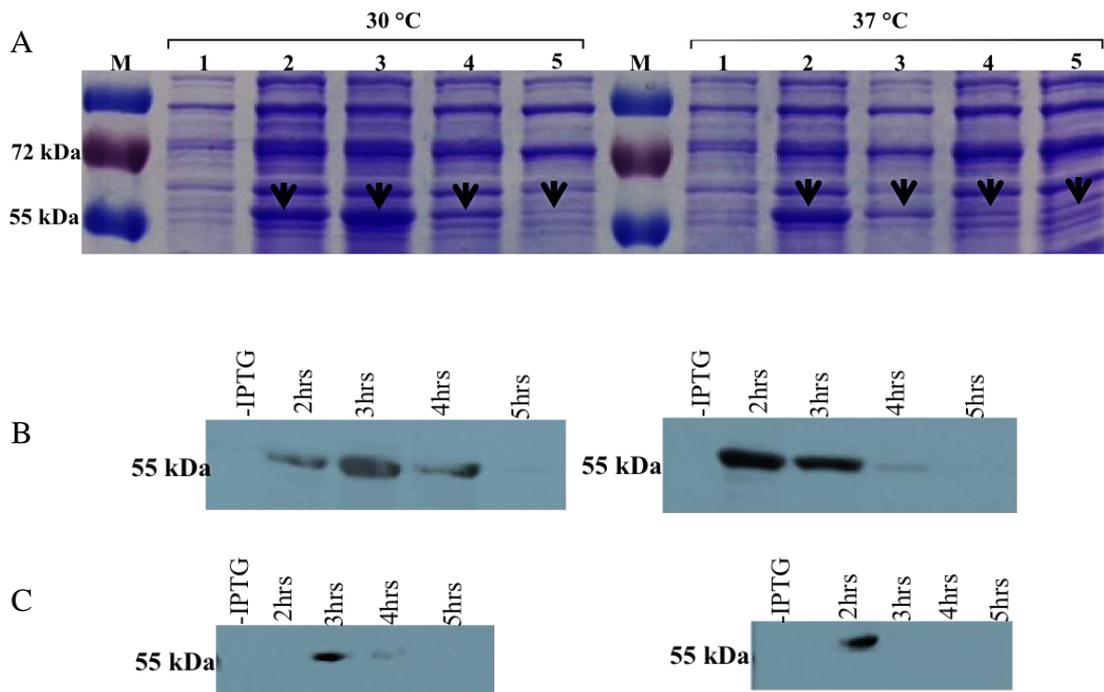


Figure 4.5. Optimising IPTG concentration to produce His tagged.

BL21 (DE3) cells containing expression plasmids were grown mid-log ($OD_{600} \approx 0.5$) before 1mM IPTG was added, mixed with 2x SDS loading buffer (100 μ l) and then boiled. (A) A 10% SDS-PAGE analysis of *C. jejuni* flagellin protein (6His-FlaA) expressed in *E. coli* BL21 (DE3) cells. Lane 2, 3, 4 and 5, induction of expression was performed for 2, 3, 4 and 5 hours respectively at 30°C (left) and 37°C (right) (arrows indicate 6His-FlaA protein). Lane M, Pre-stained protein ladder (10-250 kDa) was used as a marker. Lane1, the un-induced cells lysates containing pLEICS-01-KH*flaA* plasmid from cultures without IPTG. 6His-FlaA protein was visualised by immunoblotting with (B) D+52 *C. jejuni* serum and (C) anti-HIS-HRP antibody. Gel with a corresponding Western blots underneath it shows samples incubated with 1mM at 30°C on the left side and at 37°C on the right side.

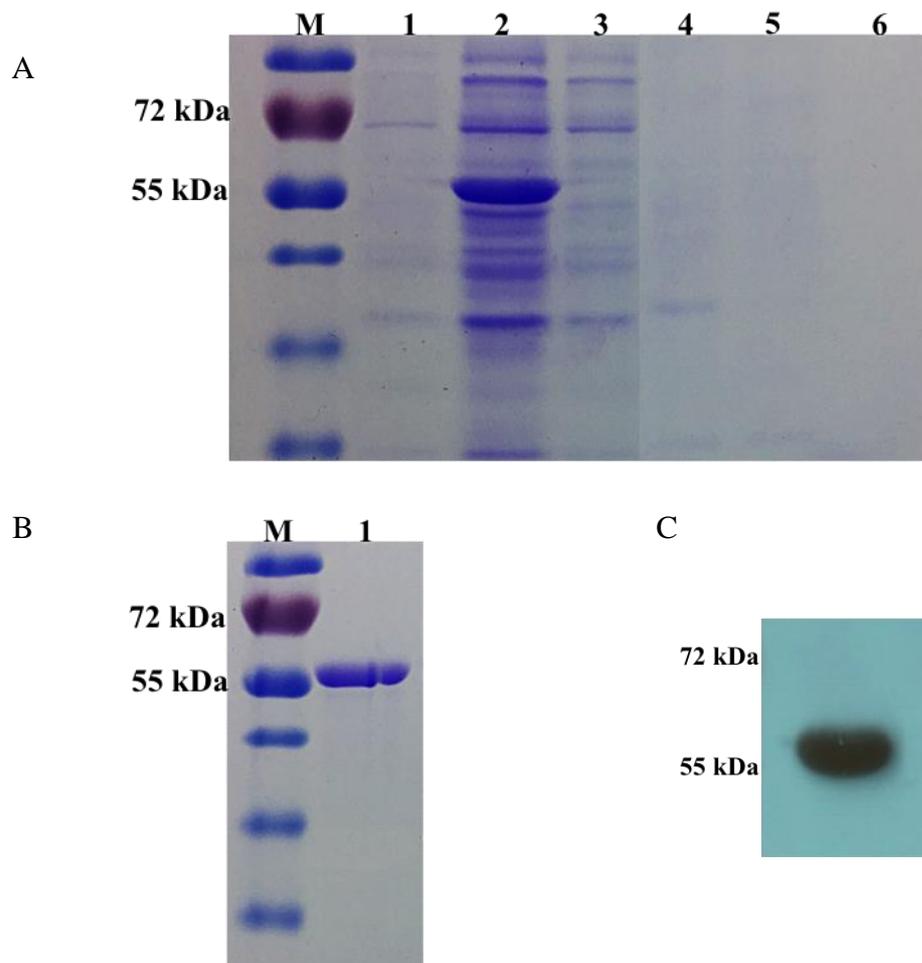


Figure 4.6. Purification of rFlaA using a nickel-affinity column.

(A) *E. coli* lysate before IPTG induction (lane 1). Induced *E. coli* lysate containing expressed rFlaA was loaded onto the column (lane 2). After equilibration of the column with 5x volume of the binding buffer, rFlaA bound to the column while unbound *E. coli* proteins flowed through (lane 3). The column was subsequently the target for several steps of washing with washing buffer containing 50mM Tris, 250 mM NaCl, 20mM imidazole pH=7.4 with decreasing concentration of urea in each step of washing, 2M urea (lane 4), 0.5M (lane 5) and zero urea (lane 6) before bound rFlaA was eluted with 300mM of imidazole (B): 1:2 dilution of re-folded purified recombinant FlaA protein (0.49 mg/ml) (lane1). (C) Purified rFlaA protein probed with anti-HIS-HRP antibody. All samples from each fraction were diluted 1 in 2 with 2x SDS loading buffer and then run on SDS-PAGE gels (10%). These gels were stained with Coomassie stain. Pre-stained protein ladder (10-250 kDa) was used as a marker (lane M).

4.5. Analysis of the immunogenicity of recombinant and native FlaA proteins

4.5.1. Identification of glycosylated FlaA protein

At the time of performing the immunoblotting assays to screen potential development of specific antibodies in bird sera against glycosylated FlaA protein, the generation of anti-FlaA antibodies was in progress. Therefore, in order to confirm that the band detected in this study represents glycosylated FlaA, the reactivity of D+52 *C. jejuni* serum (B36) with a 11168 Δ *flaACa* mutant lacking the FlaA protein by immunoblotting was examined. This mutant strain was obtained through the natural transformation of the genomic DNA from 11168 Δ *flaAH* (Table 2.2) into strain 11168Ca. Thus, whole cell lysates (WCL) of 11168Ca and 11168 Δ *flaACa* strains were probed with D+52 *C. jejuni* serum (Figure 4.7B). No reactivity was detected in 11168 Δ *flaA* strain at the expected molecular mass (65 kDa) (Figure 4.7B, lane 1, arrow) but was observed in the wild type strain 11168Ca (Figure 4.7B, lane 2, arrow). In addition, glycosylated FlaA protein in outer membrane protein (OMP) extracts was excised from the SDS-PAGE gel and subjected to MALDI-TOF mass spectrometry, which confirmed its identity (Figure 4.8).

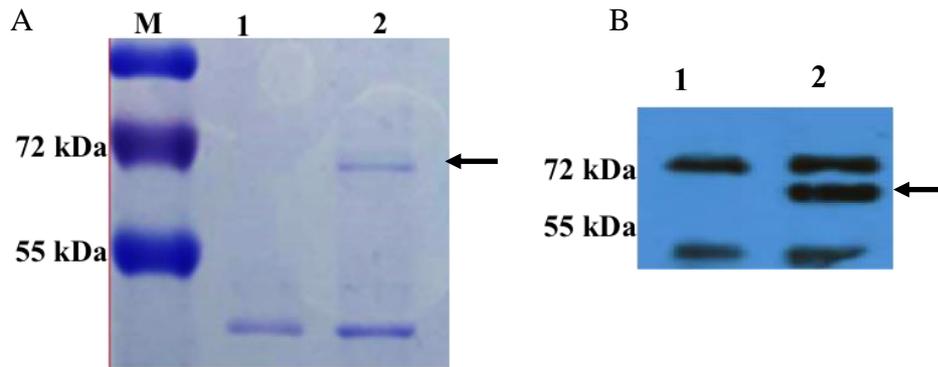
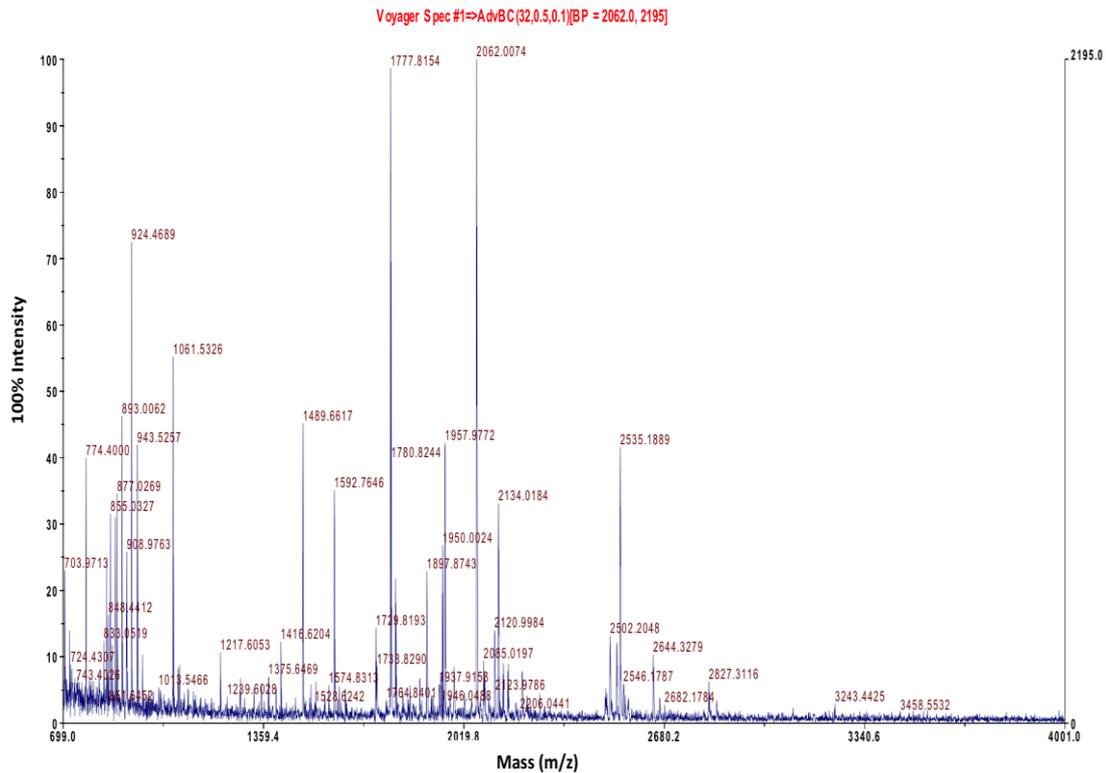


Figure 4.7. The detection of *C. jejuni* glycosylated FlaA protein in SDS-PAGE gel and Western blot.

The same amount of WCL of 11168 Wt strain and 11168Δ*flaACa* strain was loaded into SDS-PAGE (10 μl). (A) SDS-PAGE analysis showing the absence of a FlaA protein band (lane1) from 11168Δ*flaACa* as compared to the FlaA protein detected in the WT strain (lane 2, arrow). (B) Shows the reactivity of D+52 *C. jejuni* serum (B36) to glycosylated FlaA protein from 11168Ca WT in (lane 2, arrow) but no detected signal from 11168Δ*flaACa* (lane1, arrow). Pre-stained protein ladder (10-250 kDa) was used as a marker (lane M).



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1 MHHHHHSSG VDLGTENLYF QSIYFQSMGF RINTNVAALN AKANADLNSK
51 SLDASLSRLS SGLRINSAAD DASGMAIADS LRSQANTLGQ AISNGNDALG
101 ILQTADKAMD EQLKILDTIK TKATQAAQDG QSLKTRTMLQ ADINRLMEEL
151 DNIAANTTSFN GRQLLSGNFI NQEPQIGASS NQTVKATIGA TQSSKIGLTR
201 FETGGRISTS GEVQPTLKNY NGIDDPQFQK VVISTSVGTG LGALADEINK
251 NADKTGVRAT FTVETRGIAA VRAGATSDTF AINGVKIGKV DYKDG DANGA
301 LVAAINSVKD TTGVEASIDA NGQLLLTSRE GRGIKIDGNI GGAFINADM
351 KENYGRLSLV KNDGKDILIS GSNLSSAGFG ATQFISQASV SLRESKQID
401 ANIADAMGFG SANKGVVLGG YSSVSAYMSS AGSGFSSGSG YSVGSGKNYS
451 TGFANAIAIS AASQLSTVYN VSAGSGFSSG SILSQFATMK TFAFGVKDET
501 AGVTILKGAM AVMDIAETAI TNLDQIRADI GSVQNQVTST INNITVTQVN
551 VKAAESQIRD VDFAAESANY SKANILAQSG SYAMAQANSV QQNVLRLLLQ

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Figure 4.8. Peptide mass fingerprinting of glycosylated FlaA protein using trypsin digestion.

The glycosylated FlaA extracted from O.M fraction fragment was concentrated and eluted onto an anchor chip target for analysis on MALDI-ToF mass spectrometry instrument. The MS spectral data were used to search against the protein databases using the MASCOT software. The mass spectra of these peptide fragments with 55% coverage of the sequence were identified as *C. jejuni* FlaA protein. The matched peptides corresponding to the amino acid sequence are indicated red (GenBank accession number AL111168.1).

4.5.2. Reactivity of the D+14 *C. jejuni* sera against the glycosylated and non-glycosylated FlaA proteins.

At day 14 post-challenge, the analysis of the level of caecal colonisation in both groups of birds that were inoculated with either Ca variant or H variant revealed that both groups of birds showed similar low levels of caecal colonization (average number of cfu/g caecal contents was between 1×10^7 and 1×10^8), whereas no caecal colonisation was detected in the control group (Lango-Scholey and Bayliss, personal communication). An immunoblotting assay was performed to determine whether D+14 *C. jejuni* sera from broiler chickens infected with *C. jejuni* (note that these birds are 28 days of age at this time point) contained anti-FlaA antibodies that reacted with glycosylated FlaA protein (gFlaA) in comparison with recombinant FlaA protein (rFlaA). OMP extracts containing the FlaA protein of 11168Ca strain and an *E. coli* lysate containing expressed rFlaA protein were separated by SDS-PAGE, transferred to PVDF membrane, and probed with D+14 *C. jejuni* sera (Table 4.1) at a dilution of 1:100. This dilution was used for all chicken sera as it was found to be the optimal dilution factor for immunoblot analysis to detect the FlaA protein after optimisation with a range of dilutions. The secondary antibody (Rabbit anti-Chicken IgG antibody, HRP conjugate, sigma-Aldrich, UK) was similarly used a constant dilution of 1:5000.

The reactivity of D+14 *C. jejuni* sera from birds 2, 3, 23, 26 and 27 with gFlaA protein (Figure 4.9, lane 2) produced a strong specific signal band at approximately 65 kDa, whereas sera from birds 21, 45, 46 and 57 had a weak signal. A similar reactivity levels were generated with these sera from all the birds with rFlaA protein whose apparent molecular mass was 59 kDa (Figure 4.9, lane 1, arrow). Other protein bands were also observed (Figure 4.9, lane 1) in the Western blots with the whole cell lysates of *E. coli* BL21. The appearance of immunoreactive proteins of *E. coli* is not surprising because *E. coli* is an important member of the microbiota of the chicken gastrointestinal tracts and hence chicken sera may have antibodies against *E. coli* proteins (Yeh *et al.*, 2015).

These results indicated that the serum from 4-wk-old, experimentally *C. jejuni*-infected birds possessed antibodies that reacted specifically against glycosylated FlaA protein from 11168Ca OMP extracts and antibodies that reacted with

recombinant FlaA protein over-expressed in *E. coli* BL21. Interestingly, serum harvested from a control bird (B57) that was not colonised with *C. jejuni* also had reactivity with both glycosylated and non-glycosylated FlaA protein.

Table 4.1. 14 days post-challenge sera used to probe native and recombinant FlaA protein.

Bird	Group	Inoculated strain
B2 and B3	I	11168Ca2
B21 and B23	II	11168Ca1
B26 and B27	III	11168H1
B57	V	PBS

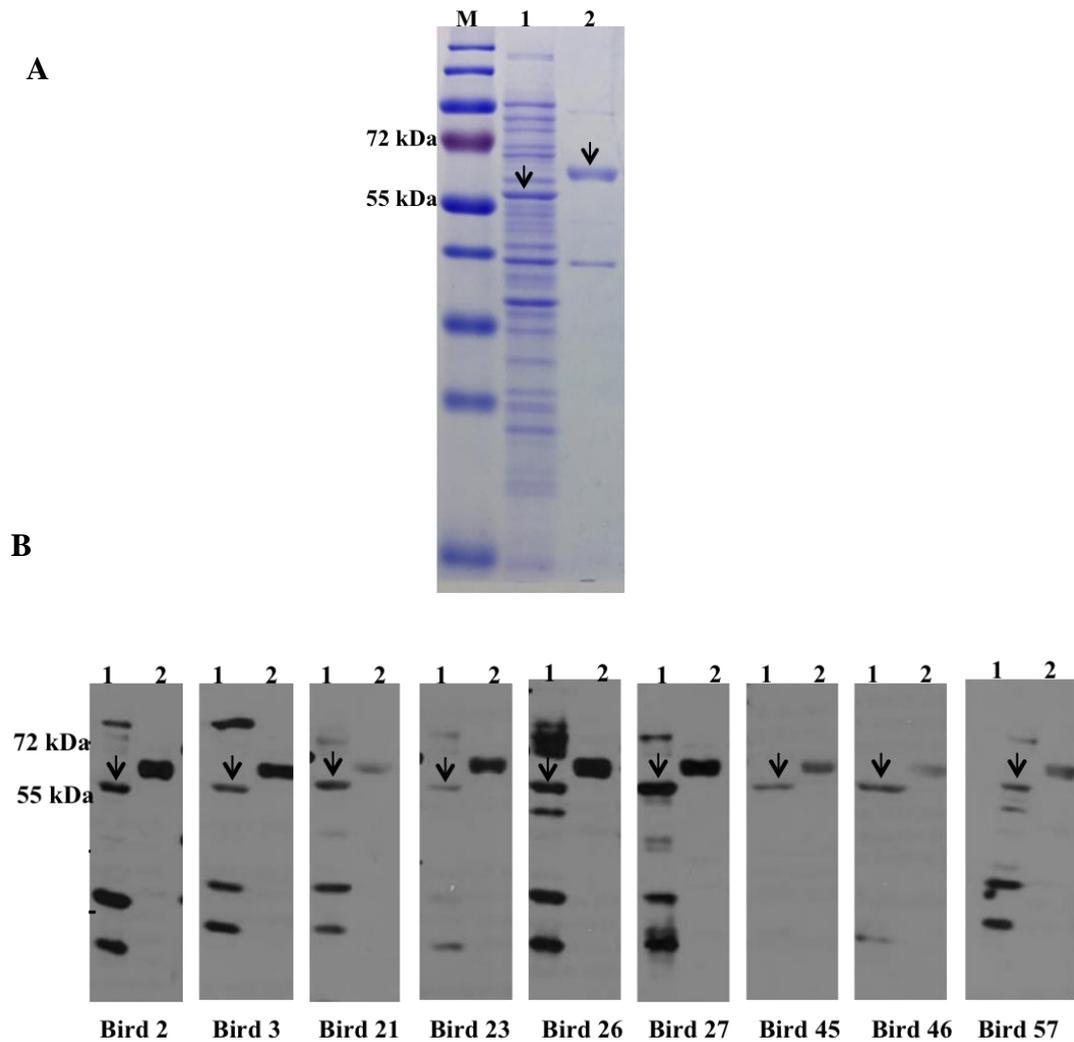


Figure 4.9. Immunoblots of rFlaA protein and native FlaA protein probed with D+14 *C. jejuni* sera.

(A) SDS-PAGE (10%) analysis of both recombinant (59 kDa) and native FlaA protein (65 kDa), lane 1, recombinant FlaA protein expressed in *E. coli* BL21 (indicated by arrow); lane 2, native FlaA protein from outer membrane fraction (OM) of *C.jejuni* (indicated by arrow). Pre-stained protein ladder (10-250 kDa) was used as a marker. (B) Immunoblotting of rFlaA protein lane1 and native FlaA protein lane 2, probed with antisera that were taken from birds after 14 days of challenge with two variants of *C.jejuni* 11168 (Ca and H).

4.5.3. The reactivity of modified and non-modified FlaA protein to chicken immune serum gets stronger over time.

The colonisation of chickens with *C.jejuni* under commercial conditions can occur after 2 to 3 weeks of chicks hatching, whereas day-old chicks can become colonised with *C. jejuni* when experimentally inoculated (Berndtson *et al.*, 1996; Cawthraw *et al.*, 1994; Sahin *et al.*, 2002). The delay in colonisation under commercial conditions has been shown to be due to the presence of maternal antibodies, which remains at high levels for 3 to 4 days after hatching. Then, these maternal antibodies gradually decrease to undetectable levels at 2 to 3 weeks of age (Sahin *et al.*, 2002). Once maternal antibodies have decreased, the intestinal tracts of chickens can be colonised with *C.jejuni* and antibodies against the bacterium are generated (Sahin *et al.*, 2001). The most important *C.jejuni* immunogen has been identified as the FlaA protein (Cawthraw *et al.*, 1994), which is known to be essential in colonisation (Wassenaar *et al.*, 1993). In addition, chicken sera have been shown to react with glycosylated forms of the FlaA or FlaB (Shoaf-Sweeney *et al.*, 2008). Moreover, Shoaf-Sweeney *et al.* (2008), found that the reactivity of sera collected from birds were inoculated with *C. jejuni* S3B with OMPs extracted from homologous (S3B) and heterologous (81-176) strains identified a range of immunoreactive proteins that are essential for chickens colonisation. While the representative banding profiles generated against the *C. jejuni* S3B and 81-176 strains were similar, some bands were unique to a particular strain. It could suggest that the generation of specific antibodies against specific antigens for a given strain lead to specific reduction in the colonisation by the same strain.

Sera samples used in this study were generated from birds infected with two different variants in terms of their expression phase states for the 28 PV genes (H and Ca) but were derived from the same strain (11168). Therefore, it was hypothesised that different forms of glycosylated FlaA can generate different antibodies during the colonization of chicken with *C. jejuni*. To test this hypothesis, forty-four serum samples were used to examine whether there were any differences in the reactivity of these sera against native FlaA proteins from WCL of 11168Ca and 11168H strains and recombinant FlaA protein. These sera

were collected at four time points from birds, which had been inoculated with either a Ca variant or H variant.

The quantification of chemiluminescent signals for these blots was performed by densitometric measurements using the software ImageJ (<http://rsb.info.nih.gov/ij/>) and normalised against the signal intensity of an external loading control that was mixed with the purified rFlaA protein and detected using a specific antibody that was mixed with the secondary antibody. This external control was the glutathione S-transferase protein (GST) that was probed with anti-Glutathione-S-transferase antibody conjugated to horseradish peroxidase (anti GST-HRP antibody, Sigma-Aldrich, UK). Anti GST-HRP antibody at a dilution of 1:5000 was mixed with the secondary antibody (Rabbit anti-Chicken IgG-HRP). As a control, the anti GST-HRP antibody was tested for reactivity with FlaA proteins and did not show any cross-reactivity with rFlaA protein or gFlaA protein from WCL of *C.jejuni* (data not shown).

To determine the reactivity of antibodies contained within bird anti-sera, WCL and rFlaA protein were separated by SDS-PAGE (Figure 4.10) and transferred to PVDF membranes, followed by immunoblot analysis with antiserum. Anti-sera collected from the same bird showed an increasing reactivity with recombinant and glycosylated FlaA protein in the sequential time points in birds inoculated with the H (see birds 28, 29, 40 and 42 in Figure 4.11) and Ca variant (see birds 6 and 16 in Figure 4.11). The D-1 *C. jejuni* sera for some birds showed reactivity with recombinant FlaA protein but not with modified FlaA protein from any of the Ca or H lysates (Figure 4.11, bird 23,28,29 and 40). In addition, the reactivity of some birds anti-sera to rFlaA was found to get stronger over time of colonisation (Figure 4.11, bird 23,29 and 31). Sera collected from some birds challenged with Ca reacted with glycosylated FlaA protein from both variants Ca and H at D+52 but not D+28 (Figure 4.11, bird 18).

These results indicated that the non-modified FlaA protein had a higher reactivity than glycosylated FlaA protein with sera from unchallenged one-day old birds (D-1). Furthermore the reactivity of these infected chicken immune sera to rFlaA gets stronger over time. Interestingly, the reactivity of anti-sera derived from birds infected with either the Ca or H variant against gFlaA protein from *C. jejuni* Ca and H lysates were similar as shown in the histograms (Figure 4.11) supplied

with each serum blot. It could be that there are sera against multiple glycans and some of the glycans have a similar structure in both variants.

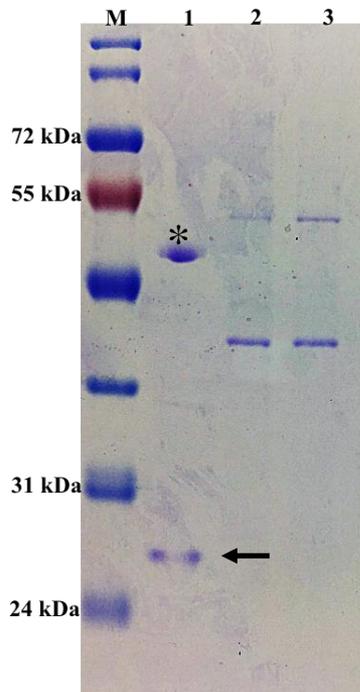
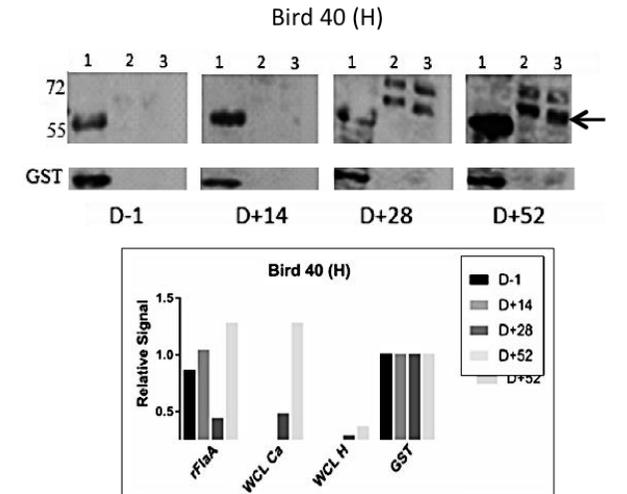
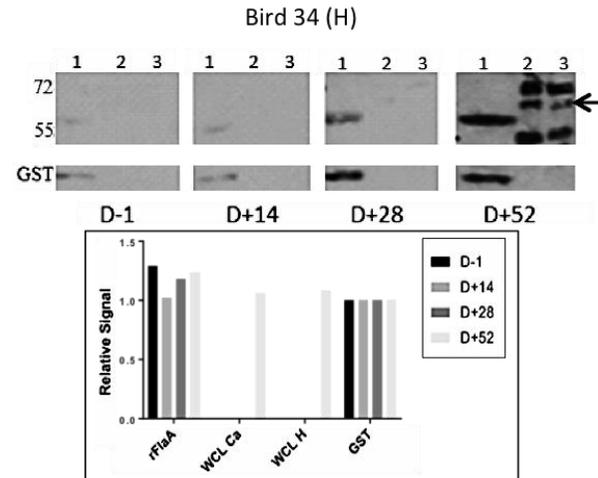
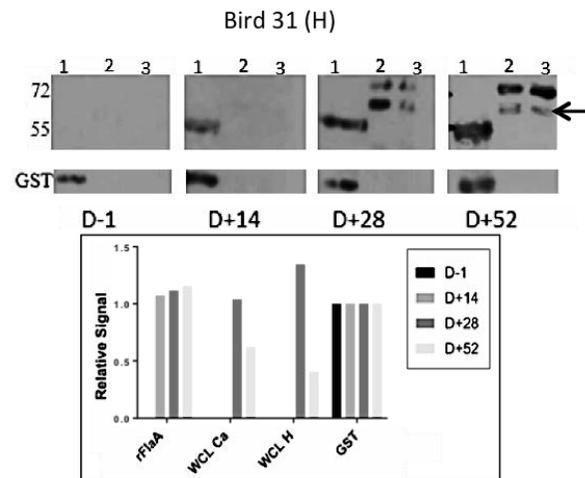
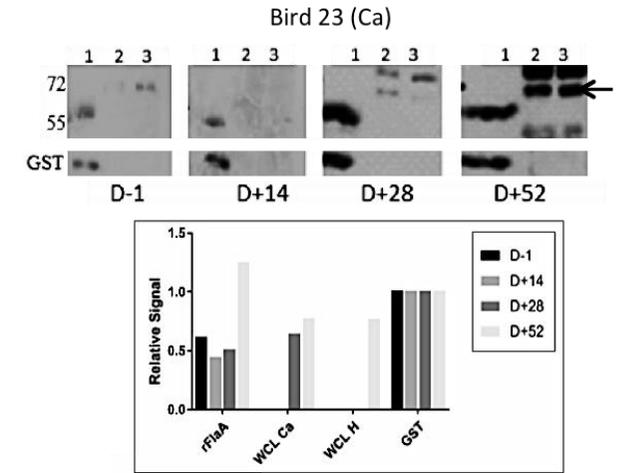
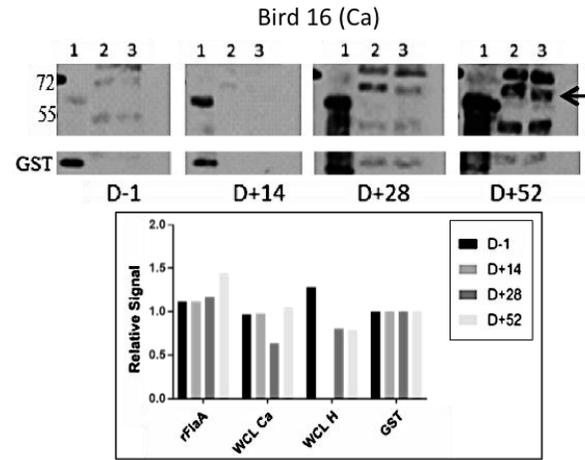
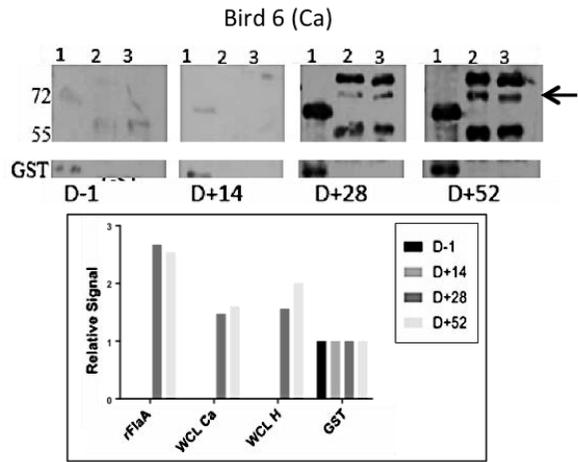


Figure 4.10. Coomassie stained SDS-PAGE gel showing the amount of recombinant protein, GST protein and glycosylated FlaA proteins used in the immunoblotting assay.

5 μ l of purified recombinant FlaA protein as indicated by asterisk (0.49 μ g/ μ l) were mixed with 5 μ l of purified GST protein as indicated by arrow (Sigma-Aldrich, UK) (lane 1); 10 μ l of WC lysate of Ca variant (0.2 μ g/ μ l) (lane 2); 10 μ l of WC lysate of H variant (0.2 μ g/ μ l) (lane 3). All samples were diluted 1 in 2 with 2x SDS loading buffer. Pre-stained protein ladder (10-250 kDa) was used as a marker (lane M).



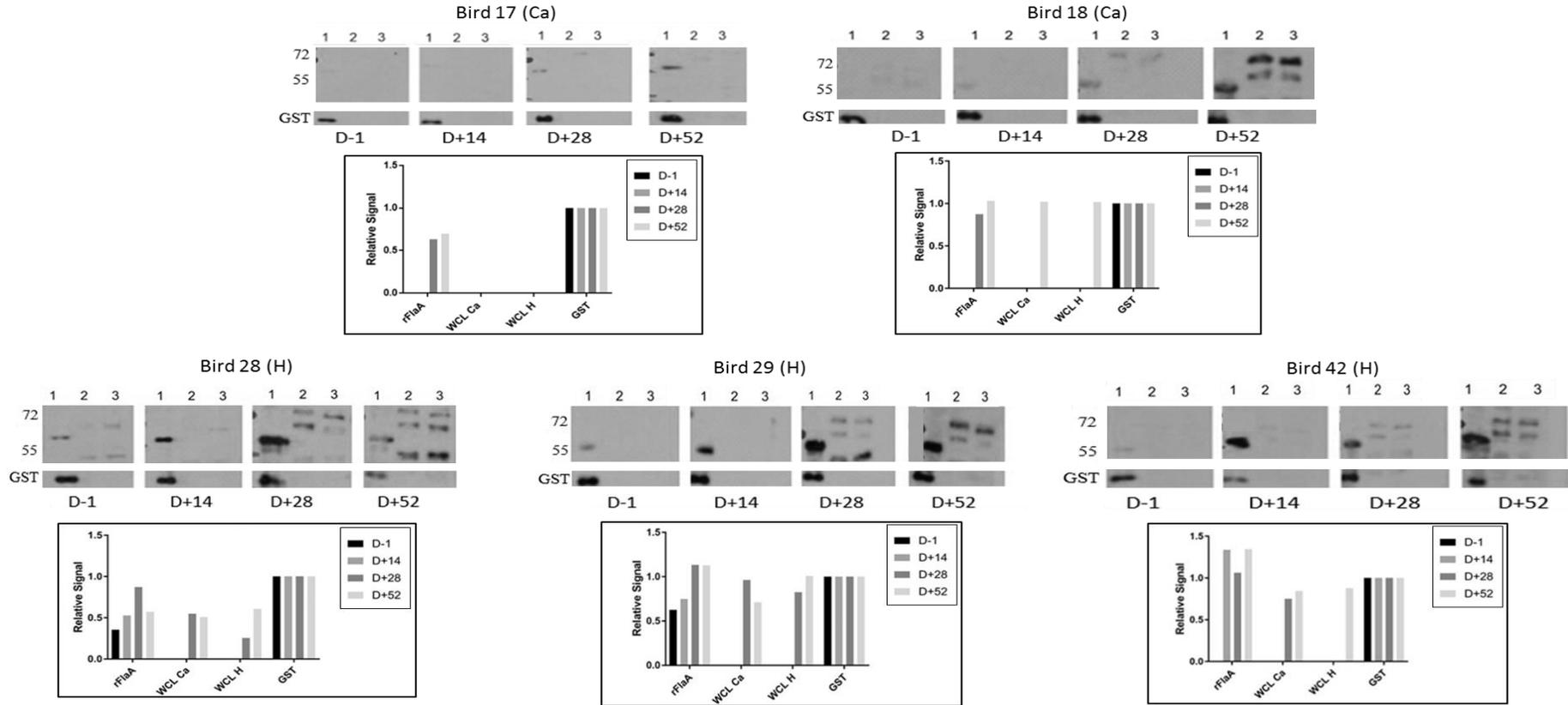


Figure 4.11. Immune response of challenge birds to native and recombinant FlaA protein of *C. jejuni*.

Western blot analysis performed to compare the reactivity of non-glycosylated FlaA protein (rFlaA, lane 1) and glycosylated FlaA protein from Ca (lane 2) and H (lane 3) variants. The intensity of each protein band detected by Western blot was normalised to the intensity of GST (external control) mixed with purified recombinant FlaA protein then probed with anti-GST antibody conjugated to horseradish peroxidase 1:5000 dilution mixed with 1:5000 dilution of anti-rabbit-HRP antibody (secondary antibody). Quantification of chemiluminescent signals was performed by densitometric measurements using the software ImageJ (<http://rsb.info.nih.gov/ij/>).

4.6. Discussion

O-glycosylated flagellin (FlaA) is an important adhesin in *C.jejuni*, initiating colonisation of the gastrointestinal tract, and is considered an immunodominant antigen recognised by the immune system of the host (Fernando *et al.*, 2008). O-linked glycans are synthesised by biosynthetic enzymes, which, through transfer by glycosyltransferases, decorate the bacterial surface. Genes encoding these enzymes are located adjacent to the flagellin structural genes (*flaA* and *flaB*) in a hypervariable region of the *Campylobacter* genome (Ewing *et al.*, 2009). Many of these genes are phase variable genes that are associated with the variability in the modification of flagellin proteins. The variability in these genes and therefore, the FlaA protein, allows this organism to rapidly generate diversity during an infection with this diversity potentially contributing to evasion of the host immune response. Several studies have identified many different *Campylobacter* immunoreactive proteins, which are recognised by maternal IgYs (Sahin *et al.*, 2003b; Shoaf-Sweeney *et al.*, 2008). These antigens include chemotaxis proteins and a few periplasmic proteins, however most antibodies are directed against surface-exposed or membrane-associated components (Yeh *et al.*, 2015; Yeh *et al.*, 2014; Shoaf-Sweeney *et al.*, 2008). More precisely, they recognise in particular the filament proteins FlaA, FlaB and the hook protein FlgE, or other surface components such as the major outer membrane protein (MOMP), LPS, CadF and PEB (Shoaf-Sweeney *et al.*, 2008; Hermans *et al.*, 2014). It is therefore likely that the different forms of the glycans on the modified FlaA protein as generated by phase variation can help *C.jejuni* to colonise the intestinal tract of chickens.

In this part of the thesis, the reactivity of the recombinant FlaA protein and glycosylated FlaA protein was analysed using sera collected from birds challenged with two variants of 11168 *C.jejuni* Ca (Chicken adapted) and H (hyper-motile). Initially, the *flaA* gene was obtained via amplification from the *C.jejuni* 11168 genome, cloned into an expression vector (pLEICS-01, PROTEX) incorporated with 6His-tag at N-terminus followed by overexpression using the construct in *E.coli* BL21 cells. Finally, FlaA protein was successfully purified under denaturing conditions using a nickel-affinity column. The purified recombinant FlaA protein was used to test the reactivity of infected chicken anti-

sera to amino acid epitopes of this protein. For comparison, the glycosylated FlaA was obtained as part of whole cell lysates or a purified outer membrane fraction. The identity of gFlaA and rFlaA proteins in these sources was confirmed by excision of bands from SDS-PAGE gel and then analysis by MALDI-TOF mass spectrometry. A 10% increase in molecular weight of gFlaA compared to rFlaA was observed, an indication of the glycosylation of gFlaA in which rFlaA and gFlaA proteins had an apparent molecular mass of 59.5 kDa and 65.5 kDa on SDS-PAGE gels, respectively.

Despite the recognition of the susceptibility of day-of-hatch chicks to *Campylobacter* challenge in the presence of maternal antibodies (MABs) (Wassenaar et al., 1993; Cawthraw et al., 1996), it is likely that experimentally challenging birds at age of 14 days old with *C. jejuni* is sufficient to allow for development of specific antibodies (IgA and IgM) against *C. jejuni* as the decreasing the levels of MABs do not prevent establishment of the colonisation of intestinal tract with *C.jejuni* (Cawthraw et al., 1994; Widders et al., 1998). Sahin et al. (2003) showed that the presence of high levels of MAB in young chickens did not interfere with the development of a humoral immune response to *Campylobacter* colonisation (Sahin et al., 2003). A key finding was that there were temporal differences between elicitation of antibodies against the glycosylated and non-glycosylated forms of the FlaA protein. There was a reactivity of recombinant FlaA with sera from unchallenged one-day old birds *C. jejuni* (D-1) while no antibodies were detected against glycosylated FlaA. This could be due to antigenic variation of FlaA whereby the variant exposed to the maternal immune system is different from that which is present in the 11168 Ca or 11168 H. The maternal antibodies induced in response to this unrecognised variant may, therefore, lack sufficient cross-reactivity preventing binding to the 11168 Ca or H FlaA variants. It is also plausible that antibodies in D-1 chicks of maternal origin targeting the glycosylated epitopes of FlaA may decay at a faster rate than those targeting conserved, non-glycosylated epitopes leading to the significant difference in reactivity to gFlaA between D-1 and D+28 or D+52 sera. The level of colonisation at day 14-post challenge had not affected by generation of high level of specific flagellin antibodies that reacted strongly with gFlaA (O.M) and rFlaA proteins. In this study, the influence of antibodies in reducing colonization was estimated using different profiles of PV genes, indicating

generating antibodies but without reduction in colonisation. This result is consistent with findings of a recently published report by Lacharme-Lora et al. (2017) showed that both groups of birds control and bursectomised birds were infected with *C. jejuni* at 21 days of age had high levels of cecal colonisation at 14 and 28 days post infection, despite the presence of antibodies in serum. This study of functional immunity to *C. jejuni* in birds show that serum antibodies are not effective in clearing *C. jejuni* from the caeca within the first nine weeks of a bird's lifetime. Therefore, in order to confirm this study observation, functional assays such as the Serum Bactericidal Activity (SBA) assay, which is used to evaluate the complement-dependent bactericidal activity of antibodies in sera against bacterial isolates (Necchi *et al.*, 2017), could be performed to evaluate the functional attributes of the anti-FlaA antibodies in D+14 *C. jejuni* sera. An enzyme-linked immunosorbent assay (ELISA) could also be performed in order to measure the titres of *C. jejuni*-specific immunoglobulins (IgY, IgA and IgM) at the time of 14-day post-challenge and hence to determine the predominant immunoglobulin. Due to the limited amount of sera available, development of a more complex micro titre assay may be required as opposed to the ELISA as performed for the evaluation of antibody induction in meningococcal carriers where limited volumes of sera were available (Alamro *et al.*, 2014).

Differences were also seen for the D+14 sera, with higher reactivity to rFlaA than gFlaA in 70% of challenged birds. However D+28 and D+52 sera showed similar or higher reactivity for gFlaA. A possible explanation for this is that in 28 day old birds the humoral immune response has not yet fully developed. Therefore, these sera can better recognise conserved domains of non-modified FlaA protein than the glycans of modified domains of FlaA. These results are consistent with those of Cawthraw and Newell (2010) who indicated that the level of *anti-Campylobacter spp.* serum IgY, IgA, and IgM increase steadily two to three weeks after experimental inoculation, with mucosal IgA being noticeably elevated three to two weeks after infection. It is possible, therefore, that these sera contain antibodies which recognised the unmodified protein but are blocked from binding to modified protein due to the presence of O-linked carbohydrate residues (Schmidt *et al.*, 2003; van Alphen *et al.*, 2008). Thus, we hypothesise that *Campylobacter* utilises glycosylated modification as a mechanism to evade

the immune response of the host by blocking binding of antibodies generated against the protein backbone.

Regarding the higher reactivity of D+14 *C. jejuni* sera with gFlaA as detected in OM fractions as compared to WCL, this was due to the much higher abundance of FlaA protein in OM fractions than WCL. The reason for using the WCL in testing sera collected from one bird in four different time points was to identify all the antigens that reacted with sera by immunoblot analysis and to determine if FlaA reactivity was dominant. The Western blots showed that more than one reactive bands was observed however in general there was only two or three dominant reactive bands. These dominant bands were shown to be FlaA/B proteins and MOMP as detected by SDS-PAGE and MALDI-TOF mass spectrometry (Figure 9.10. lane 2 and 3). The other proteins could not be identified possibly because the amounts of these proteins are very low and were not able to be detected. Other researchers have identified other proteins, which were not found here possibly because they are not expressed in standard *in vitro* cultures (Handfield *et al.*, 2000; Harris *et al.*, 2006; Alam *et al.*, 2013). Similarly, Sahin *et al.* (2003) and Shoaf-Sweeny *et al.* (2008) have used whole cell lysate of *C. jejuni* to identify antigens that reacted to chicken maternal antibodies. While chemotactic proteins were not detected in their studies, Yeh *et al.* (2014) have identified them in their preparation of WCL of *C. jejuni*.

Taken together, D-1, D+14, D+28 and D+52 *C. jejuni* sera may include specific anti FlaA antibodies that reacted to both recombinant and native FlaA proteins. D-1 and D+14 *C. jejuni* sera may contain MABs; that reacted to the recombinant FlaA protein, whereas D+14 *C. jejuni* sera reacted to both rFlaA and gFlaA proteins. The reactivity of D+14 *C. jejuni* sera to gFlaA in OM suggests the possibility of developing specific anti FlaA antibodies against modified FlaA over time as a result of adaptive immune responses. In addition, D+52 *C. jejuni* sera contain a mature antibody response as shown by the strong reactivity between the sera of this time point and all types of FlaA proteins, however, no difference in reactivity was detected between gFlaA proteins from the Ca and H variants suggestive of reactivity to multiple glycans. Finally, the reason of the variation in reactivity for each serum with gFlaA and rFlaA proteins may be due to differences in immune responses among broiler chickens.

Chapter 5: Analysis of immunology, motility and aggregation for the flagellin glycosylation mutants

5.1. Introduction and preliminary data

Many genes in the O-linked glycosylation locus of *C. jejuni* strain NCTC 11168 are predicted to encode proteins involved in flagellin glycosylation (Szymanski and Wren, 2005). Glycosylation of flagellin protein is essential for flagellar filament formation, whereas unglycosylated flagellin protein accumulates intracellularly and is not exported from the cell (Goon *et al.*, 2003). Glycosylation is also thought to provide diversity to surface-exposed proteins and help with immune evasion during persistent colonization of chickens, however there is no experimental evidence to support this theory. Consequently, in this chapter, *C. jejuni* D+52 sera were used to detect the possibility of these sera containing antibodies specific for the different forms of the glycans that decorate the FlaA protein. Sera were utilised from birds challenged with two different variants of the same strain and which differ regarding the profiles of the expression states of their phase variable genes in order to further enhance the potential for detecting differing antibody reactivities. *C. jejuni* D+52 sera were selected for this investigation due to their high reactivity with rFlaA and gFlaA, indicating the development of high levels of antibodies by that time point post-challenge (8 weeks old) (section 4.5.3).

The selection of phase-variable flagellar glycosylation genes was based on data obtained from the analysis of PV expression states for PV genes located in flagellar glycosylation locus (Table 1.1) for colonies derived from birds persistently-infected with the two *C. jejuni* NCTC11168 variants Ca and H (Bayliss *et al.*, In preparation). Briefly, the inocula and colonies isolated at different time points post-challenge (section 4.2) were screened to identify the PV states using the 28-locus-CJ11168 PV-analysis assay. As shown in Figure 5.1, there was an increase in the proportion of Ca colonies with *Cj1310c* in the ON state from 38% in the inoculum to 100% at day 52 post-challenge and while H colonies there was a rise from 22% in the inoculum to 97% in the day 52 post-challenge (d+52). A similar trend was observed in the expression of *Cj1296* within all colonies of both inocula being in the OFF state and then switching into the ON state at d+52 for the majority of colonies of both variants. Contrastingly, the

majority of the colonies in both inocula contained *Cj1295*-ON variants and this ON state persisted into the last time point (d+52) for both Ca and H colonies. These observations led to selection of these genes for investigation of their influence on the immunogenicity of the FlaA protein. Of these gene, *Cj1295* has been identified as new glycan FlaA variant, while reminders still unknown function (Hitchen *et al.*, 2010).

The *Cj1310c* gene encodes a hypothetical protein of unknown function. This gene belongs to the 617 family, which is represented by *Cj0617* and *Cj0618* but also includes four genes in the glycosylation locus (*Cj1305*, *Cj1306c*, *Cj1310c* and *Cj1342c*) that contain intragenic single nucleotide repeats indicative of phase-variable gene expression. No homologues of this family are known in other organisms (Szymanski and Wren, 2005). So far, these genes have not been characterised in mutational studies and the functions of their encoded proteins are unknown. However, these genes have no role in the biosynthesis of pseudaminic acid (Pse5Ac7Ac) in strain 81-176 (McNally *et al.*, 2006). Thus, it would be interesting to assess whether *Cj1310c* plays a role in the motility of *C. jejuni* as well as to investigate if there is an influence of the deletion of *Cj1310c* on the autoagglutination of *C. jejuni*.

Two non-phase-variable genes, *maf2* and *maf5*, present in the flagellar glycosylation locus are known to be involved in transferring different glycans into the central domain of the flagellin protein (Merino and Tomás, 2014). The product of *maf5* is involved in transferring Pse5Ac7Ac into flagellin and is required for production of a functional flagella as shown by *maf5* mutant being non-motile (Karlinsky *et al.*, 2000). The *maf2* gene product contributes to the attachment of Pse5Am7Ac into Ser or Thr residues in the flagellin protein and a *maf2* mutant strain was found to be partially motile (Butler, 2009). These mutants provide for flagellin proteins with differing glycosylation profiles for comparison to the flagellin proteins extracted for PV mutant strains ($\Delta 1295$ and $\Delta Cj1310c$).

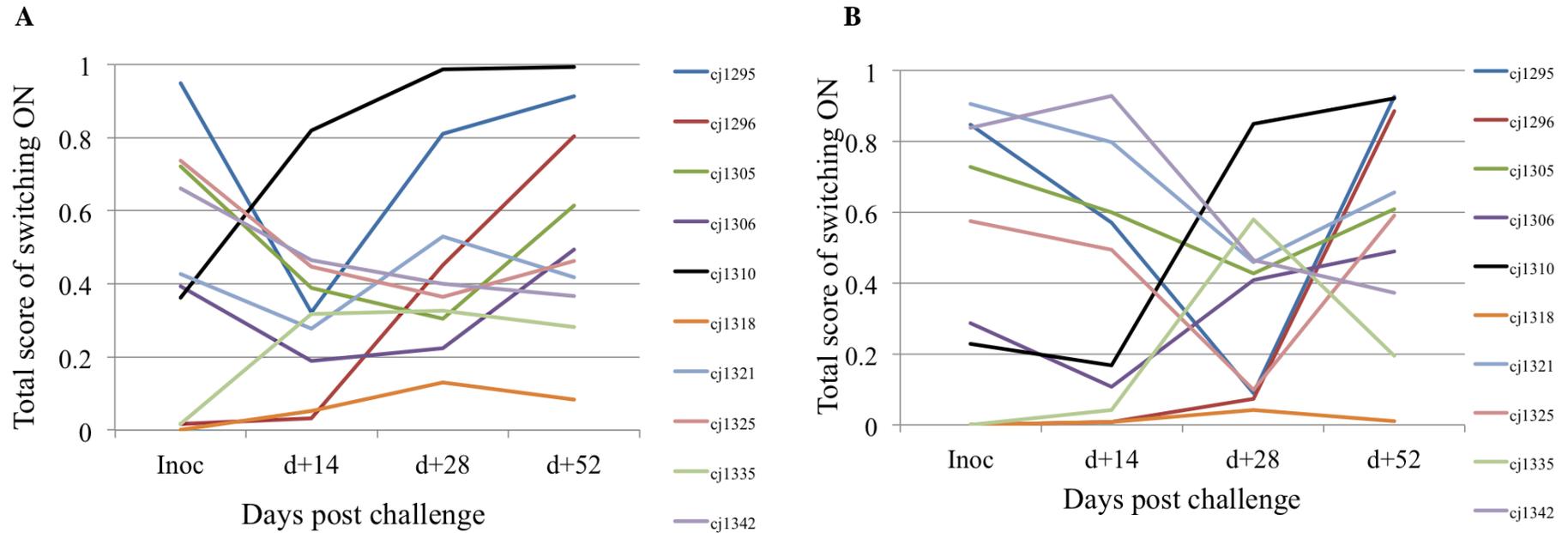


Figure 5.1. Phase states of the phase variable genes located in flagellar glycosylation locus of two-week old commercial birds challenged with two variants of *C. jejuni* (Ca and H).

Colonies were picked from serial dilutions (grown on MHB plates) of caecal or fecal swabs from multiple birds and analysed using the 11168-28-locus-PV-analysis assay (Bayliss et al., in preparation). This figure shows the proportion of colonies in the ON state (1=ON and 0=OFF) for the 10 phase variable genes located in the flagellar glycosylation locus (key indicated on the right of figure) for the inoculum and output colonies for either the Ca (A) or H (B) variants. The total numbers of analysed colonies are as follows:- inoculum, Ca; n=117 and H; n=118; output population after 14 days, Ca; n=481 and H; n=655; 28 days, Ca; n=214 and H; n=283; and 52 days, Ca; n=296 and H; n=358 (Bayliss et al., In preparation).

5.2. Verification of *C. jejuni* mutant strains.

C. jejuni genes mutated in specific glycosylation genes were used in order to investigate the induction of antibodies against specific FlaA glycosylation-protein variants and to determine the role of *Cj1310* in motility and autoagglutination of *C. jejuni*. Isogenic mutants (*maf2::kan^R*, *maf5::kan^R*, *Cj1295::kan^R*, *Cj1295::Cj0223* (*cat*-fixed ON) and Δ *flaA::kan^R*) in NCTC 11168 were a kind gift of Dr. Dennis Linton (University of Manchester) (Table 2.2). Three of these mutants (Δ *maf2*, Δ *maf* and Δ *Cj1295*) were firstly verified by plating in MHA-TV supplemented with kanamycin (50 µg/ml), then by PCR using the kanamycin F-kan and R-kan primers (Table 2.3) so that discrimination between the mutants containing the kanamycin cassette (with a resulting 795 bp amplicon) and the wild-type gene (no amplicon) could be achieved. Further confirmation of successful gene deletion was achieved by sequencing with STMinvKanF primer that is orientated so as to generate a sequence for the region adjacent to the kanamycin gene (Table 2.3). *Cj1295Comp* (*Cj1295::Cj0223*(*cat* –fixed ON)) strain was plated on MHA-TV supplemented with kanamycin (50 µg/ml) and chloramphenicol (20 µg/ml) and sequenced with the CATinvR primer. The *C. jejuni* 11168 Δ *flaA* strain was verified by plating out on MHA-TV supplemented with kanamycin (50 µg/ml) and also by analysis in a motility assay, which confirmed the loss of motility expected for this strain as a result of the deletion of the *flaA* gene.

Since the *C. jejuni* 11168Ca variant was used as a wild-type strain in the phenotypic assays and for construction mutants in this thesis, the Δ *flaA* gene was successfully transferred into *C. jejuni* 11168Ca by natural transformation (section 2.5.5) using a genome preparation from the *C. jejuni* 11168 Δ *flaA* Manchester strain.

5.3. Mutagenesis of *Cj1295/Cj1296*

5.3.1. Gene splicing by an overlap extension (SOE) PCR cloning strategy to generate a double mutant strain of *Cj1295* and *Cj1296* genes

A double mutant *C. jejuni* 11168Ca strain, defective in both *Cj1295* and *Cj1296*,

was attempted using the splicing by overlap extension (SOE) method. This method involves fusing DNA fragments without using restriction enzymes or T4 DNA ligase. The strategy is illustrated in Figure 5.2 and was designed to delete the entire open reading frame of both genes.

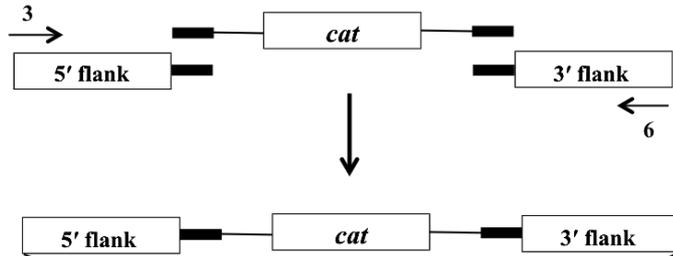
Initially, a promoterless chloramphenicol acetyl-transferase (*cat*) cassette (658bp) was amplified by PCR from the plasmid pAV35 (Table 2.2) by using Cat-pro-SOE-F and Cat-SOE-R primers (Table 2.3). In parallel, 707bp 5' flanking region (kno1295up_F and Ko1295/96SOE-R3 primers) and 756bp 3' flanking region (Ko1295/96SOE-F3 and kno1296do_R primers) were amplified from 11168Ca. Two of the PCR primers - Ko1295/96SOE-R3 (reverse primer 5' flank) and Ko1295/96SOE-F3 (forward primer 3' flank) - contain extensions that are complementary to the primers used for amplification of the *cat* cassette and allow for incorporation of the *cat* cassette in between the 5' and 3' flanking regions. The three PCR products were mixed and used as a template in a second round of amplification with primers kno1295up_F (forward primer 5' flank) and kno1296do_R (reverse primer 3' flank) primers, which flank the original gene sequences (section 2.4.1.3). The sizes of the three separate PCR products and the overlap PCR product were determined on a 1% agarose gel and were all of the predicted size (Figure 5.3). The presence and orientation of the *cat* cassette in the PCR overlap fragment was confirmed by sequencing with CATinvR and kno1296do_R (section 2.4.6 and Table 2.3). After this, the 2,121 bp fusion PCR product was A-tailed with *Taq* DNA polymerase and ligated into a linearized pGEM-T Easy vector that had thymine residue overhangs (section 2.4.5 and Table 2.2). Ligation reactions were transformed into chemically competent *E. coli* DH5 α cells (section 2.5.1) and grown on LA plates supplemented with ampicillin (100 μ g/ml), chloramphenicol (50 μ g/ml) and X-gal (40 μ g/ml) for 24 hours at 37°C. Positive white colonies were screened, as the insert would have disrupted the *lacZ* gene on the pGEM-T Easy vector. Plasmid DNA was extracted from white colonies and digested with EcoRI as this enzyme will excise the ~2 kb fusion PCR deletion fragment from the 3 kb pGEM-T vector (Figure 5.4). The recombinant plasmid was verified by DNA sequencing (CATinvF, CATinvF, pUCF and pUCR primers; Table 2.3) and subsequently designated as p Δ Cj1295/Cj1296 plasmid.

The pC Δ Cj1295/Cj1296 plasmid was transferred by electroporation into *C.jejuni* 11168Ca followed by plating on MHA-TV plates supplemented with different concentrations of chloramphenicol (5 μ g/ml, 10 μ g/ml, 15 μ g/ml and 20 μ g/ml). The pAV35 plasmid was used as a positive control to confirm that the transformation procedure was working properly. Unfortunately, no colonies were recovered from any plates supplemented with these concentrations. There are several possible explanations for this result; i) double crossover events might disrupt essential genes either upstream of Cj1295 or downstream of Cj1296; ii) the possibility of a polar effect on Cj1295 and Cj1296 flanking genes; or iii) it might be that Cj1296 is essential for growth and metabolism and hence cannot be inactivated. This latter assumption cannot be considered for Cj1295 because Hitchen et al (2010) have already created a knockout mutant of this gene, NCTC11168 Δ Cj1295.

1- Three separated PCR reactions



2- Fusion PCR reactions



3- Construction of disruption plasmid

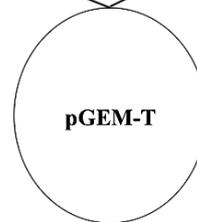


Figure 5.2. Schematic representation of overlap extension PCR and construction of disruption plasmid.

Step 1, the chloramphenicol resistance cassette was amplified using primers 1 and 2. In parallel, upstream flanking (5' flank) and downstream flanking (3') regions of the desired genes were amplified with primers 3 and 4 (primer 4 overlaps with primer 1; bolded region) and primers 5 and 6 (primer 5 overlaps with primer 2; bolded region), respectively. Note that target gene flanking region primers (4; 5' flank and 5; 3' flank) have 5'-tails that are complementary to the *cat* amplification primers (1 and 2). Step 2, the three amplified fragments were assembled into one fragment in the fusion PCR. Step 3, fusion PCR product of 2121 bp (5' flank – *cat* – 3' flank) was cloned into pGEM-T Easy Vector.

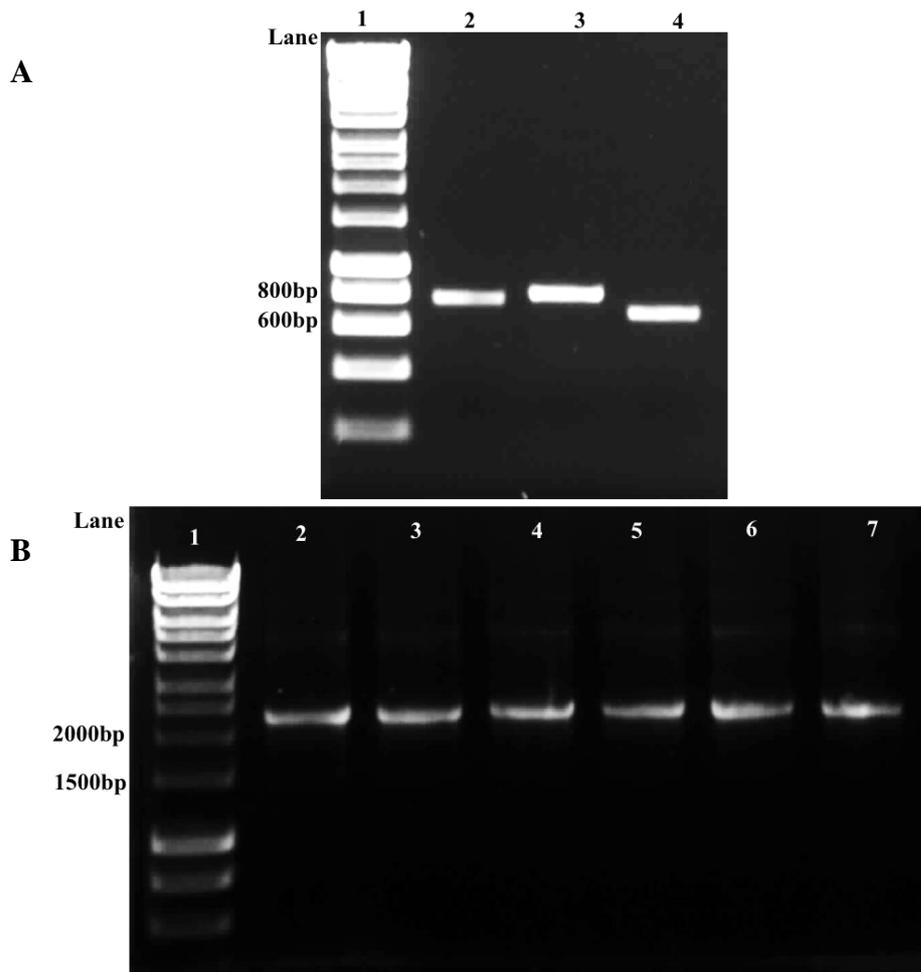


Figure 5.3. Analysis of SOE-PCR products on 1% agarose gels.

Expected sizes of PCR products are shown in brackets. (A) Lane 1, Hyper-ladder I DNA marker with fragment sizes in bp showed on left; lane 2, PCR product of 5' flanking region of target genes (707bp); lane 3, PCR product of 3' flanking region of target genes (756bp); lane 4, PCR product of chloramphenicol cassette (658bp). (B) Lane 1, Hyper-ladder I DNA marker with fragment sizes in bp showed on left. Lanes 2-7, PCR products from fusion PCR (2,121bp).

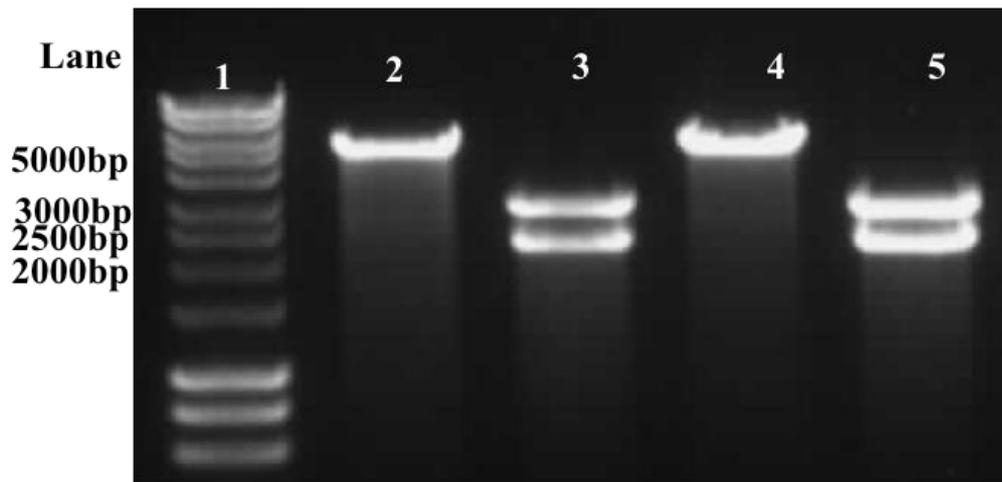


Figure 5.4. Restriction enzyme verification of positive clones by *EcoRI*.

Two clones of the pGEM Δ *Cj1295::cat* plasmid were analysed. Lane 1, Hyper-ladder I DNA marker with fragment sizes in bp showed on left. Lanes 2 and 4, undigested recombinant plasmid extracted from clone 1 and 2, respectively. Lanes 3 and 5, *EcoRI*-digested recombinant plasmid extracted from clone 1 and 2, respectively. 1% agarose gel.

5.4. Mutagenesis of *Cj1310c*

5.4.1. Construction of Δ *Cj1310c* deletion mutant

The *C. jejuni* NCTC 11168 *Cj1310* gene encodes a hypothetical protein of 404 aa (44.4 kDa) that lacks both a signal peptide sequence and transmembrane region as predicted by The TOPCONS web server <http://topcons.cbr.su.se/pred/>. This hypothetical protein belongs to Protein of unknown function DUF2920 as shown in the analysis of its amino acid using Interproscan server www.ebi.ac.uk/interpro/. So far this gene has not been characterised in mutational studies and the function of its encoded protein is unknown. The data obtained from gene scan analysis indicated that *Cj1310* was expressed within the majority of recovered colonies from both groups of birds inoculated with either Ca or H variants at 52 day post challenge (Figure 5.1). Consequently, I speculated that there was a potential that specific antibodies had been generated against either the product or an epitope modified by this gene product. In order to create a deletion in *Cj1310c*, the left and right flanking regions of *Cj1310c* were amplified using 11168 chromosomal DNA as template. These flanking regions were cloned independently and sequentially into pKM46 complementation

plasmid containing a kanamycin cassette. Figure 5.5 illustrates the strategy that was utilised to delete *Cj1310c* gene in 11168Ca. Firstly, the left flanking region of *Cj1310c* gene was cloned into pKM46 plasmid. A 754bp PCR product of left flanking region (*Cj1309c*) was amplified by PCR from 11168Ca genomic DNA with KO1310_LFlankF and KO1310_LFlankR primers containing 5'*Bss*HI and 5'*Sac*II restriction site, respectively (Table 2.3) as shown in Figure 5.6A. These purified PCR products were digested with *Bss*HI and *Sac*II, purified, and ligated to the 3,075bp *Bss*HI/*Sac*II digested-fragment of pKM46 (section 2.4.2) to produce the pKMLFlank*Cj1310* plasmid (Table 2.2). The ligation reaction was purified (section 2.4.2.1) and then transformed by heat shock into *E. coli* DH5 α E cells (section 2.5.3 and Table 2.2). Transformants were plated out onto selective media supplemented with kanamycin (Table 2.1). Positive transformants were screened by colony PCR (section 2.4.1.4) by using KO1310_LFlankF and KO1310_LFlankR primers (Figure 5.7A). Plasmids with the correct insert, and insert orientation were verified by DNA sequencing using comp1310R2, STMinvKanF and KO1310_LFlankF (section 2.4.6 and Table 2.3). After verification of the presence of left flanking in KMLFlank*Cj1310* plasmid, this plasmid was used to clone right flanking region of *Cj1310c* gene. A 799bp PCR product of right flanking region was amplified by PCR from 11168Ca genomic DNA using KO1310_RFlankF2 and KO1310_RFlankR2 primers containing 5'*Xba*I and 5'*Nde*I site, respectively (Table 2.3) (Figure 5.6B). Then, both PCR products and KMLFlank*Cj1310* plasmid were digested with *Xba*I and *Nde*I restriction enzymes (section 2.4.2). Purified digested PCR products were ligated to recovered digested *Xba*I/*Nde*I fragments from gel with size of 2849bp. All ligation reactions were transformed into *E. coli* strain by heat shock. Positive transformants were selected by plating on LB agar supplemented with kanamycin 50 μ g ml⁻¹. Plasmids with insert were verified by PCR as shown in Figure 5.7B. The final construct was verified by sequencing and designated as pKM Δ *Cj1310c* plasmid (section 2.4.6).

Finally, the pKM Δ *Cj1310c* construct was electro-transformed into competent *C. jejuni* 11168Ca strain (section 2.5.2 and section 2.5.4). The pKM Δ *Cj1310c* construct is a suicide vector and a double crossover recombination occurs between regions of homologous DNA in the construct and the *C. jejuni* 11168 chromosome. Allelic exchange leads to the replacement of the wild-type copy of

the *Cj1310c* allele with the deleted inactivated mutant allele. Consequently, final *C. jejuni* Δ *Cj1310c* mutant was kanamycin resistant (Table 2.2). The location of the kanamycin cassette was screened in positive transformants by using Cj1310-out and Cj1310-out primers that anneal outside of the cloned regions as shown in Figure 5.8 (Table 2.3). The orientation of the kanamycin cassette in *C. jejuni* Δ *Cj1310* strain was confirmed by DNA sequencing using STMinvKanF, KO1310_LFlankR and STMinvKanR (section 2.4.6)

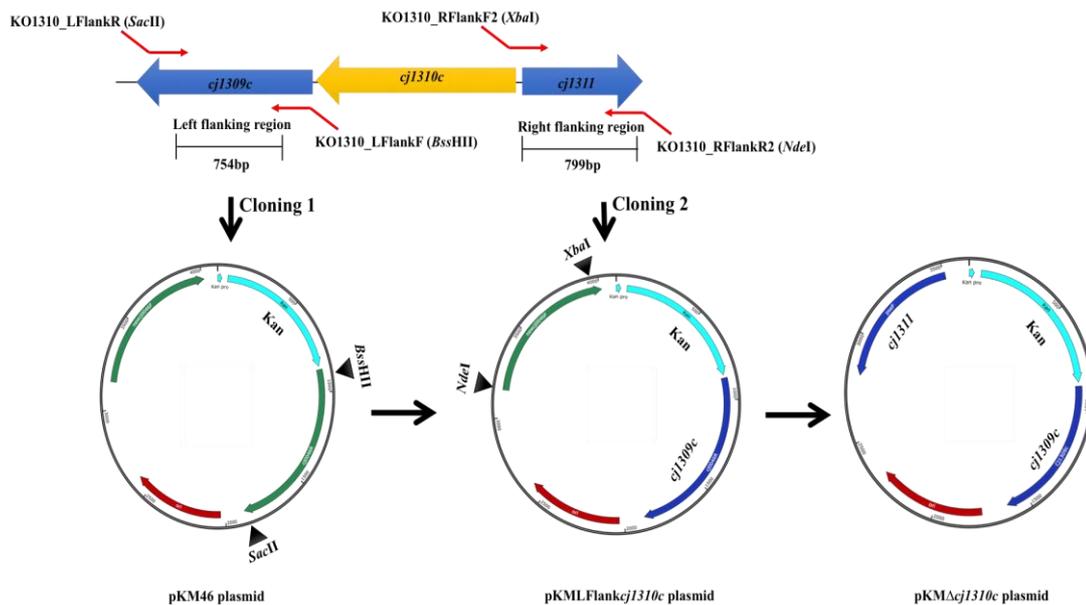


Figure 5.5. Schematic representation of the procedure followed for making pKM Δ Cj1310c.

This strategy involved two steps of cloning in which flanking regions at both ends of *Cj1310c* (left and right) were sequentially cloned into plasmid (pKM46) containing a kanamycin cassette (sky blue; Kan). Firstly, the left flank (blue open arrow; *Cj1309c*) of *Cj1310c* gene was amplified from *C. jejuni* genomic DNA by using KO1310_LFlankF and KO1310_LFlankR primers containing 5'*Bss*HII and 5'*Sac*I restriction site, respectively. Digested PCR products with *Bss*HII and *Sac*I were cloned into *Bss*HII /*Sac*I -digested plasmid pKM46 to generate plasmid containing the left flank region of *Cj1310c* designated as pKMLFlankCj1310 plasmid. Secondly, the right flanking region (blue open arrow; *Cj1311*) was amplified using *C. jejuni* chromosomal DNA as template by using KO1310_RFlankF2 and KO1310_RFlankR2 containing 5'*Xba*I and 5'*Nde*I restriction sites, respectively. Digested PCR products were ligated into *Xba*I/*Nde*I digested plasmid pKMLFlank1310 to form pKM Δ Cj1310c plasmid. Solid red arrows indicate primer position and black arrowheads indicate the location of restriction enzyme sites on the plasmid.

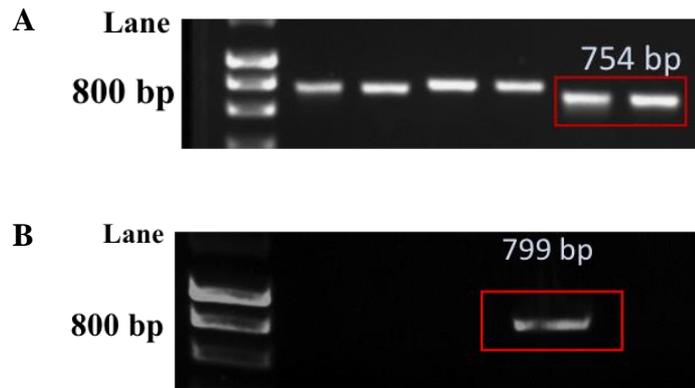


Figure 5.6. PCR amplification of left and right flanking regions of *Cj1310c* from chromosomal DNA of *C. jejuni* 11168Ca.

(A) Agarose gel electrophoresis (1%) of amplification of left flanking region (*Cj1309c*) of *Cj1310c* gene (PCR products are boxed; expected size of 754bp) by using KO1310_LFlankR and KO1310_LFlankF primers containing *Bss*HI and *Sac*II restriction sites. Lane 1, Hyper- ladder I DNA marker with fragment sizes in bp showed on left. (B) PCR product of amplification of right flanking region (*Cj1311*) of *Cj1310c* (PCR product is boxed; expected size of 799bp) by using KO1310_RFlankF2 and KO1310_RFlankR2 containing *Xba*I and *Nde*I restriction sites. Lane 1, Hyper- ladder I DNA marker with fragment sizes in bp showed on left. 1% TAE agarose gel.

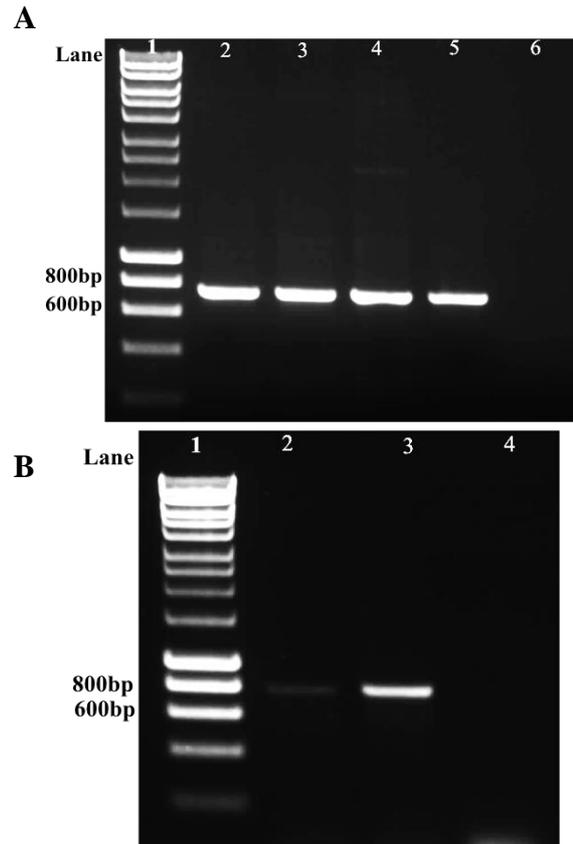


Figure 5.7. PCR verification of left flanking region (*Cj1309c*) fragment in pKMLFlank1310 plasmid and right flanking region (*Cj1311*) in pKM Δ *Cj1310c* plasmid.

(A) Lane 1, Hyper- ladder I DNA marker with fragment sizes in bp shown on left. Lanes 2-4, PCR products of left flanking region were amplified using KO1310_LFlankR and KO1310_LFlankF primers from pKMLFlank1310 plasmid (expected size of 754bp). Lane 5, PCR product of left flanking region amplification for chromosomal DNA of *C. jejuni*. Lane 6, negative control (using ddH₂O as a template). (B) Lane 1, Hyper- ladder I DNA marker with fragment sizes in bp shown on left. Lane 2, PCR product of right flanking region amplified from pKM Δ *Cj1310c* plasmid by using KO1310_RFlankF2 and KO1310_RFlankR2 primers (expected size of 799bp). Lane 3, PCR product of right flanking region amplification for chromosomal DNA of *C. jejuni*. Lane 4, negative control (ddH₂O). 1% TAE agarose gel.

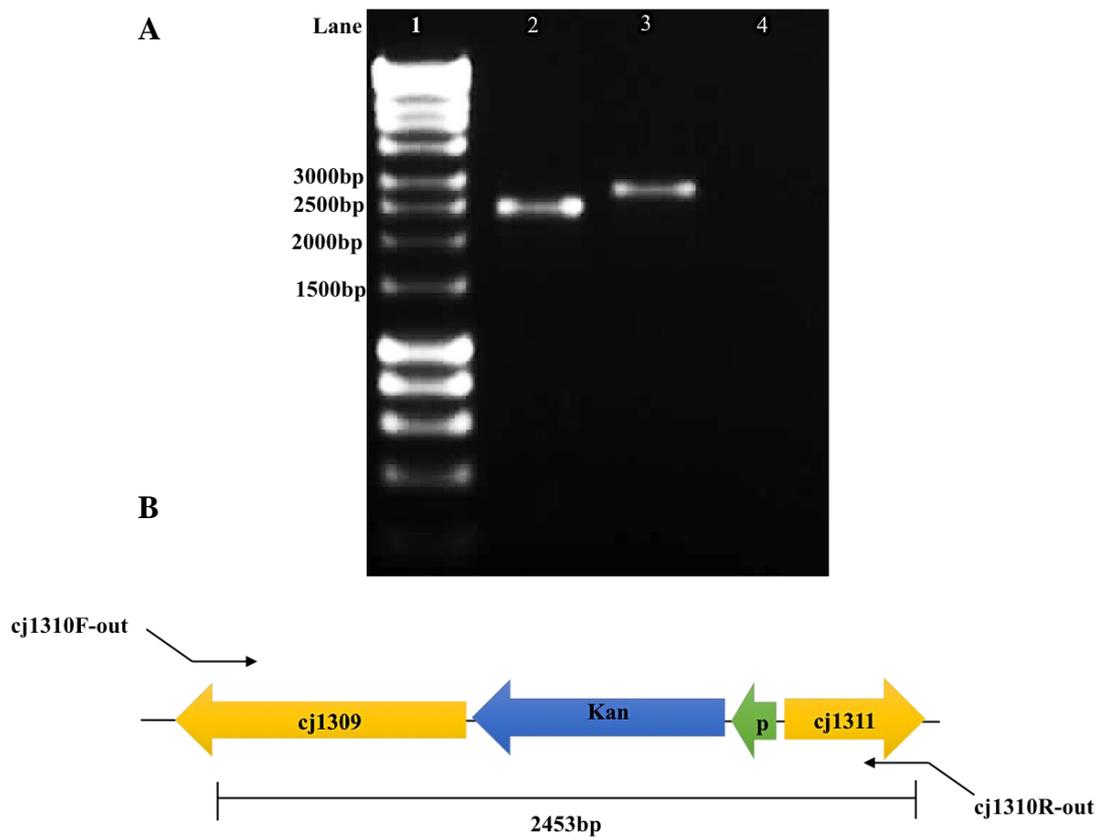


Figure 5.8. Verification of deletion of *Cj1310c* in strain *C. jejuni* Δ *Cj1310c*.

(A) Agarose gel electrophoresis of amplification of fragments derived from *C. jejuni* mutant and wild type strains using Cj1310-out and Cj1310-out primers. Lane 1, Hyperladder I marker with fragment sizes in bp shown on left. Lane 2, PCR product derived from Δ *Cj1310c* clones (predicted size 2453bp). Lane 3, PCR product amplified from wild type *C. jejuni* 11168Ca (predicted size 2754bp) lane 4, negative control (ddH₂O as a template). (B) Annealing position of primers Cj1310-out/Cj1310-out (solid arrows) used to verify the size of mutated gene. Green open arrow indicates to the orientation of kanamycin promoter.

5.4.2. Cloning of *Cj1310c* into pC46*fdxA*

C. jejuni mutants can be complemented by with the cognate wild type allele in trans using a *Cj0046* pseudogene-based insertion method (Thomas *et al.*, 2011). This approach has been successfully used for complementation of several mutants (Reuter and Vliet, 2013). *C. jejuni* Δ *Cj1310c* strain needed to be complemented with a functional copy of the phase variable gene *Cj1310c* to investigate the role of the *Cj1310c* deletion mutation in motility and autoagglutination phenotypes.

Initially, a functional copy of the *Cj1310c* gene was cloned onto the complementation vector pC46*fdxA* (Table 2.2). This vector was used to integrate a functional allele of *Cj1310c* gene into the chromosome in order to complement *C. jejuni* Δ *Cj1310c* strain (Table 2.2). There are two reasons behind using this vector: i) pC46*fdxA* contains *Cj0046* flanks for complementing *Cj1310c* into *Cj0046*, which is annotated as a pseudogene; ii) this plasmid has *fdxA* promoter that can drive expression to the complementing gene. The purpose of using a plasmid with a constitutive promoter is to ensure expression of the gene as *Cj1310c* gene is annotated as hypothetical protein and its promoter has not been identified yet. The p*fdxA* plasmid contains a constitutive medium level promoter. In order to clone *Cj1310c* gene into pC46*fdxA* plasmid, the primer pairs comp1310F2 and comp1310R2 incorporating 5' *Bsm*BI restriction sites (Table 2.3) were used to amplify the entire *Cj1310c* coding sequence of 1,405 bp using *C. jejuni* chromosomal DNA as template (section 2.4.1.2) (Figure 5.9). These PCR products were purified (section 2.4.1.5), digested with *Bsm*BI and cloned to the *Bsm*BI site of the pC46*fdxA*. The restricted purified PCR products of *Cj1310c* and pC46*fdxA* plasmid were ligated (section 2.4.4) and transformed by heat shock (section 2.5.3) into *E. coli* DH5 α E cells. Transformants were plated out onto selective media, which had been supplemented with chloramphenicol (section 2.1; Table 2.1). Recombinant clones with an insertion of the *Cj1310* fragment were identified by colony PCR (section 2.4.1.4) with primers comp1310F2 and comp1310R2 (Figure 5.10). The sequence of clone number 4 (Figure 5.10, lane 5) was analysed with CATinvR, comp1310F2 and Cj0046F primers which confirmed the location and orientation of the insert fragment (*Cj1310c* gene) with respect to the *fdxA* promoter (section 2.4.6; Table 2.3). The

resultant plasmid was named pC46fdxA-*Cj1310c* and this was used as the template for constructing the *Cj1310c* mutant plasmid.

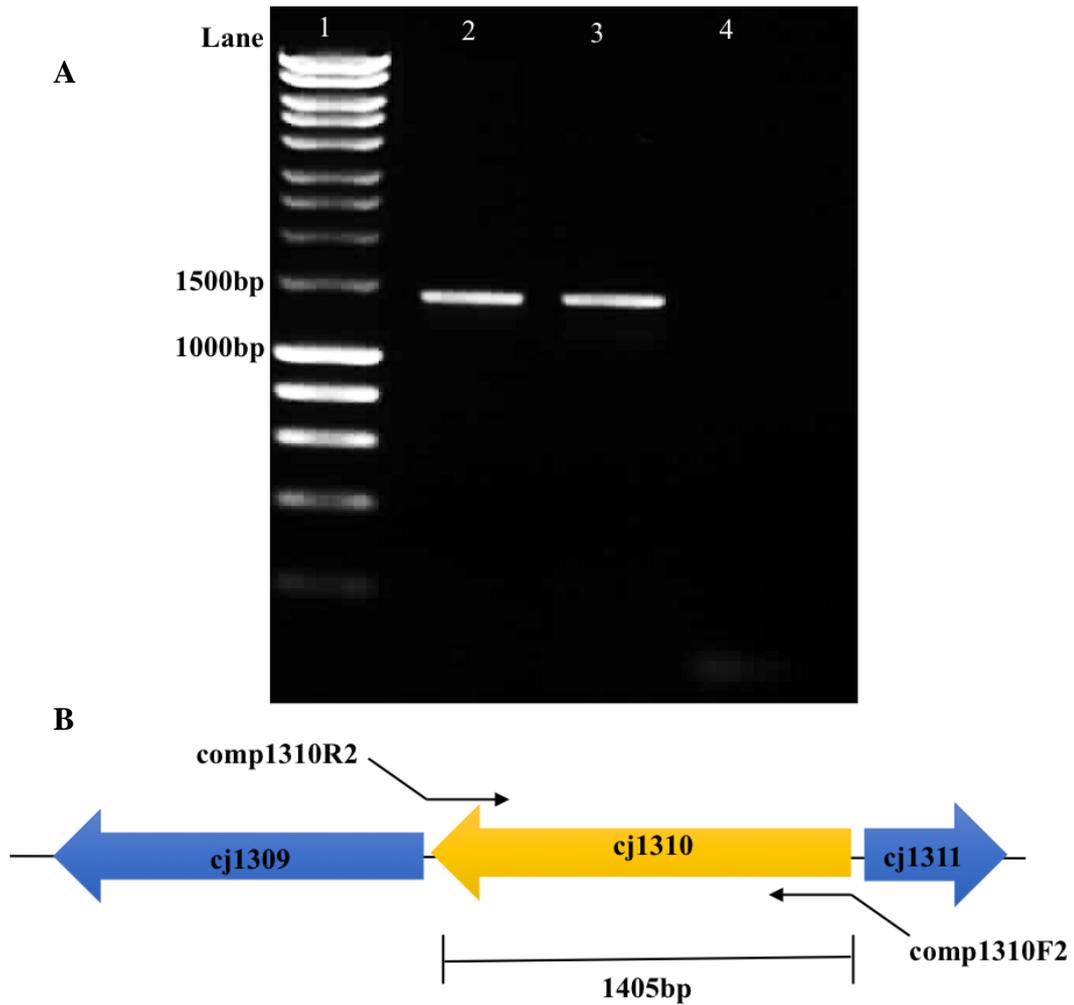


Figure 5.9. PCR amplification of *Cj1310c* from chromosomal DNA of *C. jejuni* NCTC 11168Ca.

(A) 1% TAE agarose gel electrophoresis of amplification of fragment containing the *Cj1310c* gene. The entire gene of *Cj1310c* was amplified using primers comp1310F2 and comp1310R2 incorporating 5' *Bsm*BI restriction site and cloned to pC46*fdxA*. Lane 1, Hyper- ladder I DNA marker with fragment sizes in bp shown on left. Lanes 2 and 3, PCR products of *Cj1310* amplification with comp1310F2 and comp1310R2 primers (predicted size 1405 bp). Lane 4, negative control (ddH₂O as template). (B) Diagrammatic representation of PCR amplification of *Cj1310c*. Open arrows show coding sequence orientation and solid arrows indicate primer position.

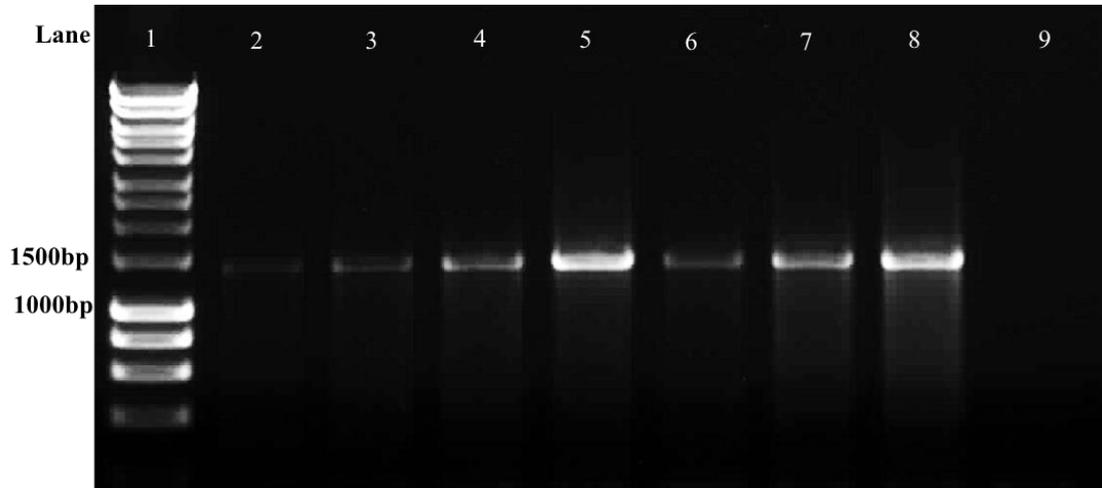


Figure 5.10. PCR verification of insert in pC46fdxA plasmid.

Transformant clones resulting from the insertion of *Cj1310c* into pC46fdxA plasmid were screened by PCR using primers comp1310F2 and comp1310R2. A fragment size of 1405bp was predicted. Lane 1 is the Hyper- ladder I marker with fragment sizes shown in bp on the left. Lane 2-7, PCR products derived from individual putative clone plasmid DNA amplified with primers comp1310F2 and comp1310R2. Lane 8, PCR product derived from wild type 11168Ca amplified with primers comp1310F2 and comp1310R2 (Positive control). Lane 9, negative control (ddH₂O). 1% TAE agarose gel.

5.4.3. Complementation of the *Cj1310* gene knockout mutant

In order to obtain a constitutively functional copy of *Cj1310c* that was not subject to PV, a point mutation was introduced into the homopolymeric tract of G residues by inserting an adenine instead of a guanine into the repeat tract employing site-directed mutagenesis. The application of this technique leads to i) fixing the gene to be in frame, ii) interruption of the run of G residues resulting in an inability of the tract to cause PV, and iii) no change to the amino acid sequence. This was achieved in *Cj1310*, which has a polyG repeat tract that contains 9 units in the ON phase, by substitution of an adenosine (A) residue to replace a G residue so that the resulting gene contained a region consisting of two G residues followed by a single A residue followed by six G residues rather than a continuous run of nine G residues.

Initially, pair of primers that are complementary to the G-repeat tract of *Cj1310c* gene were designated (Table 2.3), in which the forward and reverse primers contain adenine instead of guanine and thymine instead of cytosine, respectively. The pC46*fdxA-Cj1310* plasmid (section 5.4.2) was used as a template in the PCR reaction (section 2.4.1.2) and amplified with 1310SDM-F and 1310SDM-R2 primers (Table 2.3). After PCR amplification, parental plasmids (i.e. non-mutagenised plasmids) were removed by DpnI restriction digestion. After that, digested PCR products were alcohol precipitated (section 2.4.2.1) and transformed into *E. coli* DH5 α competent cells by heat shock. Transformant cells containing potential mutated plasmids were recovered from LB agar plates supplemented with 20 μ g/ μ l chloramphenicol. Purified plasmid DNA from potential clones was sequenced with CATinvR, newCj0046endF, Cj1310-fwd2 and Cj1310-rev primers to ensure the presence of the adenine instead of a guanine in the poly G-tract of *Cj1310c* gene. Once the single mutation was confirmed in pC46*fdxA-Cj1310* recombinant plasmid, it was designated pC46*fdxA-SDMCj1310*.

The pC46*fdxA-SDMCj1310c* plasmid was electroporated (section 2.5.4) into competent *C. jejuni* Δ *Cj1310c* (Δ *Cj1310::Kan*) cells (section 2.5.2). Transformants were selected on MHA-VT plates (section 2.1) supplemented with chloramphenicol (Table 2.1) to select for allelic exchange of the *cat-pfdxA-Cj1310* fragment into the *Cj0046* locus. Subsequently, chloramphenicol resistant

colonies were further sub-cultured on plates supplemented with kanamycin for $\Delta Cj1310::kan$ mutation confirmation and then screened by using CatInvR and Cj0046F primers. The expected PCR product size from CatInvR /Cj0046F was predicted to be 1,847bp, whereas in the wild-type the fragment was not present; recombinants producing the correct size were observed (Figure 5.11). In addition, the site of insertion of the functional *Cj1310c* gene with interrupted G/C tract was verified by sequencing with flank0046seq-F, flank0046seq-R, CATinvR, Cj0046F, Cj1310 fwd2 and Cj1310-rev primers (Table 2.3) in *C. jejuni* Comp $\Delta Cj1310c$ strain.

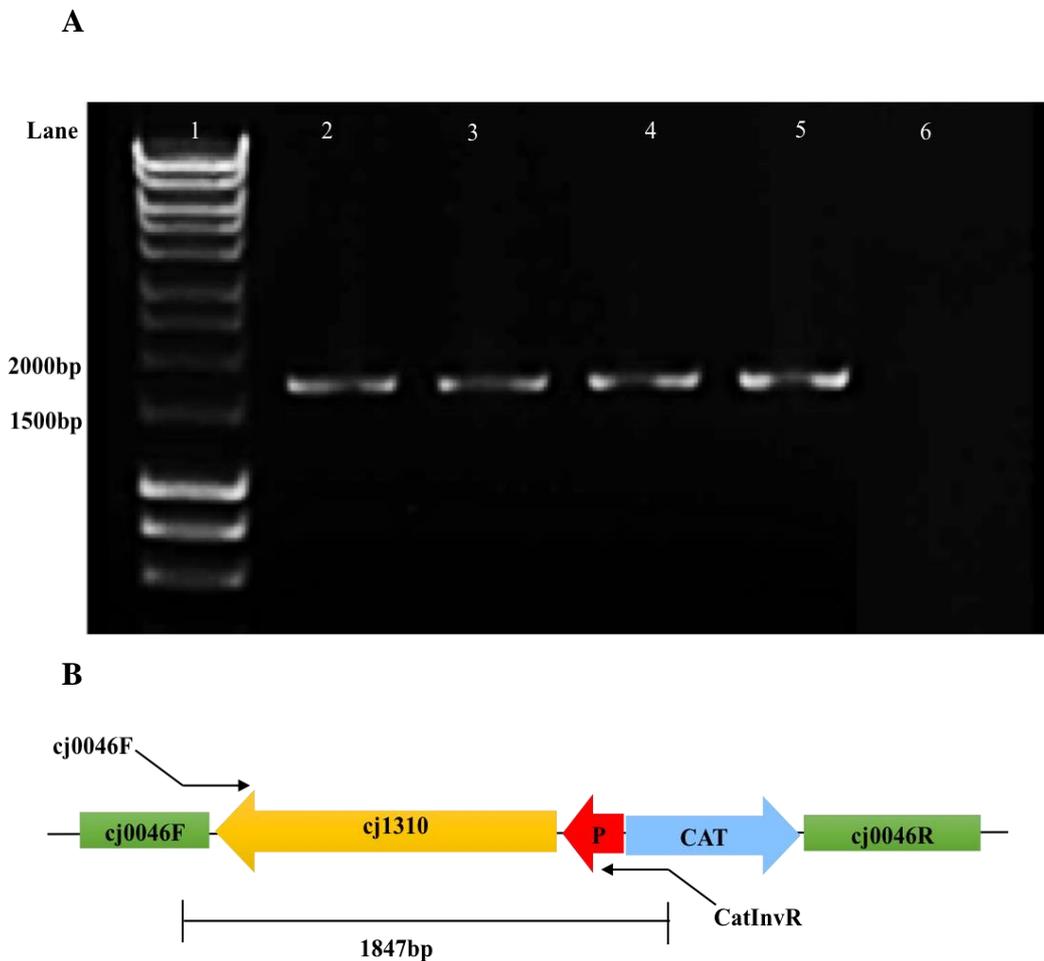


Figure 5.11. Confirmation of the location and orientation of the *Cj1310::0046* insertion in *C. jejuni* Comp- Δ *Cj1310c* strain.

CatInvR and Cj0046F primers were used to confirm the presence and determine the orientation of the *Cj1310c* inserted into the *Cj0046* pseudogene in strain Comp- Δ *Cj1310*. (A) Analysis of PCR products on a 1% TAE agarose gel. Lane 1, Hyper-ladder I marker with fragment sizes in bp shown on left. Lanes 2-5, PCR product of positive colonies by using CatInvR/Cj0046F primers (predicted size 1847bp). Lane 6, wild type *C. jejuni* 11168Ca as negative control using CatInvR/Cj0046F primers. (B) Illustration of the location of primers annealing of CatInvR in the insert and Cj0046F (solid arrows).

5.5. Immunological response against glycosylation mutants

Initially, purified recombinant FlaA protein was sent to Eurogentec (section 2.6.5) to produce rabbit polyclonal anti-FlaA primary antisera. Immunization of rabbits with the recombinant protein yielded antibodies that specifically recognised the 59.5 kDa band in the Western blot with rFlaA protein (Figure 5.12A and B; lane 1). The reactivity of this antiserum with gFlaA protein in O.M fractions, which is represented by a 65.5 kDa band, was also observed for *C. jejuni* wild type strain (11168Ca) (Figure 5.12A; lane 6) and mutant strains ($\Delta maf2$, $\Delta Cj1295$, *Cj11295*comp and *Cj1310*) but not for the FlaA-negative strains (11168 $\Delta flaA$ and $\Delta maf5$) (Figure 5.12A; lanes 7 and 3), confirming that detected bands in the immunoblotting assays performed in the present study were *C. jejuni* gFlaA protein and also confirm the purity of rFlaA protein which was used to produce this polyclonal FlaA antibodies in rabbits. Furthermore, similar results were obtained for whole cell lysate preparations of these strains (Figure 5.12B).

The reactivity of gFlaA from mutant *C. jejuni* strains was then investigated. Similar amounts of O.M fractions from each strain were used as antigen in these western blots as shown in Figure 5.13A. As shown in Figure 5.13B - F, gFlaA protein bands of wild type (Wt) and mutant strains $\Delta Cj1295$, *Cj1295*comp and $\Delta Cj1310c$ with D+52 sera had similar reactivity. Mutant *maf5* showed weak signal intensities for gFlaA in all blots, whereas there was no band in blots probed with anti-FlaA antibody. This could be due to the absence of the formation of the flagella filaments (Karlyshev *et al.*, 2002). Surprisingly, gFlaA protein in $\Delta maf2$ outer membrane extracts reacted strongly with B12, B40 and B42 serum.

Image J software was used to compare signal intensities between gFlaA protein from wild type and mutant strains with the data being normalised to the value of the signal intensity of GST protein (probed with anti GST-HRP antibody). The intensity of gFlaA in the wt in comparison with each mutant strain was not shown to be significantly different for reactivity with D+52 sera (data not shown). These results suggest that the heterogeneity in FlaA glycosylation does not induce the generation of specific antibodies.

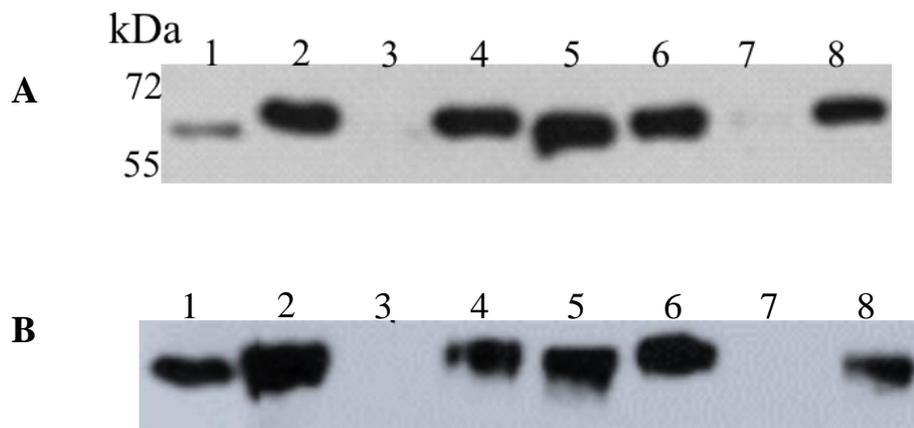
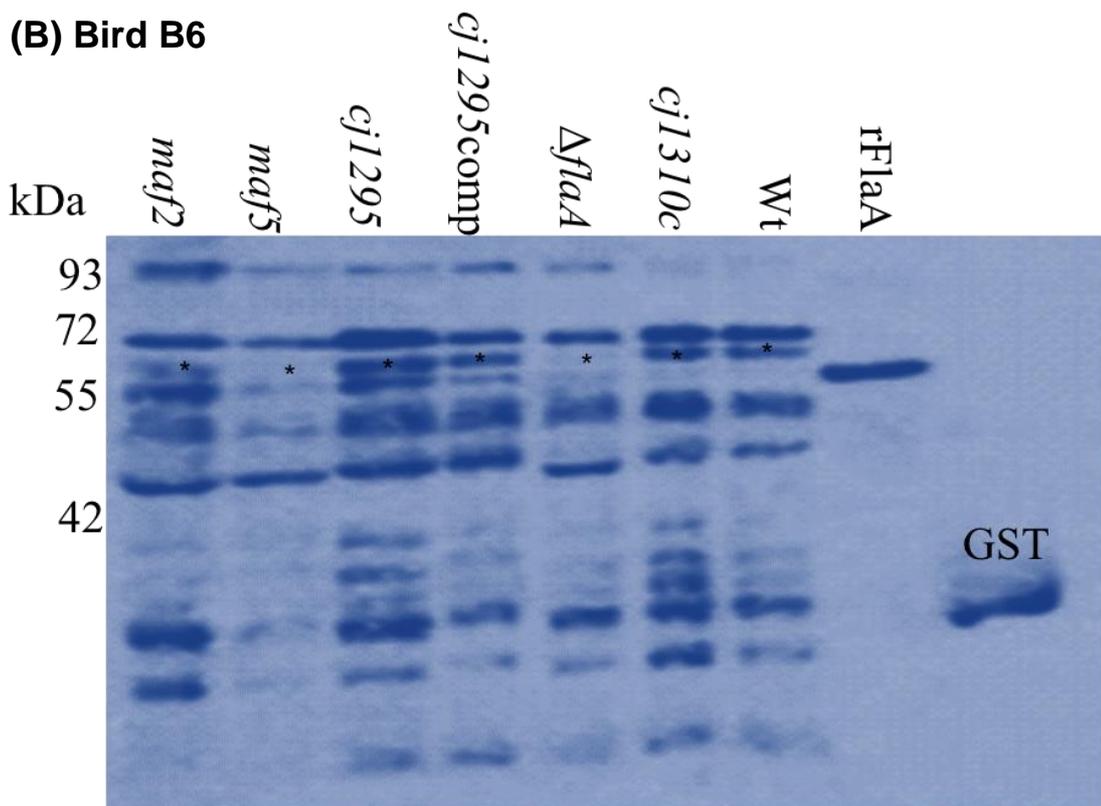


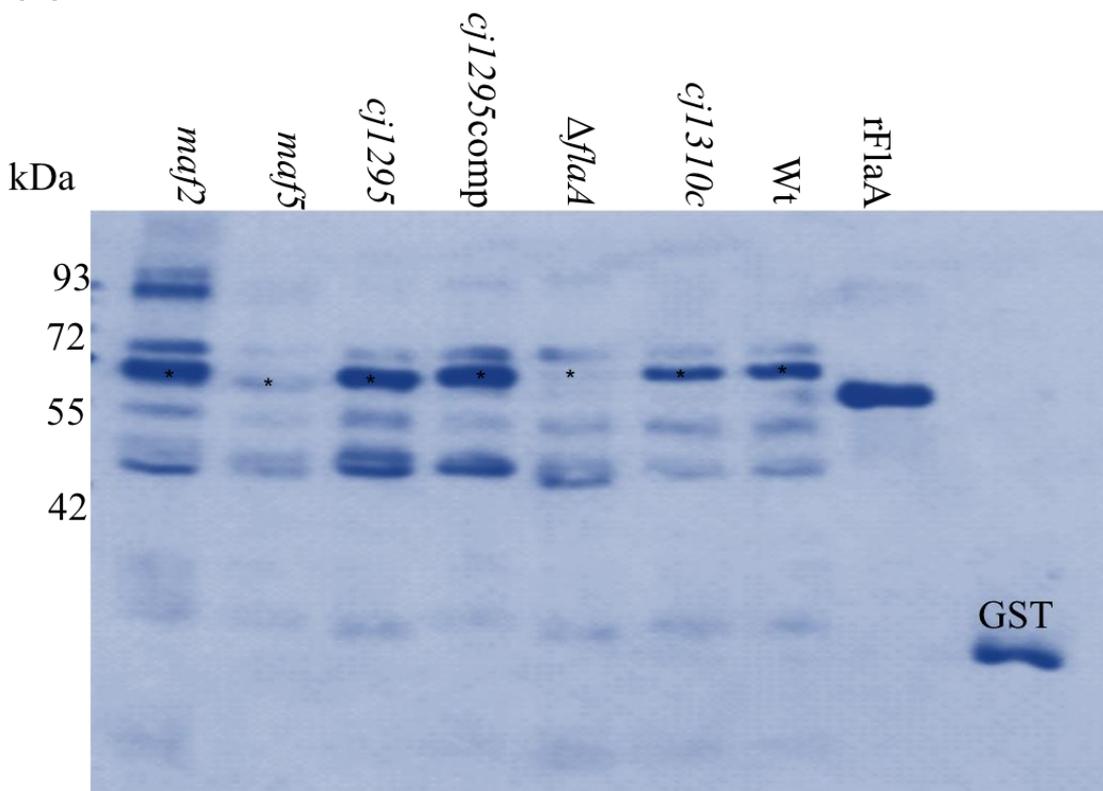
Figure 5.12. Specificity and reactivity of the generated polyclonal FlaA antibody examined by western blotting.

Purified rFlaA (lane1) and O.M. protein extracts of *C. jejuni* 11168Ca (wild type, lane 6), mutant strains ($\Delta maf2$, lane2; $\Delta Cj1295$, lane4; $Cj1295comp$, lane5; and $Cj1310c$, lane8) and FlaA-negative strains (11168 $\Delta flaA$, lane7; and $\Delta maf5$, lane3) were analyzed by Western blotting in blot (A). rFlaA and WCL preparations of similar strains were employed in blot (B). The proteins were transferred to PVDF membrane and probed with a 1:10,000 dilution of primary antibody (polyclonal rabbit anti-FlaA), washed and secondary antibody (Goat anti-rabbit HRP tagged) added with 1:5,000 dilution. The positions and sizes of the marker proteins are shown.

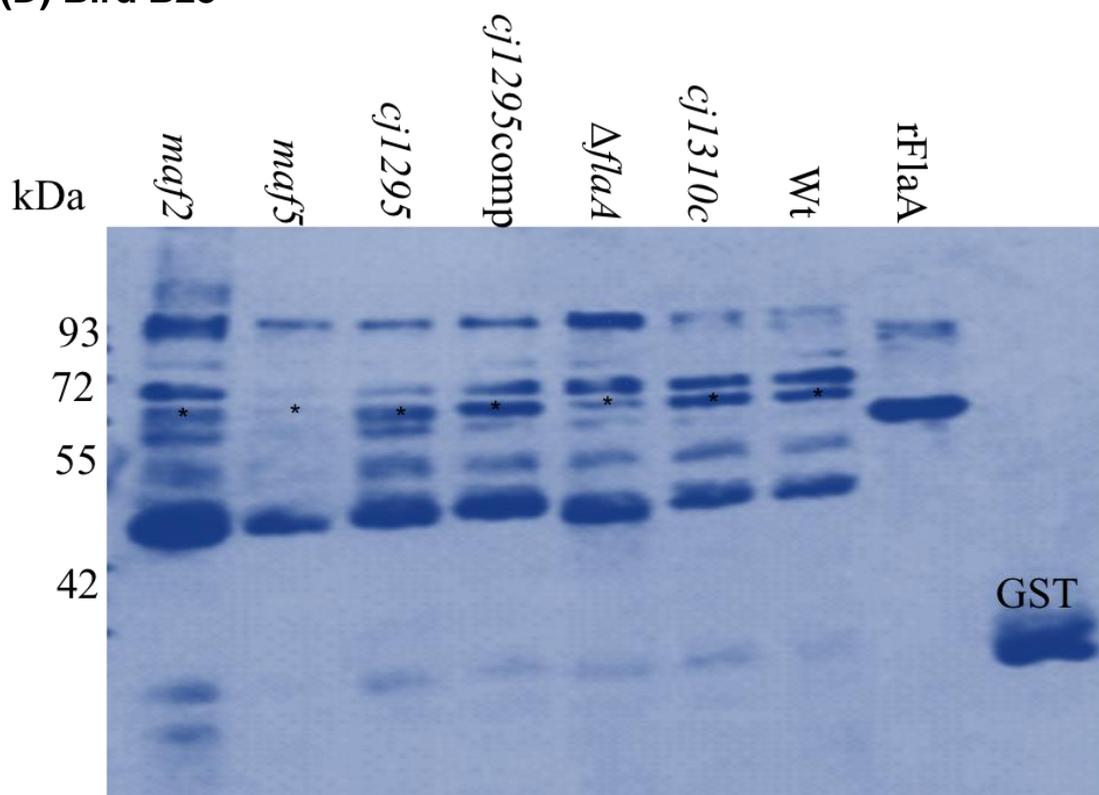
(B) Bird B6



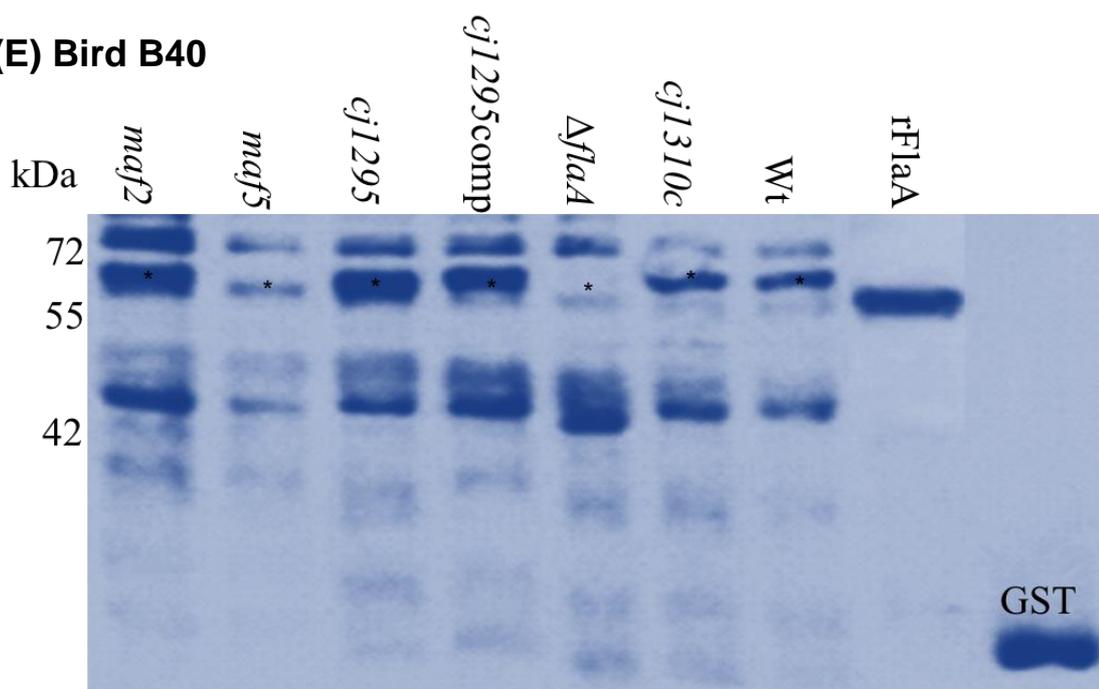
(C) Bird B12



(D) Bird B28



(E) Bird B40



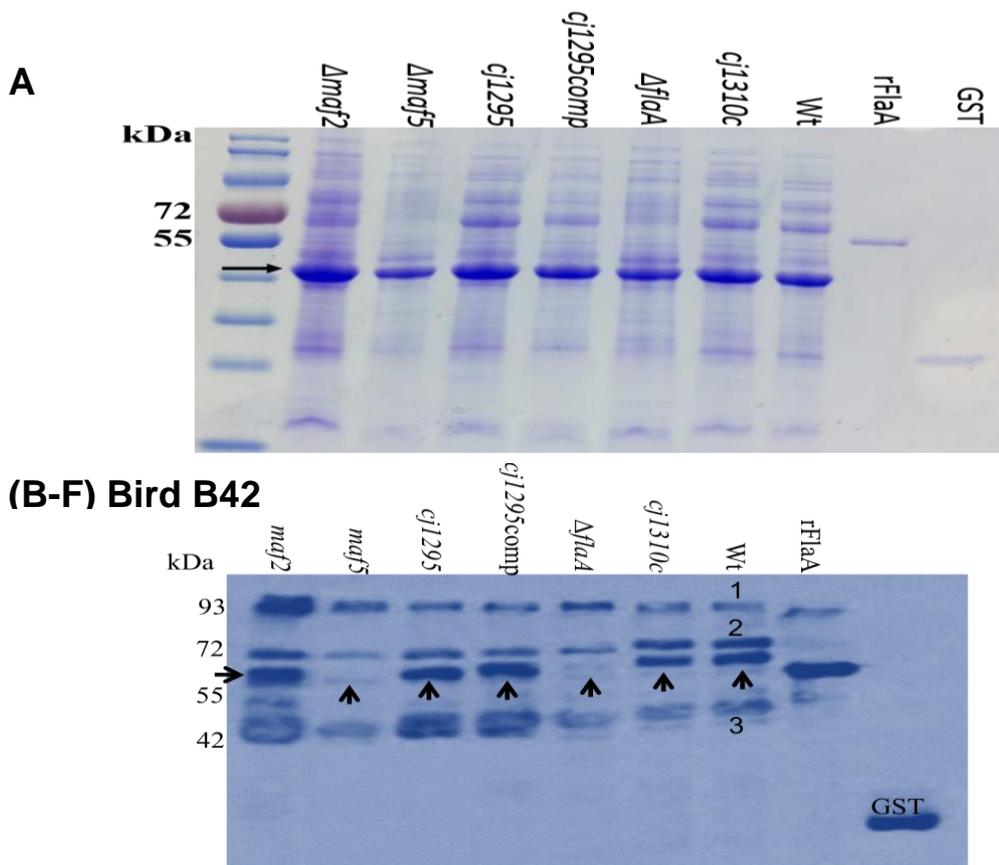


Figure 5.13. Western blot of rFlaA, wild type gFlaA (11168Ca) and the indicated mutants gFlaA protein probed with D+52 sera.

O.M. protein extracts of the *C. jejuni* wild type strain (Wt) and mutant strains ($\Delta maf2$, $\Delta maf5$, $\Delta Cj1295$ *Cj1295comp*, $\Delta flaA$ and $\Delta Cj1310c$), rFlaA protein and GST protein were: (A) analysed using SDS-PAGE; and then transferred to PVDF membranes and probed with 1:100 dilutions of primary antibody (D+52 sera), washed and then probed with a 1:5000 dilution of the secondary antibodies (a mixture of rabbit anti-chicken (IgG) HRP tagged and anti-GST-HRP). The positions of size markers are indicated in kDa on the left of the blots. Each blot was blotted with individual serum collected from different birds: (B) B6 (Ca); (C) B12 (Ca); (D) B28 (H); (E) B40 (H); and (F) B42 (H). The small black asterisk on blots (B) – (E) indicate the position of *C. jejuni* gFlaA protein in wild type strain and mutant strains. The black arrows on blot (F) indicate the position of the *C. jejuni* gFlaA protein in wild type strain and mutant strains. The numbers - 1, 2 and 3 – on blot (F) indicate FlgE2 (Flagellar hook protein), CiaB (secreted protein) and MOMP (major outer membrane protein), respectively, as estimated by MALDI-TOF mass spectrometry.

5.6. Determination of the role of *Cj1310* in motility and autoagglutination of *C.jejuni*

5.6.1. *Cj1310* does not play a role in the motility of *C. jejuni*.

As demonstrated previously, the signal intensity of gFlaA band in *Cj1310* mutant strain reacted with bird antisera was similar to the intensities bands of gFlaA wild type and rFlaA proteins. This observation suggests that the product of this gene may not have any role in the immunogenicity of FlaA. However, as shown in Figure 5.1 (A) all colonies at 52-day post challenge contained a *Cj1310*-ON variant. This interesting observation suggest that this gene may have an important function and led to performance of a motility assay to assess the potential role of this gene in controlling the functions of the flagella system. In this assay, the motility of mutant $\Delta Cj1310$ strain was compared to the motility of wild type strain (11168Ca) as a positive control. In the assay, a *Cj1310*comp strain also was included as were a range of mutant strains ($\Delta maf2$, $\Delta maf5$, $\Delta Cj1295$ and *Cj1295*comp) and a negative control (a non-motile *flaA* mutant). These strains were examined for flagella-mediated motility by point inoculation of reduced agar (0.4% w/v) Mueller-Hinton plates, a standard method for assessing motility in bacteria and in *C. jejuni* (Guerry *et al.*, 1991). Standardised inocula were used throughout and plates incubated microaerobically for 72 hours and examined. Motile organisms swarm through the agar from the inoculation point producing a zone of halo growth around the initial inoculation site. Non-motile strains did not migrate through the agar and grew as a mass of cells at the inoculation site.

As expected the diameter of the halo growth of the *maf5* mutant was significantly smaller than the growth zone of 11168Ca (Figure 5.14). The *maf2* mutant also exhibited a significant difference ($P < 0.01$) in the motility compared to the wild type, indicating that mutation of *maf2* can affect flagella filament production. This is inconsistent with the observation of a strong band of FlaA in the O.M. extract of a *maf2* mutant strain as detected by Western blot (Figure 5.14). However, mutants of *Cj1310c* and *Cj1295* were comparable with the wild type and their complementation strains (*Cj1310*comp and *Cj1295*comp). It is clear that *Cj1310c* did not contribute to the generation of an intact flagella filament or motility.

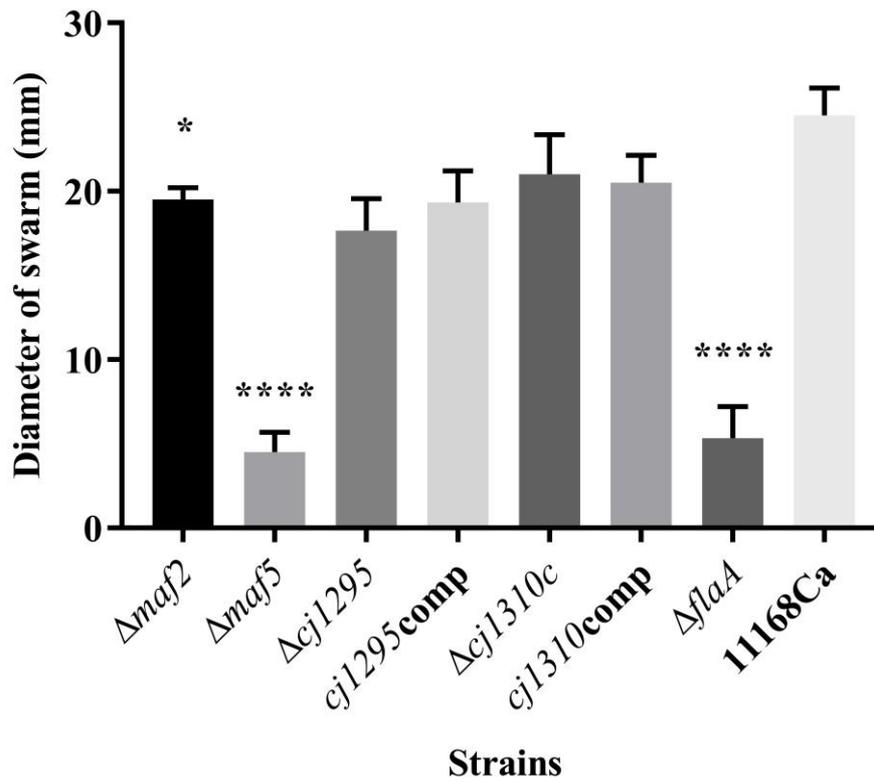


Figure 5.14. Assessment of motility phenotype of the *Cj1310* and *Cj1310comp*.

The experiment was repeated three times (n=3). Error bars show standard error, ****significant difference ($P < 0.0001$). In each plate, cells were inoculated into the centre of a 0.4% (w/v) MHA plate and grown for 72 hours at 42 °C in a microaerobic atmosphere.

5.6.2. Growth assay.

The growth assay was performed in order to investigate whether the change in swarming phenotype of the mutants seen in the swarm assay is associated with slow growth of the mutants relative to the wild type.

Growth kinetics were carried out for 20 hours in 96 micro-plates at 42°C under micro-aerobic conditions (section 2.7.1). The growth curve of 11168Ca wild-type strain, mutated strains ($\Delta maf2$, $\Delta maf5$, $\Delta Cj1295$ and $\Delta Cj1310$) and complemented mutants ($Cj1295comp$ and $Cj1310comp$) appeared indistinguishable from each other with no apparent defect in growth dynamics due to mutagenesis (Figure 5.15). However, the NCTC11168 $\Delta flaA$ mutant had a significantly faster growth rate than the wild type.

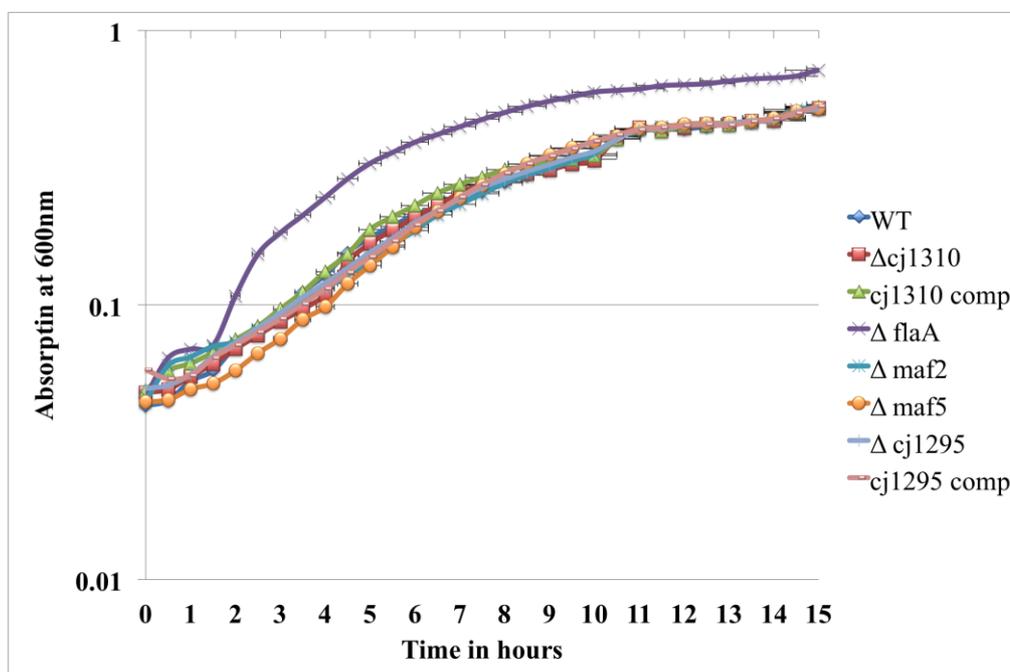


Figure 5.15. Growth of 11168Ca wild type, mutated strains ($\Delta maf2$, $\Delta maf5$, $\Delta Cj1295$, $\Delta Cj1310c$ and $\Delta flaA$) and complementation mutants ($Cj1295comp$ and $Cj1310comp$).

The graph shows the log₁₀ growth dynamics measured by OD₆₀₀ readings were taken by FLUOSTAR omega plate reader. Error bars show the S.D of the mean of data from four samples at each time point. WT = wild. A key indicating the mutants and complementation mutants is shown on the right of figure.

5.6.3. Autoagglutination of 11168Ca *Cj1310* mutant

The ability of bacterial cells to attach to each other is known as autoagglutination, which is considered as a useful indicator for assessing virulence (Misawa and Blaser, 2000). In *C. jejuni*, there is an association between autoagglutination and flagellar expression (Misawa and Blaser, 2000) that even includes specific glycan structures on the flagellin protein (Guerry *et al.*, 2006). The *maf2* and *maf5* mutants were previously observed to exhibit significant reductions in autoagglutination (Butler, 2009), which, as these genes are involved in the pathways for addition of pseudaminic acid and acetamidino pseudaminic acid onto the flagella, indicates that the glycosylation pathways have the ability to influence aggregation of bacterial cells. Therefore, the performance of this experiment aimed to assess any influence of *Cj1310* mutation on the autoagglutination of *C. jejuni*.

Suspensions of mutant and the wild type strains were prepared in PBS. The suspension was normalised to an initial OD₆₀₀ of 0.5 and incubated at RT for 24 hours. When bacteria autoagglutinate, they aggregate together and form a pellet at the bottom of the tube leaving a clarified supernatant whilst if the bacteria are unable to autoagglutinate then the solution remains turbid. The quantification of autoagglutination, was achieved by removing 1 ml from the top and re-measuring the OD₆₀₀. A decrease in OD from 0.5 indicates autoagglutination (Figure 5.16A). Agglutination results were also confirmed by performing colony counts from the supernatant and cell pellets after 24 hours of incubation, under static conditions (Figure 5.16B).

Wild-type 11168Ca autoagglutinated and exhibited an approximately 80% decrease in OD₆₀₀ (Figure 5.16A). Mutations in genes *Cj1310* and *Cj1295* did not have any effect on the level of autoagglutination and were comparable to wild type and their complementation strains (*Cj1310comp* and *Cj1295comp*). However, mutation of *maf5* resulted in statistically significant reductions in autoagglutination ability ($p < 0.0001$). Mutant *maf2*, which was partially motile (Figure 5.14) and produced a strong reactivity with sera (Figure 5.13), did not influence the level of autoagglutination. Furthermore, mutant *maf5*, which was non-motile and lacked reactivity with sera largely failed to autoagglutinate over a 24 h period, therefore indicating that full-length flagella are required for

autoagglutination.

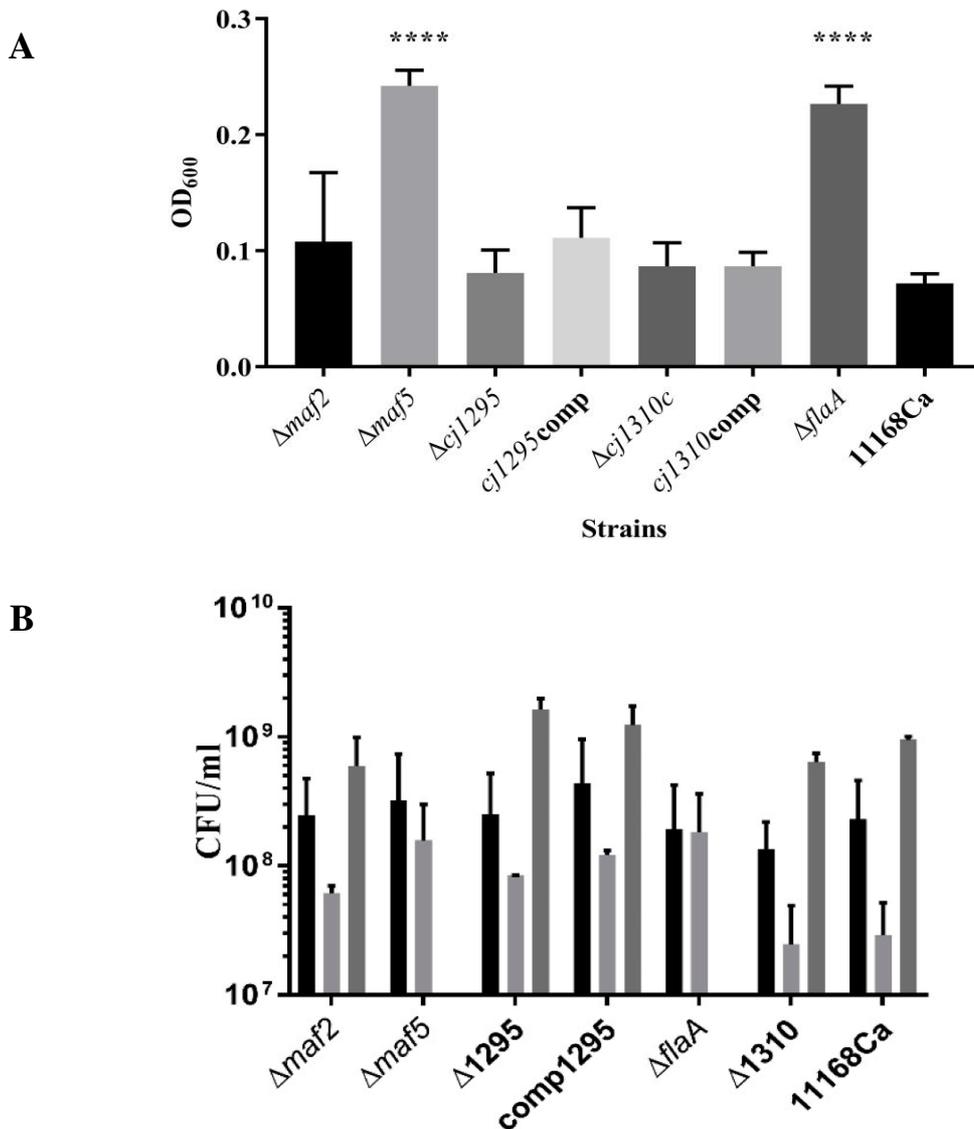


Figure 5.16. Autoagglutination of 11168ca wild type and *Cj1310c* mutant.

(A) OD measurements from supernatant; and (B) colony counts from supernatants: black bars represent inoculum, light grey bars represent supernatant after 24 hours, and dark grey bars represent colony counts of agglutinated cells. WT=wild-type 11168Ca. Error bars indicate the standard error for $n = 3$. Statistically significant differences from the wild-type values were designated with **** ($P < 0.0001$). Mutant *maf5* showed significantly reduced levels of autoagglutination ability whilst all other mutants including *Cj1310c* were comparable to wild type. The non-flagellated *flaA* mutant used as a control for flagella-dependent autoagglutination.

5.7. Discussion

The *C. jejuni* major flagella filament protein (FlaA) is a major virulence determinant but has high immunogenicity. Modification of the FlaA protein with a variety of glycans leads to increases in the complexity of specific antibodies against specific glycans and has an unknown role in reducing the load of *C. jejuni* in the digestive tract of chickens. The immunogenicity of different forms of FlaA glycans was tested in this chapter by using sera obtained from birds infected with *C. jejuni* variants that differed in their FlaA glycan profiles due to the presence of PV genes in alternate expression states. As in gene *Cj1296*, the majority of colonies had OFF-*Cj1296* variants then switched within the majority of output population to be in ON state. In a similar manner was *Cj1310* and *Cj1295*; they switched to be expressed after long time of persistence.

The sera were used in combination with a series of strains mutated in several glycosylation genes. A set of pre-existing mutants were obtained and were augmented by generation of an additional mutant with inactivation of *Cj1310*. However, there was a failed attempt to generate a *C. jejuni* *Cj1295/Cj1296* double isogenic mutant. The construction of a p Δ *Cj1295/Cj1296* plasmid was successfully achieved but the transformation of p Δ *Cj1295/Cj1296* plasmid into *C. jejuni* failed. This mutagenesis strategy would have resulted in deletion of the entire coding sequence of *Cj1295* and *Cj1296* with subsequent insertion of a chloramphenicol antibiotic cassette. The construct also resulted in deletion of 19bp of *Cj1294* and 61bp of *Cj1297*. The failure of this strategy suggests that a large deletion of the coding sequence of *Cj1295* or the small deletions in the upstream and/or downstream genes may influence the viability of *C. jejuni*, especially as a single Δ *Cj1295* knockout mutant has been achieved without any significant negative effects on the viability of *C. jejuni* (Hitchen *et al.*, 2010). It is also possible that there could have been polar effects on the two genes, *Cj1297* and *Cj1298*, that are downstream of *Cj1296*. The function of *Cj1298* found to be involved in the biosynthesis of Deoxyactagardine B as *N*-acetylaminotransferase and *Cj1297* is still unknown (Obhi and Creuzenet, 2005).

In this study, a major achievement was the generation of Anti-FlaA antisera in rabbits, which were successfully used to confirm glycosylation of FlaA in lysates and O.M fractions produced from different *C. jejuni* mutants. A further utility of

this anti-FlaA antisera would be in the purification of both glycosylated and non-glycosylated variants of FlaA, using classical immunoproteomics approaches e.g. the use of Protein A or protein G resin.

This study also found that reactivity of anti-FlaA antisera with the $\Delta maf2$ mutant was at levels similar to those observed with the wild-type strain. This finding was inconsistent with those from a Butler (2009) in which the wild-type reactivity was significantly higher than the $\Delta maf2$ mutant. The discrepancy in results between these two studies may be explained by the heterogeneity in flagellin length found within a given population of the $\Delta maf2$ cells.

The immunoblots demonstrated that there was, as expected, a strong immune response to both rFlaA and gFlaA after 52 days colonization. Surprisingly, there was no major difference in the immune responses to heterogeneous FlaA glycoforms. Also, there was no difference in reactivity between sera derived from chickens infected with the H or Ca variant when used to probe the Ca variant. It is unlikely that all of antibodies are directed against the amino acid backbone and that there are no antibodies to the FlaA glycoforms in these birds. This could be tested by performing antibody depletion experiments in which the non-glycosylated rFlaA protein could be used to deplete antibodies targeting non-glycan epitopes of FlaA. Reactivity of rFlaA-treated antisera with gFlaA would indicate the presence of antibodies specifically targeting glycan epitopes in the anti-FlaA antisera.

One issue with these experiments is the lack of appropriate controls. Antibodies are required that are specific for different FlaA glycoforms (raised in mice or rabbits) and could be used to show what glycans are present on each FlaA protein. Presently, very few antibodies targeting specific *C. jejuni* proteins are available and future studies in which these antibodies are generated are required. Liu et al. (2017) successfully generated a single-chain antibody produced from fused variable domains (scFv) to target the rare O-linked bacillosamine structure, which is expressed with bacterial pili by *Neisseria* species contributed with strains pathogenicity's.

Another problem is that output isolates were not obtained from the birds at that time point when sera were collected (i.e. 52 day post challenge) and so could not be used for the screening of potential development antibodies. Thus, if there was

a change in PV gene expression leading to variation in glycoform (inoculum to output) and a consequent immune response to the output glycoform, then these antibodies may not be detected in the Western blots. Furthermore, since glycoform derivation is dependent on a combinatorial mechanism, the glycoforms produced in the Western blot strains (*maf2* mutant, *Cj1295* mutant and *Cj1310c* mutant) may differ to the day 52 isolate glycoform.

Another somewhat surprising result was the absence of a potential role of the *Cj1310* protein with respect to motility, immunogenicity or aggregation. *Cj1310c* is not essential for flagellin production and hence the deletion of this gene did not show any effect on motility; suggesting that *Cj1310* is not involved in critical steps of synthesis pseudaminic acid and its derivatives as this derivative is required for function as shown by the lack of motility and autoagglutination observed for the *maf5* mutant (Butler, 2009, kaelyshev *et al.*, 2002). Also, *Cj1310c* and *Cj1295* did not contribute to autoagglutination and motility and showed similar behaviour in their expression states during chicken colonisation, indicating that *Cj1310c* might have a similar function as *Cj1295* that found is responsible for producing a different glycanform of FlaA protein (Hitchen *et al.*, 2010). It is assumed that this protein is involved in glycosylation of the FlaA protein. This assumption could be tested in further studies where the glycosylation status of flagellin produced in the single $\Delta Cj1310$ mutant would be assessed using 2D-PAGE. Heterogeneity in a wild-type strain is expected with respect to the isoelectric point (pI) of flagellin, as shown in van Alphen *et al.* (2008). However, if *Cj1310* is involved in glycosylation of *C. jejuni* flagellin, then a shift to a more neutral pI would be observed. Future studies could investigate also the effect of a *Cj1310c* deletion on serum resistance. However, for the generation of unequivocal data, it is required that future experiments be performed in mutant strains devoid of other glycosyltransferases in order to avoid redundancy. The growth assay was performed to validate the observation of the influence of $\Delta Cj1310c$ strain in motility, the growth of $\Delta Cj1310c$ and *Cj1310comp* strain and a range of mutant strains ($\Delta maf2$, $\Delta maf5$, $\Delta Cj1295$ and *Cj1295comp*) was measured in comparison to a negative control (a non-motile *flaA* mutant). However, the NCTC11168 $\Delta flaA$ mutant had a significantly faster growth rate than the wild type. This is consistent

with Kanji et al. (2015) who found that a non-motile NCTC11168 $\Delta Cj0390$ mutant grew faster than the wild type

Chapter 6: General discussion and future work

The FlaA protein of *C.jejuni* undergoes antigenic variation due to switches in expression of several phase variable genes that generate structural variation in the modifications of the protein backbone. This structural variation is located in the central domain of flagellin, which is post-translationally modified by attachment of O-linked glycans to serine or threonine residues. Mutants with disruption of the glycosylation pathways are affected in both their motility and colonisation. The two main aims of this project were; firstly to investigate the genetic basis of hyper-motility of *C. jejuni*; and secondly to study the immunogenicity of FlaA during asymptomatic colonisation of chickens by this bacterial species.

6.1. Novel *cbrR* variants, but not PV, are associated with hypermotility phenotypes in 11168 Ca

Individual *C. jejuni* strains were known to exhibit variations in their motility phenotypes ranging from non-motile to hyper motile cells (Karlyshev *et al.*, 2002) but the genetic basis for this variation was unknown. This study focused on investigation of the influence of the 28 phase variable genes found in *C. jejuni* strain NCTC11168 (Parkhill *et al.*, 2000) on the formation of the hyper-motility phenotype in this strain. Previous study found that the variability in the motility of NCTC11168 resulted in the formation of the hyper motile strain 11168H. Mutagenesis study on this strain revealed that the ability of the phase variable gene, *maf4* to restore the motility into *maf5* mutant strain (Karlyshev *et al.*, 2002). Their study was the fundamental research in order to improve the understanding in the correlation between phase variable genes and motility variation in *C.jejuni*. Therefore, initially, this study investigated if the motility variation mechanism that shown in *C. jejuni* due to the reversible switching of PV genes or not. As 11168Ca strain hyper-motile variant, 52 motile and hyper motile variants were isolated and analysed by GeneScan method as explained in chapter 3. However, the analysis of this large collection of isolates did not detect any correlation between the variations in motility and specific patterns of individual PV gene ON or OFF expression states. The absence of correlation was in contrast with

Karylshev group findings as maintained and that increase the curiosity for more investigations. The coming future work should consider the combinatorial expression of PV genes. It could be that the reversible expression of more one gene may have a role in this variability.

In the absence of an effect of PV, comparative whole genome sequence analysis was used to detect any specific SNPs or INDELS that might be associated with increases in motility. Sequence genome analysis of H and H.M variants in comparison to the published genome sequence of the *C.jejuni* NCTC11168 strain revealed the presence of a mix of unique variants (one SNP and ten INDELS) exclusively within the majority (60%) of H.M variants. These variants were all in active domains of the *cbrR* gene, which contains two response regulator domains and one GGDEF domain. CbrR is a known regulator of bile resistance in *C. jejuni* and is required for host colonisation as shown by a *cbrR* mutant strain exhibiting a reduction in chicken colonisation (Raphael *et al.*, 2005). The 11168Ca strain was used for this study as it was known to exhibit high-level colonisation of chickens and was presumed to be a hyper-motile variant of NCTC11168. Interestingly, this variant was hyper motile and had a G deletion in the GGDEF domain. Indicating role of this gene regarding to hyper-motility.

The results of the whole genome analysis suggest that mutations in the *cbrR* gene could affect its expression and that these mutations increase the expression of the flagella gene leading to the formation of a hyper-motility phenotype. Screening of deletion mutants in *Salmonella enterica* serotype Typhimurium for motility has shown that many genes involved in flagellar regulon, LPS biosynthesis and chemotaxis are associated with variation in motility phenotypes (Bogomolnaya *et al.*, 2014). One gene with a similar function to *cbrR* was *phoP*, which encodes the response regulator and DNA-binding protein of the PhoP/PhoQ two component system and is required for resistance to bile (van Velkinburgh and Gunn, 1999). An assessment of the motility phenotype in a Δ *phoP* mutant found that this mutant had a hyper-motility phenotype on swarming plates that was correlated with increased flagellin expression on the bacterial surface (Bogomolnaya *et al.*, 2014). It can thus be hypothesised that enhanced formation of hyper-motility in *C. jejuni* could be the

result of up-regulation of flagellin production. Future work should concentrate on experimental confirmation of this novel correlation between *cbrR* genetic variation and hyper-motility phenotypes in *C. jejuni*. This could be achieved through complementing mutations in H.M variants, and then determining the amount of FlaA protein O.M fraction of H.M variants containing mutations in the *cbrR* gene as compared to complemented strains by Western analysis and ELISA using the anti-FlaA antiserum.

6.2. The immunogenicity of glycosylated FlaA protein associated with long-term colonisation.

The flagellin protein is an immunodominant antigen in *C. jejuni*-colonised chickens (Cawthraw *et al.*, 1994) and in chickens immunised with *Campylobacter* antigens (Widders *et al.*, 1998). The generation of structural diversity in the flagellin glycan might be an important mechanism in *C. jejuni* for evading host immune responses. An initial observation was that there was higher reactivity to rFlaA than gFlaA in the D-1 sera when the birds were two weeks old. Within the first 2 weeks after hatching, chickens do not have a mature immune system, but can exhibit protection from MABs (Shoaf-Sweeney *et al.*, 2008). This speculation is supported by the observation of strong reactivity to *C.jejuni* of the D-1 serum that appeared to contain a high level of maternal antibodies with reactivity for epitopes consisting of unmodified amino acids of rFlaA but not modified FlaA. The lower reactivity of gFlaA may be due to these sera not having MABs that bind to glycan epitopes. The presence of some reactivity to both rFlaA and gFlaA could be due to conservation and lack of modification of the N-terminal and C-terminal domains whereas glycan moieties cover the central domain, which is the major surface exposed region. Collectively, it might also be that MABs contain specific anti-FlaA antibodies that reacted with unmodified epitopes in rFlaA and gFlaA proteins. It is likely also that MABs containing specific anti-FlaA antibodies can react with conserved glycanform FlaA but not the new glycan FlaA variant. In addition, there was not colonisation in

broiler flocks during first two weeks and this may justify the presence of the reactivity of gFlaA and rFlaA with D-1 sera.

A similar observation was also found for the D+14 sera, which had not developed a strong specific antibody response to the conserved epitopes or glycans of gFlaA. After 2 weeks of colonisation, the birds immune responses will have the ability to react with different glycan forms and these specific anti-glycan antibodies will have the ability to exert an effect on the glycosylation states of the colonizing *C. jejuni* strain. However the PV states of the inoculum and D+14 colonies were similar and changes only appeared at D+28. This was shown by the lack of signals being detected with gFlaA for D-1 and D+14 sera whereas reactivity was observed with the D+28 sera.

During long-term colonisation of *C.jejuni*, it was observed that there was an increase in development of antibodies against glycosylated FlaA protein. This observation is supported by the comparable reactivity of *C. jejuni* D+52 sera with rFlaA and gFlaA (Ca /H). It is a clear indication of the development of specific anti-glycan antibodies. However, these antibodies might not have a function as the level of caecal colonization at D+14 and D+52 was similar, indicating no effect of these sera on colonisation. In future work, a Serum Bactericidal Activity (SBA) assay could be performed to determine whether the sera have the potential to kill the bacteria and cause clearance of the chickens. The ability of *C. jejuni* proteins to elicit antibodies was shown, in which chickens have been vaccinated with high immunodominant proteins such as CadF and FlaA, therefore high reduction in *C. jejuni* colonization was observed in vaccinated chickens. (Neal-McKinney *et al.*, 2014)

The diversity of glycan FlaA variants could have a role in controlling elicitation of specific glycan antibodies and antibodies to different glycan phase variants. However, the reactivity to gFlaA proteins derived from Ca and H variants was indistinguishable with *C.jejuni* D+52 sera possibly due to the similarity of PV states in the output populations at this time point (Ca and H) (Figure 5.1), so that there might be some antibodies that respond to both isoforms of glycosylated FlaA proteins at similar levels.

In order to identify the dominant structural glycans that confer the immunogenicity to the FlaA proteins, it would be rationale to have mutants in different combinations of glycans. It is obvious that the similar reactivity between PV glycosylation gene mutants that have been probed with sera containing developed anti-specific anti glycan is due to the possibility of generation of anti-glycan-antibodies against combinations of glycans.

This investigation has some limitations (mentioned in section 5.7). Despite these issues, this work could be the starting point for more research on the phase variable genes that are involved in the modification of FlaA immunogenicity and hence increasing the ability of *C.jejuni* to colonise birds.

6.3. Evidence for functional contributions of *Cj1295*, *Cj1296* and *Cj1310c* PV genes.

Another interesting finding obtained in this study correlated to the *Cj1295* phase variable gene, which is involved in producing structural variation in FlaA protein, and the hypothetical phase variable gene *Cj1296* in terms of motility and colonisation.

Initially, it can be stated that the product of *Cj1295* may not be associated with flagellar synthesis and assembly and hence motility. In contrast, Gene-Scan analysis of *Cj1295* in the output population following bird's colonisation with either variant Ca or H showed that 90% of the population comprised *Cj1296*-ON variants (Bayliss *et al.*, In preparation). According to Hitchen *et al.* (2010) the product of a functional copy of *Cj1295* leads to structural glycan variation by adding a di-O-methylglyceroyl-modified version of pseudaminic acid to the major flagellin protein, FlaA. Therefore, it might be that the expression of *Cj1295* has a role in colonisation by producing a functional glycan. Moreover, the potential contribution of the *Cj1295* protein to colonisation suggests that a specific antibody against this glycan might enhance the immunogenicity of the FlaA protein. *C. jejuni* 52+D sera obtained from birds colonised with *C. jejuni* that had a high proportion of *Cj1295*-ON variants was used to detect the immunogenicity of FlaA within O.M protein of *Cj1295::kan* mutant and *Cj1295comp* strain (Fixed-ON) by the immunoblotting assay. These sera showed

similar intensity bands with these two FlaA glycan-forms as compared to gFlaA wild type and rFlaA. It might be that no specific antibodies were generated against individual glycans, alternatively there may have been specific antibodies against a combination of different glycan forms. Although changes in flagellin glycan forms can alter autoagglutination behaviour of *C. jejuni* (van Alphen *et al.*, 2008), autoagglutination assays with the $\Delta Cj1295$ mutant did not show a significant difference between $\Delta Cj1295$ mutant, complemented mutant and wild type strains. It might be that the function of this gene is to contribute to the ability of functional flagella to adhere to epithelial cells. Therefore, it would be useful to track the functional consequences of the presence of functional Cj1295 by performing adhesion and invasion assays in future work.

In terms of *Cj1296*, the double deletion inactivation strategy to create a $\Delta Cj1295/Cj1296$ *C. jejuni* mutant strain was not successful. This mutagenesis strategy included removal of part of the ORFs upstream and downstream and therefore it could be that the deletion affects the transcription of these genes. It is important to maintain up and downstream genes in frame with the target gene for mutation to prevent polar effects caused by a frameshift in a downstream co-transcribed genes. In future work a strain could be created by an insertional inactivation strategy as this strategy has been applied successfully to knock out *Cj1295* (Hitchen *et al.*, 2010).

The trend towards an ON state of expression for *Cj1296* in the colonisation experiment (Bayliss *et al.*, In preparation) and an OFF state in motility experiments was similar with *Cj1295*. In addition, they are in one operon (*Cj1293-Cj1298*) containing genes likely contributing to the biosynthesis of diacetamidobacillosamine (DAB) biosynthesis (Obhi and Creuzenet, 2005). It can therefore be assumed that the function of *Cj1296* would resemble the role of *Cj1295* and also might involve the modification of FlaA protein with a new version of pseudaminic acid. Future work needs to create a proper mutant defective in *Cj1296* and considering the polarity effect, then analysis of the profile of purified FlaA protein bands from a *Cj1296* mutant and wild type by analysis using mass spectrometry.

In a similar manner to *Cj1295*, the *Cj1310c* homopolymeric-tract-containing gene does not have an influence on motility or the autoagglutination behaviour of *C. jejuni*, presumably because the potential glycan variant may not result in an altered charge of the protein. Additionally, the *Cj1310c*-ON variant in combination with the *Cj1295*-ON and *Cj1296*-ON variants may become a dominant variant during long-term colonisation as these Glycan might prevent the immune systems from recognising other immune reactive FlaA epitopes Therefore, the contribution of these three genes being expressed in association with colonisation seems to evade the response of the immune system in generating anti-glycan antibodies against this combination of glycans. This study came out with the hypothesis that the function of the *Cj1310* gene may be to generate glycanform of the FlaA protein which have a role in the *C. jejuni* colonisation and evasion of the immune responses in combination with *Cj1295* and *Cj1296* genes.

6.4. Summary of findings

This current study has found two new observations; i) *cbrR* gene mutations mediate hypermotility in *C.jejuni*, II) the potential function of *Cj1310* in generating new glycan FlaA variants. In addition, clear evidence of the influence of the expression of glycosylated phase variable genes in increasing the immunogenicity of FlaA protein via increasing the reactivity with sera collected from infected birds in a sequential manner.

Appendix A

Primers used for 28-locus-CJ11168 PV-analysis. Primers from Lango-Scholey *et al.* (2016).

Primer name	Sequence	Primer mix	Dye
Cj0031-fwd-FAM	GGCTTTGATCTCATCATCGG	A	FAM
Cj0032-rev	GCAAAGCTTCCCCATATCCT		
Cj0045-fwd-FAM	TTTTACACTAGAACACAGAAG	A	FAM
Cj0045-rev	CCTTAAAGTGCGAAAAATGTG		
Cj0046-fwd-NED	TCAAATACTGCAAGAGCAGG	E	NED
Cj0046-rev	TAGAAGCATTAGGCGTGG		
Cj0171-fwd-NED	TGGTTGTGGAAATGGAGTGC	C2	NED
Cj0171-rev	GCTCCTTCATTGCATAGTTC		
Cj0275-fwd-NED	ATTACTCGTGATGTAAGTGG	C1	NED
Cj0275-rev	AAACCTACAACCTTTATCTCC		
Cj0565-fwd	AATTTCACTTCCCCCTTGACT	D	VIC
Cj0565-rev-VIC	TTTTGCAACATCGCGTAGAA		
Cj0617-fwd-VIC	TGGTATAATGCAAGCTATGG	D	VIC
Cj0617-rev	AAATCAATACTCCAAGGAGC		
CapA-fwd-FAM	TATTTCTAATGATGGGCAAC	A	FAM
CapA-rev	GAACGAACATTTACACCCAT		
Cj0676-fwd-NED	ATGCTTATTCCTAGTGCCTG	E	NED
Cj0676-rev	TGCATTTAAACCCAAAGAATCC		
Cj0685-fwd-FAM	GATAGCGAATATAACCTCTAAATTC	A	FAM
Cj0685-rev	GAAGAAATCCGCCAATCAAAG		

Primer name	Sequence	Primer mix	Dye
Cj1139-fwd-VIC	GCAACTTCACCTTATATC	D	VIC
Cj1139-rev	TAAATTCTTTGTTGTTGATTTTCC		
Cj1144-fwd-NED	GATGTTGTGATTCTTG	C2	NED
Cj1144-rev	GTAGCAGCGTTTAGTG		
Cj1295-fwd-NED	TTCCTATCCCTAGGAGTATC	E	NED
Cj1295-rev	ATAGGCTTCTTTAACATTCC		
Cj1296-fwd-NED	ATAAAGTGCATTCTAAAGGC	C1	NED
Cj1296-rev	CAGCAAAGGAAAAAATAGGG		
Cj1305-fwd-VIC	CAACTTTTATCCCACCTAATGGAG	D	VIC
Cj1305-rev	AAAGCCGAACCCGAATTATC		
Cj1306-fwd-NED	TTTATTCTTCGCGTGGAGA	C1	NED
Cj1306-rev	AAAAATGATCGCCCTGCAT		
Cj1310-fwd-FAM	GAACAAATTATTCCTCTTATAG	E	FAM
Cj1310-rev	TCGAAATAAAATTCCCCTTGA		
Cj1318-fwd-FAM	TCCGTGCGTCCTTCTTTTGGAC	B	FAM
Cj1335-fwd-NED	CACAATTGGTTTATCCAAGG	C2	NED
Cj1318-35-rev	GTTTGCAACTCTTTAATGGG		
Cj1321-fwd-VIC	AAAAAGGAATGATGCGTTGC	D	VIC
Cj1321-rev	CCCGCTCCTATGATGATGAC		
Cj1326-fwd-FAM	CTTTTGAATAGATATAGTTCC	A	FAM
Cj1326-rev	TTAGAGGTATGTAGTAAAGAC		
Cj1342-fwd-FAM	TTGGCAATCGTCCTCAAACC	A	FAM
Cj1342-rev	GCCAAATGCGCTAAATATCC		

Primer name	Sequence	Primer mix	Dye
Cj1420-fwd-FAM	GCTAGTTCTTTCCATTGGAC	B	FAM
Cj1420-rev	CTACAATGTGGCGAGGATTC		
Cj1421-22-fwd	TTGGGTATTTAAGTTGGGGAAA		
Cj1421-rev-VIC	TCAAACCCATCTTTATCATTTTCT	D	VIC
Cj1422-rev-NED	AATGATTTTGCTTTGCAGGAA	E	NED
Cj1426-fwd-FAM	TATAGCCGATCCACAAGG	B	FAM
Cj1426-rev	GATATAACTTTGCCCGCCAC		
Cj1429-fwd-FAM	ATGGAGATGGTGGTTATGTG	B	FAM
Cj1429-rev	ACTATCCGAAACACCCAAAG		
Cj1437-fwd-VIC	GTGCTAGGATGGAATTTGTG	B	VIC
Cj1437-rev	CAAACAAGGTGAAAACCTCC		

Six multiplexed PCRs are carried out with primers from the six mixes: A, B, C1, C2, D and E. Primers ending in 'FAM', 'NED', or 'VIC' have the named fluorescent marker attached, other primers are not marked. Note that *Cj1318* and *Cj1335* share a forward primer, and *Cj1421c* and *Cj1422c* share a reverse primer so there are only 54 primers in total, not 56.

Appendix B

Motility was assessed as diameter, in Millimeters, of growth zones after 48h of incubation. Results are shown as the mean of three independent experiments

	Samples	Swarming Mean \pm SD
Motile variants	A.1.M	21.3 \pm 1.05
	A.1.1	24.3 \pm 2.02
	A.1.2	26 \pm 1.7
	A.1.3	17.083 \pm 1.01
	A.2.1	16.88 \pm 0.70
	A.2.3	28.16 \pm 2.08
	A2.2.1	29.2 \pm 1.3
	B.1.M	25.86 \pm 1.43
	B.1.1	29.75 \pm 0.66
	B.1.2	25 \pm 1.32
	B.2.1	28.6 \pm 0.85
	B.2.2	27.93 \pm 1.35
	B2.1.M	26.83 \pm 0.76
	B3.1.1	24.88 \pm 8.3
	C.1.1	27 \pm 2.77
	C.1.2	28.3 \pm 1.9

	Samples	Swarming Mean \pm SD
Hyper-motile variants	A.2.2	43.18 \pm 0.86
	A2.1.M	42.05 \pm 0.82
	A2.1.1	43.05 \pm 1.74
	A2.2.2	54.05 \pm 3.73
	A3.1.M	47.76 \pm 67
	A3.1.1	55.41 \pm 11.18
	A3.2.1	47.41 \pm 11.38
	A3.2.2	47.68 \pm 5.80
	B.2.3	41 \pm 4.33
	B2.2.1	59.96 \pm 0.50
	B2.2.2	62.95 \pm 0.57
	B3.1.M	69.46 \pm 6.9
	B3.2.2	46.43 \pm 1.36
	C.1.M	50.75 \pm 5.9
	C.1.3	45.18 \pm 0.354
	C.2.1	52.28 \pm 0.26
	C.2.2	53.9 \pm 0.53
	C.2.3	69.03 \pm 3.190
	C2.1.M	49.33 \pm 3.329
	C2.1.1	56.15 \pm 2.10
	C2.2.1	60.61 \pm 1.077
	C3.1.M	55.816 \pm 3.4
	C3.1.1	56.63 \pm 2.9
	C3.2.1	55.16 \pm 4.6
C3.2.2	65.68 \pm 1.7	

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