Characterisation of CapC, a novel strain-specific autotransporter in Campylobacter species

By

JAI MEHAT

Submitted for the degree of Doctor of Philosophy

Faculty of Health and Medical Sciences
University of Surrey
Guildford, Surrey, UK

March 2017
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Acknowledgements

Foremost I wish to express my sincere gratitude to my supervisor Professor Roberto La Ragione for his guidance and reassurance over the last four years. Thanks also to Dr Simon Park for his supervision and advice ever since I was an undergraduate.

I am especially grateful to Dr Dany Beste for all she has done for me in enabling me to transfer from the MSc course to a PhD.

I am also extremely thankful to Tim Baker who has been a constant source of help and reassurance to me ever since I first step foot into a laboratory.

Thanks also to Dr Arnoud van Vliet, without whom this thesis would never have been completed. I would also like to thank Effa Mohd Esah, Salim Al-Hashmi and Mishaal Alanazzi for their mentorship throughout my PhD.

Thank you to Becky Clarke and Jade Passey. Your unwavering friendship and support has been a blessing and I would not have been able to do this without you. My utmost appreciation also goes to, Dr Joy Leng, Dr Susan McNally, Dr Mike Hornsey and Dr Jono Betts for their advice, attention and support. Thanks also to all members of the AX Microbial Sciences Department.

This journey would not have been possible without my parents and family. Thank you for your enduring support, belief and understanding.

And to Wendy, the last four years would not have been possible without your continual patience, positivity and compassion in the most difficult of times.

For your enthusiasm, kindness, inspiration and love throughout, thank you.
### Conference Papers and Awards

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<th>Event</th>
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<td>2016</td>
<td>Characterisation of CapC, a novel strain-specific autotransporter in Campylobacter species.</td>
<td>FHMS Festival of Research, University of Surrey, UK.</td>
<td>Winner Student Research Prize</td>
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<tr>
<td>2015</td>
<td>Characterisation of CapC, a novel strain-specific autotransporter in Campylobacter in Campylobacter species.</td>
<td>PGR Conference, University of Surrey, UK.</td>
<td>2nd Place, Oral Presentation.</td>
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<tr>
<td>2014</td>
<td>Characterisation of CapC, a novel strain-specific autotransporter in Campylobacter species.</td>
<td>Conference Poster, CampyUK14, Liverpool, UK</td>
<td></td>
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<tr>
<td>2014</td>
<td>CapC, a novel strain specific autotransporter in Campylobacter species, contributes to motility and virulence.</td>
<td>Conference Poster, Society For Applied Microbiology, Brighton, UK</td>
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Abstract

_Campylobacter jejuni_ and _Campylobacter coli_ are recognised as the principal causative agents of bacterial gastroenteritis in the developed world. However, despite the identification of factors integral to infection, characterisation of the virulence strategies employed by _Campylobacter_ remains a significant challenge. Bacterial autotransporter proteins comprise the largest and most diverse class of secretory proteins in Gram-negative bacteria; notably almost all previously characterised autotransporter proteins contribute to bacterial virulence to some extent. The aim of this study was to characterise CapC, a newly identified, strain-specific gene predicted to encode an autotransporter protein, and to examine the contribution of this factor to the virulence of _Campylobacter jejuni_.

The _capC_ gene was initially confirmed as being encoded by approximately 60% of _C. jejuni_ and _C. coli_ human clinical and poultry associated isolates. Moreover, CapC was confirmed as a member of the autotransporter family through the use of bioinformatic prediction tools and the localisation site of this protein was determined as the outer membrane of _C. jejuni_. Targeted mutagenesis of the _capC_ gene in _C. jejuni_ 81116 and _C. jejuni_ M1 and subsequent comparison of wild-type and isogenic mutant strains demonstrated that CapC contributes directly to virulence in the _Galleria mellonella_ invertebrate model (p=0.00017; p=0.002323). Furthermore, tissue culture assays using non-polarised, partially differentiated Caco-2 and T84 intestinal epithelial cells indicate that deletion of CapC resulted in significantly decreased adhesion and invasion efficiency. Additional analyses indicated that CapC primarily contributes to adhesion to intestinal epithelial cells. Additional assays showed that deletion of the _capC_ gene has no significant phenotypic effect on cytotoxicity in a Caco-2 cell model.

A secondary aim of this study was to examine the distribution of _capC_ amongst campylobacters and to establish any potential genetic associations of this virulence determinant. Using publically
available genome sequences, capC was established to be highly prevalent in *C. jejuni* (67.9%) and *C. coli* (84%). *Campylobacter* autotransporter proteins were also shown to be present in truncated and full length forms. Interestingly, full length CapC was shown to be primarily associated with the ST-45, ST-283 and ST-573 clonal complexes in *C. jejuni* and the ST-828 clonal complex in *C. coli*. Furthermore, this study detailed the identification of a novel autotransporter in *Campylobacter* species, tentatively designated as CapD. This autotransporter was found to be genetically distinct from CapC and is the most prevalent autotransporter identified in *Campylobacter* species.

The studies presented in this thesis indicate that CapC is a strain-specific virulence determinant in *Campylobacter* species that is associated with select lineages of *C. jejuni* and *C. coli*. CapC contributes to the integral infection process of adhesion however further studies are required to fully elucidate the exact nature of this interaction. Additionally, it can be concluded that possession of *Campylobacter* autotransporter proteins is dependent on genetic background.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CapC</td>
<td>Campylobacter autotransporter protein C</td>
</tr>
<tr>
<td>CapD</td>
<td>Campylobacter autotransporter protein D</td>
</tr>
<tr>
<td>CCV</td>
<td>Campylobacter-containing vesicle</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>Cia</td>
<td>Campylobacter Invasion antigens</td>
</tr>
<tr>
<td>CJIE</td>
<td>Campylobacter jejuni Insertion element</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Standards Authority</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>FSA</td>
<td>Food Standards Agency</td>
</tr>
<tr>
<td>GBS</td>
<td>Gullain-Barré Syndrome</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse-Radish Peroxidase</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial Methylated Spirit</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>kD</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>Kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple Cloning Site</td>
</tr>
<tr>
<td>MHA</td>
<td>Mueller Hinton Agar</td>
</tr>
<tr>
<td>MIST</td>
<td>Microbial In Silico Typer</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-Locus Sequence Type</td>
</tr>
<tr>
<td>mM</td>
<td>milliMolar</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>oC</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>oePCR</td>
<td>overlapping extension PCR</td>
</tr>
<tr>
<td>OMVs</td>
<td>Outer membran Vesicles</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post infection</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulse-Field Gel Electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>RM</td>
<td>Restriction Modification</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SOB</td>
<td>Standard Outgrowth Broth</td>
</tr>
<tr>
<td>spp.</td>
<td>Species</td>
</tr>
<tr>
<td>SRBCs</td>
<td>Sheeps' Red Blood Cells</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence Type</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TMPD</td>
<td>Tetra-methyl-p-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>TPF</td>
<td>1,3,5-Triphenyltetrazolium formazan</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5 Triphenyltetrazolium Chloride</td>
</tr>
<tr>
<td>UV</td>
<td>UltraViolet</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>VDC</td>
<td>Vertical Diffusion Chamber</td>
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Chapter 1: Introduction
*Campylobacter jejuni* and *Campylobacter coli* have assumed increased prominence over the last two decades as major causative agents of human gastroenteritis, making these pathogens of significant importance with regard to human and veterinary health worldwide. *C. jejuni* and *C. coli* are Gram-negative, spiral shaped (Figure 1.1), microaerophilic bacteria which have very similar physiological properties and are often referred to generically as campylobacters, though they exhibit distinct genotypic and lineage-specific differences. *C. jejuni* and *C. coli* are major bacterial species implicated in human foodborne gastroenteritis as well as diarrhoeal disease in animals.

![Figure 1.1: Scanning electron microscope image of *Campylobacter jejuni*, (indicated by the arrow) illustrating the corkscrew appearance and bipolar flagella. Cells are approximately 0.5-8um x 0.2-0.5um in size (Sourced from Virginia-Maryland Regional college of Veterinary Medicine, Blacksburg.](image)

### 1.1. History of *Campylobacter*

The initial discovery of *Campylobacter* is commonly attributed to the paediatric bacteriologist Theodor Escherich who in 1886 observed a non-culturable, spiral form bacterium in stool samples and large intestinal mucus associated with diarrhoea in children and kittens (Kist, 1986). A large number of publications followed Escherich’s seminal work describing the incidence and occurrence of “Spirilla” associated with dysenteric disease (Kist, 1986). Current information
regarding the nature and illness caused by *Campylobacter* suggest that this organism described by Escherich was in fact a member of this genus. In 1905, John Mcfadyean and Stewart Stockman, the chief investigators in an inquiry into epizoonotic abortion in the UK eventually reported the isolation of a novel spiral, motile organism from the placental cotyledon smears of aborting ewes (McFadyean and Stockman, 1913). The isolated organism was initially classed as a member of the genus *Vibrio* but is now known to have been *Campylobacter fetus*; the foremost cause of infectious abortion in the US. Human infection was first recognised in 1938 in Illinois, USA following a milk-borne outbreak of diarrhoea affecting the inmates of two adjacent state institutions. In this instance, whilst faecal cultures were negative, organisms resembling *Vibrio* species were grown in liquid medium from blood samples of 13 patients (Levy, 1946). Another outbreak of this *Vibrio*-associated gastroenteritis occurred in 1947. Once again, patients presented with mainly gastrointestinal complaints however attempts to isolate the organism from faecal samples were unsuccessful (Vinzent *et al.*, 1947).

The foundation for the modern classification of campylobacters was set by Elizabeth King in 1957. Close examination of several *Vibrio* isolates led King to propose two different types of vibrios associated with enteric disease. Thus based upon optimum growth temperature, *V. fetus* was established as distinct from thermophilic *Vibrios* which would become known as *V. jejuni* and *V. coli*. The term “*Campylobacter*” was first proposed by Sebald and Véron in 1963. Derived from the Greek language, *Campylobacter* means “curved rod” and refers to the characteristic morphology of the organism. The name was initially proposed in order to differentiate these organisms from classical cholera groups of *Vibrio* from which they differ markedly; the *Campylobacter* spp. had a different G+C content to that of the *Vibrio* spp. and could not metabolise sugars. Numerous reviews describe the naming and identification of *Campylobacters* since this discovery (Skirrow, 2006; Butzler, 2004).
An effective isolation procedure for *Campylobacter* from human stool samples was developed by Dekeyser and Butzler in 1972 (Butzler *et al.*, 1973; Dekeyser *et al.*, 1972). The procedure involved filtering stool samples through 0.64μm membrane filters which were then inoculated onto agar (Moore *et al.*, 2005), however this novel method proved too cumbersome and garnered little response from the scientific community. In 1977, Skirrow developed a selective blood based medium and a simple, direct plating technique involving culturing faecal samples onto this medium and incubation at 42° C under microaerobic conditions (Skirrow, 1977). These advances allowed microbiology laboratories to routinely isolate *Campylobacter* from clinical specimens. The frequency of human *Campylobacter* infection was subsequently made apparent leading to a wealth of epidemiological research which bought to light the true incidence of *Campylobacter* infection (Moore *et al.*, 2005). *Campylobacter* is now recognised as the leading bacterial cause of enteritis in the developed world (Moore *et al.*, 2005; Friedman *et al.*, 2000).

### 1.2. The Family Campylobacteraceae

Since the inception of the genus (Sebald and Véron, 1963), the taxonomy of *Campylobacter* has changed considerably. Despite having been shown as distinct from Vibrio species, *C. fetus* and *C. bubulus* (now called *C. sputorum*) were still widely referred to as *Vibrio* spp. by the microbiology community until the study of Butzler *et al.*, (1973) greatly increased interest in *Campylobacter* by highlighting their prevalence in human diarrhoea (On, 2001). Aided by the aforementioned improved isolation techniques, the genus *Campylobacter* grew rapidly with novel species and subspecies being isolated from a range of diseases and ecological niches (Vandamme and De Ley, 1991). The advent of more advanced genotyping tools and the potential of the 16S rRNA gene for determining phylogenetic relationships lead to extensive re-arrangement of *Campylobacter* taxonomy. The creation of the family Helicobacteraceae and the genus *Helicobacter* in 1989
allowed re-classification of *Campylobacter pylori* and *Campylobacter mustelae* and reconciled major differences from *Campylobacter* species such as flagella structure, fatty acid and methaquinone composition and 16S rRNA sequences (Goodwin *et al.*, 1989). In 1991, Vandamme and De Ley proposed the family *Campylobacteraceae* which included the genus *Campylobacter* and *Arcobacter*, thus separating this group of bacteria from other Epsilon-Proteobacteria such as *Helicobacter* and *Wolinella* and (Vandamme and De Ley, 1991). Figure 1.2 shows a representative phylogenetic tree of the kingdom Bacteria and the relative position of the genus *Campylobacter*. Figure 1.3 shows the present members of the *Campylobacteraceae*.

![Figure 1.2: A rooted phylogenetic tree of the Kingdom Bacteria showing a selection of the major phyla; Proteobacteria, Firmicutes, Cyanobacteria, Actinobacteria and Spirochaetes. The Proteobacteria are further sub-divided in to classes Alpha-Proteobacteria, Beta-Proteobacteria, Gamma-Proteobacteria and Epsilon-Proteobacteria. Delta- and Zeta-Proteobacteria are not shown. *Campylobacter* resides within the Epsilon-Proteobacteria. Adapted from Bern and Goldberg (2005).](image-url)
Figure 1.3: A Phylogenetic dendrogram based on 16S rRNA sequences showing select species of the Epsilon-Proteobacteria. The family Campylobacteraceae is comprised of the genera *Campylobacter*, *Arcobacter* and *Sulphurospirillum*. The genera *Dehalospirillum* and *Bacteroides* have since been reclassified. These genera are shown in relation to members of the family Helicobacteraceae. Figure taken from Wassenaar and Newell (2006).
1.3. Ecology of *Campylobacter jejuni* and *C. coli*

*Campylobacters* are highly successful bacteria that are able to colonise the intestinal mucosa of a broad range of hosts but they are strongly associated with the avian host in particular; an ancient association that *Campylobacter* has most likely been engaged in since before humans existed (Brendan Wren, Personal Communication). As such, *Campylobacter* has undergone considerable within-host evolution which is reflected in their biology and diversity. In spite of this close association with the avian niche, high throughput sequencing technology has shown *Campylobacter* exhibits large-scale host and habitat variation exemplified by the fact that certain lineages and sequence types are adapted to particular ecological niches (Sheppard et al., 2013). Ecological niches and resulting transmission routes of *Campylobacter* species are depicted in Figure 1.4A.

![Figure 1.4A: The various transmission routes and general ecology of *Campylobacter jejuni* and to a lesser extent, *Campylobacter coli*. Adapted from Young et al., (Young et al., 2007), the figure shows how *Campylobacter* resides in warm blooded animals, but are considered to be commensal organisms of poultry. Humans become infected with *C. jejuni* through the consumption of contaminated poultry products, animal products and contaminated water sources, where *C. jejuni* may associate with amoebae (Snelling et al., 2005).](image-url)
Interest in *C. jejuni* and *C. coli* stems from their role as zoonotic, foodborne pathogens causing infectious intestinal disease in humans via the consumption of contaminated food, particularly raw poultry. The *C. jejuni* and *C. coli* genetic lineages commonly implicated in human disease are highly prevalent in broiler flocks; as such there has been an increased focus in recent years on understanding the ecology of *Campylobacter*, in both the avian host and the environment, in order to gain new insight into potential control strategies. To date, *campylobacters* have been detected in many environments from farm and urban settings to slaughter plants, as well as being isolated from humans, wild birds and mammals, companion animals, drinking water, farm production animals and common seals (Horrocks *et al.*, 2009).

### 1.3.1. *Campylobacter* in the avian host

*C. jejuni* and *C. coli* are able to colonise the caeca of chickens in numbers of up to $10^9$ cfu/g (Newell and Fearnley, 2003). *Campylobacters* grow optimally at temperatures near 42 °C and the higher metabolic temperatures (42 °C) found in poultry species may predispose poultry to be a prominent reservoir for *Campylobacter* species (Penner, 1988). Modern broiler production is a large-scale, commercial operation in many countries in the developed world. It has been proposed that intensive farming practices involved in this production have led to increased transmission rates between broilers and throughout flocks as well as selection of robust *Campylobacter* lineages that are able to proliferate in chickens and also spread to other ecological niches (Shephard *et al.*, 2014; Newell, 2002). Figure 1.4B is a photograph of conditions within a broiler house that favour microbial proliferation.
Figure 1.4B: Broiler chickens within a broiler house. The atmospheric conditions, close proximity of other chickens and co-prophagic behaviour of chickens favour microbial proliferation and transmission (Young et al., 2007; Newell and Fearnley, 2003).

The chicken reservoir is reported to be responsible for 80% of human Campylobacteriosis which highlights the importance of the avian host to human health and disease (EFSA, 2010). In spite of this, there remains a dearth of understanding regarding *Campylobacter* interaction with the avian host. The majority of what is known regarding colonisation is derived from studies using broiler chicken breeds which are considered the model for avian colonisation. *C. jejuni* and *C. coli* are largely considered to be commensal organisms of the chicken gastrointestinal tract however it has been proposed that this apparent commensalism may in fact be controlled parasitism (Williams et al., 2014). Chicken colonisation and the paradigm for commensalism in chickens will be explored in greater depth later in this chapter.
1.3.2. *Campylobacter jejuni* and *C. coli* in humans and mammals

*Campylobacters* cause a diarrhoeal disease in humans and it is thought that disease symptoms reflect the expression of bacterial factors associated with adaption to the avian host (Newell, 2002). *Campylobacter* species readily colonise the gastrointestinal tracts of domestic and wild animals and can produce severe, acute gastroenteritis in humans (Horrocks *et al*., 2009). Human infection is most commonly acquired through the consumption of raw, undercooked poultry or poultry products, however, unpasteurized milk, raw vegetables and environmental water sources are all potential reservoirs and sources of human infection (Verhoeff-Bakkenes *et al*., 2011).

Colonisation of the human gastrointestinal tract is often transient and the infection caused by *Campylobacter* is usually self-limiting (Fearnley *et al*., 2008; Friis *et al*., 2010). Disease-susceptible human populations are generally located in industrialised countries and reports of asymptomatic carriage of *Campylobacter* species by human hosts (non-disease-susceptible populations) are most common in developing countries (Lee *et al*., 2013). The occurrence of asymptomatic infection suggests that enteritis in humans differs depending on immune responses. The incidence of asymptomatic infection reported by Lee *et al.* (2013), may reflect the emergence of *Campylobacter* lineages and genotypes that are adapted to long-term survival in the human gut. Another possible explanation for this is that repeated exposure to *Campylobacters* in the environment, particularly during infancy, generates a tolerant immune response thereby facilitating the generation of a non-disease-susceptible population (Newell and Nachamkin, 1992), though recent reports suggest a causal relationship between asymptomatic carriage and malnutrition in developing countries (Lee *et al*., 2013).

Whilst a wide array of animal hosts have been shown to be carriers of *C. jejuni* and *C. coli* comparatively little is understood regarding *Campylobacter* behaviour in these hosts. Improved genomic technologies and the development of Multi-locus Sequence Typing (MLST) has revealed
that natural populations of *Campylobacter jejuni* and *C. coli* possess host specific genotypic adoptions that are indicative of a long-term association with specific host species (Barnes *et al.*, 2007). Pigs are strongly implicated as a reservoir for *C. coli* and it is thought this species is particularly suited to the swine production environment (Thakur and Gebreyes, 2005). Epidemiological evidence suggests that the pig reservoir serves as a source of zoonotic transmission to humans resulting in disease (Manning *et al.*, 2003). Whilst *C. coli* is estimated to only account for 10-20% of human *Campylobacter* infection (Schielke *et al.*, 2014; Roux *et al.*, 2013; Ogden *et al.*, 2009), the incidence of *Campylobacter coli* mediated enteritis in England and Wales still exceeds the incidence of *Salmonella* mediated food poisoning, highlighting the importance of the swine reservoir in foodborne illness (Tam *et al.*, 2012).

There has been considerable speculation as to whether companion animals and household pets are a possible reservoir of *Campylobacter* species. Direct transmission of *C. jejuni* from canine species to humans has previously been reported and the potential transmission from other household pets is a risk factor for human *Campylobacteriosis* (Damborg *et al.*, 2004).

Ultimately, the fact that *Campylobacter* species are prevalent in a diverse range of animal hosts such as cattle, swine, companion animals, ovine hosts and more obscure hosts such as sea otters and the freshwater crustacean *Daphnia carinata* is significant as these often neglected hosts act as reservoirs of this organism (Horrocks *et al.*, 2009; Schallenberg *et al.*, 2005). Furthermore, mammalian hosts have been shown to excrete *Campylobacter* species that are lineages associated with the mammalian gut into the environment (Ogden *et al.*, 2014). The role of animals and livestock in transmission of *Campylobacter* lineages that have the potential to either colonise chickens and/or enter the food chain is an aspect that must be considered in the development of future control measures.
1.3.3. *Campylobacter* in the environment

Campylobacters are fastidious organisms with an optimum growth temperature of 42°C with specific requirements for growth including defined atmospheric conditions of 5-6% v/v oxygen and 5-10% v/v carbon dioxide (Atack and Kelly, 2009). These conditions are thought to resemble those occurring within the avian caecum, the natural habitat of *Campylobacter*. Although campylobacters do not grow outside of the host, they can remain viable for periods in environmental habitats and in food sources. This is exemplified by documented milk-borne outbreaks (Evans *et al.*, 1996).

As a result of faecal-shedding and during transmission, campylobacters inevitably encounter hostile environmental stresses, particularly oxidative stress burdens. In spite of their sensitive and fastidious nature, *Campylobacter* can be isolated from environmental water sources, crops and a range of farm and urban environments (Ogden *et al.*, 2014). In addition, campylobacters are evidently able to survive numerous stresses during poultry processing. Considering the relative lack of classic stress regulation systems encoded in the *Campylobacter* genome, its microaerophillic nature and its notorious sensitivity to oxidative stress, desiccation and temperature fluctuations, *Campylobacter* environmental survival is remarkable and testament to the ability of the organism to adapt.

The majority of *Campylobacter*-related control measures are focused on reducing carriage within broiler chickens. It is thought that broiler colonisation may be in part due to environmental factors in addition to horizontal transmission, through unchlorinated drinking water and contaminated soil (Snelling *et al.*, 2005; Bronkowski *et al.*, 2014). It is evident that *Campylobacters* vary in their ability to survive extra-intestinally (Colles *et al.*, 2003; French *et al.*, 2005) and a number of innate and environmental factors may promote *Campylobacter* environmental survival (Newell, 2002). Isolates belonging to the ST-45 clonal complex have been
shown to be more frequently isolated from environmental sources suggesting the emergence of *Campylobacter* genotypes and lineages that are better adapted for environmental survival and that can exhibit aerotolerance and starvation survival (Bronkowski *et al.*, 2014). This is evidenced by the fact that *C. coli* has been shown to be adapted to colonisation of the agricultural niche; an adaption that is the result of the exchange of *C. jejuni* and *C. coli* sequences (Sheppard *et al.*, 2013). *Campylobacter jejuni* has also been shown to survive intracellularly within waterborne protozoa (Snelling *et al.*, 2005). Such a dynamic may contribute to chicken colonisation and survival by conferring environmental protection.

1.4. Incidence and Infection of *C. jejuni* and *C. coli* in humans

1.4.1. Clinical symptoms and sequelae

Human infection with *Campylobacter* is most commonly acquired through the consumption of poultry or poultry products that become contaminated during processing (Young *et al.*, 2007). Human infection in the developed world is often symptomatic, causing severe abdominal cramps followed by gastroenteritis producing watery diarrhoea after a 24-72 hour incubation period (Zilbauer *et al.*, 2008). Infection is usually self-limiting and lasts for approximately one week. Though more severe cases can last up to three weeks and involve acute, inflammatory, bloody diarrhoea, headaches, vomiting and malaise (Zilbauer *et al.*, 2008; Young *et al.*, 2007). The discrepancies in clinical outcome are thought to arise due to strain-specific variations in virulence and differing host responses.

In a minority of *Campylobacter*-mediated gastroenteritis cases, patients can develop serious sequelae, the most common of which is Guillain-Barré Syndrome (GBS). GBS is an autoimmune disease of the peripheral nervous system and is characterised by acute polyneuropathy that is mediated by host adaptive immunity (MacCarthy and Giesecke, 2001). GBS occurs in about 0.1%
of *Campylobacter* infections but antecedent *Campylobacter* infection is only implicated in 30-40% of GBS cases (Godschalk *et al.*, 2005) and is known to be associated with select *Campylobacter* lineages that express sialylated LOS structures that mimic human ganglioside epitopes (Nyati and Nyati, 2013). *C. jejuni* is also implicated in the onset of Miller-Fisher Syndrome, a neuropathogenic syndrome causing paralysis within the eye and urinary muscles (Yu *et al.*, 2006). In addition, *C. jejuni* and *C. coli* gastroenteritis has been associated with reactive arthritis with up to 1-5% of infections developing arthritic symptoms within six months of infection (Pope *et al.*, 2007).

### 1.4.2. Incidence in humans

*Campylobacter* is the leading cause of bacterial gastroenteritis in the developed world. In the UK, *Campylobacter* is considered to be responsible for more than 280,000 cases of food poisoning per year, with in excess of 72,000 of these cases being confirmed by laboratory reports (Food Standards Agency UK, 2016; Yahara *et al.*, 2016). As infection is thought to be under-reported significantly, the true incidence of campylobacteriosis in the UK could be even greater (Wheeler *et al.*, 1999). In the E.U., *Campylobacter* is believed to cause nine million cases of food poisoning a year with 190,000 reported cases (European Food Safety Authority Report, 2015). The incidence of *Campylobacter* gastroenteritis steadily increased in the UK throughout the 1990’s and 2000’s; an increase that may reflect improved detection methods and reporting of illness at this time. Most recent figures published by the Food Standards Agency show a 17% decline in the number of laboratory reports cases of human cases of campylobacteriosis in 2016 in the UK (FSA, 2017).
Another possible explanation for the increase in the number of *Campylobacter*-mediated gastroenteritis cases in England and Wales may be the development of intensive farming practices. Modern broiler production is extremely efficient with broiler chickens grown faster and larger than ever before (Colles *et al.*, 2016). The ever increasing intensity of farming practices over the past two decades may have led to other physiological and metabolic changes to the broiler chicken which may in turn have promoted the emergence of *Campylobacter* lineages that are better adapted to colonisation and survival with the food chain and, ultimately, to cause human infection (Yahara *et al.*, 2016; Llarena *et al.*, 2016).

Campylobacteriosis exhibits a distinct seasonality with a significant increase in the number of cases in late spring (weeks 18-21) which gradually recedes in the summer and autumn (Kovats *et al.*, 2005). Seasonality was observed to be consistent over a ten year period and is common in nations with a temperate climate (Strachan *et al.*, 2013). The precise reason for this seasonality is not fully known but is thought to be primarily temperature driven (Louis *et al.*, 2005). Work carried out by Strachan and colleagues concluded that the seasonal infection peak is likely due to environmental factors rather than foodborne transmission (Strachan *et al.*, 2013).
1.4.3. Incidence in broilers and chicken carcasses

Modern broiler production is an extremely efficient process; broilers are capable of converting 3kg of food into 2kg of meat in a lifespan of 35 days (Robins and Phillips, 2011). In spite of intensive biosecurity measures on farms, between 71-77% of chicken carcasses are contaminated with *Campylobacter* in the EU (European Food Safety Authority [EFSA], 2016), and the worldwide epidemic of human *Campylobacter*-mediated gastroenteric disease continues. This suggests a lack of understanding of key intervention points (Colles et al., 2016).

A Food Standards Agency (FSA) survey carried out in 2008 reported that *Campylobacter* was present on 65% of fresh retail chicken in the UK (FSA, 2008). In 2015, FSA released a report that documented levels of *Campylobacter* on fresh retail chicken and actively stated the prevalence of contamination by individual retailer (FSA, 2015). This report showed that across all major retailers in the UK approximately 72.8% of all samples tested were positive for *Campylobacter* (FSA, 2015). More recent figures released by the FSA have shown that the proportion of chicken testing positive for the highest level of contamination (>1000 CFU/g) was reduced from 19% to 11% (FSA. Feb 2016). It is anticipated that this reduction of these heavy levels of contamination will have the greatest positive impact on public health and reduce *Campylobacter*-mediated gastrointestinal illness. Reducing *Campylobacter* in the food chain and on chicken carcasses is a priority in terms of reducing the incidence of human disease. However, whilst traditionally viewed as a commensal of poultry, recent research has suggested that the prevalence of *C. jejuni* within broiler flocks negatively impacts on bird welfare and may affect behaviour and health of broilers (Humphrey et al., 2014). Therefore, reduction of *Campylobacter* levels within flocks should not be neglected as a key intervention point.
1.4.4. Comparison to other foodborne pathogens

In most industrialized countries, *Campylobacter* is responsible for many more cases of bacterial gastroenteritis than other bacterial foodborne pathogens such as *Listeria* species, *E. coli*, and *Salmonella* species combined (Sheppard, 2014).

**Figure 1.6:** Graphical representation of microbiological findings in cohort and general practice presentation cases in the second Study of Infectious Intestinal Disease in the Community (IID2 study) (Tam et al., 2012). *Campylobacter* spp. are clearly the most common bacterial pathogens amongst the cases with gastrointestinal disease presenting to healthcare.

Two studies of infectious intestinal disease in the UK took place between 2000 and 2012 (Tam et al., 2011). The incidence of infectious intestinal disease in the community in the UK is substantial with approximately 25% of the population suffering from an episode of IID each year which is the equivalent of 17 million cases annually (Tam et al., 2012). *Campylobacter* spp. were the most commonly identified bacterial agent in stool samples in this study as shown in Figure 1.6. (Tam et al., 2012). The significantly higher incidence of *Campylobacter*-derived infectious intestinal
disease compared to all other bacterial pathogens is surprising considering Campylobacters are fastidious organisms. Despite this, the incidence of Campylobacter-derived gastroenteritis is higher than for other more resilient foodborne pathogens showing that this microaerophillic organism is able to thrive in an aerobic world.

1.4.5. Economic Burden

Aside from a considerable health burden, Campylobacter poses a significant economic burden, costing the UK economy approximately £900 million per annum in terms of healthcare costs, loss of workforce productivity and direct costs to the poultry industry (FSA, 2016). Tam and O’Brien (2016) report that the costs due to Campylobacter food poisoning to patients and the UK health service in 2008-09 was £50 million (Tam and O’Brien, 2016), considerably less than the figure reported by the FSA. The cost of Campylobacter food poisoning per case was reported to be approximately £85, mostly borne by patients and caregivers through loss of income or expenditure, considerably more than the £30 per case caused by norovirus and rotavirus (Tam and O’Brien, 2016). It should be noted, however, that overall norovirus poses a greater economic burden than Campylobacter spp. because of the increased incidence of infections caused by these viruses. The estimated cost of Campylobacter food poisoning to the UK economy is eclipsed by the estimated cost to the US economy where combined losses from healthcare and loss of productivity are thought to total $1.7 billion (~£1.3 billion) annually (Batz et al., 2011).

1.5. The Campylobacter genome

In 2000, Parkhill et al. reported the first complete sequence of the genome of a strain of Campylobacter - C. jejuni NCTC 11168, a human clinical isolate (Parkhill et al., 2000). Parkhill and colleagues reported several interesting findings; classical virulence factors such as Shiga-like toxins, cholera-like toxins, haemolysins, type III secretion systems and enterotoxins purported to be present were found not to be encoded within the genome. In addition, this genome is unusual
in that Parkhill et al. reported virtually no insertion of phage associated sequences and few long repeat sequences (Parkhill et al., 2000). Since this initial genome announcement, the genomes of other commonly used laboratory strains have also been fully sequenced (Fouts et al., 2005), including re-annotation of the original C. jejuni NCTC 11168 strain (Gundogdu et al., 2010). Furthermore, the improvement in sequencing technologies has allowed the high throughput sequencing of large datasets and subsequently, a rapid increase in information regarding C. jejuni, C. coli and other Campylobacter species and thus data is being used to inform studies on all aspects of Campylobacter biology (Johnson et al., 2014). C. jejuni has a relatively small genome of 1.6-1.8Mbp and a low guanine and cytosine nucleotide content averaging at 32% (Le and van Vliet, 2014). Comparative analysis of the genomes of the common laboratory strains of C. jejuni and the wealth of scaffold Campylobacter genomes that are publically available has shown that Campylobacter jejuni exhibits a degree of conservation of overall sequences yet also stark differences between strains and isolates (Fouts et al., 2005). Campylobacter genomes display significant levels of variation at the gene level as well as in terms of promoters, operons and gene order (Dearlove et al., 2016; Fouts et al., 2005; Miller et al., 2005). This considerable variation in terms of genome content is directly responsible for the phenotypic variation observed between strains and allows for physiological and metabolic versatility. Related to the considerable level of phenotypic variation, Campylobacters possess an unparalleled proclivity to rapidly adapt to environmental shifts as exemplified by the niche variation exhibited by these organisms (Alemka et al., 2012; Jones et al., 2004). This remarkable capacity to adapt is unusual for an organism with a comparatively small genome (Skarp et al., 2015), but this can be directly attributed to traits and behaviour of the Campylobacter genome such as phase variation, horizontal acquisition of DNA and the dearth of DNA repair mechanisms (Anjum et al., 2016; Mohawk et al., 2014; Fouts et al., 2005; Jones et al., 2004).
1.5.1. Genome plasticity

Bacterial genomes are dynamic entities that are constantly evolving in response to selective pressures (Bennett, 2001). Early analyses of Campylobacter jejuni genomes revealed significant divergence of genes at various regions (Richardson and Park, 1997). The first multi-strain comparison of C. jejuni used comparative genomic hybridisation to compare C. jejuni NCTC 11168 with 11 strains of diverse origin. Results showed that approximately 21% of the genes present in NCTC 11168 either absent or divergent in these isolates (Dorrell et al., 2001). Subsequent studies using similar techniques revealed the extent to which Campylobacter varies at the genetic level, and identified the seven hypervariable, plasticity regions which encode up to half of the variable genes (Taboada et al., 2004; Pearson et al., 2003). These studies showed that the proportion of absent or divergent genes in Campylobacter genomes ranges from 2.6% to 40% (Taboada et al., 2004; Pearson et al., 2003); moreover the majority of genes within these hypervariable regions have been shown to be associated with severity of disease, or ecological source (Champion et al., 2005; Taboada et al., 2004; Pearson et al., 2003). The regions of greatest variance contain genes involved in post-translational modifications of flagella structures, lipooligosaccharide synthesis, and capsule biosynthesis (Pearson et al., 2003). These structures, and the variance within the regions coding for them are inextricably linked with strain-specific differences in virulence capacity and clinical outcome (Dwivedi et al., 2016; Louwen et al., 2013; Godschalk et al., 2004).

More recently, a transferable plasticity region identified in C. coli allows these previously assacharolytic organisms to metabolise glucose thus potentially conferring fitness advantages in a range of niches (Vorwerk et al., 2016).
1.5.2. Plasmids and insertions

There is a population of *Campylobacter jejuni* strains that contain plasmids and integrated mobile genetic elements (Bacon *et al.*, 2000). The commonly used reference strain, *C. jejuni* 81-176 contains two plasmids, named pVir and pTet, respectively (Bacon *et al.*, 2002). The pTet plasmid confers resistance to tetracycline and pVir encodes a putative Type IV secretion system and whilst its name might suggest otherwise, the contribution of this plasmid to virulence is unclear (Louwen *et al.*, 2006). Another major plasmid commonly found in *C. jejuni* and *C. coli* is pCC31; this plasmid encodes an alternative Type IV secretion system which allows conjugative transfer of genetic material (Friis *et al.*, 2007).

*C. jejuni* NCTC 11168 is notable for the lack of insertion elements within the genome (Parkhill *et al.*, 2000). However, insertion elements and phage derived sequences are major factors contributing to the diversity of other campylobacters (Brown *et al.*, 2015; Fouts *et al.*, 2005). Four of these insertion elements (CJIE1-4) were first described in the chicken isolate *C. jejuni* RM1221 and three of these elements encode DNase proteins which can protect against exogenous genetic material that may be damaging to *Campylobacter*, but at the same time these may also prevent transfer of genetic material between *Campylobacter* strains (Brown *et al.*, 2015).

The diversity of *Campylobacter* species is due in part to the varying degrees to which these species accept genetic material from exogenous sources (Wang and Taylor, 1990). There is a clear population of *Campylobacters* that are refractive to plasmid conjugation and evidently do not encode mobile genetic elements such as pro-phage sequences as a result of genetic mechanisms that protect bacteria from predatory bacteriophages (Friis *et al.*, 2010; Parkhill *et al.*, 2000). Counterintuitively, some of these protective mechanisms are even conferred by insertion elements which by their nature are derived from exogenous sources. On the contrary, plasmids pCC31 and pVir actively promote the transfer of genetic material between strains via their Type IV secretion systems (Friis *et al.*, 2007; Batchelor *et al.*, 2004). Genetic structures such as insertion
elements and plasmids have profound effects on genomic architecture, content and the resulting phenotypic behaviour and shaping variation of *Campylobacter* species.

### 1.5.3. Phase variation

A notable feature of *C. jejuni* genomes is the presence of homopolymeric tracts of DNA (Gundogdu *et al.*, 2010). These regions are often simple sequence repeats (SSRs) consisting of mononucleotide repeats of 6-12 base, most commonly guanine, in the sense direction (Pearson *et al.*, 2007). These polyG tracts are over represented within *Campylobacter* genomes. Their distribution has been found not to be due to random sequence variation and these have been shown to be associated with higher mutability (Lin and Russell, 2012). It is thought that homopolymeric tracts in *Campylobacter* mediate a phenomenon known as phase variation (Bayliss and Palmer, 2012). Phase variation is broadly defined as the reversible, high frequency alteration in gene sequence and resulting expression as a consequence of instabilities in DNA structure; essentially a form of localised DNA hypermutation that serves as a driver for variation of gene expression in a stochastic fashion (Bayliss *et al.*, 2012). This rapid switch of genes from an actively transcribed state to a non-coding state is believed to occur through strand slippage during successive rounds of DNA replication (Levinson and Gutman, 1987). Due to their structure, guanine bases are less tightly twisted than other nucleotides. This structural difference reduces stability, therefore polyG tracts have a higher propensity for strand-slippage by DNA polymerase during replication, ultimately resulting in either the incorporation of an extra guanine nucleotide or the deletion of a base thus causing a frame-shift mutation (Karlyshev *et al.*, 2002). It has been shown that longer homopolymeric tracts have a higher mutability than shorter ones. *Campylobacter jejuni* lacks canonical DNA mismatch repair mechanisms so the localised hypermutation resulting from phase variation can have profound local and global effects on gene expression (Bayliss *et al.*, 2012). Switching of the transcriptional state of both genes and
contingency loci (a subset of phase variable genes) in a stochastic manner alters transcription in an ON/OFF manner (Moxon et al., 2006) but it has also been postulated that phase variation can also alter the activity of the proteins encoded by contingency loci (Kim et al., 2012; Moxon et al., 2006) and this may account somewhat for diversity of behaviour. The majority of contingency loci in C. jejuni carry genes involved in the synthesis or modification of surface structures (Guerry et al., 2002) so it understandable that phase variation of Campylobacter genes has shown to impact upon a range of Campylobacter behaviours mediated to some extent by outer membrane and surface structures including bacteriophage evasion, serum responses, motility, animal colonisation and invasion potential (Anjum et al., 2016; Louwen et al., 2013; Artymovich et al., 2013; Ashgar et al., 2007). The genome of Campylobacter jejuni NCTC 11168 possesses 28 homopolymeric tracts (Parkhill et al., 2000; Gundogdu et al., 2010). The presence of multiple phase variable genes in this genome equate to over a quarter of a billion potential genotypes (Aidley and Bayliss, 2014). The true degree of variation is likely to be significantly smaller however the ability of C. jejuni to adapt to novel environments stems in part from this phasotype potential.

Phase variation of Campylobacter genes can have exceptional effects on the behaviour of this organism and contributes to the diversity and variation observed by researchers. This remarkable behaviour facilitates the adaption of Campylobacter to the environmental fluctuation that these organisms inevitably face during transmission to different hosts. Owing to the selective pressure exerted by differing hosts, for example mucus constituents in mammals compared to avian hosts, temperature fluctuations and competing microbiota, phase variation gives rise to phasotypes within a population which may possess a fitness advantage leading to these phasotypes dominating the population (Bayliss et al., 2012). An understanding of phase variation in Campylobacter is still lacking, mainly due to the fact that only a small proportion of laboratory adapted strains are commonly studied in depth. Investigation of the function of individual phase
variable genes is vital however and holistic approaches for determining strain to strain variation and mutability will be required to fully understand and appreciate *Campylobacter* diversity as a consequence of phase variation.

### 1.5.4. Restriction-modification systems

Restriction modification (RM) systems are common mechanisms in bacteria that provide protection against exogenous DNA and infection by bacteriophages (Meselson *et al.*, 1972). In classical RM systems, foreign DNA is targeted for endonuclease digestion by directed methylation of certain residues (Miller *et al.*, 2005). RM systems are classified into four distinct groups based on criteria such as co-factor requirements and recognition/cleavage sites (Type I-IV) (Miller *et al.*, 2005). To date, *Campylobacter jejuni* has been found to encode Type I RM systems (Miller *et al.*, 2005), Type II and IIG (Anjum *et al.*, 2016) and Type III RM systems (Skarp *et al.*, 2015). Type I systems are composed of three different enzymes R, M and S which act in concert to exert methylation and endonuclease activity (O’Loughlin *et al.*, 2015) and cleavage on targeted DNA varies considerably in location from the recognition site. The type II systems are commonly comprised of separate restriction endonuclease and methylase enzymes with both enzymes recognising an identical target sequence (O’Loughlin *et al.*, 2015). Type III systems are also comprised of two independent enzymes; the recognition enzyme recognises and methylates a 5-6 base-pair sequence on target genetic material which is then cleaved at a position approximately 25 base-pairs from the recognition site (Rao *et al.*, 2014). A common trait of these systems is that recognition/methylation and cleavage of target sequences is a two-step process. Therefore, bacteriophages that are capable of by-passing the restriction barrier will multiply within bacterial cells using host cell machinery and thereby become insensitive to recognition and cleavage. Phase variation of the *hsd* genes involved in the RM system of *Campylobacter* has been reported and it is thought that this allows a defence against the emergence of restriction/modification resistant
phage populations (Bayliss et al., 2012). The presence of RM systems in Campylobacters, whilst protecting these organisms from bacteriophage predation, also prevents access of Campylobacter to the wider bacterial gene pool and accounts for the relative lack of plasmids, phage-derived and insertion sequences within this genus. Indirectly, RM systems in C. jejuni, account for the propensity of these organism’s high degree of mutability in the form of recombination and introgression as a necessary means of generating diversity.

1.5.5. Within-host genome evolution

de Boer and colleagues (2002) showed that the genome of Campylobacter does not remain static even during its relatively short passage through the host. Using pulse-field gel electrophoresis, it was shown that C. jejuni genomic banding patterns were altered during and after infection of chickens with an initially clonal population of C. jejuni (de Boer et al., 2002). Using the recent advances in sequencing technology, comparisons of parental and host passaged C. jejuni have shown a combination of large scale genomic recombination and more simplistic, phasotype-associated variant (Kim et al., 2012; Karlyshev et al., 2002; de Boer et al., 2002) indicating that C. jejuni undergoes considerable within host genomic evolution. The phenotypic and genotypic changes due to host passaging seem to be consistent across multiple studies and hosts. These changes are heritable and as such are evidence of genome evolution within the natural host. This is thought to have resulted in the Campylobacter lineages that are commonly observed within different reservoirs. In addition, there is evidence to suggest that passaging within the natural chicken reservoir may promote subsequent infection of humans, which fits with the notion that virulence and severity of disease in humans is the result of expression of chicken-specific factors. Ultimately, it is evident that passaging through the host results in genome evolution of Campylobacter and the emergence of progeny lineages with heritable changes that confer greater colonisation potential, altered immunogenic properties and increased severity of disease.
1.5.6. Phase variation-mediated within-host evolution

A variety of phenotypic changes have been observed after host passage of *Campylobacter jejuni* through chickens and humans (Jones *et al.*, 2004; Black *et al.*, 1988). Most of these studies report increased motility and higher frequency of motile cells within the host passaged population compared to the initial population (Bell *et al.*, 2009). Passaging of *Campylobacter jejuni* in mice, a model for human infection, can lead to increased virulence (Bell *et al.*, 2009). Serial passage through IL-10-deficient mice with clinical isolates of *C. jejuni* resulted in improved colonisation capacity and an increased virulence phenotype in terms of pathology, mortality and recovery of *C. jejuni* from faecal populations (Bell *et al.*, 2009). *Campylobacter* has also been shown to undergo antigenic variation during host passage (Prendergast *et al.*, 2004). Prendergast and colleagues experimentally administered the *C. jejuni* 81-176 reference strain that lacked LOS ganglioside mimicry structure, into human volunteers. *C. jejuni* subsequently recovered from select volunteers was shown to possess ganglioside mimicking LOS structures (Prendergast *et al.*, 2004). Though Prendergast *et al.* do not speculate on the genetic basis for this change, the LOS locus has been shown to be phase variable (Guerry *et al.*, 2002), so it is logical to presume that this antigenic variation is the direct result of phase variation. Similarly, phase variation is likely the cause of phenotypic variations such as motility and virulence during host passage. Homopolymeric tracts are common in genes involving flagella structure and biosynthesis such as *maf* and the *flgRS* (Hendrixson *et al.*, 2006), which would account for variations in motility and contributes to virulence. The importance of motility to colonisation and disease establishment has long been recognised (Hendrixson *et al.*, 2006). Likewise, LOS antigenic mimicry of human ganglioside structures confers a selective advantage to *Campylobacter in vivo* in avoiding the human serum response (Hendrixson *et al.*, 2006). It is therefore unsurprising that hypermotile variants and ganglioside mimicking variants would be selected for during passage through various hosts.
1.5.7. Recombination mediated within-host evolution

Whilst phase variation is a major driving force behind *in vivo* evolution of the *Campylobacter* genome, processes such as recombination also play a major role in the generation of *Campylobacter* diversity (Biggs *et al.*, 2011). Recombination is broadly defined as the exchange of genetic material and is an essential process by which new DNA is incorporated within an organism’s genome.

*Ridley et al.* showed that a panel of PFGE type variants of *C. jejuni* of clonal origin showed altered chicken colonisation capacity in one day old chickens when challenged in competition (Ridley *et al.*, 2008). In addition, it was reported that in certain competitive challenge experiments using variant mixtures, a population of novel PFGE types were recovered from day old chicks, suggesting that intragenomic rearrangements take place in *C. jejuni* in response to avian host selective pressures, thus generating diversity of *C. jejuni* and driving in vivo evolution (Ridley *et al.*, 2008). *Campylobacter jejuni* is a naturally competent organism (Wang and Taylor, 1990). *de Boer* and colleagues showed that co-infection of a broiler chicken model with two isogenic *Campylobacter jejuni* strains that were marked with two different antibiotic resistance markers resulted in the recovery of strains from caecal contents that encoded both resistance genes, suggesting the exchange of genetic material between strains (Boer *et al.*, 2002). Moreover, this exchange was shown to be bi-directional in that both of the original PFGE types that were used to infect broilers were recovered in addition to novel genotypes, all of which encoded double resistance (Boer *et al.*, 2002). This is strong evidence that *C. jejuni* undergoes a recombination processes in vivo and that the intrinsic genomic instability displayed is vital for generation of diversity. Similarly, Morley *et al.* (2015) showed that intra-lineage recombination of isolates belonging to the non-avian associated clonal complex ST403 led to gene gain as well as the accumulation of nonsense mutation within coding sequences which resulted in pseudogene formation (Morley *et al.*, 2015). Both intra- and intergenomic recombination in *Campylobacter* are potent drivers of genome
diversity; there is evidence that strongly supports the notion that these processes are the direct cause of the generation of host-specific lineages of *C. jejuni* and *C. coli* (Morley *et al.*, 2015; Boer *et al.*, 2002).

### 1.6. *Campylobacter* host colonisation

*Campylobacter jejuni* and *C. coli* are able to colonise chickens without the onset of the clinical symptoms of gastroenteritis observed in susceptible humans (Young *et al.*, 2007). *C. jejuni* has been shown to colonise the crypts of the caecum in number in excess of $10^9$-$10^{10}$ CFU/g of caecal contents (Young *et al.*, 2007; Newell, 2001). In spite of these high numbers of *C. jejuni* within the guts of chickens, it has been reported that these organisms do not adhere nor invade chick intestinal cells (Young *et al.*, 2007). However more recently, Byrne *et al.* (2007) showed that *C. jejuni* is able to adhere and invade primary caecal cells but intracellular survival is extremely short lived (Byrne *et al.*, 2007). These contradictions highlight the relative lack of knowledge regarding *Campylobacter* interaction with the chicken host. Moreover, this behaviour is a direct contrast to the behaviour of *C. jejuni* in the human gastrointestinal niche in which *Campylobacter* has been shown to interact directly with human intestinal epithelial cells, undergoing processes of adhesion, invasion and translocation and eliciting clinical symptoms as a result (Hu and Kopecko, 2008). Sections 1.6.1 to 1.6.3 will focus on the factors influencing *C. jejuni* colonisation of the chicken and human gastrointestinal niche with particular attention to the attributes that affect this process as well as the physical interactions with the host. In addition, the underlying reasons for the commensal and pathogenic lifestyle and subsequent discrepancies in clinical outcome in different hosts will be considered with regards to recent literature.
1.6.1. Colonisation of the avian host

Broiler chickens are the conventional model for *Campylobacter* avian colonisation as commercial broiler chickens are the primary reservoir for human illness caused by *C. jejuni* and *C. coli* (Young *et al.*, 2007). In spite of being colonised, often persistently, with up to $10^9$-$10^{10}$ CFU/g of caecal contents of *Campylobacter* spp. (Newell and Fearnley, 2003), birds commonly do not exhibit any overt signs of disease and remain asymptomatic (Dhillion *et al.*, 2006). Given this asymptomatic response, *C. jejuni* and *C. coli* have traditionally been labelled as commensal organisms of poultry (Young *et al.*, 2007; Dhillion *et al.*, 2006).

Many factors that are integral for chicken colonisation have been identified, however due to the lack of pathology in chickens, relatively little attention has been given to elucidating the precise interactions and mechanisms used by this organism to colonise the chicken intestinal tract (Pielsticker *et al.*, 2012).

1.6.1.2. The chicken gut environment

Colonisation of the gastrointestinal tract of animals involves overcoming a variety of challenges for incoming bacteria. Such microbes must evade host immune mechanisms, out-compete existing microbiota and survive harsh conditions of the GI tract. Within the avian (and human) alimentary tract *Campylobacter jejuni* is able to preferentially metabolise the nutrients found within the mucus layer as growth substrates and thereby outcompete intestinal microbiota (Stahl *et al.*, 2011). Mucus also serves as a source of glycans which are used for co- and post-translational modification of surface structures and proteins (Stahl *et al.*, 2011). *Campylobacter jejuni* has been shown to undergo a complex interaction with host mucus which will be explored in section 1.6.3. After ingestion, a proportion of bacteria reach the caecum whereupon they are able to multiply and form an established population within 24 hours. (Coward *et al.*, 2008). Unlike most enteric bacteria, *Campylobacters* have specific nutrient requirements; *C. jejuni* utilises
amino acids as sole carbon sources however, these organisms also require other compounds such as short chain fatty acids and TCA cycle intermediates for growth (Stahl et al., 2012). The majority of studies regarding Campylobacter metabolism have been performed using C. jejuni NCTC 11168 (Stahl et al., 2011). This laboratory-adapted strain has been shown to preferentially metabolise serine aspartate, glutamate, asparagine and proline (Line et al., 2010; Guccione et al., 2008). These amino acids are abundant in chicken faecal matter and thought to be used for energy generation within the GI tract. C. jejuni has traditionally been considered to be assacharolytic, however recent research in to gene variation between strains has shown that the cj0480c-cj0490 region encodes the capability for L-fucose metabolism (Muraoka and Zhang, 2011; Stahl et al., 2011). Whilst C. jejuni has previously been shown to be chemotactic for L-fucose, this is the first evidence of catabolism of a carbohydrate by Campylobacter spp. and this is thought to confer a selective advantage in vivo (Stahl et al., 2011). More recently, Vorwerk and colleagues demonstrated that several Campylobacter coli strains are able to catabolize glucose and use it for the de novo synthesis of amino acids (Vorwerk et al., 2015). This capability is encoded by a genomic island that was shown to be transferable via natural transformation and confer glycolytic activity to a recipient strain (Vorwerk et al., 2015). Not only does this finding upset years of dogma regarding Campylobacter metabolism, but it further demonstrates how strain-specific genetic differences can have profound implications for Campylobacter biology.

Campylobacter can be found throughout the alimentary tract of chickens but is most concentrated in the large blind caeca at the distal end (Humphrey et al., 2014). The caeca are protruding tubes of the chicken intestinal tract that allow the fermentation of undigested food; they are 16-18 cm long in the adult chicken and extend to the liver (Thibodeau et al., 2015). Throughout the gut, two layers of mucus secreted by the chicken gut epithelial cells protect the GI tract from invasion by microbial pathogens and resident microflora, consisting of a loosely adherent layer and an inner firmly adherent mucus layer (Flint et al., 2014). In healthy hosts,
commensal bacterial colonise the outer layer of mucus whilst the inner layer is devoid of microbes (Kim and Ho., 2010). In addition, a layer of glycocalyx is present on the epithelial cell surface, consisting of membrane bound mucins and glycoproteins (Flint et al., 2014).

### 1.6.1.3. Factors involved in colonisation of the chicken gut

Successful colonisation of the chicken gut is a multifactorial process (Newell, 2002). It has been shown that a plethora of genetic factors that regulate many areas of *C. jejuni* biology are involved in colonisation of the chicken; these factors have been reviewed in detail (Hermans et al., 2011). Table 1.1 lists most of the known genetic elements involved in colonisation of the chicken gut within the context of the functional area of *Campylobacter* biology that each factor is involved in (Hermans et al., 2011).

#### Drug and Bile resistance

Bile salts are produced within the host in order to aid the digestion of lipids and can therefore be detrimental to *Campylobacter* physiology. In order to successfully colonise the chicken, *C. jejuni* must be able to resist and overcome the effects of bile salts. The *Campylobacter* multidrug efflux pump (Cme) has previously been shown to mediate resistance to bile salts as well as a range of heavy metals and antimicrobials (Lin et al., 2003). This pump is encoded by the *cmeABC* operon comprises a periplasmic protein, and inner membrane transporter and an outer membrane protein (Lin et al., 2002). The operon is regulated by *cmeR*, a transcriptional repressor which in response to the presence of bile salts, induces the expression of *cmeABC* and the *cj0561c* gene encoding a putative periplasmic protein (Woodall et al., 2005). Various deletion mutants in genes involved in this operon and accessory genes have consistently resulted in impaired ability to colonise chicken models indicating that resistance to bile salts is crucial for *C. jejuni* survival in the chicken gut (Hermans et al., 2011).
Table 1.1: A list of most of the known genetic factors shown to be involved in colonisation of the chicken host within the context of functional areas of *Campylobacter* physiology and biology, adapted from the work of Hermans et al. (2011). Certain factors within the list have been shown to have pleiotropic roles and the majority are shown to be involved in *Campylobacter* homeostasis, motility and biosynthesis rather than direct interaction with chicken host cells and structures. Genetic factors shown to be involved in central metabolism and consequently impact on colonisation potential have been omitted.

<table>
<thead>
<tr>
<th>Functional area</th>
<th>Gene name or locus</th>
<th>Identified/predicted protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multidrug efflux pump</td>
<td><strong>cmeABC</strong></td>
<td><em>Campylobacter</em> multidrug efflux pump</td>
</tr>
<tr>
<td></td>
<td><strong>cmeR</strong></td>
<td>transcriptional repressor of <em>cmeABC</em></td>
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<tr>
<td></td>
<td><strong>cj0561c</strong></td>
<td>putative periplasmic protein</td>
</tr>
<tr>
<td></td>
<td><strong>cbrR</strong></td>
<td><em>Campylobacter</em> bile resistance orphan response regulator</td>
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<tr>
<td>Chemotaxis</td>
<td><strong>docB</strong></td>
<td>probable methyl-accepting chemotaxis protein (MCP)</td>
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<tr>
<td></td>
<td><strong>docC</strong></td>
<td>probable MCP protein</td>
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<td></td>
<td><strong>acfB</strong></td>
<td>probable MCP protein</td>
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<td></td>
<td><strong>cheY</strong></td>
<td>chemotaxis regulatory protein</td>
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<td></td>
<td><strong>tlp1</strong></td>
<td>chemoreceptor transducer-like protein</td>
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<td></td>
<td><strong>luxS</strong></td>
<td>signal autoinducer AI-2 biosynthesis enzyme</td>
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<tr>
<td></td>
<td><strong>cheB, cheR</strong></td>
<td>putative adaptation proteins</td>
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<td>Motility</td>
<td><strong>flaA</strong></td>
<td>major flagellin</td>
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<td></td>
<td><strong>maf5</strong></td>
<td>motility accessory factor (flagellar biosynthesis)</td>
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<tr>
<td></td>
<td><strong>rpoN</strong></td>
<td>RNA polymerase σ54 subunit</td>
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<td></td>
<td><strong>fliA</strong></td>
<td>RNA polymerase σ28 subunit</td>
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<td><strong>flgR</strong></td>
<td>response regulator</td>
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<td><strong>flgK</strong></td>
<td>possible flagellar hook associated protein</td>
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<td></td>
<td><strong>cj1321-cj1325/6</strong></td>
<td>flagellin O-linked glycosylation island</td>
</tr>
<tr>
<td>Capsule formation and N-linked glycosylation</td>
<td><strong>kpsM</strong></td>
<td>high molecular weight glycan</td>
</tr>
<tr>
<td>Temperature regulation and heat shock response</td>
<td><strong>pglH</strong></td>
<td>probable glycosyltransferase</td>
</tr>
<tr>
<td></td>
<td><strong>cj1496c</strong></td>
<td>glycoprotein with unknown function</td>
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<td></td>
<td><strong>dnaJ</strong></td>
<td>heat-shock protein</td>
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<tr>
<td></td>
<td><strong>racR</strong></td>
<td>reduced ability to colonize response regulator</td>
</tr>
<tr>
<td>Adhesion</td>
<td><strong>capA</strong></td>
<td>autotransporter lipoprotein</td>
</tr>
<tr>
<td></td>
<td><strong>cadF</strong></td>
<td>outer membrane fibronectin-binding protein</td>
</tr>
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<td></td>
<td><strong>pldA</strong></td>
<td>outer membrane phospholipase A</td>
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<td></td>
<td><strong>flpA</strong></td>
<td>fibronectin-like protein A</td>
</tr>
<tr>
<td>Invasion</td>
<td><strong>ciaB</strong></td>
<td><em>Campylobacter</em> invasion antigen B</td>
</tr>
<tr>
<td></td>
<td><strong>tlp1</strong></td>
<td>chemoreceptor transducer-like protein</td>
</tr>
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</table>
Motility, Chemotaxis and the Flagella

Chemotaxis is the response by which bacteria direct their movement according to chemical gradients. During infection, *Campylobacter* must compete with other organisms to colonise the mucosal lining of the gut, a process that is integral to its pathogenesis (Dasti et al., 2010). Motility allows passage of the organism through viscous milieu such as the gastrointestinal mucous but it is the process of chemotaxis that directs motility and causes the cells to migrate towards mucus (Lertsethtakarn et al., 2011; Konkel et al., 2004). A wide variety of cells throughout nature exhibit chemotactic behaviour including somatic cells, bacteria and other unicellular organisms. The process can be complex and vary considerably between species but fundamentally involves a surface receptor (chemoreceptor) that senses extracellular signal via ligand binding causing signal transduction inside the cell. This causes changes within the cell such as enzymatic activation or gene expression which ultimately leads to a chemotactic response (Lertsethtakarn et al., 2011).

Many species of bacteria, *Campylobacter* amongst them, utilise methyl-accepting chemotaxis proteins (MCP) as transmembrane chemoreceptors (Lertsethtakarn et al., 2011). Binding of a ligand to these receptors causes activation of a series of chemotactic accessory proteins which direct cellular movement (Lertsethtakarn et al., 2011). In *Campylobacter*, *docB* has been identified as an MCP and insertional mutagenesis of this gene causes a decline in colonisation of a chick model (Hendrixson and DiRita, 2004).

It has long since been established that intact and functional flagella are important colonization factors for *C. jejuni* in chickens. The flagella is composed of O-linked glycosylated flagellin and confers a rapid darting motility which is crucial for approaching attachment sites on intestinal epithelial cells (Dasti et al., 2010). The flagella of *Campylobacter* is composed of a basal body, hook and filament. The filament is comprised of two proteins, FlaA and FlaB although FlaA is the major subunit (Konkel et al., 2004). Both of these subunits are transcribed simultaneously in a concomitant fashion yet they exhibit differences in their regulation (Konkel et al., 2004). The *flaA*
gene is regulated by $\sigma^{28}$ whereas the $\text{flaB}$ gene is regulated by $\sigma^{54}$ (Hendrixson and DiRita, 2003). $\sigma^{28}$ and $\sigma^{54}$ are sigma factors that direct RNA polymerase to the flagella late promoters in the final stages of flagellum transcription and assembly (Hendrixson and DiRita, 2003). It has been reported that $\text{C. jejuni}$ isolates lacking $\sigma^{28}$ are able to assemble a truncated filament structure composed entirely of FlaB protein due to the dual regulation of filament protein transcription conferred by $\sigma^{28}$ and $\sigma^{54}$. Furthermore, Hendrixson (2006) reported that flagella biosynthesis involves phase variable and post translational mechanisms. FlgR, the response regulator of the FlgSR two-component system which is required for transcription of $\sigma^{54}$-dependent flagella genes undergoes phase variation through the loss or gain of a nucleotide in homopolymeric adenine or thymine tracts within the $\text{flgR}$ gene (Hendrixson et al., 2006). These features and the intense regulation of flagella biosynthesis highlight the importance of flagella in the pathogenesis of $\text{Campylobacter}$ species.

**Invasins**

It has been shown that the flagella apparatus in a manner analogous to a Type III secretion system, allows the secretion of $\text{Campylobacter}$-invasion antigens (Cia) into host cells (Konkel et al., 2004). Cia proteins have been shown to be important for caecal colonisation (Ziprin et al., 2001) which is unexpected as the conventional paradigm of $\text{Campylobacter}$ colonisation of the chicken portrays a non-invasive lifestyle in which these organisms transiently penetrate the inner mucus layer before replicating within the loosely adherent upper layer of mucus. Furthermore, transcription of CiaB, an integral invasion antigen, has been shown to be stimulated in response to the presence of mammalian bile salt deoxycholate (Malik-Kale et al., 2008; Raphael et al., 2005), suggesting that this factor is specific to the mammalian niche. It has been reported however that $\text{Campylobacter}$ is capable of transiently invading chick primary intestinal epithelial cells but are extremely short-lived intracellularly (Byrne et al., 2007). Whether transient invasion
of chicken epithelial cells is an actual feature of *C. jejuni* behaviour during *in vivo* colonisation is unclear and the contribution of invasins such as Cia proteins is even less so.

**Adhesins**

Adhesion to epithelial cells is believed to be an important step in *Campylobacter* colonisation and persistence within the chicken gastrointestinal tract (Hermans *et al.*, 2011) and several studies highlight the relative importance of intact flagella and adhesins to this process (Ashgar *et al.*, 2007; Ziprin *et al.*, 2001). The *Campylobacter* adhesion to fibronectin protein (CadF) is perhaps the most well characterised factor involved in host cell interactions and has been demonstrated to be integral to chicken colonisation as well as being required for full binding capacity to chicken epithelial cells (Flanagan *et al.*, 2009; Ziprin *et al.*, 2001). More recently a second fibronectin binding protein has since been identified, designated FlpA which, like CadF, was shown to be essential for chicken colonisation as well as a human INT407 epithelial line (Flanagan *et al.*, 2009). It has been suggested that fibronectin binding proteins may act cooperatively in order to facilitate access to the chicken and human niche (Eucker and Konkel., 2012). Other reported adhesive factors such as CapA, PldA and Peb proteins may be involved in establishing *Campylobacter* populations within the chicken host however, for each report detailing the contribution of these factors to chicken colonisation, there exists another suggesting the contrary (O Cróinín and Backert 2012; Flanagan *et al.*, 2009). Given the fact that these factors are not conserved between *C. jejuni* isolates, it seems likely that these factors are not integral to colonisation and this is perhaps due to redundancy. Therefore, the genuine contribution of these factors to colonisation of the chicken is unclear. The role of these adhesins in human colonisation and pathogenesis will be considered in section 1.6.2.
1.6.1.4. The chicken reservoir promotes *Campylobacter* diversity and pathogenicity

The list shown in Table 1.1 is not exhaustive and does not include more recently identified factors such as *cj1139* (*wlaN*), *cj0045* and *cj0170* which are genes that serve as contingency loci, facilitating rapid adaption to novel environments (Kim *et al.*, 2012). *wlaN* encodes the 1,3-galactosyltransferase enzyme which is involved in LOS biosynthesis and in particular with the generation of ganglioside mimicking structures (Linton *et al.*, 2000). Analysis of the homopolymeric tract within *wlaN* by Wilson *et al.* (2010) demonstrated that this locus, as well as the *flgR* gene, is involved in the two-component system regulating flagella biosynthesis, undergo increased frequency of variation suggesting that passage through the chicken reservoir promotes genetic diversity of *Campylobacter* (Wilson *et al.*, 2010). Similarly, variation in contingency genes *cj0045* and *cj0170* was also increased during chicken passage (Kim *et al.*, 2012). Kim *et al.* showed, using fragment analysis, that small insertions and deletions in these genes, occurring during passage through a broiler chicken model, had subsequent effects on successful colonisation of mice (Kim *et al.*, 2012). These findings support the notion that the chicken reservoir promotes subsequent disease in humans by promoting genetic diversity through contingency loci, thus increasing the frequency of variants that are better adapted for mammalian host colonisation.

Whilst characterisation of individual genetic factors that impact upon *Campylobacter* biology, and consequently chicken colonisation, is required to gain an accurate portrait of this organism *in vivo*, the identification of the genetic elements directly involved in the colonisation of the chicken host has been lacking. Furthermore, until the advent of improved sequencing technology, research largely has failed to address the dearth of knowledge regarding *Campylobacter* adaption to the chicken environment and whether any specific genetic factor(s) account for the commensal, non-invasive lifestyle in avian hosts compared to the pathogenic lifestyle in mammals. The study of contingency loci and the correlation of so-called “successful alleles” with increased colonisation
capacity is direct evidence of the ability of *C. jejuni* to adapt and exhibit differential behaviour in response to environmental pressures (Kim *et al.*, 2012). The response of phase variable contingency loci in these environments may shed light on additional genetic factors that may be directly involved in chicken colonisation but not in human infection.

### 1.6.1.5. Conditional commensalism

A greater understanding of *C. jejuni* interaction with the avian host is required for the development of novel control measures to reduce levels of *Campylobacter* in the food chain. Recent research into *Campylobacter* interaction with the avian host suggests that the paradigm of chicken commensalism may be flawed (Humphrey *et al.*, 2014). *Campylobacter* does not behave like a typical commensal organism; whilst most birds remain asymptomatic upon colonisation (Dhillion *et al.*, 2006), the induction of an immune response has been observed (Hermans *et al.*, 2012). Considering that this immune response fails to clear the bacteria from the GI tract, *Campylobacter* may be more aptly described as a controlled parasite than a commensal organism (Humphrey *et al.*, 2014; Hermans *et al.*, 2012).

It is recognised that *C. jejuni* can leave the chicken gut and colonise other organs such as the heart, liver, lungs, and spleen (Knudsen *et al.*, 2006). Colonisation of these organisms is indicative of an invasive pathogen rather than a commensal. *C. jejuni* also exhibits an association with certain pathologies such as hock marks and lesions resulting from wet litter (Humphrey *et al.*, 2014). Williams *et al* (2013) suggest that colonisation of broilers with *C. jejuni* causes hock marks and lesions indirectly by increasing looseness of faeces in the form of a sub-clinical diarrhoea; a symptom that is also indicative of pathogenic behaviour (Williams *et al.*, 2013).

These pathologies are only observed in select birds. Therefore commensal/pathogenic behaviour is likely to be as a result of chicken host health. Intensive farming practices due to an increased demand for poultry meat may have led to the selection of birds with defects such as microbiota
imbalance or immune dysregulation, thus making them potentially more susceptible to disease. Humphrey et al. (2014), showed that gut inflammation and hock marks were associated with only certain breeds of broiler chicken which supports the notion that Campylobacter pathogenic behaviour within chickens is likely to be determined by host predisposing factors rather than a defined virulence pathway exhibited within the avian host (Humphrey et al., 2014).

1.6.2. Colonisation of the human host

Colonisation of the human lower intestinal tract is a transient phenomenon in the majority of human infection cases in that Campylobacter causes an inflammatory disease and is subsequently cleared by the immune system (Janssen et al., 2008). This section will explore the processes and factors that allow C. jejuni to become temporarily established in this niche and cause disease. In humans, C. jejuni has been more extensively studied than C. coli, therefore the following examination of Campylobacter pathophysiology will be focussed primarily of C. jejuni. This does not preclude the same or similar disease causing mechanisms occurring in C. coli. Despite improvements in genomic tools, a complete understanding of C. jejuni pathogenicity remains elusive (Dasti et al., 2010). This is due, in part, to the fact that C. jejuni does not express a large number of classical virulence factors (Friis et al., 2010; Pearson et al., 2007; Parkhill et al., 2000). Comparisons of epidemiological data between developed and developing countries are highly suggestive that host factors should be strongly considered in efforts to understand C. jejuni pathogenicity (Havelaar et al., 2009). An example of this is the incidence of asymptomatic infection of individuals from developing countries (Newell et al., 2001). Sub-clinical infection in these individuals may be the result of C. jejuni genotypes that do not elicit disease, however smaller scale epidemiological associations are indicative of host-dependent factors, such as malnutrition, that affect the pathogenicity of these Campylobacter strains (Lee et al., 2013).
light of this, it should be noted that inferences made regarding C. jejuni colonisation of the human gut may not be universally true in vivo.

As with C. jejuni colonisation of the chicken gut, behaviours and properties of C. jejuni such as motility, chemotaxis, nutrient metabolism and stress responses all contribute to the fitness of this pathogen in vivo (Dasti et al., 2010). Properties such as motility and chemotaxis allow Campylobacter to navigate and penetrate intestinal mucus and reach the epithelium (Szymanski et al., 1995). Bile salt resistance is also a key factor, as well as robust metabolic capability and environmental stress resistance (Novik et al., 2010). Whilst deficiency in factors responsible for these behaviours will reduce colonisation potential and pathogenicity, these factors should not be classed as virulence determinants per se. The term “virulence factor” in this chapter will refer to physical factors that interact or cause damage to the human host, thereby promoting pathogen survival and proliferation within this niche.

It is recognised that there are two essential requirements to determine the virulence of C. jejuni, namely adherence to, and invasion of intestinal epithelial cells. Several studies have demonstrated the importance of host cell binding factors in the colonisation process (Flanagan et al., 2009; Konkel et al., 1999), yet the mechanisms that C. jejuni uses for adhesion and invasion are not fully understood. Moreover, whilst logically one might assume that these two processes occur sequentially, there is evidence to suggest that these species are able to adhere without subsequent invasion (Everest et al., 1992), and yet further evidence exists to indicate that adhesion and invasion occur concurrently and that adhesion is not necessary for C. jejuni invasion (O Cróinín and Backert., 2012). Ultimately, it is unclear how closely these two processes are entwined. Campylobacters also vary considerably in their capabilities to both adhere and invade (Carvalho et al., 2001). This correlates with the strain-strain discrepancies in virulence and pathogenicity that is commonly observed in this species (Fearnley et al., 2008). Given the aspects of Campylobacter biology that are considered in section 1.5.6 and 1.5.7, the assumption can be
made that differences in virulence are the result of environmental and niche adaption. There is also a degree of variation reported in C. jejuni translocation; the means by which C. jejuni is able to cross the intestinal epithelium and potentially gain access to extra-intestinal locations (Bouwmen et al., 2013; Walker et al., 1988). This pathology is evidenced by cases of Campylobacter-induced bacteraemia (Louwen et al., 2012). C. jejuni has been reported to undergo translocation either via a paracellular route, via specialised epithelial M cells or by directly invading and migrating through epithelial cells (Bouwmen et al., 2013; Walker et al., 1988). These findings typify the variation observed in C. jejuni pathology. Whereas basic adhesion and invasion potential can be affected by fine scale genetic variation resulting from phase variation and host passaging, the specificity of each of these translocation mechanisms is indicative of larger scale phenotypic variation that is likely the result of strain-specific variation in genetic content.

These processes of adhesion, invasion and translocation vary considerably in campylobacters and this variation may be exacerbated due to host responses. In spite of this, key bacterial factors involved in C. jejuni colonisation and pathogenesis and that mediate adherence and invasion have been identified and characterised (Lugert et al., 2015). Section 1.6.2.1 outlines the components and processes involved in human pathogenesis that are known and highlights areas of pathophysiology that require further study.

1.6.2.1. Pathogenesis in the human host

Early research into Campylobacter pathophysiology established a correlation between the severity of clinical symptoms in patients and the degree of adherence displayed by C. jejuni and C. coli isolated from these patient’s faeces (Pei et al., 1998). This correlation may be the result of phenotypic testing of host passaged strains, nevertheless it serves to highlight the importance of adherence in C. jejuni pathogenesis. Immortalised cell lines derived from both human and non-
human sources are most commonly used to characterise *C. jejuni* interaction with the host (Flanagan *et al.*, 2009; Everest *et al.*, 1992). The use of these models is examined in section 1.7. The increased use of these models has led to the identification of several factors that are integral to adhesion and invasion; these factors have been characterised and reviewed in detail (Lugert *et al.*, 2015; O Cróinín and Backert., 2012; Dasti *et al.*, 2010). However further work is required to clarify the sometimes contradictory role of some these factors.

**Fibronectin-binding proteins**

The role of *C. jejuni* adhesins in the chicken gut, and to some extent the human niche, is explored in section 1.6.1.3.4. Of these factors, fibronectin binding proteins are also reported to be involved in adhesion to human epithelial cells. The most well characterised *C. jejuni* factor involved in the interaction with human epithelial cells is the CadF fibronectin binding protein (O Cróinín and Backert., 2012; Hermans *et al.*, 2011). Prior to the identification of its role in chicken colonisation, CadF was shown to be involved in membrane binding to INT407 human intestinal epithelial cells (Moser *et al.*, 1997). A later study demonstrated that CadF was required for maximal binding to T84 intestinal epithelial cells (Monteville and Konkel, 2005). Fibronectin is a glycoprotein that is present in the extracellular matrix in both humans and chickens; considering this it is logical to assume that CadF is a primary colonisation/virulence factor in both humans and chickens. However, Tu *et al* (2008), demonstrated that CadF expression is down-regulated in response to human mucin, raising the possibility that its role in adherence to human cells *in vivo* may not be an integral process for infection.

The FlpA protein, the second fibronectin binding protein is similar in that it also has been demonstrated to be involved in both chicken and human intestinal cell interaction via deletion mutant comparisons (Flanagan *et al.*, 2009). Despite the wealth of literature citing the importance of these factors to colonisation and pathogenesis, neither FlpA nor CadF were
implicated in invasion related processes in a transposon mutant screen (Novik et al., 2010). However it is worth noting that this study utilised the invasive human disease isolate *C. jejuni* 81-176 (Black et al., 1988). *C. jejuni* 81-176 carries plasmids, one of which has been implicated in virulence (Bacon et al., 2000). It is unclear as to whether this plasmid, pVir, was included in the transposon mutagenesis procedure or whether redundancy of adhesion/invasion related factors contributed to the failure to identify factors previously implicated in these processes. Alternatively, this may just be indicative of strain to strain variation in the importance and role of CadF and FlpA.

**Additional adhesins**

A number of other factors encoded by *Campylobacter* are reported to function as adhesins, though additional reports question the role of some of these factors in this process. JlpA is a surface exposed lipoprotein that has been implicated as an important adhesive factor in *C. jejuni* (Jin et al., 2001). Early mutagenesis studies showed that JlpA-deficient *C. jejuni* had an impaired ability to adhere to Hep-2 cells (Jin et al., 2001). Furthermore, this phenotype was also exhibited by the wildtype strain during infection having been pre-incubated with anti-JlpA antibodies. A later study revealed the JlpA host receptor to be the heat-shock protein HSP-90α (Jin et al., 2003). Alternative models in different studies however were unable to show a role for JlpA in the infection process (Novik et al., 2010; Flanagan et al., 2009).

Reports regarding the role of the Peb1 protein are similarly contradictory. Fauchere et al. (1989) initially described the Peb proteins as immunogenic factors of *C. jejuni* and showed Peb1 to play a key role in adherence. Subsequent study and experimentation using deletion mutants and antibody inhibition confirmed this function (Pei et al., 1993). However, once again the studies performed by Flanagan et al. (2009) and Novik et al. (2010) demonstrated minimal reduction in adhesion in other cell models, calling into question the importance of Peb1 in adherence.
The CapA autotransporter protein (see section 1.8.1 for more general information on autotransporters) has been similarly implicated in adhesion and invasion of human intestinal epithelial cells as shown in a Caco-2 cell culture model. Flanagan et al confirmed this role showing CapA to be involved in *in vitro* adherence to chicken LMH hepatocellular carcinoma epithelial cells (Flanagan et al., 2009), indicating that CapA most likely serves as an auxiliary adhesin in both the chicken and human host. However, given that this protein is not conserved amongst isolates and its distribution in *Campylobacter* unknown, the relative importance of CapA to the adhesion and invasion process is unclear.

**Campylobacter invasion antigens**

Adherence and invasion are possibly two separate and independent process in *Campylobacter* (Everest et al., 1992), however the delivery of determinants influencing invasion would be considerably easier via adhesion. There are different mechanisms by which *C. jejuni* has been proposed to invade host cells yet none of these are fully understood (Lugert et al., 2015). *Campylobacter* invasion antigens were first described by Konkel et al. (1999). This report detailed the identification of CiaB, which was observed to enter host cells during *in vitro* infection (Konkel et al., 1999). In Gram-negative bacteria, effector proteins are commonly secreted utilising a type III secretion system which are not encoded by *Campylobacter* (Pearson et al., 2007; Parkhill et al., 2000). It has been proposed however that the flagella in *C. jejuni* functions as an export apparatus in a manner analogous to a type III secretion system facilitating the export of a number of effectors, including CiaB, directly in to the cytoplasm of host cells (Konkel et al., 2004). A CiaB deletion mutant showed no reduction in adherence to intestinal epithelial cells, though a significant reduction in intracellular bacteria was observed (Konkel et al., 1999). Since the discovery of CiaB, a number of other Cia proteins and flagella-excreted factors have been
identified including Cial, CiaC and FlaC (Buelow et al., 2011; Christensen et al., 2009; Song et al., 2004).

A comprehensive review of the Campylobacter cell entry process concluded that Cia proteins are not solely responsible for the invasion phenotype and may even be dispensable in certain strains (O Cróinín and Backert., 2012). Classic cellular invasion mechanisms by Gram-negative bacteria commonly fall within two general strategies; either invasion is initiated by binding of a bacterial surface protein to a host receptor thus causing internalisation, or effector proteins are delivered directly into host cells, altering signalling pathways and inducing bacterial uptake (O Cróinín and Backert., 2012). Interestingly, Campylobacter seems to share features of both of these strategies.

Cytolethal distending toxin and the delivery of virulence factors to host cells

A variety of toxic activities have been reported for C. jejuni however the cytolethal-distending toxin (Cdt) is hitherto the only characterised toxin encoded by C. jejuni, and this toxin is not conserved in all strains (Wassenar et al., 1997). Cdt has been reported to cause distension and swelling in a number of mammalian cell lines as well as cell death (Whitehouse et al., 1998). Cdt is a heat labile toxin that induces cell cycle arrest by causing DNA damage thus anchoring cells in the G2/M transition phase (Whitehouse et al., 1998). The toxin is composed of 3 subunits with CdtB the functional component. Cdt was initially considered to be a major influence on the severity of C. jejuni-mediated enteritis, however a study conducted by Mortensen et al., (2011) which included a comparison of clinical outcome between infections caused by C. jejuni Cdt-positive and -negative strains found that campylobacteriosis caused by Cdt-negative strains was indistinguishable from that of a patient infected with a Cdt-positive isolate (Mortensen et al., 2011). This calls into question the relevance of Cdt in C. jejuni infection and further exemplifies the strain-strain variation observed in Campylobacter virulence. The indistinguishable virulence
profiles reported by Mortensen et al. (2011) caused by differing genotypes suggest the lack of a defined, unified virulence and infection pathway for *C. jejuni*.

One poorly defined area of *C. jejuni* is the delivery of virulence factors to host cells. *C. jejuni* has been shown to secrete outer membrane vesicles (OMVs) containing a range of proteins including those previously implicated as integral to the infection process (Elmi et al., 2012). In the absence of archetypal secretion systems, delivery of effectors via OMVs is an attractive model. Moreover, of the 151 proteins shown to be present in the *C. jejuni* OMV fraction, Elmi and colleagues report that approximately 26% are of unknown function (Elmi et al., 2012); this represents a significant proportion of proteins, potential immunogens and virulence determinants that could be involved in pathogenesis. Characterisation of these may reveal potential virulence mechanisms and provide novel avenues of research.

**A model of *C. jejuni* adhesion and invasion**

The current understanding of *C. jejuni* adhesion and invasion of the human host intestinal epithelium is incomplete, due in part to the genomic diversity of this pathogen as well as the use of a variety of infection models which often produce conflicting findings with regards to the physical factors involved in infection. Figure 1.7 shows a very simplified, hypothetical model of the basic mechanisms and host signalling involved in *C. jejuni* binding and cell entry.
A number of putative host receptors have been identified as binding targets for *Campylobacter* factors including HSP-90α, fibronectin and the epidermal growth factor receptor (EGFR) and more are thought to be involved (Krause-Gruszczynska et al., 2011). Binding of JlpA to HSP-90α induces the activation of the MAP-kinase pathway and ultimately the nuclear transcription factor NF-κB (Jin et al., 2003). Secretion of Cdt also induces activation of Toll-like receptors leading to the transcription of pro-inflammatory cytokines and an inflammatory response via the NF-κB pathway (Zheng et al., 2008). Perhaps the most important signalling alterations shown to arise from *C. jejuni* binding are to the activity of proteins influencing cytoskeletal rearrangements such as PI3 kinase, c-Src and focal-adhesion kinase (FAK) (Eucker and Konkel., 2012). Binding of CadF or FlpA to fibronectin or receptors such as EGFR and potentially other unknown factors, causes a cellular cascade that modulates host cell cytoskeleton thereby enabling bacterial penetration (Krause-Gruszczynska et al., 2011). Activation of PI3 kinase causes Cdc42 phosphorylation, influencing
cellular actin dynamics and the formation of filopodia (O Cróinín and Backert., 2012). c-Src and FAK activation results in docking with paxillin and leads to Rac1 activation (O Cróinín and Backert., 2012). Rac1 is a Rho GTPase that functions as a molecular switch in cell signalling transduction; in this context, activation of Rac1 increases actin polymerisation causing the formation of lamelipodia, resulting in membrane ruffling and eventually, pathogen entry (O Cróinín and Backert., 2012). However cell entry is not entirely mediated by adhesins. Secreted factors also play a role in modulating cell signalling events (Eucker and Konkel., 2012); CiaC has been shown to recruit Rac1 to the paxillin docking complex, a crucial step in the induction of membrane ruffling. This constitutes a cooperative action between adhesins and secreted antigens to allow C. jejuni cell entry.

Having gained entry to epithelial cells, a proportion of the population are able to survive intracellularly, as shown in vitro (Mills et al., 2012). The levels of intracellular bacteria in vivo may be considerably higher as environmental conditions may be more favourable for C. jejuni in comparison to conventional in vitro models. C. jejuni has been shown to survive intracellularly within Campylobacter-containing vesicles (CCVs) (Buelow et al., 2011); these vesicles deviate from the archetypal endocytic pathway, however the mechanisms that allow intra-vesicle survival and that cause this deviation, are currently unknown. It is thought that CiaI plays a role by preventing vesicles from localising with the lysosomal marker Cathespin D (Buelow et al., 2011), thus allowing C. jejuni to prevent delivery of lysosomes to the CCV.

This model of C. jejuni adhesion and invasion is simplified somewhat and there are additional aspects involved, the understanding of which remains incomplete. For example, transmigration and basolateral invasion are widely recognised to occur (Bouwmen et al., 2013). Considerable work is required to identify other factors involved in cell entry and the binding processes and to evaluate the relationship of these factors to pathology and disease. Particular areas of focus
should include the investigation of the range of strategies used by *Campylobacter* to cross the epithelium and aim to deduce the reasons for the variations in these strategies. Further work is also required to identify novel virulence factors and to characterise how novel and existing factors fit into the current model of pathophysiology. An improved understanding of these mechanisms will allow the identification of novel targets in efforts to reduce levels of *Campylobacter* in the food chain and ultimately, human disease.

1.6.3. Differential host behaviour

A major peculiarity of *C. jejuni* is the differential behaviour exhibited by similar genotypes within the avian and human host. *Campylobacter* is non-invasive in the avian host but exhibits invasive behaviour and pathology in the human host (Young *et al.*, 2007). A seminal study performed by Szymanski *et al.* (1995) revealed the importance of motility to *C. jejuni* colonisation and invasion *in vitro* (Szymanski *et al.*, 1995). In addition, Szymanski and colleagues also revealed that increases in mucosal viscosity led to an increase in bacterial motility and swimming velocity (Szymanski *et al.*, 1995). This was the first suggestion that *Campylobacter* may alter its' behaviour and colonisation attributes in response to its environment. With consideration to evidence such as this, a prevailing opinion amongst researchers is that the pathology exhibited during human infection is not solely the expression of chicken-adapted factors present within an accidental host, but a differential behaviour that is regulated by environmental factors.

A study by Byrne *et al.* (2007) conclusively demonstrated that species origin of intestinal mucus can have profound effects on *C. jejuni* behaviour with regards to commensalism or pathogenicity (Alemka *et al.*, 2010; Byrne *et al.*, 2007). *C. jejuni* invasion of human primary intestinal epithelial was significantly reduced in the presence of chicken mucus by 98% (small intestinal mucus) and 54% (large intestine mucus) (Byrne *et al.*, 2007). In contrast, experiments performed with crude human mucus extracts caused an increase in invasion (Byrne *et al.*, 2007). Whether this pattern
is consistent in chicken intestinal cells is unknown, however this finding as well as those published by Van Deun et al. (2008) have prompted the proposition of a hypothetical model of \textit{C. jejuni} commensalism and pathogenicity in the chicken and human gut respectively. This model is depicted in Figure 1.8 below, adapted from Shortt et al. (2014).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hypothetical_model.png}
\caption{A hypothetical model of infection in the human and chicken gut. \textit{C. jejuni} replicates in the outer mucus layer of the chicken and may briefly invade the epithelium. In the human gut, \textit{C. jejuni} penetrates the inner mucus layer, adheres to, and invades the epithelium and is able to translocate across the epithelial barrier. Adherence and invasion in this niche is accompanied by a pro-inflammatory response resulting in the influx of macrophages, neutrophils and dendritic cells (Shortt \textit{et al.}, 2014).}
\end{figure}

It is thought that this alteration in behaviour may be due to differences in glycosylation of the mucins present within mucus (Alemka \textit{et al.}, 2010). Additional investigation is required to characterise species-specific mucin interactions and the extent of the impact of this interaction on commensalism and pathogenicity.
1.7. Models of infection

The capability of *C. jejuni* and *C. coli* to cause disease is largely host dependent as evidenced by the avirulent characteristics these organisms display in healthy avian hosts compared to the virulent phenotype observed in humans and *in vitro* infection of mice. The understanding of *Campylobacter* pathogenesis and disease causing mechanisms has been hampered by the relative lack of suitable infection models. Whilst chick colonisation and persistence models as well as transgenic mice have proven to be valuable, these models are not suitable for virulence studies and are unable to shed light on disease causing mechanisms. Mammalian infection models are the most widely used models of human disease; these models have clear value to the study of *Campylobacter* pathogenesis. To this end, *in vivo* models of the study of campylobacteriosis have been developed. A neonatal pig has been utilised has been found to be an effective model for *Campylobacter* disease and it was found that pathology in this model correlated closely with human pathology (Babakhani *et al.*, 1993). Similarly, a weanling ferret model has previously been used to study campylobacteriosis and immunity to *C. jejuni*-induced enteritis (Bell and Manning., 1990). Most recently, an infant rabbit model has been developed as a colonisation and disease model for *Campylobacter*-mediated enteritis (Shang *et al.*, 2016.) This model has more relevance to disease in humans than previous models and has been used to define hitherto unknown aspects of *Campylobacter* biology such as expression of novel genes during invasion (Shang *et al.*, 2016). Rabbits developed diarrhoeal symptoms which mimicked that observed in humans, intestinal inflammation and systemic infection (Shang *et al.*, 2016). However, the primary site of colonisation in this model was the large intestine whereas in humans *Campylobacter* preferentially colonises the small intestine and rarely becomes systemic. Despite the clear efficacy of this model, the disparity in colonisation sites calls in to question the relevance of any findings obtained as interaction with the large intestine is not necessarily representative of human colonisation.
There are a number of ethical, social and scientific limitations and restrictions that pertain to the use of these models. In most countries, the use of mammals for experimentation is highly regulated and requires specialist facilities, adequate training of staff and rigorous justification of the work to be undertaken. In addition, certain mammalian species may lack the necessary cellular features present in humans, altering pathology and decreasing the efficacy of the model. In response to the concerns regarding the widespread use of mammalian infection models, there has been a collective effort to replace these models where possible. The overwhelming majority of studies concerning *C. jejuni* pathogenesis have been performed utilising *in vitro* cultured cell infection models which include primary cells and immortalized cell lines. Such models allow high-throughput and/or in-depth analysis of *Campylobacter* cellular interactions using single cell types and has led to the identification of a number of virulence factors (Dasti *et al.*, 2010). Primary cells lines are derived from a host and are therefore short lived but may be more representative of an *in vivo* response. Immortalised cell lines are frequently utilised as they are more widely available and are able to proliferate indefinitely. The cell lines most commonly used for the study of *Campylobacter* are INT407, T84 and Caco-2. Use of these *in vitro* cell culture models has facilitated the identification of key processes in *Campylobacter* pathogenesis such as adhesion, invasion and possibly toxin secretion. In addition, the development of standardised adhesion and gentamicin protection assays has permitted the identification of integral virulence and pathogenic determinants.

These assays are also not limited to human cell lines; the development of chicken derived LMH cells and HD11 cell lines offer more readily available models for studying chicken colonisation and immune interactions, the latter being a more recent focus in *Campylobacter* research as work aimed at effective vaccine development intensifies. A major drawback in the use of multiple cell lines across many research centres is the lack of a unified cell model or protocol. This is further complicated by the innate heterogeneity of *Campylobacter* whose adhesion, invasion and toxin
secretion capabilities vary considerably. As a result, *Campylobacter* can exhibit differential behaviour between models. The suitability of such models is therefore called into question. *Campylobacter* is reported to be an invasive organism as shown by the pathology in mammals and humans. However typical invasion efficiencies of *C. jejuni* are very low, often 0.1% to 1% of an initial inoculum. In light of this, it has been questioned whether *Campylobacter* should be considered an invasive pathogen when intracellular counts obtained through gentamicin protection assays are comparatively low. The aerobic environment to which *Campylobacter* is exposed during these assays may be a contributing factor to the low invasive number of *C. jejuni*. This is certainly supported by the work of Mills et al. (2012) who demonstrated that co-culture of *C. jejuni* with intestinal epithelial cells under microaerophilic conditions resulted in an 80-fold increase in levels of invasive bacteria. In this study, the use of a Vertical Diffusion Chamber (VDC) created microaerophillic conditions at the apical surface of cultured intestinal epithelial cells and aerobic conditions at the basolateral surface. This environment more closely resembles conditions within the human small intestine. Whilst use of this model requires the use of specialist equipment such as a variable atmospheric incubator, access to which is not always possible, it represents a significant improvement in *Campylobacter in vitro* infection models.

### 1.7.1. The *Galleria mellonella* virulence model

A major drawback of *Campylobacter* pathogenesis research is that factors identified and purported to be involved in pathogenesis are rarely examined in the context of infection for the reasons outlined in section 1.7 above regarding the relative dearth of suitable *in vivo* models and the restrictions surrounding their use. The larvae of *Galleria mellonella* (the greater wax moth) have previously been shown to be a powerful and effective infection model for a variety of pathogens and shows particular promise in addressing the paucity of knowledge regarding *Campylobacter* disease causing mechanisms. This model has garnered considerable interest
owing to the simplicity and reliability of establishing infections within this insect (Debois and Coote, 2012). Owing to the demand to reduce, refine and replace mammalian infections models, the use of *Galleria mellonella* is an attractive alternative infection model for the study of *C. jejuni* virulence and pathogenesis. As well as being ethically more acceptable than mammalian models, the use of this model is comparatively easy as larvae are commercially available easy to maintain and are inexpensive whilst requiring minimal expense in terms of facilities and equipment. Use of this model is particularly advantageous as it allows the testing of antimicrobial compounds and products *in vivo* (Debois and Coote, 2012), within the context of a functional immune system that has structural and functional similarities to that of humans (Ramarao *et al.*, 2012).

![Figure 1.9](image)

**Figure 1.9:** A. The Greater Wax Moth or honeycomb moth that is native to most places in the world. B. The larvae of these moths are typically 0.2-0.3g and average 20mm in size at the stage suitable for infection. C. Infection of a single *Galleria* larva via an injection into the top right proleg (denoted by an arrow in B).

*Galleria* are capable of a cellular immune response mediated by circulating immune cells called haemocytes present within larval haemolymph (Ramarao *et al.*, 2012). These cells are phagocytic
in nature and have a number of functional similarities with mammalian phagocytes. Whilst functionally analogous to human neutrophils, they more closely resemble human eosinophils (R. Titbull-personal communication). *Galleria mellonella* are reported to possess up to six classes of haemocytes; these cell types are reported to have slightly differing killing mechanisms however it is generally recognised that haemocytes are primarily phagocytic and kill pathogens via a respiratory burst and superoxide production in a manner comparable to human phagocytes (Salem et al., 2014; Champion et al., 2010). In addition to this, *Galleria mellonella* also possess a measure of humoral immunity in the form of antimicrobial peptides such as prophenoloxidases which catalyze the production of melanin (Champion et al., 2010).

This model has previously been used to screen the virulence potential of different *C. jejuni* sequence types as well as comparisons of individual *C. jejuni* gene deletion mutants to parental strains (Champion et al., 2010). *G. mellonella* have been effectively used to show that deletions in the LOS gene cluster and of an O-methyl phosphoramidate involved in capsule modifications, both resulted in attenuation of *C. jejuni*, confirming their suspected roles in virulence (Senior et al., 2011; Champion et al., 2010). Since these initial uses of the *Galleria* model in the study of *C. jejuni*, the use of this wax-moth larvae model to screen virulence and attenuation phenotypes in *Campylobacter* has increased significantly (Junqueira 2012; Elmi et al., 2012; Gundogdu et al., 2011; Baek et al., 2011).

The potential of *Galleria mellonella* as an inexpensive tool for the study of *C. jejuni* is considerable and highly desirable. As well as being a practically viable option for research centres, this model allows a quantifiable measure of *Campylobacter* virulence within the context of a relevant immune system. The *Galleria* model should therefore be used to support research into *Campylobacter* pathogenesis in order to facilitate the identification of mechanisms of disease, thus informing the development of novel control measures.
1.8. The Type V secretion system

Autotransporter proteins are surface exposed or secreted proteins that are processed and exported by what is commonly termed the Type V secretion system or autotransporter pathway (Henderson et al., 2012). These proteins are composed of an N-terminal passenger domain which is typically 40-400 kD, a conserved C-terminal domain which forms a β-barrel structure, a signal sequence and a linker region (Tseng et al., 2009). A basic representation of this structure and the general, unified aspects export pathway of autotransporters in Gram-negative bacteria is depicted in Figure 1.10. The term autotransporter is somewhat of a misnomer. The first identification and characterisation of a protein in this family was the IgA1 protease of Neisseria meningitidis (Pohlner et al., 1989). The following years saw the identification of many of these proteins (Henderson et al., 2012). In all cases the primary structure of the protein has been reminiscent of the IgA1 protease with each possessing the N-terminal passenger domain coupled with a C-terminal passenger domain (Henderson et al., 2007). The prevailing thought within the scientific community was that proteins within this family encoded all of the machinery required for export which would be translated in to a single polypeptide; the β-barrel would form a pore in the bacterial outer membrane through which the passenger domain could be secreted (Henderson et al., 1998). However, detailed structural analysis of the autotransporter pathway in Neisseria meningitidis suggest that the β-barrel alone is incapable of forming a pore large enough for the passenger domain to pass through and that accessory, chaperone proteins may be required (Leo et al., 2012; Oomen et al., 2004). It has been proposed that folding of the secreted domain may also affect the kinetics of this process (Drobnak et al., 2015).

Bernstein proposed that export of autotransporters may be mediated by BamA, formerly referred to as YaeT, a factor that is essential for assembly of outer membrane proteins (Berstein, 2015). Bernstein also proposes that the β-barrel only targets the protein to the outer membrane of the
cell and functions as a membrane anchor (Bernstein, 2015). The closest homologue of BamA in C. jejuni is Omp85 and homologues of BamBCDE are not encoded (Watson et al., 2014) suggesting considerable variation of this pathway amongst different species. A more comprehensive characterisation of these proteins amongst specific Gram-negative bacterial species is required.
Figure 1.10: The signal sequence present at the N-terminal sequence of autotransporters directs export of the proteins into the periplasm via the Sec machinery (Tseng et al., 2009). Upon reaching the periplasm the β-barrel inserts into the outer membrane forming a pore which allows translocation of the passenger domain, which typically harbours a virulence function, into the extracellular space (Leo et al., 2012). This insertion may be mediated by the Bam complex and perhaps additional accessory proteins. Depending on the function of the passenger domain, the protein may remain anchored in the outer membrane or be cleaved and released into the supernatant (Leo et al., 2012).
1.8.1. Autotransporters in *Campylobacter*

To date, comparatively few outer membrane or secreted factors in *Campylobacter* have been ascribed functional roles (Watson *et al.*, 2014). The identification and characterisation of autotransporters in *Campylobacter* species would contribute to the analysis of novel bacterial factors that are possibly involved in pathogenesis. In 2007, Ashgar *et al.* (2007), reported the identification of CapA (*cj0628/9*) and CapB (*cj1677/8*); the first two autotransporters to be characterised in *C. jejuni*. These two proteins were reported to have classic autotransporter structure (Figure 1.10) and considerable identity in the C-terminus region. Whilst expression of CapB was not detected in any strains used in the study, Ashgar *et al.* reported that capA encodes a 116kDa protein that was demonstrated to be involved in adhesion to Caco-2 cells and colonisation of a chicken model. As mentioned in in section 1.6.1.2. this is contrary to the findings of Flanagan *et al.* (2009), who reported that a CapA-deficient mutant had no defect in its ability to colonise a chicken model. Notably however, these two studies used differing models of colonisation. Flanagan *et al.* however did confirm a role for CapA in cellular adhesion (Flanagan *et al.*, 2009).

Genomic and proteomic characterisation of *C. jejuni* 81-176 allowed the identification of an additional autotransporter encoded by *Campylobacter* (Watson *et al.*, 2014). In this study, Watson *et al.* (2014) detected the expression of a serine protease autotransporter protein encoded by the *CJ81176_1367* locus. This autotransporter is absent in *C. jejuni* strains M1, 81116 and NCTC 11168 (Watson *et al.*, 2014); strongly indicating that autotransporters are major factors contributing to *C. jejuni* heterogeneity.

These studies describe the first autotransporter proteins identified in *C. jejuni* and illustrate the considerable impact that these proteins may have on virulence and pathogenesis. Continued research to identify and characterise putative/novel virulence factors in *Campylobacter* such as autotransporters is essential to increasing our understanding of this pathogen.
1.9. Preliminary findings that led to the current project

Publication of the genome of a number of different *Campylobacter* species as well as advancements in the field of bioinformatics has yielded the identification of putative virulence factors relating to motility, adherence, invasion and toxin production. A proportion of these virulence factors have been characterised yet many aspects of *Campylobacter* pathogenesis are still largely unknown.

Soluble iron is a very limited biological resource that is required by most pathogens for successful colonisation of the host. Many such organisms secrete siderophores; iron chelating compounds with the strongest known affinity for soluble Fe$^{3+}$. Utilising the published genome sequence data for *Campylobacter*, the *ceuBCDE* operon, an iron transport system encoding an enterochelin acquisition system was characterised by Park and Richardson (Park and Richardson, 1995). More detailed analysis of the *ceuBCDE* operon in a variety of strains including *C. jejuni* NCTC 11168, *C. coli* UA585 and *C. jejuni* NCTC 11351 revealed significant strain-specific discrepancies in the arrangement of genes immediately downstream of the operon (Figure 1.11) and the presence of a novel DNA sequence. As this novel sequence has only presented itself in select strains of *Campylobacter*, it was initially thought that it may be useful as an epidemiological tool (Richardson and Park, 1998). The novel gene has since been designated *capC*. 


Figure 1.11: A comparison of the region immediately downstream of the ceuBCDE operon in C. jejuni NCTC 11168 and C. coli UA585 as determined by Richardson and Park (Unpublished Data). The regions between ceuE and ppk are largely conserved yet only the genome of C. coli UA585 possesses the open reading frame designated capC (Campylobacter Autotransporter Protein C).

1.9.1. capC encodes a putative autotransporter protein

Preliminary work performed by Richardson and Park showed that capC is a strain specific genetic feature that is present in 60% of a panel of representative C. jejuni and C. coli strains. Partial sequencing and comparative analysis using the BLAST algorithm showed that the capC gene encodes a protein, the C-terminus of which exhibit 26% sequence identity with the VacA vacuolating cytotoxin of Helicobacter pylori. The H. pylori VacA toxin plays a major role in the gastric pathology associated with this bacterium. VacA belongs to the autotransporter family of proteins; a broad and the most diverse class of secretory proteins in Gram-negative bacteria (Henderson et al., 2004). Autotransporters are so-called because they were initially thought to carry their own export machinery and signal within a single polypeptide chain (Leyton et al., 2012; Henderson et al., 1998). In this context, the C-terminal domain forms a pore-like β-barrel in the outer membrane, allowing the N-terminal or “passenger” domain to exit the cell in to the extracellular milieu (Leyton et al., 2012). A significant trait of autotransporter proteins is that all previously characterised autotransporters have an ascribed virulence function (Henderson et al., 2004).
The C-terminal domain of the predicted CapC amino acid sequence was analysed for amphipathic β-strands using the AMPHI secondary structure prediction algorithm (now replaced by a multitude of structural bioinformatic prediction algorithms and programs). Using this program, CapC was initially shown to possess 14 β-sheet transmembrane segments; a structural arrangement that corresponds to that observed for the VacA β-barrel structure and that is present in the equivalent core region of all previously characterised autotransporters.

Given these structural features, it can be predicted with a considerable degree of confidence that CapC belongs to the autotransporter family and as such is likely to play a key role in Campylobacter virulence. Considering the relative paucity in knowledge concerning C. jejuni and C. coli interactions with the host(s), characterisation of capC as a putative virulence determinant represents an attractive avenue if research into the biology of this organism.

1.10. Project Rationale and Aims

The autotransporters are a large and diverse family of proteins which are all considered to function as virulence factors in some capacity (Nishimura et al., 2010). CapC is a putative autotransporter that has been identified in select C. jejuni and C. coli strains (Richardson and Park., 1998). Considering what is known regarding the role of autotransporters in virulence there is a strong potential that CapC functions as a virulence factor in Campylobacter species. Additionally CapC is a strain-specific factor that is not conserved amongst Campylobacter strains and this differential distribution of the capC ORF is suggestive of a lineage or genotype-specific association and may indicate a role for CapC within a particular ecological niche.

With consideration to the relative lack of understanding of the precise disease-causing mechanisms of campylobacters and the documented examples of variation in behaviour amongst different strains, the overall aim of this study was to investigate the putative role of CapC in the
virulence of *Campylobacter* species which are important zoonotic pathogens. A secondary aim of this study was to investigate the distribution of the *capC* gene amongst *Campylobacter* species and determine any potential genetic associations of this factor.

Examining the proposed role of CapC as a virulence factor will enable testing of the underlying hypothesis that CapC functions as a strain-specific virulence factor in *Campylobacter* species, thereby contributing to the potential of clinical severity of *Campylobacter*-mediated gastroenteritis infections.

Therefore, the aims of this project were as follows:

- To characterise *capC* based upon bioinformatic predictions in order to infer information regarding functional traits. The use of such tools will enable classification of *capC* and confirm this factor as a member of the autotransporter family of proteins.
- To generate defined, isogenic *capC*-deletion mutants and coupled wildtype strains for subsequent use in targeted comparison assays. Use of target comparisons between wildtype and mutant strains will be used to establish the role(s) of CapC.
- To assess the impact of *capC* deficiency in mutant strains on *Campylobacter* behaviours commonly implicated in bacterial fitness.
- To compare wildtype and mutant strains in an *in vivo* insect infection model (*Galleria mellonella*) to establish the contribution of CapC to the virulence of *Campylobacter* species.
- To compare wildtype and mutant strains using *in vitro* human intestinal models of infection in order to further characterise virulence phenotypes of these strains in a more
pertinent model. This will enable the impact of CapC upon human infection to be examined.

- To assess the prevalence of the capC gene amongst Campylobacter species and determine any potential genetic associations that this factor may exhibit, for example with a particular lineage(s) or ecological niche.
Chapter 2: Materials and Methods
2.1. Bacterial Strains

All bacterial strains used in this study are listed in List of Bacterial Isolates and Strains in Appendix II. *C. jejuni* 81116 (NCTC 11828) and *C. jejuni* M1 are commonly used reference strains for *Campylobacter* research and are the main strains used in this study. *C. jejuni* 81116 is recognised as a genetically stable strain that is amenable to genetic manipulation and is infective for chickens (Pearson *et al.*, 2007; Manning *et al.*, 2001). *C. jejuni* M1 is notable for being the causative agent of a documented case of chicken to human transmission of *C. jejuni* resulting in campylobacteriosis (Friis *et al.*, 2010). Both *C. jejuni* 81116 and *C. jejuni* M1 encode capC and full, annotated genome sequences are available which makes these strains ideal for use in the present study.

2.1.1 Growth and Maintenance of Bacterial Cultures and Culture Conditions

All isolates were stored on cryoprotectant beads in 1.5ml vials (Fisher Scientific, Loughborough, UK) at -80º C until required. A number of *Campylobacter* strains were used in this study including: *Campylobacter jejuni* 81116 (NCTC 11828), a genetically amenable strain of *Campylobacter* that is infective for chickens (Pearson *et al.*, 2007). *Campylobacter jejuni* M1 is a strain that is responsible for one of the few documented cases of direct transmission of *Campylobacter jejuni* from chicken to human (Friis *et al.*, 2010). *C. jejuni* 81116 and *C. jejuni* M1 and their respective derivatives were routinely cultured on Mueller-Hinton agar supplemented with 5% sheep’s blood or in Mueller-Hinton broth and incubated at 42ºC in a microaerobic atmosphere generated utilising Campygen gas packs (Oxoid, UK) (5% O₂, 10% CO₂ and 85% N₂), unless otherwise stated. Cultures were supplemented with trimethoprim (100μg/ml), chloramphenicol (10μg/ml) and/or kanamycin (50μg/ml) where appropriate. All other *Campylobacter* strains and isolates were routinely cultured on Mueller-Hinton agar supplemented with 5% sheep’s blood or in Mueller-Hinton broth and incubated at 42ºC in a microaerobic atmosphere, unless otherwise stated.
Cloning strains such as *Escherichia coli* DH5 alpha (Invitrogen) and *Escherichia coli* JM109 (Promega) have many features that make them well suited to cloning procedures including respective endA1 and recA mutations. These features ensure these strains are able to be transformed efficiently by a variety of methods. *Escherichia coli* DH5 alpha and *Escherichia coli* JM109 were used for propagation of plasmids and constructs were maintained in Luria-Bertani broth supplemented with 25% glycerol at -80°C in 1.5ml vials. *E. coli* strains were cultivated in an aerobic environment at 37°C for 16-24 hours.

### 2.2. Media Preparation

All media used in this study are listed in List of Media (Appendix II). Media and relevant supplements excluding antibiotics were supplied by Oxoid (Thermo Scientific, UK) unless otherwise stated. Media were prepared using ddH₂O and sterilised by autoclaving at 121°C, 15psi for 15 minutes according to manufacturer’s instructions. Media was cooled to 50°C and supplements added prior to use. The list of all media used can be found in Appendix II.

### 2.3. Buffers and Reagents

All chemicals were of analytical reagent grade or of the highest purity available. All buffers were prepared with deionised water from a Triple Red Nanopure water system (Thermo Scientific) and where necessary were autoclaved at 121°C, 15psi for 15 minutes. All solutions were stored at room temperature unless otherwise stated. All buffers and reagents are listed in List of Buffers and Reagents in Appendix II.
2.4. Collection of *Campylobacter* isolates from poultry and clinical sources

2.4.1. Acquisition of Clinical Isolates

A total of 76 isolates of *Campylobacter jejuni* and *C. coli* were kindly provided by Nicola Newman, Frimley Park Hospital, Surrey, UK. Upon receipt, each isolate was cultured from cryoprotectant beads stored at -80°C and confirmed to be *Campylobacter* species based upon Gram staining, oxidase tests, catalase tests and hippurate hydrolysis tests. Only clinical data was provided with these isolates.

2.4.2. Isolation of *Campylobacter* species from supermarket poultry

Samples of chicken meat and skin were purchased from local supermarkets over a period of 6 months in 2012-13. A variety of chicken meat products were chosen including breast meat, chicken legs and chicken thighs and whole chicken. A combination of free range and enclosed chicken sources were used.

In aseptic conditions, 25g of poultry meat was weighed and added to 200ml Bolton enrichment broth (Oxoid), containing Bolton broth selective supplement but without the recommended lysed horse blood supplement, in a screw capped Duran bottle filed to within 20mm of the top. This enrichment culture was incubated aerobically at 37°C for 4 hours and then at 42°C for 24 hours in microaerophillic conditions. Following this, a loopful of this culture was streaked on to mCCDA (Oxoid) plates and incubated microaerobically for 24 hours at 42°C and examined for single colonies. Suspected *Campylobacter* colonies that showed the typical grey, moist, spreading characteristics were streaked on MHA supplemented with 5% sheep’s blood and examined for morphology by Gram staining and subjected to oxidase, catalase and hippurate hydrolysis tests.
2.5. Biochemical tests

2.5.1. Hippurate Hydrolysis

_Campylobacter jejuni_ and _C. coli_ can be differentiated on the basis of hippuricase activity as _C. jejuni_ are hippuricase positive and _C. coli_ are hippuricase negative. There are however, reports of _C. coli_ isolates that test positive for hippuricase activity. The principle of the hippurate hydrolysis test relies on the fact that hippuric acid is hydrolysed to benzoic acid and glycine by the action of hippuricase. A 1% solution of sodium hippurate was prepared. A large loopful of putative _Campylobacter_ culture was emulsified in 500μl of the substrate in 1.5ml microcentrifuge tubes (Eppendorf, UK) and incubated for 2 hours at 37°C in a heating block (Eppendorf, UK). The presence or absence of glycine was determined by addition of 200μl of ninhydrin solution, prepared as described in List of Buffers and Reagents, and further incubation for 10 minutes. A deep purple colour indicated the presence of glycine and thus of hippuricase activity.

2.5.2. Catalase Test

The catalase enzyme is encoded by the _katA_ gene in _Campylobacter_ and provides resistance to hydrogen peroxide by converting _H_2_O_2_ to water and oxygen. Testing for catalase activity is a common biochemical test in the process of identifying campylobacters. A drop of 3% hydrogen peroxide was placed on a glass slide. A small amount of bacterial colony was transferred to the _H_2_O_2_ droplet using a sterile plastic loop. The production of bubbles is indicates a positive result.

2.5.3. Oxidase Test

The oxidase test is a biochemical reaction used to determine whether a bacterium produces cytochrome c oxidases. _C. jejuni_ and _C. coli_ are oxidase positive. The test procedure utilises a reagent, _N, N, N', N'-tetramethyl-p-phenylenediamine_ (TMPD) as an artificial electron donor. When oxidised, it changes from colourless to a dark blue/purple colour. 100μl of the TMPD reagent, prepared as described in List of Buffers and Reagents, was pipetted on to a 90mm filter
paper (Whatman) in a sterile petri dish. Suspected *Campylobacter* colonies were smeared on to
the reagent using a sterile plastic loop and observed for a colour change within 15 seconds.

### 2.5.4. Gram-Staining

The Gram-stain allows for differentiation of bacteria based upon cell wall structure and is used
for the fundamental phenotypic characterisation of these microorganisms. Gram-positive cells
have a thick peptidoglycan layer and stain purple. Gram-negative cells have a thin peptidoglycan
layer and stain pink.

*Campylobacter* species are Gram-negative, spiral shaped rods that are 0.2-0.5μm in length. The
Gram-stain procedure was carried out as follows: a bacterial smear of *Campylobacter* was
prepared on a glass slide and heat fixed. The smear was flooded with Crystal Violet stain for 30
seconds and rinsed with tap water. The slide was then flooded with Lugol’s Iodine for 30 seconds.
After rinsing, the slide was flood with a decolourising agent (70% IMS) for 5 seconds and rinsed.
The slide was then flooded with Safranin for 1 minute and rinsed with tap water and blotted dry.
Slides were examined microscopically at 100x objective; several fields were observed and the
Gram-reaction determined.

### 2.5.5. Giemsa Staining

Giemsa stain is a differential stain commonly used for identification of parasites in blood smears.
In this study, Giemsa stain was used to differentiate bacterial cells from *Galleria* haemocytes.
Haemolymph samples (10μl) were smeared on glass slides and fixed with pure methanol for 30
seconds by immersion. The slide was then washed and immersed in freshly prepared 5% Giemsa
stain solution for 20–30 minutes, flushed with tap water and left to dry. Haemocytes are stained
purple whilst bacterial cells are stained pink and cell nuclei and granules are stained various shades of blue and purple.

2.6. Phenotypic Assays

2.6.1. Contact Haemolysis

The variable nature of Campylobacter haemolytic activity is well documented. Previous work in our laboratory has shown that Phospholipase A (pldA), a haemolytic factor in Campylobacter species, is a membrane bound factor and that traditional haemolysis assays are largely inadequate for accurate and consistent quantification of Campylobacter haemolytic activity. An improved assay based on the method of Pickett et al. (1992), was employed with a number of changes.

Sheep’s blood in Alsever’s containing Sheep red blood cells (SRBCs) (Oxoid) was prepared as described in List of Buffers and Reagents. Campylobacter isolates to be tested were grown on Mueller-Hinton agar supplemented with 5% Sheep’s blood for 48 hours and sub-cultured into MHB and incubated at 42°C for 18 hours prior to the start of the experiment and re-suspended in Phosphate Buffered Saline (PBS). The absorbance of the suspension was measured using a Helios spectrophotometer at a wavelength of 590nm (OD590nm) and the optical density adjusted to 1. Next, 750µl of SRBCs and 750µl of cell suspension were combined in sterile micro-centrifuge tubes in duplicate for each test strain. In addition, a spontaneous haemolysis control was prepared in duplicate in which 750µl of SRBCs are combined with 750µl of PBS. A complete haemolysis control was prepared in the same way as the spontaneous haemolysis control with addition of 10µl of Triton-X. All tubes were centrifuged at 13000 rpm for 1 minute at room temperature using a microcentrifuge (Eppendorf, UK). Bacterial cells were visible settled on top of SRBCs at bottom of the tube with clear supernatant. Tubes were incubated at 37°C for 4hrs in a heating block (Eppendorf). Following incubation, cells were resuspended by gentle pipetting and then
centrifuged again at 13000 rpm for 1 minute at room temperature. The lysed SRBCs component remained suspended in the supernatant which was aspirated into 1.5ml sterile cuvettes.

The optical density of the supernatants was measured at a wavelength of 540nm. The supernatant of the spontaneous haemolysis control was used as a blank to factor in spontaneous haemolysis. The results were expressed as percentage of complete haemolysis calculated using the formula below. This assay was repeated in duplicate on three occasions.

\[
\frac{\text{Mean OD}_{540\text{nm}} \text{ of sample}}{\text{Mean OD}_{540\text{nm}} \text{ of complete haemolysis}} \times 100 = \% \text{ Haemolysis}
\]

### 2.6.2. Motility

#### 2.6.2.1. Swarming Plate assay

All isolates and strains of *C. jejuni* to be tested were cultured on Mueller-Hinton agar supplemented with 5% Sheep’s blood for 48 hours under microaerobic conditions and sub-cultured into MHB and incubated at 42°C for 18 hours prior to the start of the experiment and re-suspended in PBS to an OD590nm of 1. 2.5μl of this suspension was stab inoculated on to MHA plates containing 0.4% agar and incubated microaerobically for 24hrs after which the resultant halo of growth is measured. The semi-solid medium allows motile bacteria to swarm through, resulting in a halo of growth dependent on how far the organism can move. Results are measured in mm and compared where appropriate using statistical analyses.

#### 2.6.2.2. Tube taxis assay

Semi-solid *Brucella* Broth containing 0.4% agar was used for tube taxis assays; an alternative motility assay prepared as described in Media Preparation. After autoclaving, media was cooled
to ~50°C and 1 ml of 1% filter sterile 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma) was added to the media, as described in List of Buffers and Reagents. After cooling to ~45°C, 10 ml of media was added to sterile 15 ml Falcon tubes (Corning) and allowed to set. If not used directly, tubes were stored at 4°C for up to one month.

TTC is a redox indicator that is commonly used as an indicator for cellular respiration. In the presence of dehydrogenases produced by metabolically active cells, TTC is reduced to a formazan product TPF (1, 3, 5-triphenylformazan).

All isolates and strains of *C. jejuni* to be tested were grown on Mueller-Hinton agar supplemented with 5% Sheep’s blood for 48 hours and sub-cultured into MHB and incubated at 42°C for 18 hours prior to the start of the experiment and resuspended in PBS to an OD590nm of 1. The top of the agar tube was inoculated with 50 µl of this cell suspension in triplicate. The tubes were re-capped and incubated at 37°C in microaerobic conditions as described in Section 2.1.1. In this assay, the red formazan product is produced as the bacteria moves through the semi-solid media towards the bottom of the tube. The distance of the dye front from the top of the agar was measured as an indicator of bacterial motility.

### 2.6.3. Biofilm Formation

All isolates and strains of *C. jejuni* to be tested for their ability to form biofilms were grown on Mueller-Hinton agar supplemented with 5% Sheep’s blood for 48 hours and sub-cultured into 10mls MHB supplemented with 100µg/ml trimethoprim and incubated at 42°C for 18 hours prior to the start of the experiment. Broth cultures were then diluted using pre-warmed MHB to obtain an optical density of 0.2 at a wavelength of 550nm. A volume of 30µl of these OD-adjusted test isolates as inoculated into the wells of a 96 well microtitre plate (Nunc) in quadruplicate. An uninoculated, negative control containing MHB only was included. An additional 100µl of MHB
containing no antibiotics was added to each well. Plates were prepared in duplicate and a single plate was incubated at 37°C and 42°C, respectively for 72 hours.

Following incubation, the supernatant of each well was removed using a multichannel pipette and each well was washed with PBS (pH 7.2). Plates were inverted to dry for 10 minutes after which 130μl of crystal violet (0.5% w/v) (Sigma) was added to each well to stain the biofilms and plates were incubated at room temperature for 30 minutes. The crystal violet was then removed using a multichannel pipette and stained biofilms were washed with sterile laboratory grade RO water in order to remove excess crystal violet. Care was taken not to leave residual crystal violet on the inner sides of each well. Each plate was once again inverted to dry for 10 minutes after which 130μl of absolute methanol was added to each well to release cell bound crystal violet and incubated once again at room temperature for 30 minutes. The absorbance of each well was immediately measured using a Wallac plate reader at a wavelength of 540nm for 1 second.

2.6.4. Growth Curve

Measuring bacterial growth over a period of time provides an indication of healthy bacterial development. *Campylobacter jejuni* is considered to be a fastidious organism with strict growth requirements, therefore assessing healthy development and growth of the strains used in this study is of paramount importance. Growth curves are an accurate and efficient means of numerically evaluating bacterial development and growth. This procedure involves cultivating a known amount of bacteria in a media and quantifying growth over a period of time using optical density and colony forming units per millilitre (CFU/ml). The growth curve procedure used in this study was adapted from Davis and DiRita (2008).

2.6.4.1. Media Preparation

*Campylobacter* growth curves were performed over a period of 48 hours periods. Cultures were cultivated in T75 flasks with vented caps (Nunc) containing biphasic media consisting of 20mls MH
agar and 20mls MH broth supplemented with trimethoprim (100ug/ml), chloramphenicol (10ug/ml) and/or kanamycin (50ug/ml) where appropriate. Flasks were prepared and incubated in a microaerobic atmosphere (5% O2, 10% CO2 and 85% N2) using Campygen atmosphere generation system and incubated at 42°C for 24 hours prior to inoculation. For each planned sample time point, individual flasks were prepared in triplicate allowing subsequent samples to be undisturbed.

2.6.4.2. Inoculation

Test isolates were cultured from freezer stocks for 48 hours and sub-cultured on to MHA supplemented with 5% sheep’s blood for 16-18 hours. The bacteria were harvested from these plates using MHB and re-suspended to OD<sub>600nm</sub> of 0.4. This suspension was diluted 1:5 and 100μl of this dilution was used to inoculate each flask. The starting inoculum was approximately 1x10<sup>4</sup> cfu/ml

2.6.4.3. Enumeration

The Miles and Misra (Miles and Misra, 1938) method is an enumeration technique developed in 1938 for determination of the exact number of colony forming units (CFUs) in a bacterial suspension or homogenate by counting colonies on the surface of agar plates. This technique was used to enumerate *Campylobacter jejuni* during controlled growth. Briefly, every 6 hours post inoculation, 3 inoculated flasks for each test isolate were removed from incubation. The optical density of each sample was measured at a wavelength of 600nm. A 1:10 serial dilution of each sample was performed in a 96 well microtitre plate and 20μl droplets of each dilution was plated on to MHA plates in triplicate supplemented with antibiotics where appropriate. Plates were kept upright to allow for uniform drying and adsorption of the culture before inversion and incubation at 42°C for 24 hours. After this time, colonies formed at each dilution were counted. For the dilution at which between 2 and 20 colonies had formed, the mean colony count was used to
calculate to CFUs in the original sample using the formula below where \( N \) is equal to the mean number of colonies, \( D \) is the dilution and \( V \) is the volume plated out (20\( \mu l \)):

\[
\frac{N}{D \cdot V}
\]

Samples were taken 8 times over 48 hours. The CFUs/ml calculated for each timepoint were plotted against time using GraphPad v6.1.
2.7. Sensitivity assays

2.7.1. Environmental stress testing

In order to ascertain whether deletion of the capC gene imparts sensitivity to environmental stress, the viable counts of both wildtype and mutant strains were assessed after incubation under experimentally relevant conditions in two different defined media. C. jejuni 81116 and M1 as well as their respective mutants were suspended in DMEM and in MHB separately to an OD$_{600nm}$ of 0.2. DMEM cultures were incubated at 37°C statically for 4 hours aerobically. MHB cultures were incubated at 37°C for 4 hours with shaking at 225rpm. Viable counts were obtained at time zero and after 4 hours incubation as described in section 2.6.4.3. Results were expressed as the percentage of bacteria remaining viable after 4 hours of aerobic incubation.

2.7.2. Triton-X 100 Sensitivity Assay

Triton-X is a non-ionic surfactant that is commonly used as a detergent in order to lyse eukaryotic cells. To determine the sensitivity of C. jejuni to Triton-X 100 and to ensure that deletion of capC did not impart a greater sensitivity to Triton-X 100 (1% vol/vol), an overnight bacterial growth of C. jejuni 81116 and M1 as well as their respective capC mutants was harvested from Mueller-Hinton agar plates supplemented with 5% sheep’s blood and resuspended in PBS to an OD$_{550nm}$ of 1. After a zero-hour viable count was obtained as described in section 2.6.4.3., Triton-X 100 was added to a final concentration of 1% (vol/vol) and the suspension was incubated at room temperature for 1 hour. After this time had elapsed viable counts were obtained by serial dilution and plating as described in section 2.6.4.3. This assay was performed in triplicate.
2.8. Nucleic Acid Methodologies

In order to study gene function in bacteria, a fundamental tool is the ability to construct mutant strains with deletions in certain genes of interest which can subsequently be used to study altered phenotypic states and elucidate gene functions. Similarly, accurate measurement of RNA transcripts is key to studying the expression of genes within the context of a given set of conditions and can be vital in deducing gene regulation.

2.8.1. DNA

2.8.1.1. Extraction of Genomic DNA

2.8.1.1.1. Extraction of DNA using Boiling Lysis

DNA from *C. jejuni*/*C. coli* and *E. coli* was extracted using a combination of two methods. For rapid genomic DNA extraction from a large number of *Campylobacter* isolates for use in Polymerase Chain Reactions (PCR) with no further downstream application, DNA was extraction using boiling lysis. Briefly, a loopful of culture from which DNA was required was mixed with 200μl of sterile water in a microcentrifuge tube and vortexed thoroughly using a SAV7 Vortex Mixer (Eppendorf). Tubes were placed in a ThermoStat Plus heating block (Eppendorf) and cultures boiled at 100°C for 10 minutes. Tubes were carefully removed from the heating block using forceps and placed on ice for 5 minutes to cool. Each tube was then centrifuged at 13000 rpm for 5 minutes using a Minispin Plus bench top microcentrifuge (Eppendorf). Cellular debris was sedimented at the bottom of the tube whilst genomic DNA released upon boiling remained in the supernatant which was subsequently used in PCR.
2.8.1.1.2. Commercial DNA Extraction Kits

For genomic DNA to be used in PCR and subsequent downstream reactions such as purification, restriction digest and sequencing and for genomic DNA to be used in Whole Genome Sequencing (WGS), DNA was extracted from log phase cultures of *C. jejuni* by use of a commercial kit. The two DNA extraction kits used in this study were the Qiagen DNeasy Blood and Tissue Kit (Qiagen, UK) and the 5Prime ArchivePure DNA Cell/Tissue Kit. All procedures were performed according to the manufacturer’s instructions with minor deviations in that DNA was eluted or re-hydrated using sterile water rather than the buffers provided.

2.8.1.2. DNA quantification

Genomic DNA extracted using commercial kits as well purified PCR products, purified plasmid preparations, and digested DNA was quantified using two spectrophotometric methods and instruments. The instrument used most often for quantification of nucleic acids was the Nanodrop 1000 (Thermo Scientific, UK). This rapid spectrophotometric method allows rapid quantification of samples ranging from 0.5-2.0 μl. The other method frequently used for quantification of genomic DNA that was sent for WGS the Qubit. This fluorometric instrument offers increased sensitivity and accuracy compared to other quantification methods. Using commercially available buffers and the Qubit 2.0 fluorometer, this technique allows accurate quantification based upon the detection of target specific fluorescence. All techniques and protocols were performed according to the manufacturer’s instructions.
2.8.1.3. Plasmid Mini-Preparations

All plasmids used in this study were propagated in *E. coli* DH5 (Invitrogen) or *E. coli* JM109 (Promega) and are listed in List of Plasmids (Appendix II). Plasmids preparations were made using the QIAprep Spin Miniprep Kit (Qiagen) with minor deviations from the manufacturer’s instructions in that DNA was eluted in sterile, nuclease-free water rather than the buffer provided.

2.8.1.4. Gel Electrophoresis

Agarose gel electrophoresis is a standard laboratory method for the separation and subsequent visualisation of nucleic acids based on size (e.g. length in base pairs). Electrophoresis uses an electrical field to move the negatively charged DNA toward a positive electrode through an agarose gel matrix. Within an agarose matrix, pores are formed, the size of which are proportional to the concentration of the agarose. The gel matrix allows shorter DNA fragments to migrate more quickly than larger ones. Thus, you can accurately determine the length of a DNA segment by running it on an agarose gel alongside a DNA ladder (a collection of DNA fragments of known lengths).

Agarose gel was prepared as described in List of Buffers and Reagents with a nucleic acid stain (Sybr-Safe, ThermoScientific, UK) and stored in a waterbath at 50°C. To cast a gel, agarose was poured into a casting tray to a uniform thickness of 0.5cm with a well comb and allowed to set. Once set, the gel was submerged in an electrophoresis chamber connected to a powerpack with the wells furthest from the positive electrode.

DNA samples were mixed 1:1 with Loading dye (Promega) in order to provide greater density to the sample and to allow visible tracking of the sample through the gel matrix. 5μl of a DNA ladder (Promega) was run in the first well of every agarose gel. Equal volumes of DNA/loading dye mixtures were loaded in to other wells. Volume was dependent on the size of the wells cast. Gels were run at 100V for 45 minutes or until the dye line was 80% of the way through the gel. After
this time, the power was turned off, the gel was removed and viewed under UV light using a Gene
Flash UV box. Band sizes were interpreted using the DNA ladder as a guide.

2.8.1.5. Polymerase Chain Reaction

Three commercially available DNA polymerases were used in this study and outlined in Table of
Enzymes (Appendix II). These enzymes, GoTaq polymerase (Promega), Phusion polymerase
(Thermo Fisher Scientific) and Q5 polymerase (NEB) all have differing properties and as such
different recommended reaction conditions, parameters and buffers. The basic PCR components
and specific conditions used for PCR with each of these enzymes are outlined below in Tables 2.1,
2.2, 2.3 and 2.4 (below). The list of primers used in this study is shown in List of Primers, (Appendix
II). All reaction mixes were prepared on ice and dispensed into 250μl PCR tubes. PCR was carried
out using an ABI Biosystems Thermocycler or a VWR Thermocycler (Fisher Scientific, UK; VWR,
UK).

Table 2.1. GoTaq Polymerase

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume per 50μl</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq 5x Buffer</td>
<td>10 μl</td>
<td>1x (1.5mMgCl2)2</td>
</tr>
<tr>
<td>Nuclease-Free H2O</td>
<td>33 μl</td>
<td>N/A</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2.5 μl</td>
<td>0.5μm</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2.5 μl</td>
<td>0.5μm</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1 μl</td>
<td>0.2mM each dNTP</td>
</tr>
<tr>
<td>Template DNA</td>
<td>0.5 μl</td>
<td>10-100ng/50μl</td>
</tr>
<tr>
<td>Polymerase</td>
<td>0.5 μl</td>
<td>2.5U</td>
</tr>
</tbody>
</table>
### Table 2.2: GoTaq Green Master Mix

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume per 50μl</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq Master Mix 2x</td>
<td>25 μl</td>
<td>1x</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2.5 μl</td>
<td>0.5μm</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2.5 μl</td>
<td>0.5μm</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1 μl</td>
<td>10-100ng/50ul</td>
</tr>
<tr>
<td>Nuclease Free H₂O</td>
<td>19 μl</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### Table 2.1 and 2.2: Denaturation using GoTaq Polymerase was performed at 95°C. Annealing Temperature (T<sub>a</sub>) was calculated as 5°C lower than the melting of the least stable primer and further optimised if required. Extension time was calculated by allowing 1min/kb of the target amplicon.

### Table 2.3: Phusion Polymerase

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume per 50μl</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phusion High Fidelity or GC Buffer</td>
<td>10 μl</td>
<td>1x</td>
</tr>
<tr>
<td>Nuclease Free H₂O</td>
<td>31 μl</td>
<td>N/A</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2.5 μl</td>
<td>0.5μm</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2.5 μl</td>
<td>0.5μm</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1 μl</td>
<td>200μm</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.5 μl</td>
<td>3%</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1 μl</td>
<td>&lt;250ng</td>
</tr>
</tbody>
</table>

### Table 2.3: Denaturation using Phusion Polymerase was performed at 98°C. Annealing Temperature (T<sub>a</sub>) was calculated as 3°C higher than the melting of the least stable primer and further optimised if required. Extension time was calculated by allowing 30 sec/kb of the target amplicon.
Table 2.4 Q5 High Fidelity Polymerase

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume per 50μl</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q5 High-Fidelity Polymerase Mix 2x</td>
<td>25 μl</td>
<td>1x</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5 μl</td>
<td>0.5μm</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5 μl</td>
<td>0.5μm</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1 μl</td>
<td>&lt;1000ng</td>
</tr>
<tr>
<td>Nuclease Free H₂O</td>
<td>19 μl</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.4: Denaturation using Q5 High Fidelity Polymerase was performed at 98°C. Annealing Temperature (Ta) was calculated as 3°C higher than the melting of the least stable primer and further optimised if required. Extension time was calculated by allowing 30sec/kb of the target amplicon. PCR products were visualised using agarose gel electrophoresis as outlined in Section 2.8.1.4 using a 10kb DNA ladder (Promega) in order to check the PCR was successful and the correct size DNA fragment had been amplified.

2.8.1.6. PCR Purification

PCR products to be used in downstream applications such as restriction digests, ligations and sequencing were purified in order to removed oligonucleotide primers, buffers, salts and polymerase that may otherwise interfere with enzymatic reactions. PCR products were purified using the Qiagen PCR Purification kit (Qiagen) or the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer’s instructions.
2.8.1.7. Purification of DNA from Agarose Gel

DNA that has been electrophoresed through agarose gels is often used as the template in a variety of downstream applications such as PCR, ligations, sequencing and hybridisations. DNA is recovered by excising a DNA band of interest directly from an agarose gel using a sterile scalpel. The DNA from the gel slice can then be extracted by various means. Gel extraction is a useful technique for separating and purifying double stranded DNA of different sizes such as products of a restriction digest.

Gel extraction was used in this study to excise and purify DNA products of interest from restriction digests. 40μl of a restriction digest reaction (Section 2.8.1.8) was mixed 1:1 with Loading dye and run on an agarose gel as in Section 2.8.1.4. The Gel was visualised on a UV transilluminator (VWR, UK) and the DNA product of interest was excised using a sterile scalpel. The gel slice was placed in to a pre-weighed microcentrifuge tube and weighed. The DNA fragment was purified using either the Qiaquick Gel Extraction Kit (Qiagen) or the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer’s instructions.

2.8.1.8. Restriction Digests

Restriction digest is the process of cleaving DNA molecules in to smaller pieces using enzymes called restriction endonucleases. This is a commonly used technique in molecular cloning to generate compatible cohesive ends on DNA capable of being ligated together to construct plasmid vectors. DNA from PCR products, plasmid preparations or genomic DNA can be digested by restriction enzymes that typically recognise a 6-8bp sequence. In this study, restriction endonucleases were used to digest PCR products or plasmids in order to introduce specific compatible ends to DNA which could then be directionally inserted into compatible plasmids.
Table 2.5 below shows the typical components of restriction digests used in this study. All enzymes used in this study are listed in Table of Enzymes in Appendix II and were obtained from Promega, UK, unless otherwise stated. All reactions were performed in 1.5ml microcentrifuge tubes at 37°C unless otherwise stated.

Certain restriction endonucleases such as Esp3I (Fermentas, UK) required additional reagents not supplied by the manufacturer, in such cases additional reagents are indicated in Table of Enzymes, Appendix II). For double digestions, commercial buffers compatible with both enzymes were used and the volume of water in the reaction adjusted accordingly. In the event that no compatible buffer was available for both enzymes, digestion was performed sequentially, with a purification step prior to the second restriction digest.

Each restriction digest was performed in quadruplicate and purified using column purification as outlined in section 2.8.1.6 with all like for like reactions purified through one spin column for use in downstream applications such as DNA ligation.

**Table 2.5**: Typical volumes and components of restriction digest reactions performed in this study. All buffers used were the enzyme compatible buffers supplied by the manufacturer. BSA was supplied by the enzyme manufacturer.

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>~1μg</td>
</tr>
<tr>
<td>Enzyme compatible buffer</td>
<td>4μl</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>0.4μl</td>
</tr>
<tr>
<td>Enzyme</td>
<td>2μl</td>
</tr>
<tr>
<td>Nuclease-Free H₂O</td>
<td>to 40μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>40μl</strong></td>
</tr>
</tbody>
</table>
2.8.1.9. Ligations

The process by which DNA is joined together covalently is termed DNA ligation. This specifically involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. The most extensively utilised enzyme used to ligate DNA fragments together is T4 DNA ligase which is derived from the T4 bacteriophage and is capable of ligating DNA with overhanging cohesive ends resulting such as those resulting from restriction digests, as well as blunt-end fragments when used at higher concentrations. T4 Ligase has a broad range of temperatures at which it is active. In this study, ligation reactions were performed at 4°C for 18-24 hours and were used to insert double stranded linear DNA fragments resulting from PCR or restriction digests into linearised plasmids in order to construct circular recombinant vectors. Table 2.6 shows typical reaction components of a DNA ligation.
An important consideration when performing DNA ligations is the molar ratio of insert DNA to vector DNA. In this study, ligations were performed with a range of molar ratios ranging from 1:3 to 1:10 (vector:insert). The mass of insert DNA required for the correct ratio was calculated using the formula: \[ \frac{\text{kb of insert}}{\text{kb of vector}} \times \text{ng of vector} = \text{ng of insert needed for 1:1 ratio} \]

Table 2.6: The reaction components of a typical ligation reaction. 50ng of vector was used and the volume of insert DNA required was calculated according to the size of the vector used and the size of the insert used. T4 Ligase was purchased from Promega (UK) and was supplied with the corresponding 10x Ligation Buffer. Ligation buffer was stored at -20°C in 10μl aliquots to avoid repeated freezing and thawing of the buffer which can result in ATP degradation.

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert DNA</td>
<td>X</td>
</tr>
<tr>
<td>Vector DNA</td>
<td>1μl (diluted to 50ng/μl)</td>
</tr>
<tr>
<td>Ligase Buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>T4 Ligase</td>
<td>1 μl</td>
</tr>
<tr>
<td>Nuclease-Free H2O</td>
<td>Up to 10μl</td>
</tr>
<tr>
<td>Total</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

2.8.1.10. Heat Shock Transformation of chemically competent *E. coli*

All plasmids and vectors used and constructed in this study were propagated in *E. coli* DH5 alpha (Invitrogen) and *E. coli* JM109 cells (Promega). Wherever possible, commercially available competent cells were used for transformations. In order to insert plasmid DNA into these strains for replication and propagation, the technique of heat shock transformation was employed. This technique uses a calcium rich environment provided by a calcium chloride based buffer to counteract the electrostatic repulsion between the plasmid DNA and the bacterial membrane. A sudden increase in temperature causes the formation of pores in the bacterial membrane and
allows plasmid DNA to enter the bacterial cell. Recipient cells are then cultured and the DNA of interest is amplified in larger quantities as a result of bacterial replication.

In this study, 5μl of ligation mixtures were combined with 50μl of competent cells and incubated on ice for 30 minutes in sterile 1.5ml microcentrifuge tubes. Transformation mixtures were heat shocked at 42°C for 45 seconds and returned to ice for 2 minutes. After this time, 450μl of Standard Outgrowth Broth (SOB) was added to the transformation mixture and tubes were incubated at 37°C with shaking for 2 hours. 100μl of the mixture was inoculated on to LB agar supplemented with appropriate antibiotics. The remainder of the mixture was spun down in microcentrifuge at 13 000rpm for 1 minutes, resuspended in 100μl of SOB and inoculated on to LB agar supplemented with appropriate antibiotics.

2.8.1.11. Overlapping Extension PCR

The traditional molecular cloning techniques that are used in this study are laborious, time consuming techniques. A technique described by Hansen et al. (2007), was also employed in this study for the rapid generation of single knockout mutants in C. jejuni. This technique, referred to in this study as Overlapping Extension PCR (oePCR), involves the individual amplification of C. jejuni sequences upstream and downstream of the gene of interest using primers with complementary leader sequences to an antibiotic cassette that is amplified separately. All amplicons are combined in a single PCR as template DNA. After an initial annealing reaction without primers to anneal the three DNA fragments together, a final PCR is performed resulting in a single amplicon that consists of the flanking regions of the target gene separated by an antibiotic resistance cassette. This linear fragment of DNA can be used as a transformation construct for C. jejuni.
2.8.1.12. Transformation of *Campylobacter jejuni*

*Campylobacter jejuni* is a naturally competent bacterial species, capable of importing macromolecular DNA from the environment and into their genomes and it is thought that this process is a potential mechanism for horizontal gene transfer that generates the remarkable genetic diversity observed in campylobacters. Whilst naturally competent, *C. jejuni* strains encode a range of restriction-modification systems that protect against exogenous DNAs such as those from bacteriophages, thus limiting the DNAs that can lead to recombination events to *Campylobacter*-derived DNA. As a result, *Campylobacter* is not as amenable to genetic manipulation when compared to other enteric pathogens however a number of methods have been developed that allow genetic manipulation of certain strains.

2.8.1.12.1. Preparation of electro-competent *C. jejuni*

A lawn culture MHA blood agar plates was flooded with 1ml MH broth per plate and harvested. This liquid culture was centrifuged for 2 minutes at >10,000 × g at 4°C in a microcentrifuge (Eppendorf). Pellets were gently resuspended in 1ml of ice cold wash buffer (272 mM sucrose, 15% glycerol) and washed four times by centrifuging at >10,000 × g at 4°C. After the last washing, the pellet was resuspended in 500µl of ice cold washing buffer and 50µl aliquots of cells were stored at -80°C. All the steps of this procedure were performed on ice.

2.8.1.12.2. Electroporation

Freshly made competent cells were used for all electroporations. 100µl of competent cells were pipetted into ice cold sterile 1.5ml microcentrifuge tubes. Transformation constructs propagated in plasmids were amplified using PCR with appropriate primers as in Section 2.8.1.5. PCR products
were purified and eluted in nuclease free water as in Section 2.8.1.6. Approximately 3µg (no more than 10µl) of purified PCR product was added to the competent cells and incubated on ice for 10 minutes. Competent cells containing no DNA served as negative controls and were included in every transformation. After incubation on ice, cells were transferred to electroporation cuvettes (2mm gap, Eppendorf, UK). Voltage pulses were delivered to the ice cold cells using a gene pulser apparatus (BioRad Gene Pulser™). The electroporation settings were adjusted to 2.5kv, 25µF, 200 ohm to a time constant of less than 4 seconds. Immediately following electroporation, the contents of each cuvette were flushed with 200µl pre-warmed SOC medium that had been incubated in a microaerobic environment. The entire contents of the cuvettes were then transferred to universals containing 2mls MHA agar supplemented with 5% sheep’s blood and, and incubated at 42°C in a microaerophillic environment for 12 hours for recovery. After this time had elapsed, the contents of the universals were flushed with 1ml pre-warmed MHB and plated on to MHA supplemented with 5% sheep’s blood and either chloramphenicol or kanamycin for selection of positive transformants. Selectivity plates were incubated at 42°C for 3-5 days. Resulting positive colonies were screened for correct insertion of transformation constructs by PCR. Successful first generation transformants were sub-cultured on to MHA supplemented as before and incubated at 42°C for 48 hours. After this, genomic DNA was extracted as described in Section 2.8.1.1 to be used for natural transformation.

2.8.1.12.3. Natural Transformation of C. jejuni

For natural transformation, C. jejuni was cultured for two days followed by an overnight culture. Bacteria were harvested from overnight agar plates and transferred to universals containing 2mls MHA agar supplemented with 5% sheep’s blood and allowed to recover by incubation at 42°C in microaerophillic conditions for 3 hours. After this time, 50ng of Campylobacter first generation
transformant chromosomal DNA was added to the appropriate universals. Contents were mixed by gentle pipetting and the universals were re-incubated for 6 hours under microaerophilic conditions at 42°C. Bacterial suspensions were then plated on to MHA plates supplemented with 5% sheep’s blood and appropriate antibiotics for selection of transformants.
2.9. Specific DNA manipulations used in this study

2.9.1. Construction of a capC-knockout via insertion of a kanamycin resistance cassette

A 1266bp capC fragment was amplified from C. jejuni 81116 genomic DNA as described in section 2.8.1.5 using the primers Cap3 and Cap4. The 1266bp capC amplicon containing a BglII restriction site was purified as in section 2.8.1.6 and ligated into the pGEM T-easy vector. The pGEM T-easy is a commercially available vector linearized with EcoRI with a single deoxythymidine nucleotide overhang added to each 3’ end, thus providing a compatible overhang for the capC fragment with a polyA tail generated by Taq polymerase.

Ligation mixtures were transformed into E. coli DH5a which were plated onto LB agar supplemented with 100µg/ml, 0.1M IPTG and 50µg/ml X-gal to perform a blue/white screen. The pGEM vector contains a gene conferring ampicillin resistance; therefore only E. coli which had taken up the plasmid were able to grow on the LBA/Amp/IPTG/X-gal plates. In addition, the vector also contains a lacZ gene encoding a β-galactosidase enzyme capable of metabolizing the chromogenic substrate X-gal thus producing a blue coloured precipitate within cells resulting in blue colonies. The insertion site for PCR products lies within the lacZ gene; successful integration of the PCR product inactivates lacZ rendering cells that successfully take up the correctly ligated plasmid unable to metabolized X-gal resulting in white colonies. Resulting white colonies were picked and screened by PCR and sequencing for successful integration of the plasmid. A plasmid which had successfully integrated the 1266bp capC fragment was designated pJM001.

pJM001 was digested with BglII as described in section 2.8.1.8. Simultaneously pJMK30 (van Vliet et al., 1998), a plasmid containing the kanamycin resistance cassette, was digested by BamH1 liberating a 1499bp fragment containing the aphA-3 kanamycin resistance gene. Restriction digest of pJM001 and pJMK30 with these enzymes resulted in sticky ends; compatible overhangs
that may be ligated together. The 1499bp kanamycin resistance fragment was ligated in to the
BglII site of pJM001 resulting in a 2765bp insert consisting of the capC fragments of 674bp and a
572bp flanking the 1499bp kanamycin cassette. This plasmid was designated pJM002. The
knockout construct 2765 knockout construct was amplified using primers Cap3 and Cap4 and
transformed into C. jejuni 81116 by electroporation and natural transformation as described in
Section 2.8.1.12.
2.9.2. Construction of capC-knockouts via insertion of a chloramphenicol resistance cassette

Additional knockout mutants in the capC gene were constructed in *C. jejuni* strains 81116 and M1 using a chloramphenicol resistance cassette insert. The capC gene was amplified using the CapCFW and CapCRV primers resulting in a 2893bp fragment. This fragment was cloned into pGEM T-easy as described in Section 2.9.1. A plasmid showing successful and correct integration was designated pJM005. pJM005 was digested with *Bgl*II and *Xba*I cleaving the capC sequence after the 1501 and 2164 nucleotide positions respectively and thus excising a 647bp capC fragment from the plasmid. The chloramphenicol resistance (*cat*) cassette was amplified from pAV103 using the primers JMCAT5 and JMCAT6. These primers were designed with *Bgl*II and *Xba*I restriction sites at the 5’ end allowing these sequences to be incorporated into the amplicon during PCR. The resulting 820bp *cat* amplicon was purified and digested with *Bgl*II and *Xba*I. The digested fragment was ligated into the digested and dephosphorylated pJM005 thereby creating a plasmid containing the capC gene in which a 647bp fragment had been replaced with the chloramphenicol resistance marker. The knockout construct was amplified using the Vac1 and Cap3 primers yielding a 1906bp fragment which was used to transform *C. jejuni* 81116 and M1 as described in Section 2.8.1.12. Coupled wildtype strains were also generated.

2.9.3. Complementation of capC

The capC gene was unable to be complemented by cloning into the *Esp*3I site of the pC46 plasmid. A complementation construct produced by overlapping PCR using the upstream and downstream flanks was also unable to be transformed into *E. coli* for propagation. The pSV009 complementation vector (de Vries *et al.*, 2015) offered an improved alternative to the pC46 plasmid. However the capC gene was only able to be ligated into pSV009 in the opposite
orientation to the cat promoter and kanamycin resistance cassette. Directional cloning yielded no transformant colonies.

capC was amplified using the primers BamCap1 and BamCap2. The BamCap primer set also contains BamHI restriction sequences at the 5’ ends of each oligo. The amplicon was cloned into pGEM-Teasy as described in Section 2.9.1, thereby generating pJM008. This plasmid was digested with BamHI releasing the capC fragment which was gel purified as described in Section 2.8.1.7.

The pSV009 plasmid (de Vries et al., 2015) is a plasmid designed for complementation of genes in C. jejuni by insertion of a gene of interest into the cj0046 pseudogene sequence. The pSV009 plasmid contains the cj0046 pseudogene fragments flanking the kanamycin resistance cassette and the cat promoter which made be used to drive expression of a gene of interest which can be inserted via the MCS. The BamHI digested capC gene was inserted into the BamHI site of pSV009 and transformed into E. coli for propagation. This construct was designated pJM009.

2.10. Multi-Locus Sequence Typing

Multi-Locus Sequence Typing (MLST) is a typing system for characterising isolates of bacterial species based on the sequences of internal fragments of housekeeping genes. MLST is based on the established principles of multi-locus enzyme electrophoresis, but differs in that it assigns alleles at multiple house-keeping loci directly by DNA sequencing, rather than indirectly via the electrophoretic mobility of their gene products. Most bacterial species have sufficient variation within house-keeping genes to provide many alleles per locus, allowing many distinct allelic profiles to be distinguished using house-keeping loci. The procedure involves the PCR
amplification of conserved house-keeping genes. Approximately 450-500bp internal fragments of these amplicons are sequenced on both strands using an automated DNA sequencer. For each house-keeping gene, an allele number is assigned based upon allelic differences. Importantly, in MLST the number of nucleotide differences between alleles is ignored and sequences are assigned different allele numbers whether they differ at a single nucleotide site or at many sites, thereby treating nucleotide changes resulting from either point mutations or recombination as a single genetic event. Using allelic profiles of the house-keeping gene, a sequence type can be assigned to each isolate. The sequence data generated by MLST can also be easily compared over large datasets lending MLST considerable discriminatory power. MLST is widely utilised as a typing system for Campylobacter jejuni and C. coli and is preferable to other typing systems for its ability to group isolates in to sequence types and clonal complexes and the fact that these sequence types correlate closely with isolate source and lineage.

Campylobacter jejuni MLST involves the amplification of seven housekeeping genes using specific primers and the subsequent sequencing of these amplicons using sequencing primers nested inside the amplification primers. House-keeping genes, amplicon sizes and amplification and sequencing primers used in this study are shown in Table 2.7. Functional gene product assignments of housekeeping genes are shown in Table 2.8.
Table 2.7: The seven house-keeping genes that are used for *C. jejuni* MLST and their respective amplicon sizes are also shown. The amplification and sequencing primer sequences used in study were obtained from http://pubmlst.org/campylobacter/info.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer type</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspA</td>
<td>Amplification</td>
<td>asp-A9, 5’-AGT ACT AAT GAT GCT TGT CC-3’</td>
<td>asp-A10, 5’-ATT TCA TCA ATT TGT TCT TTG C-3’</td>
<td>941</td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>asp-S3, 5’-CCA ACT GCA AGA TGC TGT ACC-3’</td>
<td>asp-S6, 5’-TTA ATT TGC GGT AAT ACC ATC-3’</td>
<td></td>
</tr>
<tr>
<td>glnA</td>
<td>Amplification</td>
<td>gln-A1, 5’-TAG GAA CTT GGC ATC AGC TAC TTG-3’</td>
<td>gln-A2, 5’-TTG GAC GAG CTT CTA CTG GC-3’</td>
<td>1,305</td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>gln-S3, 5’-CAT GCA ATC AAT GAA GAA AC-3’</td>
<td>gln-S6, 5’-TTC CAT AAG CTC ATA TGA AC-3’</td>
<td></td>
</tr>
<tr>
<td>gltA</td>
<td>Amplification</td>
<td>glt-A1, 5’-GGG CTT GAC TTC TAC AGC TAC TTG-3’</td>
<td>glt-A2, 5’-CCA AAT AAA GTT GTC TTG GAC GG-3’</td>
<td>1,112</td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>glt-S1, 5’-GTG GCT ATC CTA TAG AGT GGC-3’</td>
<td>glt-S6, 5’-CCA AAG CGC ACC AAT ACC TG-3’</td>
<td></td>
</tr>
<tr>
<td>glyA</td>
<td>Amplification</td>
<td>gly-A1, 5’-GAG TTA GAG CGT CAA TGT GAA GG-3’</td>
<td>gly-A2, 5’-AAA CCT CTG GCA GTA AGG GC-3’</td>
<td>1,052</td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>gly-S3, 5’-AGC TAA TCA AGG TGT TTA TGC GG-3’</td>
<td>gly-S4, 5’-AGG TGA TTA TCC GTT CCA TCG C-3’</td>
<td></td>
</tr>
<tr>
<td>Pgm</td>
<td>Amplification</td>
<td>pgm-A7, 5’-TAC TAA TAA TAT CTT AGT AGG-3’</td>
<td>pgm-A8, 5’-CAC AAC ATT TTT CAT TTC TTT TTC-3’</td>
<td>1,195</td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>pgm-S5, 5’-GGT TTT AGA TGT GGC TCA TG-3’</td>
<td>pgm-S2, 3’-TCC AGA ATA GCG AAA TAA GG-3’</td>
<td></td>
</tr>
<tr>
<td>Tkt</td>
<td>Amplification</td>
<td>tkt-A3, 5’-GCA AAC TCA GGA CAC CCA GG-3’</td>
<td>tkt-A6, 5’-AAA GCA TTG TTA ATG GGT GC-3’</td>
<td>1,133</td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>tkt-S5, 5’-GCT TAG CAG ATA TTT TAA GTG-3’</td>
<td>tkt-S4, 5’-ACT TCT TCA CCC AAA GGT GGG-3’</td>
<td></td>
</tr>
<tr>
<td>uncA</td>
<td>Amplification</td>
<td>unc-A7, 5’-ATG GAC TTA AGA ATA TTA TGG C-3’</td>
<td>unc-A2, 5’-GCT AAG CGG AGA ATA AGG TGG-3’</td>
<td>1,259</td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>unc-S5, 5’-TGT TGC AAT TGG TCA AAA GC-3’</td>
<td>unc-S4, 5’-TGC CTC ATC TAA ATC ACT AGC-3’</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.8: Table 2.8 shows the functional gene products of each of the seven house-keeping genes used for *Campylobacter jejuni* MLST.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspA</td>
<td>Aspartase</td>
</tr>
<tr>
<td>glnA</td>
<td>glutamine synthetase</td>
</tr>
<tr>
<td>gltA</td>
<td>citrate synthase</td>
</tr>
<tr>
<td>glyA</td>
<td>serine hydroxy methyl transferase</td>
</tr>
<tr>
<td>pgm</td>
<td>phospho glucomutase</td>
</tr>
<tr>
<td>tkt</td>
<td>transketolase</td>
</tr>
<tr>
<td>uncA</td>
<td>ATP synthase alpha subunit</td>
</tr>
</tbody>
</table>

Table 2.9: The components and volumes of the MLST PCR for amplification of each of the seven housekeeping gene. PCRs were performed with each primer set with the following conditions: The reaction conditions were denaturation at 94°C for 2 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min, for 35 cycles.

MLST was performed on 30 human disease isolates of *C. jejuni*. For each strain, the seven house-keeping genes were amplified in a PCR as shown in Table 2.9. PCR products amplified
using GoTaq Green Master Mix were directly run on an agarose gel as described in section 2.8.1.4 to check for correct amplification of all products. PCR products were purified as described in section 2.8.1.6 and diluted to appropriate concentrations for DNA sequencing section DNA sequencing.

Allelic profile numbers and isolate sequence types were defined by querying allele sequences individually against the *Campylobacter* database at:

2.11. Sanger DNA Sequencing and Analysis

Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. The template DNA to be sequence was diluted in nuclease-free water to an optimal concentration as advised by the sequencing service provider (Beckman Coulter Genomics). Plasmids were diluted to 100ng/μl and PCR products were diluted to 25-35 ng/μl depending on amplicon size. All sequencing primers are listed in Section 2.10. All sequencing primers were synthesised by Beckman Coulter Genomics. Sequencing was performed by Beckman Coulter Genomics using an ABI 3730XL sequencer. DNA samples were sequenced on the forward and reverse strand in the sense direction. Resulting .ab1 and .seq files were provided electronically by Beckman Coulter Genomics. .ab1 files were used to view chromatograms of each sequencing reaction using Chromas Lite v2.1.1 to check the success of the sequencing reaction and the authenticity of the bases called. For sequences where incorrect or multiple bases signals were called, sequences were curated accordingly. Forward and reverse sequences were assembled using BioEdit v7.25; consensus sequences were extracted and used for further analysis using bioinformatic tools and applications listed in the List of Bioinformatic Tools and Applications in Appendix II.

2.12. Whole Genome Sequencing and Analysis

Isolates selected for whole genome sequencing are listed in Table 6.1 in Chapter 6 and below. Isolates were cultured from -80°C stocks and sub-cultured for 24 hours. DNA was extracted from these isolates using the ArchivePure 5 Prime DNA extraction kit as described in Section 2.8.1.1.2. DNA was quantified using the Qubit 2.0 fluorometer as described in Section 2.8.1.2. DNA samples were sequenced on an Illumina MiSeq platform using the standard Illumina indexing protocol (service provided by Bioinformatics Unit, APHA, UK). 150bp paired end sequence reads
were processed and assembled into contiguous sequences using Velvet as part of the service provided by APHA. The quality of *C. jejuni* genomic assemblies was evaluated using Quast (Gurevich *et al.*, 2013).

**Table 6.1:** Table showing the 17 *C. jejuni* strains that were successfully sequenced. 11 strains that were determined to encode a *capC* variant by BLASTn search are indicated as are strains that do not encode *capC* or its sequence variant. MLST sequence type (ST) and clonal complex for each strain is also presented. Notably, only strains that belong to ST-21 clonal complex do not encode *capC* or its sequence variant.

<table>
<thead>
<tr>
<th>Sample</th>
<th>capC sequence variant</th>
<th>ST</th>
<th>Clonal Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em> HI12</td>
<td>+</td>
<td>2103</td>
<td>ST-607 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI20</td>
<td>+</td>
<td>574</td>
<td>ST-574 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI9</td>
<td>+</td>
<td>305</td>
<td>ST-574 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI1</td>
<td>+</td>
<td>52</td>
<td>ST-52 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI10</td>
<td>+</td>
<td>48</td>
<td>ST-48 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI4</td>
<td>+</td>
<td>48</td>
<td>ST-48 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI13</td>
<td>+</td>
<td>5136</td>
<td>ST-464 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI5</td>
<td>+</td>
<td>5136</td>
<td>ST-464 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI8</td>
<td>+</td>
<td>104</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI19</td>
<td>-</td>
<td>50</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI23</td>
<td>-</td>
<td>50</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI25</td>
<td>-</td>
<td>21</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI28</td>
<td>-</td>
<td>19</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI3</td>
<td>-</td>
<td>21</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI30</td>
<td>-</td>
<td>50</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI21</td>
<td>+</td>
<td>122</td>
<td>ST-206 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI17</td>
<td>+</td>
<td>1911</td>
<td></td>
</tr>
</tbody>
</table>
2.12.1 Analysis

Genomic analyses including assembly evaluation, BLAST screening, genome annotation, MLST sequence type/clonal complex designation and phylogenetic tree construction (Figures 6.7A and B) was performed by Dr. Arnoud Van Vliet.

The distribution of capA, capB, capC and capD was determined amongst a collection of 7176 complete and draft Campylobacter genome sequences (5829 C. jejuni, 1347 C. coli). These genomes are listed in Appendix VI including where available, PubMLST ID, genbank accession number, isolate source, MLST sequence type and clonal complex. Genomes were all obtained from public collections and classified on the basis of MLST clonal complex and phylogenetic clade (Cody et al., 2013; Jolley and Maiden, 2010). The nucleotide sequences of capA, capB, capC and capD were segregated into “words” of 120 nucleotides in length and used to screen of the presence of autotransporter genes amongst the collection of C. jejuni and C. coli genomes using BLASTn V 2.28 via MIST (Microbial In Silico Typer) (Kruczkiewicz et al., 2013). Autotransporter genes were classed as present if 80% identity or greater was observed across the whole gene; a threshold of 80% was found to be optimal to detect genes with and without truncations. MLST sequence types and resulting clonal complex classification of all genomes within the collection were also obtained from PubMLST via the MIST shell.

For protein based screening of these genomes, all genomes in the collection were annotated using Prokka. Annotated genomes were screened at the protein level using the amino acid sequences of full length CapA (cj0628/9), CapB (cj1677/78), CapC (C8J_1278) and CapD (compiled by amalgamation of truncated sequence from C. jejuni HI1) via the BLASTp function in BioEdit 7.25 (E value=1E-20). The prevalence of full length protein was determined by full coverage over the whole sequence when compared to query sequences where as partial identity was classed as truncation regardless of the degree of overall sequence identity. Core-genome single nucleotide
polymorphisms were identified in all genomes in the collection using ParSNP (Treangen et al., 2014) and used to generate a phylogenetic tree showing phylogenetic clustering of MLST clonal complexes and phylogenetic clades in each C. jejuni and C. coli respectively. Colour-coding of truncated and full length encoding isolates within a phylogenetic tree generated using Figtree (http://tree.bio.ed.ac.uk/software/figtree/) allowed associations of autotransporters with genotypes to be visualised.
2.13. *Galleria mellonella* Virulence Model

*Galleria mellonella* larvae were purchased from commercial suppliers Live Food or Live Foods Direct. Larvae were shipped at 5\textsuperscript{th} or 6\textsuperscript{th} instar stage (approximately between 2-3 cm in length) and were suitable for use immediately. Larvae were maintained at 17\textdegree C on wood chips for up to two weeks. If not used within this time, larvae were euthanized by freezing at -20\textdegree C. All larvae were examined prior to infection; any larvae showing discolouration or signs of pupation were immediately discarded and euthanized. Healthy larvae were selected on the basis of their uniform creamy colour with no areas of dark discoloration and that were able to right themselves quickly if turned over. Larvae were handled using ethanol-sterilised blunt-nosed forceps.

2.13.1. Preparation of *C. jejuni* for *Galleria mellonella* infection

*Campylobacter jejuni* strains were cultured from -80\textdegree C frozen stocks on to MHA supplemented with 5% sheep’s blood incubated at 42\textdegree C for 48 hours. A loopful of this culture was subcultured into 10mls of MHB supplemented with antibiotics where appropriate and incubated at 42\textdegree C for 18-24 hours. This culture was centrifuged in a Beckman Allegra X100 centrifuge for 10 minutes at 3500rpm and resuspended in PBS to an OD\textsubscript{590nm} of 1. This bacterial suspension was used to infect *Galleria* larvae.

2.13.2. Infection of *Galleria mellonella* larvae

Infection of *Galleria mellonella* larvae was performed in a microbiological safety cabinet. An injection platform was prepared by placing a 90mm filter paper (Whattman) within the lid of a Petri dish. A sterile 1000\textmu l pipette tip was taped with autoclave tape across the middle of the filter paper as shown in Figure 2.1. Larvae were positioned over the pipette tip on their back, bent over the pipette tip, and injected through the top right proleg with 10\textmu l of PBS or bacterial
cell suspension prepared as described in Section 2.13.1 above. Injection was performed using a Hamilton® 26S Micro-Syringe (Sigma). Care was taken to insert the syringe correctly without inducing injury and death. Once infected, larvae were placed on a Whattman 90mm filter paper in an inverted Petri dish. Ten larvae were infected for each isolate in triplicate. A negative control in which larvae were infected with PBS only and a non-infected control were also included.

Figure 2.1: Positioning of *Galleria* larvae on the injection platform. Larvae were infected with bacterial suspensions via microinjection through the top-right proleg using a Hamilton® Micro-Syringe with a 26S gauge needle (Sigma).
2.13.3. *Galleria* Survival Assay

*Galleria* larvae were used to assess the virulence contribution of CapC. Bacterial suspensions of *C. jejuni* 81116 and M1 as well as their respective derivatives were prepared as described in Section 2.13.1. *Galleria* larvae were infected as described in section 2.13.2. All infected larvae and controls were incubated at 37°C for 48 hours. At 6, 24, 36 and 48 hours post infection, the number of dead and live larvae were examined and mortality was assessed by the ability of larvae to move in response to physical stimuli using ethanol-sterilised blunt-nosed forceps and strong discolouration indicative of an immune response. The number of surviving larvae at each time-point was counted. This assay was repeated 3 times.

2.13.4. Morbidity and Mortality Assay

In order to gain a more sensitive measure of the contribution of CapC to virulence, *C. jejuni* 81116 and M1 and their respective capC knockouts were compared in the *Galleria* model using a morbidity and mortality assay. *C. jejuni* strains were re-suspended in PBS to an OD\textsubscript{590nm} of 1 and used to infect *Galleria* larvae as described in Section 2.13.1 including background and non-infected controls. Infected larvae were incubated for 24 hours after which they were assessed for morbidity of disease and mortality by using a scoring system developed in this study. Each individual larva was scored on the basis of mortality, colour and movement. The scoring system used in this study in outlined in Chapter 4.

2.13.5. *Galleria* Intra-Larval Replication Assay

In order to assess and compare the replication capability of *C. jejuni* 81116, M1 and their capC knockout mutants in the context of the *Galleria* host, the CFUs of *Campylobacter* within infected
Galleria larvae were quantified at 3 and 6 hours post infection. Galleria larvae were prepared and infected with each strain as described in the sections above. Viable counts of the Galleria inoculum were obtained as described in section 2.6.4.3. At 3 and 6 hours post-infection, 3 larvae in incubation were selected at random. Larvae were anaesthetised by being left on ice for 10 minutes. After this time had elapsed, larvae were placed in a sterile Petri dish and swabbed with 70% ethanol and allowed to dry. The head of the Galleria (approximately the top 4mm of each larvae) was aseptically removed using a sterile scalpel and the haemolymph drained into a sterile microcentrifuge tube. This process was repeated for each strain. Viable counts of Campylobacter jejuni were obtained as described in section 2.6.4.3 in triplicate. Bacteria were plated on to MHA supplemented with either trimethoprim, kanamycin or chloramphenicol for selection of appropriate strains and to inhibit commensal organisms present within Galleria mellonella larvae. This assay was repeated 3 times.
2.14. Tissue Culture Models of Infection

2.14.1. Preparation of *C. jejuni* for Cell Culture Infection

For experimentation with and infection of tissue culture cell lines, bacteria were grown from -80°C stocks for 48 hours and then sub-cultured for 24 hours. After this time, bacterial lawns on plates were harvested and re-suspended in PBS to an OD$_{550nm}$ of 1.1. 100µl of this suspension was used to infect cells. The CFU/ml of this initial inoculum was enumerated as described in Section 2.6.4.3.

2.14.2. Growth and Maintenance of Caco-2 colorectal cells

Caco-2 is a human epithelial colorectal adenocarcinoma continuous cell line. Although derived from a colon carcinoma, Caco-2 cells exhibit differentiation and polarisation when cultured under specific conditions such that their morphology and functionality resembles that of enterocytes of the small intestine. Caco-2 cells express tight junctions, microvilli and a number of enzymes and transporters that are characteristic of such enterocytes and thus makes Caco-2 an attractive model for the study of *C. jejuni* infection processes.

Caco-2 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, 1% Non-essential amino acids and 1%(w/v) 100 U/ml penicillin/100µg/ml streptomycin. Cells were grown routinely in 75 cm$^2$ (Nunc) tissue culture flasks at 37°C in a 5% CO$_2$ humidified incubator. Confluent stock cultures were washed, trypsinized and new stock cultures were seeded at ~ 4 x10$^4$ cells/ml. Caco-2 cells were always handled for media changes and seeding in a microbiological safety cabinet.
2.14.2.1. Caco-2 Adhesion Assay

For the adhesion assay Caco-2 cells were seeded for 1 week in 24-well plates at 1x10^6 cells/ml in 1ml of DMEM supplemented with 10% foetal bovine serum, 1% non-essential amino acids and 1% (w/v) penicillin/streptomycin. At 48 hour intervals from the time of seeding, media was removed and monolayers were washed 3 times with PBS, and 1ml of fresh DMEM added to each well. At 24 hours prior to infection of cells, monolayers were washed and 900μl of fresh antibiotic-free media was added to each well. Caco-2 cells in 24-well plates were infected with 100μl of *C. jejuni* suspensions prepared as described in Section 2.14.2 at an MOI of 1:200 and incubated at 37°C for 3 1/2 hours in a 5% CO₂ humidified incubator. After incubation, media was removed from each well and monolayers were washed three times with PBS supplemented with calcium and magnesium to removed non-associated bacteria. Monolayers were lysed with pre-warmed 1% (w/v) Triton-X 100 to release cell associated bacteria which were enumerated as described in Section 2.6.4.3. Serial dilutions were plated on to MHA supplemented with either trimethoprim (100μg/ml), chloramphenicol (10μg/ml) or kanamycin (50μg/ml) where appropriate. The number of adherent bacteria was calculated by subtracting the number of invaded bacteria (Section 2.14.2.2.) from the number of associated bacteria. Results were expressed as adhesion efficiency using the formula below:

\[
\text{Number of Adherent bacteria (cfu/ml)} \quad \frac{\text{Initial Inoculum (~}1\times10^8 \text{cfu/ml) \times 100 = Adhesion Efficiency}} = \text{Adhesion Efficiency}
\]
2.14.2.2. Caco-2 Gentamicin Protection Assay

The gentamicin protection assay was performed in conjunction with the adhesion assay. Caco-2 cells were seeded and infected as described in Section 2.14.2.1. After incubation for 3½ hours, media was removed and monolayers were washed 3 times with PBS supplemented with calcium and magnesium to remove non-associated bacteria. Complete DMEM supplemented with gentamicin to a final concentration of 150μg/ml was added to each well to kill extracellular bacteria. Plates were re-incubated for 2 hours at 37°C. Media was then removed and the infected monolayers were washed twice with PBS followed by lysis with 1% Triton-X 100. Quantification of viable intracellular bacteria was performed as described in Section 2.6.4.3. Results were expressed as invasion efficiency using the formula below:

\[
\frac{\text{Number of Invaded bacteria (cfu/ml)}}{\text{Initial Inoculum (~1x10^8 cfu/ml)}} \times 100 = \text{Invasion Efficiency}
\]

2.14.2.3. Cytotoxicity

In order to assess the toxicity of CapC to Caco-2 cells, the cytotoxicity of C. jejuni 81116 and M1 and their capC knockouts were compared in a cytotoxicity assay using the Pierce LDH Cytotoxicity Assay Kit according to the manufacturers’ instructions (Thermofisher, UK). Lactate dehydrogenase (LDH) is an enzyme found in nearly all living cells and catalyses the conversion of lactate to pyruvate via NAD+ reduction to NADH. In this assay, lactate dehydrogenase levels are used as a biomarker for cellular cytotoxicity and cytolysis as it is released into the media supernatant by Caco-2 cells upon plasma membrane damage. Extracellular LDH in the media can be quantified by a coupled enzymatic reaction in which the NADH produced by lactate dehydrogenase is used by diaphorase to reduce a tetrazolium salt to produce a red formazan
product. The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity.

Caco-2 cells were seeded at 1x10^5 per well of a 96-well plate in 100μl of complete DMEM and incubated for 48 hours. In addition to test wells, serum-free DMEM and complete DMEM without cells were included in triplicate to account for LDH activity in sera. Spontaneous LDH release and maximum LDH release were also included as directed in the manufacturers’ protocol.

2.14.2.4. Cell-Mediated Cytotoxicity Assay

Bacterial suspensions of *C. jejuni* 81116, M1 and their capC knockouts were prepared as described in Section 2.14.2. 10μl of this suspension was used to inoculate the appropriate well on the 96-well plate in triplicate. The spontaneous LDH release control wells were inoculated with PBS. Plates were incubated for 4 hours at 37°C in a 5% CO2 humidified incubator. Precisely 45 minutes before the end of this incubation period, 10μl of the 10x Lysis Buffer provided with the kit was added to the maximum LDH release control wells. After incubation, 50μl of the media supernatant was transferred to a sterile 96-well plate using a multi-channel pipette and 50μl of the Reaction Buffer supplied with the kit was added to each well. The plate was incubated in the dark at room temperature for 30 minutes followed by addition of 50μl of the Stop Buffer, also supplied with the kit. Absorbance was measure at 490nm and 680nm using a Wallac Plate Reader and the percentage cytotoxicity was calculated using the formula below:

\[
\frac{\text{Test Well LDH activity} - \text{Spontaneous LDH Activity}}{\text{Maximum LDH Activity} - \text{Spontaneous LDH Activity}} \times 100 = \text{% Cytotoxicity}
\]
2.14.2.5. Campylobacter-supernatant Cytotoxicity Assay

Cytotoxic supernatant fractions were prepared from *C. jejuni* stationary phase cultures. *Campylobacter jejuni* 81116, M1 and their respective *capC* mutants were cultured from -80°C frozen stocks on to MHA supplemented with 5% sheep’s blood incubated at 42°C for 48 hours. Bacterial lawns were harvested, re-suspended in MHB and adjusted to OD600nm of 1. 1 ml of this culture was added to 20mls of MHB in a T75 vented cell culture flask (Nunc, UK) supplemented with antibiotics where appropriate and incubated at 42°C for 36 hours. After incubation, bacterial liquid cultures were re-suspended, transferred to 50ml Falcon tubes (Fisher Scientific, UK) and centrifuged at 4000 x g for 15 minutes at 4°C. Resulting supernatants were filtered through a 0.22µm filter unit and transferred to 20ml Corning Spin-X UF centrifuge units with either a 10kDa MWCO or a 30kDa MWCO. Supernatants were concentrated by centrifugation at 5000 x g for 2 hours at 4°C to a volume of 2mls. Sterile culture medium was also concentrated for use as background control in subsequent cytotoxicity assays. Concentrated supernates were re-suspended and stored at -20°C. The concentration of the supernatants was quantified using the Qubit Protein Assay Kit (ThermoFisher Scientific, UK) and the Qubit 2.0 fluorometer. Supernatant fractions were diluted to a concentration of 10µg/ml. which was shown to be sufficient to elicit a cytotoxic response in Caco-2 cells in preliminary experimentation. 10µl of each fraction was used to infect Caco-2 cells as described in Sections 2.14.2.4 and 2.14.2.5.
2.14.4. Growth and Maintenance of T84 colonic epithelial cells

T84 cells are a continuous cell line of colonic epithelial cells derived from a lung metastasis of a colon carcinoma. These cells possess receptors for a range of peptide hormones and neurotransmitters and are capable of inflammatory responses. These cells grow as monolayers and exhibit tight junctions and desmosomes between adjacent cells.

T84 cells were maintained in DMEM/F-12 Ham’s Nutrient Mix (Invitrogen) supplemented with 10% (vol/vol) Fetal Calf Serum, 1% (wt/vol) nonessential amino acids, and 1%(w/v) 100 U/ml penicillin/100μg/ml streptomycin. Cells were grown routinely in 75cm² (Nunc) tissue culture flasks at 37°C in a 5% CO₂ humidified incubator. Confluent stock cultures were trypsinized and new cultures were seeded at ~4 x10⁴ cells/ml. For certain assays the medium from T84 cells was removed and the monolayers were washed twice with PBS and then maintained in antibiotic-free and/or serum-free DMEM/F-12 medium. T84 cells were always handled for media changes and seeding in a microbiological safety cabinet. For infection assays, cells were seeded at 1 x 10⁶ cells/ml in 24 well plates.

2.14.4.1. Enzyme-linked Immunosorbant Assay for the quantification of secreted IL-8 from Infected T-84 cells

T84 cells were infected with C. jejuni 81116, M1 and their respective capC mutants as described in Section 2.14.2.1. The levels of IL-8 secretion were assessed using a commercially available sandwich ELISA kit according to the manufacturer’s instructions (eBioscience, United Kingdom). Human IL-8 is a pro-inflammatory CXC chemokine that is secreted primarily by endothelial cells and is a potent neutrophil activator. It is commonly expressed in tissues in response to inflammatory stimuli, environmental stress and steroid hormones.
Briefly, after 4 hours of infection the infected cell supernatant was removed by pipetting and added to the wells of a 96-well Nunc Maxisorp® plate, in quadruplicate, that had been pre-blocked and exposed to a capture antibody specific for IL-8. Plates were incubated overnight and levels of IL-8 secretion were determined according to the manufacturer’s instructions in a procedure based on the principle outlined in Figure 2.2 below. Absorbance measured on a Wallac Plate Reader at 450nm and the levels of IL-8 secretion were compared using a Student’s t-test. This assay was repeated three times.

**Figure 2.2:** The principle of the sandwich ELISA used in this project. This procedure allows a quantitative measure of IL-8 between two layers of antibody (capture and detection antibodies). Plates are initially blocked overnight followed by addition of a capture antibody. Cell supernatants containing the target protein are then added to wells for a set time period and then removed. A detection antibody is then added which binds to a different conformational epitope on the target protein. This is followed by addition of a final antibody that is specific for the detection antibody conjugated to a Horse Radish Peroxidase enzyme. A TMB substrate is added to the wells that is digested by the HRP forming a pigmented product which can be colourmetrically measured as an indicator of the levels if IL-8 in a sample.
2.14.4.2. Adhesion and Invasion Assays in the T84 Cell Culture Model

Adhesion and invasion assays using the T84 colonic epithelial cell line were performed as described in Sections 2.14.2.1. and 2.14.2.2. with slight modifications. Cells were seeded for 48 hours as described in Section 2.14.4. and media was changed 12 hours prior to infection. Bacterial cells were prepared for infection as described in Section 2.14.1. Cells were infected at an MOI of 1:100. Association and gentamicin protection assays were performed as described in Sections 2.14.2.1. and 2.14.2.2. Bacterial counts were obtained as described in Section 2.6.4.3. Adhesion and Invasion Efficiencies were calculated as described in Sections 2.14.2.1. and 2.14.2.2.

2.15. Determination of CapC Cellular Localisation

2.15.1. Cellular fractionation

*C. jejuni* strains were grown from frozen stocks on MH agar supplement with trimethoprim or chloramphenicol where appropriate for 48 hours at 37°C under microaerobic conditions. Strains were then re-streaked and grown at 37°C under microaerobic conditions for 24 hours. Strains were suspended from agar plates and diluted to an optical density at 600 nm (OD600) of 0.8. These bacterial cultures were used for all fractionation procedures described below according to the method of Sommerlad and Hendrixson (2006):

Outer and inner membrane proteins were prepared and separated based on Sarkosyl insolubility by the method of Carlone *et al.* (1986). Briefly, 5ml aliquots of bacteria were pelleted, washed once in 10mM HEPES (pH 7.4), and resuspended in 1ml of the same buffer. Sonication to disrupt bacteria was achieved with a Soniprep 150 Plus using a microtip attachment. Unbroken bacteria were removed by brief centrifugation at 16,000 g for 2 minutes at room temperature. To recover total membranes, bacteria were then centrifuged for 16,000 g for 30 min at 4°C. Membrane
pellets were resuspended in 400µl 1% sodium lauryl sulfate in 10mM HEPES (pH 7.4) and incubated at room temperature for 30 min. Sarkosyl-insoluble outer membrane proteins were then removed by centrifugation for 30 min at 16,000 g at 4°C. The supernatant contained Sarkosyl-soluble inner membrane proteins.

Periplasmic and cytoplasmic proteins were collected by pelleting 20ml aliquots of bacteria at 4,200 g for 10 min. To isolate periplasmic proteins, the bacterial pellets were washed twice with 2ml of PBS containing 0.1% gelatin by centrifugation at 4,200 g at 4°C. The pellets were then suspended in 200µl of PBS containing 0.1% gelatin, and polymixin B sulfate was added to a final concentration of 2mg/ml. The bacteria were agitated for 30 min at 4°C. The preparation was then centrifuged at 16,000 g for 30 minutes at 4°C to separate the spheroplasts in the pellet from the periplasmic proteins in the supernatant. The supernatants were removed and centrifuged again at 16,000 g for 30 min at 4°C to remove any contaminating spheroplasts. The spheroplasts obtained from the periplasmic preparations were suspended in 1ml of 10mM HEPES (pH 7.4) and then disrupted by sonication as described above. Insoluble matter and unbroken bacteria were removed by centrifugation at 16,000 g for 2 min at 4°C. The supernatant was removed and centrifuged again at 16,000 g for 30 min at 4°C. The resulting supernatants represented the proteins from the cytoplasm of the bacteria.

2.15.2. Raising Anti-CapC polyclonal antibodies

Rabbit Polyclonal antibodies specific to CapC were generated. The Antigen Profiler software provided by Life Technologies (Thermo Fisher), was used to identify a region in the CapC amino acid sequence that was predicted to be highly antigenic. This sequence (EKLQQEAMQNGKIDDEKY) was identified to be ideally located in the N-terminus of CapC but excluded the predicted signal peptide region. A peptide antigen was synthesized based upon this sequence and used to generate polyclonal antibodies in two New Zealand White, specific-
pathogen free rabbits using the service provided by Thermo Scientific Custom Antibody Production. Rabbits were immunized over a period of 70 days and bled 3 times to obtain crude antibody sera. The full immunization protocol is shown in Appendix II. The synthetic peptide was conjugated to a keyhole limpet hemocyanin (KLH) carrier protein and administered subcutaneously as emulsion in Freud’s complete adjuvant (CFA) or incomplete adjuvant (IFA). At day 60 of the immunization protocol, ELISAs were performed (as part of the service offered by Thermo Scientific) to determine the antibody titre of each bleed using the unconjugated peptide. All final serum bleeds used in this study, (Day 56/58) demonstrated an antibody titer of 1:50000.

2.15.3. Western blotting

All reagents and buffers used for Western blotting is outlined in Appendix II. Bacterial samples for immune-detection were prepared as described previously with minor modifications (Sommerlad and Hendrixson, 2006). For whole cell lysates, C. jejuni strains were grown from frozen stocks on MH agar supplement with trimethoprim or chloramphenicol where appropriate for 48 hours at 37°C under microaerobic conditions. Strains were then re-streaked and grown at 37°C under microaerobic conditions for 24 hours. Strains were suspended from agar plates and diluted to an optical density at 600 nm (OD600) of 0.8. 1ml of culture was pelleted, washed once with PBS and resuspended in 500µl of PBS. 50µl of this culture was mixed 1:1 with 2x Laemmli 2x sample buffer. Membrane fractions were also mixed 1:1 with 2x Laemmli 2x sample buffer. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis using 12.5% Novex Tris-Glycine pre-cast protein gels (Thermo Scientific, UK). 20µl of samples were loaded on to each well of pre-cast gels and run using a Thermo-Scientific Mini Gel Tank at 75 V for 2 hours. Empty wells contained 1X sample buffer alone. The EZ-Run™ Prestained Rec Protein Ladder (Fisher Bioreagents, UK) was used as a protein marker in all SDS gels. Protein gels were
transferred to PVDF membranes (Fisher, UK) using a Pierce Power Blot cassette (Thermo Scientific, UK) using manufacturer programmed protocols and Pierce 1-step Transfer Buffer (Thermo Scientific, UK). PVDF membranes with transferred proteins were blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) buffer containing 10% non-fat milk for 16-18 hours at 4°C. Membranes were washed three times in TBST buffer and incubated with primary α-CapC antibody diluted 1:1000 in 10ml TBST with 3% milk for 1 hour at room temperature. Following this, membranes were washed in TBST with 3% non-fat milk three times and then incubated with Goat anti-rabbit secondary antibody conjugated to horse-radish peroxidase (HRP) diluted (1:5000) and incubated at room temperature for 2 hours. Following this, membranes were washed three times in TBST and drained thoroughly. 7mls of the ECL Clarity reagent (Biorad, UK) was applied to the membrane for 10 minutes. Following this, membranes were drained, kept moist and exposed for 30 seconds, 10 minutes and 30 minutes using a Licor Odyssey Fc imager digital images were obtained.

2.16. Data manipulation and statistical analyses

Data were tabulated using Microsoft Excel (version 2010) and Graphpad Prism 6 and 7. Means, standard error and standard deviations were calculated using these applications. Percentages for efficiency data were calculated using Microsoft Excel (version 2010). Bacterial count data were log transformed prior to further analyses. Normalisation, where appropriate, was performed using Microsoft Excel. All statistical analyses were performed using Graphpad 6 and 7. Unpaired, two-tailed Student’s t-tests and Mann-Whitney tests were used to compare two samples. Linear regression was used to analyse groups along a continuum of data. In all cases, the statistical significance critical level was p≤ 0.05
Chapter 3: Genetic, bioinformatic and phenotypic characterisation of capC
3.1 Introduction

3.1.1. capC encodes a putative autotransporter

Unpublished preliminary work carried out by Park and Richardson found that capC was encoded by 60% of Campylobacter jejuni and C. coli isolates tested, as determined by PCR and Southern blotting. Park and Richardson also found that in strains that did not encode capC, no other additional insertion was present. Using the structural prediction algorithm, AMPHI, the C-terminal region of the capC sequence was shown to translate to a protein taking the form of a β-barrel. This structural trait and the homology of capC to the vacA autotransporter of Helicobacter pylori, suggests that capC encodes a putative autotransporter protein. Indeed the presence of a β-barrel coding region has been used previously to definitively class genes as encoding autotransporter proteins (Watson et al., 2014; Henderson et al., 2012). In view of the highly documented strain-specific variation in virulence and the emergence of hypervirulent, rapidly expanding clones of C. jejuni (Wu et al., 2016), the identification of specific genetic elements that potentially impact upon pathogenesis and virulence is critical. Therefore, it is anticipated that the characterisation of capC outlined in this chapter, as well as the following chapters, will significantly enhance the current understanding of the pathophysiology of campylobacters.

The overarching objective of the work described in this chapter was to characterise CapC as a member of the autotransporter family of proteins and potential virulence factor in C. jejuni. Thus, the specific aims of this chapter were as follows:

1. To use bioinformatic prediction and classification tools to infer structural characteristics of CapC and establish whether the novel gene described by Park and Richardson is consistent with common traits of autotransporters.
2. To construct defined, isogenic capC gene deletion mutants and complements in *C. jejuni* 81116 and *C. jejuni* M1.

3. To examine phenotypes of these mutants through targeted comparisons to parental strains, with respect to *C. jejuni* behaviours and traits that are commonly implicated in bacterial fitness, survival and pathogenicity.
3.2. Results

3.2.1. Bioinformatic tools confirm capC encodes an autotransporter

3.2.1.1. Identification of conserved domains in capC

Domains within the context of proteins can be considered to be distinct structural or functional units in a protein. Identification of protein domains can provide insight into protein function. The NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) is a publically available tool that can be used to annotate conserved domains in protein sequences by querying input sequences against the database, thus allowing the identification of conserved blocks of amino acid residues that occur in molecular evolution and have previously been ascribed distinct functions in protein sequences.

![Figure 3.2](image)

Figure 3.2: The output from the Conserved Domain Database query. The C-terminal third of the CapC protein sequence is shown to possess an autotransporter domain confirming CapC as a member of the autotransporter family.

The predicted protein sequence encoded by capC (Appendix III) was downloaded in Fasta format from the CampyDB database resource at http://xbase.warwick.ac.uk/campydb/ (formerly http://www.xbase.ac.uk/campydb/). This sequence was used to identify potential conserved domains using the NCBI Conserved Domain Database. The output from the CDD query is shown in Figure 3.2. The CDD query tool allowed the identification of structural motifs in the C-terminal thirds of the CapC sequence (residues 490-750). The CDD search successfully identified an autotransporter beta-domain in this region; a domain that is specific to all autotransporter proteins, thus denoting CapC as a member of this family.
The CDD tool also annotated a portion of the N-terminal domain or “passenger” domain as belonging to the DUF342 superfamily which is a default classification for proteins with unknown function.

### 3.2.1.2. Identification of a signal peptide sequence in the capC coding sequence

Signal peptides in proteins are short sequences (5-30 residues) that serve as localisation targeting signals in many organisms including Gram-negative bacteria (Juncker et al., 2003). Autotransporter proteins bear structural resemblance to proteins secreted by the general secretory pathway (Type II secretion pathway) in that autotransporters possess a signal sequence at the N-terminal of the protein that allows targeting of the peptide to the inner membrane via the Sec machinery. The SignalP 4.1 server ([http://www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)) was successfully utilised to predict the presence and location of a signal peptide sequence motif in the CapC amino acid sequence. The protein sequence encoded by *capC* (Appendix III) was downloaded in FASTA format from the CampyDB database resource at [http://xbase.warwick.ac.uk/campydb/](http://xbase.warwick.ac.uk/campydb/). This sequence was uploaded to the SignalP server and used to search for signal peptides using default settings and parameters. Figure 3.3 shows the summary of the SignalP output. The graphical output generated by SignalP shows the results of a combination of three automatic analyses performed by the neural networks that make up the SignalP 4.1 server. These results are presented as C-, S- and Y-scores.

The C-score (red lines in Figure 3.3) denotes the raw cleavage site score and allows differentiation of cleavage sites from other sequences. This value is shown as a spike at the amino acid position immediately after the predicted cleavage site and marks the start of the mature protein. The S-score is shown as the green line in Figure 3.3; the peaks in the first 25 residues distinguish the signal peptide from the mature protein. The Y-score is a combination of the C- and S-score and
allows an improved prediction of the cleavage site than the use of the C-score alone. The graphical output from the SignalP 4.1 server clearly shows with high probability that the first 25 amino acids in the CapC sequence represent the N-terminal signal sequence of this autotransporter and that this sequence is cleaved after this residue.

![Graphical Output](image)

**Figure 3.3**: The graphical output from the SignalP tool shows a combination of the three scores generated. The Y-score is calculated taking into account both the C- and S-score. The output from this calculation clearly shows a cleavage site after the 25th residue. The high S-score in the region preceding this cleavage site indicates that the first 25 amino acids in CapC represent the signal sequence that allows CapC to be targeted to the inner membrane of *Campylobacter* species.
3.2.1.3. Prediction of the cellular localisation of CapC

The subcellular localisation prediction tool Cello V2.5 (http://cello.life.nctu.edu.tw/) is commonly used to predict the localisation of prokaryotic and eukaryotic proteins. This system uses a learning machine to group proteins into one or more of the five subcellular locations; cytoplasmic, inner membrane, periplasm, outer membrane or extra-cellular. The output table from the Cello prediction server is shown in Appendix III. Use of the Cello localisation tool suggests that CapC is ultimately an extracellular, secreted protein.

An alternative localisation prediction tool is PSORTb (http://www.psort.org/psortb/index.html) that has a wider range of analytical modules than Cello and allows for searching of multiple databases and support vector machines at once. PSORTb is designed to emphasize precision (or specificity) over recall (or sensitivity) and as such can be considered to be more accurate. CapC is predicted by PSORTb to be primarily an outer membrane protein as one might expect from an autotransporter, however there is a lower prediction score for the likelihood of CapC being an extracellular protein. Ultimately, PSORTb suggests that CapC may have multiple localisation sites.

3.2.1.4. Prediction of the partial 3D structure of CapC

Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) is a suite of tools available on the web to predict and analyze protein structure, function and mutations. Phyre2 was successfully used in this study to build a partial 3D model of the CapC protein based on remote homology detection methods. The CapC protein sequence was entered into the user interface of the Phyre2 web tool and the partial 3D structure was obtained and is depicted in Figure 3.6. The model of the CapC protein structure shows the clearly defined 14 amphipathic strands comprising the beta-barrel that is characteristic of all autotransporters, further confirming CapC as a member of this family.
Figure 3.4: Partial structure of the CapC protein modelled using Phyre2 3D modelling engine. Yellow structure denotes the β-barrel pore forming structure characteristic of autotransporters. Magenta structure is partial model of the functional passenger domain. CapC has only been partially modelled (43%) with high confidence (Kelley et al., 2015).

3.2.2. The capC gene is encoded by 60% of representative Campylobacter isolates

A number of defined reference strains of *C. jejuni* and *C. coli* were obtained from the University of Surrey Culture Collection and screened for the possession of the capC gene. Isolates were cultured as described in section 2.1.1 and genomic DNA extracted as described in section 2.8.1.1.2. PCR was performed using the Cap1/2 primers and the Hel1/Ppk1 primers and genomic DNA from *Campylobacter* isolates as template and GoTaq polymerase as described in section 2.8.1.5. Samples were subjected to thermocycling consisting of an initial denaturation period of 5 minutes at 95°C followed by 30 cycles of denaturation for 45 seconds, annealing for 45 seconds at 55°C and an extension period at 72°C for 1 minute 30 seconds. Cycling was followed by a final elongation period for 5 minutes and a hold at 4°C. After thermocycling, PCR products were visualised on agarose gel as described in section 2.8.1.4.
Figure 3.5 below shows a representative PCR showing the results from screening of the reference, typed isolates *C. jejuni* NCTC 11168, *C. jejuni* NCTC 11351 and *C. jejuni* 81-176 for possession of the *capC* ORF. Each isolate was screened with each primer set independently. If isolates encoded *capC*, a 780bp amplicon was expected when the Cap1/Cap2 primers but no fragment was expected when the Hel1/Ppk1 primers were used and vice versa if isolates did not encode *capC*, resulting in a 1097bp Hel1/Ppk1 amplicon. The agarose gel in Figure 3.5 shows that *C. jejuni* NCTC 11351 encodes *capC* as evidenced by successful amplification of a 780bp fragment in lane 4 (Cap1/Cap2 primers) and no amplicon in lane 5 (Hel1/Ppk1 primers). Conversely *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 do not encode *capC* and only show successful amplification of the Hel1/Ppk1 fragment. Table 3.1 in Appendix III summarizes the results of screening for *capC* and the proportion of reference and typed isolates that encode this gene. 45 of 77 clinical isolates and 23 of 41 poultry isolates were positive for *capC*.

**Figure 3.5A**: The relative annealing positions of oligonucleotide primers used in this study to amplify various regions of the *capC* gene. The 3’ end of each primer anneals at the following nucleotide positions of *capC*; CapCF= 6, Cap1= 1191, Cap2=1930, Cap3= 297, Cap4= 1523, Vac1= 2016. CapFW, CapRV, and Hel1 all anneal at intergenic regions. Ppk1 anneals at position 78 of the *ruvB* gene, upstream of C8J_1279. The *BglII* and *XbaI* restriction sites are located at position 945 and 1608 in the *capC* coding sequence respectively.
Figure 3.5B: A 1% agarose gel showing detection of the 780bp fragment of *capC* in the genomic template of *C. jejuni* NCTC 11351 and the absence of *capC* in *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 as indicated by the amplification of the 1097bp fragment using the HEL1/PPK1 primers. Lane 1: 1kb ladder; Lane 2-3: *C. jejuni* NCTC 11168 template showing amplification of 1097bp fragment only in lane 3; Lane 4-5: *C. jejuni* NCTC 11351 showing amplification of 780bp *capC* fragment in lane 4; Lane 6-7: *C. jejuni* 81-176 showing amplification of 1097bp fragment only in lane 7.

3.2.2.1. Determination of the proportion of poultry and human isolates encoding *capC*

In order to gain a more accurate estimate of the prevalence of *capC* in *C. jejuni* and *C. coli* that encode *capC*, *Campylobacter* isolates from human clinical disease and also from raw supermarket poultry meat were screened for possession of the *capC* ORF using the Cap1/Cap2 primers only.

3.2.2.2. Isolation of *Campylobacter* species from supermarket raw poultry samples

61 raw chicken samples from 5 different supermarket chains in Surrey, UK were assessed for the presence of *Campylobacter* using the procedures outlined in section 2.4.2. Of these samples, 41 were found to be positive for *Campylobacter* spp. Isolation and purification of *Campylobacter*
species was confirmed by Gram-staining, oxidase testing and catalase testing. *Campylobacter* species determination was determined by hippurate hydrolysis testing. These 41 *Campylobacter* poultry isolates were isolated in the UK over a period of four months in 2012. A detailed list of these isolates can be found in Appendix III.

### 3.2.2.3. Acquisition of clinical isolates of *Campylobacter* species

76 putative clinical isolates of *Campylobacter* species were kindly provided by Frimley Park Hospital Microbiology department. These isolates were all derived from stools provided by medical patients presenting to General Practitioners with symptoms of gastroenteritis which was confirmed as being *Campylobacter*-mediated. Clinical isolates were cultured and purified as described in section 2.1.1. 72 of these isolates were found to be viable. The identity of these isolates was confirmed by Gram-staining, oxidase testing, catalase testing and hippurate hydrolysis testing.

### 3.2.2.4. Evaluation of the distribution of the capC ORF

The PCR assay described above was employed to screen the 72 confirmed human derived clinical isolates of *C. jejuni* and *C. coli* obtained from Frimley Park Hospital for the possession of the capC ORF. This assay was also used to screen the poultry isolates for possession of capC.

As previously described, isolates were cultured from -80°C stocks as detailed in section 2.1.1 and sub-cultured for 24 hours on MH agar supplemented with 5% sheep’s blood. Genomic DNA was released from these isolates via boiling lysis as described in section 2.8.1.1. PCR to ascertain possession of capC was performed as described in chapter 2, using the Cap1/Cap2 primer pair only. PCR was performed using GoTaq polymerase and the thermocycler setting outlined in section 2.8.1.5. 1µl of boiled lysate served as template DNA for these reactions. 45 of 72 (62.5%) of human clinical *Campylobacter* isolates were found to encode capC as determined by PCR. 23
of 41 (56%) of Campylobacter poultry isolates were found to encode capC. Figure 3.5B shows the representative agarose gels from screening of both of the clinical and poultry isolates for possession of capC in their respective genomes. The total number of isolates screened for possession of capC was 113. Of these, 68 isolates were found to encode this gene as determined by PCR (60.2%).

Figure 3.5C: Series of 1% agarose gels showing results of PCR screening of Campylobacter clinical isolates and those isolated from supermarket poultry. All isolates were screened with the Cap1/Cap2 primers only. A 1kb interval DNA ladder is shown in Lane 1 of all gels and all additional lanes contain PCR reaction mixtures using Campylobacter species genomic DNA as template. All fragments obtained and shown on the agarose gels in this figure correspond to the 780bp fragment that was expected and indicate the presence of capC in the genomes of these isolates. Where no PCR product was generated, isolates were deemed to be capC negative.

3.2.3. Construction of capC deficient mutants

In order to elucidate the potential role of capC, a knockout mutant that was deficient in capC was constructed in C. jejuni 81116 as described in section 2.9.1 via disruption of the capC coding sequence by insertion of a kanamycin resistance cassette in to the BglII site at position 945 of the capC coding sequence. A summary of this genetic manipulation is shown in Figure 3.6.1.
Additional *capC* knockout mutants were constructed in *C. jejuni* 81116 and *C. jejuni* M1 via excision of a 663bp from the coding sequence of the *capC* gene and subsequent replacement of this sequence with a chloramphenicol resistance marker as described in section 2.9.2.

A summary of these genetic manipulations is shown in Figure 3.11.1.
3.2.3.1. Construction of a capC-knockout via insertion of a kanamycin resistance cassette

Figure 3.6.1: A flow-diagram showing the strategy used to knockout the capC gene in C. jejuni 81116 via insertion of a 1499bp DNA cassette encoding kanamycin resistance. A 1266bp portion of the capC coding sequence was cloned into pGEM T-easy to produce pJM001. The 1499bp kanR cassette was cloned into this fragment via the BglII restriction site. A linear fragment resulting from this construct was used to transform C. jejuni 81116 and generate a capC knockout.
A 1266bp capC fragment was amplified from C. jejuni 81116 genomic DNA using the primers Cap3 and Cap4. The 1266bp capC amplicon containing a BglII restriction site was purified and ligated into the pGEM T-easy vector. Figure 3.6 demonstrates amplification of this fragment and the presence of the BglII restriction site. Ligation mixtures were transformed into E. coli DH5α which were plated onto LB agar supplemented with 100µg/ml, 0.1M IPTG and 50µg/ml X-gal to perform a blue/white screen. The pGEM vector contains a gene conferring ampicillin resistance; therefore only E. coli which had taken up the plasmid were able to grow on the LBA/Amp/IPTG/X-gal plates. In addition, the vector also contains a lacZ gene encoding a β-galactosidase enzyme capable of metabolizing the chromogenic substrate X-gal thus producing a blue coloured precipitated within cells resulting in blue colonies.

**Figure 3.6:** A photograph of a 1% agarose gel demonstrating the presence of a BglII restriction site within the 1266bp capA amplicon. The 1266bp capA fragment was amplified by PCR using the CAP3 and CAP4 primers. The PCR product was digested with BglII resulting in the two expected fragments measuring 647bp and 572bp respectively, representing the sequences upstream and downstream of the restriction site. Lane 1= 1kb DNA ladder, Lane 2= BglII-digested capC resulting in two fragments, Lane 3= 1266bp capC amplicon.

The insertion site for PCR products lies within the lacZ gene; successful integration of the PCR product inactivates lacZ rendering cells that successfully take up the correctly ligated plasmid
unable to metabolized X-gal resulting in white colonies. Resulting white colonies were picked and screened by PCR and sequencing for successful integration of the plasmid. A plasmid which had successfully integrated the 1266bp capC fragment was designated pJM001. The agarose gel arising from this screening process is shown in Figure 3.7 below.

Figure 3.7: The resulting agarose gel from a PCR screen for the insertion of the capC fragment into the pGEM T Easy cloning vector. PCR using the CAP3 and CAP4 primers was using performed using selected white colony transformants as template material. Figure 3.7 shows successful amplification of the 1266bp capC insertion sequence, indicating that these colonies harboured plasmids into which the 1266bp capC sequence had been incorporated. Lane 1= 1kb ladder, Lane 2= purified capC amplicon from C. jejuni 81116, Lane 3-10= 1266bp capC fragment amplified from the pJM001 plasmid preparations.

pJM001 was digested with BglII. Simultaneously pJMK30 (van Vliet et al., 1998), a plasmid containing the kanamycin resistance cassette, was digested by BamHI liberating a 1499bp fragment containing the aphA-3 kanamycin resistance gene. Digestion and the process of gel purification of the kanamycin cassette is shown in Figure 3.8.
Figure 3.8: A photograph of a 0.8% agarose gel showing the digestion of pJMK30 with the BamH1 restriction endonuclease. BamH1 cleaves the circular 4185bp pJMK30 plasmid at two specific restriction sites resulting in a 2686bp linear fragment and a smaller 1499bp fragment (shown in red outline). The 1499bp product contains the kanamycin cassette encoding the aphA-3 gene conferring kanamycin resistance. These two fragments were separated by gel electrophoresis as shown in the gel photo and the kanamycin cassette was isolated using the Qiagen gel extraction kit. Lane 1= 1kb DNA ladder, Lane 2= circular, undigested pJMK30 plasmid, Lane 3+4, BamH1 digested pJMK30 showing isolation of the 1499bp kanamycin cassette.

Restriction digest of pJM001 and pJMK30 with these enzymes resulted in sticky ends; compatible overhangs that may be ligated together. The 1499bp kanamycin resistance fragment was ligated in to the BgIII site of pJM001 resulting in a 2765bp insert consisting of the capC fragments of 647bp and 572bp flanking the 1499bp kanamycin cassette. This plasmid was designated pJM002. The agarose gel arising from screening of transformant colonies for possession of pJM002 is shown in Figure 3.9.
Figure 3.9: 1% agarose gel showing the results of PCR of suspected pJM002 colonies. Kanamycin resistant colonies were selected for plasmid preparations. Plasmids were screened by PCR using the CAP3 and CAP4 primers to determine whether the kanamycin cassette has been successfully inserted into pJM001 resulting in pJM002. Colonies that produced the desired 2765bp fragment (shown in red outline) were shown to contain the pJM2 plasmid in which the capC sequence was disrupted by insertion of the kanamycin cassette. Lane 1= 1kb DNA ladder, Lane 2= 1266bp CapC wildtype control fragment, Lane 3, 5, 6, 7, 9= partial or no insertion of kan’, Lane 4, 8 and 10= 2765bp fragment indication insertion of kan’ cassette.

The 2765bp knockout construct was amplified using primers Cap3 and Cap4 and transformed into C. jejuni 81116 by electroporation and natural transformation as described in Section 2.8.1.12. Figure 3.10 below shows the screening of the suspected C. jejuni transformants. A single transformant (shown in red outline) which showed interruption of the capC ORF with a sequence containing a kanamycin resistance marker was sub-cultured and genomic DNA was extracted using methods outlined earlier in this chapter. This genomic DNA preparation was used to naturally transform C. jejuni 81116 wildtype strain. Transformants were screened by PCR once again and sub-cultured. A single transformant showing correct integration of kan and deletion of capC was designated the capC knockout strain ΔcapC:kanR. A representative agarose gel showing the PCR of C. jejuni 81116 and C. jejuni M1 suspected deletion mutants is shown in Figure 3.10.
Figure 3.10: 1% agarose gel showing the insertion of the kan' cassette into the capC ORF in the C. jejuni 81116 genome. The 2765bp linear amplicon from pJM002 shown in Figure 3.9 was introduced into C. jejuni 81116 by electroporation. Suspected kanamycin-resistant C. jejuni 81116 transformants were subcultured for 24 hours. The genomic DNA of these transformants was extracted and used as template DNA in PCR using the Cap3/Vac1 primer combination. PCR of a suspected transformant resulted in a 3260bp fragment whereas PCR of the parental C. jejuni 81116 strain resulted in the expected 1761bp fragment indicating successful disruption of capC and integration of the kanR resistance marker in C. jejuni 81116. Lane 1= 1kb DNA ladder, Lane 2= 1761bp capA fragment amplified from C. jejuni 81116, Lane 3-6= PCR of suspected C. jejuni transformants; Lane 4 shows the expected, clean 3260bp amplicon in red outline demonstrating insertion of the kanamycin cassette into the capC BglII site. A genomic DNA preparation from this isolate was used to naturally transform C. jejuni 81116 wildtype strain. Transformants were screened by PCR once again and sub-cultured. A single transformant showing correct integration of cat and deletion of capC was designated the capC knockout strain ΔcapC:kanR.
3.2.3.2. Construction of \textit{capC}-knockouts via insertion of a chloramphenicol resistance cassette

A 2893bp DNA fragment was amplified from \textit{C. jejuni} 81116 and \textit{C. jejuni} M1 and cloned into pGEM T-easy. These plasmids were designated pJM005.

pJM005 plasmids were digested with BglII and XbaI simultaneously, removing a 647bp region of DNA within the \textit{capC} CDS. A 820bp amplicon encoding the chloramphenicol resistance cassette was amplified with BglII and XbaI sites on the 5' ends of primers. The \textit{catR} cassette was digested and ligated into the BglII/XbaI site of pJM005, resulting in pJM006.

\( \times 2 \) 1906bp fragments were amplified from pJM006, these amplicons were used to transform \textit{C. jejuni} 81116 and \textit{C. jejuni} M1.

\textbf{Figure 3.11.1}: A flow diagram depicting the strategy used to knockout the \textit{capC} gene in \textit{C. jejuni} 81116 and \textit{C. jejuni} M1 using a chloramphenicol resistance marker. The entire \textit{capC} locus including the intergenic region upstream of this gene was cloned into pGEM T-easy to generate pJM005. The 820bp DNA amplicon encoding chloramphenicol resistance was amplified from pAV103 with BglII and XbaI restriction sites added to the 5' ends of the primers. The \textit{catR} amplicon and pJM005 were simultaneously digested with BglII and XbaI and purified to remove enzymes. The \textit{catR} cassette was ligated into the BglII/XbaI site of pJM005 to generate pJM006. A 1906bp amplicon was amplified from pJM006 consisting of the \textit{catR} cassette flanked by \textit{capC} homology regions; this fragment was used to transform \textit{C. jejuni} 81116 and \textit{C. jejuni} M1.
Additional knockout mutants in the capC gene were constructed in C. jejuni strains 81116 and M1 using a chloramphenicol resistance cassette insert. The capC gene was amplified using the CapCFW and CapCRV primers resulting in a 2893bp fragment. This fragment was cloned into pGEM T-easy as described in Section 2.9.1. Figure 3.11 below the screening of suspected transformants for integration of capC into pGEM T-easy.

Figure 3.11: 1% agarose gel that shows successful amplification of the entire capC gene. The agarose gel also shows successful integration of this amplicon into the pGEM T-easy vector. Lane 1: 1kb DNA ladder, Lane 2: amplification of a 2893bp capC fragment from C. jejuni 81116 genomic DNA template, Lane 3-6: amplification of the 2893bp capC fragment from suspected E. coli transformants. Successful amplification demonstrates integration of the capC coding sequence into pGEM T-easy. This plasmid was designated pJM005.

A plasmid showing successful and correct integration was designated pJM005a and pJM005b as separate constructs were generated for C. jejuni 81116 and C. jejuni M1. pJM005 was digested with BglII and XbaI cleaving the capC sequence after the 1501 and 2164 nucleotide positions respectively and thus excising a 663bp capC fragment from the plasmid. The chloramphenicol resistance (cat) cassette was amplified from pAV103 using the primers JMCAT5 and JMCAT6. These primers were designed with BglII and XbaI restriction sites at the 5’ end allowing these
sequences to be incorporated into the amplicon during PCR. The resulting 820bp cat amplicon shown in Figure 3.12 was purified and digested with BglII and XbaI.

**Figure 3.12**: A 1% agarose gel photograph showing amplification of an 820bp fragment from pAV103 (Baillon et al., 1999), containing the chloramphenicol resistance gene, cat, derived from a plasmid encoded by *C. coli*. This fragment was amplified using oligonucleotide primers to which *Bgl*II and *Xba*I endonuclease recognition sequences had been added to the 5′ end. Digestion of this amplicon with these enzymes generates overhangs to which compatible fragments may be ligated.

The digested fragment was ligated into the digested and dephosphorylated pJM005 thereby creating a plasmid containing the capC gene in which a 663bp fragment had been replaced with the chloramphenicol resistance marker. The knockout construct was amplified using the VAC1 and CAP3 primers yielding a 1906bp fragment which was used to transform *C. jejuni* 81116 and M1 as described in Section 2.8.1.12. Coupled wildtype strains were also generated. Figure 3.13 shows amplification of the capC coding region in the *C. jejuni* 81116 and *C. jejuni* M1 wildtype and mutant strains generated in this study using the Cap3 and Vac1 primers and demonstrates deletion of capC in *C. jejuni* 81116 and *C. jejuni* M1.
**Figure 3.13A:** A 1% agarose gel that shows successful amplification of the *capC* sequence region in the *C. jejuni* 81116 and *C. jejuni* M1 wildtype and mutant strains generated in this study using the Cap3 and Vac1 primers and shows successful deletion of 663bp sequence in mutant strains and insertion of the chloramphenicol resistance cassette. Lane 1: 1kb DNA ladder, Lane 2: 1761bp DNA fragment amplified from *C. jejuni* 81116 wildtype genomic DNA, Lane 3: 1906bp DNA fragment amplified from *C. jejuni* 81116 Δ*capC:*cat′ indicating partial deletion of *capC*, Lane 4: 1761bp DNA fragment amplified from *C. jejuni* M1 wildtype genomic DNA, Lane 5: 1906bp DNA fragment amplified from *C. jejuni* M1 Δ*capC:*cat′ indicating partial deletion of *capC*, Lane 6: 1kb DNA ladder.
Figure 3.13B: Figure 3.13B shows further evidence of the partial deletion of the capC coding sequence. The *C. jejuni* 81116 and *C. jejuni* M1 mutants confirmed as having a cat resistance marker within the capC coding sequence were used in a PCR assay using the CAP3/CAP4 and the CAP3/CC069 primers. CAP4 anneals to a capC sequence that lies within the 663bp excised sequence resulting from *BglII/XbaI* digestion. CC069 is an internal control primer that anneals to the complementary strand in the catR sequence and can thus be used as a reverse primer for amplification of the catR sequence. The agarose gel below shows amplification of an 884bp DNA fragment from *C. jejuni* 81116 ΔcapC and *C. jejuni* 81116 ΔcapC using the CAP3/CC069 primers and no amplicon using the CAP3/CAP4 primers which is conclusive evidence that the desired capC sequence portion has been removed and that the catR marker has been integrated in its place in the *C. jejuni* 81116 and *C. jejuni* M1 genomes, in the correct orientation.
3.2.3.3. Complementation of \textit{capC}

The full \textit{capC} sequence was amplified from pJM005a using the primer pair CapFEsp3I/CapREsp3I to which Esp3I endonuclease recognition sites had been added to each terminus. \textit{capC} was unable to be complemented by cloning of this fragment into the Esp3I site of the pC46 plasmid despite repeated attempts. A complementation construct produced by overlapping PCR using the upstream and downstream regions of the \textit{cj0046} pseudogene spliced to either end of the \textit{capC} coding sequence was also unable to be transformed into \textit{E. coli} for propagation.

The pSV009 complementation vector (de Vries et al., 2015) offered an improved alternative to the pC46 plasmid. However the \textit{capC} gene was only able to be ligated into pSV009 in the opposite orientation to the \textit{cat} promoter and kanamycin resistance cassette; a \textit{capC} fragment to which \textit{BamHI} sites had been added to each terminus was amplified and digested with \textit{BamHI}. This fragment was ligated into the \textit{BamHI} site of the pSV009 multiple cloning site in a site that would place \textit{capC} expression under the control of the \textit{cat} promoter included in pSV009 provided \textit{capC} was inserted in the correct orientation. This genetic manipulation is depicted in Figure 3.14. Restriction analysis and sequencing showed all subsequent transformants had incorporated \textit{capC} in the opposite orientation and was therefore unable to be expressed. Directional cloning was performed by substituting the \textit{BamHI} site on the reverse primer for a \textit{SacI} site which was also present in the MCS. This strategy would force \textit{capC} to only be ligated in the desired orientation, however this manipulation yielded no transformant colonies. The inability to insert \textit{capC} into pC46 and pSV009 in the correct orientation suggests that CapC may be toxic to \textit{E. coli}. Toxicity of \textit{Campylobacter} factors to \textit{E. coli} and consequently the inability of these genes to be complemented has been reported previously (Mamelli et al., 2007; Brown et al., 2015).
Figure 3.14: Linear representation of incorporation of the capC coding sequence into the pSV009 complementation plasmid. capC was ligated into pSV009 via the BamHI digest and by simultaneous BamHI/SacI digestion. capC was only able to be incorporated and transformed into E. coli DH5α in opposite orientation to the catR promoter indicating it was not expressed. The pSV009 schematic was provided by Stefan de Vries (de Vries et al., 2015) and was modified for use in this study.
3.2.4. Growth Curve

The growth of the capC deletion mutants relative to wildtype strain was monitored over a 48 hour period. capC deletions mutants in *C. jejuni* 81116 and *C. jejuni* M1 had no growth deficiency as compared to their coupled wildtype strains. Growth profiles of wildtype and mutant strains is shown in Figure 3. A, B and C. Whilst isolates were routinely cultivated at 42°C, growth assays were performed at 37°C. Growth of *C. jejuni* 81116 and the kanR mutant was quantified by measurement of the optical density of cultures at various timepoints over a 48 hour period. Figure 3.15A (below) shows the results of the *C. jejuni* 81116 and the kanR mutant growth dynamics as measure by optical density. Figure 3.15B and C show growth curves of *C. jejuni* 81116, *C. jejuni* M1 and their respective ΔcapC deletion mutants in which results are expressed as CFU/ml.

A

*C. jejuni* 81116 Growth curve (kanR)
Growth Profile of *C. jejuni* 81116 wildtype and *capC* mutant in MHB

**Figure 3.15A, B and C:** Figure 3.16A shows the results of the *C. jejuni* 81116 and the kan\(^R\) mutant growth dynamics as measured by optical density whereas Figure 3.15B and C show growth curves of *C. jejuni* 81116, *C. jejuni* M1 and their respective Δ*capC* deletion mutants in which results were quantified by Miles and Misra technique and are expressed as CFU/ml. *capC* deletions mutants in *C. jejuni* 81116 and *C. jejuni* M1 had no growth deficiency as compared to their coupled wildtype strains. This assay was repeated in triplicate, on three separate occasions. Doubling times for chloramphenicol resistant mutants and coupled wildtypes are shown in Appendix III.
3.2.5. Motility

The motility of *Campylobacter jejuni* is recognised as an important attribute of this organism that impacts upon a wide array of behaviours. Previously described motility assays (Section 2.6.2) were implemented to assess the motility of wildtype and mutant strains; a swarming plate assay performed as described in section 2.6.2.1. and a tube taxis assay performed as described in section 2.6.2.2.

The kanamycin resistant capC mutant was found to have a significant motility defect as shown in Figure 3.17A and Figure 3.18A respectively (p=0.0003, n=4). This motility defect was consistent over a 36 hour period. The capC deletion mutants in *C. jejuni* 81116 and *C. jejuni* M1, in which a chloramphenicol resistance marker replaced the majority of the capC coding sequence, displayed no motility defect as shown in Figure 3.17B+C and Figure 3.18B+C.

**Figure 3.16A**

Motility in SSM *C. jejuni* 81116 (kanR)

![Motility Graph](image)
Figure 3.16A, B+C: The results of the swarming plate motility assay. Figure 3.17A depicts the diameter in millimetres of the resulting motility halos of *C. jejuni* 81116 and the kan<sup>R</sup> cap<sup>C</sup> deletion mutant through semi-solid media. The cap<sup>C</sup>:kan<sup>R</sup> mutant displayed significantly impaired motility in this assay as determined by a Mann-Whitney test (p= 0.0003, n=4) The chloramphenicol resistant mutants subsequently constructed in *C. jejuni* 81116 and *C. jejuni* M1 showed no impairment in their motile capability (*C. jejuni* 81116 Δcap<sup>C</sup>:cat<sup>R</sup> p=0.6061, n=3; *C. jejuni* M1 Δcap<sup>C</sup>:cat<sup>R</sup> p=0.7446, n=3).
Figure 3.17A, B + C: Graphical representations of the results of testing the wildtype and mutant strains in the tube taxis assay performed as described in section 2.6.2.2. 50µl of bacterial suspension was inoculated at the top of 15ml Falcon tubes containing 10mls of semi-solid media supplemented with 1% TTC. This dye is reduced as a result of bacterial respiration and in this assay is used as an indicator of bacterial motility. Results are expressed as the distance of migration of the dye front from the site of inoculation in centimetres (cm). The *C. jejuni* 81116 capC:kan<sup>R</sup> mutant had an impairment in its motile capability which was consistent over 36 hours as shown in Figure 3.19A. The *C. jejuni* 81116 ΔcapC:cat<sup>R</sup> and *C. jejuni* M1 ΔcapC:cat<sup>R</sup> had no such deficiency as compared to their respective coupled wildtype strains. This assay was repeated in duplicate on three occasions. Significance was determined by two way ANOVA (A: p < 0.0001, B: p= 0.8586, C: p= 0.8006).
3.2.6. Contact Haemolysis

The variation in haemolytic activity of *C. jejuni* strains has long been acknowledged (Misawa *et al.*, 1995). The *pldA* gene, encoding a phospholipase, has previously been implicated in cell-associated haemolysis of *C. jejuni* and *C. coli*. The contribution of *capC* to cell-associated haemolysis was determined using the method outlined in section 2.6.1. The optical density of the *Campylobacter/sheep* red blood cell co-incubation supernatant is directly proportional to the degree of haemolysis caused by *Campylobacter*. This optical density is expressed as a percentage of complete haemolysis. Figure 3.18A shows the results of this assay when performed with *C. jejuni* 81116 and the Δ*capC:kan*B mutant. Figure 3.19B and C show the results of this assay when performed with *C. jejuni* 81116, *C. jejuni* M1 and their respective *capC:cat*B deletion mutants. All *capC* deletion mutants consistently exhibited decreased haemolytic activity, however this decrease was not statistically significant.

![Figure 3.19A](image)

**Figure 3.19A**

Haemolysis *C. jejuni* 81116 (kanB)

<table>
<thead>
<tr>
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<th>Mean % of Complete Haemolysis</th>
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<tbody>
<tr>
<td>Wildtype</td>
<td>15</td>
</tr>
<tr>
<td>Mutant</td>
<td>5</td>
</tr>
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*C. jejuni* 81116 Wildtype  
*C. jejuni* 81116 capC Mutant
Figure 3.1 A B and C: Graphical representation of the results of the contact haemolysis assay. Mean OD$_{540\text{nm}}$ of the respective *Campylobacter* sheepr blood cell co-incubation supernatants was calculated. This optical density is a measure of the degree of lysed red blood cells after 4 hours co-incubation with *Campylobacter* strains. Results are expressed as a percentage of complete haemolysis. All *capC* deletion mutants consistently exhibited decreased haemolytic activity, however this decrease was not statistically significant as determined by a Mann-Whitney test compared to respective coupled wildtype strains (*C. jejuni* 81116 Δ*capC:kan* $p=0.0571$, n=4; *C. jejuni* 81116 Δ*capC:cat* $p=0.4$, n=3; *C. jejuni* M1 Δ*capC:cat* $p=0.1$, n=3). Data shown is the mean percentage of haemolysis and SEM. This assay was repeated in duplicate on three occasions.
3.2.7. Biofilm Formation

The capability of *C. jejuni* to form biofilms is known to vary between different strains (Brown *et al.*, 2015). Given that *capC* is only present in select strains, it is an ideal candidate to examine for involvement in biofilm formation. Furthermore, as an autotransporter protein that is highly likely to be surface exposed, CapC has high potential to be involved in interactions with the *Campylobacter* immediate environment. This potential is exemplified by the large number of previously characterised autotransporters that have been reported to be involved in biofilm formation (Wells *et al.*, 2007).

Only the *C. jejuni* 81116 Δ*capC:*cat<sup>8</sup> and the *C. jejuni* M1 Δ*capC:*cat<sup>8</sup> mutants as well as their respective wildtype strains were assayed for their ability to form biofilms. The motility defect in the *C. jejuni* 81116 Δ*capC:*kan<sup>8</sup> mutant may have impacted upon subsequent biofilm formation and thus confounded results. Deletion of *capC* did not affect biofilm formation in *C. jejuni* 81116 as shown in Figure 3.19A and B at both 37°C and 42°C. Deletion of *capC* in *C. jejuni* M1 lead to an increase in biofilm formation, this increase in biofilm formation was even greater at 42°C.

![Biofilm Formation (42°C)](image)

**Figure 3.19A:** Graphical representation of biofilm formation assay results performed as described in section 2.6.3 in 96-well plates incubated at 42°C.
Figure 3.19B: Graphical representation of biofilm formation assay results performed as described in section 2.6.3 in 96-well plates incubated at 37°C. Results of the biofilm formation assay are expressed as the OD540nm of crystal violet stained biofilms, normalised to complete growth media background controls. Deletion of capC had no effect on the degree of biofilm formation of C. jejuni 81116 at either 37°C or 42°C. Deletion of capC resulted in an increase in biofilm formation in the C. jejuni M1 ΔcapC mutant as compared to the coupled wildtype strain. This assay was repeated in quintuplet on three occasions. Differences in biofilm formation were not significant as determined by an Unpaired t-test (42°C - C. jejuni 81116 p= 0.8541, n=5; C. jejuni M1 p= 0.1571, n=5. 37°C- C. jejuni 81116 p= 0.9967, n=5; C. jejuni M1 p= 0.4561, n=5).

3.2.8. Aeration Stress

In order to ascertain whether deletion of capC imparts a sensitivity to environmental stress, the viable counts of both wildtype and mutant strains were assessed after incubation under experimentally relevant conditions in Dulbecco’s Modified Eagle Medium as described in section 2.7.1. Only the C. jejuni 81116 ΔcapC:catR and the C. jejuni M1 ΔcapC:catR mutants as well as their respective wildtype strains were assayed. DMEM cultures were incubated at 37°C statically for 4 hours aerobically and viable counts were obtained immediately before and after this incubation. Figure 3.20A and B show the results of this aeration stress assay. After 4 hours exposure to aerobic conditions, all strains showed a decrease in their viable count. This decrease was consistent across all wildtype and mutant strains. The mutant strains did not show a greater
decrease in viable count due to aeration stress. Proportional to the initial inoculum, the mutant strains exhibited greater levels of survival that the coupled wildtype strains (C. jejuni 81116: 13% survival, C. jejuni 81116 ΔcapC: 28% survival, C. jejuni M1: 12% survival, C. jejuni M1 ΔcapC: 21% survival).

Figure 3.2A and Figure 3.2B: Figure 3.21A+B show the results of the aeration stress assay. After 4 hours exposure to aerobic conditions, all strains showed a decrease in their viable count. This decrease was consistent across all wildtype and mutant strains (C. jejuni 81116 ΔcapC: p>0.999; C. jejuni M1 ΔcapC: p>0.999). This assay was repeated in duplicate on three occasions.
3.3. Discussion

The studies detailed in this chapter have confirmed through the use of bioinformatic tools that the capC open reading frame found in the genomes of various C. jejuni and C. coli strains, encodes an autotransporter protein. This is evidenced by the presence of a β-barrel domain at the C-terminal which structurally denotes CapC as belonging to this class of secretory proteins. The predicted β-barrel structure has been partially modelled as shown in Figure 3.4. The Phyre2 engine relies upon sequence homology to existing sequences to interpret secondary and tertiary structure of query sequences and to provide a prediction of protein structure. This essentially utilises an alignment between two sequences; one of unknown structure and one of known structure. This alignment provides a basis to construct a model of one sequence based upon the other. The Phyre2 partial model of CapC is based upon the 3D structure of VacA of Helicobacter pylori and also on the EstA autotransporter encoded by Pseudomonas aeruginosa. Given that the N-terminal of autotransporters dictates the function of these proteins and the lack of sequence identity in this region between CapC, VacA and EstA, is it apparent that these factors do not necessarily share functional homology and merely share similarities in their export process. Therefore, the majority of publically available Campylobacter genomes may be incorrect in their annotation of capC as a vacuolating cytotoxin encoding gene.

Further use of bioinformatic tools has defined common features of autotransporter-encoding genes within capC such as the presence of an N-terminal signal sequence. The SignalP program has been successfully utilised to identify the N-terminal signal sequence in capC. Use of this tool has predicted with high probability that the first 25 residues of the capC protein represents the signal machinery that allows the full protein to be targeted to the inner membrane via the Sec machinery. The Y-score calculated by SignalP, which represents the final output, predicts a highly charged region indicating a cleavage site immediately after the 35th amino acid in the CapC sequence. The concurrence of both the C- and S-score suggests that this identification of the
cleavage site is highly like to be correct. Local alignments of CapC with multiple, publically available genomes of *Campylobacter jejuni* and *C. coli* show that this signal peptide region is highly conserved within the *capC* sequence and represents an integral structural component of this autotransporter protein.

This chapter also describes the distribution of the *capC* gene amongst a sample of representative strains. Preliminary analysis of *capC* showed variation within the N-terminal portion of the gene. The extent of this variation is considered in Chapter 6. Primers were designed to target conserved regions of *capC*. 68 of 113 isolates tested in this study were found to encode *capC*. This is a comparatively small sample size and as such is limited in terms of conclusions that can be drawn from it. In addition, these samples are representative of only a small population of *Campylobacter* that survive poultry processing and are present on supermarket poultry and also of those are able to cause infections in humans. As considered in Chapter 1, in order to reach these respective niches, campylobacters must overcome a wide array of environmental hostilities. The proportion of *Campylobacter* species introduced into these niches, namely poultry meat surfaces during processing and the human small intestine that survive exposure to these stresses is very small. Therefore the samples of *Campylobacter* species isolated and obtained in this study represent only a minority of the *Campylobacter* population. Nonetheless, these samples are clinically relevant in that they are derived directly from human disease and also from supermarket poultry samples; what is considered to be the primary reservoir of infection. Establishing the distribution of *capC*, as a putative virulence factor, in these isolates is therefore of value. This study has found that *capC* is present in 60% of isolates tested yet it is unclear as to whether this is true for the wider population of *Campylobacter jejuni* and *C. coli*.

Chapter 3 also describes the construction of three defined knockout mutants in which the *capC* gene had been interrupted or partially deleted, as well as the generation of coupled wildtype
strains for each knockout constructed. Initially, a capC knockout was constructed in C. jejuni 81116 via insertion of a 1499bp fragment encoding a kanamycin resistance marker into the capC coding sequence. Subsequent phenotypic assays comparing the wildtype and mutant were highly suggestive that the ΔcapC:kanR mutant had impaired motility resulting from insertion of the kanR cassette. Given that CapC, as an autotransporter, is predicted to be an outer membrane or extracellular protein (Section 3.2.1.3) this effect upon motility was unexpected and is suspected to have arisen as a result of a polar mutation, although this suspicion has not been confirmed by whole genome comparison of the wildtype and ΔcapC:kanR mutant strains. Such a mutation may impact upon a trait such as motility could subsequently influence other phenotypic behaviours; particularly those associated with virulence, and thus confound results.

In order to assess the contribution of CapC to motility further, additional capC knockouts were therefore generated in C. jejuni 81116 and C. jejuni M1 by partial deletion of the capC coding sequence and insertion of a 798bp marker conferring chloramphenicol resistance. Motility testing of these mutants demonstrated that deletion of capC had no effect on motility in semi-solid media suggesting the motility defect observed in the ΔcapC:kanR mutant may have been as a result of a polar effect arising from genetic manipulation.

In this study, a strain in which the capC gene had been successfully complemented was unable to be constructed. A variety of approaches were used in order to attempt complementation of capC via the cj0046 pseudogene. However the results generated in this thesis, results are suggestive that expression of capC was toxic to the E. coli strain used for intermediary cloning steps. The capC coding sequence fragment was unable to be cloned into the pC46 plasmid or the pSV009 plasmid in the correct orientation using the catR promoter. capC was successfully cloned into pGEM T-easy and maintained in E. coli in both orientations as judged by restriction analysis (data not shown), however it is unclear as to whether this plasmid permits the expression of this
inserted sequence. The failure to construct a genetic complement for capC in order to fully examine phenotypic associations of capC and the precise role of this putative virulence factor is a major limitation of this study.

All capC deletion mutants showed marginally decreased haemolytic activity in the contact haemolysis assay including the kanR mutant, as compared to their coupled wildtype strains. Haemolysis is a behaviour of Campylobacter species that is ill-defined and highly variable. The phospholipase A (PldA) of Campylobacter has previously been implicated as contributing to Campylobacter-mediated haemolysis. The decrease in haemolytic activity displayed by the mutants may suggest CapC functions as an auxiliary haemolysin. However, a previous study regarding haemolytic activity of campylobacters demonstrated that a knockout of PldA in a C. coli strain that was positive for capC demonstrated levels of haemolysis that were equal to background, spontaneous haemolysis controls which is indicative of a complete cessation in haemolytic activity. This is highly suggestive that CapC does not contribute directly to haemolysis and that the phenotypic effects observed in this study are the corollary of another behaviour that CapC impacts upon. Furthermore, CapC displays no sequence homology with known bacterial haemolysins.

CapC deficient mutant strains show no growth or growth rate deficiency as determined in Mueller-Hinton Broth, a comparatively rich media, indicating that deletion of capC confers no growth deficiency. CapC was not expected to be involved in catabolism or impact upon C. jejuni nutrient acquisition or growth as it lacks any homology to known factors involved in metabolism. Previous studies have established that mutations that confer metabolic defects in Campylobacter have adverse effects on the behaviour and virulence phenotypes in vitro (Novik et al., 2010). Growth profiles presented in this chapter can be used to directly inform other assays that examine
behaviour the contribution of \textit{capC} to aspects of \textit{Campylobacter} behaviours. One such behaviour is biofilm formation.

As a presumptive outer membrane factor, CapC would be ideally placed to be involved in the attachment of \textit{Campylobacter} to cells and surfaces; crucial aspects of \textit{Campylobacter} biofilm formation. Deletion of \textit{capC} did not cause a decrease or cessation in biofilm formation, and the degree of biofilm formation was found to vary between \textit{C. jejuni} 81116 and M1. Interestingly, deletion of \textit{capC} was found to increase biofilm formation in the \textit{C. jejuni} M1 Δ\textit{capC} strain only; whereas the wildtype \textit{C. jejuni} M1 attached to the polyprolene surface very poorly. The \textit{C. jejuni} M1 wildtype growth profile matched that of the mutant so a disparity in growth cannot account for differential biofilm formation. Likewise, motility of the mutant strain is unimpaired. A possible explanation is that a polar mutation is responsible for this phenotype, or possibly that deletion of a putative outer membrane protein results in a transcriptomic response resulting in increased biofilm formation. In a genome-wide, transposon mutagenesis screen for genes involved in biofilm formation in \textit{C. jejuni}, \textit{capC} was found not to significantly affect biofilm formation (R. Clarke, Personal Communication). Taking this into account along with the fact that deletion of \textit{capC} in \textit{C. jejuni} 81116 had no effect on biofilm formation, the exact role, if any, of CapC in biofilm formation is likely to be minimal.
Chapter 4: CapC is an outer membrane protein that contributes to *Campylobacter* virulence in the *Galleria mellonella* virulence model
4.1. Introduction

Chapter 3 described the confirmation of capC as belonging to the autotransporter family of proteins. This classification was made based on a combination of sequence homology to existing autotransporters, the identification of conserved domains consistent with CapC being an autotransporter and three-dimensional structure prediction. All previously characterised autotransporters are reported to be involved in pathogenicity to some extent, functioning as virulence determinants that are responsible for a wide array of behaviours that occur at the bacterium/host interface including cell adhesion, toxin secretion and host defence avoidance (Nishimura et al., 2010).

The term virulence is commonly defined as the degree of tissue damage a microbe may cause to the host (Henderson and Nataro, 2001). Virulence is often determined by the extent to which an organism can invade, elicit pathology or destroy cells and tissues; fatality rates are also used as an indicator of virulence. Given the plethora of literature citing the involvement of these secretory proteins in virulence, it is likely that CapC serves a comparable role in Campylobacter. The overarching purpose of this chapter was to elucidate any potential role CapC may have in the virulence of C. jejuni. The use of the Galleria mellonella infection model is ideal for this investigation, as this model provides the basis for a rapid screening of virulence phenotypes within the context of a functional immune system comparable to that of mammals. Factors previously implicated as integral to Campylobacter infection of the human host have also shown to contribute to insecticidal activity in this model (Naz, 2014), demonstrating that Galleria mellonella are an effective model for Campylobacter virulence.
The specific aims of this chapter, therefore, are:

1. To build on previous bioinformatic predictions and determine the cellular localisation of CapC. Chapter 3 outlines use of Cello software to predict the localisation site of CapC as the outer membrane. Using immunoblotting, localisation of CapC will be investigated and an indication of its potential role in host interaction will be ascertained.

2. To use targeted comparison of wildtype and isogenic capC mutants to compare virulence capability of each strains in the Galleria mellonella virulence model. A combination of studies will be employed to assess whether deletion of capC impacts upon virulence of these C. jejuni strains.
4.2. Results

4.2.1. Immunodetection of CapC and determination of its cellular localisation.

Determination of the cellular localisation site of the CapC protein is an important part of the characterisation of this factor as an autotransporter and may give insight into the role this factor plays in Campylobacter biology. C. jejuni 81116, C. jejuni M1 and their respective isogenic ΔcapC mutants were fractionated using the procedure described in Chapter 2. Briefly, outer and inner membrane fractions were prepared based on Sarkosyl insolubility and solubility respectively as described by Carlone et al. (1986). Cytoplasmic fractions were prepared by centrifugation followed by gelatin and polymixin B treatment, as previously described (Sommerland and Hendrixson, 2007). Extracellular fractions were also prepared as described in section 2.14.2.5. Western blotting analysis for detection of the CapC protein was performed according to the protocol outlined in section 2.15 and exposure and image capture was performed on a Licor Odyssey Fc imager (Licor, USA). The CapC protein is predicted to be ~72KDa taking into account cleavage of the signal peptide but barring any other form of processing.
Figure 4.1A: Western blot of *C. jejuni* 81116 wildtype, *C. jejuni* M1 wildtype, *C. jejuni* 81116 ΔcapC:cat' and *C. jejuni* M1 ΔcapC:cat' whole cell lysate probed with α-CapC rabbit polyclonal primary antibody and goat anti-rabbit secondary antibody conjugated to HRP. ECL Clarity reagent (Biorad, UK) was applied to PVDF membranes and exposed to X-ray film for 10 minutes and developed. Bands are visible in lanes run with wildtype strains only indicating that mutant strains do not express CapC. Interestingly, probing of whole cell lysate resulted in two bands from wildtype strains at ~50kDa and ~25kDa suggesting that *capC* may undergo a measure of post-translational modification.
Figure 4.1B: Western blot demonstrating detection of CapC in the outer membrane fractions of wildtype *C. jejuni* 81116 and *C. jejuni* M1. *C. jejuni* cells were fractionated and extracts were separated by SDS-PAGE, blotted on to a PVDF membrane which was probed with α-CapC antibody (Thermo Scientific) followed by probing with goat α-rabbit IgG conjugated to horse-radish peroxidase (HRP). Reagent addition and membrane exposure was performed as described in section 2.15.3. Lane 1 contains the EZ-Run™ Protein MW marker; Lane 2 and 3 contain extracts from *C. jejuni* 81116 (Lane 2) and *C. jejuni* M1 (Lane 3) extracellular preparations; Lane 4 contains sample buffer only; Lane 5-6 were loaded with samples from *C. jejuni* 81116 (Lane 5) and *C. jejuni* M1 (Lane 6) outer membrane fractions. Labelled on Figure 4.1B are two bands of circa. 50 KDas which are visible in lanes loaded with the outer membrane fractions of *C. jejuni* 81116 and *C. jejuni* M1, indicating detection of CapC in the outer membrane but not in the supernatant.
Figure 4.1C: Western blot demonstrating absence of CapC within inner membrane and cytoplasmic fractions of C. jejuni 81116 wildtype and C. jejuni M1 wildtype. C. jejuni cells were fractionated and extracts were separated by SDS-PAGE, blotted on to a PVDF membrane which was probed with α-CapC antibody (Themo Scientific) followed by probing with goat α-rabbit IgG conjugated to horse-radish peroxidase (HRP). Reagent addition and membrane exposure was perform as described in section 2.15.3. Lane 1 contains the EZ-Run™ Protein MW marker; Lane 2 and 3 were loaded with inner membrane fractions of C. jejuni 81116 and C. jejuni M1 respectively. Lanes 4 and 5 were loaded with 1x sample buffer only and contain no C. jejuni fraction material. Lanes 6 and 7 were loaded with cytoplasmic fractions of C. jejuni 81116 and C. jejuni M1 respectively. Lane 8 is loaded with the EZ-Run™ Protein MW marker. No protein bands are visible in the inner or cytoplasmic fractions prepared from C. jejuni 81116 and C. jejuni M1.
Figure 4.1A shows an X-ray film showing detection of CapC in C. jejuni 81116 and C. jejuni M1 wildtype strains and absence in the mutant strains, confirming that CapC has been successfully knocked-out in these strains. Unexpectedly, two protein bands of ~25KDa and ~50KDa were obtained when blotting whole cell lysate with antibodies specific for CapC indicating that CapC undergoes processing and a measure of post-translational modification. Two different band sizes may also indicate different localisation sites.

Figure 4.1B shows a photo of the PVDF membrane after exposure depicting detection of the CapC protein in the outer membrane fractions of wildtype C. jejuni 81116 and C. jejuni M1. Figure 4.1B also shows the absence of CapC in the Campylobacter supernatant fractions, indicating that CapC is an outer-membrane anchored protein and does not appear to be secreted extracellularly in its native form. Additional Western blots were performed on inner membrane and cytoplasmic fractions of C. jejuni 81116 and C. jejuni M1 however CapC was not detected as shown in Figure 4.1C.

The fact that CapC is detected in whole cell lysate and in the outer membrane preparation by rabbit antisera, shows that this protein is capable of eliciting an immunogenic response and is therefore likely to serve as an immunogenic factor in vivo.
4.3. Comparison of virulence of wildtype and ΔcapC mutant strains in the *Galleria mellonella* virulence model

The use of insect models of infection as a replacement for conventional mammalian models has gained in popularity in recent years. The larvae of the greater wax moth (*Galleria mellonella*) have previously been shown to be an effective virulence model for a variety of bacterial and fungal pathogens including *C. jejuni* (Senior *et al.*, 2010). In addition to being an efficient model that is an ethical alternative to animal models, use of *Galleria mellonella* provides a quantifiable measure of virulence within the context of a functional immune system. Use of this model is considered in detail in section 1.7.1.

4.3.1. *Galleria* Larvae Survival Assay

The survival of larvae infected with either *C. jejuni* 81116 or *C. jejuni* M1 and their respective isogenic ΔcapC mutants in parallel was assessed over a 48 hour period according to the method stated in section 2.13.3. In brief, standardised suspensions of *C. jejuni* were used to infect larvae by microinjection. Larvae were incubated at 37°C for 48 hours. At various times post infection, larval survival was assessed. Fatality was confirmed by the inability of larvae to respond to physical stimuli and the degree of discolouration. The number of surviving larvae at each time point was counted. This assay was performed with 10 larvae in duplicate for each strain and repeated on three separate occasions.

Figure 4.2A+B show results of the larval survival screen post infection with *C. jejuni* 81116 and *C. jejuni* 81116 ΔcapC (Figure 4.2A) and *C. jejuni* M1 and *C. jejuni* M1 ΔcapC (Figure 4.2B). Both figures show 100% survival of larvae that were mock infected with PBS and incubated under identical conditions to those infected with PBS (green line). The proportion of surviving larvae infected with wildtype strains (red lines on Figure 4.2A+B) was consistently and significantly decreased as compared to mutant strains (blue lines) from 6 to 48 hours post infection. This
disparity between the wildtype and mutant was consistent across both strains. Figure 4.2A show these results when larvae were infected with *C. jejuni* 81116 and *C. jejuni* 81116 ΔcapC; at 24 hours post infection, only 10% of larvae infected with wildtype *C. jejuni* 81116 remained alive whereas up to 70% of larvae infected with the capC mutant remained alive (p=0.0027, n=3). Similarly, Figure 4.2B depicting survival of larvae infected with *C. jejuni* M1 wildtype and mutant, shows that at 24 hours post infection 0-10% of larvae infected with the wildtype strain survived whereas approximately 60% of larvae infected with the mutant strain survived (p=0.0034, n=3). In this context survival of larvae is used as an indicator of virulence. The results presented here suggest that deletion of capC results in decreased *C. jejuni* virulence in the *Galleria* model as indicated by survival rates. Furthermore, this preliminary screen of virulence shows that the optimal time-point for observing differences between strains used in this study is 24 hours post infection. These results provide a basis for further testing of the contribution of capC to virulence in the *Galleria mellonella* virulence model.

*Figure 4.2A*
Figure 4.2B Graphical representation of the larval survival assay performed with C. jejuni 81116, C. jejuni M1 and their respective ΔcapC mutants. PBS, mock infections controls were also used. Survival curves of larvae infected with wildtype strains over 48 hours is depicted by red lines; survival curves of larvae infected with mutant strains is represented by blue lines; survival of mock infected larvae is represented by green lines. Deletion of capC in both C. jejuni 81116 (Figure 4.2A) and C. jejuni M1 (Figure 4.2B) causes increased survival of Galleria larvae over 48 hours. This decrease in becomes apparent as early as 4 hours post infection with the onset of larval death which decreases further thereafter. Comparison of mean survival at 24 hours post infection shows deletion of capC causes significantly decreased survival (C. jejuni 81116: p=0.0027, n=3; C. jejuni M1: p= 0.003, n=3. ** denotes P<0.01). These assays were performed in duplicate (10 larvae per replicate) on three different occasions; error bars show standard deviation. P-values obtained using an unpaired t-test.

4.3.2. Galleria Morbidity and Mortality Assay

Results from the larval survival screen indicate that deletion of capC causes a decrease in virulence suggesting that capC contributes to virulence and infection in some capacity. To investigate the role of capC in virulence in greater depth, a more sensitive assay was employed in which the morbidity and mortality elicited by C. jejuni wildtype and mutant strains was assessed at 24 hours post infection. The assay was performed as described in section 2.13.4. Larval morbidity and mortality was scored as outlined in section 2.13.4. Briefly, after 24 hours incubation, larvae were assessed by eye based on three criteria; mortality, discolouration and ability to move. Live larvae
were allocated a score of 0 whereas dead larvae were given a score of 1. The degree of colour change of larvae from a healthy pale yellow to a dark discolouration was also assessed and scored. Larvae with a healthy yellow colour were given a score of 0. Larvae with slight brown or grey discolouration were given a score of 1 and larvae that displayed complete discolouration were given a score of 2. Figure 4.3 (below) shows representative colour changes of *Galleria mellonella* in response to infection. Larvae were given a score of 0 if able to turn over having been placed on their back on filter paper with a pair of sterile forceps; those unable to move were given a score of 1. For each of these criteria, the total scores for each plate (10 larvae) were converted to percentages of the maximum possible virulence score to give the final virulence percentage shown in Figure 4.4A and B. This assay was performed in triplicate (3 plates of 10 larvae) and repeated three times.

Figure 4.3: Four larvae of *Galleria mellonella* that demonstrates representative discolouration during the course of infection with *C. jejuni*. Larva 1 shown on Figure 4.3 would be allocated a score of 1.5; Larva 2 would be allocated a maximum score of 2; Larva 3 would be allocated a score of 0.5 having shown slight discolouration and Larva 4 would be allocated a score of 0.
Figure 4.4A and B shows the results of the morbidity and mortality assay in the *Galleria* model as performed with *C. jejuni* 81116 wildtype and ΔcapC mutant and *C. jejuni* M1 wildtype and mutant. Across both sets of strains the ΔcapC mutants exhibited significantly decreased virulence based on mortality rates, discolouration and the ability to move. The wildtype strains consistently elicited mortality rates of 90-100% whereas the mortality rates of 10-33% for *C. jejuni* 81116 ΔcapC (p=0.00017) and 0-57% for the M1 ΔcapC strain (0.00232). These results indicate that capC contributes to mortality and virulence in this model. Notably, deletion of capC does not cause a cessation in virulence, merely a decrease.
**Figure 4.4A**

*C. jejuni* 81116 Morbidity/Mortality screen in *Galleria*

![Graph showing morbidity and mortality screen of C. jejuni 81116 and 81116 ΔcapC.](image)

**Figure 4.4B**

*C. jejuni* M1 Morbidity/Mortality screen in *Galleria*

![Graph showing morbidity and mortality screen of C. jejuni M1 and M1 ΔcapC.](image)

**Figure 4.4A** (above) and **4.4B** (below): Morbidity and mortality screen of *C. jejuni* 81116 and 81116 ΔcapC (Figure 4.4A) and *C. jejuni* M1 and M1 ΔcapC (Figure 4.4B). Mean virulence scores of biological replicates for each criterion, obtained as described above, were converted to percentages of the maximum possible score and compared. Figure 4.4A shows significant differences in the morbidity and mortality elicited by *C. jejuni* 81116 and *C. jejuni* 81116 ΔcapC. Morbidity and mortality scores were compared using an unpaired t-test (Mortality: p=0.00017, n=3; Discolouration: p=0.000019, n=3; Ability to Move: p=0.000624, n=3). Figure 4.4B shows significant differences in the morbidity and...
mortality elicited by *C. jejuni* M1 and M1 ΔcapC (Mortality: p= 0.002323, n=4; Discolouration: p=0.002323, n=4; Ability to move: p=0.004571, n=4). These assays were performed in triplicate (10 larvae per replicate) on four different occasions; error bars show standard error of the mean.

4.3.3. Assessment of intra-larval bacterial load

It was observed in previous assays that wildtype strains induced a discolouration in *Galleria* much more rapidly than mutant strains. This pigmentation is due to melanin production caused by the anti-microbial peptide prophenoloxidase. The differences observed in morbidity between wildtype and mutant infected *Galleria* likely corresponds with a particular pathology. More detailed examination of *Campylobacter* behaviour in this model may provide insight into the role CapC in the virulence phenotype with which it has been correlated.

To this end, the replication kinetics of both the wildtype and mutant strains were compared *in vivo* using the *Galleria* model. Results are shown in Figure 4.5A and 4.5B. This assay was performed as described in section 2.13.5.

Notably, within 6 hours of infection, the viable count of the wildtype strains exhibited almost a 2 log reduction, a significantly greater reduction than exhibited in the mutant strains. This phenotype was consistent in both *C. jejuni* 81116 and *C. jejuni* M1. In spite of the decrease in the viable count, wildtype *C. jejuni* remained more virulent in these assays, as determined by fatality rates.
Figure 4.5A and 4.5B: A comparison of bacterial viable counts from *Campylobacter* present in the haemolymph from larvae infected with either wildtype of Δ*capC* mutant *C. jejuni* 81116 and M1. Within 6 hours of infection, the wildtype strains exhibited greater reduction in viable count as compared to the mutant strains. This disparity between strains in terms of the viable count is more pronounced in *C. jejuni* M1 (Figure 4.5B), though the decrease in viable count of the wildtype is consistent across both *C. jejuni* M1 and 81116. Time 0 viable counts were taken by harvesting haemolymph immediately (within 10 minutes) after infection. Larvae were anaethetised by incubation on ice for 8-10 minutes and 20μl haemolymph was extracted as described in section 2.13.5, and viable counts obtained as described in section 2.6.4.3. These assays were performed in triplicate (10 larvae per replicate) on four different occasions; error bars show standard error of the mean.
4.3.4. Visualisation of the interaction of *C. jejuni* with *Galleria* haemocytes from infected larvae

One possible explanation for the phenotype observed in Figure 4.5A and B is that the wildtype strains, with CapC intact on their outer membranes were able to elicit a greater inflammatory immune response from *Galleria* larvae and were subsequently killed via the increase bacteriocidal action of the *Galleria* immune system. In order to investigate this hypothesis, the interaction between *C. jejuni* wildtype and mutant strains with haemocytes was visualised by light microscopy. Briefly, haemolymph from infected larvae was extracted at 3 hours post infection. 10μl of haemolymph was fixed on a glass slide; this preparation was fixed with methanol and stained with Giemsa stain according to the method outlined in section 2.5.5. Slides were observed and examined for any potential differences in interaction with haemocytes and potential pathology. No difference in interaction with haemocytes was observed between wildtype and mutant strains. Figure 4.6 shows representative photographs of the light microscopy performed on haemolymph preparations. From these images, it is clearly evident that *C. jejuni* does interact with *Galleria* haemocytes during infection.
Figure 4.6: A series of annotated micrographs of giemsa-stained haemocytes from healthy, mock-infected larvae (A-D), and larvae infected with *C. jejuni* 81116, *C. jejuni* M1 and their respective ΔcapC mutants (E-H). No qualitative difference was observed in the interaction of *Campylobacter* with *Galleria* haemocytes between wildtype and mutant strains. Nevertheless, the annotated images in this figure confirm that *C. jejuni* does interact with the *Galleria* immune system. A-D= *Galleria* haemocytes from PBS infected, healthy larvae at x40 objective (A) and x100 objective (B, C, D) showing healthy morphology of haemocytes and intact cell membranes. E-H= *Galleria* haemocytes from larvae infected with *C. jejuni* 81116 (E), *C. jejuni* M1 (G) and their respective ΔcapC mutants (F and H). Haemocytes from infected larvae show lysed or abhorrent cell membrane and morphology and disrupted intracellular structures. Images were taken with a Motic RED233 microscope at x40 and x100 objective.
4.4. Triton-X sensitivity

Triton-X is a non-ionic surfactant that is commonly used as a detergent in order to lyse eukaryotic cells. Triton-X 100 was used in the assay described in section 4.3.3. to lyse Galleria haemocytes to ensure and intracellular Campylobacter was included in viable counts and not victim to bacteriocidal action of the Galleria haemocytes. A relative sensitivity of the wildtype or mutant strains to Triton-X 100 would confound results obtained from the intralarval replication experiment; therefore, to determine the sensitivity of C. jejuni wildtype and mutant strains to Triton-X 100 and to ensure that deletion of capC did not impart a greater sensitivity to Triton-X 100 (1% vol/vol), the sensitivity of all wildtype and ΔcapC mutant strains to this detergent were compared. Assays were performed as described in section 2.7.2. Figure 4.13A and B show the results of these assays.

Figure 4.7A and 4.7B show viable counts of C. jejuni 81116 and C. jejuni M1 wildtype and ΔcapC mutant strains with and without Triton-X 100 treatment for 1 hour. There are no differences in the viable counts between treated and untreated cultures and no difference in viable count between wildtype and mutant strains indicating that no strains used in this study were unusually sensitive to Triton-X 100.
Figure 4.7A: Viable counts of *C. jejuni* 81116 wildtype and *C. jejuni* 81116 ΔcapC mutant suspensions in PBS with and without treatment with 1% Triton-X 100 for 1 hour at room temperature (p>0.999). Figure 4.7B: Viable counts of *C. jejuni* M1 wildtype and *C. jejuni* M1 ΔcapC mutant suspensions in PBS with and without treatment with 1% Triton-X 100 for 1 hour at room temperature (p>0.999). Treatment with Triton-X 100 has no effect on the viable count of these strains. These assays were performed in triplicate on three different occasions; error bars show standard error of the mean.
4.5. Discussion

**CapC is localised to the outer-membrane of *C. jejuni***

This chapter has detailed the successful determination of the CapC cellular localisation site as the outer membrane in both *C. jejuni* 81116 and *C. jejuni* M1 by immunoblotting with α-CapC antibody raised in Specific Pathogen Free New Zealand White rabbits against a peptide antigen designed with using the CapC amino acid sequence. A 50KDa protein was detected, in outer membrane fractions of *C. jejuni* wildtype strains only. Notably, this band was not present when probing concentrated supernatant fractions, inner membrane preparations or cytoplasmic fractions. This finding is consistent with preliminary bioinformatic predictions that implicate CapC as an outer membrane protein and with the wealth of knowledge regarding autotransporters that implicate these proteins as outer membrane factors (Leo *et al.*, 2012). This study failed to detect CapC in extracellular fractions however this does not preclude the possibility that CapC may be cleaved and secreted, or even secreted as a component of an outer membrane vesicle. The size of the protein band consistently detected is ~50KDa; smaller than the theoretical 72KDa protein predicted by the ExPASy MW tool ([http://web.expasy.org/compute_pi/](http://web.expasy.org/compute_pi/)) based on the CapC amino acid sequence. This may indicate an error in the predicted size of CapC or else may suggest that CapC undergoes a measure of post translational modification. This is supported by the fact that an additional ~25KDa protein band was detected in *C. jejuni* wildtype whole cell lysate that was absent from the mutant strains. This smaller protein is likely a processed form of CapC and may have an alternative localisation site. Significantly, CapC has been detected here in the outer membrane of *C. jejuni* using immunodetection, indicating that CapC is likely to be an immunogenic antigen in vivo that plays a significant role in the interaction with the host; a trait consistent with autotransporter proteins.
CapC contributes to virulence in the *Galleria mellonella* virulence model

Chapter 4 has also outlined the use of a combination of different assays in the *Galleria mellonella* virulence model which, taken together, indicate that CapC is a major virulence determinant encoded by *Campylobacter jejuni*. Larval survival assays were used to demonstrate that larvae infected with ΔcapC strains exhibited significantly increased survival of *Galleria* as compared to those infected with coupled wildtype strains indicating that deletion of capC attenuates *C. jejuni* to some extent. A more sensitive virulence assay was also employed in which the mortality and morbidity of infection was quantified at 24 hours post inoculation. On the basis of all three criteria examined; mortality, ability to move and degree of melanisation, the ΔcapC mutant strains caused significantly decreased virulence, reinforcing the outcome of previous survival assays and signifying that CapC contributes to both insecticidal activity, presumably through increased tissue damage and severity of disease. Given that ΔcapC deletion mutants have no impairment in their growth rates or motility capability, it is thought that CapC impacts upon virulence in this model directly. There is also the possibility that deletion of this outer membrane protein confers an *in vivo* fitness impairment that is not apparent in *in vitro* assays described in Chapter 3, and that the reduction in virulence observed is due to this. However, the increased survival of mutant strains up to 6 hours post infection renders this possibility unlikely.

Notably, whilst deletion of capC significantly decreased virulence, it did not result in a total cessation of insecticidal activity and disease. This is likely due to other virulence factors encoded by *C. jejuni* 81116 and *C. jejuni* M1; whilst the evidence in this case for the contribution of CapC to virulence is compelling; this contribution must be taken in context with respect to these other virulence factors which may still cause a degree of disease in *Galleria*, independent of CapC. These include the immunogenic flagella of *C. jejuni*, adhesins and the production of CDT (Dasti et al., 2010). Therefore it can be concluded that CapC merely contributes to virulence in this model, but
is not essential. The conclusions that can be drawn from this model regarding CapC contributions to severity of disease are limited as this model, whilst ideal for rapid screens of fitness and virulence factors, is not representative of the *C. jejuni* site of infection in humans, nor of its route to this site.

*C. jejuni* wildtype strains encoding CapC may show greater immunogenicity in *Galleria*

This study also quantified bacterial load between wildtype and mutant-infected larvae up to six hours post infection. In these studies, the viable count of bacteria decreased immediately after inoculation and no viable *C. jejuni* were recovered at 24 hours post infection. Therefore infection progression cannot be monitored over an extended period of time and infection modelling is limited to a matter of hours. Nonetheless, valuable insight regarding *Campylobacter* behaviour during initial contact with tissues and immune cells can be gleaned.

This study has shown that the viable count of both *C. jejuni* 81116 and *C. jejuni* M1 wildtype strains decreased up to six hours post infection at a greater rate than the coupled ΔcapC mutant strains. A possible reason for this phenotype is that the wildtype strains are able to localise to a different area of the *Galleria* body such as the fatty tissue and therefore not be present within larval haemolymph to be enumerated, in which case capC deficiency would result in increased levels of *C. jejuni* in the haemolymph. A likelier explanation for the observed phenotype is that the wildtype strains stimulate a greater immune response from *Galleria* larvae and subsequently fall victim to the bacteriocidal action of the haemocytes. Light microscopy indicates *Campylobacter* interacts with haemocytes and causes considerable morphological changes, though no difference was observed between wildtype and mutant strains. Melanisation is the term used to describe the pigmentation observed in challenged larvae. This pigmentation is caused by melanin deposition around sites of infection or intruding pathogens (Cerenius and Söderhäll, 2004).
Melanin production is catalysed by phenoloxidase which is activated by recognition of foreign material in *Galleria mellonella* (Cerenius and Söderhäll, 2004). Thus, the degree of melanisation observed in infected *Galleria* is indicative of the greater immune response elicited by wildtype strains. This suggests that CapC may function as a major immunogenic factor in *Campylobacter*. 
Chapter 5: The contribution of \textit{capC} to virulence in intestinal epithelial cell models
5.1. Introduction

Chapter 4 described the identification of CapC as an outer membrane protein and demonstrated that this factor contributes to virulence in the Galleria mellonella in vivo model. Virulence and infection mechanisms in the Galleria model have been shown to have direct parallels to those observed in human infection in other pathogens (Harding et al., 2012), however more suitable and better characterised infection models were also used to assess the role of CapC in virulence.

In the absence of convenient small animal models, tissue culture represents a viable and reproducible means of investigating C. jejuni infection of the human host. C. jejuni has been shown to interact with various polarised and non-polarised intestinal epithelial cells in vitro including INT407, T84 and Caco-2 (Monteville and Konkel, 2002; Everest et al., 1992; Konkel et al., 1992). These models have proven useful in deciphering the factors and mechanisms involved in C. jejuni adherence, invasion and transmigration (Bouwmen et al., 2013; Javed et al., 2012; Monteville and Konkel, 2002). Cell line choice is an important consideration in the investigation of C. jejuni interaction with intestinal epithelial cells; it has been demonstrated for example, that C. jejuni infects Caco-2 less and T84 more efficiently than INT407 cells (Poly et al., 2008; Wine et al., 2008). Moreover, these cell lines each exhibit unique characteristics that are useful in investigations such as adherence, transcytosis and intracellular localisation (Backert and Hofreuter, 2013).

A combination of Caco-2 and T84 cells were used in the present study. Caco-2 cells are reminiscent of enterocytes, expressing enterocyte-specific brush border enzymes and hydrolases such as LCT and DDP4 and long microvilli (Devriese et al., 2017). By comparison, T84 cells are a more appropriate model for colonocytes, expressing colonocyte-specific structures such as the MCT1 transporter and shorter microvilli than Caco-2 cells (Devriese et al., 2017). Both cell lines also differ in their TLR expression; whilst this appears not to impact upon functionality, it is
apparent that T84s exhibit a more robust inflammatory response and cytokine expression that Caco-2 cells (Devriese et al., 2017; Eckmann et al., 1993).

In order to further investigate the virulence phenotype with which CapC has been correlated, tissue culture assays represent ideal models to examine the C. jejuni infection processes in vitro (Mills et al., 2012; Javed et al., 2012; Everest et al., 1992).

The specific aims of this chapter, therefore, were as follows:

1. To use more established in vitro models of bacterial infection such as Caco-2 and T84 intestinal cell lines to assess virulence-associated phenotypes. Through the use of these models, aspects of C. jejuni pathogenesis such as the processes of adhesion and invasion will be assessed using association and gentamicin protection assays.

2. To assess the contribution of CapC to cytotoxicity and ascertain whether CapC functions in any capacity as a specific cytotoxin. This will be performed in a Caco-2 cell culture model using a commercially available kit.

3. To build on observations regarding the contribution of CapC to immunogenicity in the Galleria mellonella virulence model and determine whether CapC contributes to the inflammatory process in a T84 cell culture model. This will be performed by comparison of the levels of secretion of the pro-inflammatory chemokine IL-8 upon infection with wildtype and mutant strains.
5.2. The contribution of CapC to adhesion and invasion in a Caco-2 cell culture model

The cell line Caco-2 has been used as a relevant model for the study of Campylobacter pathogenicity, from as early as 1992 when investigations in to the mode of Campylobacter pathogenesis began (Everest et al., 1992). Partially differentiated Caco-2 cells were used as a tissue culture model to determine the contribution of CapC to the processes of adherence and invasion. Association and gentamicin protection assays were carried out in parallel as described in section 2.14.2.1 and 2.14.2.2.

Briefly, Caco-2 cells were seeded for 7 days at 1x10^6 cells/ml until confluent and partially differentiated. Confluent monolayers were infected with 10^8 cfu/ml of C. jejuni at an MOI of 1:100. Infection lasted for 3½ hours, after which media was removed and cells washed. For association assays monolayers were then lysed using the detergent Triton-X 100 and the number of C. jejuni were enumerated. For invasion assays, media supplemented with gentamicin was added to the cells and 24-well microplates were incubated for a further 2 hours to kill extracellular bacteria. Monolayers were then washed as before and lysed for enumeration of bacteria. Invasion and adhesion efficiencies were calculated as described in Chapter 2, section 2.14.2.1 and 2.14.2.2.

Figure 5.1A and 5.1B show adhesion and invasion efficiencies C. jejuni 81116 wildtype, C. jejuni 81116 ΔcapC and C. jejuni NCTC 11168H ΔfliD mutant which was used as an aflagellate, low adhesion and invasion control. As depicted by Figure 5.1A, the wildtype C. jejuni 81116 strain exhibited an adhesion efficiency of 0.174% whereas the ΔcapC mutants exhibited an adhesion efficiency of 0.014%. This decrease in adhesion efficiency of the ΔcapC mutant strain was statistically significant as determined by an unpaired Student’s t-test (p=0.0067, n=3). Significantly, the wildtype C. jejuni 81116 strain also exhibited greater invasion efficiency (0.002%) compared to the mutant strain (0.00039%) (p=0.048, n=3).
Figure 5.1A and 5.1B show adhesion and invasion efficiencies of *C. jejuni* 81116 (red bars), *C. jejuni* 81116 ΔcapC (blue bars) and *C. jejuni* NCTC 11168 ΔfliD mutant (black bars) in a Caco-2 cell culture model. *C. jejuni* 81116 exhibited an adhesion efficiency of 0.174% whereas the ΔcapC mutants exhibited an adhesion efficiency of 0.014% (p=0.0067, n=3). The wildtype *C. jejuni* 81116 strain also exhibited greater invasion efficiency (0.002%) compared to the mutant strain (0.00039%) (p=0.048, n=3). These assays were performed in triplicate on three different occasions; error bars show standard error of the mean. Cfu/ml viable counts and 100% efficiency values are shown in Appendix V.

Similarly, as shown in Figure 5.2A and 5.2B, *C. jejuni* M1 wildtype, demonstrated an adhesion efficiency of 0.173% whereas the *C. jejuni* M1 ΔcapC mutant exhibited an adhesion efficiency of 0.0188%; a statistically significant reduction (p=0.033, n=3). In addition, the *C. jejuni* M1 ΔcapC
mutant had a significant impairment in its ability to invade Caco-2 cells as compared to the wildtype strain (p=0.03, n=3).

**Figure 5.2A**

![Graph showing adhesion efficiency](Image)

- **C. jejuni NCTC11168 fliD mutant**
- **C. jejuni M1 Wildtype**
- **C. jejuni M1 capC Mutant**

**Figure 5.2B**

![Graph showing invasion efficiency](Image)

- **C. jejuni NCTC11168 fliD mutant**
- **C. jejuni M1 Wildtype**
- **C. jejuni M1 capC Mutant**

**Figure 5.2A** (above) and **5.2B** (below): Figure 5.2A and 5.2B show adhesion and invasion efficiencies, respectively, of **C. jejuni M1** (red bars), **C. jejuni M1 ΔcapC** (blue bars) and **C. jejuni NCTC 11168 ΔfliD mutant** (black bars) in a Caco-2 cell culture model infected with an MOI of 100. **C. jejuni M1** wildtype demonstrated a mean adhesion efficiency of 0.173% whereas the **C. jejuni M1 ΔcapC** mutant exhibited an adhesion efficiency of 0.0188 (p=0.033, n=3). As shown in **Figure 5.2B**, the **C. jejuni M1 ΔcapC** mutant had a significant impairment in its ability to invade Caco-2 cells as compared to the wildtype strain (p=0.03, n=3). These assays were performed in triplicate on three different occasions; error bars show standard error of the mean. Cfu/ml viable counts and 100% efficiency values are shown in Appendix V.
5.3. The contribution of CapC to adhesion and invasion capability of *Campylobacter jejuni* in a T84 cell culture model

T84 cells are a continuous cell line of human colonic epithelial cells derived from a lung metastasis of a colon carcinoma. They display phenotypic similarity to colonic crypt cells and possess receptors for a range of peptide hormones and neurotransmitters. Notably, they are capable of inflammatory responses. When grown under specific conditions, T84 cells form polarised cell monolayers, however in this study, T84 cells were utilised as a non-polarised colonic cell model.

Figure 5.3A

*C. jejuni* Adhesion assay in T84 cell model
Figure 5.3B

C. jejuni Invasion assay in T84 cell model

Figure 5.3A and 5.3B: Adhesion and invasion efficiencies, respectively, of C. jejuni 81116, C. jejuni M1 as well as their respective ΔcapC mutants in a non-polarised T84 cell culture model infected with an MOI of 100. C. jejuni 81116 wildtype and C. jejuni M1 wildtype demonstrate adhesion efficiencies of 0.59% and 1.6% respectively, as compared to 0.078% and 0.24% for their respective coupled ΔcapC mutants (C. jejuni 81116 p=0.11, n=3; C. jejuni M1 p=0.121, n=3). C. jejuni 81116 invasion efficiency was reduced from 0.019% to 0.002% as a result of capC deletion and C. jejuni M1 invasion efficiency was reduced from 0.039% to 0.014% (p=0.24, n=3). These assays were performed in triplicate on three different occasions; error bars show standard error of the mean. Cful/ml viable counts and 100% efficiency values are shown in Appendix V.

T84 cells were seeded at 1x10^6 cells/ml for 48 hours. Adhesion and invasion assays were performed as described above and adhesion and invasion efficiencies of C. jejuni 81116, C. jejuni M1 and their respective isogenic ΔcapC mutants were calculated and compared. Once again a ΔfliD in C. jejuni NCTC 11168H was used as a negative control. Figure 5.3A and 5.3B show adhesion and invasion efficiencies respectively of the negative control, C. jejuni 81116 C. jejuni M1 and their respective isogenic ΔcapC mutants in the non-polarised T84 cell culture model. C. jejuni 81116 wildtype and C. jejuni M1 wildtype demonstrate adhesion efficiencies of 0.59% and 1.6% respectively, as compared to 0.078% and 0.24% for their respective coupled ΔcapC mutants. These reductions in adhesion efficiency exhibited by the ΔcapC mutant strains were not
statistically significant as ascertained by an unpaired t-test (C. jejuni 81116 p=0.11, n=3; C. jejuni M1 p=0.121, n=3). A reduction in invasion efficiency was also observed in the ΔcapC deletion mutants as compared to wildtype strains; C. jejuni 81116 invasion efficiency was reduced from 0.019% to 0.002% as a result of capC deletion and C. jejuni M1 invasion efficiency was reduced from 0.039% to 0.014% (p=0.24, n=3).

5.3.1. CapC is required for maximal adhesion to Caco-2 and T84 intestinal epithelial cells

In both a non-polarised Caco-2 and a non-polarised colonic epithelial cell model (T84), ΔcapC deletion mutants exhibited decreased adhesion efficiencies at levels comparable to the aflagellate negative control. Though this reduction was not statistically significant in the T84 cell culture model, it was a consistent phenotype observed for both C. jejuni 81116 and C. jejuni M1. Notably, deletion did not abolish adherence of C. jejuni 81116 or C. jejuni M1 to intestinal epithelial cells however the significant reduction in the efficiency of adherence suggests CapC may promote adhesion to human cells. Considering that capC deletion mutants are equally as capable as coupled wildtype strains in surviving aeration stress in tissue culture media as demonstrated in Chapter 3, it is highly likely that the adhesion-deficiency phenotype observed here is a result of a direct role in this process as opposed to a relative inability of mutant strains to survive tissue culture assay conditions. A role for CapC as an auxiliary adhesin in C. jejuni is logical given that CapC has been confirmed as an outer membrane autotransporter protein that is likely involved in the interaction with the host.
5.3.2. The impact of deletion of capC on invasion is the result of impaired adhesion

Additionally, in both models ΔcapC deletion mutants demonstrated an impaired ability to invade cells as determined by a gentamicin protection assay. As observed with the decrease in the adhesion efficiencies, deletion of capC does not result in total cessation of cellular invasion. This reduction in cellular invasion may be the corollary of a reduction in cell association; fewer Campylobacter cells associating and adhering to Caco-2 and T84 cells initially would result in a fewer number of campylobacters available to invade these cells. To determine if this was the case, the number of invaded bacteria from each strain (i.e. the number surviving gentamicin treatment) was represented as a proportion of the total associated bacteria, and compared. Figure 5.4A and 5.4B below show these proportions in both cell culture models.
Figure 5.4B
Proportion of adhered *C. jejuni* that survive gentamicin treatment in a T84 cell model

![Bar chart showing the proportion of adhered *C. jejuni* that survive gentamicin treatment](image)

- **C. jejuni** 11168 *flhD* Mutant
- Wildtype
- *capC* mutant

Figure 5.4A and 5.4B: The proportion of associated *C. jejuni* that survive gentamicin treatment in a Caco-2 cell model (Figure 5.4A) and a T84 cell culture model (Figure 5.4B). The viable count in CFU/ml of *C. jejuni* surviving gentamicin treatment is taken as the number of invaded bacteria which is used to calculate invasion efficiency. Values shown in Figure 5.4A and 5.4B are calculated as the number of invaded bacteria, divided by the number of associated bacteria and converted to percentages. Δ*capC* mutant strains show a slight increase in the proportion of invaded bacteria as compared to their coupled wildtype strains indicating that CapC likely does not cause a proportional decrease in invasion capacity (Mann-Whitney Test: *C. jejuni* 81116 *p*>0.999; *C. jejuni* M1 *p*>0.999). Error bars show standard error of the mean. Cfu/ml viable counts and 100% efficiency values are shown in Appendix V.

Figure 5.4A and 5.4B serve to show the number of invaded bacteria as a proportion of the number of associated bacteria does not decrease in the mutant strain and may be increased in the T-84 cell model albeit with a high degree of variation. Taken in context of the adhesion and invasion efficiency data, these calculations serve to suggest that deletion of *capC* does not impact directly upon *C. jejuni* 81116 and *C. jejuni* M1 invasion capability. The reduction in invasion efficiency that has been observed is likely to be a consequence of reduced adhesion efficiency. Therefore, results from adhesion and invasion assays in Caco-2 and T84 cell lines indicate that CapC directly impacts upon adhesion of *C. jejuni* 81116 and *C. jejuni* M1 in *vitro*, thereby reducing invasion efficiency indirectly.
5.4. The contribution of CapC to cytotoxicity

The capC ORF is annotated in many C. jejuni publically available draft genome sequences as a functioning cytotoxin, perhaps due to the sequence and motif similarity with the vacA cytotoxin encoded by Helicobacter pylori that capC displays. As discussed previously, the sequence identity is only observed in the C-terminal region of these two factors rather than in the N-terminal, functional domain of the autotransporters. Therefore, these proteins, CapC and VacA do not necessarily share any functional homology.

In order to establish whether CapC functions as a cytotoxin or impacts upon cytotoxic activity of Campylobacter, wildtype and mutant strains were compared for cytotoxic activity against Caco-2 cells using the Pierce LDH Cytotoxicity Assay Kit (ThermoScientific) according to the manufacturer’s instructions. Caco-2 cells have previously been used as a model for Campylobacter cytotoxicity (Coote et al., 2007), however other cells lines have also been used with varying degrees of sensitivity.

A cell mediated cytotoxicity assay was performed using C. jejuni wildtype and ΔcapC mutant preparations suspended in PBS as used for adhesion and invasion assays. 10 μl of this cell suspension was used to infect 1x10⁵ cells per well of a 96 well microtitre plate. Assays establishing the cytotoxicity of C. jejuni supernatant preparations were also performed using concentrated C. jejuni supernatant fractions prepared in two ways. Cytotoxicity is the quality of being toxic to cells in a manner that causes cell damage and may induce cell death. The Pierce Cytotoxicity kit enables an indirect quantification of the levels of lactate dehydrogenase released by cells, an enzyme commonly used as a biomarker for cell cytolysis and damage. Utilising a diaphorase enzyme, the NADH produced by lactate dehydrogenase causes reduction of a tetrazolium dye to produce a red formazan product. The level of formazan formation is directly proportional to the amount of LDH released in to the media which, in turn is indicative of levels of cytotoxicity. In all
assays, appropriate negative, media background, serum-free media, and maximum cytotoxicity controls were used in triplicate. These assays were repeated three times. Cytotoxicity was determined using the method outlined in section 2.14.2.4.

Figure 5.5

Cell Mediated Cytotoxicity Assay in Caco-2 cell model

![Bar chart showing cytotoxicity values for C. jejuni 81116 and C. jejuni M1 with respective ΔcapC mutant strains.]

Figure 5.5: The percentage of complete cytotoxicity elicited by C. jejuni 81116, C. jejuni M1 and their respective ΔcapC mutant strains in a non-polarised Caco-2 cell culture model. Assays were performed using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, UK). C. jejuni 81116 elicited greater cytotoxicity than its ΔcapC deletion mutant counterpart, though this difference was found not to be statistically significant (p=0.09, n=3). There is very little difference in cytotoxicity elicited between C. jejuni M1 and C. jejuni M1 ΔcapC (p=0.389, n=3). These assays were performed in quintuplet on three different occasions; error bars show standard error of the mean.

Figure 5.5 shows the results of the cell mediated cytotoxicity assay in the Caco-2 cell model. C. jejuni 81116 elicited marginally greater cytotoxicity than its ΔcapC deletion mutant counterpart, though this difference was found not to be statistically significant (p=0.09, n=3). As Figure 5.5 shows, the difference in cytotoxicity elicited between C. jejuni M1 and C. jejuni M1 ΔcapC is minimal (p=0.389, n=3). These data suggest that CapC does not mediate cytotoxicity.
The identification of CapC as an outer membrane protein does not preclude the possibility that this protein may be secreted extracellularly in either its native or a processed form or even as a component of an outer membrane vesicle (Elmi et al., 2012). In order to investigate further any potential impact that CapC may have on cytotoxicity, further assays were performed using C. jejuni supernatant preparations as described in section 2.14.2.5. Cell free supernatants were filter sterilised, and concentrated using 20ml Corning Spin X UF centrifuge units with a molecular weight cut-off of 10KDa. 10μl of a 10μg/ml supernatant concentration was used to infect Caco-2 cells.

Figure 5.6A

Supernatant-mediated Cytotoxicity assay in Caco-2 cell model (10KDa MWCO)

Figure 5.6A: The percentage of complete cytotoxicity in a non-polarised Caco-2 cell culture model elicited by concentrated supernatant fractions of C. jejuni 81116, C. jejuni M1 and their respective ΔcapC mutant strains prepared using a 10KDa MWCO. Cytotoxicity elicited by all strain supernatants was very similar (10% +/- 1.4%), indicating that deletion of capC had no effect on cytotoxicity. These assays were performed in quintuplet on three different occasions; error bars show standard error of the mean.
Figure 5.6A shows results of the supernatant-mediated cytotoxicity assay. Cytotoxicity elicited by all strain supernatants was very similar (10% +/- 1.4%), indicating that inactivation of capC had no effect on cytotoxicity. A possible reason for this is that the cytolethal distending toxin (Cdt) encoded by both *C. jejuni* 81116 and M1 and by extension their ΔcapC mutants may be contributing to a high level of background cytotoxicity and thereby confounding results.

The contribution of cell supernatants to cytotoxicity independent of Cdt was determined by preparing supernatants as described previously and concentrating using a 30KDa MWCO. CdtB, the functional subunit of the Cdt toxin has a molecular weight of 28-29KDa. Concentration using the MWCO effectively filtered out the effects of Cdt, allowing cytotoxicity of supernatants to be assayed in a low background environment. Figure 5.6B below shows the resulting cytotoxicity elicited by each strain preparation. The percentage cytotoxicity was considerably lower as a result of the higher molecular weight cut off. There was no significant difference between the levels of cytotoxicity elicited by *C. jejuni* 81116 and *C. jejuni* 81116 ΔcapC (p=0.8, n=3). *C. jejuni* M1 shows increased cytotoxicity (12.5%) as compared to the *C. jejuni* ΔcapC mutant (5.1%) though with a significant greater degree of variation. Statistical comparison using a Mann-Whitney test shows this result is not statistically significant and that the difference observed between these two strains is due to a single outlying result (p>0.9, n=4).

These results indicate that the contribution of CapC to cytotoxicity is minimal. Whilst evidence indicates that CapC is not secreted extracellularly, this possibility remains. Regardless, supernatant-mediated cytotoxicity assays show deletion of CapC does not impact upon cytotoxicity elicited by these preparations. The cell mediated cytotoxicity assay also indicates that as a cell-bound factor, CapC does not contribute to cytotoxicity in the assays employed in this chapter.
5.5. The contribution of CapC to secretion of IL-8 from T84 cells

Interleukin-8 is a pro-inflammatory chemokine that is produced by epithelial cells in response to inflammatory stimuli. IL-8 secretion, as well as IL-6 and TNF-α are part of the well characterised early host response to *C. jejuni* infection in humans and *C. jejuni* has been previously been shown to induce the secretion of the chemokine from polarised T84 cells via activation of the NF-κB pathway (Zheng *et al.*, 2008). The contribution of CapC to part of the early immune response of human cells was investigated in a T84 cell culture model by assessment of the levels of IL-8 produced by these cells after 4 hours infection. Levels of IL-8 were quantified by ELISA using the Human IL-8 ELISA ReadySetGO! 2nd Generation kit according to the manufacturer’s instructions.
Briefly, T84 cells were seeded at 1x10^6 cells/ml in 24-well plates and infected with *C. jejuni* 81116, *C. jejuni* M1 and their respective isogenic ΔcapC mutants at an MOI of 1:100 as described above. After four hours infection, media supernatant samples were obtained and frozen at -20°C for up to three days and thawed on ice when required. ELISAs were performed in 96 well-Nunc Maxisorp Plates as described in section 2.14.4.1. Absorbance values were measured on a Wallac Plate Reader and normalised to uninfected T84 cell supernatant controls. Levels of IL-8 in samples were interpolated from known standards using standard curves. Figure 5.7 shows levels of IL-8 secreted from T84 cells infected with wildtype and ΔcapC mutants.

**Figure 5.7**

Levels of IL-8 Secretion from T84 cells

*Figure 5.7: The decrease in IL-8 (pg/ml) secretion from T84 cells in response to infection with *C. jejuni* wildtype and ΔcapC mutant strains (MOI=1:100). Infection of T84s with CapC-deficient mutant caused a decrease in levels of IL-8 secretion from T84 cells. The greatest decrease was observed in *C. jejuni* M1 ΔcapC (p<0.0001, n=3). Error bars show standard error of the mean. This assay was performed in quadruplicate on three occasions.*
As Figure 5.7 shows, CapC deletion mutants in *C. jejuni* 81116 and *C. jejuni* M1 elicited decreased IL-8 secretion from T84 intestinal epithelial cells as compared to their coupled wildtype strains. T84 cells infected with *C. jejuni* M1 ΔcapC show a greater reduction in IL-8 secretion (p<0.0001) than the *C. jejuni* 81116 ΔcapC mutant relative to their respective coupled wildtype strains. These phenotypes are reflective of adhesion and invasion phenotypes observed in the T84 cell model. Whist the magnitude of the decrease in IL-8 secretion from T84 cells is not consistent between the two ΔcapC mutant strains, the observed decrease indicates that CapC may play a role in elicitation of immune responses from intestinal epithelial cells.
5.6. Discussion

The use of two cell culture models have been described in this chapter. Caco-2 cells are a human epithelial colorectal adenocarcinoma continuous cell line that undergo spontaneous differentiation \textit{in vitro} after reaching confluence and display characteristics of mature enterocytes such as microvilli and a brush border as found in the small intestine (Ferruzza \textit{et al.}, 2012). We also included confluent, non-polarised T84 cell monolayers. The T84 cells used here differ from the Caco-2 model in that these cells do not differentiate in the conditions used here and T84 cells are reported to be more robust in inflammatory responses to infection (Eckman \textit{et al.}, 1993). In addition, \textit{C. jejuni} has been shown to invade T84 cells to a greater extent than Caco-2 cells, and is therefore used here as an alternative model for studying interaction. Deletion of the \textit{capC} gene has been shown to result in decreased adhesion and invasion efficiencies; this decrease was greater in the Caco-2 cell model than the T84 cell model which typifies the variation previously reported in interaction with different cell lines (Konkel \textit{et al.}, 1992), and the efficiency of infection in these two particular cell lines (Poly \textit{et al.}, 2008). It is concluded that CapC primarily contributes to adhesion and that the decrease in invasion efficiency is a result of this impaired adhesion. This decrease was consistent in both Caco-2 cells and T84 cells but was greater in the former; this may reflect the physiological differences in the two cell lines used.

\textbf{CapC contributes to adhesion in a human colorectal cell model}

Deletion of CapC has been shown to significantly decrease adhesion and invasion efficiencies in this study to levels comparable to that of an aflagellate negative control. As described in Chapter 1, the processes of adhesion and invasion do not necessarily occur sequentially. However in these assays, the number of invading bacteria are dependent on the capability of \textit{C. jejuni} to interact with intestinal cells. The significant reduction in invasion efficiency observed in \textit{ΔcapC} deletion
strains is thought to be the result of the decrease in adhesion efficiency of the strains. This is evidenced by the fact that representation of invaded bacteria as a proportion of associated bacteria indicates that ΔcapC mutants actually exhibited slightly increased intracellular survival in both cell lines proportional to their wildtype counterparts. This proportion is calculated from viable counts obtained from what are in effect two separate assays and relies on the assumption that the number of associated bacteria calculated in the adhesion assay is identical to associated bacteria in the gentamicin protection assay. This drawback to this calculation is mitigated to an extent by the fact that the inoculum between each assay is kept identical. Furthermore, the method of combining viable counts from different assays in calculation has been used previously to calculate adhesion efficiency. With consideration to this result, it is thought that CapC contributes primarily to the process of adhesion and the impact of capC deletion on invasion efficiency is the consequence of reduced adhesion capability.

Many proteins encoded by Campylobacter have been reported to function as adhesins either directly or by contributing to adhesive activity indirectly. The identification of CapC as a potential auxiliary adhesin complicates an area of C. jejuni pathogenesis that is already poorly understood. This lack of comprehension is due in part to the perhaps erroneous reports of certain factors functioning as adhesins whose primary function has since been reported to not be pathogenesis. Considering this, it would be premature to conclude that CapC functions as an adhesin though this is an attractive prospect. Future work should analyse the interaction of CapC with the host in greater depth through the use of polarized intestinal cells with a defined apical and basolateral surface and aim to ascertain whether CapC is directly involved in cell attachment.

Complemented strains of the capC mutants were unable to be generated so reversion of these phenotype was unable to be observed. Considering the decrease in adhesion and invasion efficiency is consistent between both C. jejuni mutant strains, it is likely this phenotype is the
result of inactivation of capC. This phenotype is unlikely to be the result of a polar mutation, especially considering the capC flanking gene encode a polyphosphate kinase (ppk) and a DNA helicase (ruvB); these factors are not surface exposed and second-site mutations in these genes are unlikely to impact upon host cell interaction.

**C. jejuni** wildtype strains show greater capability to elicit secretion of IL-8 from T84 cells

The secretion levels of the pro-inflammatory chemokine, interleukin 8, from T84 cells infected with either wildtype or mutant strains was investigated. It has been demonstrated that inactivation of capC causes a decrease in the levels of IL-8 secreted from these cells. Previous work performed by Zheng et al. (2008), showed that FlaA and Cdt toxin are major immunogens of *C. jejuni* and elicit the secretion of IL-8 via TLRs and activation of the NF-kB pathway. All strains used in this study possess intact Fla flagella subunits and are positive for Cdt, factors which can confound results regarding immunogenesis. Ultimately it is unclear as to whether deletion of capC causes a decrease in IL-8 secretion directly by an inability of these strains to activate a particular pathway leading to secretion of this chemokine, or indirectly by limiting interaction with epithelial cells and lessening the effects of other immunogens. Future work should seek to clarify this and take into account the role of other cytokines involved in the early response to *C. jejuni* infection. Given that the decrease in IL-8 secretion reflects the decrease in adhesion and invasion efficiencies, it is likely that differences between wildtype and mutant ability to elicit IL-8 secretion is due to limited interaction resulting from CapC deficiency.
**CapC does not contribute to cytotoxicity**

Results from cytotoxicity assays indicate that the contribution of CapC to cytotoxicity is minimal. CapC has been shown to be a membrane anchored protein. Cell mediated cytotoxicity assays show that deletion of CapC has no effect on cytotoxicity in a Caco-2 cell model. Similarly, supernatant-mediated cytotoxicity showed deletion of CapC has no effect on cytotoxicity, though a high degree of variation was observed in these assays. This may be a reflection of the sensitivity of the cell model or of a lack of uniformity in the supernatant preparations. Possible future work should confirm that CapC is not involved in cytotoxicity by examination of the contribution in Cdt-negative strains to provide a low background of cytotoxicity to more accurately determine the contribution of CapC to host cell damage.
Chapter 6: Genomic associations of autotransporters in *C. jejuni* and *C. coli*
6.1. Introduction

The initial identification of the capC genomic island by Park and Richardson (1998), demonstrated that the capC sequence was not conserved amongst C. jejuni and C. coli strains. Using a combination of PCR and Southern blotting, Park et al. demonstrated the presence of the C-terminal region of the capC sequence in six of ten C. jejuni strains (Unpublished data). In addition, analysis of the genome sequence of C. jejuni NCTC 11168, the only published sequence available at the time, revealed this strain did not contain capC. The present study has extended these preliminary findings and established the prevalence of capC by PCR in a range of clinical and supermarket poultry isolates as 60.2% indicating a variable distribution of this factor. Moreover, in Chapter 4 it has been shown that CapC functions as a virulence factor in C. jejuni 81116 and C. jejuni M1. In 2007, Ashgar et al. reported the identification of CapA and CapB; two phase-variable autotransporter proteins encoded by C. jejuni NCTC 11168 (Ashgar et al., 2007). Deletion of CapB was reported to have no phenotypic effect and no function has been ascribed, however CapA has been reported to function as an adhesin with a potential role in chicken colonisation (Flanagan et al., 2009; Ashgar et al., 2007). As with CapC, CapA and CapB are also strain-specific autotransporters and are not encoded by the reference strains utilised for experimental analyses in this study.

In light of the reported functions of CapA in both adhesion to human intestinal cells and chicken colonisation, as well as the role of CapC in virulence as determined in Chapter 4, the importance of these factors is evident. With consideration to the relative absence of classical virulence factors within Campylobacter genomes (Dasti et al., 2010), autotransporter protein contributions to virulence and infections may address the lack of understanding of these processes. Furthermore, the fact that these autotransporter proteins show strain-specific distribution may account for Campylobacter phenotypic variation; given the reported roles of these autotransporters,
presence or absence of these factors may have a profound impact upon Campylobacter infection and colonisation processes.

The identification and characterisation of these autotransporters in C. jejuni and C. coli, raises interesting questions regarding Campylobacter sequence diversity and the potential genetic associations of these genes.

Therefore the aims of this chapter were as follows:

1. To determine the proportion of C. jejuni and C. coli strains that encode capA, capB and capC genes amongst a wider and more representative population of campylobacters.

2. To establish whether possession of autotransporter genes is associated with other genetic features, MLST sequence type or ecological niche.

3. To elucidate the extent of sequence variation within the N-terminal domain of CapC.
6.2. Selection and Whole genome Sequencing of C. jejuni isolates

Thirty clinical isolates of C. jejuni that had been previously phenotypically characterised (Appendix VI) were selected and designated C. jejuni HI1-30). Isolates were typed using the MLST typing scheme as described in section 2.10 (Dingle et al., 2001). Briefly, approximately 800 to 1200bp fragments of seven, conserved “housekeeping” genes were amplified by PCR using and purified. Using sequencing primers whose annealing site lay nested within amplification fragments, 400-500bp portions of the amplified housekeeping gene fragments were sequenced via Sanger sequencing (Genewiz [formerly Beckman Genomics], Takeley, UK). Resulting sequences were used to obtain allelic profile assignments and presumptive isolate sequence types (ST) were defined by querying allele sequences individually using the Sequence Type finder tool at https://pubmlst.org/campylobacter/. With consideration to relatedness of isolates, 10 C. jejuni isolates from this collection found to encode CapC, as determined by PCR, and 10 isolates that did not encode CapC were selected for whole genome sequencing; a list of these selected isolates can be found in Table 6.1. DNA from selected isolates was extracted as outlined in section 2.8.2.1.2. and sequenced using an Illumina MiSeq platform and the standard Illumina indexing protocol (service provided by Bioinformatics Unit, APHA, UK). Raw sequence reads were processed and assembled into contiguous sequences using Velvet as part of the service provided by APHA. The quality of C. jejuni genomic assemblies was evaluated using Quast (Gurevich et al., 2013). Analysis of the number of contigs arising from each isolate indicated that three assemblies (C. jejuni HI2, HI16 and HI18) were likely derived from mixed samples and have thus been excluded from further analyses.
Table 6.1: Table showing the 17 *C. jejuni* strains that were successfully sequenced. 11 strains that were determined to encode a *capC* variant by BLASTn search are indicated as are strains that do not encode *capC* or its sequence variant. MLST sequence type (ST) and clonal complex for each strain is also presented. Notably, only strains that belong to ST-21 clonal complex do not encode *capC* or its sequence variant.

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>capC</em> sequence variant</th>
<th>ST</th>
<th>Clonal Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em> HI1</td>
<td>+</td>
<td>52</td>
<td>ST-52 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI3</td>
<td>-</td>
<td>21</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI4</td>
<td>+</td>
<td>48</td>
<td>ST-48 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI5</td>
<td>+</td>
<td>5136</td>
<td>ST-464 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI8</td>
<td>+</td>
<td>104</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI9</td>
<td>+</td>
<td>305</td>
<td>ST-574 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI10</td>
<td>+</td>
<td>48</td>
<td>ST-48 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI12</td>
<td>+</td>
<td>2103</td>
<td>ST-607 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI13</td>
<td>+</td>
<td>5136</td>
<td>ST-464 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI17</td>
<td>+</td>
<td>1911</td>
<td></td>
</tr>
<tr>
<td><em>C. jejuni</em> HI19</td>
<td>-</td>
<td>50</td>
<td>ST-21 complex</td>
</tr>
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<td><em>C. jejuni</em> HI20</td>
<td>+</td>
<td>574</td>
<td>ST-574 complex</td>
</tr>
<tr>
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<td>122</td>
<td>ST-206 complex</td>
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<tr>
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<td>-</td>
<td>50</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI25</td>
<td>-</td>
<td>21</td>
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<tr>
<td><em>C. jejuni</em> HI28</td>
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<td>19</td>
<td>ST-21 complex</td>
</tr>
<tr>
<td><em>C. jejuni</em> HI30</td>
<td>-</td>
<td>50</td>
<td>ST-21 complex</td>
</tr>
</tbody>
</table>

6.3. Identification of CapD; a sequence variant of the CapC autotransporter

Alignment of the *capC* coding sequence from *C. jejuni* 81116 (annotated as C8J_1278 in the *C. jejuni* 81116 genome sequence [Pearson et al., 2007]) with the remaining *C. jejuni* 17 genomes using BLASTn V 2.28 revealed that only 6 genomes did not encode an autotransporter as opposed to the 10 genomes determined to be positive for *capC* by PCR. This calls into question the suitability and efficacy of the PCR assay to detect strains encoding this gene as well as the reported prevalence of *capC* reported in Chapter 3.
Furthermore, within the 11 genomes found to be positive by PCR, the coding sequence of the detected autotransporter does not show 100% identity with the *capC* coding sequence from *C. jejuni* 81116. The autotransporter gene detected by alignment of the *capC* nucleotide sequence with these genomes shows the presence of a gene with significant divergence. The protein sequences of CapC and the predicted protein sequence of the variant sequence encoded by the 11 genomes previously thought to encode CapC (Table 6.1), were aligned using the Cobalt protein alignment tool (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi).

Results from this alignment are shown in Figure 6.1A and a schematic of the alignment is shown in Figure 6.1B. The two sequences show 71% identity with each other; identity is indicated by the red colouring of residues in Figure 6.1A whereas blue coloured residues indicate sequence divergence. This alignment shows the two autotransporter proteins share significant identity in their C-terminal domains and also within the first 25 residues of the N-terminal. These two domains represent the β-barrel structure that is characteristic of autotransporters and the signal peptide, respectively. Identification of these domains is described in Chapter 3. CapC and the novel sequence variant show considerable sequence variation in the N-terminal domain. Given that the N-terminal or “passenger” domain represents the secreted, membrane exposed component of autotransporter proteins that exerts effector functions (Leo *et al.*, 2012), the fact that this sequence variant possesses a distinct N-terminal sequence from CapC suggests that the alternative allele of CapC may exert a different effector function. With consideration to this significant sequence variation, the novel sequence detected in in 11 clinical isolates listed in Table 6.1 has been tentatively designated as *capD*. 
Figure 6.1A and B: Alignment of the CapC amino acid sequence with that of the variant encoded by the clinical isolates sequenced in this study. Alignment was performed using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) and coloured manually (Edgar et al., 2004). Sequence identity between protein sequences is indicated by red colouring of residues and sequence divergence and insertions by blue colouring. CapC and CapD display 71% amino acid sequence identity. (*) indicates positions which have a single, fully conserved residue; (: ) indicates conservation between groups of weakly similar properties.
Figure 6.1B: Linear illustration of the regions of homology between CapC and CapD. Dark shading indicates the signal peptide portion of the protein, light grey shading indicates the C-terminal β-barrel and the white regions of each protein represent the N-terminal domain. Vertical stripes indicate regions of homology and numbers indicate the amino acid residues.
6.3.1. The N-terminal domain of CapD likely confers different phenotypic function

Figure 6.2A and B illustrate the predicted core, structural homology of CapC and CapD in the form of the C-terminal β-barrel, and Figure 6.3 illustrates the significance of sequence divergence within the N-terminal domain.

A. CapC partial 3D structure

B. CapD partial 3D structure

Figure 6.2A and B: Figure 6.2A and B show the partial modelling of the CapC and CapD proteins respectively and were generated using the Phyre2 3D structure modelling engine. The yellow denotes the β-barrel structure of both of these autotransporters and magenta colouring shows partial modelling of the N-terminal domain. These proteins’ structures have only been partially predicted with respect to their homology to sequences with known 3D structures. The prediction of the CapC and CapD β-barrel structure has been modelled on that of the EstA autotransporter encoded by Pseudomonas aeruginosa. The partial models of CapC and CapD show significant structural similarity between the two proteins in terms of the β-barrel, which reflects the homology illustrated in Figure 6.1A and B. Divergent N-terminal sequences have not been successfully modelled.
**Figure 6.3:** Illustration of membrane topology of EstA autotransporter. The predicted structure of the CapC and CapD C-terminal is based upon structural homology to the estA autotransporter. Membrane topology of EstA was illustrated using Protter V1.0 (http://wlab.ethz.ch/protter/start/). The C-terminal domain of EstA is clearly shown as membrane embedded whilst the N-terminal domain is extracellularly exposed. Signal peptide residues of EstA are shown in red.

Using the Phyre2 3D structure modelling engine, the 3D structure of CapD was partially predicted. Comparison of this structure with the predicted partial structure of CapC demonstrates that these autotransporters have highly similar β-barrel domains. CapC has already been shown to localise to the outer membrane of *C. jejuni* 81116 and *C. jejuni* M1. Considering the predicted structural homology, it can be assumed that CapD would also localise to the outer membrane. Figure 6.3 depicts the membrane topology of the EstA esterase autotransporter of *Pseudomonas aeruginosa*, a protein whose 3D structure is known and for whom both *in silico* and experimental evidence is readily available. The predicted structure of the CapC and CapD C-terminal, is based upon structural homology to the EstA autotransporter. Membrane topology of EstA was illustrated using Protter V1.0 (http://wlab.ethz.ch/protter/start/). The C-terminal domain of EstA is clearly shown as membrane embedded whilst the N-terminal domain is extracellularly exposed. Whilst CapC and CapD have no homology to the N-terminus of EstA, it is extremely likely that, as autotransporters the N-terminal of these proteins would be surface exposed. The sequence variation in CapD may well confer an alternative phenotypic function that that observed in CapC.
6.3.2. CapC and CapD occur at the same genomic locus

In all genomes in which CapC and CapD have hitherto been detected, these genes are not present in tandem suggesting the possibility that CapD represents a variant allele, i.e. strains either encode either CapC or CapD, and not both. Moreover, these genes are located in the same genomic locus in all strains in which they are encoded. Figure 6.4 depicts a schematic representation of the visual alignment of the capC encoding region of the C. jejuni HI8 draft genome assembly with genomes of commonly used laboratory strains (C. jejuni NCTC 11168, 81-176, 81116 and M1) and shows that capD is located at the same genomic locus as capC.

![Figure 6.4A](image)

**Figure 6.4A**: A linear representation of *C. jejuni* genomes showing the CapC and CapD insertion locus. CapC or CapD are located at the same genomic position in strains that encode these genes; downstream of the *ppk* gene in the reverse orientation. The equivalent site in CapC and CapD negative strains encodes a tmRNA as shown in the figure in the *C. jejuni* NCTC 11168 and 81-176 genomes (white blocks). The CapC coding sequence is shown highlighted in yellow and the CapD coding sequence locus is shown highlighted in blue. The CapD coding sequence in *C. jejuni* HI8 was found to have two mutations resulting in an inactivated gene indicated by dotted red outline, presumably resulting in a truncated protein. In all genomes examined thus far, genomes encode either CapC or CapD at the same locus.
Both \textit{capC} and \textit{capD} are located downstream of the \textit{ppk} gene (encoding a polyphosphate kinase) and are transcribed in opposite orientation to this gene. CapC and CapD autotransporters seem to be encoded in between the \textit{ppk} gene and the tmRNA encoding region. The regions upstream and downstream of the CapC/D encoding locus are largely conserved between strains with the exception of the presence of \textit{cj1365c}; a gene downstream of \textit{fumC} in CapC/D negative strains. The fact that CapD is genetically distinct from CapC and that these genes do not occur simultaneously is highly suggestive that CapD is an alternative allele to CapC that may be phenotypically distinct.
Figure 6.4B: Alignment of the capC (C8J_1278) amino acid sequence in C. jejuni 81116 with the truncated capD sequence in C. jejuni HI8. C. jejuni HI8 is a human clinical isolate sequenced as part of the present study. The alignment depicts the position of truncations in the C. jejuni HI8 capD amino acid sequence, shown in red. These truncations are the result of two premature stop codons resulting in a frameshift mutation and an interrupting mutation respectively. Alignment was performed using the BLAST algorithm.
6.4. Prevalence and distribution of autotransporters in *Campylobacter jejuni* and *C. coli*

The identification of CapD, an additional strain-specific autotransporter in *Campylobacter jejuni* highlights the need for understanding of the genotypic associations of these factors, particularly in light of the proposed role that autotransporters play in the pathophysiology of many Gram-negative pathogens (Henderson *et al.*, 2004). In addition, alignment of the predicted protein sequences of CapC and CapD (Figure 6.1A) encoded by all of the 11 clinical isolates shown in Table 6.1 revealed that all 11 genomes possessed mutations in capD that resulted in a frameshift mutation and an interrupting mutation arising from premature stop codons. Truncation of capD is depicted in Figure 6.4 in *C. jejuni* by a dotted outline. The identification of inactivated, truncated CapD sequences amongst all sequenced isolates raises the question as to whether there are any sequences encoding an active form of CapD. Additionally do truncated forms of other *Campylobacter* autotransporter proteins exist and, if so, what is their distribution?

**DNA sequence based analysis**

The distribution of capA and capB, and independently, capC and capD was determined amongst a collection of 7176 complete and draft *Campylobacter* genome sequences (5829 *C. jejuni*, 1347 *C. coli*). These genomes are listed in Appendix VI including where available, PubMLST ID, genbank accession number, isolate source, MLST sequence type and clonal complex. Genomes were all obtained from public collections (Cody *et al.*, 2013; Jolley and Maiden, 2010) and previous iterations of this collection were utilised for the identification of DNase genes (Brown *et al.*, 2015), CRISPR repeats and cas genes (Pearson *et al.*, 2015), and the fucose utilisation cluster (Dwivedi *et al.*, 2016). Determination of the distribution of the autotransporter genes within these genomes was initially performed with no distinction between the capA and B autotransporter genes and also with no distinction between the distribution of capC and D autotransporter coding
sequences. Each gene within these two pairs share significant sequence identity and therefore were treated as two distinct groups of genes in this preliminary analysis.

Briefly, the nucleotide sequences of capA, capB, capC and capD were segregated into “words” of 120 nucleotides in length and used to screen the presence of autotransporter genes amongst the collection of C. jejuni and C. coli genomes using BLASTn V 2.28 via MIST (Microbial In Silico Typer) (Kruczkiewicz et al., 2013). Autotransporter genes were classed as present if 80% identity or greater was observed across the whole gene. MLST sequence types and resulting clonal complex classification of all genomes within the collection were also obtained from PubMLST via the MIST shell. Figure 6.5A and B illustrate the prevalence of capA/B and capC/D in C. jejuni and C. coli.

Protein sequence based analysis

More sensitive analysis was performed to determine the prevalence and distribution of full length and truncated CapA/B, CapC and CapD autotransporters amongst the collection of 7176 Campylobacter jejuni and C. coli genomes. Given that CapA and CapB are very difficult to distinguish in terms of sequence and the lack of a phenotypic function for CapB, these autotransporters were once again grouped together in analysis output.

Briefly, all genomes in the collection were annotated using Prokka (Seemann et al., 2014). Annotated genomes were screened at the protein level using the amino acid sequences of full length CapA (cj0628/9), CapB (cj1677/78), CapC (C8J_1278) and CapD (compiled by amalgamation of truncated sequence from C. jejuni/HI1) via the BLASTp function in BioEdit 7.25 (E value=1E-20). Prevalence of full length protein was determined by full coverage over the whole sequence when compared to query sequences where as partial identity was classed as truncation regardless of the degree of overall sequence identity. The prevalence of truncated and full length autotransporters in C. jejuni and C. coli was determined and presented in Figure 6.6A and B. Core-
genome single nucleotide polymorphisms were identified in all genomes in the collection using ParSNP (Treangen et al., 2014) and used to generate a phylogenetic tree showing clustering of MLST clonal complexes and phylogenetic clades in each *C. jejuni* and *C. coli* respectively. Colour-coding of truncated and full length encoding isolates using Figtree (http://tree.bio.ed.ac.uk/software/figtree/) allowed associations of autotransporters with genotypes to be visualised. Figure 6.7A and B show the associations of full length and truncated *Campylobacter* autotransporter proteins with particular genotypes of *C. jejuni* and *C. coli*. Information regarding the ecological source of isolates is included in these figures.
6.4.1. Prevalence of capA/B and capC/D genes in *C. jejuni* and *C. coli*

Figure 6.5A and B demonstrate, autotransporter genes encoding CapA/B and CapC/D are highly prevalent in both *C. jejuni* and *C. coli*. In both species, a relative minority of genomes within this collection were found not to encode these genes, using the parameters outlined above.

![Venn diagram illustrating the proportion of *C. jejuni* genomes in the public collection used in this study that possess nucleotide sequences of autotransporters. For this preliminary analysis, capA/B autotransporters and capC/D autotransporters are treated as two groups due to sequence similarity. Autotransporter genes were classed as present if 90% identity or greater was observed across the whole gene. Figure 6.5A shows autotransporters are highly prevalent with 25.7% of strains encoding capA/B, 37.6% encoding capC/D and 30.3% of genomes encoding both capA/B and capC/D. Darker shading indicates greater proportions. Figure was generated using Venny 2.1 (http://bioinfolegp.cnb.csic.es/tools/venny/).](image-url)
Figure 6.5B: Venn diagram showing the prevalence of capA/B and capC/D nucleotide sequences in *C. coli*. For this analysis, capA/B autotransporters and capC/D autotransporters are treated as two groups due to sequence similarity. Autotransporter genes were classed as present if 90% identity or greater was observed across the whole gene. Figure 6.5B illustrates that 14.9% of *C. coli* strains do not possess these genes, 0.8% possess solely capA/B and an overwhelming 84% possess capC/D genes. Darker shading indicates greater proportions. Figure was generated using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/).

In *C. jejuni* only 6.5% of all genomes were determined to not encode any of the autotransporter genes that are the focus of this study. CapC and D autotransporter genes were determined to be the most prevalent autotransporter genes with 37.6% of genomes encoding a version of these sequences and a further 30.3% of *C. jejuni* genomes possess capC/D in addition to capA/B. In comparison, only a quarter of *C. jejuni* genomes in this collection possess only capA/B.

In *C. coli* on the other hand, 14.9% of genomes were ascertained not to possess any of the autotransporter genes that were screened for, however an overwhelming majority (84%) possess capC/D only with a small minority found to possess capA/B sequences. With consideration to the
associated virulence phenotype of CapC established in Chapter 3, the prevalence of capC/D in C. jejuni and C. coli is suggestive of a relative functional importance.

Most notably, this preliminary analysis of the distribution and association of capA/ and capC/D has suggested that possession of Campylobacter autotransporter proteins is correlated with MLST clonal complex and phylogenetic clade.

In C. coli, capA/B genes are highly underrepresented and only possessed by a total of 15 genomes in the collection. Significantly possession of autotransporters is confined to the introgressed, phylogenetic clades 1a and 1b. There are loose correlations of these genes with isolate sources; for example, in the C. jejuni collection, capA/B and capC/D genes are highly prevalent in environmental isolates and capC/D positive strains are associated with agricultural isolates. Contrastingly however, in the C. coli collection, environmental isolates are largely negative for these genes.

These preliminary analyses demonstrate the widespread presence of these autotransporter genes in C. jejuni and C. coli which is suggestive of a relative importance to the genetic lineages with which they are associated. More stringent analyses with regards to the association of individual autotransporter genes with Campylobacter genotypes was performed to glean further insight as to the contribution of these autotransporters to Campylobacter biology.

6.4.2. C. jejuni and C. coli encode full-length and truncated autotransporter genes

The number of C. jejuni and C. coli genomes encoding full length and truncated autotransporter proteins is shown in Figure 6.6A and B respectively. These figures exclude all genomes that do not encode autotransporters.
Figure 6.6A: Figure 6.6A is a Venn diagram depicting the number of \textit{C. jejuni} genomes in the present collection that encode full length and truncated forms of CapA/B, CapC and CapD. Genomes that do not encode these autotransporters are not included in the figure. Genomes were annotated using Prokka and screened using BLASTp via BioEdit V 7.25 for autotransporter sequences. Numbers of truncated autotransporters outweigh the numbers of full length proteins in \textit{C. jejuni}. Figure was generated using Venny 2.1 ([http://bioinfogp.cnb.csic.es/tools/venny/](http://bioinfogp.cnb.csic.es/tools/venny/)).

As Figure 6.6A shows, within the \textit{C. jejuni} collection, truncated forms of all autotransporter proteins are highly pervasive. Truncation in CapA/B are by far much more common that their full length counterparts; of the 2752 genomes encoding CapA/B, only 53 encode full length proteins. Likewise of the 1779 genomes encoding CapD, only a minority (2%) encode the full length form of this protein. Similarly, truncated forms of CapC are common in \textit{C. jejuni} with 335 genomes of the 617 encoding \textit{capC} sequences indicating possession of a truncated protein.

In \textit{C. coli} however (Figure 6.6B), the full length form of autotransporters is most prevalent, with the exception of CapA/B which are only encoded by a minority of strains in a truncated form. Of the 1132 genomes in which CapC or CapD is present a mere 56 (4.9%) genomes possess truncated proteins. The fact that truncated, inactive autotransporters are most common in \textit{C. jejuni} and
that full length versions are highly restricted suggests that these proteins may have a limited role in the pathophysiology of *C. jejuni*. Although autotransporter sequences have been determined to be common in *C. jejuni* (Figure 6.5A and B), truncated autotransporters by their nature are unlikely to be functional; therefore identification of widespread truncated autotransporter forms may indicate a somewhat more diminished role in *Campylobacter* biology than previously suggested. Contrastingly, the fact that full length CapC and CapD are so common in *C. coli*, suggests that these autotransporters may play a more significant role in the biology of this species.

**Figure 6.6B:** Figure 6.6B is a Venn diagram depicting the number of *C. coli* genomes in the present collection that encode full length and truncated forms of CapA/B, CapC and CapD. Genomes that do not encode these autotransporters are not included in the figure. Genomes were annotated using Prokka and screened using BLASTp via BioEdit V 7.25 for autotransporter sequences. *C. coli* shows extremely low prevalence of CapA/B and highest levels of CapD. Remarkably, there is a comparatively low amount of truncated forms of these proteins. Figure was generated using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/).
6.5. Possession of *Campylobacter* autotransporter protein is associated with MLST clonal complex

Figure 6.7A and B serve to demonstrate the association of individual *Campylobacter* autotransporter proteins with *C. jejuni* (Figure 6.7A) and *C. coli* (Figure 6.7B) genetic backgrounds and the lack of an association with an isolate source. Preliminary analysis that established the prevalence of groups of autotransporter genes using nucleotide BLAST searches was indicative of an association between autotransporters and MLST clonal complex. Furthermore, there was a loose association between possession of certain autotransporters and isolate source. In both *C. jejuni* and *C. coli*, as Figure 6.7A and B show, there are clear genotypic associations of CapA/B, CapC and CapD. Figure 6.7A shows that full length forms of CapA/B are largely confined to ST-574 clonal complex with instances of this protein also present in ST-353 and ST-21 clonal complexes. The predominant association of CapA/B is with the generalist ST-21 and ST-48 lineages, though the present analysis reveals that these genotypes exhibit truncated CapA/B autotransporters.

Preliminary analysis indicated that certain genotypes do not encode autotransporters. Figure 6.7A shows a distinct lack of any of the autotransporter proteins screened for in this study in genomes belong to the ST-61 clonal complex. This evident disassociation with the ST-61 clonal complex with autotransporters may be a consequence of a barrier to recombination that prevents transfer of autotransporter genes. The absence of autotransporters in ST-61, considering that closely phylogenetically-related genomes encode CapA/B and CapD, is strong evidence that possession of particular autotransporters is correlated with the genetic background of *Campylobacter* species.
Figure 6.7A: Prevalence and genotypic associations of autotransporter proteins in *C. jejuni*. 5829 genomes were phylogenetically clustered using on the basis of core genome SNPs. This clustering was depicted in a phylogenetic tree constructed using Figtree. The 1st row beneath the resulting tree labelled isolation source indicates the source of isolation for each genome within the collection via colour coding with labels directly beneath this row. Rows 2, 3 and 4 labelled CapA/B, CapC and CapD respectively indicate the corresponding genomes possessing either full length (blue colouring) or truncated (red colouring) autotransporter proteins. Where no colouring is present in these rows indicates the absence of a particular autotransporter gene. The final row shows the associated MLST clonal complex of the corresponding *C. jejuni* genomes with each coloured clonal complex linked with the designation below this row.
Figure 6.7B: Prevalence and genotypic associations of autotransporter proteins in *C. coli*. 1347 genomes were phylogenetically clustered using on the basis of core genome SNPs. This clustering was depicted in a phylogenetic tree constructed using Figtree. The 1st row beneath the resulting tree labelled isolation source indicates the source of isolation for each genome within the collection via colour coding with labels directly beneath this row. Rows 2, 3 and 4 labelled CapA/B, CapC and CapD respectively indicate the corresponding genomes possessing either full length (blue colouring) or truncated (red colouring) autotransporter proteins. Where no colouring is present in these rows indicates the absence of a particular autotransporter gene. The final row shows the associated MLST clonal complex of the corresponding *C. coli* genomes with each coloured clonal complex linked with the designation below this row and is linked with the 1st row above the phylogenetic tree indicating the phylogenetic clades and sub-clade in which *C. coli* is phylogenetically classed.
CapC and CapD also exhibit clear associations with specific MLST sequence types; CapD is shown to be the most prevalent autotransporter however it is not present in Clonal complex ST-21, ST-42 and ST-61 and ST-22. As observed with CapA/B, there are obvious lineages with which the full length CapC and CapD are associated, and others that predominantly possess the truncated format. Clonal complexes ST-45, ST-283 and ST-573 are those that are associated with full length CapC and CapD. Notably, a clearly demarcated sub-population of ST-45 genomes are positive for full length CapD, whilst all other ST-45 other genomes encode full length CapC. This clear-cut association within a single clonal complex reinforces the notion that possession of individual autotransporters is associated, and perhaps dependent on, MLST clonal complex.

Defined associations of CapC and CapD within particular genetic background are also observed in C. coli. Screening at the DNA and protein level revealed that CapD is the most prevalent autotransporter in C. coli and that it occurs in a predominantly full length format (Figure 6.7B). C. coli is phylogenetically clustered into distinct clades; those strains belonging to Clade 1 are largely comprised of agricultural isolates and exhibit high levels of introgression with C. jejuni sequences. Possession of CapC and CapD is confined exclusively to the introgressed phylogenetic clade 1a and 1b, suggesting that these autotransporters may have spread between these two species via recombination resulting in lineages that have proliferated in other niches. Moreover, the fact that CapC and CapD occur in Clade 1a (ST-828) and Clade 1b (ST-1150) in their full length form may indicate that the autotransporters are conserved in these lineages and therefore may play an important functional role in the biology of these lineages.
6.6. Discussion

This chapter has described the distribution of *Campylobacter* autotransporters encoded amongst a representative range of *C. jejuni* and *C. coli* genomes and identified the genotypes that these autotransporters are associated with. In addition, the identification of a novel variant autotransporter gene, *capD*, is presented and the prevalence and genotypic associations of this autotransporter is determined.

Autotransporter proteins comprise many important bacterial virulence factors in Gram-negative pathogens. Hitherto, CapA represents the only characterised autotransporter encoded by *Campylobacter jejuni*, and this factor is widely regarded as an important mediator of the process of adhesion to epithelial cells with a potential role in avian colonisation (Gripp *et al.*, 2011; Flanagan *et al.*, 2009; Ashgar *et al.*, 2007).

Perusal of the genomes of the commonly utilised laboratory *Campylobacter* strains *C. jejuni* 81116 and M1 show that neither CapA or CapB are encoded by these strains which are infective for both human and chicken hosts, respectively (Friis *et al.*, 2010; Pearson *et al.*, 2007), thereby demonstrating that CapA is not an integral factor to pathogenesis. With consideration to the phenotype of *capC* deletion mutants established in Chapter 4, the present study sought to elucidate the relative importance of *Campylobacter* autotransporters by the use of comparative genomics to examine the distribution of these factors.

Sequencing of clinical isolates of *Campylobacter jejuni* revealed that presence of an autotransporter gene with significant sequence divergence from the *capC* coding sequence, primarily in the region encoding the N-terminal domain, leading to the designation of this novel autotransporter as CapD. Overall, sequence identity of CapC and CapD is quite high and given that CapC and CapD only occur in the same genomic location independently, it could be argued
that differential designation of a sequence variant may only serve to complicate *Campylobacter* gene nomenclature, and therefore the sequence variant should just be considered a *capC* variant. However, the variation of the N-terminal domain is significant. The circumstance is reminiscent of that observed in *Campylobacter* methyl-accepting chemotaxis proteins (MCPS); these often exhibit high sequence identity and functional homology within the C-terminus of the mature protein yet differ in the N-terminal domain which is specific for a particular ligand. In a corresponding manner, the variation in the N-terminus of CapD likely confers a differing function to that observed in CapC as depicted in Figure 6.2A, 6.2B and Figure 6.3.

This study has effectively established the prevalence of *Campylobacter* autotransporters CapA/B, CapC and CapD in *Campylobacter* species and identified CapD and to a lesser extent CapC as the most prevalent of these proteins amongst the collection used in this study. The collection of publically available *Campylobacter* genomes used in this study, whilst diverse and encompassing a wide array of isolates from a range of lineages, is heavily comprised of human clinical isolates, as these are naturally more readily available than isolates from other sources, for example than those from environmental or riparian sources. It is known that certain genotypes are associated with particular hosts (Dearlove *et al.*, 2016). An exception to this is the existence of so-called “generalist” lineages (ST-21 clonal complex, ST-45 clonal complex and ST-828 in *C. coli*) which exhibit flexibility in host range (Gripp *et al.*, 2011). Full length CapC and CapD are highly prevalent in ST-45 in *C. jejuni* and ST-828 in *C. coli* but are totally absent in ST-21 with is CapA/B positive. The ST-21 clonal complex is most frequently isolated from human infections and therefore makes up about 20% of the present collection of genomes. The frequency with which these lineages are isolated can skew interpretations regarding the prevalence of the autotransporters that are the focus of this study. Similarly, isolates from the introgressed clade 1a in *C. coli* are more readily
available that those from environmental sources, thus skewing the prevalence of CapC and CapD in this species. Therefore, the prevalence of autotransporters presented are not necessarily representative of the wider *C. jejuni* and *C. coli* as a whole, yet their importance to the most common and important reservoirs is evident.

A significant finding of this study is the high levels of truncated forms of *Campylobacter* autotransporters in *C. jejuni*. Initial analysis of the 11 CapD positive genomes shown in Table 6.1 revealed the presence of two premature stop codons in CapD resulting in the coding of separate peptides thus causing inactivation of CapD. No transcriptional studies have been performed on either CapC or CapD so the possibility that these mutations result in an active tripartite system cannot be precluded. However due to autotransporter structure to function dynamic, this is extremely unlikely. Further studies should focus on the nature of these truncations and determine whether these are the result of phase switching of genes that were previously expressed or whether these truncated forms represent pseudogenes. The clear associations of full length and truncated formats, and the ubiquity of full length autotransporters in *C. coli* suggests the latter scenario is the more likely. This in itself is of interest as it may suggest redundancy of autotransporters, particularly of CapA which is reported to function as an adhesin, which is widely reported in *C. jejuni*.

Analysis of the distribution of full length autotransporter proteins confirmed preliminary BLAST screens that autotransporters are associated with specific clonal complexes. CapA/B autotransporters are predominantly associated with ST-21, ST-48, ST-206, and ST-353 however the active form is only associated with ST-574, a lineage that has previously been reported to be mostly restricted to UK clinical isolates (McCarthy *et al.*, 2012). CapC and CapD are encoded by
most clonal complexes with the exception of ST-21, ST-42 and ST-61. Notably these clonal complexes are reported to be the most frequently recovered genotypes recovered from cattle (Kwan et al., 2008). Whilst ST-21 is a multi-host adapted lineage, it is also commonly isolated from bovine sources (Kwan et al., 2008). Complete absence of CapC and CapD from these genotypes may suggest that these proteins confer a selective disadvantage in bovine hosts. Interestingly, the ST-22 clonal complex is the only recognised lineage that does not encode any of the autotransporters that are the focus of this study. Further work should seek to define the barriers to recombination and explain the genotypic associations of autotransporters observed here.

Full length CapC and CapD have clear links to the ST-45 and ST-283 clonal complexes, with a clear, defined sub-population within ST-45 encoding CapD rather than CapC. The degree of demarcation between lineages that encode certain autotransporters and those that do not is exemplified by this sub-population and is evidence of strong genotype associations rather than with isolate source as was initially suggested by preliminary analysis. Further work should aim to investigate this subpopulation and identify the other genetic factors involved in this differential distribution of CapC and CapD.

As previously mentioned, linkage of genotype and ecological niche is widely observed in Campylobacter; therefore any observed association of a particular autotransporter with a lineage may cause an indirect association with an isolate source that is the lineage preference. However, ecological preference does not preclude events leading to transmission of isolates to different niches. For example clinical isolates causing human disease may be derived from chicken-adapted strains, or environmental isolates may be originally derived from agricultural sources. True source attribution is difficult in Campylobacter species (Dearlove et al., 2016), particularly with multi-host lineages which display weak genetic signals of host specificity thus making potential correlations with isolate source difficult to accurately determine. Ultimately it is clear that
possession of CapC and CapD in \textit{C. jejuni}, as well as the CapA/B autotransporters, is correlated with genetic background of \textit{C. jejuni} and \textit{C. coli}.

\textit{Campylobacter coli} is phylogenetically clustered into distinct clades which are in turn linked to environmental niches, Clade 1 of \textit{C. coli} represent the majority of agricultural isolates and display 1\% (clade 1a) and up to 20\% (clade 1b) introgression with \textit{C. jejuni} type alleles (Sheppard \textit{et al.}, 2013). Clades 2 and 3 represent the non-agricultural, environmental reservoir, which show minimal incorporation of \textit{C. jejuni} sequences (Sheppard \textit{et al.}, 2013). Sheppard \textit{et al.} (2013) reported that as a result of interaction via a shared niche, \textit{C. coli} lineages have progressively accumulated \textit{C. jejuni} genetic material. Resulting introgressed lineages dominate the agricultural niche, as evidenced by the fact that \textit{C. coli} dominate gastroenteritic infections in swine (Thakur \textit{et al.}, 2006). Introgression is therefore considered to be a major driving force in determining the population structure of \textit{Campylobacter} species.

Evidence of the association between possession of CapC or CapD and genetic background is more readily available in \textit{C. coli} as shown in Figure 6.7B. Possession of full length CapD is prevalent in phylogenetic clades 1a and 1b which are associated with the agricultural niche. There are also instances of full length CapC in these clades, while CapA is always truncated and restricted to the highly introgressed clade 1b. Whilst the sample size of genomes belonging to clade 2 and 3 is relatively small, thus skewing the dataset to an extent, the total absence of any of the autotransporters screened for in these clade is telling. The notion of dependence or association of genetic features with a particular genotype has been proposed and observed previously with a differential distribution of CRISPR-cas systems in \textit{C. jejuni} and \textit{C. coli} (Pearson \textit{et al.}, 2015), of the oxidative stress regulators RrpA and RrpB (Gundogdu \textit{et al.}, 2016) and association of the \textit{fuc} locus (L-fucose metabolism) with particular MLST types (Dwivedi \textit{et al.}, 2016).
The near ubiquity of capC and capD in Clade 1a, which shares a niche with certain C. jejuni lineages, and the select distribution of these factors in C. jejuni suggest that these autotransporters have transferred from C. coli clade1 lineages to C. jejuni by interspecies recombination. This scenario would indicate that these factors originate in C. coli implicating them determinants in C. coli colonisation of the agricultural niche and perhaps in swine. This notion is further reinforced by the absence of autotransporters in the riparian clades and it is tempting to suggest that these lineages exhibit very little ecological overlap. Alternatively, these genes may have passed from certain C. jejuni lineages to C. coli which have then proliferated via clonal expansion or further recombination. This movement of genetic material is consistent with previously proposed models of introgression and derived population structure (Pearson et al., 2015; Sheppard et al., 2013). Considering the dataset is skewed towards agricultural isolates linked with human infection the high prevalence of CapD may suggest that this autotransporter may facilitate the generalist, multi-host lifestyle associated with ST-828. Further analysis of CapD may provide insight into the phenomenon of gene flow between C. jejuni and C. coli in an effort to understand evolutionary and epidemiological relationships, and the adaptive effects of recombination.
Chapter 7: General Discussion
7.1. General Discussion

*Campylobacter jejuni* and *C. coli* are two important human and animal pathogens that are the foremost bacterial causes of human gastroenteritis in the developed world and pose a significant economic burden (Tam and O’Brien, 2016). Many *Campylobacter* genetic factors have been proposed to be involved in the integral infection mechanisms of adhesion, invasion and translocation, and factors integral to chicken colonisation have been identified (Hermans *et al*., 2012; Backert and Hofreuter, 2012; Van Deun *et al*., 2008). It is also known that these behaviours vary considerably between different *Campylobacter* strains (Humphrey *et al*., 2015); for example, invasion of intestinal cells by *Campylobacter* has been observed to occur preferentially at basolateral surfaces (Bouwmen *et al*., 2013; Backert *et al*., 2013; Monteville and Konkel, 2002). However, confusion exists as to whether transmigration of *Campylobacter* across epithelial monolayers occurs via a transcellular or paracellular route (Backert *et al*., 2013).

This thesis has demonstrated that certain factors proposed to be involved in these processes are not conserved between different strains. In addition, the role of host-specific factors has been shown to affect infectious behaviour of campylobacters (Alemka *et al*., 2010). Therefore it is clear that much remains to be understood regarding the pathogenic mechanisms of campylobacters. The overarching aim of this thesis was to characterise the CapC, a novel strain-specific autotransporter in *Campylobacter* species and to establish any potential role in the virulence of these pathogens.

CapC was initially characterised and confirmed as an autotransporter as described in Chapter 3. Using the conserved domain database, the C-terminal domain of CapC was identified as consisting of the β-barrel structure that is characteristic of all autotransporters and therefore denotes CapC as a member of this protein family. The Conserved Domain Database is a widely used resource
hosted by the National Centre for Biotechnology Information (NCBI) and is actively maintained making this classification of CapC highly reliable and accurate (Marchler-Bauer et al., 2017).

capC gene deletion mutants were effectively constructed in this study in order to relate observed phenotypic differences to function. Initially, a capC deletion mutant constructed in C. jejuni 81116 (belonging to the ST-283 Clonal Complex) was determined to have a motility impairment that was thought to be due to a polar mutation. Additional, capC deletion mutants were constructed via partial deletion of the capC coding sequence and insertion of a chloramphenicol resistance marker in C. jejuni 81116 and C. jejuni M1 (ST-45 Clonal Complex). These strains are from two relatively closely related clonal complexes as shown in Figure 5.7A. Both are defined reference strains that encode the full length form of CapC. Deletion mutants were determined not to express CapC and to have no impairment in fitness. These isogenic mutants were subsequently used in phenotypic assays. A limitation of the present study is the failure to generate genetically complemented strains of the capC wildtype gene in order to demonstrate reversion of the observed phenotypes. Attempts to complement deletion mutants with the wildtype capC genes were unsuccessful in that the capC coding sequence was unable to be cloned into the pC46 and pSV009 complementation vectors in the correct orientation. This suggests that CapC may be toxic to the E. coli cloning strains used in this study. This circumstance has been reported previously with Campylobacter outer membrane proteins and enzymes (Brown et al., 2015; Mamelli et al., 2007). Lack of complementation limits interpretations of the results obtained here however this has been mitigated by the fact that capC-deficient phenotypes are similar between two different strains.

A combination of assays using the Galleria mellonella virulence model used in this study show that deletion of capC in C. jejuni 81116 and C. jejuni M1 causes significantly impaired virulence in
these strains. The use of *Galleria mellonella* as a model has been used previously as an effective and powerful model to identify putative virulence determinants in a range of pathogens including *C. jejuni* (Champion *et al.*, 2010). This model has been used to demonstrate that mutants with deletions in factors that have previously been implicated as virulence determinants (CadF, FlpA and CiaB) elicit decreased mortality in the *Galleria* model, relative to wildtype strains (Naz, 2014). Additionally, the model has been used to identify factors that contribute to bacterial fitness without being a virulence factor *per se*, as in the case of Cj1416 which enables O-methyl phosphoramidate capsule modification (Champion *et al.*, 2010). However, use of this model can lead to contrasting results to those obtained in mammalian models (Shang *et al.*, 2016; Senior *et al.*, 2011). Moreover, the use of *Galleria* is limited in terms of its applicability to *Campylobacter* infection of human hosts; infection of *Galleria* larvae is not representative of the course of human infection in terms of the physiological barriers campylobacters encounter during infection. Additionally, the intra-larval environment does not resemble the site of *Campylobacter* infection. Nonetheless, *Campylobacter* does exhibit insecticidal activity which in this instance has been quantified as a measure of virulence.

The present study has shown that *capC* deficiency impairs virulence in *Galleria* larvae as determined by fatality/survival rates and severity of disease. Various assays have indicated that Δ*capC* deletion mutants have no relative fitness impairment that may contribute to this phenotype suggesting that this decrease is the result of a direct interaction.

A combination of non-polarised, partially differentiated Caco-2 cells and non-polarised T84 cells were used to demonstrate that CapC contributes to adhesion in these models. Adhesion and invasion efficiencies of Δ*capC* mutants were decreased in the T84 cells model and significantly decreased in the Caco-2 cell model. Subsequent comparison of the levels of invaded *C. jejuni* as a proportion of associated bacteria revealed that mutant strains showed marginally increased
proportional invasion. Therefore, the observed phenotypes indicate that CapC is required for maximal adhesion to human intestinal cells.

CapC has been established to play a role in virulence however this study has not identified a precise nature of this role in human intestinal cell models. Despite the association of CapC with a virulence phenotype, the non-polarised cell models used here are restricted in terms of the conclusions that can be drawn from them. The architecture of non-polarised cells grown on plastic surfaces differs markedly from cells in vivo. In order to accurately assess the role of CapC in the interaction with human intestinal cells, a more pertinent model would be the use of polarised cells whose architecture more closely resembles that of in vivo enterocytes. Furthermore, use of polarised cells would enable the relative contribution of CapC to be assessed in context with other reported adhesins of C. jejuni. For example, the fibronectin-binding proteins CadF and FlpA may confound results in adhesion assays (Flanagan et al., 2009; Monteville and Konkel, 2002). Fibronectin is localised to the basolateral surface of polarised intestinal cells; binding to this factor is thought to follow transmigration across the epithelium and perhaps facilitate the process of “subvasion” observed in C. jejuni (Bouwmen et al., 2013; O’Croinin and Backert, 2012; Monteville and Konkel, 2002). Use of polarised cell models to examine apical/basolateral interaction would be hugely beneficial therefore and should be performed in order to elucidate whether CapC plays a direct role in adhesion.

As stated throughout this thesis, a plethora of factors have been reported to contribute to the adhesion process including CadF (Monteville and Konkel, 2002), FlpA (Flanagan et al., 2009), JlpA (Jin et al., 2003), Peb1a (Pei et al., 1993), and CapA (Ashgar et al., 2007) amongst others. This multitude of reported adhesins has complicated understanding of the adhesion process, and is further complicated by the failure to distinguish between factors contributing to bacterial fitness and “true” adhesins involved in direct interaction with the host as in the case of Peb1a (Backert and Hofreuter, 2013; Flanagan et al., 2009; Pei et al., 1993). Furthermore, most of the reported
adherence-impaired single gene mutants in genes encoding the aforementioned factors, exhibit only reduced rather than abolished interactions with eukaryotic cells (Van Alphen et al., 2008). This observation suggests that redundancy of factors may exist in the adhesion process, a notion reinforced by the fact that certain reported adhesins are not conserved amongst C. jejuni lineages (Ashgar et al., 2007). Identification of CapC as an additional strain-specific autotransporter contributing to adhesion may indicate that C. jejuni does not have a defined, uniform method of adhesion and invasion of intestinal cells and that this process may vary between different lineages. Ultimately further studies are required to establish whether CapC interacts direct with host cells.

A secondary aim of this study of this study was to examine the distribution of CapC amongst campylobacters and to establish any potential genetic associations of this virulence determinant. The distribution and prevalence of the Campylobacter autotransporters capA/B and capC are detailed in Chapter 6. In addition, this study has identified a novel autotransporter in C. jejuni and C. coli, designated CapD. Identification of full length and truncated forms of CapA/B, CapC and CapD facilitated the successful determination of genetic associations of Campylobacter autotransporters.

The high prevalence of truncated autotransporters (Figure 5.7A), of which both CapA and CapC are reported to be adhesins, exemplifies the notion of functional redundancy of adhesins in certain lineages. Full length forms of both CapC and CapD are shown to be predominantly associated with the ST-45 and ST-283 clonal complexes, amongst others. Full length CapC and CapD are also associated exclusively with phylogenetic Clade 1a in C. coli, which is primarily comprised of isolates belonging to ST-828 clonal complex which adapted to agricultural animals but also with a strong capability to infect humans and are considered to be generalist lineage
(Dearlove et al., 2016; Thakur et al., 2006). The relative absence of truncated forms of CapC and CapD in ST-45 and ST-828 suggests that these factors are functionally important in *C. coli*.

The presence of CapC and CapD in the majority of isolates of *C. coli* in only Clade 1a and presence in select *C. jejuni* lineages suggests the genes encoding these autotransporters originated in *C. coli* and were able to cross the species boundary and recombine with *C. jejuni* via shared ecological niches. This is likely to have occurred within the agricultural niche which has been shown to be associated with genome-wide introgression of these two species. (Sheppard et al., 2013).

Interspecies recombination of the *capC* and *capD* genes may have helped facilitate *Campylobacter* species colonise the agricultural niche and it is feasible that genetic exchange of autotransporters from *C. coli* to *C. jejuni* contributes to genetic diversity in *C. jejuni* and the resulting discrepancies in virulence capability within this species. The present study has shown that horizontal transfer of virulence genes between species may give rise to strains and lineages with greater pathogenic potential; therefore transfer of *capC* and *capD* may have the potential to be used to study recombination between these two species.

In conclusion, this thesis indicates that CapC is a strain-specific virulence determinant in *Campylobacter* species. CapC has been shown to contribute to the integral infection process of adhesion however, its precise role in this process is yet to be determined. Future studies on exactly how CapC fits into the current understanding of *Campylobacter* pathophysiology is therefore an attractive avenue of research. A secondary aim of this study was to investigate the distribution of the *capC* gene and determine any genetic associations of this gene. CapC, as well as CapA, CapB and the CapD autotransporter, newly identified herein, were all established to exhibit associations with specific MLST clonal complexes and it is concluded that possession of *Campylobacter* autotransporters is dependent on genetic background.
Future Perspectives

The results presented here provide the foundation for further investigation particularly with regards to the exact role of CapC and CapD in the adhesion process as well as functional and genomic studies of these factors in *C. coli*. Specific areas of further study are:

1. **Confirmation of the ΔcapC deletion phenotype in a polarised human intestinal epithelial cell model.** A limitation of the present study is the use of non-polarised epithelial cell models. Use of polarised cells that more accurately model *in vivo* host cell architecture should be performed in order to confirm the role of CapC in adhesion. Such a model may also give insight as to whether CapC interacts with the apical or basolateral surface of host cells. This model would also facilitate investigation of the contribution of CapC to the process of transmigration across the epithelium.

2. **More detailed analysis of the interaction of CapC with human intestinal cell lines is required.** In order to characterise any potential interactions of CapC more closely, employing the use of techniques such as immunofluorescence to enable visualisation of any potential interaction is also needed. Additionally, the use of specific competitive inhibitors for host cell receptors may provide insight into a potential ligand or site of action for CapC.

3. **Characterisation of the novel autotransporter, CapD.** This project has detailed the identification of CapD a sequence variant of CapC. CapD has been established to be highlight prevalent in *C. jejuni* and *C. coli*. Characterisation of CapD using a targeted mutagenesis approach and subsequent comparison using aforementioned models will give insight to CapD function.

4. **Phenotypic characterisation of CapC and CapD in alternative host models and *in vivo* studies.** The present study has focused predominantly on the characterisation of CapC in
the context of human infection. The role of CapC and CapD in the avian host is an appealing route of further study given the importance of the chicken reservoir. Additionally, swine are implicated as a major reservoir of *C. coli* (Thakur and Gebreyes, 2005). Considering the high prevalence of full length CapC and CapD in *C. coli* associated with the agricultural niche, examination of the role of CapC and CapD in porcine hosts may further increase our understanding of infection of these hosts and proliferation of *C. coli* in this niche.

5. More detailed analysis of genetic associations of *capC* and *capD*. A clear, defined sub-population of genomes within ST-45 encode CapD rather than CapC as shown in Figure 5.7A. A more detailed investigation as to the associations of CapC and CapD with genetic background would be interesting in order to determine why a sub-population with the ST encode CapD rather than CapC. This analysis may lead to the identification of epistatic factors that may facilitate autotransporter export or even suggest a niche preference for these genomes.
Appendices and Bibliography
Appendix II

List of Isolates and Strains

Campylobacter jejuni NCTC 11828 (81116) (Pearson et al., 2007)

Campylobacter jejuni NCTC 11828 (81116) ΔcapC

Campylobacter jejuni M1 (Friis et al., 2010)

Campylobacter jejuni M1 ΔcapC

E. coli DH5α (MAX Efficiency® DH5α™ Competent Cells, ThermoFisher Scientific, UK)

E. coli JM109 (Promega, UK)

Salmonella Typhimurium SL1344 (SAP16)

Campylobacter jejuni NCTC 11168 ΔfliD

Campylobacter jejuni 81-176

Campylobacter jejuni NCTC 11351

Clinical and poultry isolates of Campylobacter species used in this study are listed in table 3.1 in Appendix III
**List of Media**

**A. Mueller-Hinton Broth/Agar**

Mueller-Hinton agar 38g:

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<td>Beef, dehydrated infusion from</td>
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<td>Casein hydrolysate</td>
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Distilled water 950mL

Defibrinated Sheep’s blood 50ml

**B. mCCDA**

CCDA dehydrated formulation 22.75g:

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Distilled water 500ml

CCDA selective supplement dissolved in 5ml distilled water (Cefoperazone (16mg), Amphotericin B (5mg).
C. Bolton Broth

Bolton Broth (dehydrated formulation) 13.8g:

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Distilled water 500ml

Bolton Broth Selective supplement dissolved in 5ml ethanol (cefoperazone (10mg), vancomycin (10mg), trimethoprim (10mg), cycloheximide (25mg).

D. Brucella Broth

Brucella Medium Base 45g:

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</table>

Distilled water 1L

E. L-Broth/Agar

5 g Tryptone
2.5 g Yeast extract
2.5 g NaCl

Distilled water to 500 ml

[1.5 % Agar]
List of Buffers, Reagents and Supplements

10x TBE Buffer
Tris base  108 g
Boric acid  55 g
0.5M EDTA  40 mL
800 ml of deionized water
10X buffer solution stored at room temperature. The solution is diluted before use.

TE Buffer
1 ml of 1 M Tris-HCl (pH 8.0) and 0.2 ml EDTA (0.5 M) and made up with double distilled water up to 100ml.

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)
0.08g X-gal added to 2ml dimethyl formamide in a fume hood.

IPTG (Isopropyl-beta-D-thiogalactopyranoside)
238mg was dissolved in 10mls MilliQ water and filter sterilised to obtain a 100mM stock solution.

TTC (2,3,5-Triphenyl-tetrazolium chloride)
0.2g was dissolved in 20mls of filter sterile MilliQ water and filter sterilised to obtain a 1% W/W stock solution. 1ml is added to 200mls molten media. Solution is kept from direct sunlight.

Ampicillin 100mg/ml
1g ampicillin was added to 10ml deionised water and then filter sterilised.

Kanamycin 50mg/ml
0.5 g kanamycin was added to 10ml deionised water and then filter sterilised.

Chloramphenicol 25mg/ml
250mg is added to 10mls absolute ethanol and filter sterilised. Stored at -20 °C
**Trimethoprim 100mg/ml**

1g trimethoprim was added to 10ml absolute ethanol and then filter sterilised.

**Campylobacter jejuni wash buffer**

9g of sucrose,

15ml glycerol

Distilled water to a final volume of 100ml and then filter sterilised.

**Sodium Hippurate**

Dissolve 0.5 g in 50 ml water and sterile filter through a 0.2 μm membrane filter. Dispense aseptically into screw-cap tubes (0.5 ml/tube).

**Ninhydrin reagent**

*ninhydrin*, 3.5g; *acetone-butanol* mixture (l:l), 100ml

**200mM Tetra-methyl-p-phenylenediamine dihydrochloride (TMPD)**

0.2ml of 800mM ascorbate was added to 0.8ml of H2O. 47.4mg of TMPD was added to this solution and dispensed into 0.2ml aliquots and stored away from sunlight.

**3% Hydrogen Peroxide (H2O2)**

1ml of 30% methanol was added to 9ml absolute methanol to obtain 3% H2O2 (v/v). Stored at 4°C

**Phosphate Buffered Saline (PBS) Dulbecco A**

1 tablet of Dulbecco A PBS (Oxoid) was added to 100mls of double distilled H2O and autoclaved according to the manufacturer’s directions.

**Giemsa Stain**

0.5g of Giemsa powder was added to 33ml of glycerol and heated to 55°C in a water bath. Once dissolved, 33mls of methanol was added to this mixture. This resulting 20x solution was diluted 1:20 before use.

**2,3,5-Triphenyl-tetrazolium chloride (TTC)**

0.2g was dissolved in 20mls of filter sterile MilliQ water and filter sterilised to obtain a 1% v/w stock solution.
## List of Plasmids

<table>
<thead>
<tr>
<th>Plasmid ID</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM T-easy®</td>
<td>pGEM T-easy® is a 3065bp TA vector that has been linearised by EcoR V digestion and a thymidine added to both 3’ terminals. The vector contains an ampicillin resistance cassette upstream of a T7 promoter region and a beta-galactosidase coding region into which a Multiple Cloning Site has been inserted.</td>
<td>Kobs, G., 1997 Promega</td>
</tr>
<tr>
<td>pUC19</td>
<td>pUC19 is a commonly used 2686bp, circular, plasmid cloning vector in E.coli. Like pGEM T-easy®, pUC19 contains an ampicillin resistance cassette upstream of a beta-galactosidase coding region into which a Multiple Cloning Site has been inserted.</td>
<td>Yanisch-Perron, C. <em>et al</em>, 1985</td>
</tr>
<tr>
<td>pJM001</td>
<td>The pGEM t-easy® backbone into which a 1266bp capC fragment amplified by CAP3 and CAP4 primers has been inserted by TA ligation.</td>
<td>This study</td>
</tr>
<tr>
<td>pJM002</td>
<td>The pJM001 backbone plasmid into which the 1499bp kanamycin cassette yielded from BamHI restriction digest of pJMK30 had been inserted via the BgIII restriction site.</td>
<td>This study</td>
</tr>
<tr>
<td>pJMK30</td>
<td>The source of the kanamycin cassette. The Kanamycin resistance (kan’) gene <em>aphA</em>-3 from <em>C. coli</em> cloned into pUC19.</td>
<td>van Vliet, A. <em>et al</em>, 1998</td>
</tr>
<tr>
<td><strong>pJM005</strong></td>
<td>The full length 2921bp <em>capC</em> amplicon cloned into pGEM T-easy with Esp3I restriction sites at each terminal.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pJM005.1</strong></td>
<td>The full length 2921bp <em>capC</em> amplicon cloned into pGEM T-easy with BamHI and XbaI restriction sites at each terminal.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pJM006</strong></td>
<td>The <em>capC</em> knockout construct with a chloramphenicol marker. The <em>cat</em> cassette from pAV103 inserted to the BglII/XbaI digested pJM005.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pC46</strong></td>
<td>The chloramphenicol cassette flanked by <em>cj0046</em> pseudogene sequences with an Esp3I (BsmBI) restriction site immediately upstream of the <em>cat</em> promoter.</td>
<td>Gaskin et al, 2007</td>
</tr>
<tr>
<td><strong>pSV009</strong></td>
<td>The kanamycin resistance gene flanked by the <em>cj0046</em> pseudogene sequences and a multiple cloning site and the <em>cat</em> cassette promoter upstream of the kanamycin resistance gene.</td>
<td>De Vries SPW et al, 2007</td>
</tr>
<tr>
<td><strong>pJM007</strong></td>
<td>The BamHI and XhoI-digested <em>capC</em> amplicon cloned into the corresponding sites in pSV009.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pJM009</strong></td>
<td>A shorter, BamHI-digested <em>capC</em> amplicon cloned into pSV009</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pJM008</strong></td>
<td>The <em>cat</em> cassette inserted between the flanks of the <em>cadF</em> gene in a pGEM T-easy® backbone.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>piLOVporA</strong></td>
<td>iLOV</td>
<td>Elgamoudi and Ketley, 2015</td>
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### Table of Restriction Endonucleases

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<th>Recognition Site</th>
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<tr>
<td><strong>BglII</strong></td>
<td>5’...AGATCT...3’</td>
</tr>
<tr>
<td></td>
<td>3’...TCTAGA...5’</td>
</tr>
<tr>
<td><strong>BamHI</strong></td>
<td>5’...GGATCC...3’</td>
</tr>
<tr>
<td></td>
<td>3’...CCTAGG...5’</td>
</tr>
<tr>
<td><strong>XhoI</strong></td>
<td>5’...CTCGAG...3’</td>
</tr>
<tr>
<td></td>
<td>3’...GAGCTC...5’</td>
</tr>
<tr>
<td><strong>XbaI</strong></td>
<td>5’...TCTAGA...3’</td>
</tr>
<tr>
<td></td>
<td>3’...AGATCT...5’</td>
</tr>
<tr>
<td><strong>Esp3I</strong></td>
<td>5’...CGTCTC(N)_x...3’</td>
</tr>
<tr>
<td></td>
<td>3’...GCAGAG(N)_x...5’</td>
</tr>
<tr>
<td><strong>NotI</strong></td>
<td>5’...GCGGCCGC...3’</td>
</tr>
<tr>
<td></td>
<td>3’...CGCCGCGG...5’</td>
</tr>
<tr>
<td><strong>NcoI</strong></td>
<td>5’...CATGG...3’</td>
</tr>
<tr>
<td></td>
<td>3’...GGTAC...5’</td>
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<tr>
<td><strong>SacI</strong></td>
<td>5’...GAGCTC...3’</td>
</tr>
<tr>
<td></td>
<td>3’...GTCGAG...5’</td>
</tr>
</tbody>
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### List of Primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Restriction Site</th>
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</thead>
<tbody>
<tr>
<td>Cap1</td>
<td>5’ CAA TTA GGA AGT ATA GGC GCA 3’</td>
<td>N/A</td>
</tr>
<tr>
<td>Cap2</td>
<td>5’ AGC ACC CAC TTC AAC ACT TAC 3’</td>
<td>N/A</td>
</tr>
<tr>
<td>Vac1</td>
<td>5’ GTG TAA TCA TCA CCA CTA TTG ATA 3’</td>
<td>N/A</td>
</tr>
<tr>
<td>Vac2</td>
<td>5’ TAT AGG TTC TCG TGT AGC AAT GCT 3’</td>
<td>N/A</td>
</tr>
<tr>
<td>Hel1</td>
<td>5’ TAC AGA TCA AAG TCG TGC AAC GCA 3’</td>
<td>N/A</td>
</tr>
<tr>
<td>Ppk1</td>
<td>5’ CTT TCT TGA CCT ATA TAA CCA 3’</td>
<td>N/A</td>
</tr>
<tr>
<td>JMCAT5</td>
<td>5’ AAA CTGAG TGC TCG GCG GTG TTC CTT TCC AAG</td>
<td>XhoI</td>
</tr>
<tr>
<td>JMCAT6</td>
<td>5’G AT AGATCT TGC TCG GCG GTG TTC CTT TCC AAG</td>
<td>BglII</td>
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<tr>
<td>Cdt-F</td>
<td>5’ GCG GAA AAT TAT AAT GAA ATT 3’</td>
<td>N/A</td>
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<tr>
<td>Cdt-R</td>
<td>5’ GCA AGG GGC TAT TCC AAA GC 3’</td>
<td>N/A</td>
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<td>Cat C1</td>
<td>5’ CTCGGCGGTGTCCCTTTTCAAAG 3’</td>
<td>N/A</td>
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<tr>
<td>Cat C2</td>
<td>5’-CGCCCTTTAGTCCCTAAAGG-3’</td>
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<tr>
<td>CC069</td>
<td>ATATGTGCAAGGCGTATATTG</td>
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<td>Cat C1</td>
<td>5’ GAT AGATCT CTCGGCGGTGTCCCTTTTCAAAG 3’</td>
<td>BglII</td>
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<td>Cat C2</td>
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<tr>
<td>Cj0046 F</td>
<td>CAC TAC CCA ATT GAA AAT CTA AG</td>
<td>N/A</td>
</tr>
<tr>
<td>Cj0046 R</td>
<td>GTG AGT TAA TGC CAT CAT AAC</td>
<td>N/A</td>
</tr>
<tr>
<td>CAP3</td>
<td>5’ CCA AAT GGA TTA TCA AGG C 3’</td>
<td>N/A</td>
</tr>
<tr>
<td>CAP4</td>
<td>5’ GCT TGT TTA TCT ACT CCC ACG G 3’</td>
<td>N/A</td>
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<tr>
<td>CapC-FW</td>
<td>ACTTGGCTTCGACAGAGG</td>
<td>N/A</td>
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<tr>
<td>CapC-RV</td>
<td>AAGATAAAGCCTAGGAGATTTC</td>
<td>N/A</td>
</tr>
<tr>
<td>BamCapC-FW</td>
<td>GAT GGATCC TGAAGGAGAAACTTATGAAG</td>
<td>BamHI</td>
</tr>
<tr>
<td>BamCapC-RV</td>
<td>GAT GGATCC AAGATAAAGCCTAGGAGATTTC</td>
<td>BamHI</td>
</tr>
<tr>
<td>pSV009FW1</td>
<td>TAATAGAAATTCCCAGAATCCCA</td>
<td>N/A</td>
</tr>
<tr>
<td>pSV009RV1</td>
<td>CTATTGCCATAGTAGCTCTTAGTG</td>
<td>N/A</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>CapCF Bam</td>
<td>5’ GAT <strong>GGATCC</strong> ACTTGGCTTTGACAGGAGT 3’</td>
<td><strong>BamHI</strong></td>
</tr>
<tr>
<td>CapCR Xho</td>
<td>5’ GAT <strong>CTCGAG</strong> AAGATAAAGCCCTAGGAGATTTC 3’</td>
<td><strong>XhoI</strong></td>
</tr>
<tr>
<td>SacCapC-RV</td>
<td>GAT <strong>GAGCTC</strong> AAGATAAAGCCCTAGGAGATTTC</td>
<td><strong>SacI</strong></td>
</tr>
<tr>
<td>pC46P2 Bam</td>
<td>5’ GAT <strong>GGATCC</strong> AGTAGAAGCATAGCGTGGA 3’</td>
<td><strong>BamHI</strong></td>
</tr>
<tr>
<td>pC46P3 Xho</td>
<td>5’ GAT <strong>CTCGAG</strong> TCTCATGGATTGAAAAGTGG</td>
<td><strong>XhoI</strong></td>
</tr>
<tr>
<td>Tool/Application</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Mauve</td>
<td>An application for generation of multiple genome sequence alignments</td>
<td>Darling et al., 2010</td>
</tr>
<tr>
<td>Phyre2</td>
<td>service for predicting the 3-dimensional structure of a protein amino acid sequence</td>
<td>Kelley et al., 2015</td>
</tr>
<tr>
<td>Conserved Domain Database</td>
<td>A collection of well-annotated multiple sequence alignment models</td>
<td>Marchler-Bauer et al., 2017</td>
</tr>
<tr>
<td>SignalP</td>
<td>Program for Identification of prokaryotic and eukaryotic signal peptides</td>
<td>Peterson et al., 2010</td>
</tr>
<tr>
<td>Cello</td>
<td>Program for Subcellular Localization Prediction</td>
<td>Yu et al., 2014</td>
</tr>
<tr>
<td>pSORT</td>
<td>A computer program for the prediction of protein localization sites in cells</td>
<td>Horton et al., 2007</td>
</tr>
<tr>
<td>Insilico PCR</td>
<td>In-Silico PCR searches a sequence database with a pair of PCR primers</td>
<td>Bikandi et al., 2004</td>
</tr>
<tr>
<td>MUSCLE</td>
<td>MUltiple Sequence Comparison by Log-Expectation</td>
<td>Edgar et al., 2004</td>
</tr>
<tr>
<td>Serial Cloner 2.0</td>
<td>Software that assists in DNA cloning, sequence analysis</td>
<td><a href="http://serialbasics.free.fr/Serial_Cloner.html">http://serialbasics.free.fr/Serial_Cloner.html</a></td>
</tr>
<tr>
<td>BioEdit V7.25</td>
<td>Sequence alignment editor and sequence analysis program</td>
<td>Hall et al., 2007</td>
</tr>
<tr>
<td>Figtree</td>
<td>graphical viewer of phylogenetic trees</td>
<td><a href="http://tree.bio.ed.ac.uk/software/figtree/">http://tree.bio.ed.ac.uk/software/figtree/</a></td>
</tr>
<tr>
<td>MIST</td>
<td>a tool for generation of molecular data from bacterial genome sequences</td>
<td>Kruczkiewicz et al., 2013</td>
</tr>
<tr>
<td>BLAST</td>
<td>BLAST finds regions of similarity between biological sequences.</td>
<td>N/A</td>
</tr>
<tr>
<td>Quast</td>
<td>Tool for evaluation of genome assemblies</td>
<td>Gurevich et al., 2013</td>
</tr>
<tr>
<td>Prokka</td>
<td>A software tool for the rapid annotation of prokaryotic genomes</td>
<td>Seemen, 2014</td>
</tr>
<tr>
<td>ParSNP</td>
<td>Tool for aligning the core genome of bacterial genomes</td>
<td>Treangen et al., 2014</td>
</tr>
</tbody>
</table>
**Immunisation Protocol for Raising α-CapC antibodies**

Antigen preparation, such as peptide synthesis and/or immunogen conjugation, occurs before Day 0. Protocol Days are approximate. Immunization, serum collection and bleeding were performed by technicians from ThermoScientific.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protocol day</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control serum collection</td>
<td>Day 0</td>
<td>Pre-immune bleed (5 mL per rabbit)</td>
</tr>
<tr>
<td>Primary injection</td>
<td>Day 1</td>
<td>Immunize with 0.50 mg of antigen in CFA, 10 SQ sites</td>
</tr>
<tr>
<td>1st booster</td>
<td>Day 14</td>
<td>Boost with 0.25 mg of antigen in IFA, 4 SQ sites</td>
</tr>
<tr>
<td>2nd booster</td>
<td>Day 28</td>
<td>Boost with 0.25 mg of antigen in IFA, 4 SQ sites</td>
</tr>
<tr>
<td>Serum collection</td>
<td>Day 35</td>
<td>bleed (~25 mL per rabbit)</td>
</tr>
<tr>
<td>3rd Booster</td>
<td>Day 42</td>
<td>Boost with 0.25 mg of antigen in IFA, 4 SQ sites</td>
</tr>
<tr>
<td>Serum collection</td>
<td>Day 56, 58</td>
<td>Two bleeds (~50 mL total per rabbit)</td>
</tr>
<tr>
<td>ELISA and shipping</td>
<td>Day 60</td>
<td>ELISA titration (results available online); Verify disposition of rabbits; decide to continue or terminate</td>
</tr>
</tbody>
</table>
Appendix III

capC nucleotide sequence

>lcl||C8J_1278||87711454 hypothetical protein
ATGAGAGAAAAATCTCTGCGAACAGATATTGCTAAGCTTAGTGTTACCACTTTGCTTTAT
TCGGGTCCTTTAACGTCATTTTCTTTTTTATTGCGTAGTAGTATAGAAATATGCATGTT
GATAAACATAGAAAGCAATTGCTGATTCTTTTAAAAATGAAAAATTATAAAAAATACGA
GATGATTTATATATCGTCCTTGAACGCTTTTGGGAATGTCAGCTATGAACTACGTT
ACGCTGCTTTAACGACAATAGATAAAAAAAATGCTCATTTTGAATATTGACAGACT
GGATTATATATGCTTTAACGACATACATAATATCATTGATATATGACAGATCTA
GAGCAGCTTTAAAGAAGGATTTGCTTTAACAGCTGATAAATGCTTTATTTATTT
GCTACATCTGTTCTTACAAATTAAAATATATTGCTTATGGTATAAAAATTATCTTCT
AACGAGCTTTAAAGAAGGATTTGCTTTAACAGCTGATAAATGCTTTATTTATTT
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CapC amino acid sequence

>lcl||C8J_1278||87711454 hypothetical protein
MKKNASSKILLSLGVATLLYSGAFAAEFNGDSDLKDYFDINEKDNVATFKNENYKNQ
DVTNFISTSAFDADPEDTKINIDGLGNNSLTLLKQMDYQKTAALVKNFNVDKAKDFKTTDI
GLSYFNAGINANFTMGEKFDLGNIDKNKASSLLIFGNSRENNDTVGLSNTVNGDF
STTNSAIVSKTDFTKVGNTATLKEAGLGFSSYSNLVNDIFIALRAKDIKTDTLNDT
NAGALIKTASSYINENLNGDDDAAYLTVTDDKYYYYGAFVYDYLKSLKNCGDKICLVDING
GATAAAKLNQIIVDLEAIKITIIGDLNEQAKALKQEQKTELEKLLQEMMQNGGKIDDE
KYIDLVNKNENLNLASNDKASVLLRDTSEQLGSIADLRSREGVQLALQIKKTDNTGK
SVSNFSNASAVNMTMISNCVSGSRVAMLNNPFTYASKMNGLKFAALDSDMRPSYNV
EYNTSVWANAFFGANIIIGDGSGMYGATVGDKQANDNVLWGAYFTYANAKIKDNLEKQ
SDNFQLGMYSTINIAFPQWELNLKAYAQVSPTKQDNVQVDGAYNDSYTSKFLGSLAPAGRV
FDLSDNPTFISKPFAGVNYFSYTPSHTENGAIKDIDSMKNNSVSEVGAERKYNENS
YIFVTPKIEQFVINSGDDYTANLAVNNAFTSVEANKKKTYGGQIIVGNNVDFTNQLSMN
LGFGAKQILAGKVDNKNETYLSQVQLKYKF

Cello Subcellular localisation prediction output

CELLO RESULTS

SeqID: lcl|C8J_1278||87711454 hypothetical protein

<table>
<thead>
<tr>
<th>Analysis Report</th>
<th>LOCALIZATION</th>
<th>RELIABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acid Comp.</td>
<td>Extracellular</td>
<td>0.941</td>
</tr>
<tr>
<td>N-peptide Comp.</td>
<td>Extracellular</td>
<td>0.549</td>
</tr>
<tr>
<td>Partitioned seq. Comp.</td>
<td>Extracellular</td>
<td>0.752</td>
</tr>
<tr>
<td>Physico-chemical Comp.</td>
<td>OuterMembrane</td>
<td>0.928</td>
</tr>
<tr>
<td>Neighboring seq. Comp.</td>
<td>Extracellular</td>
<td>0.687</td>
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</table>

<table>
<thead>
<tr>
<th>Cello Prediction:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
</tr>
<tr>
<td>OuterMembrane</td>
</tr>
<tr>
<td>Plasmatic</td>
</tr>
<tr>
<td>InnerMembrane</td>
</tr>
<tr>
<td>Cytoplasmic</td>
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</table>

******************************************************************************
Table 3.1: Reference and Typed Isolates that encode capC/capD

Table 3.1 shows common species of C. jejuni that are used as reference strains and whether or not they encode capC. Possession of capC was determined by a combination of PCR (shown in bold font) and in silico pcr.

<table>
<thead>
<tr>
<th>Laboratory and Typed Campylobacter strains</th>
<th>Possession of CapC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter jejuni subsp. jejuni NCTC 11168</td>
<td>X</td>
</tr>
<tr>
<td>Campylobacter jejuni subsp. jejuni NCTC 11351</td>
<td>✓</td>
</tr>
<tr>
<td>Campylobacter jejuni subsp. jejuni NCTC 11168H</td>
<td>X</td>
</tr>
<tr>
<td>Campylobacter jejuni subsp. jejuni NCTC 81116</td>
<td>✓</td>
</tr>
<tr>
<td>Campylobacter jejuni 480</td>
<td>X</td>
</tr>
<tr>
<td>Campylobacter coli NCTC 11350</td>
<td>✓</td>
</tr>
<tr>
<td>Campylobacter coli NCTC 11437</td>
<td>✓</td>
</tr>
<tr>
<td>Campylobacter coli NCTC 12110</td>
<td>✓</td>
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<tr>
<td>Campylobacter jejuni RM1221</td>
<td>✓</td>
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<tr>
<td>Campylobacter jejuni subsp. doylei</td>
<td>-</td>
</tr>
<tr>
<td>Campylobacter jejuni subsp. jejuni 81-176</td>
<td>X</td>
</tr>
<tr>
<td>Campylobacter jejuni subsp. jejuni IA3902</td>
<td>X</td>
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Table of Growth Curve Doubling times

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Doubling Times were calculated using the formula:

\[\text{Doubling Time} = \frac{\text{duration} \times \log(2)}{\log(\text{Final Concentration}) - \log(\text{Initial Concentration})}\]
Clinical isolate screening for possession of *capC* gene  Original isolate designation and species as confirmed by hippurate hydrolysis is shown. “x” denotes that the isolate was found to encode *capC* as determined by PCR.

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Poultry isolate screening for possession of the capC gene

Original isolate designation and species as confirmed by hippurate hydrolysis is shown. “x” denotes that the isolate was found to encode capC as determined by PCR.

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Appendix IV

*Galleria mellonella* invertebrate infection model morbidity and mortality scores

Evaluation and scoring of *Galleria mellonella* after infection with wildtype and mutant strains. The scoring system is outlined in Chapter 4. Morbidity and mortality scores across technical replicates are combined and shown in the table below.

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<td>0/10</td>
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<td>21/60</td>
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Appendix V

CFU/ml counts from adhesion and invasion assays

The two graphs below show the raw Colony forming units per ml counts from association and invasion assays performed in Caco-2 and T84 cell models. Data has been present on a log scale. Error bars show standard deviation.
Appendix VI

Phenotypic evaluation of 30 clinical isolates for selection for whole genome sequencing

30 clinical isolates of *Campylobacter jejuni* were selected for phenotypic testing. These isolates were tested for virulence profiles in the *Galleria mellonella* model, haemolytic activity and motility as described in Chapter 2.
Multi-locus Sequence Typing of 30 clinical isolates

Multi-locus Sequence Typing of the 30 characterised clinical isolates of *C. jejuni* was performed as described in Chapter 2. Due to the high error rate of Sanger sequencing and limited funding availability, complete MLST profiles for all isolates was not obtained however, putative clonal complex designations were determined. Isolates were selected for whole genome sequencing on the basis of phenotypic properties and relative relatedness of isolates.

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List of Publically available genomes

The publically available genome collection used in this study to ascertain the distribution of, and genomic associations of *Campylobacter* autotransporters is available at:

https://www.dropbox.com/s/ziyxpq5vhzh80ak/List%20of%20Publically%20available%20Genomes.xl

This file, alternatively available on CD-ROM 1, containing all available information including, where available, PubMLST ID, genbank accession number, isolate source, MLST sequence type and clonal complex. Information regarding possession of truncated or full length autotransporters is also included.


Dwivedi, Ritika, Harald Nothaft, Jolene Garber, Lin Xin Kin, Martin Stahl, Annika Flint, Arnoud H.


Junqueira, Juliana Campos. 2012. “Galleria Mellonella as a Model Host for Human Pathogens:


Lee, Gwennyth, William Pan, Pablo Peñataro Yori, Maribel Paredes Olortegui, Drake Tilley,


Meselson, Matthew, Robert Yuan, Janet Heywood, and Modification B METHYLASES Modification methylease. n.d. “RESTRICTION AND MODIFICATION OF DNAI Hemophilus Endonuclease Sites.”


Miller, W. G., Bruce M. Pearson, Jerry M. Wells, Craig T. Parker, Vladimir V. Kapitonov, and Robert E. Mandrell. 2005. “Diversity within the Campylobacter Jejuni Type I Restriction-


Skirrow, M. B. 2006. “John McFadyean and the Centenary of the First Isolation of Campylobacter


Woodford, Neil, and Alan P Johnson. n.d. “Genomics, Proteomics, and Clinical Bacteriology Methods and Reviews METHODS IN MOLECULAR BIOLOGY METHODS IN MOLECULAR BIOLOGY Genomics, Proteomics, and Clinical Bacteriology Methods and Reviews.”


