Hyperinvasiveness in the major food-borne pathogen *Campylobacter jejuni*

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ABSTRACT

Campylobacter jejuni is a common cause of human gastrointestinal infections. Invasion of host epithelial cells is believed to be an important virulence mechanism of this bacterium. *C. jejuni* strains vary in their ability to invade the human epithelial cells and some of the strains are hyperinvasive. The aim of this work was to find the molecular basis of this hyperinvasive phenotype. The previously studied hyperinvasive phenotype of *C. jejuni* 01/51 in INT-407 cells was verified using Caco-2 cell based invasion assay and the assay was set up at Nottingham Trent University by selecting blood agar for 48 hours as the pre-assay bacterial growth conditions and 100 multiplicity of infection as the starting inoculum. Seven hundred and sixty eight mutants generated in this strain by random transposon insertional mutagenesis were screened for their ability to invade human intestinal epithelial cells in an *in vitro* model and 174 mutants were selected for further studies. The motility of selected mutants was determined and 40 mutants that showed more than 75% motility compared to wildtype strain, with a very low level of invasion were selected for reconfirmation of this reduced invasion phenotype using standard INT-407 and Caco-2 cells based invasion assays. Localisation of the transposon insertion site by plasmid rescue was attempted in 15 mutants that showed very low levels of invasion in both eukaryotic cell lines. Preliminary DNA sequencing data from these mutants has identified, amongst others: *cipA*; an anion-uptake ABC-transport system permease; a glycosyltransferase; a membrane protein; a capsule polysaccharide biosynthesis protein, a putative histidine triad (HIT) family protein; a putative restriction modification enzyme; a *put*A; a putative cytochrome C and 2 hypothetical proteins. DNA sequence from one mutant had no database match. Targeted mutagenesis was done in 6 genes to verify the reduced invasion phenotype observed in the transposon mutants and Caco-2 cell-based adhesion and invasion assays were performed. All the six targeted mutants showed reduced invasion of Caco-2 cells compared to the wildtype strain but interestingly adhesion was variable. Complementation of 4 mutated genes partially restored the reduced invasion phenotype in the mutants. The reduced invasion of the mutants into human intestinal epithelial cells was not due to a reduction in their growth, motility, atmospheric air survival, culture media or intracellular survival of the mutants. The *Cj1136* mutant showed significantly reduced ability to colonise the chick gut and the gene was also found to be crucial for lipooligosaccharides biosynthesis in *C. jejuni.*

STATEMENT

Experimental work contained in this thesis is original research carried out by the author, unless otherwise stated, in the School of Science and Technology at the Nottingham Trent University. No material contained herein has been submitted for any other degree, or at any other institution.

Muhammad Afzal Javed

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ABC	ATP binding cassette
AFLP	Amplified fragment length polymorphism
ATP	Adenosine triphosphate
BA	Blood agar
BHI	Brain heart infusion broth
CDS	Coding sequence
CDT	Cytolethal distending toxin
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxynucleoside 5'-triphosphate
ECACC	European collection of animal cell cultures
EDTA	Ethylenediamine tetra-acetic acid
FCS	Foetal calf serum
GBS	Guillain Barré syndrome
G + C	Guanine and cytosine
kbp	Kilobase pair
LB	Luria-Bertani broth
LEP	Laboratory of entric pathogens
LPS	Lipopolysaccharide
LOS	Lipooligosaccharide
MCP	Methyl-accepting chemotaxis protein
MEM	Minimal essential media
MFS	Miller Fisher syndrome
MLST	Multi locus sequence typing
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
NCTC	National collection of type cultures

NEAA	Non-essential amino acids
NF-kB	Nuclear factor-kappa- enhancer of activated B cells
OD	Optical density
ORF	Open reading frame
P value	Probability value
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline solution
PFGE	Pulsed field gel electrophoresis
p.s.i.	Pounds per square inch
RAPD	Random amplified polymorphic DNA
rcf	Relative centrifugal force
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
sIgA	Secretory immunoglobulin A
σ^{28}	Sigma factor 28 or *fliA* (promoter)
σ^{54}	Sigma factor 54 or *rpoN* (promoter)
TSAP	Thermostable alkaline phosphatase
TAE	Tris/acetate/EDTA
TLR	Toll-like receptor
Tm	Melting temperature
v/v	Volume per volume
WT	Wildtype
w/v	Weight per volume

Chapter One

INTRODUCTION

1. INTRODUCTION

1.1 General description

Campylobacters are small (0.2 – 0.9 μm wide and 0.2 – 5.0 μm long), curved or spiral shaped non-sporulating rod-shaped, Gram-negative bacteria that exhibit rapid darting and spinning motility. They generally are microaerophilic, growing best in an atmosphere containing 3 - 13% CO_2 and 3 - 5% O_2 though some grows under aerobic and anaerobic conditions. Most *Campylobacters* have a rather narrow temperature range for growth with a maximum temperature of ~46^{o}C and a minimum of 30^{o}C and are classified as thermophiles. *Campylobacters* have a low G + C content and are unable to ferment or oxidise carbohydrates due to the absence of a key glycolytic enzyme 6-phosphofructokinase, instead the main carbon sources used by *Campylobacters* are amino acids especially serine; pyruvate and tricarboxylic acid cycle intermediates can also be utilised as the sources of carbon (Guccione *et al.*, 2008; Kelly, 2005; Wright *et al.*, 2009).

1.2 Historical Perspectives

Although *Campylobacters* were not recognised as human pathogens until the 1970s, they probably caused illness in man for centuries. In 1886, Escherich published a series of articles describing microscopical examinations suggesting the presence of non-culturable spiral bacteria in the colon of infants who died of what he called “cholera infantum” as well as the stool of infants suffering from diarrhoea (Escherich, 1886).

Campylobacter has been recognised as a cause of disease in animals particularly in bovines and ovines. In 1909, McFadyean and Stockman reported the presence of an unknown vibrio-like bacterium that was frequently isolated from aborted foetuses in ovines (McFadyean and Stockman, 1913). In 1919, Smith who was investigating infectious abortions in the bovine also identified the spiral shaped bacteria, spirallum (Smith, 1919). Later, Smith became acquainted with the work of McFaydean and Stockman, and assumed they had been studying the same bacteria and proposed the name “*Vibrio fetus”* for this bacteria (Smith and Taylor, 1919). In 1931, winter

dysentery in calves was attributed by Jones *et al.* to infection with a 'vibrio' that was called *Vibrio jejuni* (Jones *et al.*, 1931), and in 1944 Doyle described a similar organism associated with swine dysentery (Doyle, 1944). The pathogenic role of *V. fetus venerealis* in enzootic sterility in cows was demonstrated in 1949 (Stegenga and Terpstra, 1949). In 1959, Florent was able to distinguish two types of *V. fetus* by their biochemical and pathogenic characteristics, namely *V. fetus venerealis* and *V. fetus intestinalis* (Florent, 1959).

Campylobacter was not isolated from human blood until the 1940s; in 1946 the *Vibrio*-like bacteria were isolated from human cases which involved a milk-borne outbreak of diarrhoea (Levy, 1946) and a year later Vinzent *et al.* isolated *V. fetus* from the blood of three pregnant women having raised temperature (Vinzent *et al.*, 1947). In 1957, King described a vibrio with several features in common with the agents described by Vinzent and termed the organism as "related vibrio" (King, 1957) which was later renamed as *Campylobacter* (Sebald and Véron, 1963). The isolation of *Campylobacter* from faeces was accomplished in 1972 using a filtration technique (Dekeyser *et al.*, 1972). In 1977, Skirrow described a simpler technique of culturing *C. jejuni* and *C. coli* from stool specimens (Skirrow, 1977) and later, the development of selective media brought the isolation of *Campylobacter* into the realm of routine microbiology (Bolton *et al.*, 1984; Butzler *et al.*, 1983; Endtz *et al.*, 1991; Goossens *et al.*, 1989; Karmali *et al.*, 1986).

1.3 Taxonomy of campylobacters

The taxonomy of *Campylobacter* has changed significantly since its inception in 1963. The vibrio-related organisms *Vibrio fetus* and *V. bubulus* were classified into the novel genus *Campylobacter* by Sebald and Vernon in 1963 (Sebald and Véron, 1963). In a comprehensive study on taxonomy of the microaerophilic *Vibrio*-like organisms four distinct species were described in the genus *Campylobacter*: *C. jejuni*, *C. coli, C. fetus* and *C. sputorum.* Since the acceptance of divergence in 16S rRNA gene sequences as a model for determining phylogenetic relationships between bacteria in the 1980s, several studies of 16S rRNA gene sequence divergence in *Campylobacter* demonstrated the diversity of the genus (Lau *et al.*, 1987; Romaniuk *et al.*, 1987). On the basis of 16S rRNA gene sequence, cellular fatty acid composition and flagella morphology two

Campylobacter species, *C. pylori* and *C. mustelae,* were assigned to a new genus *Helicobacter* (Goodwin *et al.*, 1989). The 16S rRNA sequence divergence, protein profiling and immunotyping data was the basis of reclassifying the so-called aerotolerant campylobacters under new genus *Arcobacter,* as *A. nitrofigilis* and *A. cryaerophilus* (Vandamme *et al.*, 1991; Vandamme *et al.*, 1992). Using the 16S rRNA sequence divergence two oral anaerobic species *Wolinella curva* and *Wolinella recta* were also shown to belong to genus *Campylobacter.* Finally the genera *Arcobacter* and *Campylobacter*, *Sulfurospirillum* and *Bacteroides ureolyticus* were accommodated in the new bacterial family *Campylobacteraceae* (Vandamme and De Ley, 1991). All the above taxa form a distinct but diverse phylogenetic group referred to as rRNA superfamily VI (Vandamme *et al.*, 1991), the epsilon division of the *Proteobacteria* or the Epsilobacteria (Cavalier-Smith, 2002). Recently, whole-genome sequences of species belonging to the family *Campylobacteraceae* are becoming available; whole-genome comparison using 25 complete and incomplete genome sequences of 15 species from *Campylobacteraceae* generated a phylogenetic tree similar to that generated using 16S rRNA gene sequences, although whole-genome comparison gave more resolution between closely related species like *C. jejuni* subspecies *jejuni, C. jejuni* subspecies *doylei* and *C. coli* (Debruyne *et al.*, 2008).

There are currently 17 species and 6 subspecies within the *Campylobacter* genus and the number of species has continued to rise (Debruyne *et al.*, 2008). Currently well known *Campylobacter* species are *C. jejuni*, *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. upsaliensis*, and *C. lari*. *C. jejuni* comprises two subspecies: subsp. *jejuni* and subsp. *doylei*. *C. jejuni* subsp. *jejuni* represents the taxon first described by Jones *et al.* as *Vibrio jejuni* (Jones *et al.*, 1931) from bovine intestinal contents and commonly occurs as a commensal in a wide range of animal hosts. On the other hand, no animal host has been found for *C. jejuni* subsp. *doylei* (Steele and Owen, 1988) although it has been found in human cases of gastroenteritis and septicaemia (Lastovica and Skirrow, 2000). Subspecies *doylei* differ biochemically from subsp. *jejuni* by the absence of nitrate reduction, cephalothin susceptibility and this subspecies also fails to grow at 42^{o}C. Many molecular-based methods are not suitable for subspeciation (On and Jordan, 2003), but recently a multiplex PCR based on the *nap* locus was proposed for *C. jejuni* subspecies differentiation (Miller *et al.*, 2007).

1.4 Typing of *Campylobacter*

Detection and diagnosis of *Campylobacter* infections is based upon culturing on selective media, Gram's stain and phenotyping (e.g. biochemical tests, fatty acid and/or protein profiles) or genotyping (e.g. DNA fingerprints, sequences, PCR).

1.4.1 Phenotyping

In the absence of the carbohydrate fermentation ability in *Campylobacter,* most phenotyping schemes are focused on differentiating *Campylobacter* according to production of catalase, growth at 42^{o}C or 25^{o}C and resistance to nalidixic acid or cephalothin. The hippurate hydrolysis test is used to distinguish *C. jejuni* from *C. coli* as the latter is generally unable to hydrolyse hippurate (On, 1996). The tolerance of *C. fetus* to glycine and the ability to produce hydrogen sulphide distinguishes this organism from the rest of the taxa. Other typical biochemical characteristics of *Campylobacter* species are reduction of fumarate to succinate; negative methyl red reaction and acetoin and indole production; and for most species, reduction of nitrate, and presence of oxidase activity. A computerised scheme of nearly 70 biochemical tests readily differentiated virtually all *Campylobacter* species (On and Holmes, 1995; On, 1996). Cellular fatty acid and protein profiling, and mass spectrometry have also been used for the identification of bacterial organisms including *Campylobacter* (Costas *et al.*, 1990; Mandrell *et al.*, 2005; Steinbrueckner *et al.*, 1998).

1.4.2 Serotypting

Serotyping is a type of phenotyping and mainly three serotyping schemes are used for *Campylobacter* species, the Penner, Lior and LEP schemes. The Penner and LEP schemes are based on the soluble heat stable (HS) antigens while the Lior scheme is based on heat labile antigens of *Campylobacter.* The Penner serotyping scheme (Penner and Hennessy, 1980) involves the passive haemagglutination of the erythrocytes sensitised with the supernatant of a boiled *Campylobacter* cell suspension; sensitised erythrocytes are then mixed with antisera to demonstrate agglutination. Penner serotype by means of microarrays have revealed that the capsule is the likely Penner serodeterminant (Dorrell *et al.*, 2001). This scheme recognises 65 serotypes and comprises 47 antisera for *C. jejuni* and 15 antisera for *C. coli* (Penner *et al.*, 1983). The Lior scheme, a slide agglutination technique, can recognise up to 130 serotypes of *C.*

jejuni, C. coli and *C. lari* (Lior *et al.*, 1982). A Penner serotyping scheme based LEP (*L*aboratory of *E*nteric *P*athogens) serotyping scheme was described by Frost *et al.* in which passive haemagglutination used in the Penner scheme was replaced by detection of heat-stable antigens by direct bacterial agglutination using absorbed antisera (Frost *et al.*, 1998). This was not proven to be any better than the Penner scheme, with cross reacting isolates still a problem (Oza *et al.*, 2002). The sensitivity of serotyping is poor with over 20% of human strains and 40% of veterinary strains still non-typable (Newell *et al.*, 2000).

1.4.3 Phage typing

The other typing scheme commonly used for *Campylobacter* is phage typing. The phage particles bind to the phase-variable surface structures including capsular polysaccharide and flagella of *Campylobacter* to enter the bacterial cells (Coward *et al.*, 2006) and elicit bacterial cell lysis. Phage typing relies on the lytic reaction patterns of an isolate to different virulent typing bacteriophages. Different phage typing schemes have been used in the USA, Canada and UK, however, 76 defined phage types have been recognised using the UK scheme (Frost, 2001). A simple phage type is defined as two or more unrelated isolates that have the same bacteriophage pattern reaction (Frost *et al.*, 1999). Phage typing is often used in conjunction with heat stable antigen based serotyping.

1.4.4 Molecular typing

Molecular typing or genotyping schemes mainly rely on nucleotide sequence differences between strains. Numerous molecular typing systems have been developed in recent years such as PCR based techniques including restriction fragment length polymorphism, nucleotide sequencing, restriction endonuclease analysis, ribotyping, pulsed-field gel electrophoresis of restricted chromosomal fragments and amplified fragment length polymorphism (Newell *et al.*, 2000).

Restriction fragment length polymorphism (RFLP) is a PCR based method that detects variation in a particular locus e.g. flagellin (*fla* typing) through amplification and subsequent digestion with one or more restriction enzymes (*fla*-RFLP) or direct sequencing (*fla*-amplified short variable regions [*fla*-SVR]) (On *et al.*, 2008b). Amplified fragment-length polymorphism (AFLP) is a RFLP related method.

Conventional AFLP profiling involves digesting DNA with two enzymes and ligation of specific adaptors, which allow PCR amplification of a subset of DNA fragments using fluorescent-labelled primer pairs (On *et al.*, 2008b). Comparative studies of molecular typing techniques suggest that AFLP is equally as discriminatory as pulsed-field gel electrophoresis and multi-locus sequence typing (Lindstedt *et al.*, 2000; Miller *et al.*, 2005; Siemer *et al.*, 2005). Random amplification of polymorphic DNA (RAPD) has also been applied for *Campylobacter* typing. In this technique, PCR of genomic DNA using a single 10 to 15 bp long primer, which binds to multiple sites in the genome, will generate different amplicon patterns in different strains (Acik and Cetinkaya, 2006; On *et al.*, 2008b). Ribotyping detects polymorphism in the ribosomal-DNA loci. Genomic DNA is digested, then hybridised with rRNA-specific probes followed by visualisation of the labelled patterns. This technique is technically demanding, difficult to standardise (On *et al.*, 2008b) and less discriminatory than pulsed-field gel electrophoresis using *Sma*I (Ge *et al.*, 2006).

Pulsed field gel electrophoresis (PFGE) detects polymorphism in the restriction patterns of enzymes, such as *Sma*I, *Kpn*I and *Sal*I that cut the *Campylobacter* chromosome infrequently (Cornelius *et al.*, 2005; Gilpin *et al.*, 2006; Malik-Kale *et al.*, 2007). Although it is a technically demanding and expensive technique, it is widely regarded as among the most discriminatory typing methods for bacterial typing (On *et al.*, 2008b). Comparison between large numbers of isolates have been facilitated by software such as BioNumerics (Applied Maths, Ghent, Belgium), which is electronically able to compare the electrophoresis patterns generated.

Multi-locus sequence typing (MLST) compares DNA sequence differences in several (usually seven) *Campylobacter* house-keeping genes. For each gene; alleles are assigned an arbitrary number. The allele number for all the genes are then combined and given a unique sequence type number. MLST has shown *C. jejuni* to be genetically diverse yet weakly clonal (Dingle *et al.*, 2002; Maiden *et al.*, 1998; Manning *et al.*, 2003b).

In this decade, the sequencing of genomes of *Campylobacter* species has opened up the post-genomic era, and DNA microarrays and down-scaling of detection processes to the Lab-on-chip scale have been found helpful in determining the differences between *Campylobacter* strains (Keramas *et al.*, 2003; Keramas *et al.*, 2004; Poly *et al.*, 2004).

The disadvantage of microarrays is the high cost of the technique and the technically demanding nature of the methodology (Dorrell *et al.*, 2001).

1.5 The *C. jejuni* genome

The progress in the whole-genome sequencing of *Campylobacter* and related bacteria presents an opportunity to deduce all biological aspects of these bacteria. The first full genome-sequence of *C. jejuni* was published in 2000 (Parkhill *et al.*, 2000) which produced many interesting observations including a capacity for polysaccharide biosynthesis and the presence of hypervariable homopolymeric tract sequences. Since then the number of whole genome sequences has increased and currently at least 25 completed and incomplete genome sequences have been obtained for 15 species belonging to the *Campylobacteraceae* (www.genomesonline.org). Based on the completed genome sequences of 6 *C. jejuni* strains NCTC11168 (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000), RM1221 (Fouts *et al.*, 2005), 81-176 (Hofreuter *et al.*, 2006), 269.97, 81116 (Pearson *et al.*, 2007) and CG8486 (Poly *et al.*, 2007b), the average size of the *Campylobacter jejuni* genome is 1.68 Mb, which encodes ~1756 genes and has a G + C content of ~30.57%. Strain to strain variation in genetic content of *C. jejuni* has been observed; the genome of strain NCTC11168 encodes for 1,643 genes while 2,037 genes are encoded by that of *C. jejuni* subsp. *doylei* 269.97. The genome of three human clinical isolates shared 1,474 core genes, with 35 genes unique to 81-176, 38 genes unique to CG8486, and 8 genes unique to NCTC11168; the number of pseudogenes also varied greatly from strain to strain e.g. no pseudogene was found in *C. jejuni* 81-176 while 251 pseudogenes were present in the *C. jejuni* subsp. *doylei* 269.97 genome (Champion *et al.*, 2008).

The genome of all the four clinical isolates of *C. jejuni* that have been sequenced so far did not show the presence of orthologues of virulence factors that have been well characterised in other enteric pathogens (Champion *et al.*, 2008). Unlike *C. jejuni* 81-176 which possesses the pVir and pTet plasmids (Bacon *et al.*, 2000; Batchelor *et al.*, 2004), the genome of strain NCTC11168, RM1221, 81116 and CG8486 do not contain plasmids. The size of plasmids in *Campylobacter* varies from 1.3 to 208 Kb (Aquino *et al.*, 2002; Lee *et al.*, 1994; Miller, 2008) and all plasmids are not associated with virulence (Schmidt-Ott *et al.*, 2005), however, pVir may be important for pathogenesis

in some *C. jejuni* strains (Champion *et al.*, 2008). Pathogenicity islands or insertion sequences were not found in the genome of strain NCTC11168 and CG8486, however, the genome of strain RM1221 is disrupted by four large integrated elements and a *Campylobacter* Mu-like phage (CMLP1) (Fouts *et al.*, 2005). The sequence of 81-176 chromosome also revealed a 6 Kb element with characteristics of an integrated plasmid (Champion *et al.*, 2008).

The genome sequence of *Campylobacter jejuni* NCTC11168 revealed the presence of repeat sequences or homopolymeric G/C tracts that were proposed to be responsible for gene slip in and out of frame or phase variation (Parkhill *et al.*, 2000). The clusters of genes involved in biosynthesis or modification of surface structures e.g. lipooligosaccharide, capsule polysaccharide and flagella biosynthesis and *O*-linked glycosylation are among the most hypervariable regions in the *Campylobacter* genome (Miller, 2008). Pearson *et al.* reported the identification of at least 7 hypervariable plasticity regions (PR1 to PR7) in *C. jejuni* genome using whole genome microarrays. The authors found that 16.3% (269) of the genes present in the first genome sequenced *C. jejuni* NCTC11168 were either absent or highly variable in sequence among the examined *C. jejuni* strains. Most of the variable genes were clustered in groups designated plasticity regions which contained 136 (50%) of the variable gene pool (Pearson *et al.*, 2003).

1.6 Clinical and epidemiological aspects of *Campylobacter* infection

1.6.1 Clinical aspects of disease in humans

Campylobacter cause a diverse range of infections including diarrhoea, abortion, infertility, periodontal diseases or may live commensally without harming their host. *C. jejuni, C. coli* and infrequently *C. upsaliensis, C. lari* and *C. fetus,* are considered human pathogens (Humphrey *et al.*, 2007). *C. jejuni* and *C. coli* mainly are major causes of human bacterial diarrhoea worldwide. *Campylobacter jejuni* infections vary from asymptomatic to severe gastroenteritis characterised by fever, abdominal cramps and inflammatory watery or blood-stained diarrhoea. *C. jejuni* gastroenteritis is usually self-limiting but bacteria may enter blood and cause septicaemia and meningitis especially in immunocompromised patients (Black *et al.*, 1988). The first sign is often

fever, which may be followed by abdominal pains, myalgia and nausea. This is rapidly followed by an acute attack of diarrhoea lasting for 2 to 7 days. Stools are usually extremely watery and often contain blood and mucous (Blaser and Engberg, 2008). In developed countries, young adults are the most common group with disease and the diarrhoea is often associated with an inflammatory colitis and bloody faeces, whereas in developing countries enteritis usually occurs in infants and the diarrhoea is generally profuse and watery (Young and Mansfield, 2005).

Some debilitating neuropathies including Guillain-Barré syndrome (GBS), Miller-Fisher syndrome and septic arthritis have been seen in humans previously infected with *C. jejuni* (Young and Mansfield, 2005; Yuki *et al.*, 1995). GBS is a rare complication but the most common form of acute neuromuscular paralysis. The symptoms generally develop 1-3 weeks after a *C. jejuni* infection and begin with motor and sensory deficits of the lower extremities, which can subsequently spread to the upper extremities and trunk (Mishu and Blaser, 1993). It is estimated that 1 in 1000 infections lead to GBS but the risk increases to 1:200 for a patient infected with a particular *C. jejuni* Penner type HS:19 (Nachamkin, 2002). It has been hypothesised that the patients who develop GBS after *C. jejuni* infection develop antibodies against the bacterial lipooligosaccharides. These antibodies cross-react with the peripheral nerve cell surface gangliosides, leading to an autoimmune response and causing acute neuromuscular paralysis (Penner and Aspinall, 1997; Willison and Yuki, 2002). The less common variant of GBS is Miller-Fisher syndrome (MFS) which is also associated with *C. jejuni* (Yuki *et al.*, 1995). MFS is characterised by acute onset of unsteadiness of gait (ataxia), absence of reflexes and an inability to move the eyes (Ang *et al.*, 2002). Other sequelae of *Campylobacter* infections are the development of septic arthritis which commonly affects the knee joints (Blaser and Engberg, 2008).

The infective dose of *C. jejuni* is often low and infection has been induced with doses as low as 500 bacteria in experimental human infection (Black *et al.*, 1988; Robinson, 1981). Thus campylobacters are much more infectious than enteropathogenic *E. coli* and *Salmonella* Typhi, for which the infective doses are 10^8 – 10^9 and 10^3, respectively (Blaser and Newman, 1982; Donnenberg *et al.*, 1993). On the contrary, *C. jejuni* is not as infectious as other enteric pathogens such as *Shigella* and enterohaemorrhagic *E. coli* for which as few as 10 – 100 microorganisms can cause infection (DuPont *et al.*, 1989; Ge *et al.*, 2002). The typical period from transmission of microorganisms to appearance

of symptoms ranges from 1 to 7 days and the mean incubation period is 3 days (Blaser and Engberg, 2008).

1.6.2 Habitat and sources of infection

Campylobacters are widespread in nature and have been isolated from many water sources including rivers, wells, domestic water supplies contaminated with sewage effluent, as well as surface water from farms (Engberg *et al.*, 1998; Moore *et al.*, 2001). *Campylobacter* species have also been found in a wide range of animals including chickens, turkeys, ducks, geese, ostrich, sheep, cattle, deer, goats, pigs, cats, dogs, rodents, wild birds, shell fish, and even reptiles harbour them (On *et al.*, 2008a). Whilst a diverse range of animals carries campylobacters, each *Campylobacter* species may favour colonisation of different hosts. *C. jejuni* is predominantly found in the guts of poultry, domestic and wild birds whilst *C. coli* is mainly found in pigs and birds (Humphrey *et al.*, 2007).

Human campylobacteriosis is primarily a food-borne disease. *C. jejuni* is usually a gut commensal in most of the food-producing animals and birds, and animal faecal contamination of the food is a well characterised route of transmission to humans. Infection is generally spread by consumption and handling of raw or under-cooked poultry meat and water or milk contaminated with bacteria. Natural hosts of *Campylobacter* species are the reservoir and primary sources of infection in humans as poultry meat accounts for up to 70% of *C. jejuni* infections (Deming *et al.*, 1987). *C. jejuni* lives in the intestine of wild and domestic birds and animals and infection is not only transmitted by contact with/handling these birds or eating contaminated meat but the bacteria released through faeces into the environment contaminate water sources and transmit infection to new hosts consuming this water (Blaser *et al.*, 1980).

1.6.3 Disease burden

The disease burden has been described in different ways. In the US, it has been estimated that food-borne *Campylobacter* infections number around 2.5 million each year (Mead *et al.*, 1999). The World Health Organisation (WHO) estimates that ~1% of Western Europe will be infected with campylobacters each year (Humphrey *et al.*, 2007). This estimate is supported by findings that only 10% of *Campylobacter* cases are reported in England and Wales (Wheeler *et al.*, 1999) . In England and Wales there

were 46,603 culture-confirmed cases of *Campylobacter* reported to health protection agency centre for infection in 2006 (Health Protection Agency, UK) which is 3.4 times higher than the combined cases of *Salmonella*, *Shigella* and *Yersinia* in the same year. The actual numbers of infections in the community are not known, the actual cases of *Campylobacter* infection in 2006 could be as high as 465,000 assuming Wheeler's estimate was correct. The economic burden of *Campylobacter* infection is large, the average cost of a case of acute *Campylobacter* infection (excluding long-term sequelae) in England in 1995 was estimated to be £1315 (Humphrey *et al.*, 2007). Therefore, *Campylobacter* infection costs the UK at least £61 million per annum and the true figure is probably 10-times higher even if we ignore inflation since 1995. Generally duration of illness is not longer than 10 days but restlessness and psychological disturbance also contribute to the disease burden. Patients should be excluded from working as food handlers until they have been symptom-free for 48 hours, however, some food businesses may ask infected employees to submit stool samples for testing in line with their own occupational health requirements which will keep them away from work for longer (Humphrey *et al.*, 2007).

1.7 Pathogenesis and host response to *Campylobacter* infection

Campylobacter jejuni infection is generally transmitted to humans by consumption of undercooked poultry meat, contaminated water and unpasteurised milk (Allos and Blaser, 1995). The bacteria pass through an acidic environment of the stomach and colonise the small intestine in the early stage of infection, they later move to the large intestine and may cause local acute inflammatory changes in both the small and large intestine but the large intestine is the major target (Black *et al.*, 1988). Bacterial adhesion and invasion of the intestinal epithelium is a well-established early event before initiation of inflammatory processes and diarrhoeal development (Ketley, 1997). *C. jejuni* colonises a range of animals commensally without causing a disease similar to that observed in humans, thus the lack of a convenient animal model that mimics the disease in humans has severely hampered investigations into the molecular mechanisms of *C. jejuni* infection. However, some host processes and bacterial determinants involved in the pathogenesis of the bacteria have been determined using studies including animal models and *in vitro* cell culture. The host response to *C. jejuni*

infection and the bacterial factors that contribute to its pathogenesis will be briefly discussed below.

1.7.1 Host response to *C. jejuni* infection

C. jejuni mainly colonise the human and chicken gut; it can also colonise the intestinal tract of other animals. The bacteria have to bypass the mechanical and immunological barriers of the gastrointestinal tract to establish an infection. The human stomach's highly acidic environment provides the first line of defence against infection (Black *et al.*, 1988), the mucous layer overlying the intestinal epithelium serves as another line of defence in the intestine. *C. jejuni* has developed many traits that contribute to its ability to pass through these barriers. These include the motility and corkscrew morphology of *C. jejuni* (Young *et al.*, 2007) and the relatively short *O*-side chain of its lipooligosaccharide (LOS), which is proposed to reduce its non-specific binding to the mucin glycoproteins (McSweegan and Walker, 1986). The high concentrations of bile salt in the intestine can kill bacteria by disrupting bacterial cell membranes (Sung *et al.*, 1992), however, *C. jejuni* is resistant to bile salts present in the human intestine (Lin *et al.*, 2003).

In addition to the acidic pH of the stomach and the physical barrier of the intestinal epithelial layer, the human body has also acquired immune defences to defend itself from microbial insult. The human acquired immune defence can be divided into two categories: cell-mediated and humoral or antibody-mediated, which will be described briefly.

1.7.1.1 Cell-mediated immunity

C. jejuni infection in naïve immunocompetent adults clears before the adaptive humoral immune response is mounted (Blaser and Engberg, 2008); thus, the major immune response to clear *C. jejuni* from human body is cell-mediated immunity and presumably involves the dendritic cells (DCs) and macrophages (Rathinam *et al.*, 2009; Young *et al.*, 2007). The epithelial lining of the gut not only provides a physical barrier protecting the underlying mucosa from the external environment, but it also plays an important role in microbial sensing and mounting appropriate innate immune responses by the production of chemokines, cytokines and antimicrobial peptides (Eckmann, 2005; Kagnoff and Eckmann, 1997).

The NF-κB family of transcription factors are the central regulators in early host immune responses to infection. The production of NF-κB in intestinal epithelial cells is stimulated in response to *C. jejuni* infection (Chen *et al.*, 2006; Mellits *et al.*, 2002) which consequently initiates expression of several innate immune genes (Sansonetti, 2006). In a previous study, NF-κB-deficient mice that were orally infected with *C. jejuni* 81-176 developed gastritis and duodenitis in contrast to wildtype mice suggesting the role of NF-κB in innate defence against *C. jejuni* (Fox *et al.*, 2004).

C. jejuni infection of human monocytes has shown the induction of proinflammatory cytokines IL-1α, IL-1β, IL-6, tumour necrosis factor alpha (TNF- α) and IL-8 (Hickey *et al.*, 2005; Jones *et al.*, 2003). Interleukin-8 (IL-8) is a proteolytic chemokine produced by myeloid and epithelial cells. It is a potent chemoattractant known to recruit lymphocyte and neutrophils to the site of local inflammation. Activation of extracellular signal-regulated kinase (ERK) and p38 mitogen activated protein (MAP) kinase pathways by *C. jejuni* infection is essential for IL-8 production (Watson and Galan, 2005). IL-8 production triggered by enteric pathogens also requires signalling through NF-κB (Hobbie *et al.*, 1997; Mellits *et al.*, 2002; Philpott *et al.*, 2000). *C. jejuni* adhesion/invasion (Hickey *et al.*, 1999) and the presence of cytolethal distending toxin (Hickey *et al.*, 2000; Zheng *et al.*, 2008) are crucial mediators of IL-8 production in intestinal epithelial cells. The degree of IL-8 induction varies in *in vitro* models of infection and the response is dependent on both the bacterial strain and cell line used (MacCallum *et al.*, 2006). Recently a study showed a correlation between the ratio of pro-inflammatory and anti-inflammatory cytokine levels with histological changes in a mouse lung model of *C. jejuni* infection (Al-Banna *et al.*, 2008) that suggests the important role of cytokines in *C. jejuni in vivo* infection.

Defensins are a major family of antimicrobial, cysteine-containing cationic peptides produced by neutrophils and mammalian epithelia (Iovine, 2008). Like human α defensins, β defensins 1 (HBD1) are constitutively expressed in cells throughout the gastrointestinal tract (Lehrer *et al.*, 1993), whereas expression of the inducible β defensins 2, 3 and 4 is activated by proinflammatory cytokines (Iovine, 2008). *C. jejuni* induced the expression of human β defensins 2 and 3 in human intestinal epithelial cell lines Caco-2 and HT-29 but did not affect expression of the β defensins 1 (Zilbauer *et al.*, 2005). Defensins contribute to innate defence against *Campylobacter* in poultry as

well. The avian defensins called gallinacins may also contribute to the resistance of chickens to *Campylobacter* infection (van Dijk *et al.*, 2007).

C. jejuni pass through the mucous layer, adhere to and invade the intestinal epithelial lining (Ketley, 1997). Where invasion of bacteria in intestinal epithelial cells protects them from phagocytosis by neutrophils, monocytes/macrophages and dendrictic cell, it also exposes them to Toll-like receptors (TLRs). TLR5, which is expressed on the apical side of human intestinal epithelial cells, recognises flagellin of enteropathogens such as *Salmonella* leading to activation of NF-κB (Hayashi *et al.*, 2001). TLR5-dependent signalling is activated by conserved amino acid residues on positions 89 through 96 in bacterial flagellin (Andersen-Nissen *et al.*, 2005); however, *C. jejuni* and *Helicobacter* use alternative amino acids at these positions rendering them unrecognisable by TLR5 (Andersen-Nissen *et al.*, 2005). TLR9, which recognises unmethylated CpG dinucleotides, is also not stimulated during *C. jejuni* infection (Dalpke *et al.*, 2006), most probably due to the AT-rich genome of this organism. TLR4 senses *C. jejuni* LOS and triggers an NF-κB-dependent cascade leading to the production of proinflammatory cytokines (Iovine, 2008). The role of TLR-2 and TLR-4 in inducing an immune response against *C. jejuni* has recently been suggested using murine dendritic cells (Rathinam *et al.*, 2009). The role of other TLRs in eliciting host responses during *C. jejuni* infection is yet unknown; however, the cytoplasmic pathogen-recognition receptor NOD1 plays an important role in immune stimulation by *C. jejuni* (Zilbauer *et al.*, 2007).

C. jejuni infection in humans is characterised by the influx of neutrophils and other phagocytic cells (van Spreeuwel *et al.*, 1985b). The direct antimicrobial activities of these phagocytes are attributable to the production of antimicrobial peptides/proteins and reactive oxygen species (Iovine, 2008). The ability of phagocytes to ingest and kill *Campylobacter* is strain-dependent and varies with the host source of the phagocytes (Wassenaar *et al.*, 1997; Wooldridge and Ketley, 1997). Generally phagocytosis of *C. jejuni* is enhanced by opsonins such as complement (Blaser *et al.*, 1985). Extremely limited availability of iron inside host tissue is thought to represent a non-specific host defence system against pathogenic bacteria (Schaible and Kaufmann, 2004). The intestinal mucous layer contains the iron-sequestering protein lactoferrin which makes the majority of iron unavailable for bacteria (Ward and Conneely, 2004). *C. jejuni* have to cope with iron-restricted conditions for successful colonisation of the intestine and

they acquire iron using the iron chelator protein FeoB and iron released from dying host cells during the inflammatory process (Stintzi *et al.*, 2008).

1.7.1.2 Antibody-mediated immunity

Epidemiological studies have provided the evidence of protective humoral immunity against *Campylobacter*-mediated diarrhoea. A survey in breast-fed Mexican infants showed a lower incidence of *Campylobacter*-associated diarrhoea in those infants whose mothers had *Campylobacter*-specific secretory IgA (sIgA) antibodies in breast milk (Ruiz-Palacios *et al.*, 1990). In the developing world where *Campylobacter* exposure is frequent, campylobacteriosis is more common in early childhood and a *Campylobacter*-specific antibody-mediated immune response starts developing within the first 2 years of life which is followed by continued increase in IgA levels and it is accompanied by lower incidence of illness and milder symptoms of *Campylobacter* infection in adults (Tribble *et al.*, 2008). On the other hand, in the industrialised world where hygienic conditions are much better than the developing regions, *Campylobacter* enteritis generally occurs in adult patients who manifest moderate to severe symptoms and dysentery consistent with naïve or a semi-immune status (Tribble *et al.*, 2007). A secretory immunoglobulin A (sIgA) response at the intestinal mucosa is considered the primary specific defence against *Campylobacter*. In the case of *Campylobacter*, flagellin, outer-membrane proteins and LOS elicit sIgA and IgG responses (Black *et al.*, 1988; Cawthraw *et al.*, 2002) and *Campylobacter*-specific secretory IgA and serum IgA antibodies levels correlate with protection against disease (Tribble *et al.*, 2008). Immunoglobulin G which mainly provides protection in blood and tissue can be detected in serum a few days post-infection and their levels peak 2-3 weeks after infection (Cawthraw *et al.*, 2002) which persisted after clearance but elevated levels of IgA were detected only from onset of symptoms until the clearance of *C. jejuni* (Kaldor *et al.*, 1983).

In chickens, both the innate and humoral responses are activated against *C. jejuni;* new born chicks carry *Campylobacter*-specific maternal antibodies for the first two weeks of their life. Such antibodies which recognise LOS, outer membrane proteins and flagellin (Cawthraw *et al.*, 1994), lead to complement-mediated killing of *C. jejuni* in a strain specific manner (Sahin *et al.*, 2003). The chick produces flagellin-specific antibodies by the age of 3 weeks (Cawthraw *et al.*, 1994) but unlike humans, no antibodies are

produced against cytolethal distending toxin (Abuoun *et al.*, 2005) and a poor immune response is elicited against the *C. jejuni* capsule (Jeurissen *et al.*, 1998).

1.7.2 Bacterial virulence determinants

The bacterial pathogens use their weapons, the bacterial determinants, to establish an infection and develop the pathological changes in their host. A reasonable understanding of clinical and epidemiological aspects of *C. jejuni* infection has been achieved. Though molecular basis of *C. jejuni* pathogenesis is not fully understood, many bacterial factors that contribute to the pathogenesis of this organism have been identified; following is the brief description of these determinants.

1.7.2.1 Cytolethal distending toxin

Cytolethal distending toxin (CDT) is produced by many Gram-negative bacteria, including *E. coli*, *Haemophilus ducreyi*, *Shigella dysenteriae*, and *Helicobacter* species (Cope *et al.*, 1997; Okuda *et al.*, 1995; Peres *et al.*, 1997; Young *et al.*, 2000b). CDT was first described in *C. jejuni* in 1987 (Johnson and Lior, 1988) and since then considerable progress has been made in determining its role in disease and pathogenesis. The toxin causes the arrest of the eukaryotic cell cycle at the G_2/M transition, preventing them to enter mitosis, leading to cell death (Lara-Tejero and Galan, 2000; Whitehouse *et al.*, 1998). The toxin was named after the morphological changes associated with its action: cytoplasmic distension. The active holotoxin is a tripartite complex of CdtA, CdtB and CdtC (Lara-Tejero and Galan, 2001). The CdtB is the toxic component and microinjection or transfection of this subunit alone into host cells leads to the effects that are observed with the holotoxin (Lara-Tejero and Galan, 2000). The B subunit of CDT shares similarity with a family of DNase I-like proteins and is thought to act as a DNase. It localises to the nucleus of host cells and causes DNA damage by double-strand breaks (Hassane *et al.*, 2003). The functions of CdtA and CdtC subunits are not clear but they might mediate binding to host cells. CdtA and CdtC have some similarity to the B chain of the ricin toxin, which is responsible for receptor-mediated endocytosis of ricin (Lara-Tejero and Galan, 2001). Moreover, CdtA and CdtC bind HeLa cells with specificity, probably using the same receptor (Lee *et al.*, 2003).

CDT is responsible for the secretion of interleukin (IL)-8, a hallmark of *C. jejuni* pathogenesis, but there are also CDT-independent mechanisms of IL-8 stimulation (Hickey *et al.*, 1999; Hickey *et al.*, 2000). CDT might have a role in asymptomatic, commensal infections, which would provide a way to either avoid host immune-response mechanisms or redirect them towards tolerance. *In vitro*, *C. jejuni* CDT induces cell death by apoptosis in human monocytic cells (Hickey *et al.*, 2005). In a study that used a mouse model of *H. hepaticus* colonisation it was suggested that CDT has a function in immune modulation and persistent colonisation (Ge *et al.*, 2005; Pratt *et al.*, 2006). In a murine model experiment, the persistent colonisation of NF-κB-deficient, but not wildtype mice, with wildtype *C. jejuni* 81-176 and its CdtB mutant was associated with a significantly impaired humoral antibody response in NF-κB-deficient mice which indicates that CDT might allow *C. jejuni* to escape immune surveillance in an NF-κB-dependent manner (Fox *et al.*, 2004).

1.7.2.2 Lipooligosaccharides

Like many Gram-negative pathogenic bacteria, *C. jejuni* has surface polysaccharides including lipooligosaccharides (LOS) and capsule. Lipopolysaccharides (LPS) and LOS are anchored to the outer membrane of bacteria by lipid A and are considered toxic with potent immuno-modulating and immuno-stimulating properties (Gilbert *et al.*, 2008). High molecular weight LPS is composed of a polymer of repeating oligosaccharide units linked to the core oligosaccharide of 10 -15 sugar residues which are anchored to lipid A. In contrast to LPS, LOS has a low molecular weight and lacks repeating oligosaccharide chain and consists of an outer core of non-repeating oligosaccharides anchored to lipid A (Gilbert *et al.*, 2008). The outer core of *C. jejuni* LOS is highly variable (Dorrell *et al.*, 2001; Parker *et al.*, 2008) and is capable of mimicking human gangliosides. This mimicry can induce the post-infection autoimmune neurological disorders Guillain-Barré syndrome and Miller-Fisher syndrome (Guerry and Szymanski, 2008; Willison and Yuki, 2002; Yuki *et al.*, 1995). *C. jejuni* is capable of the endogenous synthesis of sialic acid for incorporation into ganglioside-like LOS cores. Mutants that lacked LOS core sialylation showed reduced reactivity with GBS patient serum and failed to induce an anti-ganglioside antibody response in mice (Godschalk *et al.*, 2007). LOS and its sialylation are important for the virulence of this organism. Strains expressing sialylated LOS were more invasive in cultured epithelial cells than strains expressing non-sialylated LOS (Louwen *et al.*, 2008). Moreover, mutation in

sialyltransferase (*Cst*-II) reduced the invasiveness in the mutants (Louwen *et al.*, 2008). In *C. jejuni* the inactivation of *galE*, a gene important for biosynthesis of LOS, resulted in organisms with a reduced potential to invade epithelial cells *in vitro* (Fry *et al.*, 2000). Mutants in *cgtA*, which encodes an *N*-acetylgalactosaminyltransferase in *C. jejuni* 81-176, exhibited increased bacterial attachment and invasion of human cells, and increased protection from complement mediated killing (Guerry *et al.*, 2002). Deletion of the LOS biosynthesis locus in *C. jejuni* NCTC11168 showed attenuated growth, loss of invasion and natural transformation efficiency of the mutant (Marsden *et al.*, 2009). LOS is also involved in natural transformation competence and antibiotic resistance in *C. jejuni*. LOS truncation resulted in increased sensitivity to antibiotics and an increased natural transformation efficiency in the mutant (Jeon *et al.*, 2009; Marsden *et al.*, 2009).

1.7.2.3 Capsule

Many bacteria contain cell surface structures called capsules which are generally polysaccharide in nature however the capsule of bacteria including *Yersinia pestis* (Karlyshev *et al.*, 1992), *Bacillus anthracis* (Ezzell and Welkos, 1999), *Francisella tularensis* (Su *et al.*, 2007) and the S-layer of *C. fetus* (Blaser *et al.*, 1988) are made up of polypeptides. Unlike LPS and LOS, capsule polysaccharides are loosely attached to the bacterial cell surface via a phospholipid moiety and are susceptible to the action of phospholipases (Karlyshev *et al.*, 2008). Until recently, many strains of *C. jejuni* were thought to produce both LOS and a high molecular weight LPS. Sequencing of the first *C. jejuni* genome showed the presence of capsule biosynthesis genes and subsequent mutagenesis of these genes demonstrated that these high molecular weight LPS were actually capsular polysaccharides (Karlyshev *et al.*, 2000). Capsule is also the major serodeterminant of the Penner serotyping scheme (Karlyshev *et al.*, 2000). Capsule polysaccharides (CPS) play an important role in resistance to desiccation, the bactericidal activity of serum, as well as phagocytic uptake and may also contribute to intraphagocytic survival of the organism (Guerry and Szymanski, 2008). CPS can exhibit structural variation and confer resistance to complement-mediated killing of the bacteria. Indeed, loss of the *C. jejuni* capsule through inactivation of *kpsM* in *C. jejuni* resulted in organisms which were more sensitive to complement and which displayed reduced virulence in the ferret model (Bacon *et al.*, 2001). A study found that the capsule provides no greater protection to *C. jejuni* against the potent antimicrobial action of human β-defensins 2 or 3 (Zilbauer *et al.*, 2005), however, capsule may

provide protection against β-defensins 1 and lysozyme (Zilbauer *et al.*, 2008). Capsule also confers other virulence traits to bacteria. Inactivation of *kpsE* in *C. jejuni* 81116 resulted in reduced adhesion and invasion in cultured epithelial cells; however, the ability to colonise the chicken gut was not affected in the mutant (Bachtiar *et al.*, 2007). Bacterial cell-surface polysaccharides including capsule are required for biofilm formation (Moran and Annuk, 2003), which contributes to virulence of bacteria and leads to increased resistance to antibacterial drugs (Jeon *et al.*, 2009). In a recent report, the expression of *C. jejuni* capsule polysaccharides, bacterial invasion and serum resistance were reduced when bacterial cells were co-cultured with epithelial cells (Corcionivoschi *et al.*, 2009) which shows the interesting link between bacterial capsule and virulence.

1.7.2.4 Glycosylation system

Glycosylation of proteins is necessary for extending protein life, protease resistance and biological activity (Nothaft *et al.*, 2008). It had long been believed that post-translational *N*-linked glycosylation of proteins is a eukaryotic phenomenon and that bacteria only express non-glycosylated proteins. In 1989, post-translational modification of flagellin in *C. jejuni* was discovered (Logan *et al.*, 1989). Following this observation, other studies found that *Campylobacter* express two protein glycosylation systems: one modifies serine or threonine residues on flagellin (*O*-linked glycosylation) and the other modifies asparagine residues on many proteins (*N*-linked glycosylation) (Szymanski *et al.*, 1999; Szymanski *et al.*, 2003; Szymanski and Wren, 2005).

O-linked glycosylation in *C. jejuni* was first observed in flagellin and the gene cluster encoding for *O*-linked glycosylation mapped adjacent to the flagellin structural genes in one of the most hypervariable regions of the *C. jejuni* chromosome. The flagellin in *C. jejuni* 81-176 is glycosylated with pseudaminic acid (Guerry and Szymanski, 2008), whereas, the flagellin of *C. coli* VC167 is modified with legionaminic acid (Young *et al.*, 2007). More recently, in addition to the pseudaminic acid and legionaminic acid, two unusual 2,3-di-O-methylglyceric acid modifications of a nonulosonate sugar were identified in *C. jejuni* 11168 (Logan *et al.*, 2009). *O*-linked glycosylation of flagellin is necessary for the proper assembly of the flagellar filament (Goon *et al.*, 2003), thus defects in *O*-linked glycosylation result in a loss of motility and autoagglutination, a

decrease in the adherence to and invasion of host cells and decreased virulence in ferrets (Guerry *et al.*, 2006).

Unlike other polysaccharide surface structures of *C. jejuni*, the *pgl* genes encoding for the *N*-linked glycosylation system are conserved in *C. jejuni* and *C. coli* (Dorrell *et al.*, 2001; Szymanski *et al.*, 2003). The *N*-linked glycan which is assembled by the Pgl system in *Campylobacter* consists of a heptasaccharide, unlike the tetrasaccharide that is transferred by the eukaryotic glycosylation system (Young *et al.*, 2007). The role of *N*-linked glycosylation in the biology of *C. jejuni* is not clear. However, strains with an inactivated Pgl system show defects in natural competence (Larsen *et al.*, 2004) as well as reduced adherence and invasion in INT-407 cells and colonisation in chicken and mouse models (Kakuda and DiRita, 2006; Karlyshev *et al.*, 2004; Szymanski *et al.*, 2002). The *pgl* gene mutation affects the glycosylation of numerous proteins and the resulting pleiotropic effects also include reduced protein reactivity to both human immune and Penner typing antisera (Nothaft *et al.*, 2008).

1.7.2.5 Flagella

In humans, campylobacters mainly colonise the mucus filled crypts of the intestine. To survive and replicate in this viscous environment, flagella and the spiral-curved shape of *Campylobacter* aid its movement giving it a corkscrew motion (Shigematsu *et al.*, 1998). A rapid and darting motility of *Campylobacter* is mediated by polar flagella which is also one of the most well-characterised virulence determinants. The flagellum consists of an unsheathed polymer of flagellin subunits encoded by *flaA* and *flaB* that share 95% sequence homology (Nuijten *et al.*, 1990) and are independently transcribed. Transcription of *flaA* is regulated by σ^{28} (encoded by *fliA*) whereas *flaB* expression is regulated by σ^{54} (encoded by *rpoN*) and this regulation is controlled by two component system FlgRS (Carrillo *et al.*, 2004; Hendrixson and DiRita, 2003; Hendrixson, 2006; Sommerlad and Hendrixson, 2007; Wösten *et al.*, 2004). Mutation of *flaA* results in the production of a truncated flagellar filament composed of FlaB and having a severe reduction in motility (Wassenaar *et al.*, 1991). Mutants in *flaB*, in contrast, showed no significant change in motility and produced flagellar filament that appears structurally normal (Guerry *et al.*, 1991; Nuijten *et al.*, 1990; Wassenaar *et al.*, 1991). Flagella of *C. jejuni* are not only essential for motility but the importance of flagella in virulence of *C. jejuni* was realised in early studies. Experimentally intact flagella are necessary for

optimal colonisation of birds, model animals and humans (Black *et al.*, 1988; Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993). *C. jejuni* mutants in motility were reduced in adhesion and invasion in cultured eukaryotic cells (Grant *et al.*, 1993; Wassenaar *et al.*, 1991; Yao *et al.*, 1994). There are several reports that in the absence of a type-III secretion system in *C. jejuni,* flagella can function to secrete Cia proteins that might modulate virulence (Konkel *et al.*, 1999; Konkel *et al.*, 2004; Rivera-Amill *et al.*, 2001). One of the secreted proteins, CiaB, was found important for *C. jejuni* F38011 invasion into INT-407 cells (Konkel *et al.*, 1999). Secretion of FlaC which shares 77% similarity to FlaA but has no role in motility is proposed to be secreted through flagella. Its secretion was not observed in mutants in *flgE* (encoding the flagellar hook) and *flgC, flgI* or *flgK* which encodes the basal-body (Poly *et al.*, 2007a; Song *et al.*, 2004). The *flaC* gene is involved in virulence as mutation of *flaC* in *C. jejuni* TGH9011 resulted in a 86% reduction in invasion in HEp-2 cells (Poly *et al.*, 2007a), however a *flaC* mutant of *C. jejuni* stain 81-176 showed 58% less invasion in INT-407 cells compared to the wildtype (Goon *et al.*, 2006). Though not well-established in *C. jejuni,* autoagglutination is often associated with virulence of other pathogens (Chiang *et al.*, 1995; Frick *et al.*, 2000; Sherlock *et al.*, 2005). Flagella of *C. jejuni* have been shown to mediate autoagglutination (Golden and Acheson, 2002) and glycosylation of flagellins is also important for autoagglutination in this organism (Guerry *et al.*, 2006). The functional aspects of *Campylobacter* flagella have recently been reviewed elsewhere (Guerry, 2007).

1.7.2.6 Chemotaxis

Bacteria respond to changes in their environmental conditions and change their temporal position to avoid unfavourable conditions and move towards favourable niches. Chemotaxis, where the bacterial cell moves in response to a chemical signal, is regulated by alternating between the clockwise and counter-clockwise rotation of the flagellum (Korolik and Ketley, 2008; Young *et al.*, 2007). *C. jejuni* displays chemotactic motility towards amino acids that are found in high levels in the chick gastrointestinal tract and towards components of mucous (Hugdahl *et al.*, 1988; Tu *et al.*, 2008). Chemotactic motility has an important role in the commensal lifestyle and is essential for colonisation and pathogenicity of *C. jejuni*. Non-chemotactic mutants failed to colonise in a suckling mouse model (Takata *et al.*, 1992). Mutants in methyl-accepting chemotaxis receptors (*Cj0019c* or *Cj0262c*) showed decreased chicken

colonisation ability (Hendrixson and DiRita, 2004). Strains that lack CheY, a response regulator that controls flagellar rotation, showed decreased virulence in a ferret disease model (Yao *et al.*, 1997), reduced chicken colonisation (Hendrixson and DiRita, 2004) but appeared to be hyperinvasive (Golden and Acheson, 2002). Bioinformatics based genetic analysis showed that *C. jejuni* transduces an energy taxis (aerotaxis) signal using two proteins, CetA and CetB, in place of the single Aer protein used by *E. coli* and other species (Hendrixson *et al.*, 2001; Young *et al.*, 2007). *C. jejuni* mutants of chemoreceptor CetB (*Cj1189*) were deficient in their ability to invade cultured human intestinal epithelial cells (Golden and Acheson, 2002). In a recent report invasion of cells via the subcellular route (subvasion) was increased in *C. jejuni* in which expression of CheW, a scaffolding protein, was decreased (van Alphen *et al.*, 2008). This all clearly demonstrates that there are some interesting connections between the *C. jejuni* chemotactic response and host cell interactions.

1.7.3 Animal models of disease

C. jejuni causes gastroenteritis in humans but the infection is asymptomatic in chickens and most other animals. The basis of the different outcomes in different hosts are not well understood (Zilbauer *et al.*, 2008). This is partly due to lack of a good small animal model that reproduces human disease. Different animal models including chickens (Biswas *et al.*, 2007; Hendrixson and DiRita, 2004; Jones *et al.*, 2004), hamsters (Humphrey *et al.*, 1985), ferrets (Bell and Manning, 1990), dogs (Olson and Sandstedt, 1987), primates (Russell *et al.*, 1989; Russell *et al.*, 1993), rabbits (Burr *et al.*, 1988; Caldwell *et al.*, 1983; Walker *et al.*, 1988), mice (Baqar *et al.*, 2008; Watson *et al.*, 2007; Ziprin *et al.*, 2001), guinea pigs (SultanDosa *et al.*, 1983) and swine (Boosinger and Powe, 1988; Harvey *et al.*, 2001; Mansfield and Gauthier, 2004) have all been used to study different aspects of *Campylobacter* infection. These models have been useful in understanding mechanisms of *Campylobacter*-induced disease and in determining different bacterial factors that contribute to disease by using bacterial strains in which different potentially virulence-related genes were mutated (Abuoun *et al.*, 2005; Baqar *et al.*, 2008; Fox *et al.*, 2004; Hendrixson and DiRita, 2004; MacKichan *et al.*, 2004), but limitations exist with these models. Monkeys and ferrets infected with pathogenic *C. jejuni* isolates can exhibit symptoms of disease that are seen in humans, including

inflammation of the gut and diarrhoea (Fox *et al.*, 1987), but high cost and lack of knockout technology to study the host factors that are involved in disease reduced the attractiveness of these models. However, knock-out mice models have recently been used to study *Campylobacter* disease mechanisms and host immune responses (Fox *et al.*, 2004; Mansfield *et al.*, 2007; Watson *et al.*, 2007) and may prove useful in understanding the host factors that contribute to *C. jejuni* infection. Chicken is a good model for studying the host colonisation and is potentially a good target for designing strategies to reduce *Campylobacter* infection in humans. Animal models of disease have their own limitations including high cost, inconsistency in results, ethical and legal processes involved in these studies as well as lack of disease and post-infection symptoms that mimic human disease. In addition to natural and experimental models of animal infection, much of the data on pathogenic mechanisms used by *C. jejuni* has come from *in vitro* studies using cultured human and animal cells.

1.7.4 Interaction of *Campylobacter jejuni* with host cells

Bacterial diseases of the gastrointestinal tract typically result from multifactorial processes of interactions between the offending bacteria and the host. Humans have developed a variety of innate mechanisms including mucous layer, epithelial barrier and gastric acidity to protect themselves from pathogenic organisms but at the same time bacteria have also evolved pathogenic attributes to circumvent these innate host defences. The understanding of interactions of *C. jejuni* with host cells is very important for understanding the mechanisms of pathogenesis. A brief description of interactions of *C. jejuni* with the host is given below.

1.7.4.1 Colonisation

Campylobacter is transmitted to the host through contaminated food and after passage through the stomach these organisms colonise the intestinal mucosa of most vertebrate hosts (Newell and Fearnley, 2003). In chickens, this colonisation is commensal, with *C. jejuni* found at highest levels in the mucosal crypts of caeca and to a lesser extent in the small intestine (Beery *et al.*, 1988). However, in humans *C. jejuni* mainly colonises the colon and to a lesser extent the small intestine, and colonisation can lead to acute enteritis (Black *et al.*, 1988). The surface of the intestinal tract is covered with a

viscous, mucin-rich mucous layer that provides lubrication and protection to underlying epithelial cells against chemical, enzymatic and microbial insult (Tu *et al.*, 2008). Mucins are major chemoattractants for *C. jejuni* (Hugdahl *et al.*, 1988) and the bacterium exhibits higher adherence and invasion in Caco-2 cells in the presence of mucous (Szymanski *et al.*, 1995). Most pathogenic bacteria including *C. jejuni* that colonise the gastrointestinal tract subvert the mucous barrier by producing mucolytic enzymes and effective motility (Wassenaar *et al.*, 1993). The flagella and flagellar motility is very important in host gut colonisation by *C. jejuni* (Black *et al.*, 1988; Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993). Exposure of *C. jejuni* to mucin up-regulates the expression of *flaA* but not *flaB* and genes encoding mucin-degrading enzymes including *Cj1344c* and *Cj0843c* (Tu *et al.*, 2008). Additionally, mutants in genes that encode the flagellin and flagellar biosynthesis regulators FlgR, σ^{54} and σ^{28} all display defects in host colonisation (Fernando *et al.*, 2007; Hendrixson and DiRita, 2004; Hendrixson, 2006; Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993; Wösten *et al.*, 2004). Two component regulatory systems of *C. jejuni* are important for chicken gut colonisation; the DccRS regulated genes whose function is yet unknown are important for growth and/or chicken gut colonisation (MacKichan *et al.*, 2004), and the RacRS regulatory system of *C. jejuni* is required for the organism's survival at avian body (higher) temperature and colonisation in the chicken gut (Bras *et al.*, 1999). Another two-component regulator that regulates the ability of enteric bacteria to resist the antimicrobial effects of bile is also important for their colonisation of the host intestinal tract. A mutant in CprS part of the two-component regulatory system, CprRS which regulates the *Campylobacter* planktonic growth showed a reduction in chicken colonisation (Svensson *et al.*, 2009). Inactivation of the *Campylobacter* bile resistance regulator, CbrR encoded by *Cj0643*, resulted in the reduction in deoxycholate resistance and chicken colonisation in *C. jejuni* strain F38011 (Raphael *et al.*, 2005).

Glycosylation systems, chemotaxis and the ability to degrade reactive oxygen species are also important for host gut colonisation in *C. jejuni.* The mutants with inactivated *O*-linked and *N*-linked glycosylation system were severely affected in their ability to colonise chickens (Howard *et al.*, 2009; Kakuda and DiRita, 2006; Karlyshev *et al.*, 2004). Signature tagged-mutagenesis of *C. jejuni* in a chicken model of infection resulted in the identification of two adjacent genes, one was a methyl-accepting chemotaxis protein (MCP) and the other was a putative cytochrome peroxidase

(Hendrixson and DiRita, 2004). A *C. jejuni* 81-176 mutant of *luxS* encoding for an autoinducer-2 biosynthesis enzyme showed reduced chicken gut colonisation potential and chemotaxis toward organic acids (Quinones *et al.*, 2009) suggesting the importance of chemotaxis in host gut colonisation. Recently two genes, *docA* and *Cj0382*, encoding for a putative cytochrome C peroxidase showed a modest decrease in chick colonisation ability of *C. jejuni* 81-176 (Bingham-Ramos and Hendrixson, 2008). Other bacterial determinants involved in chicken gut colonisation include *znuA* (*Cj0143c*) encoding for a periplasmic component of a putative Zinc ABC transport system (Davis *et al.*, 2009), γ-glutamyl transpeptidase (Barnes *et al.*, 2007), an autotransporter protein, CapA (Ashgar *et al.*, 2007), CmeR, a transcriptional repressor modulating the expression of the multidrug efflux pump CmeABC in *Campylobacter jejuni* (Guo *et al.*, 2008), *kpsM* encoding for capsule polysaccharide export protein (Jones *et al.*, 2004) which is in contrast to another report that found that capsular polysaccharide is not important for chicken gut colonisation (Bachtiar *et al.*, 2007), *Campylobacter* outer membrane protein, CadF (Ziprin *et al.*, 1999) and *Campylobacter* secreted protein, CiaB (Ziprin *et al.*, 2001).

Human intestine colonisation by *C. jejuni* is usually accompanied by inflammatory disease whereas the chicken gut colonisation is asymptomatic. This difference in the outcome of gut colonisation may be due to differences in body temperature and/or immune responses of both the hosts. Recently a study suggested that the expression of *Cj0414* and *Cj0415* encoding for a putative gluconate dehydrogenase in *C. jejuni* 81-176 was higher at chicken body temperature (42^{o}C) than at human body temperature (37^{o}C). The mutant in *Cj0415* had defective chicken colonisation but not in mice whose body temperature is 37^{o}C and the authors suggested that the ability of *C. jejuni* to use gluconate as an electron donor via GADH activity is an important metabolic characteristic that is required for full colonization of avian but not mammalian hosts (Pajaniappan *et al.*, 2008). A recent study in which the immune response of chicken against *C. jejuni* colonisation was investigated, found that *Campylobacter* infection significantly reduced expression of seven antimicrobial peptide (AMP) genes (Meade *et al.*, 2009). These antimicrobial peptides play an important role in clearing the bacteria from host intestinal surfaces (Zilbauer *et al.*, 2008) and the reduced expression of the antimicrobial peptides in chickens infected with *Campylobacter* may be involved in prolonged chicken colonisation of bacteria (Meade *et al.*, 2009).

1.7.4.2 Adhesion and invasion

Bacterial disease of the enteric tract generally results from a set of complex interactions between the causative bacterial organism and the host. Many bacterial pathogens including *Yersinia enterocolitica*, *Salmonella, Shigella* species and *C. jejuni* develop specific interactions e.g. adherence and/or invasion with host mucosa to initiate disease (Hu and Kopecko, 2008). *Campylobacter* invasion into the epithelial mucosa appears to be an essential process leading to the disease outcome i.e. gastroenteritis (Ketley, 1997). The results of analysis of intestinal biopsy samples of patients infected with *C. jejuni* (van Spreeuwel *et al.*, 1985b), as well as experiments with infected primates (Russell *et al.*, 1989; Russell *et al.*, 1993) and other experimentally infected model animals (Babakhani and Joens, 1993; Newell and Pearson, 1984; Yao *et al.*, 1997), together with *in vitro* experiments that used human epithelial cells (De Melo *et al.*, 1989; Ketley, 1997; Konkel and Joens, 1989; Oelschlaeger *et al.*, 1993), have clearly demonstrated that *C. jejuni* can adhere to and invade the cells of the intestinal tract. Invasion studies with intestinal epithelial cells as well as other cell lines revealed that the relative ability of different *C. jejuni* isolates to invade cultured cells is strain dependent (Everest *et al.*, 1992; Konkel and Joens, 1989; Newell *et al.*, 1985) and a statistically significant correlation was observed between the ability of *C. jejuni* to invade cultured cells and clinical symptoms of infection (Everest *et al.*, 1992). A previous study found that *C. jejuni* recovered from patients with diarrhoea and fever adhered to cultured human cells with higher efficiency than those isolated from asymptomatic individuals (Fauchere *et al.*, 1986). Everest *et al.* reported that 86% of isolates of *C. jejuni* from patients suffering from colitis were able to invade and translocate across Caco-2 polarised cells compared to 48% of strains isolated from individuals with non-inflammatory disease (Everest *et al.*, 1992). Immunofluorescent and electron microscopy examination of HeLa cells infected with *C. jejuni* showed that the strains isolated from the environment were much less invasive than clinical isolates (Newell *et al.*, 1985). Fearnley *et al.* identified a higher ratio of hyperinvasive strains among clinical isolates of *C. jejuni* than those obtained from poultry or environmental sources (Fearnley *et al.*, 2008). These observations emphasize the importance of bacterial invasiveness as a virulence factor for *Campylobacter* pathogenesis as different *Campylobacter* isolates vary in their virulence properties and that those having a higher ability to invade intestinal epithelial cells are more virulent.

1.7.4.2.1 Cytoskeleton required for invasion

Invasive bacteria are those that are capable of inducing their own uptake into non-phagocytic cells. The mechanisms underlying bacterial entry, host cytoskeleton requirements and dissemination reveal common strategies as well as unique tactics used by individual species to establish infection. Some enteropathogens including *Yersinia enterocolitica* and *Listeria monocytogenes* express surface ligands that bind host cell receptors often involved in cell-cell adherence. Binding of the bacterial ligand to the cell receptor leads to the formation of a vacuole that engulfs bacteria through a zippering process in which relatively modest cytoskeleton rearrangements and membrane extensions occur in response to engagement of the receptor (Cossart and Sansonetti, 2004). Other pathogens such as *Shigella* and *Salmonella* species use a trigger mechanism of entry in which components of a bacterial type-III secretion system make a bridge between the bacterium and host cell transmembrane receptor which then mediates the entry process. The pathogens bypass the adhesion step and interact directly with the host cell machinery, inject effector molecules through a dedicated secretory system that causes massive cytoskeletal changes triggering the formation of a macropinocytic pocket and bacterial cell internalisation (Cossart and Sansonetti, 2004). Microfilaments are the major cytoskeleton exploited in both the pathways described above for the entry of enteric pathogens into host cells. In contrast to the processes described above, some microorganisms including *C. jejuni* enter host cells via a microtubule and/or a microfilament dependent pathway (Hu and Kopecko, 2008).

C. jejuni strains have been reported to exhibit different host cytoskeletal requirements for invasion, which are dependent upon the bacterial strain and host cell utilised. Only a few *C. jejuni* strains have been studied in detail for the molecular mechanism of host cell invasion but results suggest that *Campylobacter* may utilise either microfilament dependent (De Melo *et al.*, 1989; Konkel and Joens, 1989) or microtubule dependent (Hu and Kopecko, 1999) or both microtubule and microfilament dependent (Biswas, 2003; Monteville *et al.*, 2003) pathways for entry into host cells. During INT-407 cell invasion, finger like epithelial cell membrane protrusions were formed in response to signal transduction from *C. jejuni* strain 81-176 bound to cells. The formation of these membrane protrusions is a result of actin filament depolymerisation and polymerisation of microtubule bundles (Hu and Kopecko, 2008). Once internalised *C. jejuni* remains

within a membrane-bound endosome and the endosome containing *C. jejuni* moves along microtubules to the perinuclear region of the cell (Hu *et al.*, 2008).

1.7.4.2.2 Translocation

The occurrence of extra-intestinal *C. jejuni* infections and isolation of the bacteria from human blood implies that the bacteria pass through the normally impermeable intestinal epithelial barrier to reach the lamina propria. Translocation of bacteria in *in vitro* systems has been reported for *C. jejuni* (Everest *et al.*, 1992; Konkel *et al.*, 1992). The pathways used by bacteria to traverse the intestinal barrier are transcellular where bacteria pass through the epithelial cell cytoplasm or paracellular where bacteria pass through the tight junctions of cell layers (Hu and Kopecko, 2008; Konkel *et al.*, 1992). *Campylobacter* species have been observed to use the paracellular route to translocate across polarised epithelial cell monolayers (Everest *et al.*, 1992; Grant *et al.*, 1993; Konkel *et al.*, 1992). Once internalised, *C. jejuni* does not escape from the endosome and these bacteria undergo limited intracellular replication during the first 8 to 10 hours post infection (Hu and Kopecko, 2008). The endosome carrying the internalised bacteria moves towards and fuses with the basolateral membrane to release the bacteria by exocytosis (Hu and Kopecko, 2008). *C. jejuni* infection does not disrupt tight junction integrity at early times after infection (Hu and Kopecko, 2008); however, infection of polarised Caco-2 cells with high multiplicities of infection (i.e. 10,000) was found to cause loss of transmembrane electrical resistance 24 hours after infection (MacCallum *et al.*, 2005). The loss of transmembrane electrical resistance was accompanied by rearrangement of tight junction proteins (MacCallum *et al.*, 2005) and similar observations were also made during infection of polarised T84 cells with *C. jejuni* (Chen *et al.*, 2006). Recently a paracellular pathway of *C. jejuni* mucosal translocation termed subvasion has been described (van Alphen *et al.*, 2008). During infection of non-polarised epithelial cells, *C. jejuni* were observed to pass between cells and proceed basally before internalisation (van Alphen *et al.*, 2008).

1.7.4.2.3 Bacterial determinants involved in host cell adhesion and invasion

Microorganisms generally require surface molecules such as pili and adhesins for their adherence to molecules on the host cell surface (i.e. receptors) to successfully bind and colonise their host (Young *et al.*, 2007). In some instances, such binding is also required for host cell invasion, where the organisms are protected from the immune responses.

Moreover, pathogenic bacteria typically utilise host cell molecules to promote their own pathogenic ability; for example *Salmonella* species and *Shigella* species secrete effector molecules through type-III secretion machinery into eukaryotic host cells. These bacterial adherence factors and secreted effector proteins initiate host signal transduction events that lead to internalisation of the pathogen (Hu and Kopecko, 2008). The genome sequences of many strains reveal that *C. jejuni* does not contain an orthologue of any obvious pilus or classical adhesins, invasins or pathogenicity islands (Fouts *et al.*, 2005; Parkhill *et al.*, 2000). The virulence-associated factors of the genetically related *Helicobacter pylori* are absent in *C. jejuni,* with the only revealed orthologues being housekeeping genes (Hu and Kopecko, 2008).

In the absence of identifiable adherence organelles and a type-III secretion system, several proteins have been reported that contribute to *C. jejuni* adherence to and invasion of eukaryotic cells. The reported adhesins of *C. jejuni* include flagella, outer membrane proteins and surface polysaccharides (Fauchere *et al.*, 1986; Jin *et al.*, 2001; Karlyshev *et al.*, 2004; McSweegan and Walker, 1986). The role of flagella in colonisation of host gut, cultured epithelial cell adherence and internalisation of *C. jejuni* into host epithelial cells has been suggested by many studies (Grant *et al.*, 1993; Konkel *et al.*, 1992a; Wassenaar *et al.*, 1991; Yao *et al.*, 1994). Mutation in genes encoding for flagella biosynthesis alternate sigma factors *fliA* (Sigma 28) and *rpoN* (sigma 54) resulted in reduced motility and invasion in cultured HeLa cells (Fernando *et al.*, 2007). *C. jejuni* mutants with paralysed flagella showed 30- to 40-fold reduced adhesion and invasion in INT-407 cells *in vitro* when compared to the wildtype strain (Yao *et al.*, 1994).

Other than flagellar motility, chemotaxis also plays a very important role in *C. jejuni* adhesion to host cells (Hendrixson and DiRita, 2004). Insertional inactivation of *cheY*, a gene thought to have a role in chemotaxis, results in a more adherent and invasive phenotype compared to the wildtype strain (Golden and Acheson, 2002; Yao *et al.*, 1997). Duplication of the *cheY* gene resulted in attenuation of virulence in the ferret model, however colonisation in the mouse model was not affected (Yao *et al.*, 1997); these observations suggest *cheY* to be a repressor of adhesion and invasion. *C. jejuni* mutants of the chemoreceptor CetB (*Cj1189*) were deficient in their ability to invade human epithelial cells *in vitro* (Golden and Acheson, 2002). In a recent report invasion of cells via the subcellular route (subvasion) was increased in *C. jejuni* in which

expression of CheW, a chemotaxis-associated protein, was decreased (van Alphen *et al.*, 2008). It suggests an interesting link between the *C. jejuni* chemotactic response and host cell invasion.

The CadF (*Campylobacter* adhesion to fibronectin), a *C. jejuni* outer membrane protein mediates the binding of the pathogen to the host extracellular matrix component, fibronectin (Konkel *et al.*, 1997). Anti-CadF antibody reduced the binding of *C. jejuni* to immobilised fibronectin by more than 50%. CadF is required for maximal binding and invasion of cultured eukaryotic cells by *C. jejuni* (Monteville *et al.*, 2003). The major outer membrane protein of *C. jejuni* is also thought to bind to INT-407 cells (Schroder and Moser, 1997). JlpA is another surface exposed adhesin of *C. jejuni*; it is lipoprotein in nature and is important for *C. jejuni* binding to HEp-2 cells (Jin *et al.*, 2001). JlpA binds to the host cell's heat shock protein Hsp90α that consequently activates NF-κB and p38 mitogen-activated protein (MAP) kinase, both of which contribute to proinflammatory responses (Jin *et al.*, 2003). This indicates how some of the adhesins contribute to the inflammation that is observed during *C. jejuni* pathogenesis. Another lipoprotein, CapA, is a *Campylobacter* autotransporter protein which was implicated as a possible adhesin. A CapA-deficient mutant showed decreased attachment to Caco-2 cells and reduced colonisation in a chick model (Ashgar *et al.*, 2007).

Some putative adhesins of *C. jejuni* are located in the periplasm. PEB1 is a 28-kDa *Campylobacter* periplasmic protein and it belongs to other related proteins, PEB2-4 (Pei *et al.*, 1991). PEB1 is conserved in *C. jejuni* and *C. coli* strains (Pei and Blaser, 1993; Pei *et al.*, 1998). PEB1 and PEB3 are antigenically similar; PEB3 shows partial homology with class 1 pili of *Neisseria meningitidis* and heat-labile enterotoxin B-unit of *E. coli*, both of which are involved in adhesion to human mucosal epithelia (Pei *et al.*, 1991). PEB1 is crucial for *C. jejuni* binding to HeLa cells and shares homology with the periplasmic-binding proteins involved in nutrient uptake i.e. ATP-binding cassette (ABC) transporters (Leon-Kempis Mdel *et al.*, 2006; Pei and Blaser, 1993). Although PEB1 has not been localised to the inner or outer membrane, some has been observed in culture supernatants (Leon-Kempis Mdel *et al.*, 2006). The glycoprotein *Cj1496c* is another periplasmic protein of *C. jejuni*, which has homology to a magnesium transporter and is also required for maximal adherence and invasion in cultured INT-407 cells (Kakuda and DiRita, 2006).

C. jejuni invasion is dependent both on host cell signal transduction and bacterial nascent protein synthesis (Konkel *et al.*, 1999; Wooldridge and Ketley, 1997). *C. jejuni* secrete a set of proteins called Cia (*Campylobacter* invasion antigen) proteins when grown in the presence of human intestinal epithelial cells; CiaB is one of Cia proteins and has been characterised in detail (Konkel *et al.*, 1999). CiaB is predicted to be 73.1 kDa in size and shows no similarity to other known proteins. Mutation of the *ciaB* gene in *C. jejuni* strain F38011 resulted in the loss of secretion of this protein and a 50-fold reduction in invasion of the mutant into INT-407 cells compared to wildtype strain (Konkel *et al.*, 1999); however, mutation of this gene in *C. jejuni* strain 81-176 had no significant effect on the invasion of INT-407 cells (Goon *et al.*, 2006).

The surface polysaccharide molecules including the LOS and capsule of *C. jejuni* also play important roles in the interaction of the bacteria with hosts. The high-molecular weight glycan capsule and LOS of *C. jejuni* are also required for bacterial attachment and invasion in host cells *in vitro* and full virulence in the ferret animal model (Bacon *et al.*, 2001; Kanipes *et al.*, 2004; Karlyshev *et al.*, 2000). Purified LOS can bind to INT-407 cells which can be inhibited by periodate oxidation of LOS; moreover, epithelial cell binding of purified LOS subsequently inhibits adhesion of whole *C. jejuni* cells (McSweegan and Walker, 1986). Inactivation of *galE*, a gene involved in LOS biosynthesis, also caused a reduction in *C. jejuni* adhesion to and invasion in INT-407 cells, however, the ability of chicken colonisation of the mutant was not affected (Fry *et al.*, 2000). Mutation in the LOS locus of *C. jejuni* results in the loss of invasiveness in cultured intestinal epithelial cells (Kanipes *et al.*, 2004). A large deletion of the whole LOS biosynthesis locus showed that LOS was not essential for the viability of *C. jejuni* but it abolished invasion of the mutant in Caco-2 cells (Marsden *et al.*, 2009). Sialylation of LOS is also important for *C. jejuni* invasion in cultured epithelial cells; inactivation of LOS sialyltransferase encoded by *Cst-II* in three GBS-associated *C. jejuni* strains reduced their ability to invade cultured epithelial cells (Louwen *et al.*, 2008).

The *N*-linked general protein glycosylation pathway (pgl) of *C. jejuni* is also important for host cell adherence and invasion. The inactivation of *pglH* in *C. jejuni* resulted in a significant reduction in the adherence and invasion in Caco-2 cells *in vitro* (Karlyshev *et al.*, 2004). *C. jejuni pgl* mutants are reduced in their ability to adhere to and invade cultured Caco-2 cells (Hu and Kopecko, 2008). Inactivation of the glycosylation

associated genes *pglB* and *pglE* encoding for an oligosaccharide transferase and a putative aminotransferase in *C. jejuni*, respectively, resulted in reduction in adhesion and invasion of the mutants in INT-407 cells *in vitro* (Szymanski *et al.*, 2002). Mutation of *Cj1121c* encoding for an aminotransferase which is involved in protein glycosylation in *C. jejuni* impaired the motility of the mutant and reduced its ability to invade intestinal epithelial cells *in vitro* (Vijayakumar *et al.*, 2006). A *C. jejuni* glycoprotein encoded by *Cj1496c* is important for invasion in intestinal epithelial cell line INT-407, however, glycosylation of the protein is not important for virulence (Kakuda and DiRita, 2006). A previous report showed a monoclonal antibody that bound to the flagella and the surface of a less passaged (invasive) strain only bound to the flagella of the same strain that had lost its invasiveness by repeated passage. Western blots of lysates of the invasive and repeatedly passaged strains identified antigens of 38-42 kDa, possibly glycoproteins, which were only expressed by the invasive strain (Konkel *et al.*, 1990). These results suggest that glycosylation pathway is important for pathogen-host cell interactions and glycoproteins of *C. jejuni* may act as adhesins and promote invasion in epithelial cells.

Other *C. jejuni* factors involved in adherence to and/or invasion in cultured host cells include *Cj1461* encoding for a methyltransferase (Kim *et al.*, 2008), *luxS* encoding for an autoinducer-2 biosynthesis enzyme (Quinones *et al.*, 2009), a putative posttranslational regulator CsrA of *C. jejuni* 81-176 (Fields and Thompson, 2008), *clpP* and *lon* encoding for ATP-dependent proteases (Cohn et al., 2007), *Cj0125c* encoding the transcription regulator DskA-like protein in *C. jejuni* (Yun *et al.*, 2008) and SpoT, a stringent response regulator of *C. jejuni* (Gaynor *et al.*, 2005). Characterisation of some the proteins found on a *C. jejuni* plasmid, pVir, has suggested their role in virulence of *C. jejuni* strain 81-176. All the four plasmid-encoded proteins (*comB1, comB2, comB3* and *virB11*) have some homology to components of type-IV secretion systems and similarity to *Helicobacter pylori* proteins (Bacon *et al.*, 2000). These proteins have been shown to be important in INT-407 cell adhesion and invasion, as well as contributing to virulence in the ferret model (Bacon *et al.*, 2000).

1.7.5 Project background and aims of study

Clinical observations and experimental evidence indicate that the ability of *C. jejuni* to invade the host intestinal epithelium is an important determinant of its ability to cause focal necrosis of the colonic mucosa (van Spreeuwel *et al.*, 1985a). However, the molecular mechanisms involved in *C. jejuni* invasion of host epithelial cells remain largely uncharacterised. Moreover, the ability of *C. jejuni* to invade cultured epithelial cells varies from strain to strain and the strains isolated from individuals suffering from inflammatory disease have shown a higher ability of host cell invasion compared to those isolated from asymptomatic individuals. This suggests that invasiveness is an important marker of virulence in *C. jejuni.* Many studies have been undertaken to investigate the bacterial factors that contribute to invasiveness exhibited by *C. jejuni*. However, the molecular basis underlying the variation in invasiveness in *C. jejuni* strains is not yet fully understood.

In a previous study, the variation in invasiveness of *C. jejuni* was investigated and a number of strains were found to be hyperinvasive *in vitro* (Fearnley *et al.*, 2008). One of these strains, 01/51, a clinical isolate, was studied further by using transposon insertion mutagenesis. A random transposon mutant library (*ca.* 1000) was generated in this strain and a number (n=96) of these mutants were screened for invasion using the human intestinal epithelial cell line INT-407 in a pilot study (Manning *et al.*, 2003a). A considerable number of the mutants were yet to be screened to find the mutants with reduced invasion compared to wildtype strain and hence identify the genes involved in invasion.

The overall aim of this investigation was to identify the molecular basis of hyper-invasiveness in *Campylobacter jejuni*. A number of approaches were adopted to achieve this aim:

1. To establish a *Campylobacter jejuni* invasion assay using human colonic epithelial (Caco-2) cells and to confirm the invasion phenotype of selected *C. jejuni* strains from a previous study.
2. To screen a random transposon mutant library constructed in hyper-invasive strain *C. jejuni* 01/51 (as determined in a previous study using INT-407 cells) for genes involved in host cell invasion.
3. To confirm the host cell invasion-associated role of the identified genes

using further mutagenesis and complementation tools.

4. To characterise the phenotypic properties of selected identified genes using defined and complemented mutants in a variety of assays including chick colonisation, motility, growth and stress survival assays.
5. To determine the functional aspects of the selected gene(s) to determine the molecular basis of the invasion-related role.

Chapter Two

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Chemicals, bacterial strains, plasmids and primers

All the chemicals were purchased from Sigma-Aldrich (Poole, UK) unless specified.

All bacterial strains were previously isolated and identified and were obtained from the Veterinary Laboratories Agency, Weybridge, UK. Bacterial genetic modifications made in this study will be mentioned.

2.1.1 Bacterial strains used in this study

All the bacterial strains used in this study were kindly provided by Prof. DG Newell, Veterinary Laboratories Agency (VLA), Weybridge, UK. Details of these strains are given in Table 2.1.

Table 2.1 Bacterial strains and mutants used in this study.

Source	Strain	Isolated from	Invasion potential	Comment/reference
Human	*C. jejuni* 01/04	Blood	Hyperinvasive	(Fearnley *et al.*, 2008)
	C. jejuni 01/10	Blood	Hyperinvasive	(Fearnley *et al.*, 2008)
	C. jejuni NCTC11168	Faeces	Highly invasive	This strain of *C. jejuni* was first isolated from the feaces of a human patient suffering from diarrhoea by Martin Skirrow in 1977 (Gaynor *et al.*, 2004). The NCTC11168 is a laboratory adapted strain that was the first fully sequenced genome *C. jejuni* (Parkhill *et al.*, 2000).
	C. jejuni 81116	Faeces	Low invasive	Isolated from patient with diarrhoea during a water-borne outbreak in a boarding school (Palmer *et al.*, 1983). The whole genome sequence of this strain has been published (Pearson *et al.*, 2007).
	C. jejuni 01/51	Faeces	Hyperinvasive	(Fearnley *et al.*, 2008)
	C. jejuni 01/32	Faeces	Hyperinvasive	(Fearnley *et al.*, 2008)
	C. jejuni 01/35	Faeces	Hyperinvasive	(Fearnley *et al.*, 2008)
	C. jejuni	Faeces	Hyperinvasive	(Fearnley *et al.*, 2008)

	01/41			
	C. jejuni 01/46	Faeces	Low invasive	(Fearnley *et al.*, 2008)
	C. jejuni 01/50	Faeces	Hyperinvasive	(Fearnley *et al.*, 2008)
Environment	*C. jejuni* EX114	Puddle	Hyperinvasive	This strain was isolated from a puddle near a poultry farm (Fearnley *et al.*, 2008).
	C. jejuni 01/51Δ*cipA*			The *C. jejuni* 01/51 mutant in *cipA* (this study).
	C. jejuni 01/51 Δ*Cj0690c*			The *C. jejuni* 01/51 mutant in *Cj0690c* (this study).
	C. jejuni 01/51 Δ*Cj1136*			The *C. jejuni* 01/51 mutant in *Cj1136* (this study).
	C. jejuni 01/51 Δ*Cj1245c*			The *C. jejuni* 01/51 mutant in *Cj1245c* (this study).
	C. jejuni 01/51 Δ*Cj1305c*			The *C. jejuni* 01/51 mutant in *Cj1305c* (this study).
	C. jejuni 01/51 Δ*Cj1539c*			The *C. jejuni* 01/51 mutant in *Cj1539c* (this study).
	C. jejuni 01/51 Δ*cipA*+*cipA*			The *C. jejuni* 01/51 mutant in *cipA* complemented with *cipA* along with presumptive promoter (this study).
	C. jejuni 01/51 Δ*Cj0690c*+ *Cj0690c*			The *C. jejuni* 01/51 transposon mutant in *Cj0690c* complemented with *Cj0690c* along with presumptive promoter (this study).
	C. jejuni 01/51 Δ*Cj1136*+ *Cj1136*			The *C. jejuni* 01/51 transposon mutant in *Cj1136* complemented with *Cj1136* along with presumptive promoter (this study).
	C. jejuni 01/51 Δ*Cj1539c*+ *Cj1539c*			The *C. jejuni* 01/51 transposon mutant in *Cj1539c* complemented with *Cj1539c* along with presumptive promoter (this study).
Chicken	*C. jejuni* RM1221	Carcass	Not known	This strain is a genome sequenced *C. jejuni* strain (Fouts *et al.*, 2005).
Invitrogen, UK	*E. coli* TOP10	Commercial	Not known	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74*rec*A1 *ara*D139 Δl(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

2.1.2 Plasmids used in this study

Table 2.2 Plasmids used in this study.

Plasmid name	Plasmid description	Antibiotic resistance cassette	Original source
pGEM®-T Easy	PCR products cloning vector	Ampicillin	Promega, UK
pUOA18	*Campylobacter* shuttle vector	Chloramphenicol	(Wang and Taylor, 1990)
pMA24	Source of kanamycin cassette	Kanamycin Ampicillin	(Abuoun, 2007)
pGEM:685	pGEM®-T Easy with cloned *cipA* gene	Ampicillin	This study
pGEM:685::kan	*cipA* mutation construct in pGEM®-T Easy vector	Ampicillin Kanamycin	This study
pGEM:690	pGEM®-T Easy with cloned *Cj0690c* gene	Ampicillin	This study
pGEM:690::kan	*Cj0690c* mutation construct in pGEM®-T Easy vector	Ampicillin Kanamycin	This study
pGEM:1136	pGEM®-T Easy with cloned *Cj1136* gene	Ampicillin	This study
pGEM:1136::kan	*Cj1136* mutation construct in pGEM®-T Easy vector	Ampicillin Kanamycin	This study
pGEM:1245	pGEM®-T Easy with cloned *Cj1245c* gene	Ampicillin	This study
pGEM:1245::kan	*Cj1245c* mutation construct in pGEM®-T Easy vector	Ampicillin Kanamycin	This study
pGEM:1305	pGEM®-T Easy with cloned *Cj1305c* gene	Ampicillin	This study
pGEM:1305::kan	*Cj1305c* mutation construct in pGEM®-T Easy vector	Ampicillin Kanamycin	This study
pGEM:1539	pGEM®-T Easy with cloned *Cj1539c* gene	Ampicillin	This study
pGEM:1539::kan	*Cj1539c* mutation construct in pGEM®-T Easy vector	Ampicillin Kanamycin	This study
pAJ20	Part of *Cj0652*, *Cj0653c* and intergenic region cloned in PGEM®-T Easy	Ampicillin	This study
pAJ21	pAJ20 with additional *Bam*HI site introduced in intergenic region.	Ampicillin	This study
pAJ22	*C. jejunji* complementation suicide vector with Cm^r cassette cloned in pAJ21.	Ampicillin Chloramphenicol	This study
pAJ23	*C. jejunji* complementation suicide vector with kan^r cassette cloned in pAJ21.	Ampicillin Kanamycin	This study
pAJ22-1136	pAJ22 with cloned *Cj1136* along with its promoter	Ampicillin Chloramphenicol	This study
pAJ22-*cipA*	pAJ22 with cloned *cipA* (*Cj0685c*) and its promoter	Ampicillin Chloramphenicol	This study
pAJ22-1245	pAJ22 with cloned *Cj1245c* along with its promoter	Ampicillin Chloramphenicol	This study
pAJ22-690	pAJ22 with cloned *Cj0690c* along with its promoter	Ampicillin Chloramphenicol	This study
pAJ23-690	pAJ23 with cloned *Cj0690c* along with its promoter	Ampicillin Kanamycin	This study
pAJ22-1539	pAJ22 with cloned *Cj1539c* along with its promoter	Ampicillin Chloramphenicol	This study

2.1.3 Primers used for amplification and sequencing

All the primers were purchased from Sigma-Aldrich (UK) and are shown in Table 2. 2.

Table 2. 3 Oligonucleotides used in this study.

Oligo	Tm* (°C)	Sequence 5' to 3'	Comments
Transeq	62.8	CCCGGGAATCATTTGAAG	Transposon sequencing primer.
KCN-F	75.4	ggaggatccGATAAACCCAGCGAACC	*Kan*^{r} PCR primer, *Bam*HI site near 5' end.
Kan-Fex	72.5	gcaggatccGACATCTAAATCTAGGTAC TAAAACAATTC	*Kan*^{r} PCR reverse primer, *Bam*HI site near 5' end.
KNHD-F	72.3	caaaaaagcttgATAAACCCAGCGAACC	*Kan*^{r} PCR primer, *Hind*III site near 5' end.
KNHD-R	69.8	caaaaaagcttgACATCTAAATCTAGGTAC TAAAACAATTC	*Kan*^{r} PCR primer, *Hind*III site near 5' end.
KanBgl-R	73	cacaagatctGACATCTAAATCTAGGTAC TAAAACAATTC	*Kan*^{r} PCR reverse primer, *Bgl*II site near 5' end.
aj1-FOR	60.6	GCTTGATAGTGTTACTGAACAACTG	PCR primer for *Cj1245c* binds at 69 bp downstream to start codon.
aj1-REV	59.0	CTACTTACATCACTTAAAACTTCGC	PCR primer for *Cj1245c* binds at 1151 bp downstream to start codon.
1136-F	62.8	GCTCGAAATCAATCATCAAATTT	*Cj1136* PCR primer binds at 66 bp upstream to start codon.
1136-R	63.0	CAACTCTTTGGGAAGAAATTCAA	*Cj1136* PCR primer binds at 90 bp downstream to termination codon.
1136-INF	60.2	caaaaggatccGCACTATCACTCCAAGAG ACTATG	*Cj1136* inverse PCR primer binds at 566 bp downstream to start codon, *Bam*HI site near 5' end.
1136-INR	60.5	caaaaggatccCCATCATCATAAATACAA GCATG	*Cj1136* inverse PCR primer binds at 509 bp downstream to start codon, *Bam*HI site near 5' end.
685-F	62.5	CATCGATAAATTCAGCACAATATAC AG	*Cj0685c* PCR primer binds at 400 bp upstream to start codon.
685-R	63.4	CACCCATATCATTACATAGGTCGTT	*Cj0685c* PCR primer binds at 1141 bp downstream to start codon.
690-F	61.4	GAAGCTTTTGCGTATTTTTATAGTGT	*Cj0690c* PCR primer binds at 586 bp downstream to start codon.
690-R	64.0	CCATCTCCACTGCCTTTGTT	*Cj0690c* PCR primer binds at 2483 bp downstream to start codon.
1305-F	62.5	GATCATGGTTGTGGTATACCTGAT	*Cj1305c* PCR primer binds at 188 bp upstream to start codon.
1305-R	62.4	CAAATTTGTCACCCACATCTTT	*Cj1305c* PCR primer binds at 1198 bp downstream to start codon.
1539-F	62.8	GGTATTGCCACTTTCTTTTTCATAA	*Cj1539c* PCR primer binds at 188 bp upstream to start codon.
1539-R	64.5	GTAGATTTTCCACTTCCATTCGC	*Cj1539c* PCR primer binds at 126 bp downstream to stop codon.
1539-INF	59.5	caaggatccGCAGTTTATCTTAGCGCTTC CTAT	*Cj1539c* inverse PCR primer binds at 351 bp downstream to start codon, *Bam*HI site near 5' end.
1539-INR	60.2	caaggatccGTAAAAAGCAATCCAAATT CTCCC	*Cj1539c* inverse PCR primer binds at 329 bp downstream to start codon, *Bam*HI site near 5' end.
CML-F	60	caaggatccGTCGGTATCGTATGGAGCG	CAT gene PCR primer, *Bam*HI site near 5' end.
CML-R	60	cacaagatctCCTAAAGGGTTTTTATCAG TGCG	CAT gene PCR primer, *Bgl*II site near 5' end.

C1136-F	60	caaggatccCGCAAAGTAATTTTACAAG GTTCA	*Cj1136* PCR primer binds at 458bp upstream to start codon, *Bam*HI site near 5' end.
C1136-R	60	caaggatcctCAACTCTTTGGGAAGAAAT TCAA	*Cj1136* PCR primer binds at 90 bp downstream to stop codon, *Bam*HI site near 5' end.
C1245-F	61	caaggatcctCGCGTAGGGCTTTGGACT	*Cj1245c* PCR primer binds at 476 bp upstream to start codon, *Bam*HI site near 5' end.
C1245-R	59	caaggatccGCTTCATTTGCGCTCACA	*Cj1245c* PCR primer binds at 135 bp downstream to stop codon, *Bam*HI site near 5' end.
C690-F	62	caaggatccCACAAGCAGTAACCATAAC AGCG	*Cj0690c* PCR primer binds at 428 bp upstream to start codon, *Bam*HI site near 5' end.
C690-R	60	caaggatccGTGCTTGTTATTCCTACAGA TGAAGA	*Cj0690c* PCR primer binds at 99 bp downstream to stop codon, *Bam*HI site near 5' end.
C1539-F	60	caaggatccCAGGAACACCGCCAAGAT	*Cj1539c* PCR primer binds at 507 bp upstream to start codon, *Bam*HI site near 5' end.
C1539-R	59	caaggatccCCATTCGCTCCCATTAAGAT	*Cj1539c* PCR primer binds at 110 bp downstream to stop codon, *Bam*HI site near 5' end.
C685-F	58	caaggatccGCCGTGATTTACACCGATA C	*Cj0685c* PCR primer binds at 506 bp upstream to start codon, *Bam*HI site near 5' end.
C685-R	57	caaggatccGAAGTGAAAATCATAAAGC TTTAGTG	*Cj0685c* PCR primer binds at 186 bp downstream to stop codon, *Bam*HI site near 5' end.
652-F	60	GAACTTACGATAGATATAGAGC	*Cj0652* PCR primer binds at 697 bp downstream to start codon
653-F	62	TTATGATGAAATCATCATGGAGC	*Cj0653c* PCR primer binds at 737 bp downstream to start codon.
IN652-R	54	caaggatccCAAGATTTTTAAACCAATT GC	Inverse PCR primer binds 42 bp downstream to stop codon of *Cj0652*.
IN653-R	50	caaggatccAATTATAAGTTTAATACAA GG	Inverse PCR primer binds 72 bp downstream to stop codon of *Cj0652*.
652Seq-F	59	GGCAACTGTAGCTAAAATTTATCAA A	Sequencing primer binds at 1752 downstream to start codon i.e. 54 bp upstream to stop codon of *Cj0652*.
653Seq-F	59	CCTAAGCTAGGTGATTATCCAAAAG	Sequencing primer binds at 86 bp upstream to stop codon of *Cj0653c*.
CML-Frev	60	CGCTCCATACGATACCGAC	Reverse complement of CML-F designed to sequence insert in pGEM643CML.
napAL2	74	CTT TAG AAG GGC TTT TAG CTC GTG C	*C. jejuni* subspeciation primer binds the *nap*A upstream region.
napAR4	74	ATT TCC CTG CAA GAT AAA ATC TGT AGC	*C. jejuni* subspeciation primer binds the *nap*A downstream region.

*Tm denotes the melting temperature of the oligonucleotide. Tm given in the table were calculated on the basis of *Campylobacter*-specific sequences and do not consider the additional endonuclease restriction sites along with extra nucleotides at 5' ends of the primers, where added, and these additional restrictions sites and extra nucleotides are shown in lower case.

2.1.4 General buffers

All buffers were prepared using distilled water (dH_2O) unless specified.

0.1 M Phosphate buffered saline (pH 7.2)

Phosphate buffered saline (PBS) was purchased from (Sigma, UK). The composition of PBS was 23.4 mM disodium hydrogen phosphate, 6.6 mM potassium dihydrogen orthophosphate and 70.0 M sodium chloride. PBS was sterile and ready to use.

50x Tris-acetate-EDTA buffer

50x Tris-acetate-EDTA (TAE) buffer was purchased National diagnostics, England. The composition of TAE buffer was 2.0 M Tris-acetate and 100 mM sodium EDTA. For use x1 TAE buffer was prepared by diluting 20 ml of the concentrated buffer in 980 ml distilled water.

2.2 Culture media

All culture media were obtained from Oxoid (Basingstoke, UK) unless specified and prepared using distilled water. Culture media sterilisation was carried out by autoclaving at 121^{o}C, 15 psi for 15 min. All the culture media used were adjusted to pH 7.4. Sheep blood was added to the media after it had cooled to 52^{o}C. Media plates were stored at 4^{o}C.

5% (v/v) blood agar plates

Columbia agar base*	39 g

(* Contains peptone 23 g, starch 1.0 g, sodium chloride 5.0 g and agar 10 g per litre)

Defibrinated sheep blood (TCS Biosciences, UK)	50 ml
Distilled water	1 L

Mueller-Hinton agar/ Mueller-Hinton broth biphasic media

10 ml of Mueller-Hinton (MH) agar dispensed onto 250 cm^3 tissue culture flasks left in standing position for 30 minutes to solidify, then overlaid with 20 ml of MH broth.

Mueller-Hinton Agar

MH agar powder*	38 g

(*Contains beef, dehydrated infusion from 300 gm, casein hydrolysate 17.5 gm, starch 1.5 gm and agar 17.0 gm per litre)

Distilled water	1 L

Mueller-Hinton broth

MH broth powder*	21 g
Distilled water	1 L

2.3 Bacterial culture methods

All strains were stored at -80^{o}C in 1% (w/v) proteose peptone water containing 10% (v/v) glycerol until required. Strains were routinely grown from frozen on 5% (v/v) blood agar (BA) plates for 48 hours at 37^{o}C in the compact anaerobic work station (Don Whitley scientific, UK) attached to a gas cylinder supplying 10 % CO_2, 5% O_2 and 85% (v/v) N_2 or in a gas jar where a CampyGen® pack (Oxoid, UK) sachet was added to generate microaerobic conditions. Other culture media including MH agar and MH broth were also used to grow *C. jejuni* and are mentioned, where used.

2.4 Bacterial growth curve by viable count method

Strains were grown overnight on BA plates and the cells were harvested for preparing the inoculum. One hundred microlitres of bacterial suspension in 0.1 M phosphate buffered saline pH 7.2 was inoculated into pre-warmed MH broth and incubated overnight in microaerobic conditions on a shaker. Then each of nine pre-warmed (37^{o}C) MH/MH biphasic broth flasks was inoculated to an OD (550 nm) of 0.05 with the overnight growth and left to grow at 37^{o}C under microaerobic conditions. A viable count of the bacterial culture was taken, in triplicate, after 0, 3, 6, 9, 12, 20, 24 and 30 hours of growth. The number of viable bacteria was determined by serially diluting the bacterial culture with PBS and plating by spreading out 20 µl of each dilution onto pre-dried blood agar plates. The plates were incubated for 48 hours at 37^{o}C under

microaerobic conditions and the colonies counted. The bacterial growth curve experiment was done in duplicate.

2.5 Estimating the number of bacteria by measuring DNA content

In a standard gentamicin protection assay, an estimated number of bacterial cells were added to the cell cultures. There are a number of methods available to measure bacterial numbers in a solution and measuring the bacterial DNA content is one way, however, this method does not take into account the number of live and dead bacteria. The actual viable count was determined retrospectively by serial dilution of the bacterial inocula in PBS and plating onto blood agar plates.

An estimate of the number of bacteria per ml was made by lysing 50 µl of the bacterial suspension in 950 µl of 1 M sodium hydroxide. The optical density (OD) of the released nucleic acid was measured at λ260 nm. A calibration curve (Figure 2.1) was prepared using different dilutions of *C. jejuni* 01/51 cell suspension in phosphate buffered saline whereby OD (260 nm) was plotted against actual bacterial numbers, which were measured by the viable cell count method. When the values of this experiment were applied (y = 2E+10x - 4E+08) using the straight line equation, (y=mx+c, where the y is Y-axis value (cfu/ml in this case), x is the X-axis value (OD_{260nm}), m is gradient of straight line and c is the Y-intercept), an OD (260 nm) of one was equal to 1.96×10^{10} bacterial cells per millilitre and this equation was used for calculating the approximate cell count (cfu/ml) of *C. jejuni* strain 01/51. Because calibration curve was plotted with bacterial cell suspensions having DNA content with optical density (260nm) between 0.06 and 0.45; for the gentamicin protection assay bacterial cell suspension having DNA content with optical density (260 nm) between 0.1 and 0.4 was used to keep within known linear range and cell suspension density was adjusted accordingly by diluting or concentrating the suspension if was found outside this range.

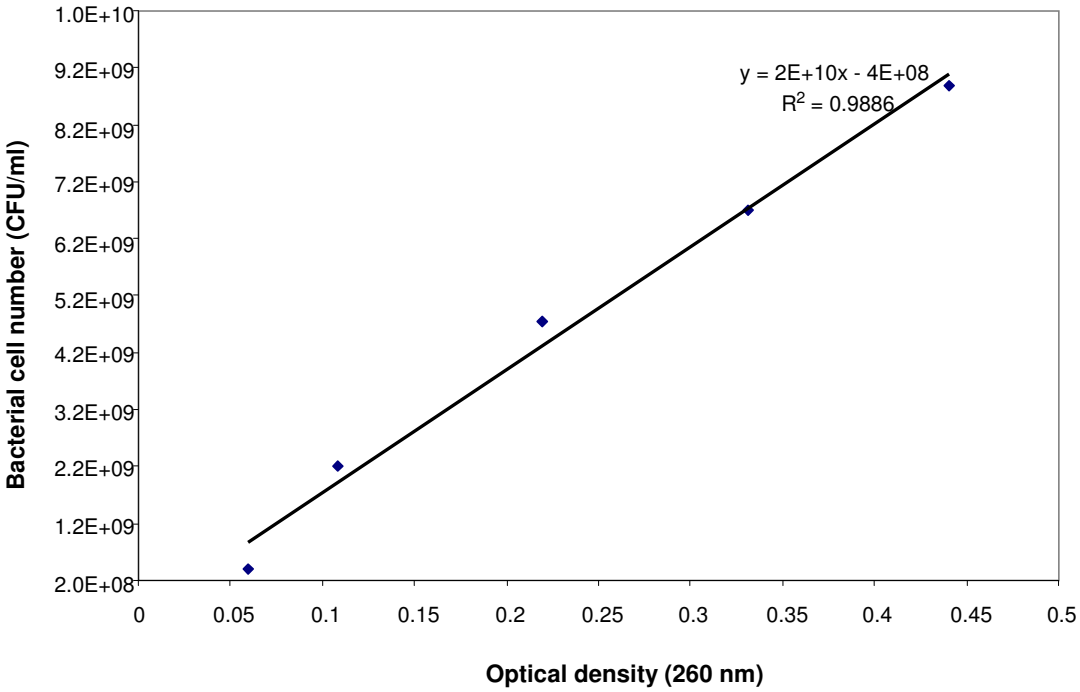

Figure 2.1 A calibration curve of *C. jejuni* 01/51 cell number determined by measuring the DNA content.

C. jejuni cell number was estimated by measuring its DNA content after lysing the cells in 1 M sodium hydroxide. A calibration curve using different dilutions of a known cell number of *C. jejuni* 01/51 was prepared. The calibration curve was drawn by plotting optical density (260 nm) of DNA content of different dilutions of bacterial cell suspension prepared in sterile phosphate buffered saline against actual bacterial cell numbers, which were measured by the viable count method. This calibration curve was used to calculate the cell number of *C. jejuni* in a cell suspension by measuring the DNA content.

2.6 Culture of mammalian cell lines

All the cell culture media and supplements were purchased from Sigma, United Kingdom unless specified. INT-407 cells (Human Caucasian embryonic intestinal epithelial, ECACC# 85051004) and Caco-2 cells (Human colonic carcinoma epithelial, ECACC# 86010202) were obtained from the European Collection of Cell Cultures (Porton Down, Salisbury, UK). Frozen ampoules were thawed quickly and added to 10 ml of pre-warmed complete cell culture media consisting of Eagle's minimal essential medium with glutamine supplemented with 10% foetal calf serum and 1% non-essential amino acids. Gentamicin (200 µg/ml) was added to the media in the first two passages. The cells were pelleted by centrifugation at 1200 rpm for 5 min using CENTAUR2 centrifuge (MSE, UK). The supernatant was removed and the cells resuspended in 25 ml of complete media and added to a sterile 250 cm^3 polypropylene tissue culture flask. The cells were incubated for 48 hours at 37°C in 5% (v/v) CO_2 until a confluent

monolayer was present. Confluent monolayers were trypsinised with trypsin-EDTA solution (Sigma) and split in the ratio of 1:3 to 1:6 and the growth continued.

2.7 The gentamicin protection invasion assay

A twenty four well tissue culture plate was seeded with INT-407 or Caco-2 cells at a concentration of 1 x 10^5 cells per well in complete cell culture media without antibiotics and incubated at 37^{o}C with 5% (v/v) CO_2 for forty eight hours to establish confluent monolayers (3 x 10^5 cells per well).

Bacteria were harvested from blood agar plates after 48 hours incubation in a microaerobic atmosphere. A few colonies were resuspended in PBS and the bacterial count was estimated by measuring the DNA (2.5). Bacterial counts in the final inoculum were confirmed retrospectively by viable cell count method. The bacterial suspension was diluted in pre-warmed complete cell culture media to a multiplicity of infection (MOI) of 100; MOI is the ratio of bacterial cell number in the initial inoculum to the eukaryotic cell number in the well. Cell culture media covering the monolayers was replaced with 1 ml of bacterial suspension. Infected monolayers were incubated for three hours at 37^{o}C in 5% (v/v) CO_2 to allow the bacteria to invade the cells. The monolayers were washed three times with PBS. To remove any external bacteria that had not invaded, 1 ml of complete cell culture media supplemented with 250 µg gentamicin ml^{-1} was added to each well and incubated for a further two hours. Following incubation the monolayers were washed three times with PBS as before and lysed with 1 ml of 1% (v/v) Triton-X100 in PBS. After 10 minutes the lysate was mixed well by pipetting up and down and the total number of viable bacteria per well was determined by serially diluting the cell lysate with PBS and spread plating out 20 µl of the undiluted and dilutions of lysate onto pre-dried blood agar plates. Plates were incubated at 37^{o}C in microaerobic conditions and the colonies were counted after 48 hours. Each colony was assumed to represent one bacterium that had become internalised.

The invasion efficiency of each isolate was calculated using the formula below, where the number of bacteria internalised per well was expressed as a percentage of the bacterial cell number added to the well at the start of the experiment. Assays were done in triplicate. A non-invading flagellar double mutant *C. jejuni* 81116 *fla*A$^-$/*fla*B$^-$

(Wassenaar *et al.*, 1994) was used as negative control and was processed in the same way as the test strains in the assay. The standard error of the mean (SEM) was calculated for the invasion efficiencies of each isolate tested in triplicate wells. Error bars were used to represent the SEM of invasion efficiencies.

$$\text{Invasion efficiency: } \frac{\text{Total number (c.f.u.) of internalised bacteria}}{\text{Total number (c.f.u.) of bacteria in inoculum}} \times 100$$

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2.8 Adhesion assay

Adhesion assays were done to check the ability of bacterial strains and mutants to adhere to INT-407 or Caco-2 cells. Adhesion and invasion assays were done at the same time using the same batch of cultured eukaryotic cells and the same bacterial cell suspensions to minimise the assay to assay variation. Adhesion assays were done in triplicate for each strain.

INT-407 or Caco-2 cells were grown as described above (2.6). The bacterial suspension was diluted in pre-warmed complete cell culture medium to a multiplicity of infection (MOI) of 100. Cell culture media covering the monolayers was replaced with 1ml bacterial suspension. Infected monolayers were incubated for three hours at 37^{o}C in 5% (v/v) CO_2. Following incubation the monolayers were washed three times with PBS and lysed with 1 ml of 1% (v/v) Triton-X100 in PBS. The lysate was mixed well by pipetting up and down and the total number of viable bacteria per well was determined by serially diluting the cell lysate with PBS and spreading out 20 µl onto pre-dried blood agar plates. Plates were incubated at 37^{o}C in microaerobic conditions and the colonies counted. Each colony was assumed to represent one bacterium that had become internalised. A second cell infection plate was processed for the invasion assay at this point.

The adhesion efficiency of each isolate was calculated using the formula below, where the number of bacteria adhered per well was expressed as a percentage of the bacterial cell number added to the well at the start of the experiment. A non-adhering flagellar double mutant *C. jejuni* 81116 *fla*A^{-}/*fla*B^{-} was used as negative control and was processed in the same way as the test strains in the assay. The standard error of the

mean (SEM) was calculated for the adhesion efficiencies of each isolate tested in triplicate wells. Error bars were used to represent the SEM of adhesion efficiencies.

$$\text{Adhesion efficiency: } \frac{\text{Total bacterial numbers counted - Total number of internalised bacteria}}{\text{Total number (c.f.u.) of bacteria in inoculum}} \times 100$$

2.9 General molecular biology techniques used in gene manipulation

2.9.1 Separation of DNA fragments by agarose gel electrophoresis

Analysis of DNA fragments was done in 1% (w/v) agarose in 1 X TAE buffer. A DNA molecular marker of established DNA band sizes was run alongside the DNA samples. The 1 kbp DNA ladder (Promega, Southampton, UK) was the DNA molecular marker used and 250 ng of marker was loaded on the gel. 2 µl of 6 x DNA loading buffer (Promega) was added to the sample (10 µl) before loading the agarose gel. The agarose gel was placed in a Mini-Sub® Cell GT tank (Bio-Rad Laboratories Ltd., Hertford, UK) and submerged in 1 x TAE buffer and electrophoresed at 100 V. The gel was incubated in an ethidium bromide solution (1 mg/ml) for 30 min and the DNA bands were visualised under ultra-violet light using InGenius® gel documentation system (Syngene, UK).

2.9.2 Estimation of DNA concentration

DNA concentration was measured by using a UV spectrometer at 260 nm. DNA concentration was calculated using the formula: OD x 50 x dilution factor = DNA concentration in µg/ml.

The NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, USA) was also used at the University of Nottingham to quantify the DNA concentration prior to sequencing. A one microlitre sample was used to record the absorbance at OD (260 nm) and a reading of 1.0 at this wavelength was equivalent to 50 µg/ml of double stranded DNA. The purity of the DNA preparation was determined by measuring the optical density at λ260 nm and 280 nm and the ratio of 1.8 defined a pure preparation.

2.9.3 Restriction digests of DNA

Genomic, plasmid and other DNA fragments were digested using restriction endonucleases obtained from Promega, Southampton, UK. Digestions were carried out according to the manufacturer's instructions with the appropriate reaction buffer. Generally, 1-2 µg DNA was incubated in the presence of 10-20 units of restriction enzyme for 1-4 hours at 37ºC in 20-100 µl reaction volumes.

2.9.4 Dephosphorylation of DNA at the 5' end

To avoid self-ligation, the restriction digest plasmid DNA was dephosphorylated at 5' ends. Thermostable alkaline phosphatase (TSAP) was obtained from Promega, Southampton, UK. Immediately following restriction digestion, 2 units of TSAP was added directly to the restriction digest which was then incubated at 37ºC for 15-30 min. The dephosphorylated DNA was then either purified using the GenElute® PCR clean-up kit (Sigma, Dorset, UK) or the restriction endonuclease and TSAP were inactivated by incubating at 74ºC for 15 min depending upon the endonuclease used.

2.9.5 Ethanol precipitation of DNA

DNA was precipitated by the addition of 0.1 x vol 3M sodium acetate (pH 4.6) and 2 x vol absolute ethanol. The mixture was incubated at -20ºC for 30 min and then centrifuged at 15,115 rcf at 4ºC for 15 min. The pellet was washed with ice-cold 70% (v/v) ethanol (500 µl), air dried at room temperature for 5-10 min and resuspended in 10-20 µl of water.

2.9.6 Extraction of DNA from agarose gels

Digested DNA was run on a 1% (w/v) agarose gel and the DNA band of interest was excised using a sterile scalpel blade and subjected to the QIAEX II DNA extraction procedure (QIAGEN, Crawley, UK). Briefly, the gel slice was solublised and the DNA adsorbed to silica particles. The bound DNA was washed a number of times and then eluted with water.

2.9.7 Ligation of DNA

T4 DNA ligase 1 U/µl was obtained from Promega, Southampton, UK and was supplied with a 10x ligase buffer (300mM Tris-HCl (pH 7.8), 100mM $MgCl_2$, 100mM DTT and

10mM ATP). Reactions were carried out according to manufacturer's instructions; generally 120 ng of insert and 20 ng of vector was used in a reaction volume of 10 µl and incubated overnight at 4ºC. The ligation mixture was used as such in the transformation of chemically competent cells or was ethanol precipitated for use in electro-transformation reactions.

2.9.8 Cloning into pGEM®-T Easy vector

The pGEM®-T Easy vector (Promega, Southampton, UK) (Figure 2.2) was used for general cloning and to generate a suicide vector due to its ease of use to directly clone PCR products by TA cloning and its inability to replicate inside *Campylobacter* species. It allows blue/white selection of colonies and carries the ampicillin resistance gene. *Bam*HI, *Bgl*II, *Bcl*I and *Hind*III are amongst many other endonucleases that do not cut inside the vector and their restriction sites in the gene of interest can be exploited to generate insertional mutations by inserting an antibiotic cassette.

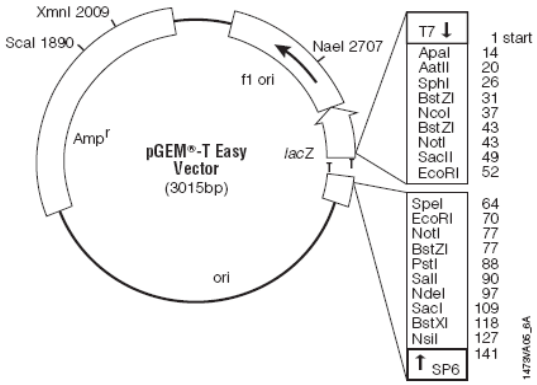

Figure 2.2 Circular map of pGEM®-T Easy vector (Promega, UK).

Cloning of PCR products was carried out using the pGEM®-T Easy vector according to the manufacturer's instructions. Briefly, it involved incubating the pGEM®-T Easy vector (50 ng) with the PCR product to be cloned (50 ng) in the presence of T4 DNA ligase (supplied with the kit) for one hour at room temperature or 4ºC overnight. The ligation mixture (2-5 µl) was used to transform One Shot® chemically-competent TOP10 *E. coli* cells (Invitrogen, Paisley, UK) and selection was made on LB agar containing 100 µg ampicillin ml^{-1} and 30 mg X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) ml^{-1}. After overnight incubation at 37ºC, white or light blue colonies were picked and subcultured onto LB agar containing ampicillin.

2.10 Preparation of competent cells and transformation

2.10.1 Preparation of electrocompetent *E. coli*

Electroporation with high voltage is the most efficient method for transforming *E. coli* with large plasmid DNA (Dower *et al.*, 1988) and plasmids generated in the plasmid rescue technique 5.2.1) were likely to have large molecular weights so electroporation was done to transform electrocompetent *E. coli* S-17λpir in this study. Electrocompetent *E. coli* cells were prepared following the method of Ausubel (Ausubel *et al.*, 1994). Briefly, *E. coli* S-17λpir was grown in LB broth overnight at 37ºC with moderate shaking. 500 ml pre-warmed LB broth was inoculated with 2.5 ml of the culture and incubated at 37ºC with shaking at 300 rpm to an OD (600 nm) of ~0.5 to 0.6. Cells were chilled in ice-cold water for 10 to 15 min and centrifuged for 20 min at 4000 rcf at 2ºC. The supernatant was poured off and cells were washed 3x by resuspending in 160 ml ice-cold water followed by centrifugation. Finally an opaque dense (~10^9 CFU/ml) suspension of the pelleted cells was prepared in ice-cold water and aliquots (50 µl) were used immediately or they were resuspended in 10% ice-cold glycerol and aliquots (50 µl) were frozen down rapidly in an absolute ethanol bath on dry-ice and stored at -80 ºC.

2.10.2 Electroporation of *E. coli* cells

Electrocompetent *E. coli* S-17λpir cells (fresh or thawed on ice) were mixed with 5 µl of DNA (0.5 µg) by gentle tapping and then incubated on ice for 10 min. The bacteria were transferred to a pre-chilled 2 mm cuvette (Bio-Rad laboratories, Hertford, UK) and pulsed at the preset programme for *E. coli* transformation (2.5 kV, 25 µF, 400 ohms) using the Bio-Rad Gene Pulser Xcell™. Immediately following the pulse the contents of the cuvette were flushed with 500 µl pre-warmed SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose) and transferred to a sterile Eppendorf tube and incubated for 60 min with moderate shaking at 37ºC. The cells were plated out onto LB agar plates supplemented with the appropriate antibiotic. The plates were incubated at 37ºC overnight to select for transformants.

2.10.3 Preparation of electrocompetent *C. jejuni* cells

Campylobacter strains were cultured on blood agar plates for 17 hours at 37 ºC in a microaerobic atmosphere. The culture plates were then placed at 4ºC for 20 min to allow the agar to become firm. *Campylobacter* cells were harvested from the plates and resuspended in ice-cold sucrose solution (272 mM sucrose in 5% (v/v) glycerol). Cells were washed in sucrose solution three times by centrifugation at 4000 rcf for 15 min at 4ºC. Finally the pelleted cells were resuspended in sucrose solution to give an opaque density. Electrocompetent *Campylobacter* cell (50 µl) aliquots were immediately frozen as described above and stored at -80ºC.

2.10.4 Electroporation of *C. jejuni* cells

Two 50 µl aliquots of the electrocompetent *Campylobacter* cells were thawed on ice, one was mixed with 5 µl of DNA (0.5 µg) and other with 5 µl of sterile water to act as a negative control. They were incubated on ice for 10 min and then were transferred to 2 mm cuvettes (Bio-Rad laboratories, Hertford, UK) and pulsed at 2.48 kV, 25 µF, 600 ohms using Bio-Rad Gene Pulser Xcell™. The contents of the each cuvette wase flushed with 500 µl pre-warmed BHI broth (37ºC) and plated onto two pre-dried BA plates. Cells were recovered for 5 hours at 37ºC in a microaerobic atmosphere. The bacteria were harvested from the recovery plates using 1 ml pre-warmed PBS and plated onto pre-dried BA plates containing the appropriate antibiotic and incubated for 72 hours at 37ºC. Plates were examined for the presence of colonies for up to 72 hours. Colonies were sub-cultured onto the appropriate antibiotic-containing media.

2.10.5 Heat shock transformation of *E. coli* cells

Ten microlitres of plasmid DNA (10 ng) or ligation reaction mixture was added to 50 µl of thawed One Shot® chemically-competent TOP10 *E. coli* cells (Invitrogen, Paisley, UK) and incubated on ice for 30 minutes. The cells were heat shocked for 30 seconds at 42ºC, before being recovered for one hour at 37ºC with shaking (200 rpm) in 250 µl of SOC medium. After recovery the cells (20, 50 and 100 µl) were plated out onto LB agar plates supplemented with the appropriate antibiotic(s). The plates were incubated at 37ºC overnight to select for transformants.

2.11 DNA extraction procedures

2.11.1 Genomic DNA preparation from *Campylobacter*

Genomic DNA from the *Campylobacter* cultures was extracted using the GenElute®Bacterial Genomic DNA kit (Sigma, Dorset, UK) according to the manufacturer's instructions. Briefly, *Campylobacter* strains were grown on BA plates for 48 hours under microaerobic conditions at 37ºC. The growth from half of the plate was resuspended in lysis solution T (360 µl); RNase A solution (40 µl) was added, mixed and incubated for 2 min at room temperature. Then Proteinase K solution (40 µl) was mixed in and incubated for 30 min at 55ºC before adding lysis solution C (400 µl), followed by vortex mixing and incubation for 10 min at 55ºC. After preparing the DNA binding columns (provided with the kit) the cell lysate was loaded and centrifuged and the DNA was allowed to bind to the columns. The columns were washed twice with wash solution and finally DNA was eluted with 200 µl elution buffer and stored at -20^{o}C for further use.

2.11.2 Mini DNA plasmid preparations from *E. coli*

Following transformation (Section 2.10), an *E. coli* colony was picked and a small culture (5 ml) was grown in Luria-Bertani broth supplemented with appropriate antibiotic for 18-20 hours in a shaker at 37ºC. The culture was used to extract plasmid DNA from the *E. coli* cells using QIAprep spin miniprep kit (QIAGEN, Crawley, UK) as described by the manufacturer. Biefly, *E. coli* cell pellete was resuspended in 250 µl of buffer P1 followed by alkaline lysis with an equal volume of buffer P2. Then lysate was neutralised and cell proteins and genomic DNA were precipitated by mixing with 350 µl of buffer N3. After centrifugation for 10 minutes at ~17900 x g, the supernatant was moved into a silica column provided with the kit. After washing the column with buffers PB and PE, the plasmid DNA was eluted in 50-100 µl of water or 10 mM Tris-HCl (pH 8.0) and stored at -20^{o}C for further use.

2.12 Polymerase chain reaction (PCR)

2.12.1 Standard PCR

PCR reactions were generally carried out in 50 µl volumes. GoTaq® Flexi DNA polymerase (1.5 unit) (Promega, Southampton, UK), $MgCl_2$ (2.5 mM), dNTPs (0.2 mM), forward and reverse primers (0.5 µM each) and DNA template (0.1 µg) were used in the PCR reaction mixture. DNA was denatured by incubating at 94ºC for 4 min; followed by 30 cycles of 94ºC for 45 sec, 50-65ºC for 45 sec (depending on the Tm of primers) and 72ºC for 1-5 min (depending on expected PCR product size i.e. 1 min/1 kb). After all the cycles were completed a final incubation at 72ºC for 10 min was performed and the PCR products visualised by gel electrophoresis (Section 2.9.1).

2.12.2 Proof reading PCR

Where high fidelity was required *Pfu* DNA polymerase (Promega, Southampton, UK) was used for DNA polymerisation. The PCR reaction mixture was prepared as mentioned above and kept on ice to avoid exonuclease activity of *Pfu* DNA polymerase and the extension time in the reaction was 2 min for every 1 kbp to be amplified.

2.12.3 Colony PCR

A loopful of freshly grown bacteria was suspended in 300 µl of water. The bacterial cell suspension was boiled for 10 minutes and centrifuged at 15,500 rcf for 5 min and one microlitre of the supernatant was used as template in a standard PCR (Section2.12.1).

2.12.4 Purification of PCR products

PCR amplification products were purified from other components in the reactions, such as excess primers, nucleotides, DNA polymerase and salts. The GenElute®PCR clean-up kit (Sigma, Poole, UK) was used for this purpose. Purification was done according to manufacturer's instructions. Briefly, PCR reaction products were mixed with loading buffer and a silica membrane column was loaded with mixture. After centrifugation, the column was washed with wash buffer and finally the PCR products were eluted with 50-200 µl of elution buffer.

2.13 Generation of transposon mutant library

The *mariner*-based *Himar* transposon was used to generate a transposon random insertion mutant library in a hyperinvasive clinical isolate *C. jejuni* strain 01/51. The transposon library was generated in the Professor Duncan Maskell's laboratory at the Cambridge University following the protocol described previously (Grant *et al.*, 2005).

Briefly, the plasmid pEnterprise2 (Hendrixson *et al.*, 2001) which contains a *mariner*-based transposon (derived from *Himar1*) on a pUC19 backbone, was used to construct the transposons. Transposition reactions were performed as previously detailed (Hendrixson *et al.*, 2001), using total bacterial DNA (2 µg) and ~250 ng *Himar1* transposase purified as described by Lampe *et al.* (Lampe *et al.*, 1996) from *E. coli* harboring pET29b+C9, an expression vector containing the C9 hyperactive *Himar1* transposase (Lampe *et al.*, 1999). The transposed DNA was introduced into *C. jejuni* by natural transformation using a plate biphasic method. Transposon mutants were recovered on selective agar after growth under standard conditions for 60 to 72 h.

Chapter Three

OPTIMISATION OF THE GENTAMICIN PROTECTION ASSAY

3 OPTIMISATION OF THE GENTAMICIN PROTECTION ASSAY

3.1 Introduction

Campylobacter jejuni generally infects humans by colonising the mucous layer of the intestine followed by adherence and invasion of epithelial cells (Ketley, 1997). Clinical observations and experimental evidence indicate that the ability of *C. jejuni* to invade the host intestinal epithelium is an important determinant of its ability to cause focal necrosis of the colonic mucosa (Black *et al.*, 1988; Russell *et al.*, 1989). Studying the mechanisms of *Campylobacter* pathogenesis is complicated by the lack of simple animal models that mimic the disease in humans. *In vitro* cell culture methods provide a useful alternative to investigate the interaction between *Campylobacter* and the host epithelium that occur during infection. The invasiveness of *Campylobacter* in cultured epithelial cell lines has, in some cases, been correlated with the disease outcome (Bacon *et al.*, 2000; Fauchere *et al.*, 1986; Newell and Pearson, 1984) supporting the view that cell culture models are useful indicators of pathogenicity. Different cell lines of human intestinal origin including Caco-2 (Hanel *et al.*, 2004), INT 407 (Hu and Kopecko, 1999), HT29 and T84 (MacCallum *et al.*, 2006; Van Deun *et al.*, 2007), and non-intestinal origin including HeLa, HEp-2 (de Melo and Pechere, 1990; Prasad *et al.*, 1996) and Vero (Johnson and Lior, 1986) have been used to study the pathogenesis of *C. jejuni* but INT 407 and Caco-2 cells have recently been most extensively used to study the adhesion and invasion in this organism. The gentamicin protection assay (Elsinghorst, 1994) has become a standard method to measure cultured eukaryotic cell penetration by bacteria. There are many different parameters in this assay that independently affect optimal invasion and, consequently, methods described in the literature often differ and the assay has been review well in Friis' paper (Friis *et al.*, 2005). The aim of this part of the study was to check and standardise some of the important factors in the gentamicin protection assay and to find the parameters that give optimal invasion in *C. jejuni.*

3.2 Results and discussion

3.2.1 Growth curve of *Campylobacter jejuni* 01/51

Hu and Kopecko reported that *Campylobacter jejuni* inoculum taken from exponential phase of growth show maximum invasion in cultured INT-407 cells (Hu and Kopecko, 1999). A growth curve of *C. jejuni* 01/51 was done to find the growth phases over time periods so that inoculum for gentamicin protection assay could be taken from exponential phase of bacterial growth. *C. jejuni* grown in Mueller-Hinton agar/Mueller-Hinton broth (MHA/MHB) biphasic medium has been used in gentamicin protection assay in many previous studies (Gaynor *et al.*, 2004; Hu and Kopecko, 1999; Kakuda and DiRita, 2006; Monteville *et al.*, 2003). So, initially *C. jejuni* 01/51 was grown in MHA/MHB biphasic medium and samples were taken at different time intervals to measure the bacterial cell number by the serial dilution method. The bacterial growth curve (Figure 3.1) showed progressive growth up to 25 hours followed by a decline in growth with visible aggregation of the bacterial cells. It has previously been reported that *C. jejuni* 81-176 autoagglutinates at higher densities (Misawa and Blaser, 2000) and this visible aggregation might be the result of autoagglutination of the *C. jejuni* cells. There were no cultureable bacterial cells in the samples taken at 48 hours of growth (not shown), which was an unusual finding. Generally, the bacterial growth curve consists of initial lag phase where bacteria adjust themselves to the new environment followed by exponential growth phase, then a comparatively long stationary phase and finally a decline phase. In this experiment the stationary phase was not obvious. This experiment was also repeated separately using brain heart infusion agar/brain heart infusion broth (BHIA/BHIB) biphasic medium, standard brain heart infusion broth and standard Mueller-Hinton broth where the growth curves (not shown) were similar to that observed in MHA/MHB biphasic medium. The lack of stationary phase observed in the growth curve of *C. jejuni* 01/51 correlates with an other report stating that *C. jejuni* lacks a 'classic' stationary phase (Kelly *et al.*, 2001). One of the possible reasons for lack of the stationary phase in *C. jejuni* growth is the lack of stationary phase sigma factor *rpoS* in the genome sequence of *C. jejuni* NCTC11168 (Parkhill et al., 2000), which modulates stress and stationary phase responses in many Gram-negative bacteria (Ishihama, 2000; Venturi, 2003). It has been reported that *C. jejuni* changes its morphology during different phases of growth being spiral in early exponential phase

and changes its shape to coccoid as the culture ages or is exposed to stress (Thomas *et al.*, 1999). The conversion to coccoid morphology is accompanied by a rapid loss of culturability (Boucher *et al.*, 1994). This loss of *in vitro* culturability of *C. jejuni* might be responsible for the lack of stationary phase and rapid decline in viable plate counts.

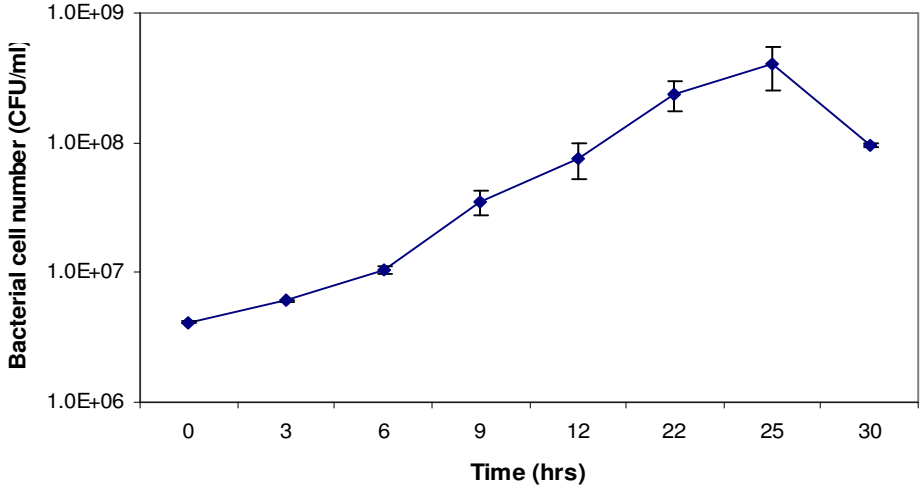

Figure 3.1 A growth curve of *C. jejuni* 01/51 in MHA/MHB biphasic media.

Growth of *C. jejuni* 01/51 was checked in Mueller-Hinton agar/Mueller-Hinton broth (MHA/MHB) biphasic medium. For the growth curve pre-warmed MHA/MHB was inoculated to an OD (550 nm) of 0.05from an overnight culture of *C. jejuni* 01/51 in MH broth. The bacterial culture was incubated at 37^{o}C on an orbital shaker at 200 rpm and samples were taken at different time intervals for up to 48 hours and the viable cell count was done. The assay was done in duplicate and the error bars show the standard deviation.

3.2.2 Pre-assay bacterial optimal growth conditions

The effect of the bacterial growth conditions prior to the invasion assays, including culture media and incubation period was studied to select the optimal conditions for bacterial invasion in the gentamicin protection assay. Bacteria were grown in the Mueller-Hinton agar/Mueller-Hinton broth (MHA/MHB) biphasic medium for 22 hours and on blood agar plates for 24 hours and 48 hours before the invasion assay. The growth curve of *C. jejuni* 01/51 grown in MHA/MHB biphasic media (Figure 3.1) showed that the bacteria were in exponential phase for up to 25 hours after inoculation and later entered into the decline phase and there was no growth observed after 48 hours of incubation thus 48 hours growth in MHA/MHB biphasic medium was not included in this experiment.

Gentamicin protection assay using *C. jejuni* 01/51 grown on blood agar and MHA/MHB biphasic media for different time periods showed that bacteria grown on blood agar plates for 48 hours gave the maximum invasion efficiency in cultured Caco-2 cells (Figure 3.2) moreover, there was a little growth on blood agar plates after 24 hours as compared to 48 hours incubation and it was easy to pick the colonies from a 48 hours growth plate, thus 48 hours growth on blood agar plates was used in further assays. Blood agar grown *C. jejuni* has also been used in gentamicin protection assays by others (Malik-Kale *et al.*, 2008; Monteville *et al.*, 2003; Pei *et al.*, 1998). It has previously been reported that a group of *Campylobcater* secreted antigens (*cia* proteins) are required for the maximal host cell invasion by *C. jejuni* and secretion of these proteins was enhanced by addition of serum in the growth media (Konkel *et al.*, 1999). It was thought that by growing *C. jejuni* on blood agar the expression of such proteins will also be enhanced and the a change in invasion efficiency shown by *C. jejuni* will remain at their maximum level and will not be affected by a change in the expression levels of *cia* protein.

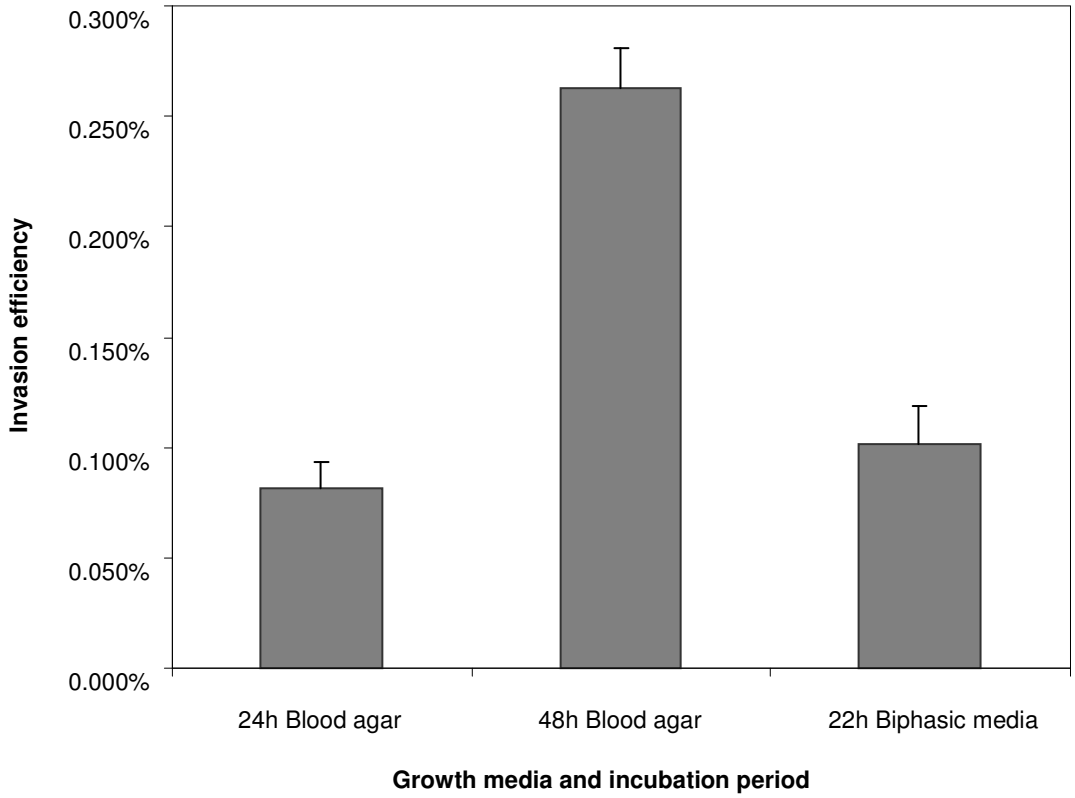

Figure 3.2 The efficiency of invasion of *C. jejuni* 01/51 grown on different media from 22 to 48 hours prior to assay.

To determine the bacterial growth conditions that give maximum invasion efficiency in the gentamicin protection assay, *C. jejuni* 01/51 growth on blood agar for 24 hour and 48 hour as well as Mueller-Hinton biphasic medium for 22 hours was used. Maximum invasion efficiency was shown by *C. jejuni* 01/51 grown on blood agar for 48 hour and it was used in further gentamicin protection assays. The efficiency of invasion was the number of bacteria internalised expressed as a percentage of total bacterial cell number added to the Caco-2 cell monolayer at the start of the assay.

3.2.3 Optimal multiplicity of infection (MOI) for invasion assay

Evidence exists from previous assays that variation in the inocula could affect the invasion levels measured (Hu and Kopecko, 1999). A dose response curve was produced to find out the optimal number of bacteria per Caco-2 cell that give the best invasion efficiency in the assay. Thus the total number of bacteria internalised per well was measured against a range of MOI from 25 to 1000.

The results showed that the number of internalised bacteria increases with increasing MOI up to 200 after that there is no significant increase (Figure 3.3). Fearnley *et al.* reported a progressive increase in internalised *C. jejuni* cell number in INT-407 cells up

to the multiplicity of infection of 200 followed by a plateau (Fearnley *et al.*, 2008); similar findings were reported in a previous study where INT-407 cells were used to assess the *C. jejuni* invasion (Hu and Kopecko, 1999). Invasion efficiency of each MOI was also calculated and the averaged invasion efficiency was maximum (0.34) at the minimal MOI used *i.e.* 25 and invasion efficiency in Caco-2 cells was steadily decreased with increasing MOI of *C. jejuni* 01/51 (Appendix 1). The inverse relationship of invasion efficiency with MOI of *C. jejuni* 81-176 in cultured INT-407 cells *i.e.* decrease in invasion efficiency with increasing MOI was also reported by Hu and Kopecko (Hu and Kopecko, 1999) and suggests that this organism is a highly efficient solitary invader; *i.e.* a single *C. jejuni* can induce its own uptake into host cells. This contrasts sharply with *S. typhi* invasion, invasion efficiency is suboptimal at lower and higher MOIs, but reaches a broad optimum at an MOI 40 (Huang *et al.*, 1998). The MOI of 100 was selected for further invasion assays so that an experimental variation in adjusting the MOI could not have a major effect on the invasion efficiency; as the MOI above 200 is not part of straight line and thus was expected to give lower invasion efficiency than the actual.

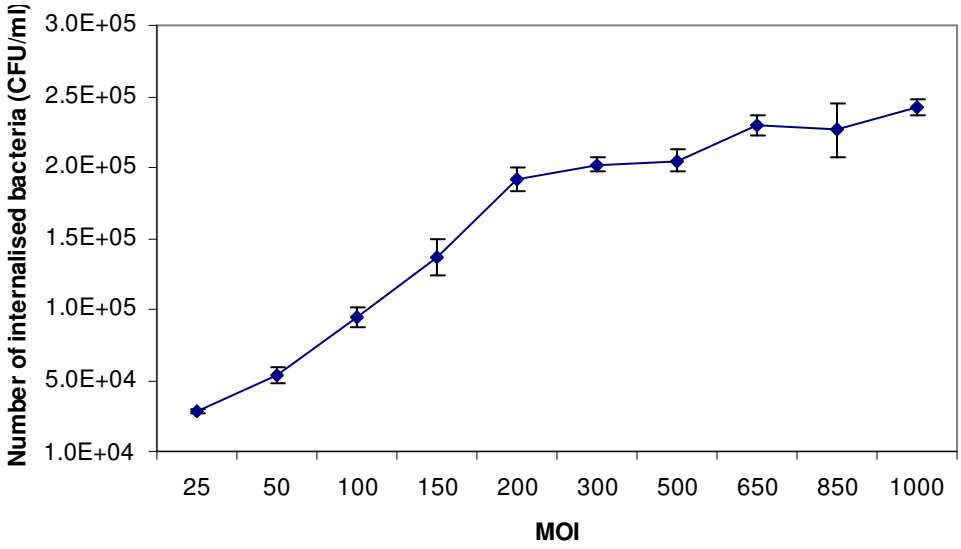

Figure 3.3 The number of internalised *C. jejuni* 01/51 cells against different MOIs in the Caco-2 cell invasion assay.

Multiplicity of infection (MOI) of *C. jeuni* 01/51 that gives maximum invasion level in Caco-2 cell based gentamicin protection assay was determined. A range of MOIs from 25 to 1000 was used in the assay and there was an increase in the number of internalised bacteria with increasing MOI but maximum level of invasion was observed at MOI 200 after which was not a big increase in the number of internalised bacteria with increasing MOI. The MOI 200 was selected as maximum MOI for further gentamicin protection assays.

Briefly, *C. jejuni* 01/51 grown on blood agar for 48 hour and MOI 200 were found to give optimal level of invasion in Caco-2 cells and were thus used in gentamicin protection assays in future. *C. jejuni* with known invasion potential in INT-407 cells were tested in Caco-2 cell gentamicin protection assay to check if the invasiveness in INT-407 cells could be conferred in Caco-2 cells.

3.3 Confirmation of the invasion phenotype of selected *C. jejuni* strains from a previous study

Now that optimal conditions for Caco-2 cell based gentamicin protection assay were determined and the assay was used to test and validate the invasion potential of *C. jejuni* strains with known invasion capability in INT-407 cells. Most of the *Campylobacter* strains used in this study at Nottingham Trent University were previously isolated and their ability to invade human intestinal cells was studied using INT-407 at the Veterinary Laboratories Agency, Weybridge, UK. In the previous studies *C. jejuni* strains were up to x10, x10-25 and more than x25 invasive in INT-407 cells compared to *C. jejuni* 81116 were classified as low, high and hyperinvasive, respectively. In those previous studies *C. jejuni* strains 01/04, 01/10, 01/35, 01/41, 01/51 and EX114 were found to be hyperinvasive and *C. jejuni* strains 01/32, 01/46, 01/50 and 81116 were low invaders of INT-407 cells (Fearnley *et al.*, 2008). In order to corroborate these results and to see whether this phenotype would exist in an alternative cell line, these strains were re-tested for invasion in Caco-2 cells. Gentamicin protection assays using Caco-2 cells were performed to see how these bacterial strains invade Caco-2 cells. Results showed that four (01/04, 01/41, 01/51 and EX114) out of six strains that were hyperinvasive in a previous study using INT-407 cells were still hyper or highly invasive in this assay with Caco-2 cells (Figure 3.4). *C. jejuni* 01/51 was hyperinvasive in both the studies so was still best strain to use. The strains (01/10 and 01/35) were hyperinvasive in INT-407 cells but not in Caco-2 cells which could be due to inherent differences in different cell lines. The same *Campylobacter* strain can vary in invasion efficiency for different host cell lines and this dependence appears to be host cell line-specific (Konkel *et al.*, 1992a; Oelschlaeger *et al.*, 1993). In addition, the reduced invasion phenotype of a number of transposon mutants of *C. jejuni* 01/51 that had

shown reduced invasion in a previous study with INT-407 cells (Dr. Georgina Manning, personal communication) was also tested using Caco-2 cells. Generally, the level of invasion in Caco-2 cells was similar to that previously observed in INT-407 cells as all the mutants showed very low level of invasion in Caco-2 cell but mutant 1E8 which was non-invasive in INT-407 cells showed 64% invasion in Caco-2 cells as compared to wildtype strain 01/51 (Table 3.1). Broadly, the reduced invasion phenotype observed in INT-407 cell was persistent in Caco-2 cells.

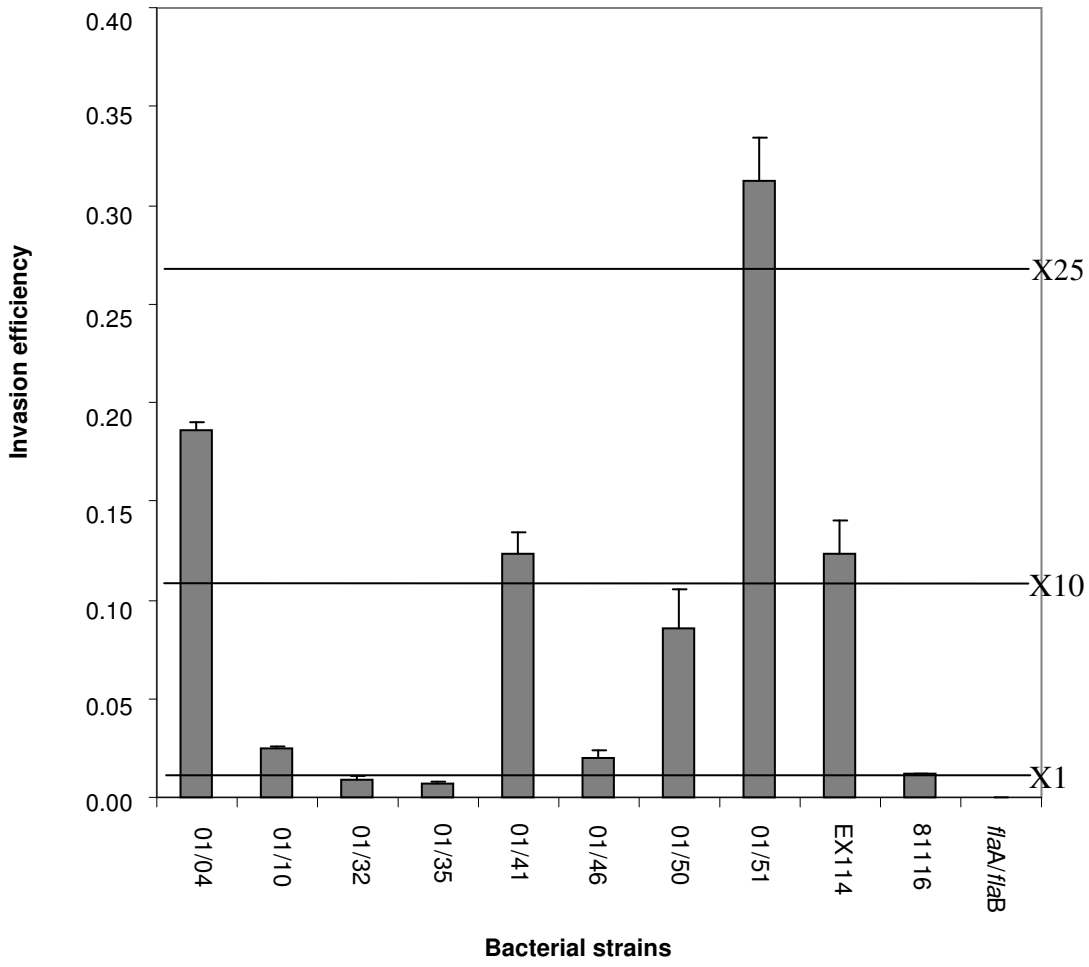

Figure 3.4 The invasion efficiency of different *C. jejuni* strains into Caco-2 monolayers.

C. jejuni strains whose invasiveness in INT-407 cells was previously known were checked for invasion in Caco-2 cells. The X1 band indicates the invasion efficiency of *C. jejuni* 81116 used as a reference low invasive strain in this assay. Bands X10 and X25 indicates the invasion efficiency of test strains as 10 fold and 25 fold, respectively, higher than the reference strain. Non-invasive *C. jejuni* 81116 Δ*fla*A/*fla*B strain was used as a negative control in the assay. The strains that show invasion above x10 were high and above x25 compared to *C. jejuni* 81116 were hyperinvasive (Fearnley *et al.*, 2008).

Table 3.1 The invasion efficiency of mutants of *C. jejuni* 01/51 into INT-407 and Caco-2 monolayers.

Mutants	Invasion (INT-407)	Invasion (Caco-2)	Comments
1C1	19	5	Transposon mutant of Cj0490: *ald*, aldehyde dehydrogenase C-terminus
1D7	10	3	Transposon mutant of Cj0486: putative sugar transporter
1E8	0	64	Transposon mutant: gene not yet determined
1G5	4	3	Transposon mutant: gene not yet determined
1H10	0	1.6	Transposon mutant of *dtp*T gene in 01/51: di-tripeptide transporter protein not present in NCTC11168
ΔCj0497	0.8	10	Targeted mutant of Cj0497: putative lipoprotein
Δ*fla*A/*fla*B	0	0	*C. jejuni* 81116 double mutant of *fla*A and *fla*B.

Mutants of *C. jejuni* 01/51 whose invasion potential in INT-407 cells was previously known were tested for their invasiveness in Caco-2 cells using optimised gentamicin protection assay. Invasiveness is presented as a percentage of the invasiveness of the wild-type 01/51. The figure given is the mean of three replicates from within a single assay. Non-invasive *C. jejuni* 81116 Δ*fla*A/*fla*B strain was used as a negative control in the assay.

3.4 Summary and conclusions

Cultured eukaryotic cell invasion assays have become standard technique for the study of bacterial internalisation. The gentamicin protection assay was optimised and *C. jejuni* 01/51 grown on blood agar for 48 hours gave maximal invasion at MOI 100 in Caco-2 cells. When selected *C. jejuni* strains and transposon mutants of *C. jejuni* 01/51 with previously known invasion capability into INT-407 cells were tested for invasion into Caco-2 cells using optimised gentamicin protection assay, invasion in Caco-2 cells was found generally similar to INT-407 cells though two *C. jejuni* strains out of eleven and one *C. jejuni* 01/51 mutant out of six showed major differences in invasiveness in Caco-2 than INT-407 cells. Generally bacterial invasion phenotype in INT-407 was transferable into Caco-2 which is a widely accepted human intestinal epithelial cell-line and on the basis of these findings INT-407 cells were selected to screen a transposon mutant library generated in a hyperinvasive clinical isolate *C. jejuni* 01/51 for invasion.

Chapter Four

SCREENING OF THE TRANSPOSON MUTANT LIBRARY

4 SCREENING OF THE TRANSPOSON MUTANT LIBRARY

4.1 Introduction

Campylobacter jejuni strains vary in invasiveness in eukaryotic cells. A number of hyperinvasive clinical strains of *C. jejuni* have recently been identified. These isolates were found to invade the human epithelial cell lines INT-407 and Caco-2 to high levels compared with a low-invasive control strain *C. jejuni* NCTC81116 (Fearnley *et al.*, 2008). Despite the fact that many *C. jejuni* genome sequences have been completed, the molecular mechanisms by which *Campylobacter jejuni* invade the intestinal epithelial cells and cause disease in human are not yet well understood. The analysis of the *C. jejuni* genome sequences produced many interesting observations, including an unappreciated capacity for polysaccharide biosynthesis and the presence of hypervariable homopolymeric tract sequences, but there was apparent lack of genes encoding virulence determinants similar to those expressed in other more extensively characterised enteric pathogens such as *Escherichia coli* and *Salmonella enterica* serovars (Fouts *et al.*, 2005; Parkhill *et al.*, 2000; Pearson *et al.*, 2007). This paucity of information is also due to a lack of genetic tools; in particular the lack of a transposon mutagenesis method for the efficient generation of random mutants of *Campylobacter* spp. Previous attempts to generate mutants have relied on shuttle mutagenesis and homologous recombination (Bleumink-Pluym *et al.*, 1999; Labigne *et al.*, 1992; Yao *et al.*, 1994). However, more recently a highly permissive *mariner*-based transposon known as *Himar*1 has been utilized for efficient random transposon mutagenesis in *C. jejuni* (Golden *et al.*, 2000; Grant *et al.*, 2005; Hendrixson *et al.*, 2001); the transposon mutant libraries were screened for mutants with reduced motility (Hendrixson *et al.*, 2001), motility and autoagglutination (Golden and Acheson, 2002), and chicken colonisation (Grant *et al.*, 2005). Therefore, an *in vitro mariner*-based transposon mutagenesis was used to produce a random insertional mutant library in a hyperinvasive clinical isolate of *C. jejuni*, strain 01/51. The chloramphenicol acetyltransferase (*cat*) gene was used as selection marker for the generation of transposon mutant library. The library consisting of 867 mutants was previously generated in collaboration with Prof. Duncan Maskell, Cambridge University, United Kingdom and the randomness of

transposon insertion was also confirmed by Southern blotting and sequencing of a selection of mutants. In this study, this transposon mutant library was screened in assays of invasion to find those mutants having reduced invasion levels in human intestinal epithelial cells as compared to parent strain *C. jejuni* 01/51.

4.2 Methods

4.2.1 Screening of the transposon mutant library

The invasion assay used was an adaptation of the gentamicin protection assay, using the human intestinal epithelial cell line, INT-407 (Elsinghorst, 1994; Fearnley *et al.*, 2008). For screening of the transposon mutants the semi-quantitative 96-well plate assay was adapted from that of Golden and Acheson (Golden and Acheson, 2002). Briefly, the frozen transposon mutant library was thawed on ice and 10 µl transferred to Columbia agar (100 µl) supplemented with sheep blood (5%) and chloramphenicol (10 µg/ml) in a 96-well plate. The plates were incubated at 37^{o}C in microaerobic conditions for 48 hours. At the same time INT-407 cells were resuspended in complete cell culture media (Modified Eagle's Medium, MEM, supplemented with 1% non-essential amino acids and 10% calf serum (Sigma)) at a density of 1 X 10^5 cells per ml. Two hundred microlitres of cell suspension was added to each well in the 96-well plates and incubated for 48 hours at 37^{o}C in 5% (v/v) CO_2. On the day of the assay, the cell culture media covering the INT-407 cell monolayers was replaced with 200 µl fresh pre-warmed complete cell culture media. Complete cell culture media (100 µl) was added to each well of the plate containing bacterial growth and left for 5-10 min for colonies to soften. Bacterial cells were resuspended by pipetting and 20 µl of the suspension was added to each well of the INT-407 monolayers. Infected plates were incubated for 3 hours at 37^{o}C in 5% (v/v) CO_2. After incubation monolayers were washed 3X with PBS and were covered with 200 µl complete cell culture media with 250 µg gentamicin ml^{-1}. Following a 2 hour incubation, monolayers were washed 3X with PBS and cells were lysed with 100 µl 1% (v/v) Triton-X100. The number of viable bacteria per well was determined by serial dilution with PBS and spotting 3 µl onto large pre-dried BA plates. Plates were incubated at 37^{o}C in microaerobic conditions and the colonies counted. *Campylobacter jejuni* 01/51 and *Campylobacter jejuni* 81116 *fla*A^{-}/*fla*B^{-} were also used as reference parent strain and negative control, respectively, in the assays. The invasion

screening assay was repeated three times independently. The mutant invasion results were compared with those of the parent strain 01/51.

4.2.2 Motility

Like many other enteric pathogens, motility in *C. jejuni* is important for invasion into cultured epithelial cells and those strains with reduced motility are reduced in invasion. The *C. jejuni* 01/51 and those transposon mutants that showed reduction in invasion than parent strain 01/51 were tested for their motility so that those mutants with low motility could be excluded from further studies. Motility of the bacterial strains was tested by measuring growth on 0.4% MH agar plates. *C. jejuni* 01/51 and its selected transposon mutants were initially grown on blood agar plates and harvested cells were suspended in sterile phosphate buffered saline to an OD (550 nm) of 1.0. Approximately 1 µl of this suspension was stabbed into the centre of the MH agar plate. The low density of the agar allowed the bacteria to move within the agar forming a halo of growth around the point of inoculation. Following inoculation the bacteria were incubated microaerobically at 37ºC for 48 hours. The diameter of the ring of growth formed by the mutants was measured and compared with that formed by the *C. jejuni* 01/51. The flagellar double mutant *C. jejuni* 81116 *fla*A$^-$/*fla*B$^-$ was used as negative control. The assay was carried out in triplicate and mean of values was plotted.

4.3 Results and discussion

4.3.1 Results of screening of the transposon mutant library

A transposon mutant library previously generated in *Campylobacter jejuni* 01/51 was screened for invasion potential using INT-407 cells to find the mutants with a reduced invasion phenotype. INT-407 cells were used because they are easy to grow and *C. jejuni* invade them at higher levels than Caco-2 cells (Hu *et al*., 2008) thus it gives a better resolution for screening the transposon mutant library. Moreover, INT-407 cells were used to screen 96 mutants out of this library in a previous study at the Veterinary Laboratories Agency, Weybridge, UK so these cells were used for comparative studies at Nottingham Trent University. The reduced invasion phenotype of previously identified transposon mutants using INT-407 cells was generally transferable to Caco-2 (Table 3.1) which also supported the selection of INT-407 cells for transposon mutant

library screening. The invasion screening assay was repeated three times. Mutants that showed up to six colonies in undiluted lysate spots (3 µl) compared to too many to count by the parent strain 01/51 were selected for further testing of their motility. It is known that reduced motility in *C. jejuni* results in reduced invasion into in vitro cultured cells (Wassenaar et al., 1991) and thus motility was determined to rule out those mutants with reduced motility. In this study, 768 transposon mutants were screened for their capability to invade INT-407 cells and 174 mutants consistently showed reduced invasion in screening assays and were therefore selected for further characterisation (appendix 2). Figure 4.1 shows the invasion screening results for one 96-well plate of mutants and those mutants indicated by shaded boxes were selected for motility tests.

Figure 4.1 The combined results recorded for each well of one of the 96-well plates included in the invasion screen.

	1	2	3	4	5	6	7	8	9	10	11	12
A	+	++	+++	+++	+	+++	+++	+++	++	+++	+	NG
B	3	+++	+++	+	X	++	+	+++	+++	X	+++	NG
C	+++	+	2	++	+++	+++	+	++	+++	+	NG	NG
D	+++	++	+++	+++	+++	+++	+++	+++	+++	++	X	NG
E	++	+++	+++	+++	++	+++	++	++	X	+	+	X
F	++	+++	++	+++	+++	+++	+++	++	+++	X	+++	X
G	++	++	++	+++	+++	+++	+++	++	+++	+++	++	+++
H	+++	+++	+++	+	+++	+++	+++	++	+	X	+++	X
	C. jejuni 01/51: +++								*C. jejuni* 81116 Δ*fla*A/*fla*B: X			

The transposon mutant library was screened for invasion in INT-407 cells. Mutants that showed up to six colonies in the undiluted lysate were selected (shaded) for motility testing. Key: NG: no growth in the inoculum; X: no colonies seen in test lysate; 1,2 or 3: exact number of colonies counted; **+:** 4 to 6 colonies counted; **++:** 7 to 9 colonies counted; **+++:** more than 9 colonies per spot.

4.3.2 Motility

Motility has been implicated in intestinal colonisation and virulence in many enteric pathogens including *Yersinia enterocolitica* (McNally *et al.*, 2007; Young *et al.*, 2000a), *Salmonella* Typhimurium (Schmitt *et al.*, 1994) and *C. jejuni* also depends on flagellar motility for its pathogenicity (Wassenaar *et al.*, 1991). One study showed both *fla*A and *fla*B mutants adhere to cultured intestinal epithelial cells but they were deficient in their ability to invade Caco-2 cells (Grant *et al.*, 1993). Therefore motility was tested in the transposon mutants (Section 4.2.2) with a low invasion capability in the screening

assays and only those mutants that were highly motile were included in further studies to confirm the reduced invasion phenotype in standard gentamicin protection assays.

The motility was tested in the 174 transposon mutants that showed reduced invasion by growing the bacteria on semisolid Mueller-Hinton broth supplemented with 0.4% agar and measuring the diameter of growth after 48 hours of incubation in microaerobic conditions. A non-motile mutant *C. jejuni* 81116 *fla*A$^-$/*fla*B$^-$ (Wassenaar *et al.*, 1991) was used as negative control in the motility assay. There was a range in motility shown by the mutants as compared to wildtype strain *C. jejuni* 01/51, most of the mutants showed motility at approximately the same level as wildtype strain but five of them were non-motile (Appendix 3). The motility and invasion data for a selection of mutant is given in (Table 4.1). Previously, during the generation of random transposon mutagenesis library of *C. jejuni* strain 480, mutants with 0 – 70% motility of the wildtype strain, were characterised; the ability to invade cultured INT-407 cells in these mutants was reduced (Golden *et al.*, 2000; Golden and Acheson, 2002). So an arbitrary level of motility (75%) was selected and forty mutants that showed motility above 75% as compared to wildtype strain 01/51 and up to three colonies in the screening assays were selected for confirmation of the reduced invasion phenotype by standard gentamicin protection assays using INT-407 and Caco-2 cells.

Table 4.1 An example of the combined results of the invasion and motility screen.

Mutant	Invasion	Diameter of growth (cm)	% motile*
3A8	X	0.6	14.6
3A10	2	4.3	87.8
3B10	3	5.2	106.1
3C1	X	3.5	71.4
3C2	+	3	61.2
3C5	+	4.4	89.8
***C. jejuni* 01/51**	+++	4.9	100
***C. jejuni* 81116 Δ*fla*A/*fla*B**	X	0.5	10.2

*%motile: percentage of motility shown by the mutants as compared to that shown by *C. jejuni* 01/51. Selected mutants were tested for motility and the table shows the results of 6 out of 174 mutants. The mutants that showed up to three colonies in the invasion screening assay and more than 75% motility as compared to wildtype strain were selected (shaded) for confirmation of the reduced invasion phenotype in INT-407 and Caco-2 cells. *C. jejuni* 81116Δ*fla*A/*flaB* was used as negative control in the assays. Key: X: no colonies; 1, 2 or 3: exact number of colonies counted; +: 4 to 6 colonies; ++: 7 to 9 colonies; +++: more than 9 colonies counted in 3 µl spot of the undiluted lysate in invasion screening assay.

4.3.3 Confirmation of phenotype using a standard gentamicin protection assay

The reduced invasion phenotype in the selected transposon mutants was confirmed by a standard gentamicin protection assay. The standard 24-well plate gentamicin protection invasion assay using INT-407 cells was performed as described earlier in Section 2.7 and the forty transposon mutants that showed very low or no invasion in INT-407 cells in the semi-quantitative invasion screening assays but good motility were tested for their invasion potential in the standard invasion assays. For ease of handling the forty mutants were divided into three groups and their invasion potential was tested in three independent gentamicin protection assays. Wildtype strain *C. jejuni* 01/51 and some of the non-motile mutants identified in this study were used as reference and negative controls, respectively, in the assays. Most of the mutants showed reduced invasion in INT-407 cells as determined in the previous screens. Interestingly five of the mutants showed invasion levels higher than the wildtype strain 01/51 including 5E12 and 5F12 that showed more than 40% higher invasion than the wildtype strain (Appendix 4). The

five motility mutants (3G4, 3H9, 9B2, 6G1 and 6G3) identified in this study were used as negative controls in the standard invasion assays and all of them showed very little invasion in INT-407 cells that was in agreement with previous reports (Grant *et al.*, 1993; Malik-Kale *et al.*, 2007; McNally *et al.*, 2007; Wassenaar *et al.*, 1991).

The invasion efficiency of the wildtype strain was variable in the standard invasion assays and to make the comparison uniform the mean values of invasion efficiencies of the mutants from triplicate wells were compared with those of the wildtype strain in the same assay and the percentage of mutant invasion was calculated considering the wildtype mean invasion efficiency as 100% (Appendix 4). Therefore the invasion potential of each mutant was presented as a percentage of the invasion efficiency of the wildtype strain. To further shortlist the transposon insertion mutants with very low invasion potential, a stringent cut off value of 80% reduction in invasiveness into INT-407 cells was used *i.e.* those mutants with up to 20% of invasion of the parent strain 01/51. Mutants showing a reduction in invasiveness of more than an 80% compared to wildtype were selected for reconfirming their reduced invasion phenotype. Fifteen out of forty mutants tested showed more than an 80% reduction in invasion *i.e.* having up to 20% invasion of that shown by wildtype strain 01/51 in the standard invasion assays and were selected for further characterisation (Figure 4.2). These selected fifteen transposon mutants were tested to reconfirm their reduced invasion potential into INT-407 as well as Caco-2 cells.

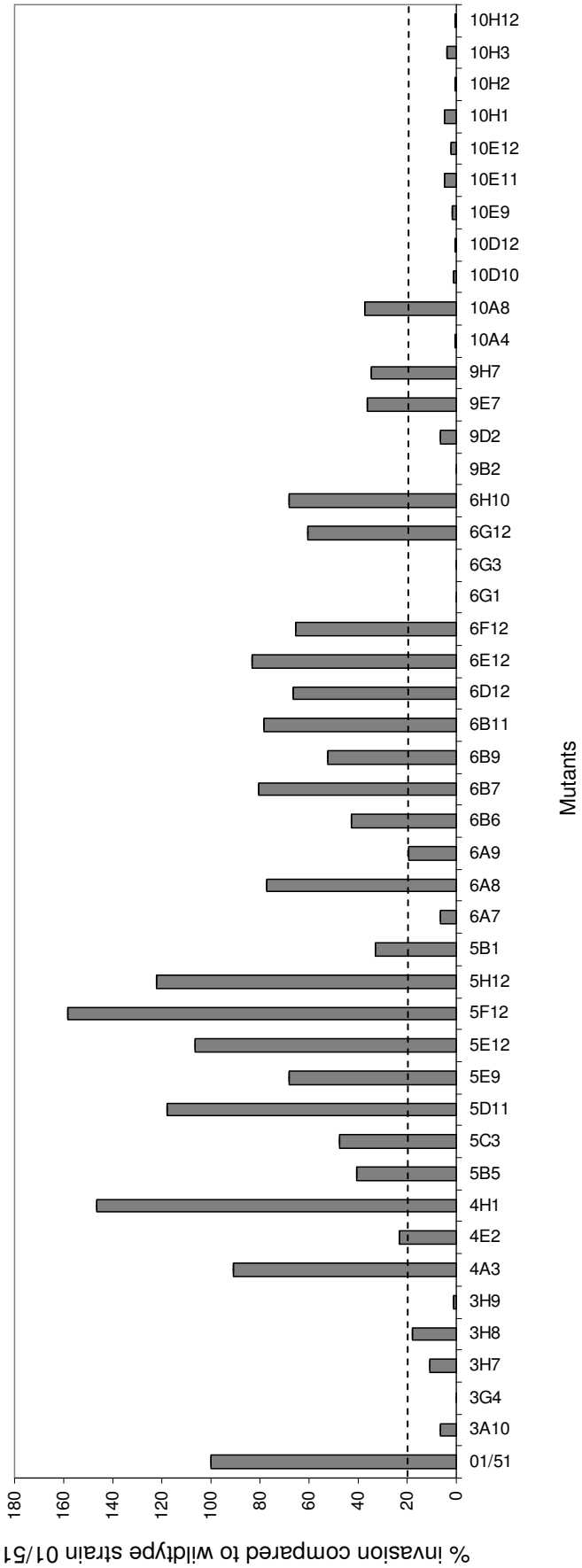

Figure 4.2 The INT-407 invasion potential of the selected transposon mutants.

Invasion potential of wildtype strain 01/51 and selected transposon mutants was tested in gentamicin protection assays. Mean value of invasion efficiencies shown observed in triplicate wells used for each mutant were compared with those of 01/51 and percentage of invasion efficiency was calculated considering that shown by 01/51 as 100%. Most of the mutants showed reduced levels of invasion in INT-407 cells, however, five mutants *i.e.* 4H1, 5D11, 5E12, 5F12 and 5H12 showed greater invasion capability than wildtype strain. Those fifteen mutants with invasion potential below 20 % (dashed line) were reconfirmed for their reduced invasiveness in INT-407 and Caco-2 cells. Motility mutants 3G4, 3H9, 9b3, 6G1 and 6G3 were used as negative controls in the assay and they showed minimal invasion.

The selected fifteen transposon mutants were reconfirmed for their reduced invasiveness in INT-407 and Caco-2 cells and all the mutants, apart from two, maintained the reduced invasion phenotype in both the INT-407 (Figure 4.3) and Caco-2 cells (Figure 4.4). Mutant 6A9 showed nearly a 50% reduction in invasion efficiency compared to wildtype strain in both cell lines in this assay and mutant 9D2 showed a higher invasion in Caco-2 cells than INT-407 cells which might be due to its differential interaction with Caco-2 cells. It has previously been reported that the same *Campylobacter jejuni* strain can vary in invasion efficiency for different host cell lines and this dependence appears to be host cell line-specific (Konkel *et al.*, 1992a; Oelschlaeger *et al.*, 1993) however in this study the selected mutants in general do behave similarly in both cell lines.

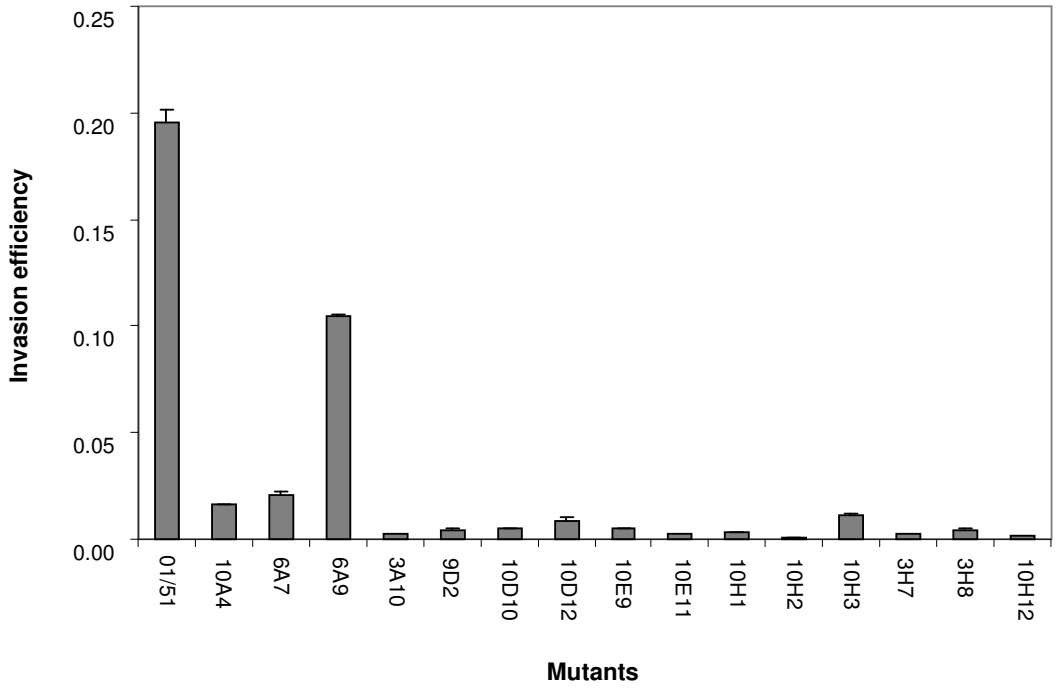

Figure 4.3 Invasion of *C. jejuni* 01/51 transposon mutants in INT-407 cells.

The fifteen selected mutants that showed more than an 80% reduction in invasion compared to the wildtype strain 01/51 were retested for their invasion potential in INT-407 cells. All of them maintained the reduced invasion phenotype apart from mutant 6A9 which showed about a 50% reduction in invasion.

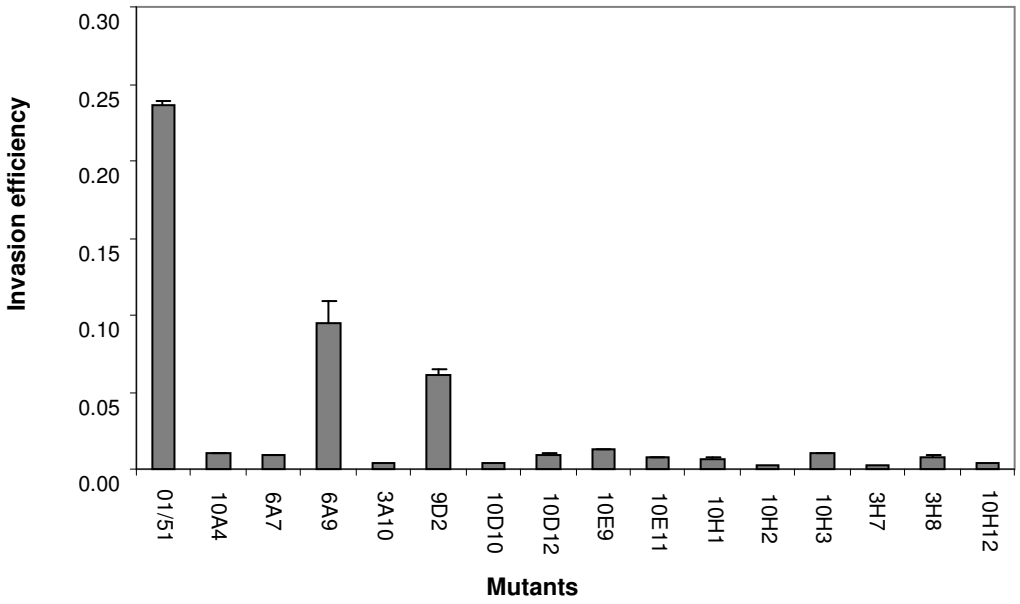

Figure 4.4 Invasion of *C. jejuni* 01/51 transposon mutants in Caco-2 cells.

The fifteen selected mutants that showed greater than an 80% reduction in invasion in INT-407 cells were also checked for their invasion potential in another human intestinal epithelial cell-line Caco-2. Most of the mutants showed invasion potential in Caco-2 cells similar to that in INT-407 cells (Figure 4.3) except mutant 9D2 which showed slightly higher invasion in Caco-2 cells than INT-407 cells.

4.4 Summary and conclusions

Transposon mutagenesis was applied to a hyperinvasive *Campylobacter jejuni* 01/51 and a library consisting of 867 mutants was generated previously. In this study, 768 transposon mutants were screened to find those mutants in which transposon insertion resulted in reduced invasiveness. One hundred and seventy four mutants showed very low invasion in INT-407 cell-based semi-quantitative screening assays and they were subsequently tested for motility. It is believed that reduction in motility results in reduced invasion of culture eukaryotic cells in many enteric pathogens so the mutants in which reduced invasion was due to reduced motility were excluded from further studies. Forty mutants that showed very low invasiveness but maintained good motility were checked for invasion in INT-407 cells using standard quantitative gentamicin protection assays. Most of the mutants showed reduced invasion in INT-407 cells however five mutants showed increased invasion. Fifteen out of the forty mutants with greater than an 80% reduction in invasion into INT-407 cells were re-confirmed using this cell line and were also tested in a second cell line Caco-2. All of the fifteen mutants maintained very low levels of invasiveness in INT-407 cells except one mutant (6A9) that showed just

below fifty percent reduced invasion in both INT-407 and Caco-2 cells. These fifteen mutants with reduced invasion were selected for further study to determine the location of transposon insertion.

Chapter Five

LOCALISATION OF TRANSPOSONS, BIOINFORMATICS ANALYSIS AND SUBSPECIATION

5 LOCALISATION OF TRANSPOSONS, BIOINFORMATICS ANALYSIS AND SUBSPECIATION

5.1 Introduction

Epithelial cell invasion is considered to be an essential step in *Campylobacter* infection (Hu and Kopecko, 2005; Konkel *et al.*, 2000). Invasion studies with intestinal epithelial cells as well as other cell lines revealed that the relative ability of different *C. jejuni* isolates to invade cultured cells is strain dependent (Everest *et al.*, 1992; Konkel and Joens, 1989; Newell *et al.*, 1985) and a statistically significant correlation was observed between *C. jejuni*'s ability to invade cultured cells and clinical symptoms of infection (Everest *et al.*, 1992). Fearnley *et al.* identified a higher ratio of hyperinvasive strains among clinical isolates than those from poultry or environmental sources (Fearnley *et al.*, 2008). These observations suggest that different *Campylobacter* isolates vary in their virulence properties and that those having a higher ability to invade intestinal epithelial cells are more virulent. Some bacterial cell components that affect the adhesion and invasion in *C. jejuni* have previously been reported *i.e.* flagella (Grant *et al.*, 1993; Wassenaar *et al.*, 1991; Yao *et al.*, 1994), capsule (Bacon *et al.*, 2001) and lipooligosaccharide (Guerry *et al.*, 2002); moreover, *C. jejuni* also express different proteins to promote their own attachment followed by uptake by non-phagocytic cells such as intestinal epithelial cells, these proteins include PEB1, Cad-F, Iam, JlpA (Carvalho *et al.*, 2001; Jin *et al.*, 2001; Jin *et al.*, 2003; Konkel *et al.*, 1997; Pei and Blaser, 1993; Pei *et al.*, 1998). Despite the identification of these factors, the mechanism of invasion and the molecular basis of this observed variation in invasiveness in *C. jejuni* is not fully understood. A transposon mutant library generated in a hyperinvasive clinical isolate *C. jejuni* 01/51 was studied to determine those genes that promote invasiveness in strain 01/51. The use of a hyperinvasive strain in this study will provide more insight into the molecular mechanisms underlying this phenotype in *C. jejuni.* So the transposon mutant library was screened to find those mutants with a reduced invasion phenotype and invasion-related genes interrupted by transposon insertion were identified.

5.2 Methods

5.2.1 Localisation of transposon insertion sites

Those genes disrupted by transposon insertion in the *C. jejuni* 01/51 mutants were identified by the plasmid rescue technique (Grant *et al.*, 2005). Mutants were selected that showed very low invasion of Caco-2 and INT-407 cells in standard gentamicin protection invasion assays but good motility. Briefly, genomic DNA was isolated from the mutants using the GenElute® Bacterial Genomic DNA kit (Sigma, Dorset, UK) and digested with *Bgl*II (Promega, Southampton, UK) or *Ssp*I (Sigma, Dorset, UK). Digested DNA fragments were self-ligated using T4 DNA ligase (Promega, Southampton, UK) and *E. coli* S17λ*pir* was transformed with these self-ligated DNA fragments by electroporation. Transformants were selected on LB agar containing chloramphenicol (10 µg/ml). The resulting plasmids were likely to be large and low copy number so the plasmid DNA was extracted from 10 ml overnight culture and plasmid DNA was eluted in pre-warmed (70ºC) elution buffer using the QIAprep spin miniprep kit (QIAGEN, Crawley, UK). Plasmids were sent for DNA sequencing at the University of Nottingham using the transposon specific Transeq primer (Table 2.3) on the BigDye cycle sequencing system (ABI Prism; PE Biosystems). The sites of transposon insertion into the *C. jejuni* 01/51 mutant genome were determined by subjecting the obtained DNA sequences to *B*asic *L*ocal *A*lignment *S*earch *T*ool (BLAST) searches in the database of published *Campylobacter* genome sequences using web-based local alignment search tools on http://xbase.bham.ac.uk/campydb/blast.pl and http://blast.ncbi.nlm.nih.gov/Blast.cgi.

5.2.2 Bioinformatics analysis

Bioinformatics analysis was carried out to further characterise the genes identified in the selected transposon mutants with a reduced invasion phenotype. The analysis including finding the restriction sites in a DNA sequence, nucleotide-nucleotide and protein-protein sequence alignment and generation of DNA reverse complementary strands were done using the CCL sequence viewer software downloaded from http://www.clcbio.com/index.php. Pfam domains, the functional regions in the translational products of the gene of interest were searched online at

http://pfam.sanger.ac.uk/search/ and transmembrane helices that give an idea about the position of a protein in the cell were also checked online at http://www.cbs.dtu.dk/services/TMHMM/.

5.2.3 Subspeciation of *C. jejuni*

The subspecies of *C. jejuni* 01/51 was determined both biochemically using a nitrate reduction test and by PCR. *C. jejuni* subspecies *jejuni* reduces nitrate to nitrite while subspecies *doylei* cannot reduce nitrate (Steele and Owen, 1988) which is due to a deletion of 2.8 kbp in the *nap*A gene that encodes for large subunit of nitrate reductase (Miller *et al.*, 2007).

5.2.3.1 Nitrate reduction test

C. jejuni 01/51 (test strain), *C. jejuni* subspecies *jejuni* NCTC11168 (reference strain), *Citrobacter koseri* (positive control) and *Acinetobacter baumannii* (negative control) were grown overnight in Bacto® nitrate broth (Difco) at 37^{o}C; *C. jejuni* was incubated in microaerobic conditions while *C. koseri* and *A. baumannii* were grown in aerobic conditions. Five millilitres of these broth cultures was transferred into test tubes and 100 µl of sulfanilic acid and an equal amount of α-naphthylamine were added to each tube. Development of red colour indicated nitrate reduction; if no colour change was observed, a little amount of zinc powder was added to tubes to confirm whether nitrate was reduced to ammonia or N_2. Development of red colour on addition of zinc powder indicated negative result.

5.2.3.2 Determination of *C. jejuni* subspecies by PCR

The subspecies of *C. jejuni* 01/51 was also determined using the PCR method described elsewhere (Miller *et al.*, 2007). PCR using napAL2 and napAR4 primers (Table 2.3) was performed following the standard PCR protocol described in Section 2.12. napAL2 and napAR4 are *nap*A flanking primers that generate a 4 kbp PCR product from a subspecies *jejuni* DNA template while the amplicon resulting from subspecies *doylei* is 1.2 kbp. Genomic DNA extracted from *C. jejuni* 01/51 (test strain) and *C. jejuni* subspecies *jejuni* NCTC11168 (reference strain) was used as template and the PCR amplification time was kept at 4 minutes. A PCR reaction mixture containing all those components present in PCR for 01/51 except DNA template was used as the PCR

negative control. PCR products were run on an agarose gel (0.8%) and PCR product size was compared with a 1 kbp DNA marker (Promega, UK).

5.3 Results and discussion

5.3.1 Localisation of the transposon insertion

A transposon mutagenesis library generated in a hyperinvasive clinical isolate *C. jejuni* 01/51 was screened and fifteen mutants that showed very low levels of invasion into INT-407 and Caco-2 cells were selected to identify the genes that play a role in promoting invasion of the organism in cultured human intestinal epithelial cells. Location of transposon insertion that caused reduction in invasiveness in the selected mutants was determined in only those mutants that were more than 75% motile as compared to the parent strain 01/51 to focus the effort on identifying those genes with a role in invasion rather than motility.

The transposon insertion point was identified by the plasmid rescue technique (Grant *et al.*, 2005). Resulting plasmids carried the transposon along with the transposon insertion site flanking *Campylobacter jejuni* 01/51 genomic DNA. Plasmids were sequenced using Transeq (Table 2.3), a transposon derived primer and DNA sequences were searched for matching sequences at http://xbase.bham.ac.uk/campydb/. The transposon insertion point was identified in 12 out of 15 selected mutants; the transposon insertion site could not be determined in three mutants even after several attempts. The fact that no insertion site was determined for these mutants (10A4, 10E11 and 10H2) was either due to a failure in the plasmid rescue process or nucleotide sequencing. Initially the selected transposon mutants' genomic DNA was digested using *Bgl*II for plasmid rescue and DNA sequencing was successful in seven mutants (3A10, 3H7, 3H8, 10D10, 10E9, 10H1, and 10H12). It was possible that those plasmids resulting from *Bgl*II digestion where sequencing failed might have been very big in size and a good signal may not have been generated in the sequencing reactions. Plasmid rescue was attempted at least two times more from those mutants in which transposon localisation was initially failed using *Bgl*II digestion. So, later genomic DNA was digested with *Ssp*I an enzyme that also does not cut inside the transposon sequence, and using this enzyme genes mutated by transposon insertion in another five mutants (6A7, 6A9, 9D2, 10D12 and 10H3)

were identified. Thus those 12 genes into which transposon insertion had caused a reduction in invasion of the *C. jejuni* 01/51 mutants into INT-407 and Caco-2 cells were identified (Table 5.1).

Table 5.1 The genes mutated by transposon insertion in 15 selected transposon mutants.

Mutant	Transposon location	Mutant	Transposon location
3A10	*Cj0685c*: Invasion protein *cipA*	**10D12**	No database match found (date of last search March 2009)
3H7	*Cj1020c*: Putative cytochrome C	**10E9**	*Cj1136*: Putative galactosyltransferase
3H8	*Cj0499*: Putative HIT family protein	**10E11**	Not determined yet
6A7	*Cj1539c*: Putative anion-uptake ABC-transport system permease protein	**10H1**	*Cj1245c*: Putative membrane protein
6A9	*Cj0690c*: Putative restriction/modification enzyme	**10H2**	Not determined yet
9D2	*JJD26997_1801*: Capsular polysaccharide biosynthesis protein of *C. jejuni* subsp. *doylei* 269.97	**10H3**	*Cj1503c*: *put*A; Putative proline dehydrogenase
10A4	Not determined yet	**10H12**	*Cj1305c*: Hypothetical protein
10D10	*Cj1555c*: Hypothetical protein		

The genes in selected transposon mutants of a hyperinvasive clinical isolate *C. jejuni* 01/51 where transposon insertion had reduced invasiveness into INT-407 and Caco-2 cells were identified by the plasmid rescue technique. All the identified genes except those in mutant 9D2 and 10D12 are also present in the first genome sequenced *C. jejuni* strain NCTC11168. The gene mutated in mutant 9D2 showed homology to *JJD26997_1801*, a gene encoding capsule polysaccharide biosynthesis protein in *C. jejuni* subspecies *doylei* 269.97 whereas the gene mutated in 10D12 did not show match to any of the genes in the available sequenced genome databases. Genes shaded (n=6) were selected for generating targeted mutations and further characterisation.

All but two of the transposon insertions were found to be in genes which are also present in NCTC11168, the original genome-sequenced strain (Parkhill *et al.*, 2000). Of the remaining two insertions one in mutant 9D2 showed a match with the gene annotated as a capsule polysaccharide biosynthesis protein (*JJD26997_1801*) in *C. jejuni* subspecies *doylei* strain 269.97. Interestingly in the second mutant (10D12) the sequence obtained had no database match at the time of searching, which may indicate that this region is unique to strain 01/51. None of the transposons had inserted into orthologues of any of the known invasion-related genes found in other bacteria, such as

in *Salmonella* or *Yersinia*. This is not too surprising, as from the numerous genome sequences that are now available it is apparent that *C. jejuni* does not contain any such invasion-related genes, however this does not preclude there being novel invasion-related genes present in strain 01/51.

DNA sequence obtained from transposon mutant 3A10 showed a match with the *cipA* gene of *Campylobacter* species. *cipA* is a 1353 bp (450 amino acids) long gene present in genome-sequenced *Campylobacter jejuni* strains NCTC11168, 81-176 and RM1221 but is absent from *C. jejuni* strains 81116 and 269.97. It contains a variable poly-G tract near its 3' end, and shows some similarity with *Cj1421c* (28.0%) and *Cj1422c* (25.3%) both of which contain variable poly-G tracts. Originally it was annotated as a putative sugar transferase based on family clustering (BLASTP) with other *C. jejuni* predicted sugar transferases e.g. *Cj1434c* and *Cj1438c*. The *cipA* amino acid sequence has no transmembrane helices. A previous study reported that a mutation in the *cipA* gene of *C. jejuni* TGH9011 resulted in the reduced invasion of HEp-2 cells but there was no change in invasion of INT-407 and Caco-2 cells (Lynett, 1999). This gene has now been patented as encoding an invasion protein of *Campylobacter* species, which can be accessed at http://www.freepatentsonline.com/6087105.html. This gene was identified in our study to be involved in invasion of eukaryotic cells and was selected for further characterisation by targeted mutagenesis and complementation to study its role in the virulence of *C. jejuni* 01/51.

A putative cytochrome C family protein (*Cj1020c*) was mutated in mutant 3H7. *Cj1020c* is a 453 bp (144 amino acids) long gene, also present in the genome-sequenced *C. jejuni* strains NCTC11168, RM1221, 81-176 and 269.97. The cytochrome C (CytC) domain was identified by Pfam search and one probable transmembrane helix was identified by TMHMM2.0 in the amino acid sequence of the translation product of this gene. This gene might be involved in energy production and electron transport but has not yet been characterised in *C. jejuni.*

DNA sequence obtained from the mutant 3H8-derived plasmid showed a match with a gene (*Cj0499*) encoding for a putative histidine triad (HIT) family protein. *Cj0499* is a 486 bp (161 amino acids) long gene and its predicted amino acid sequence has no transmembrane helix. In addition to HIT, Pfam identified a molybdopterin oxidoreductase, Fe4S4 domain which is found in a number of dehydrogenases. This gene has not yet been characterised in *C. jejuni.*

Mutant 6A7 contained the transposon insertion at nucleotide 160 in *Cj1539c*, a putative anion-uptake ABC-transport system permease protein. *Cj1539c* is a 720 bp (239 amino acids) long gene present in genome-sequenced *C. jejuni* strains NCTC11168, 81-176, RM1221 and 269.97. Its translation product is similar to many involved in anion uptake including the sulphate transport system permease protein of *E. coli* (23.6%), the ABC-transport permease protein of *C. coli* RM2228 (91%), *C. upsaliensis* RM3195 (85%), *Wolinella succinogenes* DSM1741 (57%) and *Desulfovibrio vulgaris* (48%). Pfam identified an inner membrane component domain of bacterial binding-protein-dependent transport in predicted amino acid sequence of *Cj1539c* and five probable transmembrane helices were also predicted by TMHMM2.0. It is established that ABC transport system components are involved in virulence in *Mycobacterium* species (Collins *et al.*, 2003; Peirs *et al.*, 2005), *Streptococcus pneumoniae* (Dintilhac et al., 1997), *Yersinia pestis* (Bearden and Perry, 1999) and many other micro-organisms. The exact role of this gene in *Campylobacter jejuni* pathogenesis is not known and the gene was selected for targeted mutagenesis and complementation to further characterise and confirm its role in *C. jejuni* pathogenesis.

A gene encoding for a putative restriction/modification enzyme (*Cj0690c*) was inactivated in the transposon mutant 6A9. *Cj0690c* is a 3753 bp (1250 amino acids) long gene of *Campylobacter jejuni* NCTC11168. The actual function of this gene is not known yet but in addition to a type I restriction enzyme domain, this gene also contains the signature of the N-6 adenine-specific DNA methylase (Prosite motif PC00092), indicating that it might have a regulatory role in the expression of virulence-associated genes, similar to other Dam methylases (Balbontin *et al.*, 2006; Falker *et al.*, 2005; Low *et al.*, 2001). Indeed another gene (*Cj1461*, a predicted methyl transferase gene) containing this same signature was recently reported to have a role in the regulation of virulence in *C. jejuni*. In that study a mutation in the *Cj1461* gene resulted in reduced invasion and motility, but increased adhesion (Kim *et al.*, 2008). *Cj0690c* was also interrupted by transposon insertion in *C. jejuni* 81-176 in a separate study, however the mutant was only tested for chicken colonisation where it did not show significant reduction compared to the wildtype strain (Grant *et al.*, 2005). A good correlation with chicken colonisation and invasion in INT-407 cells has been reported previously (Hanel *et al.*, 2004), however, a *galE* deletion mutant of *C. jejuni* 81116 showed reduction in adherence and invasion of INT-407 cells but it was still able to colonise chickens to the

same level as the wildtype strain (Fry *et al.*, 2000). This gene was selected for further characterisation to study its role in virulence of *C. jejuni* 01/51.

Two of the mutants (10D10 and 10H12) had transposon insertions in genes encoding putative or hypothetical proteins (*Cj1555c* and *Cj1305c* respectively), the function of which is not yet known. *Cj1555c* is 636 bp (211 amino acids) long. TMHMM2.0 did not find any transmembrane helix in the amino acid sequence of this hypothetical protein but DapB, a Dihydrodipicolinate reductase N terminus domain, which binds NADPH in the biosynthesis of lysine, was identified by Pfam. *Cj1305c* is 1218 bp (405 amino acids) in size and it shows similarity with *Cj1306c* (78.1%), *Cj0617* (38.9%), *Cj0618* (37.1%), *Cj1310c* (63.7%), *Cj1342c* (38.9%) and hypothetical proteins of other bacteria including *Aeromonas hydrophila* (27%). It is a member of the 617 family of c.j. proteins (Szymanski and Wren, 2005). This 617 family of proteins contains homopolymeric tracts and are part of *C. jejuni O*-linked glycosylation locus (*Cj1293 – Cj1342*); no orthologues of this family are found in other bacterial species (Szymanski and Wren, 2005). Mutation of the *Cj1305c* homologue in *C. jejuni* 81-176 resulted in fully motile bacteria with no effect on flagellin glycosylation (Guerry *et al.*, 2006). *Cj1305c* was selected for further characterisation.

Cj1136, a putative galactosyltransferase was interrupted by transposon insertion in mutant 10E9; *Cj1136* is 1173 bp (390 amino acids) in size. A glycosyltransferase family domain was identified in the amino acid sequence and it has homology (29%) to a glycosyltransferase of *Streptococcus pneumoniae,* however, it has not yet been characterised in *Campylobacter* species. This gene is part of the *Campylobacter jejuni* lipooligosaccharides (LOS) biosynthesis locus (*Cj1131c - Cj1152c*). The LOS is an important virulence factor in Gram-negative bacteria and has been reported to play a role in *Campylobacter jejuni* adhesion and invasion into human intestinal epithelial cells (Fry *et al.*, 2000; Kanipes *et al.*, 2008). Previous PCR based analysis of *C. jejuni* strains with differential Caco-2 cell invasion potential showed two genes, *cgtB* and *wlaN*, encoding putative β-1,3-galactosyltransferases, were detected in most of the strongly invasive strains and rarely in non-invasive strains (Hanel *et al.*, 2007; Muller *et al.*, 2006; Muller *et al.*, 2007). However, Muller *el al.* found no correlation between the presence or absence of other galactosyltransferases including *Cj1136* and invasion capability (Muller *et al.*, 2007). *Cj1136* was selected for further characterisation.

DNA sequence obtained from transposon mutant 10H1 showed a match with a putative membrane protein (*Cj1245c*) of *Campylobacter jejuni*. *Cj1245c* is 1197 bp (398 amino acids) in size and conserved in all the genome-sequenced *C. jejuni* strains. TMHMM2.0 predicted two transmembrane helices in the amino acid sequence of the translation product of *Cj1245c* and no significant domain was identified by Pfam. Membrane proteins have been reported to be involved in the virulence of many bacterial pathogens; outer membrane proteins Rck of *Salmonella* Typhimurium and Ail of *Yersinia enterocolitica* were shown to have role in invasion/adhesion and serum resistance of the pathogens (Heffernan *et al.*, 1994; Pierson and Falkow, 1993), MOMP (major outer membrane protein), JlpA, PEB1 and CadF (*Campylobacter* adhesion to fibronectin) of *C. jejuni* were involved with adhesion and invasion of the organism in cultured eukaryotic cells (Jin *et al.*, 2001; Monteville *et al.*, 2003; Moser *et al.*, 1997; Pei *et al.*, 1998). Though *Cj1245c* did not show homology to any of the characterised virulence-associated membrane proteins it would be interesting to study the role of this gene in the virulence of *C. jejuni* 01/51 and thus this mutant was selected for further characterisation.

In mutant 10H3 the transposon was inserted in *put*A (*Cj1503c*), a putative proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase, which catalyses the oxidation of proline into glutamate. *Cj1503c* is 3489 bp (1162 amino acids) in size. TMHMM2.0 found no transmembrane helix in the amino acid sequence however Pfam identified domains matching to entry "PF00171 aldedh, Aldehyde dehydrogenase, PS00017 ATP/GTP-binding site motif A (P-loop), PS00687 Aldehyde dehydrogenases glutamic acid active site, and PS00070 Aldehyde dehydrogenases cysteine active site and domain PF01619 Proline dehydrogenase". This gene might be involved in amino acid metabolism but functional characterisation has not been carried out in *C. jejuni.* The lack of well-defined virulence mechanisms in *C. jejuni* and the involvement of metabolism associated genes with virulence phenotypes suggest that: *C. jejuni* is an opportunistic pathogen and does not possess specific virulence factors as in other bacteria, and that disease results as a consequence of the need of the organism to grow and survive within the human host.

Briefly, to identify the genes that enhance invasiveness, the transposon insertion site was determined in twelve out of fifteen selected mutants of *C. jejuni* 01/51 with reduced invasion in INT-407 and Caco-2 cells. All of the identified genes except two were

present in the first genome-sequenced strain *C. jejuni* NCTC11168; among the two genes that were absent from NCTC11168, the gene mutated in 9D2 showed homology to *JJD26997_1801*, a capsule polysaccharide biosynthesis protein of *C. jejuni* subspecies *doylei* 269.97 while sequence obtained from mutant 10D12 did not show a match that could be a sequence specific to 01/51. None of the genes identified in this study showed homology to invasion-related genes reported in other bacteria and most of the genes identified in this study have yet not been characterised apart from *cipA* (mutant 3A10) which had previously been reported to be involved in invasion of *C. jejuni* TGH9011 (Lynett, 1999). Six of the genes mutated by transposon insertion in *C. jejuni* 01/51 were selected on the basis of their potential relevance to virulence and ease of genetic manipulation for further characterisation through targeted mutation and complementation to confirm their role in invasion in cultured eukaryotic cells.

5.3.2 Subspeciation of *C. jejuni* 01/51

C. jejuni has been subdivided into two subspecies: subspecies *jejuni* and subspecies *doylei*. Most of the isolated strains are subspecies *jejuni,* and subspecies *doylei* has infrequently been isolated. Subspecies *doylei* has primarily been isolated from human clinical samples and in addition to gastroenteritis (Kasper and Dickgiesser, 1985; Lastovica and Skirrow, 2000), it has been found associated often with bacteraemia in children (Musmanno *et al.*, 1998). In a recent study subspecies *doylei* showed divergence from subspecies *jejuni* in many hypervariability regions including the capsule biosynthesis locus (Parker *et al.*, 2007). Previously microarray and PCR based analysis has shown that in *C. jejuni* 01/51 many of the capsule genes are missing or the nucleotide sequence of capsule biosynthesis locus is different from the first genome sequenced strain *C. jejuni* NCTC11168 (Manning, personal communication). In the current study, the gene mutated in transposon mutant 9D2 showed a match with *JJD26997_1801*, a capsule biosynthesis protein of *C. jejuni* subspecies *doylei* 269.97. Moreover, the frequency of the observed hyperinvasive phenotype was previously reported to be higher amongst clinical isolates than those from poultry (Fearnley *et al.*, 2008). There is also evidence that subspecies *doylei* was isolated more often from blood cultures (24%) than from stool cultures (9.7%) (Lastovica, 1996); furthermore, subspecies *doylei* was isolated from 85.2% of *Campylobacter*/*Helicobacter*-related

bacteraemia cases (Morey, 1996). In light of these tenable links between its capsule biosynthesis gene, invasion and a clinical source of isolation, the subspecies of *C. jejuni* 01/51 was determined both biochemically and by PCR and it was confirmed to be *C. jejuni* subsp. *jejuni.* The nitrate reduction test showed that *C. jejuni* 01/51 has a functional nitrate reductase enzyme (Figure 5.1) which is found in *C. jejuni* subsp *jejuni* but not *C. jejuni* subsp *doylei* (Steele and Owen, 1988). *C. jejuni* subsp *jejuni* NCTC11168 was used as a reference strain and it showed nitrate reductase activity as expected. In the absence of a known *C. jejuni* subsp *doylei* strain*, Citrobacter koseri* and *Acinetobacter baumannii* were used as positive and negative control respectively and they validated the test (Figure 5.1).

Recently, a PCR method for subspeciation of *C. jejuni* was described elsewhere (Miller *et al.*, 2007). In this method, primers napAL2 and napAR4 were designed based on the conserved sequences of the nitrate reductase (*nap*) locus of the genome-sequenced *C. jejuni* strains NCTC11168, RM1221 and *C. coli* RM2228. The napAL2 and napAR4 primers bind in the flanking regions of *nap*A and generate a 4 kbp product in *C. jejuni* subspecies *jejuni* but a 1.2 kbp DNA fragment is amplified in subspecies *doylei*. The subspecies of *C. jejuni* 01/51 was also confirmed as *jejuni* by PCR using napAL2 and napAR4 primers and both *C. jejuni* 01/51 and *C. jejuni* subspecies *jejuni* NCTC11168 generated a 4 kbp product (Figure 5.2). No PCR product was obtained in PCR mixture without DNA template that validated the PCR reaction and confirmed that products were not due to primer dimerisation or contamination. Unfortunately, *C. jejuni* subspecies *doylei* was not available to be used as a reference strain in the assays.

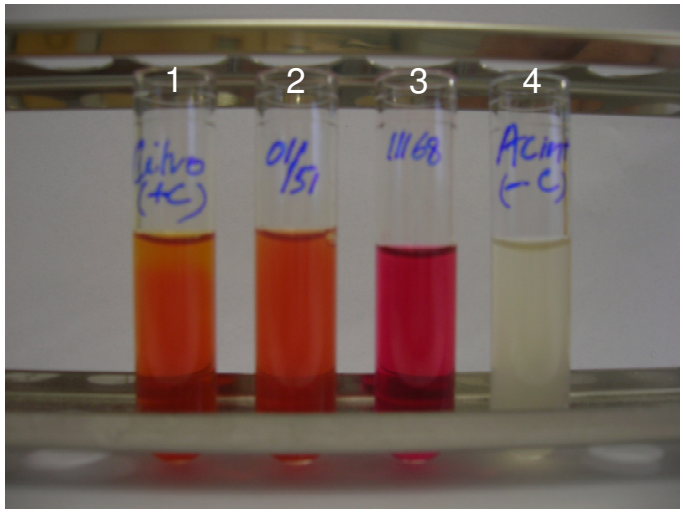

Figure 5.1 Nitrate reduction test confirmed that *C. jejuni* 01/51 is *C. jejuni* subspecies *jejuni*.

Subspecies of *C. jejuni* 01/51 (tube 2) was determined biochemically using the nitrate reduction test. Development of a red colour on addition of sulfanilic acid and α-naphthylamine to an overnight broth culture indicated a positive result. Nitrate was reduced to nitrite by *C. jejuni* 01/51 which confirmed that the subspecies of 01/51 is *jejuni*. *Citrobacter koseri* (tube 1) and *Acinetobacter baumannii* (tube 4) were used as positive and negative controls, respectively, for the nitrate reduction test while *C. jejuni* NCTC11168 (tube 3) was used as reference subspecies *jejuni*. As expected, nitrate activity was also shown by *Citrobacter koseri* and *C. jejuni* NCTC11168 while nitrate reduction was negative in *Acinetobacter baumannii*.

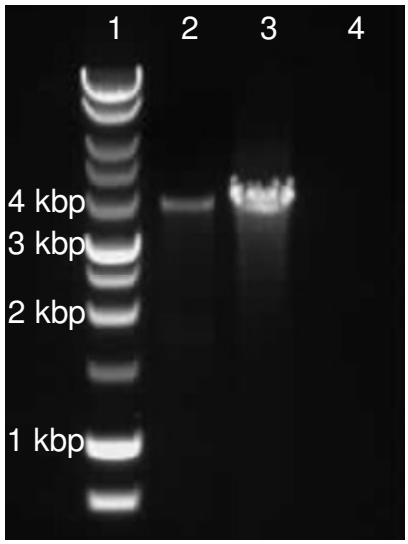

Figure 5.2 PCR confirmed the *jejuni* subspecies of *C. jejuni* 01/51.

The subspecies of *C. jejuni* 01/51 was determined by using a subspeciation PCR described by Miller *et al*. PCR using napAL2 and napAR4 primers generated a 4 kbp product for *C. jejuni* 01/51 (lane 2) and from reference strain *C. jejuni* subsp. *jejuni* NCTC11168 (lane 3). PCR confirmed that 01/51 is *C. jejuni* subspecies *jejuni.* PCR reaction control without template (lane 4) did not show a product as was expected. Lane 1: 1.0 kbp DNA markers (Promega, UK); lane 2: PCR product generated by 01/51 template, lane 3: PCR product generated by 11168 DNA template, and lane 4: PCR control with no template.

5.4 Summary and conclusions

A transposon mutant library generated in *C. jejuni* 01/51 was studied and identification of the genes mutated by transposon insertion that resulted in the reduction in invasiveness but not motility was attempted in fifteen selected mutants. The site of transposon insertion was determined in twelve of the mutants and many previously uncharacterised genes with a potential role in host cell invasion have been identified in this study. Six of the genes were selected for further characterisation through targeted mutagenesis as well as complementation studies which it was hoped would provide greater insight into the molecular basis of the observed variation in virulence in *C. jejuni*. Moreover, despite this hyperinvasive strain possessing a capsular biosynthetic gene with similarity to one found in *C. jejuni* subspecies *doylei* the subspecies of *C. jejuni* 01/51 was confirmed to be subspecies *jejuni* both biochemically and by PCR.

Chapter Six

MUTAGENESIS AND COMPLEMENTATION OF THE GENES

6 MUTAGENESIS AND COMPLEMENTATION OF THE GENES

6.1 Targeted mutagenesis: an introduction

The reduced invasion phenotype of the six transposon insertion mutants that were confirmed as poor invaders of both INT-407 and Caco-2 cell lines and were motile, was reconfirmed by generating targeted mutations. This was done to reconfirm that the reduced invasion phenotype in the mutants was not due to a polar effect of transposon insertion on the transcription of downstream gene(s). Targeted mutations were generated in the selected genes and the kanamycin resistance cassette (*kan*^{r}) was inserted in a suitable restriction site in the gene *i.e.* a restriction site that was not present in the cloning vector, pGEM®-T Easy in this case. The *kan*^{r} cassette was amplified from pMA24 (Abuoun, 2007); this cassette can be expressed in *E. coli* and *C. jejuni* and has no transcription termination sequences and thus was unlikely to have any polar effects. If a suitable restriction site was not present in the gene for insertion of *kan*^{r} cassette then it was introduced by inverse PCR (Wren *et al.*, 1994) of the gene with primers carrying the restriction sequences near their 5'ends. Though all the invasion associated genes identified by transposon mutagenesis were interesting, six genes (*cipA*, *Cj0690c*, *Cj1136*, *Cj1245c*, *Cj1305c* and *Cj1539c*) were selected initially for generating targeted mutations. The following points were considered when selecting the genes; a) a previous study has shown that the gene might play a role in either virulence or chicken colonisation; b) the gene product has transmembrane helices; and c) the gene should be more than 0.7 kbp in size for ease of genetic manipulation. The genome sequence of the *C. jejuni* strain 01/51 is not yet known thus the genome sequence of *C. jejuni* strain NCTC11168 (Parkhill *et al.*, 2000) was used as the template for PCR primer designing and for bioinformatics analysis. The mutants generated by targeted mutations were tested for their adhesion and invasion into Caco-2 cells to confirm the reduced invasion phenotype. Mutations in these genes were also complemented with their wildtype alleles to satisfy Koch's molecular postulates (Falkow, 1988) with an attempt to restore the invasion phenotype and verify the virulence-associated role of the genes.

6.1.1 Mutagenesis of *Campylobacter* invasion protein, *cipA* (*Cj0685c*)

6.1.1.1 Introduction

DNA sequence obtained from transposon mutant 3A10 showed a match with the *cipA* (*Cj0685c*) gene in *Campylobacter* species. *cipA* (1353 bp) contains a variable poly-G tract near its 3' end and originally it was annotated as a putative sugar transferase. A previous study showed that a *cipA* deletion mutant of *C. jejuni* TGH9011 had reduced invasion in Hep-2 cells but there was no change in invasion of INT-407 and Caco-2 cells (Lynett, 1999). This gene was identified in our study to be involved in invasion of eukaryotic cells, and interestingly the *cipA* mutation in *C. jejuni* 01/51 resulted in reduced invasion of both INT-407 and Caco-2 cells. The invasion-related role of *cipA* was reconfirmed by generating a targeted mutation in this gene.

6.1.1.2 Cloning and generation of the mutant

To study the role of *cipA* in the invasion potential of *C. jejuni,* a suicide vector was generated to mutate the gene in the strain 01/51. Standard *E. coli* vectors including pGEM®-T Easy have been frequently used as suicide vectors in *Campylobacter* because they are unable to replicate in it. Normally a double crossover event takes place leading to the elimination of vector sequence and replacement of the wildtype gene with disrupted gene. The *cipA* gene has a *Hind*III site at 108 bp in the CDS that was selected for insertion of a kan^r cassette and inactivate the gene. Initially, a 1.5 kbp DNA fragment from 0.4 kbp upstream of the *cipA* gene to 1141 bp inside the *cipA* CDS was amplified by PCR (Section 2.12.1) using primers 685-F and 685-R (Table 2.3). The PCR product with A overhangs was ligated into the cloning vector pGEM®-T Easy (Promega, UK) (Section 2.9.7) and the resulting plasmid pGEM:685 was digested with *Hind*III and dephosphorylated (Section 2.9.4) with thermostable alkaline phosphatase (TSAP) to avoid plasmid recircularisation. The kan^r cassette was amplified by PCR from pMA24 using the KNHD-F and KNHD-R primers that harbour *Hind*III restriction sites near their 5'ends. The *Hind*III digested kan^r PCR products were then ligated with linearised pGEM:685. *E. coli* TOP10 cells were transformed with the resulting plasmid pGEM:685::kan and colonies were selected on LB agar supplemented with kanamycin (50 µg/ml) and ampicillin (100 µg/ml). Plasmid was extracted (Section 2.11.2) from those colonies that had grown after 24 hrs incubation and the resultant construct was confirmed by PCR using 685-F and 685-R primers. The PCR generated a 2.9 kbp

product in the plasmid having *kan*^{r} inserted in the cloned *cipA* gene, the wildtype genomic DNA was used as a control in this PCR that generated an expected 1.5 kbp product (Figure 6.1). The mutation construct was also confirmed by nucleotide sequencing of pGEM:685::kan with SP6 and T7 primers. pGEM:685::kan was used to transform *C. jejuni* 01/51.

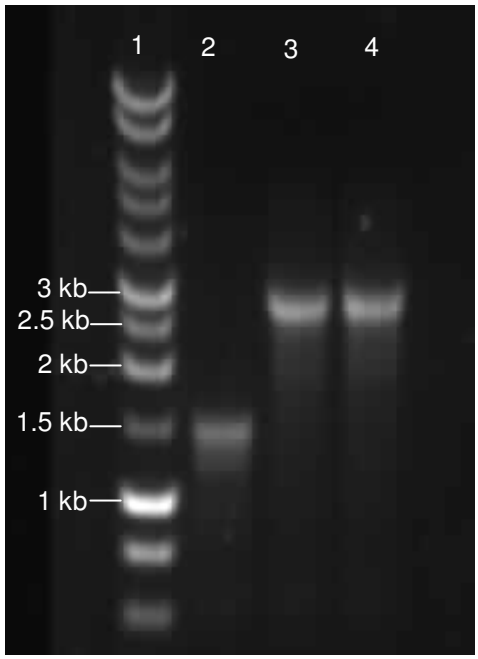

Figure 6.1 PCR analysis of pGEM:685::kan.

PCR with 685-F and 685-R primers generated a 1.5 kbp product in 01/51 wildtype genomic DNA (lane 2) and a 2.9 kbp product in pGEM:685::kan (lane 3 and 4) and) that confirmed the insertion of *kan*^{r} cassette in the cloned *cipA*. 1.0 kbp DNA markers (Promega, UK) were loaded in lane 1.

C. jejuni 01/51 cells were made electrocompetent (Section 2.10.3) and electroporation was done to transform 01/51 (Section 2.10.4) with pGEM:685::kan and colonies were selected on blood agar supplemented with kanamycin (50 µg/ml). Three out of fifteen colonies grown after 72 hours incubation were sub-cultured and genomic DNA was extracted (Section 2.11.1). PCR of the genomic DNA of *C. jejuni* 01/51 wildtype and potential mutants was done using 685-F and 685-R primers. PCR products showed a single 1.5 kbp band in the wildtype and 2.9 kbp bands in the mutants (Figure 6.2) that confirmed the replacement of the wildtype *cipA* with the interrupted *cipA* by homologous recombination. Another PCR using M13-F and M13-R primers was also done to check if the PCR products were from the replicating suicide vector rather than recombination crossover. PCR using M13-F and M13-R primers did not generate a

product in mutants confirming that the suicide vector was not replicating in the *C. jejuni*Δ*cipA* mutant and that the gene had been mutated as a result of crossover (not shown). *C. jejuni*Δ*cipA* mutant was tested in the gentamicin protection assay to reconfirm the *cipA* involvement in invasion in cultured eukaryotic cells.

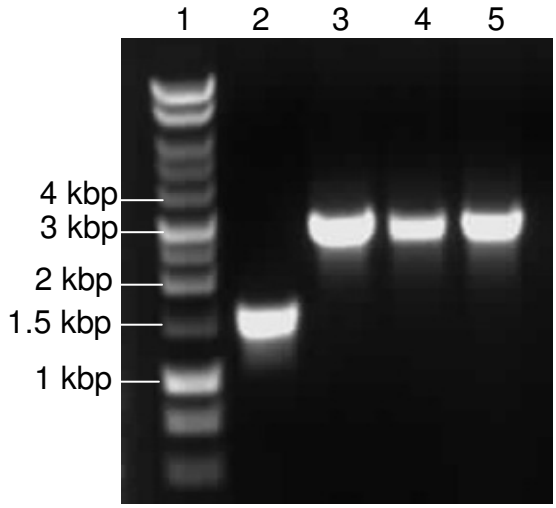

Figure 6.2 PCR analysis of the *cipA* mutation in *C. jejuni* 01/51.

PCR with 685-F and 685-R primers generated a 1.5 kbp product in 01/51 wildtype (lane 2) and a 2.9 kbp product in three *cipA* mutants (lane 3, 4 and 5) that confirmed the successful mutation of *cipA* in *C. jejuni* 01/51. Lane 1 shows 1.0 kbp DNA markers (Promega, UK).

6.1.2 Mutagenesis of a putative restriction/modification enzyme (*Cj0690c*)

6.1.2.1 Introduction

DNA sequence obtained from transposon mutant 6A9 showed the inactivation of a putative restriction/modification enzyme (*Cj0690c*) of *Campylobacter* species. *Cj0690c* is 3753 bp (1250 amino acids) in size and the actual function of this gene is not yet known. This gene contains an N-6 adenine-specific DNA methylase signature and a type I restriction enzyme domain was identified within the coding sequence so it might be involved in the methylation/restriction of DNA. The DNA methyltransferases of *C. jejuni* and some other enteric pathogens have been reported to play a role in their virulence (Balbontin *et al.*, 2006; Falker *et al.*, 2005; Heusipp *et al.*, 2007; Kim *et al.*, 2008). *Cj0690c* was mutated by insertional inactivation to reconfirm its involvement in invasiveness in *C. jejuni* 01/51.

6.1.2.2 Cloning and generation of the mutant

A targeted mutation was generated in *Cj0690c* to further investigate its role in invasion. This gene has a *Bcl*I site at 1578 bp in the CDS that was selected to insert the *kan*^{r} cassette and inactivate the gene. Initially, a 1.9 kbp DNA fragment from 586 bp to 2483 bp inside the gene was amplified using 690-F and 690-R primers. The PCR product with A overhangs was ligated with the pGEM®-T Easy vector and the resulting plasmid pGEM:690, carrying the cloned DNA fragment, was confirmed by PCR and restriction digest (not shown).

Then pGEM:690 was digested with *Bcl*I to generate the mutation construct by inserting the *kan*^{r} cassette; *Bcl*I generates compatible ends to *Bam*HI whose restriction sites were flanking the amplified *kan*^{r} cassette. The *kan*^{r} cassette was amplified by PCR from pMA24 using KCN-F and Kan-Fex primers that harbour *Bam*HI restriction sites near their 5'ends. *Kan*^{r} amplicons were digested with *Bam*HI and ligated with *Bcl*I linearised pGEM:690. *E. coli* TOP10 cells were transformed with ligation products and colonies were selected on LB agar supplemented with kanamycin (50 µg/ml) and ampicillin (100 µg/ml). Plasmids were extracted from four out of more than one hundred colonies that grew after 24 hour incubation and were tested for the correct construct by PCR with *Cj0690c* specific (690-F and 690-R) and plasmid specific (M13-F and M13-R) primers. PCR generated a 3.3 kbp product in plasmid extracted from two of the colonies which confirmed that *Cj0690c* was interrupted with the *kan*^{r} cassette (Figure 6.3). The mutation construct was also confirmed by *Eco*RI restriction analysis that released the 3.3 kbp band of the interrupted *Cj0690c* from the 3.0 kbp plasmid vector. On a 1% agarose gel the insert and vector bands co-migrated and appeared as single band in the correct construct (Figure 6.3). PCR using M13 primers generated no product in reaction without a DNA template which was used as a negative control; an expected 3.3 kbp product in the successful construct (pGEM:690::kan) confirmed interruption of cloned *Cj0690c*. Two bands of 3.3 and 1.9 kbp in one of the plasmids indicated that the *E. coli* cell might had been transformed with two plasmids one carrying interrupted insert and the other intact *Cj0690c*. Lane 6 in the figure showed a 1.9 kbp PCR product indicating that it was not the correct construct and the plasmid carried intact *Cj0690c*. PCR using *Cj0690c* specific primers showed a 1.9 kbp product in the wildtype 01/51 genomic DNA used as PCR reference positive control; the PCR product band pattern in the plasmids carrying right construct was same as shown by M13 PCR reconfirming those

plasmids in lane 8 and 9 of figure 6.3 were correct constructs. These constructs were also tested by restriction digest with *Eco*RI that generated two products of 3.3 kbp and 3.0 kbps that co-migrated in the gel and a single band was observed in the correct constructs; while two bands of 1.9 kbp and 3.0 kbp were observed in those plasmids in which cloned *Cj0690c* was not interrupted by *kan*^{r}. So the plasmid extracted from the colony which was shown in lane 3, 8 and 12 was selected for transformation of *C. jejuni* 01/51.

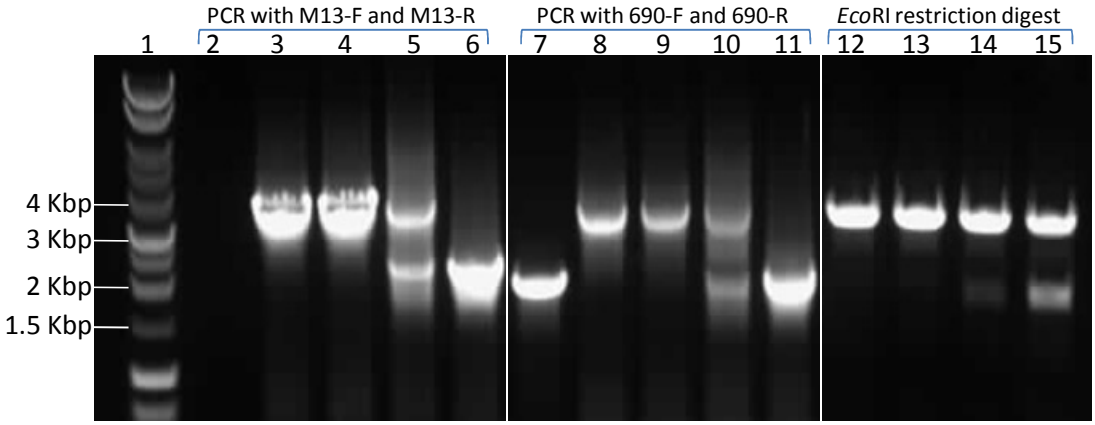

Figure 6.3 Analysis of the pGEM:690::kan.

PCR using M13-F and M13-R primers as expected generated no product in PCR negative control (lane 2), a 3.3 kbp product in plasmids carrying interrupted *Cj0690c* insert (lane 3 and 4) but the PCR products generated in plasmids from other two clones (lane 5 and 6) were not of right size so they were discarded. PCR using *Cj0690c* specific primers 690-F and 690-R generated an expected 1.9 kbp product in 01/51 genomic DNA (lane 7), a 3.3 kbp product in the plasmid having the correct construct (lane 8 and 9) but products generated in other two plasmid preps were again not right (lane 10 and 11) so they were discarded. PCR results were verified by *Eco*RI restriction analysis that generated co-migrating products of 3.0 kbp (vector) and 3.3 kbp (insert) in the correct constructs (lane 12 and 13) while products from two other plasmids (lane 14 and 15) confirmed that they were not the correct constructs, so the construct from lane 12 was used further for generating mutation in *C. jejuni* 01/51. Lane 1 shows the 1.0 kbp DNA markers (Promega, UK)

Electrocompetent *C. jejuni* 01/51 cells were transformed with the suicide vector carrying the mutation construct of *Cj0690c* and the colonies that grew on BA plates supplemented with kanamycin were sub-cultured and genomic DNA was extracted. PCR using 690-F and 690-R primers showed a 3.3 kbp band in eight selected colonies that corresponds to the *Cj0690c* gene interrupted with *kan*^{r} cassette (Figure 6.4). PCR with pGEM®-T Easy vector specific M13-F and M13-R primers generated no product (not shown) that confirmed the *Cj0690c* mutation took place by allelic exchange and the vector was lost in the event.

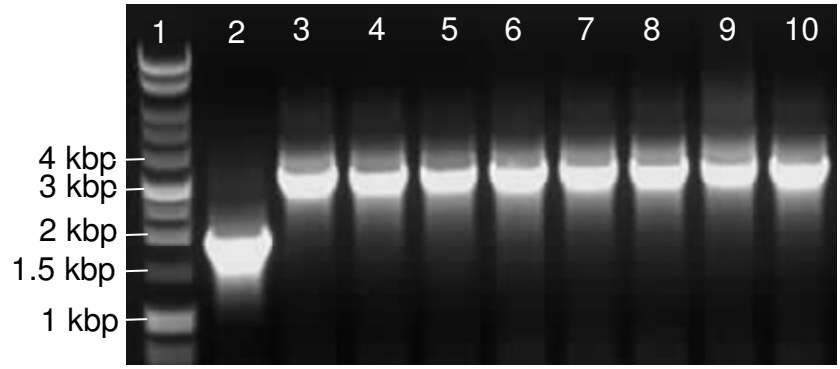

Figure 6.4 PCR analysis of *Cj0690c* mutation in *C. jejuni* 01/51.

PCR using 690-F and 690-R primers generated a 1.8 kbp product in 01/51 wildtype genomic DNA (lane 2) and a 3.3 kbp product in all the eight presumptive mutants (lane 2 – 10) that corresponds to interrupted *Cj0690c*. It confirmed that *Cj0690c* had been replaced by interrupted *Cj0690c* thus the mutation had been generated in all the eight tested mutants. Lanes: L1: 1.0 kbp DNA markers (Promega); L2: PCR product in wildtype strain 01/51; L3 to L10: PCR products in *C. jejuni* 01/51Δ*Cj0690c*.

6.1.3 Mutagenesis of a putative galactosyltransferase (*Cj1136*)

6.1.3.1 Introduction

DNA sequence obtained from transposon mutant 10E9 showed a match with a putative galactosyltransferase (*Cj1136*) of *Campylobacter* species. *Cj1136* is a 1173 bp (390 amino acids) gene that was part of LOS biosynthesis locus in *C. jejuni* NCTC11168, however, its role has not yet been characterised.

6.1.3.2 Cloning and generation of the mutant

A suicide vector was generated to mutate *Cj1136* in *C. jejuni* 01/51. Initially, a 1.3 kbp DNA fragment, from 66 bp upstream of the *Cj1136* start codon to 90 bp down stream of the termination codon, was amplified using primers 1136-F and 1136-R. The PCR product with A overhangs was ligated with pGEM®-T Easy and the resulting plasmid pGEM:1136 carrying the cloned DNA fragment was confirmed by restriction digest with *Eco*RI, which cut the 1.3 kbp insert from the vector (Figure 6.5).

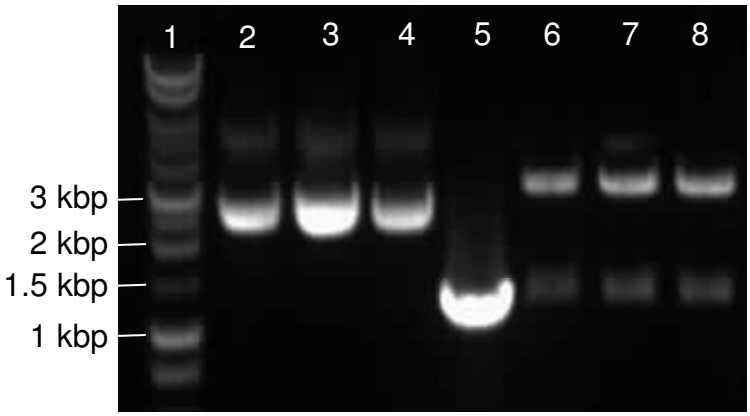

Figure 6.5 Restriction analysis of pGEM:1136.

*Eco*RI digest released the 1.3 kbp insert from 3.0 kbp vector (lanes 6, 7 and 8) that confirmed the cloning of *Cj1136* in pGEM®-T Easy. Lanes: L1: DNA markers; L2, L3, and L4: untreated plasmid; L5: PCR product of *Cj1136*; and L6, L7, and L8: *Eco*RI digested plasmid.

Cj1136 does not have a suitable restriction site that could be used for inserting the *kan*^{r} cassette. Previously, the development of the plasmid based inverse PCR technique has aided the construction of insertional mutants when no suitable restriction site was present within the gene sequence for insertion of an antibiotic resistance cassette (Wren *et al.*, 1994). The length of the flanking sequences on either side of the insertion site is of prime importance for recombination of the mutant gene with its wildtype chromosomal counterpart (Wassenaar *et al.*, 1993). Recombination integration events have been possible with as little as 202 bp of homologous DNA sequence, however, the frequency of recombination increased with longer homologous sequences (Wassenaar *et al.*, 1993). For that reason, a *Bam*HI restriction site was introduced ~0.6 kbp from the start codon of the *Cj1136*, using primers 1136-INF and 1136-INR. Inverse PCR generated a 4.3 kbp PCR product flanked by *Bam*HI restriction sites and it also deleted a 57 bp sequence in *Cj1136*. The inverse PCR product (5 µl) was run on a 1.0% agarose gel to check the size and the remaining volume (95 µl) was purified using PCR purification kit (Sigma, UK), digested with *Bam*HI and treated with TSAP to avoid self-ligation. The digestion mixture was purified again before ligation with the *kan*^{r} cassette. The *kan*^{r} cassette was PCR amplified as above and *Bam*HI digested; *kan*^{r} PCR products were ligated with *Bam*HI digested and dephosphorylated inverse PCR product of pGEM:1136. *E. coli* TOP10 cells were transformed and colonies were selected on kanamycin (50 µg/ml) and ampicillin (100 µg/ml). Colony PCR using 1136-F and 1136-R primers was performed to look for those colonies carrying the pGEM:1136::kan plasmid, the mutation construct was also confirmed by restriction analysis (not shown).

Then the suicide vector pGEM:1136::kan was introduced into *C. jejuni* 01/51 as mentioned above. Eight out of more than a hundred colonies that grew on BA supplemented with kanamycin (50µg/ml) were checked by colony PCR using 1136-F and 1136-R primers. PCR products showed a single 1.3 kbp band in the wildtype and three of the selected colonies and a 2.7 kbp band in five other colonies that confirmed the replacement of the wildtype gene with interrupted *Cj1136* by recombination in these five out of eight tested colonies (Figure 6.6). PCR using M13-F and M13-R primers was also done and again no product was present in any of mutant lanes confirming that the suicide vector was not replicating in *C. jejuni* and that the gene had been mutated as a result of a double crossover event (not shown).

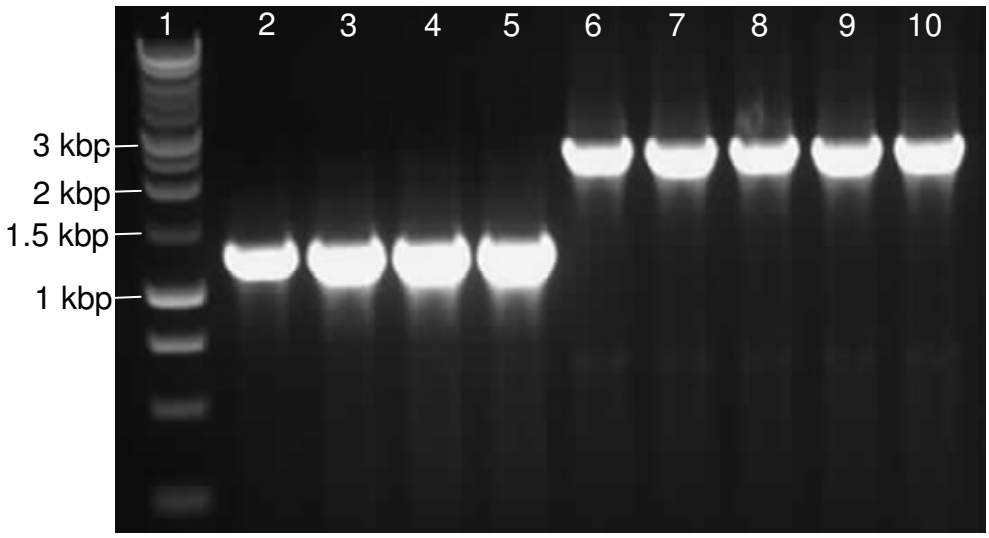

Figure 6.6 PCR analysis of *Cj1136* mutation in *C. jejuni* 01/51.

PCR using 1136-F and 1136-R primers generated a 1.3 kbp product in 01/51 genomic DNA (lane 2) and three presumptive mutants (lane 3, 4 and 5) that confirmed that *Cj1136* was not mutated in these three colonies but a 2.7 kbp product was generated in five other colonies of presumptive mutants (lane 6, 7, 8, 9 and 10) that confirmed the successful mutation of *Cj1136* in these five colonies. Lanes: L1: DNA markers; L2: PCR product of *C. jejuni* 01/51 wildtype; L3 - L10: PCR products of eight of presumptive mutant colonies.

6.1.4 Mutagenesis of a putative membrane protein (*Cj1245c*)

6.1.4.1 Introduction

DNA sequence obtained from transposon mutant 10H1 showed a match with a putative membrane protein (*Cj1245c*) of *Campylobacter* species. *Cj1245c* is 1197 bp (398 amino

acids) long gene of *Campylobacter jejuni* NCTC11168 and is conserved in other strains of *Campylobacter jejuni*. *Cj1245c* has not yet been further characterised.

6.1.4.2 Cloning and generation of the mutant

The transposon insertion mutation in the possible membrane protein (*Cj1245c*) gene of *Campylobacter jejuni* 01/51 showed poor invasion of INT-407 and Caco-2 cells. A targeted mutation was generated in *Cj1245c* to confirm its role in invasion. This gene has a *Bgl*II site at 781 bp in the CDS that was selected for insertion of the *kan*^{r} cassette to inactivate the gene. Initially, a 1.1 kbp DNA fragment from 69 bp to 1151 bp inside the *Cj1245c* CDS was amplified using aj1-FOR and aj1-REV primers. The PCR product was ligated with the vector pGEM®-T Easy and the resulting plasmid pGEM:1245 carrying the cloned DNA fragment was confirmed by PCR and restriction digest (not shown).

pGEM:1245 was digested with *Bgl*II to generate the mutation construct by inserting the *kan*^{r} cassette. *Bgl*II generates compatible ends to *Bam*HI whose restriction sites were flanking the *kan*^{r} cassette. The *kan*^{r} cassette (1.4 kbp) was PCR amplified from the pMA24 template using KCN-F and Kan-Fex primers harbouring *Bam*HI restriction sites near 5'ends and was digested with *Bam*HI. The *kan*^{r} PCR product was ligated with linearised pGEM:1245. *E. coli* TOP10 cells were transformed with the ligation products and colonies were selected on LB agar supplemented with kanamycin (50 µg/ml) and ampicillin (100 µg/ml). Colony PCR using aj1-FOR and aj1-REV primers was done to find colonies carrying pGEM:1245::kan. The mutation construct was also confirmed by restriction analysis with *Eco*RI which cuts the vector on both sides of the insert and release it. *Eco*RI digested products were run on an agarose gel and two bands of 3.0 kbp and 2.5 kbp sizes corresponding to the vector and *Cj1245c* with *kan*^{r} insert respectively, were observed. A PCR product of 2.5 kbp and restriction analysis confirmed that the mutation construct was correct (Figure 6.7).

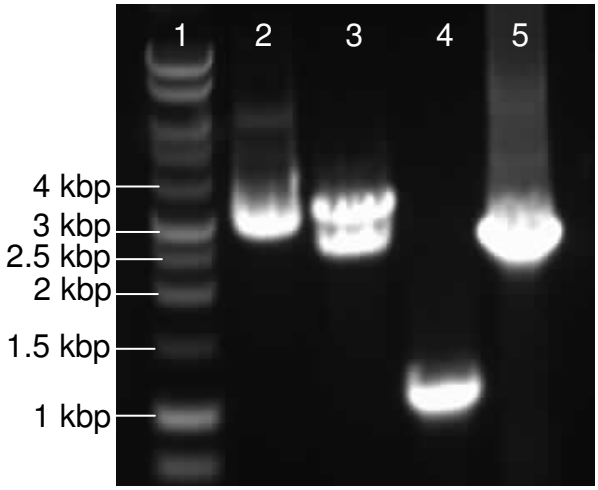

Figure 6.7 The confirmation of pGEM:1245::kan.

Mutation construct of *Cj1245c* was analysed by restriction digestion with *Eco*RI that released a 2.5 kbp insert from the 3.0 kbp vector (lane 3) and confirmed the construct was correct. PCR using aj1-FOR and aj1-REV generated a 1.1 kbp product in 01/51 genomic DNA (lane 4) and 2.5 kbp product in pGEM:1245::kan (lane 5) that verified that *Cj1245c* mutation construct was correct. Lanes: L1: 1.0 kbp DNA markers (Promega); L2: untreated plasmid; L3: *Eco*RI digested plasmid; L4: *Cj1245c* PCR product from 01/51 genomic DNA; L5: PCR product from pGEM:1245::kan.

The suicide vector pGEM:1245::kan was introduced into electrocompetent *C. jejuni* 01/51 by electroporation in order to disrupt the wildtype *Cj1245c*. After a 5 hour recovery on BA plates, the transformants were selected by plating onto BA plates supplemented with kanamycin (50 µg/ml). After 48 hours, there were no colonies observed on the negative control plates, however the test plates gave tens of colonies. Three colonies were sub-cultured and their genomic DNA was extracted. PCR of the genomic DNA extracted from the *C. jejuni* 01/51 wildtype and its (*Cj1245c*) mutants was done using aj1-FOR and aj1-REV primers. PCR products showed a single 1.1 kbp band in the wildtype and 2.5 kbp bands in the mutant lanes that confirmed the replacement of the wildtype gene with interrupted *Cj1245c* by homologous recombination (Figure 6.8). PCR products with M13 primers did not give a product in the *C. jejuni* mutant DNA template but it gave the expected 1.1 kbp size band with pGEM:1245 template, which was used as PCR control (not shown), suggesting the *Cj1245c* mutation by a double crossover event and that the vector was lost.

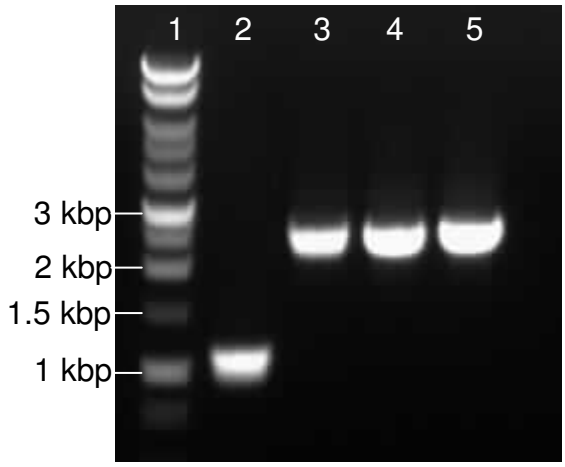

Figure 6.8 PCR analysis of *Cj1245c* mutation in *C. jejuni* 01/51.

PCR using aj1-FOR and aj1-REV primers generated a 1.1 kbp product in 01/51 wildtype genomic DNA (lane 2) and a 2.5 kbp product in three of the presumptive *Cj1245c* mutants (lane 3, 4 and 5) that confirmed that the wildtype *Cj1245c* gene was replaced by allelic exchange with the interrupted *Cj1245c* in the mutants. Lanes: L1: 1.0 kbp DNA markers; L2: PCR product of *C. jeuni* 01/51 wildtype; L3, L4 and L5: PCR products of three *C. jejuni* 01/51Δ*Cj1245c* colonies.

6.1.5 Mutagenesis of a hypothetical protein (*Cj1305c*)

6.1.5.1 Introduction

DNA sequence obtained from transposon mutant 10H12 showed a match with a hypothetical protein (617 family) of *Campylobacter* species. *Cj1305c* is a 1218 bp (405 amino acids) long gene of *Campylobacter jejuni* NCTC11168. The actual function of this gene is not known yet. It shows similarity with *Cj1306c* (78.1%), *Cj0617* (38.9%), *Cj0618* (37.1%), *Cj1310c* (63.7%), *Cj1342c* (38.9%) and hypothetical proteins of other bacteria including *Aeromonas hydrophila* (27%). It is a member of the 617 family of *C. jejuni* proteins. This 617 family of proteins contains homopolymeric tracts and are part of *C. jejuni* O-linked glycosylation locus (*Cj1293 – Cj1342*); no homologues of this family are found in other bacterial species (Szymanski and Wren, 2005). Mutation of the *Cj1305c* homologue in *C. jejuni* 81-176 resulted in fully motile bacteria with no effect on flagellin glycosylation (Guerry *et al.*, 2006). The targeted mutation was generated in this gene to further characterise its involvement in invasion.

6.1.5.2 Cloning and generation of the mutant

Cj1305c has a *Bgl*II site at 531 bp in the CDS that was selected to insert the *kan*^{r} cassette and inactivate the gene. Initially, a 1.4 kbp DNA fragment from 188 upstream

of the start codon to 1198 bp inside the *Cj1305c* was amplified using 1305-F and 1305-R primers. The PCR product was ligated with the pGEM®-T Easy vector and the resulting plasmid pGEM:1305 was confirmed by PCR and restriction digest analysis. There was no *Bgl*II restriction site in pGEM®-T Easy vector but *Bgl*II cuts at 531 bp inside *Cj1305c* CDS so one *Bgl*II restriction product of 4.4 kbp was observed. pGEM:1305 was also digested with *Eco*RI that cut the vector on flanking sides of the insert and released the insert; there were *Eco*RI restriction sites in the *Cj1305c* CDS as well where it cut 378 bp in the CDS and three expected restriction products of 3.0 kbp, 0.84 kbp and 0.56 kbp were obtained (Figure 6.9). The correct sized products of restriction digest were observed confirming the cloning of *Cj1305c* into the pGEM®-T Easy vector (Figure 6.9).

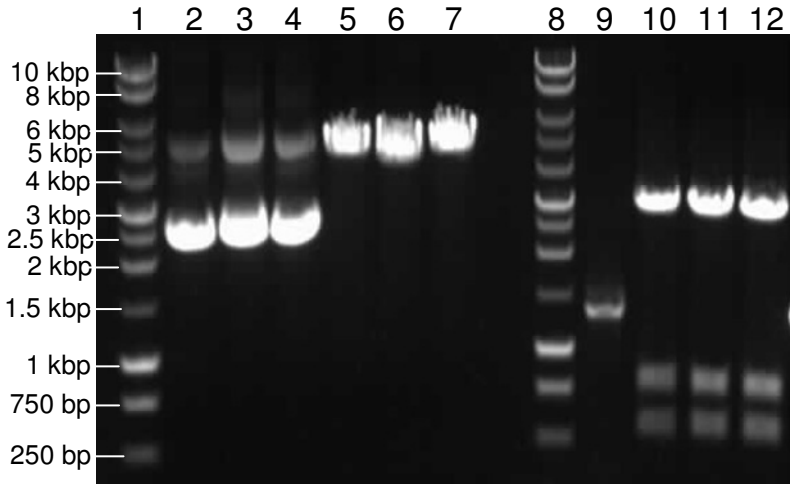

Figure 6.9 Restriction analysis of pGEM:1305.

The plasmid carrying *Cj1305c* gene was analysed by *Bgl*II restriction digestion that linearised the vector and a 4.4 kbp band was observed in digest pGEM:1305 (lane 5, 6 and 7). *Eco*RI restriction of pGEM:1305 generated three bands of 3.0 kbp, 0.8 kbp and 0.5 kbp (lane 10, 11 and 12). Restriction analysis confirmed the cloned *Cj1305c* in pGEM®-T Easy in all the three tested plasmid extracts. Lanes: L1 and 8: DNA markers; L2, L3 and L4: untreated plasmid; L5, L6 and L7: *Bgl*II cut pGEM:1305; L9: PCR product of *Cj1305c*; and L10, L11 and L12: *Eco*RI digested pGEM:1305.

pGEM:1305 was digested with *Bgl*II in order to generate the mutation construct by inserting the *kan*^{r} cassette. The *kan*^{r} cassette PCR product having *Bam*HI restriction sites near the 5'ends was digested with *Bam*HI and was ligated with linearised pGEM:1305. *E. coli* TOP10 cells were transformed with the resulting plasmid pGEM:1305::kan and colonies were selected on LB agar supplemented with kanamycin (50 µg/ml) and ampicillin (100 µg/m). Colony PCR using 1305-F and 1305-R primers

was done to select the colonies carrying the pGEM:1305::kan plasmid. The mutation construct was also confirmed by restriction analysis (not shown).

The suicide vector pGEM:1305::kan was introduced into electrocompetent *C. jejuni* 01/51. Colony PCR indicated one of the five checked mutants was correct and it was sub-cultured and genomic DNA was extracted. PCR of the genomic DNA of the *C. jejuni* 01/51 wildtype and the *Cj1305c* mutant was done using 1305-F and 1305-R primers. PCR products showed a single 1.4 kbp band in the wildtype and a 2.8 kbp band in the mutant lane that confirmed the mutation of *Cj1305c* (Figure 6.10). PCR using M13-F and M13-R primers did not show a product in the mutant lane confirming that the suicide vector was not replicating in the *C. jejuni*Δ*Cj1305c* and the gene had been mutated as a result of a double crossover event (not shown).

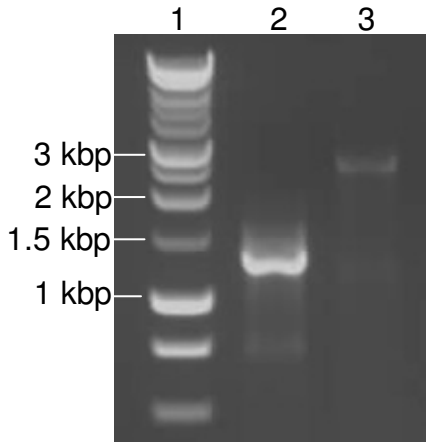

Figure 6.10 PCR analysis of *Cj1305c* mutation in *C. jejuni* 01/51.

PCR using 1305-F and 1305-R primers generated a 1.4 kbp band in 01/51 wildtype genomic DNA (lane 2) and a 2.8 kbp product in the *Cj1305c* mutant (lane 3) that confirmed that *Cj1305c* was mutated in *C. jejuni* 01/51. Lanes: L1: DNA markers; L2: PCR product of *C. jejuni* 01/51 wildtype; L3: PCR product of *C. jejuni* 01/51Δ*Cj1305c*.

6.1.6 Mutagenesis of a putative anion-uptake ABC-transport system permease protein (*Cj1539c*)

6.1.6.1 Introduction

Cj1539c is a 720 bp (239 amino acids) long gene of *C. jejuni* NCTC11168. Its translation product is similar to many involved in anion uptake including the sulphate transport system permease protein and the ABC-transport permease protein It is

established that ABC transport system components are involved in virulence in *Mycobacterium* species (Collins *et al.*, 2003; Peirs *et al.*, 2005), *Streptococcus pneumoniae* (Dintilhac et al., 1997), *Yersinia pestis* (Bearden and Perry, 1999) and many other micro-organisms . It would be interesting to study how the ABC transport permease plays a role in *C. jejuni* pathogenesis.

6.1.6.2 Cloning and generation of mutant

Cj1539c does not have a suitable restriction site and so one was generated using the inverse PCR technique. Initially, a 1.0 kbp DNA fragment, from 188 bp upstream of the *Cj1539c* start codon to 126 bp down stream of the termination codon, was amplified using primers 1539-F and 1539-R. The PCR product was ligated with the vector and the resulting plasmid pGEM:1539 was confirmed by PCR and restriction digest analysis with *Eco*RI. Restriction products were run on the agarose gel and two bands of 3.0 kbp and 1.0 kbp were observed corresponding to the cloning vector and the insert, respectively (Figure 6.11).

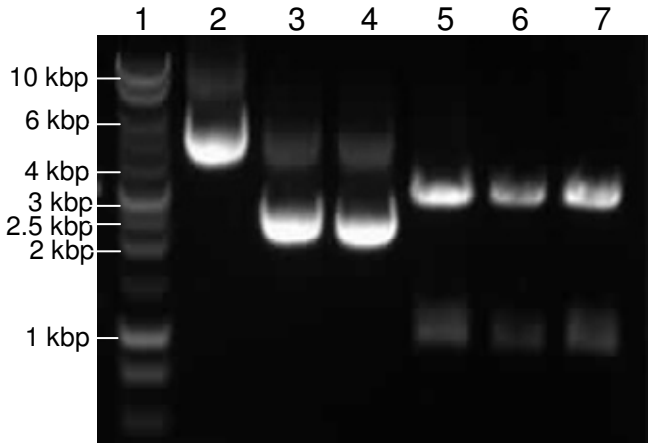

Figure 6.11 Restriction analysis of pGEM:1539.

Cloning of *Cj1539c* was confirmed by restriction with *Eco*RI that released 1.0 kbp insert from 3.0 kbp vector (lane 5, 6 and 7) and confirmed the *Cj1539c* cloning in pGEM®-T Easy. Lanes: L1: DNA markers; L2, L3 and L4: untreated plasmid; L5, L6 and L7: *Eco*RI digested pGEM:1539.

A *Bam*HI restriction site was introduced ~0.3 kbp from the start codon of *Cj1539c*, using primers 1539-INF and 1539-INR. Inverse PCR generated a 4.0 kbp PCR product flanked by *Bam*HI restriction sites and it also deleted a 21 bp sequence in *Cj1539c*. The inverse PCR product was purified, digested with *Bam*HI and treated with TSAP to

dephosphorylate and avoid self-ligation. The digestion mixture was purified again before ligation with the *kan*^{r} cassette.

The *kan*^{r} cassette was PCR amplified and the *Bam*HI digested *kan*^{r} PCR products were then ligated with the *Bam*HI digested inverse PCR product of pGEM:1539. *E. coli* TOP10 cells were transformed with the resulting plasmid and colonies grown on LB agar supplemented with kanamycin and ampicillin. Colony PCR using 1539-F and 1539-R primers was done to select colonies carrying pGEM:1539::kan plasmid. The mutation construct was also confirmed by restriction analysis (not shown).

The homologue of *Cj1539c* in *C. jejuni* 01/51 was mutated by transforming *C. jejuni* 01/51 with pGEM:1539::kan. The colonies grown on blood agar plates supplemented with kanamycin (50 µg/ml) after 48 hour incubation were analysed by PCR using C1539-F and C1539-R primers. The PCR generated a 1.3 kbp product in the wildtype strain and a 2.7 kbp product in the three selected mutant colonies corresponding to *Cj1539c* interrupted with the *kan*^{r} cassette along with an unexpected 4.3 kbp band; there was no 1.3 kbp band corresponding to intact *Cj1539c* in the mutants (Figure 6.12). PCR using the KCN-F and Kan-Fex primers was also done to check if the *kan*^{r} cassette was inserted in the chromosome of the mutants and this showed a 1.4 kbp band corresponding to the *kan*^{r} cassette in all the three mutants (Figure 6.12). The colony PCR of the mutants using M13 primers did not generate a product (not shown). These mutants were further investigated by PCR using *kan*^{r} specific and *Cj1539c* specific primers to confirm the *kan*^{r} insertion in *Cj1539c*. Both ends of mutated *Cj1539c* were tested using primer combinations comprising of *Cj1539c* specific primer and *kan*^{r} specific primer. The PCRs generated expected size products and confirmed the *Cj1539c* mutation in *C. jejuni* 01/51 (Figure 6.13).

The mutant in *cipA*, *Cj0690c*, *Cj1136*, *Cj1245c*, *Cj1305c* and *Cj1539c* were successfully generated. These mutants were tested in the gentamicin protection assays for adhesion and invasion phenotype.

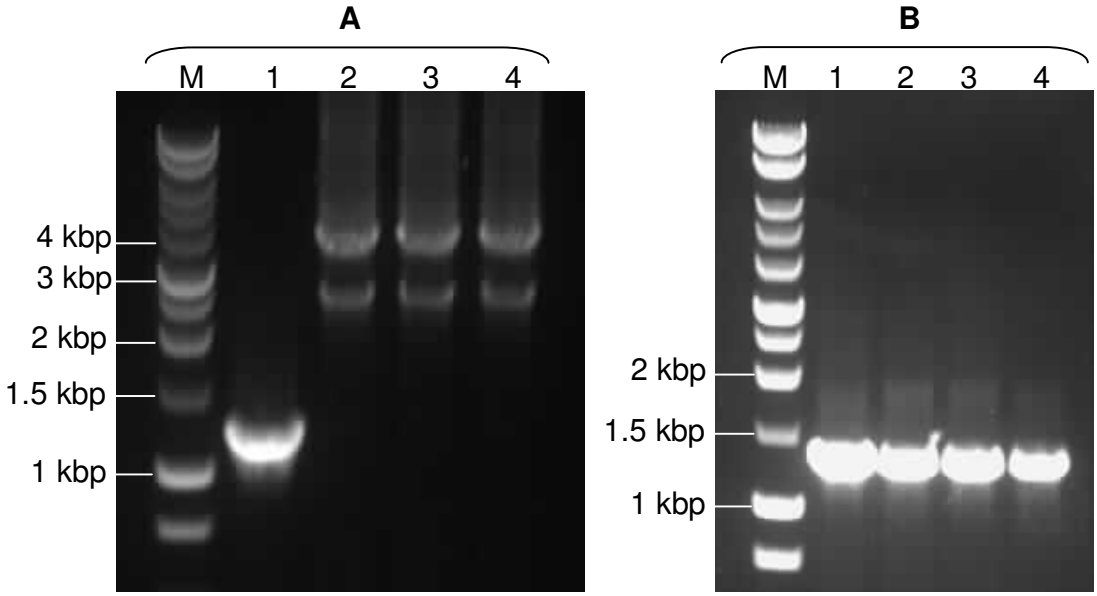

Figure 6.12 PCR analysis of *Cj1539c* mutation in *C. jejuni* 01/51.

A: PCR using C1539-F and C1539-R primers generated a 1.3 kbp band in 01/51 wildtype genomic DNA (lane 1) and a 2.7 kbp product corresponding to interrupted *Cj1539c* along with an unexpected 4.3 kbp band in the mutants (lanes 2 to 4) suggesting the mutation of *Cj1539c* which was further investigated. **B:** PCR using *kan*^{r} cassette specific primers KCN-F and Kan-Fex showed an expected 1.4kbp product in pMA24 (lane 1) and the same sized product in three mutants (lane 2, 3 and 4) that confirmed that the *kan*^{r} cassette was inserted into the mutant's chromosome. Lane M shows the DNA markers.

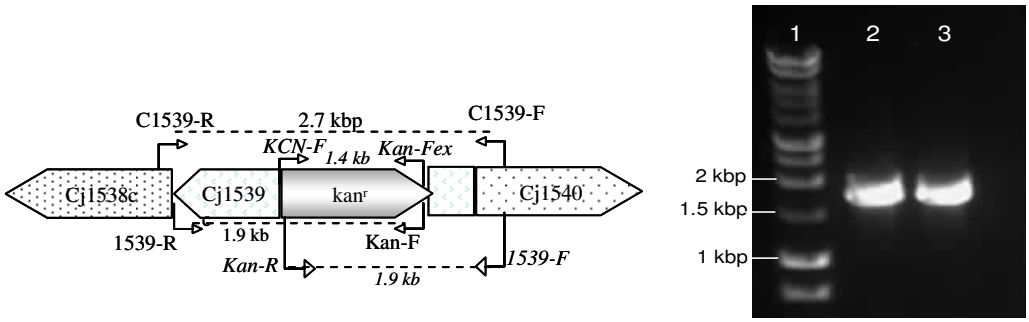

Figure 6.13 Schematic diagram and PCR confirmation of *Cj1539c* mutation in *C. jejuni* 01/51.

The schematic diagram (left) shows the postions of the primers and expected size of the PCR products used in the analysis of *Cj1539c* mutation. The right image shows the PCR products using Kan-F and 1539-F which generated 1.9 kbp product in *Cj1539c* mutant (lane 2) and PCR using Kan-R and 1539-R primers also generated a 1.9 kbp product in mutant (lane 3) that confirmed the generation of *C. jejuni*Δ*Cj1539c*. Lane 1 shows DNA markers.

6.2 Adhesion and invasion assays using Caco-2 cells

C. jejuni 01/51 was previously known to be hyperinvasive in INT-407 cells (Fearnley *et al.*, 2008) and a transposon mutant library was generated in this strain to study the molecular basis of hyperinvasiveness. Mutants that showed reduced invasion of INT-407 and Caco-2 cells were selected and genes interrupted by transposon insertion were identified. Transposons are known to have polar effects (Dymov *et al.*, 2004) and to check if the genes identified in the mutants were responsible for the reduced invasion phenotype or it was due to a polar effect, targeted mutations were generated in the selected genes.

Adhesion to the epithelial cell layer (Konkel *et al.*, 1992a) can be followed by invasion of epithelial cells, but this has been reported to be strain-dependent, since some strains adhered to cultured eukaryotic cells without invasion, while the capacity of others for invasion was 10^2–10^4 times higher (Everest *et al.*, 1992; Oelschlaeger *et al.*, 1993). The ability of the targeted mutants to adhere and invade Caco-2 cells was therefore tested. The mutants showed variable levels of attachment to Caco-2 cells and interestingly some of the mutants showed higher adhesion than the parent strain *C. jejuni* 01/51 (Figure 6.14). The change in adhesion efficiency was statistically significant in mutants of *cipA* (P = 0.01) and *Cj1539c* (P = 0.008) but the adhesion efficiency was not significantly different in other mutants *i.e. Cj0690c* (0.08), *Cj1136* (0.09), *Cj1245c* (0.19) and *Cj1305c* (0.57). All the six mutants showed reduction in invasion in Caco-2 cells (Figure 6.15). The invasion efficiency of *C. jejuni* 01/51 was 0.068 while mutants in *cipA* (0.014), *Cj0690c* (0.033), *Cj1136* (0.010), *Cj1245c* (0.025), *Cj1305c* (0.04) and *Cj1539c* (0.007) showed statistically significant reduced invasion compared with the parent strain 01/51. The probability value in all the mutants was <0.001 except mutant of *Cj1305c* which showed a value of P = 0.001. *C. jejuni* 81116Δ*flaA/B* was used as negative control that as expected showed very low invasion efficiency (P = <0.001). This targeted mutagenesis followed by gentamicin protection assays reconfirmed the invasion-related role of the selected six genes that were previously identified in transposon mutants of *C. jejuni* 01/51. On the basis of these observations it can be inferred that other genes identified by transposon mutagenesis are likely to be involved in the invasiveness in 01/51, however, further characterisation is needed to confirm this assumption. *C. jejuni* 01/51 mutants in *cipA*, *Cj0690c*, *Cj1136*, *Cj1245c* and *Cj1539c*

were selected for complementation studies to rule out any effect of *kan*^{r} cassette insertion on transcription of flanking genes and to further discount any polar effects.

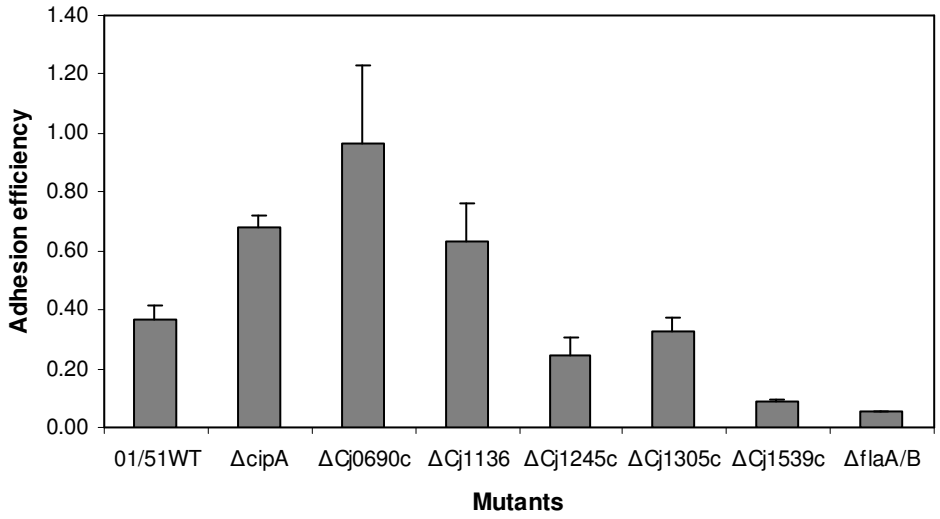

Figure 6.14 Adhesion of *C. jejuni* 01/51 and mutants to Caco-2 cells.

The adhesion assay was performed in triplicate and the mean efficiency of adhesion (Y-axis) shows the percent of initial inoculum that had attached to the Caco-2 cells over the course of three hours. *C. jejuni* 81116Δ*flaA/B* was used as the negative control.

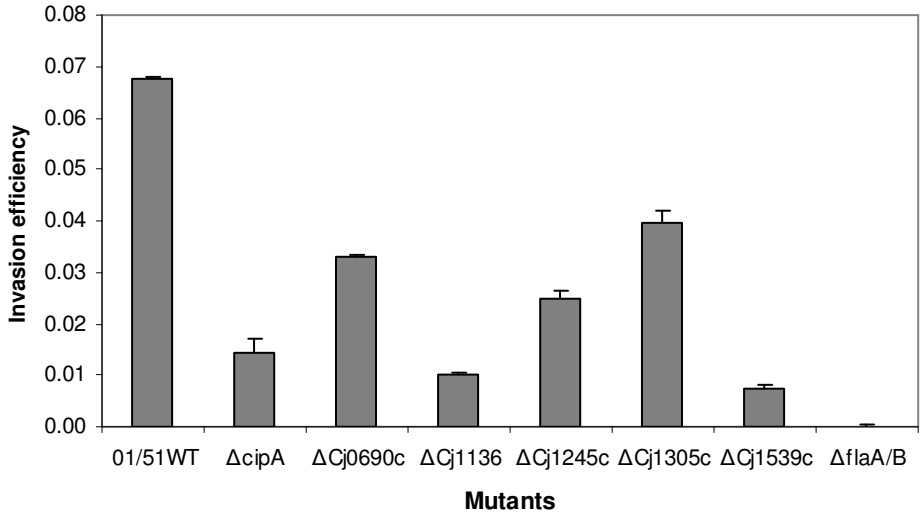

Figure 6.15 Invasion of *C. jejuni* 01/51 and mutants in Caco-2 cells.

The invasion potential of *C. jejuni* 01/51 and mutants generated by targeted mutagenesis was tested by gentamicin protection assay using Caco-2 cells; all the six mutants showed reduction in invasion as compared to parent strain 01/51. *C. jejuni* 81116Δ*flaA/B* was used as negative control in the assays and it showed very low invasion efficiency that confirmed the validity of the assay.

6.3 Complementation of mutated genes

In total 12 genes were identified that potentially play a role in enhancing the invasion in Caco-2 cells by *C. jejuni* 01/51. Generation of targeted mutations in six of the selected genes followed by observation of reduced invasion in Caco-2 cells by the mutants further strengthened the initial finding that these genes were involved in the hyperinvasive phenotype. Mutated genes were complemented to satisfy Koch's molecular postulates, to firmly establish a gene as a virulence factor (Falkow, 1988) which states the complementation of the mutated gene should reverse the reduced virulence-related phenotype in the mutant. Moreover, insertion of an antibiotic cassette into the gene may have terminated transcription of the flanking genes and the observed reduced invasion phenotype might be a result of lack of expression of those other genes rather than the mutated gene itself. Thus in order to prove that the mutated genes were responsible for the reduced invasion, the wildtype copy of the mutated gene was expressed in the mutant and if the reduced invasion was restored, it could be confirmed that the mutated gene was responsible for the loss of the phenotype in *C. jejuni* 01/51.

6.3.1 Complementation strategies

Two strategies have commonly been used to complement mutated genes in *C. jejuni;* one is *trans*-complementation in which the wildtype copy of the mutated gene is expressed extra-chromosomally in a plasmid, and other is *cis*-complementation in which the wildtype copy of the mutated gene is expressed at such a place in the mutant's chromosome where insertion of the gene does not affect the transcription of its flanking genes. Complementation in *trans* is an easy and quick way and is especially suitable when many genes are to be complemented as was the case in this study but the higher copy number of the gene complement is an issue. On the other hand complementing the mutated gene with its wildtype copy on the chromosome of the mutant is time demanding but the difference in expression levels of the wildtype copy due to higher copy number is not a problem with this method. For its ease of use complementation of the mutated genes in *C. jejuni* 01/51 was initially attempted using the *trans*-complementation method.

6.3.1.1 *Trans*-complementation using pUOA18

Initially, complementation of the genes mutated in this study was attempted in *trans* using pUOA18, a 7.4 kbp plasmid, that can replicate in both *E. coli* and *Campylobacter* species (Wang and Taylor, 1990) and had successfully been used to express genes in *C. jejuni* (Abuoun *et al.*, 2005; Lin *et al.*, 2003; Whitehouse *et al.*, 1998). This method was not successful in this study so will be described briefly. The mutated genes *i.e. cipA*, *Cj0690c*, *Cj1136*, *Cj1245c*, *Cj1305c* and *Cj1539c* that were interrupted by insertion of the kanamycin resistance cassette in this study (Section 6.1) were PCR amplified using gene specific primers. A *Bam*HI cloning site in pUOA18 was used to clone the inserts and all the primers (Table 2.3) used to amplify the mutated genes were designed to contain an additional *Bam*HI site near their 5' ends. *Bam*HI digested PCR products were ligated with *Bam*HI cut and dephosphorylated pUOA18. Cloning of the genes in pUOA18 was confirmed by restriction digest analysis (Figure 6.16). Transformation of the mutants of *C. jejuni* 01/51 with their respective complementation constructs was attempted by both electroporation and natural transformation methods. Unfortunately, transformation was not successful in any of the six mutants. To test if the failure in transformation was due to the inability of pUOA18 to replicate inside the mutants or whether it was due to loss of the competence in the mutants, *C. jejuni* 01/51 wildtype strain, a known transformation efficient strain, was transformed with pUOA18 but this was not successful either. It confirmed that pUOA18 was not an efficient vector for use in *C. jejuni* 01/51 derived mutants. This finding was similar to another report in which Karlyshev and Wren (2005) previously reported that the use of the shuttle vectors was *Campylobacter* strain specific. They found that shuttle vectors including pRY112 (Yao *et al.*, 1993), pMW10 (Wösten *et al.*, 2004), pMEK91 (Mixter *et al.*, 2003) and pGUO0202 (Alfredson and Korolik, 2003) which had previously been successfully used in some *Campylobacter* strains did not replicate in other strains of *C. jejuni* (Karlyshev and Wren, 2005).

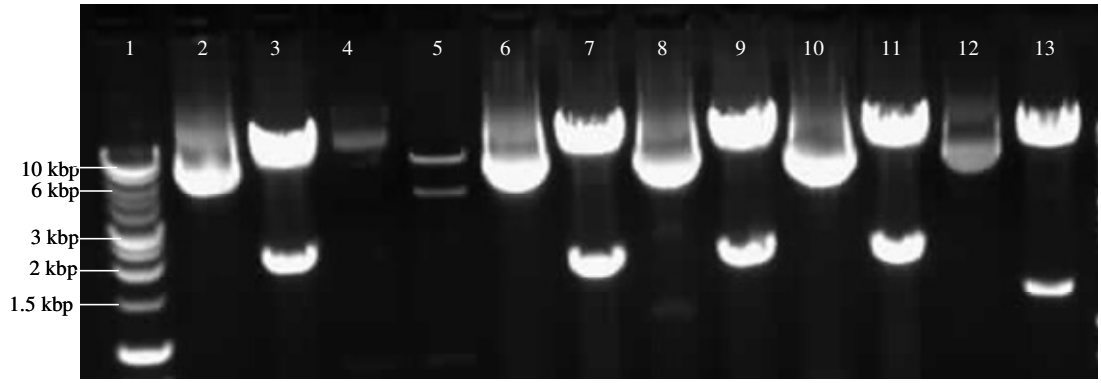

Figure 6.16 Restriction digest analysis of genes cloned in pUOA18.

Wildtype copies along with presumptive native promoters of the *cipA*, *Cj0690c*, *Cj1136*, *Cj1245c*, *Cj1305c* and *Cj1539c* were cloned in pUOA18 to complement the mutated alleles of these genes in the respective mutants of *C. jejuni* 01/51 and cloning of the genes in PUOA18 was confirmed by restriction digest with *Bam*HI. *Bam*HI digest which released the cloned *cipA* (2.0 kbp), *Cj0690c* (4.3 kbp), *Cj1136* (1.7 kbp), *Cj1245c* (1.8 kbp), *Cj1305c* (1.9 kbp) and *Cj1539c* (1.3 kbp) from pUOA18 (7.4 kbp) confirmed cloning of the genes in this vector. Lane 1: 1.0 kbp DNA marker (Promega); lane 2: undigested pUOA18-*cipA*; lane 3: digested products of pUOA18-*cipA*; lane 4: undigested pUOA18-*Cj0690c*; lane 5: digested products of pUOA18-*Cj0690c*; lane 6: undigested pUOA18-*Cj1136*; lane 7: digested products of pUOA18-*Cj1136*; lane 8: undigested pUOA18-*Cj1245c*; lane 9: digested products of pUOA18-*Cj1245c*; lane 10: undigested pUOA18-*Cj1305c*; lane 11: digested products of pUOA18-*Cj1305c*; lane 12: undigested pUOA18-*Cj1539c*; and lane 13: digested products of pUOA18-*Cj1539c*.

6.3.1.2 *Cis*-complementation

The mutated genes in *C. jejuni* 01/51 were then complemented in the bacterial chromosome by allelic crossover following a method suggested by Dr. Neil Oldfield, University of Nottingham (Dr. Oldfield, personal communications). In the first genome-sequenced *C. jejuni* strain NCTC11168 two adjacent genes *Cj0652* that encodes a putative penicillin-binding protein and *Cj0653c* that encodes a putative aminopeptidase sit tail-to-tail and there is a 121bp intergenic spacer region between them; a gene of interest can be inserted in this spacer region without affecting the transcription of the flanking genes. Previous studies have demonstrated that as little as 202 bp of homologous sequence are required for the allelic crossover integration of transformed DNA into the *Campylobacter* genome (Baillon *et al.*, 1999; Richardson and Park, 1997; Wassenaar *et al.*, 1993). A 2.3 kbp DNA molecule consisting of ~1 kbp from each of *Cj0652* and *Cj0653c* along with the intergenic spacer region was PCR amplified from 01/51 genomic DNA using 652-F and 653-F primers (Table 2.3) and high fidelity *Pfu* DNA polymerase (Promega, UK). The purified PCR products were cloned into pGEM®-T Easy (Promega, UK) by TA cloning and pAJ20 was generated. The nucleotide sequence of the cloned DNA fragment was checked by sequencing of the

pAJ20 plasmid using T7 and SP6 primers that bind pGEM®-T Easy vector in the flanking region of the insert and sequence towards the insert (Figure 2.2). Sequencing confirmed that the nucleotide sequence of this region in 01/51 was homologous to NCTC11168. There was not a suitable restriction site for cloning in the intergenic region between *Cj0652* and *Cj0653c* and so a *Bam*HI restriction site was then introduced in the middle of the intergenic region by inverse PCR of pAJ20 using IN652-R and IN653-R primers (Table 2.3). The amplicons were digested with *Bam*HI and then self-ligated using T4 DNA ligase (Promega, UK). The self-ligation products were transformed into *E. coli* Top10 (Invitrogen, UK) and the resulting plasmid pAJ21 was then confirmed by nucleotide sequencing using 652Seq-F primer which binds near the 3' end of *Cj0652* and the resulting sequence reads through the intergenic region between *Cj0652* and *Cj0653c*.

Two pAJ21 derived shuttle vectors carrying either the chloramphenicol resistance cassette (Cm^r) or the kanamycin resistance cassette (kan^r) were generated for complementing genes in targeted mutants of 01/51 which harbour the kan^r cassette or transposon mutants that carry Cm^r, respectively. The Cm^r cassette, originally identified in *C. coli* plasmid pNR9589 (Sagara *et al.*, 1987), was PCR amplified from pUOA18 using CML-F and CML-R primers (Table 2.3). CML-F harbours a *Bam*HI and CML-R had *Bgl*II restriction sites near their 5'ends; these restriction sites were added to the Cm^r primers to allow the *Bam*HI/*Bgl*II double digested PCR products of Cm^r to ligate with *Bam*HI digested pAJ21. *Bam*HI/*Bgl*II double digest PCR products of Cm^r were ligated with *Bam*HI digested and dephosphorylated pAJ21 and the ligation products were used to transform *E. coli* TOP10 (Invitrogen, UK). Colonies were selected on LB agar supplemented with ampicillin (100 µg/ml) and chloramphenicol (15 µg/ml). The resulting plasmid pAJ22 was extracted from colonies and cloning of the Cm^r cassette in the vector was confirmed by PCR (Figure 6.17), restriction analysis (Figure 6.20) and nucleotide sequencing. As expected the restriction site at the 3'end of the Cm^r gene was lost and a *Bam*HI site was left at the 5'end to allow cloning of the genes in pAJ22 (Figure 6.18). This was done to express the cloned gene under its native promoter and to avoid the effect of the constitutive Cm^r promoter on the transcription levels of the cloned genes. pAJ22 was used in complementing the *C. jejuni* genes mutated by kan^r cassette.

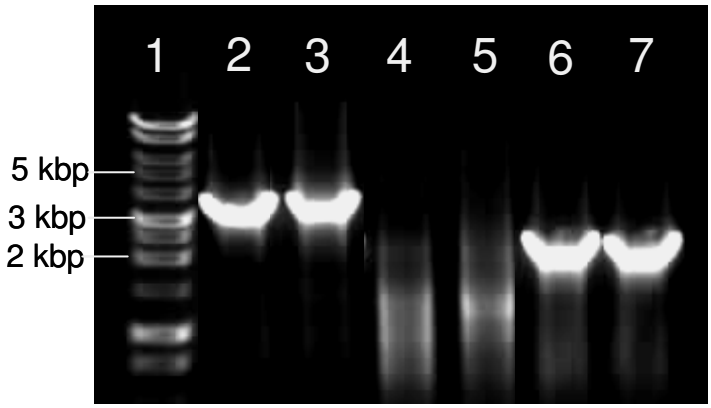

Figure 6.17 PCR analysis of pAJ22.

pAJ22 extracted from two of the colonies was analysed by PCR using 652-F/653-F primers that generated a 3.2 kbp product (lane 2 and 3) and confirmed that *Cm*^{r} was cloned in both of them; no PCR products were generated by 652-F/CML-F primers (lane 4 and 5) as expected but primers 653-F/CML-F generated ~2 kbp bands (lane 6 and 7) that confirmed the orientation of *Cm*^{r} in pAJ22 was directed towards *Cj0653c*. Sizes of the bands were compared with 1.0 kbp DNA markers (lane 1).

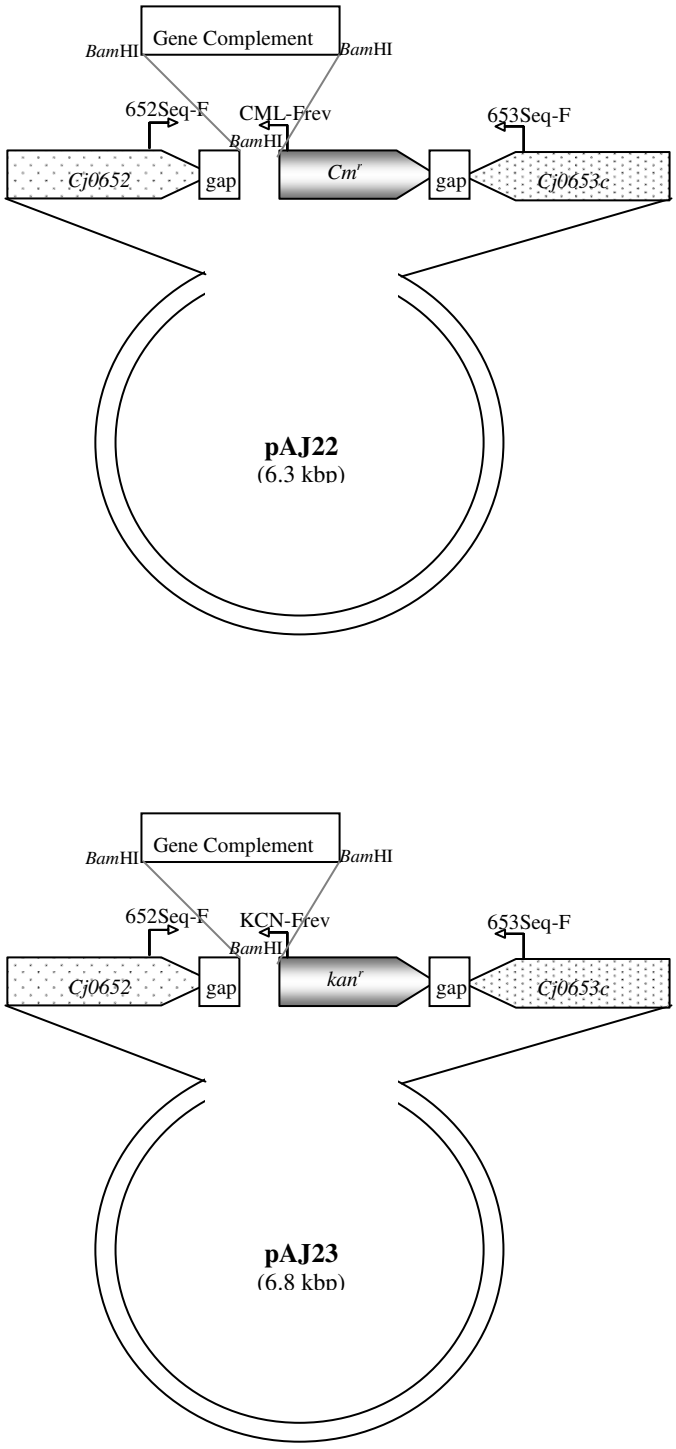

Figure 6.18 Schematic diagram of complementation suicide vectors pAJ22 and pAJ23.

pAJ22 (upper) and pAJ23 (bottom) have a pGEM®-T Easy backbone shown as a double circle and it can replicate in *E. coli* but not *C. jejuni*. A DNA molecule can be cloned at the *Bam*HI site near 5'end of the antibiotic resistance cassette and cloning can be confirmed by *Bam*HI restriction analysis as well as nucleotide sequencing using 652Seq-F and CML-Frev or KCN-Frev primers. *Cj0652* and *Cj0653c* (*C. jejuni* genomic DNA fragments) help in the integration of the cloned DNA molecule along with Cm^r antibiotic selection marker in the *C. jejuni* chromosome by allelic exchange.

A *kan*^{r} carrying suicide vector pAJ23 was generated the same way as pAJ22 except that the *Cm*^{r} cassette was replaced by the *kan*^{r} cassette that was amplified from pMA24 using KCN-F and KanBgl-R primers (Table 2.3); KCN-F had *Bam*HI while KanBgl-R had *Bgl*II sites near their 5'ends. *kan*^{r} PCR products were double digested using *Bam*HI and *Bgl*II and were ligated with *Bam*HI digested and dephosphorylated pAJ21. Chemically competent *E. coli* Top10 were transformed with the ligation products and colonies were selected on LB agar supplemented with ampicillin (100 µg/ml) and kanamycin (50 µg/ml). Five out of more than hundred colonies obtained after overnight incubation were tested by colony PCR using 652-F/653-F primers. All the five colonies showed a 3.7 kbp product confirming the insertion of the *kan*^{r} cassette in pAJ21 and pAJ23 was generated. PCR results of two selected plasmids are shown in Figure 6.19; construct of pAJ23 was also confirmed by nucleotide sequencing with 653Seq-F primers and restriction with *Bam*HI analysis (Figure 6.20). pAJ23 was used to complement those mutants in *C. jejuni* 01/51 in which *Cm*^{r} was used to inactivate the gene.

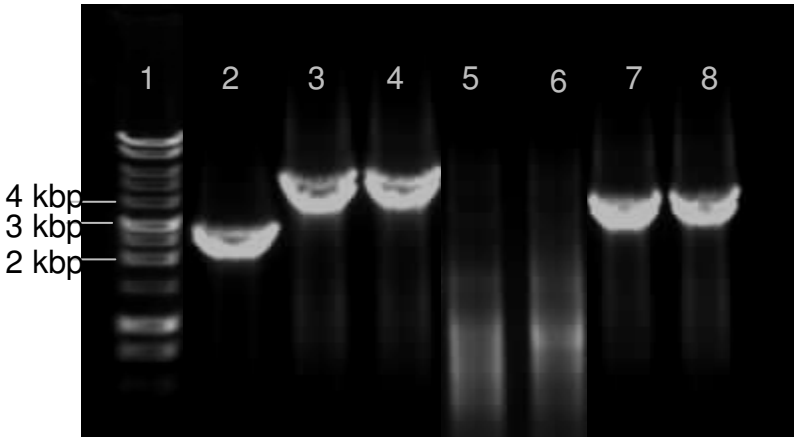

Figure 6.19 PCR analysis of pAJ23.

The pAJ23 construct was confirmed by PCR using 652-F/653-F primers which showed a 2.3 kbp product in the pAJ21 control (lane 2) and 3.7 kbp products in two of pAJ23 minipreps (lane 3 and 4) confirming that the *kan*^{r} cassette (1.4 kbp) was cloned in pAJ21 and pAJ23 was constructed. PCR with 652-F/KCN-F primers showed no products (lane 5 and 6) but primers 653-F/KCN-F showed 2.5 kbp products (lane 7 and 8) that confirmed the orientation of *kan*^{r} cassette was towards C0653c in the plasmid. Lane 1 shows 1.0 kbpDNA markers (Promega).

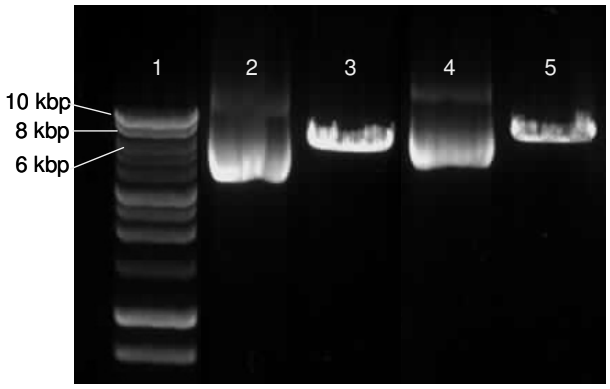

Figure 6.20 Restriction analysis of pAJ22 and pAJ23.

The pAJ22 and 23 were digested with *Bam*HI to test the presence of a *Bam*HI cloning site at the 5' of the *Cm*^{r} and *kan*^{r} cassettes. Restriction analysis confirmed the presence of the restriction site in both the vectors as single bands of 6.2 kbp (lane 2) and 6.7 kbp (lane 5) were observed in restricted pAJ22 and pAJ23, respectively. Lane 1 was 1.0 kbp DNA markers, lane 2 and 3 were undigested pAJ22 and 23, respectively.

6.3.2 Complementation of *cipA* (*Cj0685c*)

In this study, *cipA* was identified in transposon insertion mutant 3A10 and was found to play a role in the invasiveness in *C. jejuni* 01/51. Targeted mutation of this gene by insertion of kan^r cassette verified the initial findings from the transposon mutant studies that this gene promotes the invasion of *C. jejuni* 01/51 in cultured human intestinal epithelial cells. The interrupted *cipA* was complemented with its wildtype allele along with presumptive promoter to confirm that the reduced invasion phenotype in the *cipA* mutant was not due to a polar effect of transposon or the *kan*^{r} cassette.

Complementation of *cipA* was done by inserting its wildtype allele on the chromosome of the targeted mutant using the complementation suicide vector pAJ22. *cipA* along with its presumptive promoter was amplified from *C. jejuni* 01/51 genomic DNA by PCR using C685-F/C685-R primers (Table 2.3) and high fidelity *Pfu* DNA polymerase; these primers had *Bam*HI restriction sites near their 5' ends and the purified PCR products were digested with *Bam*HI. The digested *cipA* amplicons were ligated with *Bam*HI digested and dephosphorylated pAJ22. Chemically competent *E. coli* TOP10 were then transformed with ligation products and colonies were selected on LB agar supplemented with ampicillin and chloramphenicol. Twelve out of more than three hundred colonies grown after overnight incubation were screened for the successfully transformed clone by colony PCR using 652Seq-F and 653Seq-F primers and two colonies generated the

expected 3.0 kbp product. Further testing by PCR confirmed that *cipA* was cloned at the expected site in pAJ22 and its orientation in the resulting plasmid pAJ22-685 was directed towards the Cm^r cassette (Figure 6.21). The PCR using C6854-F and C685-R primers generated ~1.9 kbp product while the expected size was 2.0 kbp but nucleotide sequencing of pAJ22-685 with 652Seq-F and CML-Frev primers confirmed that the whole expected product was cloned.

Electrocompetent *C. jejuni* 01/51Δ*cipA* was transformed with pAJ22-685 and colonies were selected on blood agar supplemented with kanamycin (50 µg/ml) and chloramphenicol (15 µg/ml). PCR based analysis confirmed the successful integration of the wildtype *cipA* allele in the mutant's chromosome (Figure 6.22) in the only colony grown after 72 hours of incubation. PCR with 685-F and 685-R primers generated a 1.5 kbp product in both the wildtype 01/51 and complemented mutant. With these primers a 3.0 kbp product from the kan^r interrupted *cipA* was also expected in addition to the observed 1.5 kbp product from complemented mutant, however this was not observed which may have been due to the biased preference of PCR towards smaller products. To confirm that the mutated *cipA* was not replaced by its wildtype copy in the complemented mutant, another PCR with primers specific to the kan^r cassette and the *cipA* gene was performed which confirmed that the mutated *cipA* was not replaced in the complemented mutant (Figure 6.23). The *cipA* complemented *C. jejuni* 01/51Δ*cipA* was characterised further in the adhesion and invasion assays to test if complementation of the gene had reversed the reduced invasion phenotype.

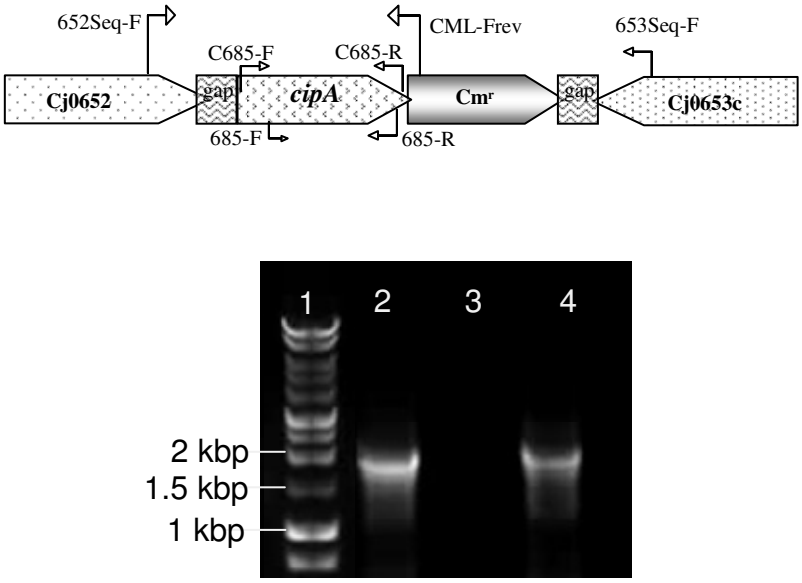

Figure 6.21 Schematic diagram and PCR analysis of *cipA* cloning in pAJ22.

The schematic diagram (upper) shows the position of primers used in the analysis of *cipA* cloning and complementation. The bottom image shows the PCR of pAJ22-685 with C685-F/C685-R primers that generated ~1.9 kbp product (lane 2) that confirmed the *cipA* cloning; no product was generated with C685-F/652Seq-F primers (lane 3) and a 2.0 kbp product was generated with C685-R/652Seq-F primers (lane 4) that confirmed the orientation of *cipA* in pAJ22-685 was directed towards *Cm*^{r}.

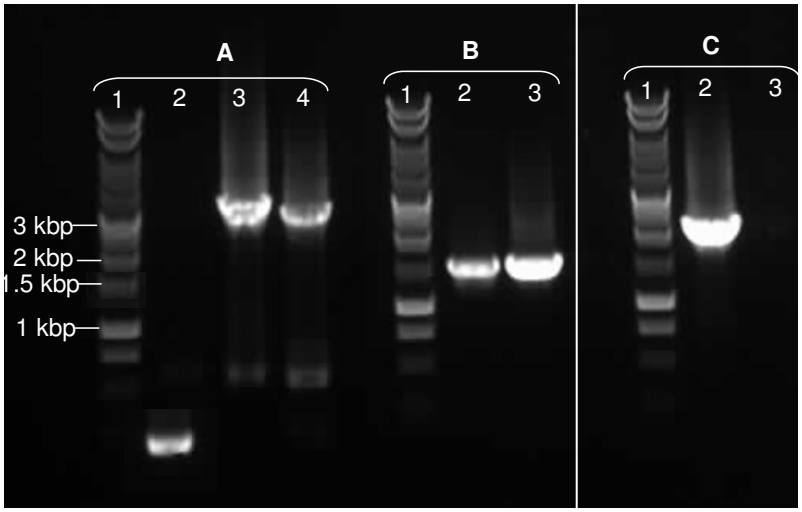

Figure 6.22 PCR confirmed the complementation of *cipA* in *C. jejuni* 01/51Δ*cipA*.

A: PCR with 652Seq-F/653Seq-F primers generated a 250 bp product in the 01/51 wildtype (lane 2), a 3.0 kbp product was generated in the pAJ22-685 control (lane 3) and the same size product was generated in complemented mutant (lane 4) confirming the integration of *cipA* in the expected site of the bacterial chromosome. **B:** PCR with 685F/685-R primers generated the expected 1.5 kbp products in both 01/51 wildtype (lane 2) and complemented mutant (lane 3) that confirmed the presence of the wildtype copy of *cipA* in the complemented mutant but further investigation was needed to confirm the presence of the mutated copy of the gene in the complemented mutant. **C:** PCR with vector specific primers M13-R/CML-F generated a 2.0 kbp product in pAJ22-685 (lane 2) and as expected no product was generated in the complemented mutant (lane 3) that confirmed the simultaneous allelic exchange and loss of vector lead to the integration of wildtype *cipA* in intergenic spacer region between *Cj0652* and *Cj0653c* in *C. jejuni* 01/51Δ*cipA*. Lane 1 shows the 1.0 kbp DNA marker (Promega).

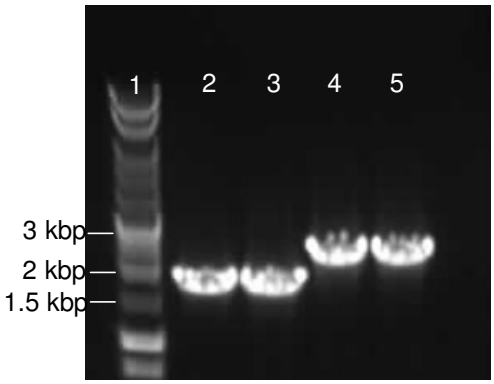

Figure 6.23 Mutated *cipA* was not replaced by its wildtype allele in complemented mutant.

PCR with Kan-F and 685-F primers generated a 1.9 kbp product in 01/51Δ*cipA* (lane 2) and complemented mutant (lane 3) while PCR with Kan-R/685-R primers generated 2.4 kbp product in both the mutant (lane 4) and complemented mutant (lane 5). It confirmed that mutated *cipA* was not replaced by its wildtype allele during complementation. Lane 1 shows the 1.0 kbp DNA marker (Promega).

6.3.3 Complementation of *Cj0690c*

The reduced invasion phenotype observed in the transposon and targeted mutant of *Cj0690c* in *C. jejuni* 01/51 was verified by complementing the mutation. *Cj0690c* along with its presumptive promoter was PCR amplified with C690-F and C690-R primers and the amplicon (4.3 kbp) was initially cloned in pAJ22 (Figure 6.24) to complement the targeted mutant of this gene but complementation was not successful despite many attempts. Few colonies appeared each time on blood agar supplemented with kanamycin and chloramphenicol but each time the mutated *Cj0690c* was replaced with its wildtype allele (not shown). This failure might be due to the large size (4.3 kbp) of *Cj0690c* along with its presumptive promoter sequence that provided at least 2.0 kbp sequence on each side of the *kan*^{r} cassette in the targeted mutant thus increasing the chance of crossover in this region rather than the region between *Cj0652* and *Cj0653c*. *Cj0690c* was then cloned in the other complementation vector pAJ23 so that complementation could be attempted in the transposon mutant 6A9 in which *Cj0690c* was mutated by transposon insertion. So *Cj0690c* was PCR amplified along with its presumptive promoter and *Bam*HI digested PCR products were ligated with *Bam*HI digested and dephosphorylated pAJ23. *E. coli* Top10 was transformed with these ligation products and colonies were selected on LB agar supplemented with ampicillin and kanamycin. After 24 hours incubation ten colonies were tested by colony PCR with 652Seq-F and

653Seq-F primers and two of them generated a product of the expected size (4.5 kbp). The resulting plasmid pAJ23-690 was extracted from one of these colonies and tested by PCR using gene specific primers C690-F and C690-R that generated a 4.3 kbp product (Figure 6.25) confirming the *Cj0690c* complementation vector was constructed; plasmid pAJ23-690 was also confirmed by nucleotide sequencing. The electrocompetent transposon mutant 6A9 was transformed with pAJ23-690 by electroporation and colonies were selected on blood agar supplemented with chloramphenicol and kanamycin. All the twelve colonies that appeared after 72 hours of incubation were tested by colony PCR with 652Seq-F and 653Seq-F primers and one colony that showed the expected 5.9 kbp product was further confirmed by PCR. PCR analysis confirmed that the *Cj0690c* wildtype allele was integrated in the region between *Cj0652* and *Cj0653c* of *C. jejuni* 01/51 mutant 6A9 (Figure 6.26). This *Cj0690c* complemented mutant was further characterised in adhesion, invasion, motility, growth curve and different stress survival assays.

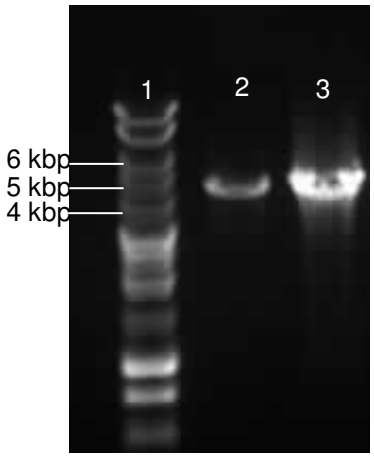

Figure 6.24 *Cj0690c* was cloned in pAJ22.

PCR using C690-F and C690-R primers generated expected 4.3 kbp product in 01/51 wildtype genomic DNA (lane 2) and the plasmid extracted from *E. coli* transformed with ligation products of pAJ22 and *Cj0690c* (lane 3). PCR confirmed that *Cj0690c* was cloned in pAJ22 and the complementation vector (pAJ22-690) was constructed which was also confirmed by nucleotide sequencing. pAJ22-690 was used to transform *C. jejuni* 01/51Δ*Cj0690c*. Lane 1 shows the 1.0 kbp DNA marker (Promega).

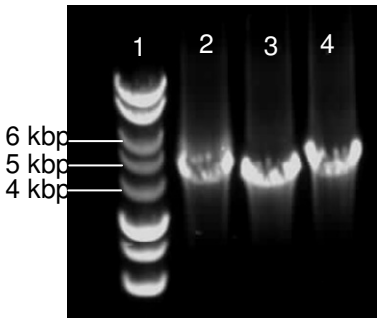

Figure 6.25 *Cj0690c* was cloned in complementation vector pAJ23.

PCR using C690-F and C690-R primers generated 4.3 kbp products in 01/51 wildtype genomic DNA (lane 2), pAJ23-690 extracted from colony-2 (lane 4) and a slightly smaller product was generated from the plasmid extracted from colony-1 (lane 3). pAJ23-690 extracted from colony-2 was further tested by nucleotide sequencing and was used to complement the mutated *Cj0690c*. Lane 1 shows the 1.0 kbp DNA markers (Promega).

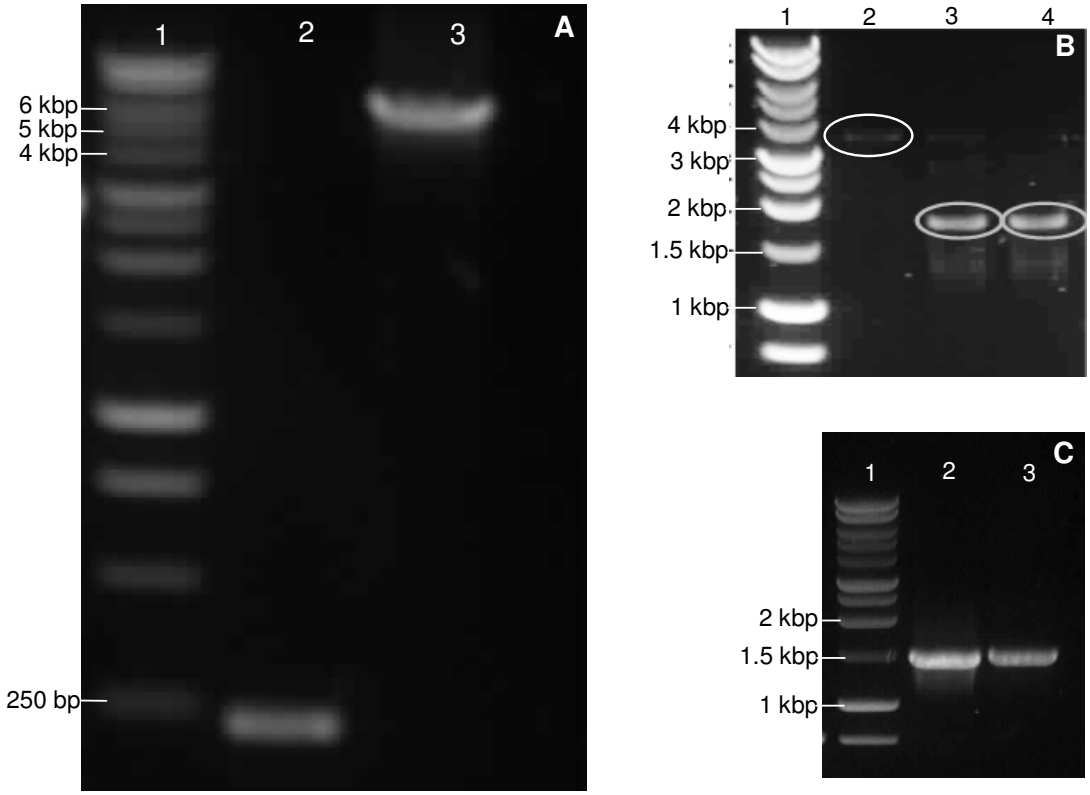

Figure 6.26 *Cj0690c* mutation was complemented in transposon mutant 6A9.

A: PCR using 652Seq-F and 653Seq-F primers generated an expected 250 bp product in the 01/51 wildtype (lane 2) and a 5.9 kbp product in the complemented mutant (lane 3) that confirmed the integration of *Cj0690c* in the spacer region between *Cj0652* and *Cj0653c*. **B:** PCR with *Cj0690c* specific (690-F and 690-R) primers generated a 3.8 kbp product in the 6A9 transposon mutant control template (lane 2) and a 1.8 kbp product in both the 01/51 wildtype (lane 3) and complemented mutant (lane 4) that confirmed the complementation of *Cj0690c* in the mutant. There should have been two PCR products of 1.8 kbp and 3.8 kbp in the complemented mutant but the appearance of a 1.8 kbp band demanded further investigation to confirm that the mutated copy of *Cj0690c* was also present in the complemented mutant. **C:** PCR with transposon specific (Transeq) and *Cj0690c* specific (690-F) primers generated a ~1.5 kbp product in both the mutant 6A9 (lane 2) and complemented mutant (lane 3) that confirmed the presence of mutated *Cj0690c* gene in the complemented mutant. 1.0 kbp DNA markers (lane 1) were used for size comparison in all the gels. The PCR confirmed the successful complementation of *Cj0690c* in its mutant and it was also confirmed that mutated *Cj0690c* was not replaced by its wildtype allele and the wildtype allele was integrated in the intergenic region between *Cj0652* and *Cj0653c*.

6.3.4 Complementation of *Cj1136*

The mutated *Cj1136* was complemented with a wildtype copy of the gene to rule out any polar effects of the *kan*^{r} cassette on the downstream genes. Complementation was accomplished using suicide vector pAJ22. *Cj1136* along with its presumptive promoter was amplified by PCR using C1136-F and C1136-R primers (Table 2.3) and high fidelity *Pfu* DNA polymerase (Promega, UK). Primers C1136-F and C1136-R had additional *Bam*HI site near their 5' ends, therefore, purified PCR products were digested with *Bam*HI and were ligated with *Bam*HI digested and dephosphorylated pAJ22 to generate pAJ22-1136. Chemically competent *E. coli* TOP10 cells were transformed with pAJ22-1136 and colonies were selected on ampicillin (100 µg/ml) and chloramphenicol (15 µg/ml). Twelve out of more than a hundred colonies were screened by colony PCR with 652Seq-F and 653Seq-F primers (Table 2.3) and one colony showed an expected 2.8 kbp product which was further tested by PCR using gene specific primers that confirmed the successful cloning of *Cj1136* in the vector pAJ22. PCR of the resulting vector pAJ22-1136 with C1136-F and C1136-R primers generated a 1.7 kbp product confirming that *Cj1136* was successfully cloned in pAJ22 (Figure 6.27); cloning was also confirmed by nucleotide sequencing using 652seq-F and CML-Frev primers.

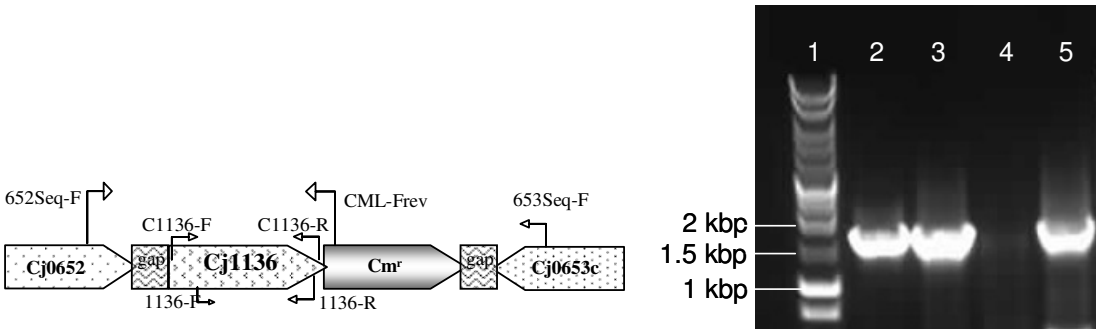

Figure 6.27 Schematic diagram and PCR analysis of *Cj1136* cloning in pAJ22.

The schematic diagram (left) shows the binding positions of primers used in the analysis of *Cj1136* cloning and complementation. The right image shows the PCR using C113-F and C1136-R primers that generated a 1.7 kbp product in 01/51 genomic DNA (lane 2) and pAJ22-1136 (lane 3) confirming that *Cj1136* was cloned in pAJ22. PCR with C1136-F/652seq-F did not generate a product (lane 4) but C1136-R/652Seq-F primers generated a 1.8 kbp product (lane 5) that confirmed that the orientation of *Cj1136* in pAJ22-1136 was directed towards the *Cm*^{r} cassette. Lane 1 shows 1.0 kbp DNA marker (Promega).

Following confirmation of the construct *C. jejuni* 01/51Δ*Cj1136* was made electrocompetent following protocol described in Section 2.10.3 and were transformed with pAJ22-1136. Colonies were selected on blood agar supplemented with kanamycin (50 µg/ml) and chloramphenicol (15 µg/ml) and the only two colonies grown after 48-72 hours of incubation were picked and genomic DNA was extracted and analysed by PCR. PCR with 652Seq-F and 653Seq-F primers generated a 2.8 kbp band in both the colonies tested while an expected 250 bp band was observed in 01/51 genomic DNA that was used as control (Figure 6.28). Thus confirming the integration of *Cj1136* in the intergenic region between *Cj0652* and *Cj0653c*. PCR with C1136-F/C1136-R and 1136-F/1136-R primer sets generated 1.8 kbp and 1.3 kbp products, respectively, which were correct for the wildtype (complement) copy of *Cj1136* but bigger bands of 2.7 kbp and 2.2 kbp from the mutated *Cj1136* were missing (Figure 6.28). The generation of only smaller bands was most probably due to PCR bias because easy (smaller) templates are preferentially amplified in multitemplate PCR (Hori *et al.*, 2007). PCR with Kan-F/1136-R and Kan-R/1136-F sets was performed separately that generated 2.0 kbp and 1.9 kbp products respectively to the primer set used in the mutant and both complemented mutants (Figure 6.29). It was confirmed by PCR that mutated *Cj1136* was not replaced by its wildtype copy in the complemented mutants. The *kan*^{r} cassette was in the same orientation as that of the mutated *Cj1136*. The *Cj1136* mutant in *C. jejuni* 01/51 and its complemented mutant were further studied in the assays of adhesion and invasion to test if complementation had restored the invasion phenotype.

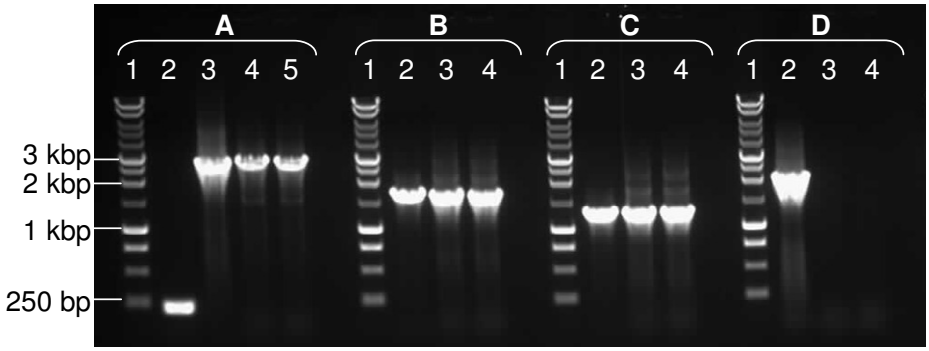

Figure 6.28 PCR analysis of *Cj1136* complementation in *C. jejuni* 01/51Δ*Cj1136*.

A: PCR using 652Seq-F and 653Seq-F generated a ~250 bp product in 01/51 genomic DNA (lane 2), 2.9 kbp products in pAJ22-1136 control template (lane 3) and both complemented mutants (lane 4 and 5) confirming that the wildtype copy of *Cj1136* was integrated in the intergenic region between *Cj0652* and *Cj0653c*. **B:** PCR with C1136-F and C1136-R generated a 1.7 kbp product in 01/51 wildtype genomic DNA (lane 2) and both the complemented mutants (lane 3 and 4) that confirmed the presence of the wildtype copy of *Cj1136* in the complemented mutants but further investigation was needed to confirm the presence of the mutated copy. **C:** PCR with 1136-F and 1136-R generated 1.3 kbp products in 01/51 control (lane 2) and both complemented mutants (lane 3 and 4) that reconfirmed the results shown by C1136-F/R primers (B). **D:** PCR with Cm^r specific CML-F and pGEM®-T Easy specific M13-F primers generated ~2.0 kbp product in the pAJ22 control template (lane 2) but no product was generated in the complemented mutant genomic DNA templates (lane 3 and 4) that confirmed the simultaneous homologous cross over and loss of vector resulting in the integration of *Cj1136* in *C. jejuni* 01/51Δ*Cj1136* chromosome. Lane1 in all the blocks shows 1.0 kbp DNA marker (Promega).

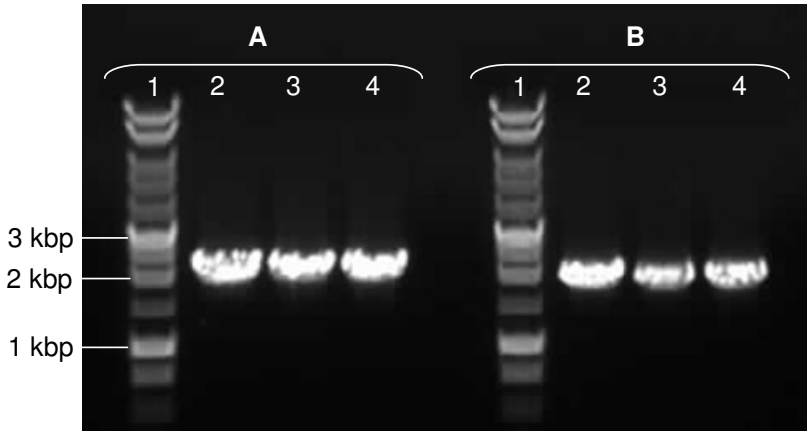

Figure 6.29 PCR confirmed the presence of mutated *Cj1136* in complemented mutant.

A: PCR using Kan-F and 1136-R primers generated a ~2.0 kbp product in 01/51Δ*Cj1136* mutant (lane 2) and both of its complemented mutants (lane 3 and 4) confirming that the mutated *Cj1136* was present in the complemented mutants. **B:** PCR with Kan-R and 1136-F primers which binds in the *Cj1136* and the kan^r cassette also generated ~1.9 kbp products in 01/51Δ*Cj1136* control template (lane 2) as well as in both the complemented mutants (lane 3 and 4) which reconfirmed that the interrupted *Cj1136* was not replaced by the wildtype copy and also confirmed that both parts of the gene flanking kan^r were present in the complemented mutants. Lane 1 in A and B blocks in the above figure were 1.0 kbp DNA markers.

6.3.5 Complementation of *Cj1245c*

Interruption of *Cj1245c*, a putative membrane protein encoding gene in *C. jejuni* 01/51 resulted in reduced invasion in its transposon insertion mutant 10H1 and the invasion-related role of *Cj1245c* observed in the transposon mutant was then reconfirmed by inactivating the gene by *kan*^{r} insertion. To verify the involvement of *Cj1245c* in *C. jejuni* virulence, mutated *Cj1245c* was complemented by expressing the wildtype allele of this gene under its presumptive native promoter and the complemented mutant was tested for the restoration of invasion.

Cj1245c was PCR amplified from 01/51 with C1245-F and C1245-R primers and high fidelity *Pfu* DNA polymerase and the *Bam*HI cut PCR products were ligated with *Bam*HI digested and dephosphorylated pAJ22. *E. coli* TOP10 was transformed with ligation products and colonies that were grown on LB agar supplemented with ampicillin and chloramphenicol were screened by PCR for the resulting plasmid pAJ22-1245. PCR with C1245-F and C1245-R primers generated a 1.8 kbp product in both the 01/51 wildtype control and plasmid extracted from *E. coli* transformed with pAJ22-1245 (Figure 6.30). PCR with C1245-F/652Seq-F and C1245-R/652Seq-F primers confirmed that the orientation of *Cj1245c* in the plasmid was directed towards *Cm*^{r}. Cloning of *Cj1245c* in plasmid pAJ22 was also confirmed by nucleotide sequencing with CML-Frev and 652Seq-F primers and the complementation vector pAJ22-1245 was used to transform and complement the mutated *Cj1245c* in *C. jejuni* 01/51Δ*Cj1245c*. Transformation of *C. jejuni* 01/51Δ*Cj1245c* with pAJ22-1245 was not successful despite many attempts as no colonies were observed on blood agar supplemented with ampicillin and chloramphenicol. Transformation was attempted by both electroporation of electrocompetent, and natural transformation of normally growing *C. jejuni* 01/51Δ*Cj1245c*. It was possible that mutation of *Cj1245c* in 01/51 might have affected its transformation efficiency or ability.

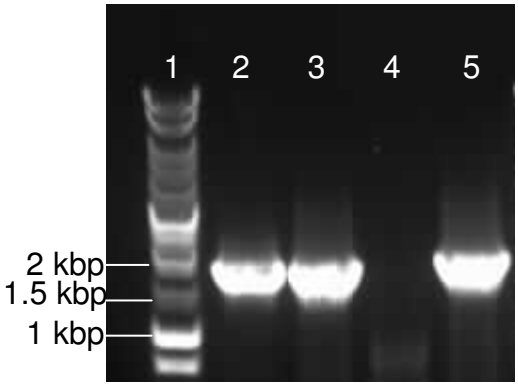

Figure 6.30 *Cj1245c* along with its presumptive promoter was cloned in pAJ22.

PCR with C1245-F and C1245-R primers generated a 1.8 kbp product in the 01/51 wildtype control (lane 2) and pAJ22-1245 (lane 3) confirming that *Cj1245c* was cloned in pAJ22. PCR with C1245-F and C652Seq-F primers generated no product in pAJ22-1245 (lane 4) but primers C1245-R and 652Seq-F generated a 1.9 kbp product in pAJ22-1245 (lane 5) that confirmed the orientation of *Cj1245c* in pAJ22-1245 was directed towards Cm^r. Lane 1 shows the 1.0 kbp DNA marker (Promega).

6.3.6 Complementation of *Cj1539c*

The reduced invasiveness observed in the 6A7 mutant due to transposon insertion in *Cj1539c* was reconfirmed by generating a targeted mutation in the gene in *C. jejuni* 01/51. The invasion-related role of *Cj1539c* was verified by complementing the gene in *C. jejuni* 01/51Δ*Cj1539c*.

Cj1539c along with its presumptive promoter was PCR amplified from 01/51 genomic DNA with C1539-F and C1539-R primers and high fidelity *Pfu* DNA polymerase (Promega). Purified PCR products were digested with *Bam*HI as the primers used to amplify *Cj1539c* had *Bam*HI restriction site their near 5'ends. Digested PCR products were ligated with *Bam*HI digested and dephosphorylated pAJ22. *E. coli* Top10 was transformed with ligation products and colonies were selected on LB agar supplemented with ampicillin and chloramphenicol. Twenty four colonies grown after overnight incubation were screened for the presence of the resulting plasmid pAJ22-1539 by colony PCR using 652Seq-F and 653Seq-F primers. Three of the colonies generated expected 2.4 kbp products (not shown) and plasmid extracted from one of them was further analysed by PCR. PCR with C1539-F and C1539-R primers generated a 1.3 kbp product that confirm that whole the gene along with promoter had been cloned; PCR with C1539-F/652Seq-F and C1539-R/652Seq-F primers confirmed that *Cj1539c* was cloned at the expected *Bam*HI cloning site and the orientation of the gene in pAJ22-1539 was directed towards the Cm^r cassette (Figure 6.31). *Cj1539c* cloning in pAJ22-

1539 was also confirmed by nucleotide sequencing with 652Seq-F and C1539-R primers. pAJ22-1539 was used to complement mutated *Cj1539c* in *C. jejuni* 01/51Δ*Cj1539c*.

C. jejuni 01/51Δ*Cj1539c* was transformed with pAJ22-1539 and a single colony grown on blood agar supplemented with kanamycin and chloramphenicol was tested by PCR for successful complementation. PCR with 652Seq-F/653Seq-F and 1539-F/1539-R primers generated 2.4 kbp and 1.3 kbp products, respectively and confirmed that *Cj1539c* was integrated at the expected place in the bacterial chromosome by homologous recombination (Figure 6.32). The complemented *C. jejuni* 01/51Δ*Cj1539c* was characterised further in gentamicin protection, growth, motility, autoagglutination and different stress assays.

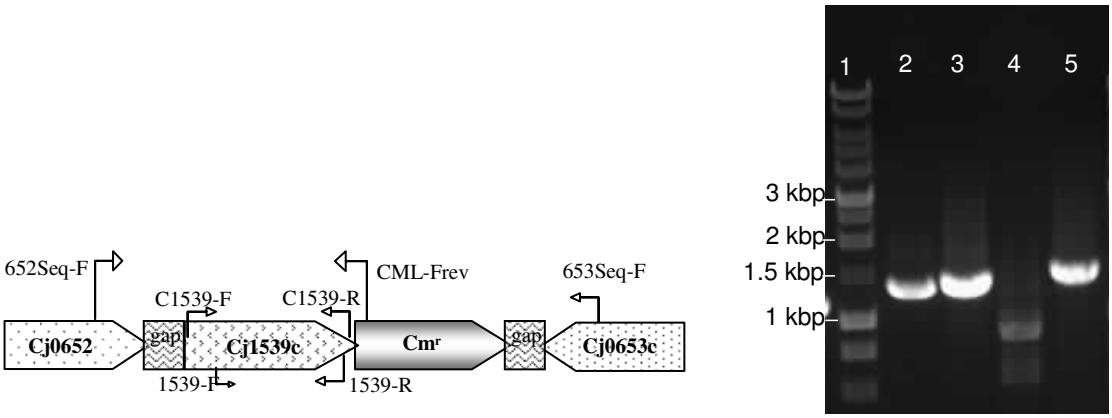

Figure 6.31 Schematic diagram and PCR confirmation of the *Cj1539c* cloning in pAJ22.

The schematic diagram (left) shows the binding positions of primers used in the analysis of *Cj1539c* complementation. The image on the right side shows the PCR with C1539-F/C1539-R primers that generated a 1.3 kbp product in both the 01/51 wildtype control (lane 2) and vector pAJ22-1539 (lane 3) that confirmed the *Cj1539c* cloning in the vector. PCR with C1539-F/652Seq-F primers did not show a band of 1.5 kbp (lane 4), a faint band of ~1 kbp was probably a single primer PCR product. PCR with C1539-R/652Seq-F showed a 1.5 kbp product (lane 5) and confirmed that orientation of *Cj1539c* in the vector was directed towards *Cm*^{r}.

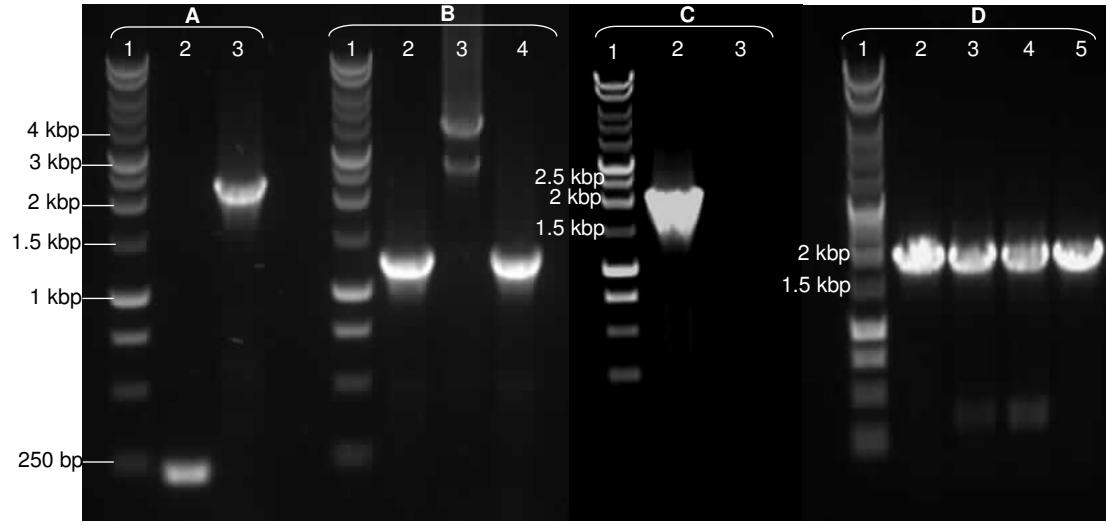

Figure 6.32 Confirmation of *Cj1539c* complementation in *C. jejuni* 01/51Δ*Cj1539c* by PCR.

A: PCR with 652Seq-F and 653Seq-F primers generated ~250 bp product in the 01/51 (lane 2) and 2.4 kbp in the complemented mutant (lane 3) that confirmed the integration of *Cj1539c* in the expected site between *Cj0652* and *Cj0653c* in the mutant's chromosome. **B:** PCR with C1539-F and C1539-R primers generated a 1.3 kbp product in 01/51 (lane 2), *Cj1539c* mutant control generated 2.7 kbp and ~4.0 kbp products (lane 3) and the complemented mutant generated 1.3 kbp product (lane 4) that reconfirmed the *Cj1539c* complementation but the expected higher size band of the mutated *Cj1539c* was not generated in the complemented mutant. **C:** PCR with pGEM®-T Easy specific M13-F and *Cj1539c* specific C1539-R primers generated a 2.3 kbp product from the pAJ22-1539 template (lane 2) but no product was generated from the complemented mutant (lane 3) that confirmed that the vector was not replicating in the complemented mutant. **D:** PCR with Kan-F/1539-F generated a 1.9 kbp product in *Cj1539c* mutant (lane 2) and the complemented mutant (lane 3) and Kan-R/1539-R primers also generated a 1.9 kbp product in mutant (lane 4) and complemented mutant (lane 5) that confirmed the presence of the mutated *Cj1539c* in the complemented mutant. Lane 1 in the above images was 1 kbp DNA markers.

6.4 Summary and conclusions

A transposon mutant library was screened for mutants showing reduced invasion and the genes mutated in thirteen selected mutants were identified. The role in invasion in *C. jejuni* 01/51 of six selected genes (*cipA*, *Cj0690c*, *Cj1136*, *Cj1245c*, *Cj1305c* and *Cj1539c*) was reconfirmed by generating insertion mutations using a kan^r cassette which does not have transcription termination sequences thus was unlikely to have polar effects. These six mutants of *C. jejuni* 01/51 were tested for adhesion and invasion in cultured Caco-2 cells using gentamicin protection assays; all the six targeted mutants showed statistically significant reduction in invasion in Caco-2 cells compared to the wildtype strain 01/51 but interestingly adhesion was variable with some of the mutants showing a higher level of adhesion as compared to the wildtype strain *C. jejuni* 01/51. Complementation of the mutated genes was attempted in five of the mutants and four of the mutated genes (*cipA*, *Cj0690c*, *Cj1136* and *Cj1539c*) were successfully

complemented. The mutants and complemented mutants were selected for adhesion and invasion in Caco-2 cells and further characterisation.

Chapter Seven

PHENOTYPIC CHARACTERISATION OF THE MUTANTS AND COMPLEMENTED MUTANTS

7 PHENOTYPIC CHARACTERISATION OF THE MUTANTS AND COMPLEMENTED MUTANTS

7.1 Introduction

Attachment to target host cells is a critical early step in the pathogenesis of many bacterial infections including *C. jejuni*, since adherent bacteria can release enzymes, toxins and trigger changes in the host cell cytoskeleton that may lead to invasion into epithelial cells (Ketley, 1997). Invasiveness in cultured eukaryotic cells has been correlated with virulence of *C. jejuni* in many previous studies (Everest et al., 1992; Fearnley et al., 2008; Newell et al., 1985). The use of Caco-2 cells as a model for studying adhesion and invasion of the intestinal epithelial cells by *C. jejuni* and *C. coli* was previously proposed (Everest et al., 1992). The gentamicin protection assay is a standard method of determining the bacterial invasion in cultured eukaryotic cells (Elsinghorst, 1994). There are different variables in the gentamicin protection assay and bacterial cells are exposed to different environmental stresses that may affect the bacterial survival in the assay. The adhesion and invasion ability of *C. jejuni* 01/51 wildtype, selected mutants and complemented mutants was tested to verify the virulence-associated role of the mutated genes that were previously identified by transposon mutagenesis. *C. jejuni* 01/51 and mutants were also tested for their growth and resistance to different stresses to establish if the reduced invasion phenotype of the mutants was related to the lack of survival of mutants in different stresses encountered during gentamicin protection assays.

Campylobacter jejuni generally colonises in the chicken gut which results in contamination of the raw poultry meat, which is considered as the major source of food-borne campylobacteriosis (Butzler, 2004; Park, 2002). The mechanisms by which *C. jejuni* initiates colonisation, either in humans or in the avian host, are poorly understood, but a putative correlation between the ability of *C. jejuni* to invade the cultured Caco-2 cells and colonisation of chicks was previously proposed (Hanel *et al.*, 2004). Such a correlation between invasion of cell cultures and colonisation ability was also found in other investigations. Defined mutants of *cia*B, which encodes for a *Campylobacter*

secreted protein required for the maximal invasion of *C. jejuni* in cultured eukaryotic cells were unable to colonise the chick gut (Ziprin *et al.*, 2001). Inactivation of an auto-transporter protein *capA* in *C. jejuni* NCTC11168 resulted in the reduced invasion of the mutant in Caco-2 cells as well as reduction in colonisation in chick gut compared to parent strain NCTC11168 (Ashgar *et al.*, 2007). Virulence association of some of the genes in *C. jejuni* 01/51 was confirmed in this study and three invasion-related genes *cipA*, *Cj1136* and *Cj1245c* were tested to determine whether mutation in these genes impaired the organism's ability to colonise chicks.

Moreover, lipooligosaccharide (LOS) of *C. jejuni* has previously been found to be important for invasion in cultured eukaryotic epithelial cells (Marsden *et al.*, 2009) and chicken intestine colonisation (Gilbert *et al.*, 2002). The *Cj1136* homologue in *C. jejuni* 01/51 was found to be important for invasion in Caco-2 cells in this study and this gene is part of the LOS biosynthesis locus (*Cj1131c – Cj1152c*) in first genome-sequenced *C. jejuni*, NCTC11168. LOS from *C. jejuni* 01/51, the *Cj1136* mutant and the complemented mutant was also analysed to determine if *Cj1136* was involved in LOS biosynthesis in 01/51, and whether an altered LOS biosynthesis pathway in the mutant led to the reduced invasion and chick colonisation phenotypes.

7.2 Gentamicin protection assay with complemented mutants

The reduced invasion phenotype observed in transposon mutants of *C. jejuni* 01/51 (Section 4.3) was reconfirmed by generating targeted mutations in the selected genes (*cipA*, *Cj0690c*, *Cj1136*, *Cj1245c*, *Cj1305c* and *Cj1539c*) that confirmed the invasion-related role of these genes (Section 6.2). The four targeted mutants (Δ*cipA*, Δ*Cj0690c*, Δ*Cj1136* and Δ*Cj1539c*) showed lowest levels of invasion in Caco-2 cells (Section 6.2) and thus were selected for complementation (Section 6.3) to verify the involvement of these genes in invasion by *C. jejuni* 01/51. The complementation of the mutated genes was attempted to rule out a polar effect of *kan*^{r} cassette insertion on the transcription of downstream genes. After complementing these genes, the *C. jejuni* 01/51 wildtype, mutants and complemented mutants were tested in the adhesion and gentamicin protection assays (Section 2.7 and 2.8) to determine if complementation of the mutated genes had restored the reduced invasion phenotype in mutants.

The complementation of all mutated genes increased adhesion to Caco-2 cells (Figure 7.1). *C. jejuni* 01/51-CML, the 01/51 wildtype strain with the Cm^r cassette integrated in the intergenic gap between *Cj0652* and *Cj0653c*, was used as a control to test if the change in adhesion levels was affected by the Cm^r cassette. *C. jejuni* 01/51-CML showed similar results to the 01/51 wildtype confirming that the increase in adhesion phenotype was due to complementation of the mutated gene and that Cm^r insertion had no effect on the adhesion phenotype. The change in adhesion to Caco-2 of mutants in *cipA*, *Cj0690c* (mutant 6A9), *Cj1136* as well as complementation of these mutated genes was not statistically significant. Reduction in adhesion of the *Cj1539c* mutant compared to parent strain 01/51 remained statistically significant ($P < 0.001$) as was observed in the previous assays (Section 6.2) and the increase in adhesion by complementation of the mutated *Cj1539c* significantly restored adhesion ($P < 0.05$). However, the level of restoration was far below the parent strain 01/51 which might be due to low levels of expression of *Cj1539c* in the complemented mutant compared to wildtype strain. *C. jejuni* 81116Δ*flaA/B* mutant (Wassenaar *et al.*, 1991) was used as negative control in the adhesion assays and it showed very low levels of adhesion as was expected.

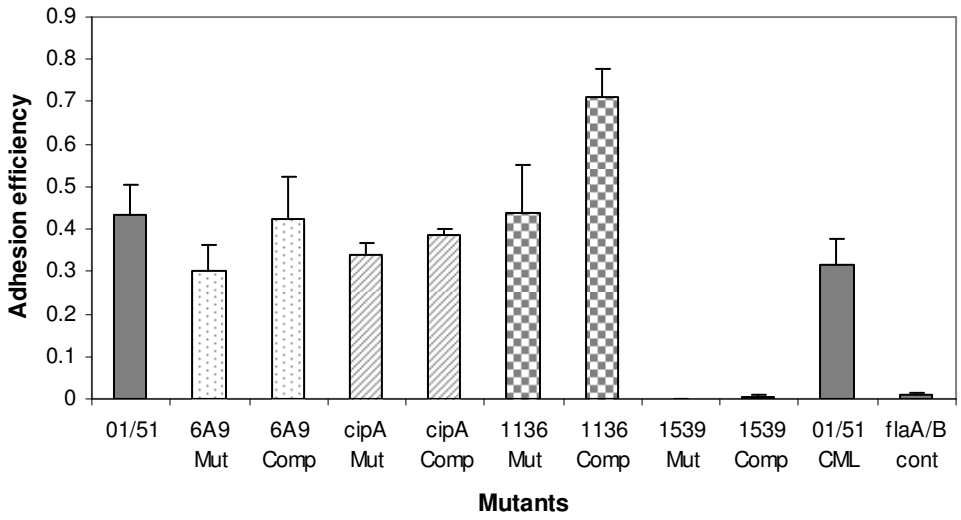

Figure 7.1 Adhesion of *C. jejuni* 01/51, mutants and complemented mutants to Caco-2 cells.

Adhesion of *C. jejuni* 01/51 wildtype (01/51) and *Cj0690c* transposon insertion mutant (6A9 Mut), complemented mutant 6A9 (6A9 Comp), targeted *cipA* mutant (*cipA* Mut), complemented *cipA* mutant (*cipA* Comp), targeted *Cj1136* mutant (1136 Mut), complemented *Cj1136* mutant (1136 Comp), targeted *Cj1539c* mutant (1539 Mut), complemented *Cj1539c* mutant (1539 Comp), *C. jejuni* 01/51 with Cm^r inserted in the intergenic gap between *Cj0652* and *Cj0653c* (01/51CML) and a double mutant of *C. jejuni* 81116 in *flaA* and *flaB* (flaA/B cont) was tested in Caco-2 cells. Complemented mutants showed higher levels of adhesion than their respective mutants. 01/51CML was used as a control in the adhesion assay that confirmed that integration of Cm^r in the 01/51 chromosome had no affect on the adhesion potential. *C. jejuni* 81116Δ*fla*A/B was also used as negative control in the assay and it showed very low adhesion that confirmed the validity of the adhesion assay.

Complementation of the mutated genes in *C. jejuni* 01/51 partially restored the invasion phenotype in the complemented mutants (Figure 7.2). The levels of invasion restored in the complemented mutants were statistically significant as compared to their respective mutants but the invasion phenotype was not fully restored as compared to the 01/51 wildtype strain. This might be due to lower levels of expression of the complemented genes in the complemented mutants. *C. jejuni* 01/51-CML had a similar invasion efficiency to the 01/51 wildtype which confirmed that the change in invasion levels observed in the complemented mutants was not due to Cm^r cassette insertion but was due to complementation of the mutated genes. A non-invasive *C. jejuni* 81116Δ*flaA/B* was used as control in the gentamicin protection assay and it showed very low levels of invasion that confirmed the validity of the assay.

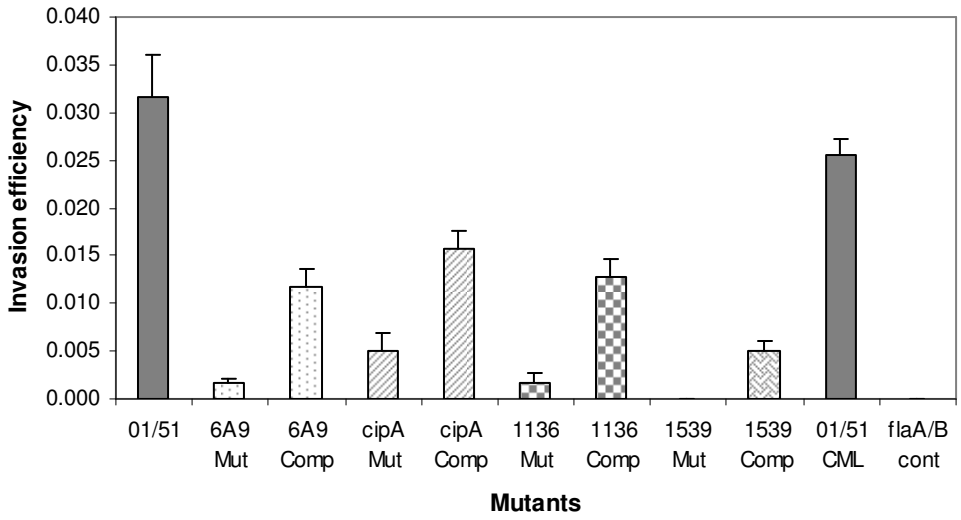

Figure 7.2 Invasion of *C. jejuni* 01/51, mutants and complemented mutants to Caco-2 cells.

The invasion phenotype of *C. jejuni* 01/51 wildtype strain (01/51) and *Cj0690c* transposon insertion mutant (6A9 Mut), complemented mutant 6A9 (6A9 Comp), targeted *cipA* mutant (cipA Mut), complemented *cipA* mutant (cipA Comp), targeted *Cj1136* mutant (1136 Mut), complemented *Cj1136* mutant (1136 Comp), targeted *Cj1539c* mutant (1539 Mut), complemented *Cj1539c* mutant (1539 Comp), *C. jejuni* 01/51 with Cm^r inserted in intergenic gap between *Cj0652* and *Cj0653c* (01/51 CML) and a double mutant of *C. jejuni* 81116 in *fla*A and *fla*B (flaA/B cont) was tested in Caco-2 cells. Complemented mutants showed higher levels of invasion than their respective mutants. 01/51 CML was used as control in the gentamicin protection assay that confirmed the invasion potential was not affected by the integration of Cm^r in 01/51 chromosome. *C. jejuni* 81116Δ*fla*A/B was also used as negative control in the assay and it showed very low level of invasion that confirmed the validity of the gentamicin protection assay.

Both adherence and invasion are multifactorial and motility-dependent processes (Wassenaar *et al.*, 1991) but a strong correlation between them has not been established. A previous study found that all investigated *C. jejuni* strains were able to adhere to Caco-2 cells but some strains were deficient in invasion (Hanel *et al.*, 2004). Adherence is most likely a prerequisite for invasion of epithelial cells by any bacterial pathogen. However, the pathogen may bind to an epithelial cell membrane without subsequent internalization and the number of adherent bacteria does not correlate with the number of intracellular bacteria (Hanel *et al.*, 2004). Non-viable and metabolically inactivated campylobacters were still able to adhere to host cells (Konkel and Cieplak, 1992) while *Campylobacter* invasion did require nascent protein synthesis (Konkel and Cieplak, 1992; Oelschlaeger *et al.*, 1993). This means that the factors required for adhesion might be different from those needed for invasion. A gene involved in invasion may not necessarily be important for adhesion as well. This study showed that mutation in *cipA*, *Cj0690c*, *Cj1136*, *Cj1245c* and *Cj1305c* resulted in reduced invasion in cultured human

intestinal epithelial cells without significant reduction in adhesion potential but *Cj1539c* was found to be involved in both adhesion and invasion since inactivation of *Cj1539c* reduced adhesion and invasion in this mutant.

7.3 Further phenotypic characterisation of the mutants

Further studies of the *C. jejuni* 01/51 wildtype, mutants and complemented mutants were done to test if the observed changes in invasion potential in the mutants were due to differences in their atmospheric oxygen tolerance, intracellular survival, sensitivity to Triton X-100, growth rates or motility.

7.3.1 Methods for phenotypic characterisation

7.3.1.1 Atmospheric stress assay

During gentamicin protection assays, bacterial cells have to go through different atmospheric conditions including 5% CO_2 and air. Atmospheric stress assays were performed to check whether the change in invasiveness in cultured eukaryotic cells shown by mutants and complemented mutants of *C. jejuni* 01/51 was due to a difference in their survival under atmospheric oxygen concentration. For the assay, bacterial growth was harvested from blood agar plates as above, and suspended in 2.5 ml sterile PBS. The bacterial suspension was inoculated into pre-warmed complete cell culture media (Eagle's minimal essential medium supplemented with 10% foetal calf serum and 1% non-essential amino acids) to an optical density (570 nm) of 0.1 and this suspension was incubated at 37^{o}C in the cell culture incubator supplied with 5% CO_2. Samples were taken at 0, 1, 2, 3, 4 and 5 hour time points and serial dilutions were plated out for viable cell count. The assay was carried out in duplicate.

7.3.1.2 Hydrogen peroxide sensitivity assay

The toxic reactive oxygen species including hydrogen peroxide are released in the eukaryotic cells as a result of oxidative metabolism. *C. jejuni* 01/51, selected mutants and complemented mutants were also tested for hydrogen peroxide sensitivity to check

if the change in hydrogen peroxide sensitivity was responsible for the observed changed in their interaction with INT-407 and Caco-2 cells. For the hydrogen peroxide sensitivity assay, bacterial growth was harvested from blood agar plates as above and suspended in 2.5 ml sterile PBS. The bacterial cells were resuspended in 1 ml of pre-warmed Mueller-Hinton (MH) broth (Merck, UK) to an optical density (570 nm) of 0.1 in a 24-well cell culture plate. The plate was placed in an airtight plastic container with Campygen pack and incubated at 37^{o}C on a shaker with 150 rpm agitation. After taking a zero hour sample for viable count, hydrogen peroxide was added to a final concentration of 0.5 mM (calculation given below) to each well. The plate was again placed on the shaker with agitation at 150 rpm under microaerobic conditions and further samples were taken at 0.5, 1, 1.5, 2 and 3 hours for serial dilutions and viable cell count. The assay was carried out in duplicate.

Calculation of H_2O_2 concentration for the assay:

Molarity of the 30% hydrogen peroxide solution (Sigma, UK) was calculated using the formula

$$\text{Molarity} = \frac{\% \times \text{density} \times 1000}{100 \times \text{Mol. Weight}}$$

Molarity of 30% H_2O_2 was 9.7 M.

A fresh stock of hydrogen peroxide was prepared every time by diluting 30% H_2O_2 1000-fold in water (10 µl of 30% H_2O_2 in 10 ml water made it 9.7 mM solution) and for making 0.5 mM H_2O_2, 52 µl of H_2O_2 stock solution was diluted in 950 µl of MH broth.

7.3.1.3 Survival in Triton X-100

The Triton X-100 (1% v/v) was used in gentamicin protection assays to release internalised bacteria from the cultured epithelial cells. *C. jejuni* 01/51, selected mutants and complemented mutants were also tested for Triton X-100 (1% v/v) sensitivity to check if the change in sensitivity to the detergent was responsible for the observed changed in their interaction with INT-407 and Caco-2 cells. For the Triton X-100 sensitivity assay, bacterial growth was harvested from blood agar plates as above and suspended in 2.5 ml sterile PBS. The bacterial cells were resuspended in 1 ml of distilled water to an optical density (570 nm) of 0.1 in 24-well plate. The sterile Triton

X-100 (10 µl) was added to each well and after taking a zero hour sample for viable count, the bacterial cell suspension was incubated at room temperature and further samples were taken after 30 minutes and 1 hour incubation for serial dilutions and viable cell count. The assay was carried out in triplicate.

7.3.1.4 Measurement of bacterial growth by spectrophotometer

To check if the reduction in the levels of invasion in *C. jejuni* mutants was due to a change in their growth as compared to parent strain *C. jejuni* 01/51, growth of the mutants was measured overtime by a spectrophotometric method in a 96-well plate. For the assay bacterial strains were grown for 48 hours on blood agar with appropriate antibiotics added where needed. The bacterial growth was harvested from the plates and resuspended in 2.5 ml sterile PBS. An appropriate volume of this suspension was added to Mueller-Hinton (MH) broth in a 96-well plate to make a final volume of 100 µl in a well and an absorbance of ~ 0.1 (OD 570nm). The plate was then placed in an air-tight container with a Campygen gas pack (Oxoid, UK) and incubated on a shaker at 37^{o}C and 150 rpm agitation. Absorbance was read at 570 nm using an ELISA plate reader. Regular absorbance readings were taken at 1, 2, 3, 4, 5, 6, 7, 8, 23, 26, 28 and 30 hrs. Each mutant was tested in triplicate and the mean of these values was plotted.

7.3.1.5 Motility assay

The *C. jejuni* 01/51 and its mutants were tested for their motility to check if a reduction in motility was responsible for the low invasion. Motility of the bacterial strains was tested as described earlier (Section 4.2.2). The assay was carried out in triplicate and the mean of these values was plotted.

7.3.1.6 Autoagglutination assay

The autoagglutination assay was performed following the protocol described elsewhere (Golden and Acheson, 2002). Briefly, the growth of each strain was harvested from blood agar plates as above and suspended in 2.5 ml sterile phosphate buffered saline (PBS). The absorbance of the solution was adjusted to ~1.0 at 600 nm in PBS and the

actual absorbance was measured again. The bacterial suspension (2.0 ml) was transferred into sterile bijoux tubes with caps and left standing undisturbed at 37^{o}C in microaerobic conditions for 24 hrs. One millilitre of the upper aqueous phase formed was then taken gently with a micropipette and the absorbance was again measured at 600 nm. The level of autoagglutination was calculated by subtracting the absorbance shown by the upper aqueous phase collected after 24 hour incubation from the initial absorbance measured at the start of incubation. The assay was carried out in triplicate.

7.3.1.7 Chick colonisation

The chick colonisation experiment with *C. jejuni* 01/51 and its mutants in *cipA*, *Cj1136* and *Cj1245c* were carried out at the Veterinary Laboratories Agency, Weybridge, United Kingdom. The chick colonisation assay was performed using a quantitative oral chick-colonisation model as previously described (Wassenaar *et al.*, 1993). Briefly, groups of 10 specific pathogen-free chicks used in this study, hatched from eggs obtained from Charles River SPAFAS Inc., Hanover, Germany, were housed in isolators and were dosed at day 1 of age. Two doses of each of *C. jejuni* 01/51 wildtype, *C. jejuni* 01/51Δ*cipA*, *C. jejuni* 01/51Δ*Cj1136* and *C. jejuni* 01/51Δ*Cj1245c* at 3.4 ± 0.9 x 10^4 and 3.4 ± 0.9 x 10^6 CFU in 100 µl 0.1M PBS pH 7.2 were given by oral gavage. Doses were prepared by harvesting bacteria grown overnight on blood agar plates into sterile PBS and the actual doses were determined retrospectively by viable count. At 5 days post-inoculation the chicks were sacrificed by cervical dislocation and the levels of colonisation into the caeca were determined by plating out dilutions of caecal contents onto blood agar plates that were supplemented with kanamycin (50 µg/ml) in the case of the mutants. The degree of chick colonisation was determined as CFU per gram of caecal contents, for individual birds. The minimal level of detection was 100 CFU/gram caecal contents. The chick colonisation assays were performed in accordance with the local ethics committee and UK home office licence guidelines. Statistical analysis of the data was carried out using Minitab software and the data was analysed by using nonparametric Mann-Whitney (two-tailed) test to assess statistical significance and a statistical P value of <0.05 was statistically significant.

7.3.1.8 Lipooligosaccharide analysis

7.3.1.8.1 Extraction of lipooligosaccharide by phenol-water extraction method

Lipooligosaccharide was extracted from *C. jejuni* 01/51, *C. jejuni* 01/51Δ*Cj1136* and *C. jejuni* 01/51Δ*Cj1136*:*Cj1136* using a modification of the phenol-water extraction procedure described elsewhere (McNally *et al.*, 2007; Prendergast *et al.*, 2001). Briefly, confluent bacterial growth from blood agar plates was washed three times in sterile PBS (Sigma, UK). After the last wash cells were resuspended in sterile distilled water to an optical density (600 nm) of 1.0. One millilitre of the bacterial cell suspension was added to an equivalent volume of phenol (pre-heated to 65^{o}C; Sigma, UK) in an eppendorf tube and mixed for one minute using a vortex mixer. Then samples were incubated at 65^{o}C for 10 minutes, during which the samples were mixed at regular intervals. After cooling on ice, the samples were centrifuged (12000 X g for 3 min). At this stage separated layers were visible and the upper aqueous layer containing LOS was removed to a new tube. The LOS preparation was purified by adding 0.1 x vols of 20% sodium acetate (pH 4.5) plus 5 x vols of absolute alcohol and LOS was allowed to precipitate at 4^{o}C overnight. Precipitated LOS was harvested by centrifugation (5000 x g) for 10 minutes and the pellet was washed with ice-cold 70% ethanol. Dried LOS was resuspended in 500 µl distilled water.

7.3.1.8.2 Sodium dodecyl sulphate polyacrylamide electrophoresis

The LOS extracts were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). LOS samples (20 µl) were mixed with an equivalent volume of 2x sample loading buffer (100 mM Tris-HCl [pH 8.0], 2% β-mercaptoethanol, 4% SDS, 0.02% bromophenol blue, 20% glycerol) and mixtures were boiled for 10 minutes before loading (20 µl) onto an SDS-PAGE gel consisting of a stacking gel of 4% (v/v) acrylamide and a separating gel of 15% (v/v) acrylamide; composition of the gels is shown in Table 7.1. The gel was run in SDS-Tris-glycine buffer (0.025 M Tris base, 0.192 M glycine and 0.1% SDS, pH 8.3) at 200 V constant and 30 mAmp per gel until the dye front reached the bottom of the gel (BIO-RAD, Protean II system).

Table 7.1 Components for preparation of SDS-PAGE gels.

Reagents	Volume for stacking gel (4%)	Volume for separating gel (15%)
Acrylamide/bis-acrylamide 30%, 29:1	650 µl	5 ml
Sodium dodecyl sulphate 10% (w/v)	50 µl	100 µl
1.5M Tris-HCl (pH 8.8)	-	2.5 ml
0.5M Tris-HCl (pH 6.8)	1.25 ml	-
Distilled water	3.0 ml	2.5 ml
Ammonium persulphate 10% (w/v)	50 µl	50 µl
Tetramethylethylenediamine (TEMED)	5 µl	10 µl

7.3.1.8.3 Silver staining of lipooligosaccharide SDS-PAGE gel

The LOS SDS-PAGE gel was stained by a sensitive LPS silver staining method (Tsai and Frasch, 1982). The silver staining was done in glass trays that were soaked in 2% (v/v) nitric acid overnight and were washed with plenty of distilled water before use. After running, the gel was washed once with fixative solution (40% isopropanol and 5% glacial acetic acid) then was fixed overnight in the fixative solution. The LOS was oxidised by soaking the gel in periodic acid solution (0.7% periodic acid, 40% isopropanol and 5% acetic acid) for 5 minutes. Then the gel was washed with distilled water on an orbital shaker for two hours and the water was changed every ten minutes. The gel was stained by shaking for 10 minutes in a freshly prepared staining solution (2 ml concentrated ammonium hydroxide [fresh frozen stock], 28 ml 0.1 N NaOH, 115 ml distilled water and 5 ml of 20% silver nitrate [freshly prepared silver nitrate solution was added drop-wise to the above with vigorous stirring and the staining was done in the dark). After staining, the gel was washed 3 times in distilled water (15 min/wash). Then the gel was transferred to a new tray and pre-warmed (37^{o}C) developer (citric acid 10 mg, 30% formaldehyde 0.1 ml and distilled water 200 ml) was added. The tray was agitated by hand for few minutes until the LOS bands became visible, then it was washed with distilled water and the staining reaction was stopped by adding 0.35% acetic acid.

7.3.2 Results and discussion of phenotypic characterisation of mutants

The selected mutants and complemented mutants of *C. jejuni* 01/51 were characterised further to check whether the change in invasiveness was due to other factors that can potentially affect invasion including atmospheric stress, survival in hydrogen peroxide and Triton X-100, growth and motility. Selected mutants were also tested for their chick gut colonisation ability and role in LOS biosynthesis.

7.3.2.1 Atmospheric stress

C. jejuni has to go through a diversity of atmospheric conditions during the gentamicin protection assay. The bacterial cell survival and growth was determined under the conditions encountered in the gentamicin protection assay to assess the effects of these diverse environmental conditions on the invasion potential of *C. jejuni* 01/51 and its mutants in genes *Cj0690c* (6A9), *Cj1245c*, *cipA* (*Cj0685c*), *Cj1136* and *Cj1539c*. The number of wildtype cells recovered over the course of 5 hours was comparable to those of the initial number of cells tested (0 hours) and mutants showed survival very similar to parent strain 01/51 (Figure 7.3). This suggested that the survival rate under atmospheric conditions was similar for the parent strain and its tested mutants. It also confirmed that the cell numbers of *C. jejuni* 01/51 and its tested mutants did not significantly change by growth or death in the complete cell culture media over the 5 hours time period. A posttranslational regulator *csrA* was found to be important for oxidative stress resistance and virulence of *C. jejuni* (Fields and Thompson, 2008) and *H. pylori* (Barnard *et al.*, 2004). The invasion-related genes in *C. jejuni* 01/51 that were tested in this stress assay do not seem important for the atmospheric stress in this bacterium.

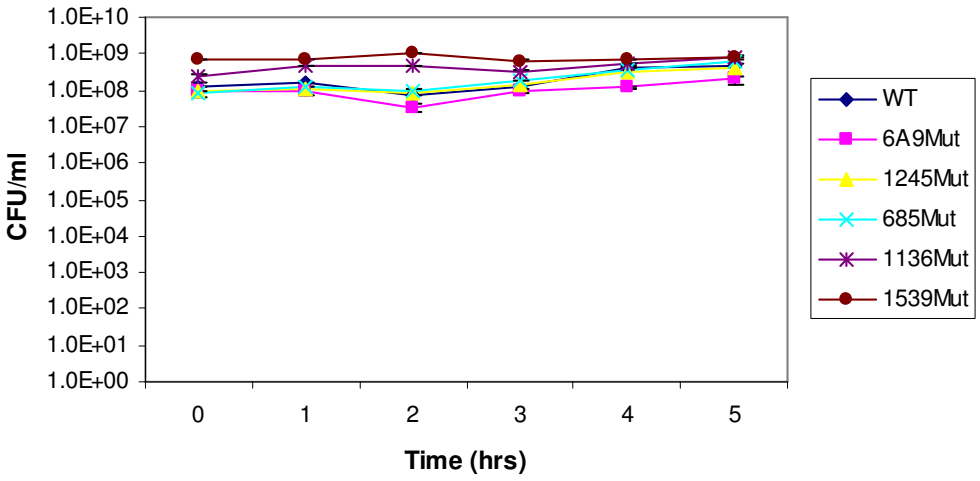

Figure 7.3 Atmospheric stress in *C. jejuni* 01/51 and mutants.

Effect of atmospheric stress on *C. jejuni* 01/51 wildtype (01/51), mutants: transposon insertion mutant in *Cj0690c* (6A9 Mut), targeted *Cj1245c* mutant (1245 Mut), targeted *cipA* mutant (685 Mut), targeted *Cj1136* mutant (1136 Mut) and targeted *Cj1539c* mutant (1539 Mut) was determined by incubating the bacteria in complete cell culture media in 5% CO_2 at 37°C and determining the viable bacterial cell number (CFU/ml). There was no significant effect of atmospheric stress on the survival of *C. jejuni* 01/51 wildtype and its tested mutants.

7.3.2.2 Hydrogen peroxide sensitivity

Intracellular (internalised) bacteria may be exposed to hydrogen peroxide (H_2O_2) which is a by-product of oxidative metabolism in aerobic organisms (Alvarez-Peral *et al.*, 2002). Hydrogen peroxide resistance in *C. jejuni* may play a role in the intracellular survival of the bacteria after invading the host cells. Mutation of γ-glutamyl transpeptidase in *C. jejuni* strain 81116 resulted in increased H_2O_2 resistance and higher invasion of INT-407 cells by the mutant compared to its wildtype strain (Barnes *et al.*, 2007). Resistance to hydrogen peroxide was tested in *C. jejuni* 01/51 and its mutants in *Cj0690c* (mutant 6A9), *Cj1245c*, *cipA* (*Cj0685c*), *Cj1136* and *Cj1539c* to establish if reduced intracellular survival of the mutants was responsible for the reduced invasion phenotype shown by the mutants in this study. Resistance to hydrogen peroxide in *C. jejuni* 01/51 and its mutants was tested in 0.5 mM H_2O_2 solution in MH broth and bacterial cell numbers were determined every 30 minutes for three hours. The *C. jejuni* 01/51 wildtype strain and its mutants showed similar levels of resistance to H_2O_2 (Figure 7.4) confirming that the reduction in invasion in cultured human epithelial cells shown by the mutants was not due to an increased sensitivity to H_2O_2 thus reducing intracellular survival of the mutants.

Some studies suggested that *C. jejuni* can survive within eukaryotic cells for several days (Babakhani and Joens, 1993; Black *et al.*, 1988). Inside host cells, organisms may be exposed to a variety of host killing mechanisms, including reactive oxygen species like hydrogen peroxide (Alvarez-Peral *et al.*, 2002). To circumvent the harmful reactive oxygen species, like many organisms *C. jejuni* has evolved specific defence mechanisms including the ability to remain bound in an endosome inside the cell (De Melo *et al.*, 1989) and/or synthesise protective enzymes like catalase encoded by *katA* (Day *et al.*, 2000), alkyl hydroperoxide reductase encoded by *ahpC*, superoxide dismutase encoded by *sodB* (Iovine, 2008) and cytochrome C peroxidase (Bingham-Ramos and Hendrixson, 2008). Therefore resisting hydrogen peroxide stress and subsequently interrupting the formation of the toxic intermediate products may allow improved persistence and survival of *C. jejuni* within eukaryotic cells. The genes encoding for catalase (*kat*A) and cytochrome c peroxidase are present in C. *jejuni* (Bingham-Ramos and Hendrixson, 2008; Day *et al.*, 2000; Parkhill *et al.*, 2000; Purdy and Park, 1994) and intracellular survival of a *C. jejuni* mutant in *kat*A was reduced compared to parent strain (Day *et al.*, 2000). Catalase activity in *C. jejuni* 01/51 and its mutants was also tested by dipping a part of bacterial colony with a plastic loop into 30% H_2O_2 and the appearance of bubbles indicated catalase activity. The wildtype strain 01/51 and mutants were all positive for catalase activity so they were less likely to be sensitive to 0.5 mM hydrogen peroxide which was supported by the results (Figure 7.4).

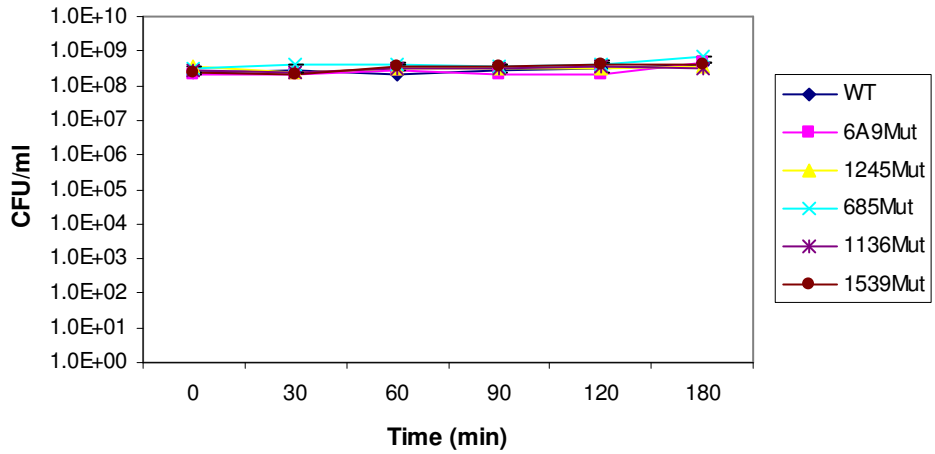

Figure 7.4 Sensitivity of *C. jejuni* 01/51 and mutants to hydrogen peroxide.

C. jejuni 01/51 wildtype (01/51) and mutants: transposon insertion mutant in *Cj0690c* (6A9 Mut), targeted *Cj1245c* mutant (1245 Mut), targeted *cipA* mutant (685 Mut), targeted *Cj1136* mutant (1136 Mut) and targeted *Cj1539c* mutant (1539 Mut) were tested for their sensitivity to H_2O_2 by incubating the bacteria in 0.5 mM H_2O_2 and determining the viable bacterial cell count (CFU/ml) at different time points. *C. jejuni* 01/51 wildtype and its tested mutants showed similar levels of resistance to 0.5 mM H_2O_2.

7.3.2.3 Survival in 1% Triton X-100

Triton X-100 (4-octylphenol polyethoxylate) is a non-ionic detergent which has a hydrophilic polyethylene oxide group and a hydrocarbon lipophilic group. Though Triton X-100 has no antimicrobial properties (Sigma, product information guide), commercial preparations of Triton X-100 have been found to contain peroxides up to 0.22% hydrogen peroxide equivalents (Ashani and Catravas, 1980) which may interfere with biological reactions. Moreover, Triton X-100 can solubilise lipids and proteins, and the membrane of Gram-negative bacteria is made up of lipoproteins that can be affected by the detergent. Sensitivity of *C. jejuni* to sodium deoxycholate and Triton X-100 was increased in *cbrR*, a response regulator, mutant of *C. jejuni* strain F38011 (Raphael *et al.*, 2005). Triton X-100 (1% v/v) was used in the gentamicin protection assays in this study to lyse eukaryotic cells and release internalised *C. jejuni.* Therefore the *C. jejuni* 01/51 wildtype and its mutants in *Cj0690c*, *Cj1245c*, *Cj0685c*, *Cj1136* and *Cj1539c* were tested for survival in Triton X-100 (1% v/v) to determine if mutation in the genes rendered them sensitive to Triton X-100 and the observed reduction in invasion in Caco-2 cells observed in mutants was due to their inability to survive in the detergent.

C. jejuni 01/51 and its mutants were suspended in 1% Triton X-100 and incubated for one hour at room temperature. The viable count (CFU/ml) was determined at the start, 30 minutes and 60 minutes of incubation. There was no significant reduction in the bacterial cell numbers in first 30 minutes in wildtype and all the mutants but the *Cj1539c* mutant showed a one log reduction in viable cell numbers in second half of the one hour incubation (Figure 7.5). This increased sensitivity of *C. jejuni* 01/51Δ*Cj1539c* to Triton X-100 (1%, v/v) is unlikely to have an effect on their invasiveness in Caco-2 cells determined by the gentamicin protection assay because generally bacteria are diluted and plated out within 30 minutes after eukaryotic cell lysis in the detergent, during which period the survival of *Cj1539c* mutant was not affected. The translational product of *Cj1539c*, a putative anion-uptake ABC-transport system permease protein, has five transmembrane helices and is expected to be a membrane protein. It is possible that the inactivation of this protein might have reduced the bacterial cell membrane permeability allowing Triton X-100 to enter the cell and cause cell lysis; however, this process seems very slow as there was no reduction in bacterial cell numbers in first half an hour of challenge with the detergent and there was only one log reduction after one hour incubation in 1% (v/v) Triton X-100.

A previous study found that *C. jejuni* 81-176 mutants in putative glycosyltransferases encoded by *waaF, lgtF* and *galT* had reduced INT-407 cells invasion potential as compared to wildtype strain, and this reduction in invasiveness was due to increased sensitivity to Triton X-100 used for lysis of the cultured cell monolayer. When invasion capability of the mutants was determined using a modified invasion assay in which the monolayer was lysed with water rather than detergent, there was no statistically significant difference in the invasion levels of wild-type strain 81-176 and mutants (Kanipes *et al.*, 2008). The mutant in a putative glycosyltransferase encoded by *Cj1136* did not show higher levels of sensitivity to the detergent used in this study that confirmed the reduction in invasion levels exhibited by the mutant was not due to killing of the mutant cell in 1% Triton X-100.

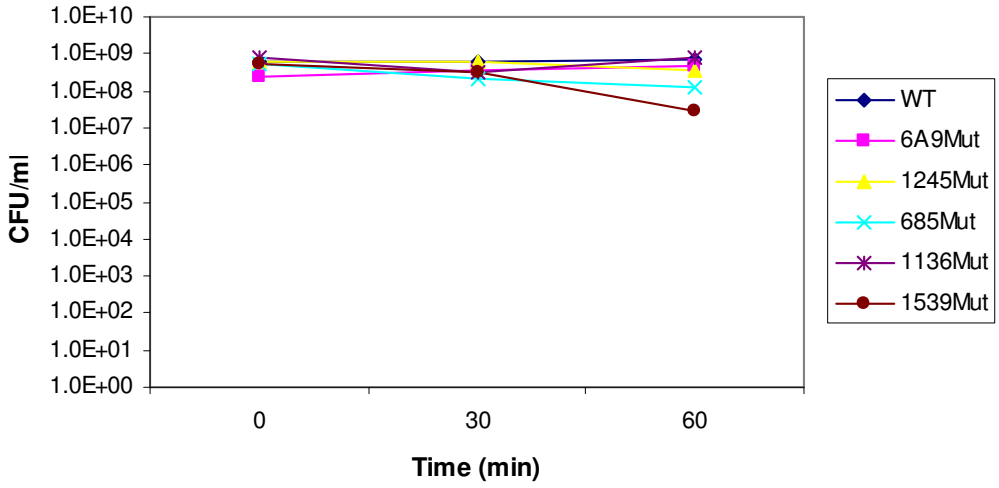

Figure 7.5 Survival of *C. jejuni* 01/51 and mutants in Triton X-100.

Survival of *C. jejuni* 01/51 wildtype (WT), *Cj0690c* transposon insertion mutant (6A9Mut), targeted *Cj1245c* mutant (1245Mut), targeted *cipA* mutant (685Mut), targeted *Cj1136* mutant (1136Mut) and targeted *Cj1539c* mutant (1539Mut) was determined in Triton X-100 (1%, v/v) by viable cell count (CFU/ml). There was not a decrease in bacterial cell numbers in first 30 minutes but *Cj1539c* mutant showed a log decrease in viable cells between 30 and 60 minutes incubation in the detergent.

7.3.2.4 Growth curves of mutants and complemented mutants

Growth curves of *C. jejuni* 01/51 wildtype, its mutants in *Cj0690c* (6A9Mut), *cipA* (*Cj0685c*), *Cj1136*, *Cj1245c* and *Cj1539c*, and the complemented mutants of *Cj0690c*, *Cj0685c*, *Cj1136* and *Cj1539c* were carried out to check if the change in their growth was responsible for the observed changes in their invasion phenotype. Growth was tested in Mueller-Hinton (MH) broth by measuring optical density at different time intervals. The wildtype strain 01/51 and tested mutants showed similar growth curves (Figure 7.6) though lag phase in mutants of *Cj0685c*, *Cj1539c* and *Cj0690c* was slightly longer than *C. jejuni* 01/51 wildtype. This suggested that the mutation of *Cj0685c*, *Cj0690c*, *Cj1136*, *Cj1245c* or *Cj1539c* in *C. jejuni* 01/51 did not have a detrimental effect on growth. Growth of complemented mutants of *Cj0685c*, *Cj0690c*, *Cj1136* and *Cj1539c* was also determined and they showed similar results to wildtype strain (not shown).

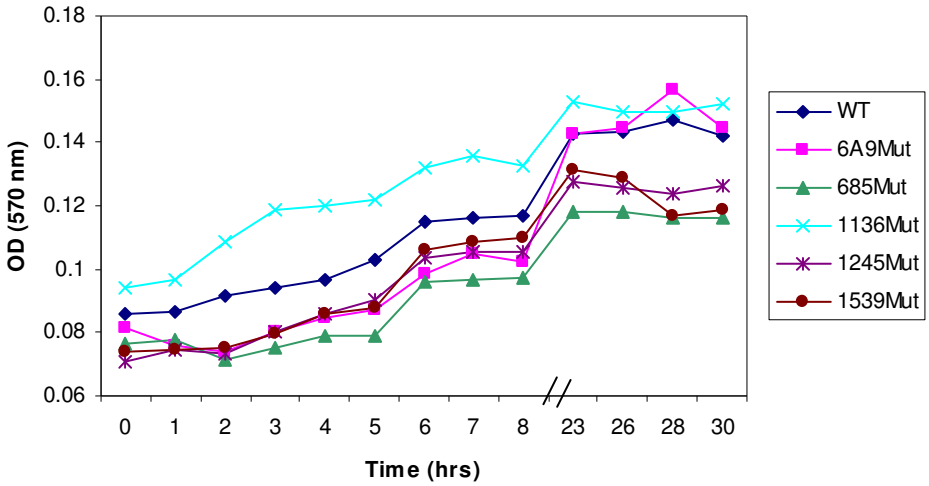

Figure 7.6 Growth curves of *C. jejuni* 01/51 and its mutants.

Growth curves of *C. jejuni* 01/51 and its mutants in invasion-related genes were determined in MH broth by measuring optical density (OD) at different time intervals. 01/51 Wildtype strain and mutants showed similar growth curves. Legends: WT: *C. jejuni* 01/51 wildtype; 6A9Mut: transposon insertion mutant in *Cj0690c*; 685Mut: targeted mutant in *cipA* (*Cj0685c*); 1136Mut: targeted mutant in *Cj1136*; 1245Mut: targeted mutant in *Cj1245c*; 1539Mut: targeted mutant in *Cj1539c*.

7.3.2.5 Motility

Motility in *C. jejuni* and other enteric pathogens has been implicated in intestinal colonisation and invasion by several classical studies (Schmitt *et al.*, 1994; Wassenaar *et al.*, 1993; Yao *et al.*, 1994; Young *et al.*, 2000a). Motility of the *C. jejuni* 01/51 wildtype strain and transposon mutants was measured earlier and those mutants selected for transposon insertion site localisation, were having at least 75% motility as compared to the wildtype strain (Section 4.3). The motility of the mutants in *cipA*, *Cj0690c* (mutant 6A9), *Cj1136*, *Cj1245c*, *Cj1305c* and *Cj1539c*, and complemented mutants was tested to re-confirm the previous findings but with targeted mutants. All the tested mutants and complemented mutants showed motility similar to the wildtype strain 01/51 confirming that a change in the invasion phenotype in these mutants and complemented mutants was not due to a change in motility (Figure 7.7).

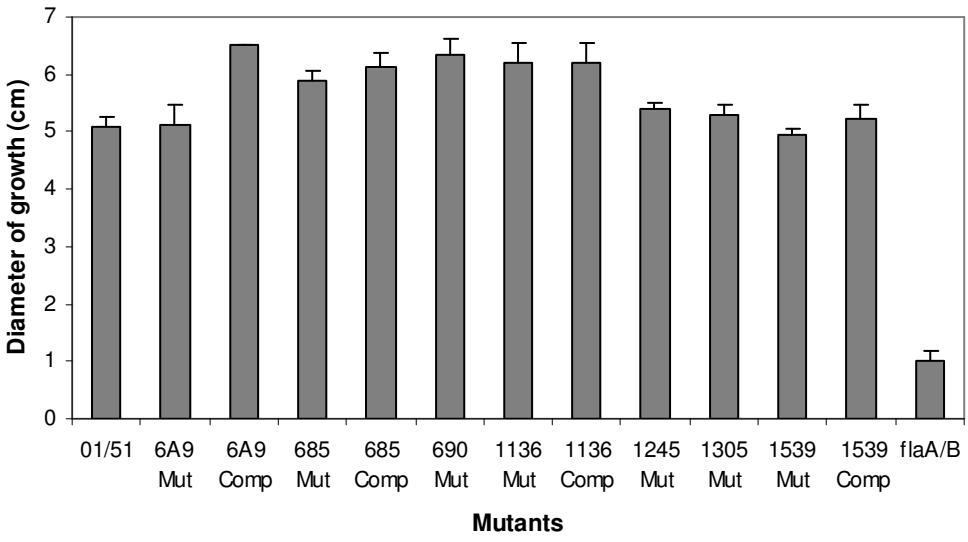

Figure 7.7 Motility of *C. jejuni* 01/51, mutants and complemented mutants.

Motility of *C. jejuni* 01/51 wildtype (01/51), mutants: transposon insertion mutant in *Cj0690c* (6A9 Mut), complemented mutant 6A9 (6A9 Comp), targeted *cipA* mutant (685 Mut), complemented *cipA* mutant (685 Comp), targeted *Cj0690c* mutant (690 Mut), targeted *Cj1136* mutant (1136 Mut), complemented *Cj1136* mutant (1136 Comp), targeted *Cj1245c* mutant (1245 Mut), targeted *Cj1305c* mutant (1305 Mut), targeted *Cj1539c* mutant (1539 Mut), complemented *Cj1539c* mutant (1539 Comp) and a non-motile *C. jejuni* 81116Δ*flaA/B* (flaA/B) control was determined in MH broth supplemented with 0.4% agar. All the *C. jejuni* 01/51 mutants and complemented mutants showed a level of motility similar to the 01/51 wildtype. *C. jejuni* 81116Δ*flaA/B* was used as a negative control in the test that showed very low levels of motility.

7.3.2.6 Autoagglutination

The ability of *C. jejuni* to autoagglutinate has been associated with changes in invasion and/or adherence (Golden and Acheson, 2002; Misawa and Blaser, 2000) and *C. jejuni* 81-176 mutants defective in autoagglutination showed a modest reduction in invasion of INT-407 cells. So the autoagglutination ability of the *C. jejuni* 01/51 and its mutants used in this study was tested to determine if the reduced invasion in Caco-2 cells shown by mutants was related to their inability to autoagglutinate. Interestingly, a statistically significant inverse correlation between the ability to autoagglutinate and invasion potential was found in this study (Figure 7.8). The autoagglutination in *C. jejuni* 01/51 was lower than its mutants and complemented mutants which was statistically significant ($P = <0.05$).

Previously, a transposon mutant library generated in *C. jejuni* 480 was studied to find those mutants with reduced motility and autoagglutination; a link between expression of functional flagella and autoagglutination was suggested as 20 mutants that lost motility, were not able to autoagglutinate (Golden and Acheson, 2002). Four of the mutants

having lost their ability of motility and autoagglutination expressed FlaA, and two out of these four mutants were unable to invade cultured INT-407 cells (Golden and Acheson, 2002). Motility was not reduced in any of the mutants tested in this study that may account for a lack of reduction in autoagglutination in these mutants.

Moreover, *Cj1333* (*pseD*) was mutated in one of the autoagglutination mutants in which invasion in INT-407 cells was abolished (Golden and Acheson, 2002). The *pseD* is part of glycosylation locus (*Cj1293- Cj1342*) in *C. jejuni* NCTC11168 (Parkhill *et al.*, 2000) and is involved in attachment of PseAm (pseudaminic acid) to flagellin during glycosylation (Guerry *et al.*, 2006). Glycosylation in *C. jejuni* is important for autoagglutination (Guerry *et al.*, 2006) and host cell invasion (Karlyshev *et al.*, 2004) and the loss of invasion and autoagglutination was most probably due to impaired glycosylation in *pseD* mutant. No glycosylation related genes were mutated in any of the mutants in which autoagglutination was tested in this study so autoagglutination was not affected. A non-motile double mutant *C. jejuni* 81116Δ*flaA/B* was also used in the autoagglutination assay, interestingly it did not show a reduction in autoagglutination (Figure 7.8) which was contrary to a previous report that motility is important for autoagglutination (Golden and Acheson, 2002).

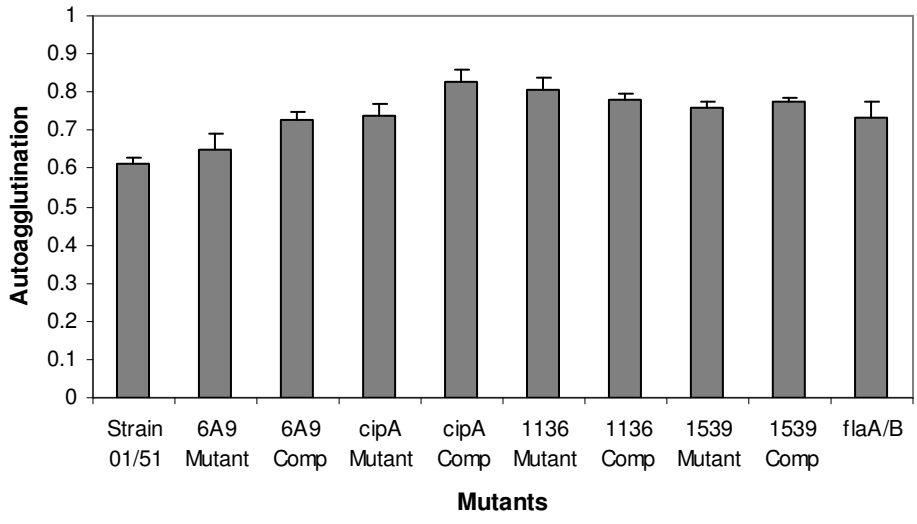

Figure 7.8 Autoagglutination in *C. jejuni* 01/51 and mutants.

The ability to autoagglutinate in *C. jejuni* 01/51 wildtype (strain 01/51), transposon insertion mutant in *Cj0690c* (6A9 mutant), complemented 6A9 mutant (6A9 comp), targeted *cipA* mutant (cipA mutant), complemented *cipA* mutant (cipA comp), targeted *Cj1136* mutant (1136 mutant), complemented *Cj1136* mutant (1136 comp), targeted *Cj1539c* mutant (1539 mutant), complemented *Cj1539c* mutant (1539 comp) and non-motile *C. jejuni* 81116Δ*fla*A/B was tested. No strong correlation between autoagglutination and invasion was found.

7.3.2.7 Chick colonisation

Chicken colonisation potential of *C. jejuni* 01/51 wildtype and targeted mutants in *cipA* (*Cj0685c*), *Cj1136* and *Cj1245c* was tested in the chick colonisation model. Colonisation results showed that the *cipA* mutant colonised the birds at the similar levels (2.62 x 10^8 CFU/gram of caecal contents) to the parent strain 01/51 whose mean level of colonisation was 2.44 x 10^8 (Figure 7.9). This suggests that the inactivation of *cipA* (*Cj0685c*) does not affect colonisation potential of *C. jejuni* 01/51. The mutants of *Cj1245c* showed a reduction in chick colonisation potential as compared to parent strain 01/51 (Figure 7.9) but this was not statistically significant ($P > 0.05$) suggesting that this gene does not play a role in chick colonisation by *C. jejuni* 01/51. *C. jejuni* 01/51 wildtype strain colonised 100% of the birds and colonisation level was high but the mutant in *Cj1136* showed a significantly ($P < 0.001$) low level of chick colonisation as 30% of birds (n = 3/10) infected with the lower dose (4.3 x 10^4 CFU) of the *Cj1136* mutant were not colonised. The colonisation levels in those birds infected with higher dose (4.3 x 10^6 CFU) of the mutant were significantly reduced compared to the lower dose of the wildtype strain 01/51 (Figure 7.9), which strongly suggests that *Cj1136* is important for the chick colonisation by *C. jejuni*.

Successful colonisation of the chicken gut is a multifactorial process. Mutations in genes of RacR-RacS, a two component regulatory system involved in temperature regulation (Bras *et al.*, 1999), dnaJ involved in the heat shock response (Ziprin *et al.*, 2001), *cbrR*, a gene involved in bile tolerance (Lin *et al.*, 2003; Raphael *et al.*, 2005), iron transport and regulation (Cawthraw *et al.*, 1996a; van Vliet *et al.*, 2002), serine catabolism (Velayudhan *et al.*, 2004), and glycosylation (Karlyshev *et al.*, 2004; Kelly *et al.*, 2006) have all been shown to reduce or abolish the ability of *C. jejuni* to colonise the chicken gut. Flagella have been shown to be required for passage through the gastrointestinal tract but not for caecal colonisation (Wösten *et al.*, 2004). Mutations in the *pldA* gene and the *ciaB* gene (Ziprin *et al.*, 2001) also lead to a noncolonising phenotype. In *Salmonella* species, a reduction in the ability to colonise chickens correlated with changes in the lipopolysaccharide profile (Craven *et al.*, 1993; Turner *et al.*, 1998). Like many other pathogens LOS is important for invasion in *C. jejuni* and deletion of the LOS biosynthesis locus in *C. jejuni* NCTC11168 abolished invasion of the mutant in Caco-2 cells however chicken colonisation of this mutant was not tested (Marsden *et al.*, 2009). *Cj1136* is part of the LOS biosynthesis locus in *C. jejuni*

NCTC11168 (Parkhill *et al.*, 2000) and is potentially involved in LOS biosynthesis. The impaired ability of colonisation in chicks shown by the *Cj1136* mutant might be due to altered LOS biosynthesis in this mutant. *Cj1136* encodes for a putative galactosyltransferase and the importance of this enzyme for chicken colonisation in *C. jejuni* has previously been reported. Mutation of *cgtB* that encodes for a putative galactosyltransferase in *C. jejuni* 81-176 reduced the invasion in INT-407 cell and chick colonisation in the mutant (Gilbert *et al.*, 2002).

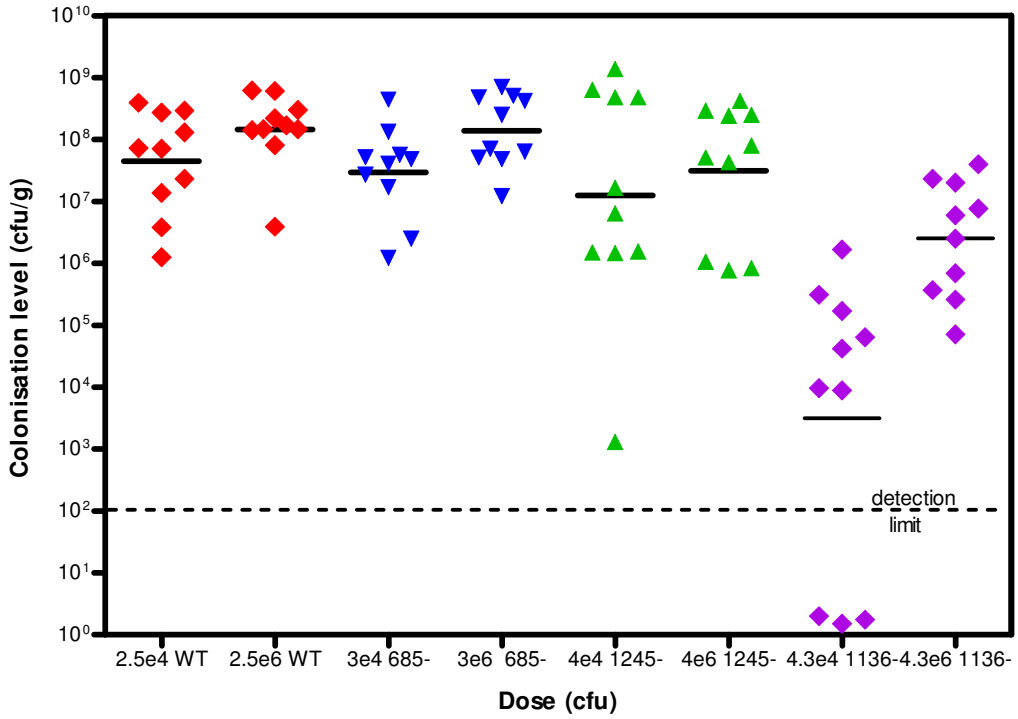

Figure 7.9 Colonisation of chicks by *C. jejuni* 01/51 WT and mutants.

The *C. jejuni* 01/51 wildtype, its mutants in *cipA* (*Cj0685c*), *Cj1136* and *Cj1245c* were tested for their ability to colonise gut of chicks. A low and a high dose of the wildtype strain or mutants was given to 10 birds in each group. Numbers of bacteria that had colonised the birds were determined in caecal contents five days after infection. The *C. jejuni* 01/51 wildtype colonised to ~ 10^8 (cfu/g) and *cipA* (*Cj0685c*) mutants showed a similar level of colonisation to wildtype strain. *C. jejuni* 01/51 mutant in *Cj1245c* showed a reduction in colonisation but it was not statistically significant. Both low and high doses of the mutant in *Cj1136* showed significantly lower levels of chick colonisation compared to the lower dose of the wildtype strain 01/51 confirming that *Cj1136* plays a role in chick colonisation in *C. jejuni* 01/51.

Acknowledgement: Dr. Shaun Cawthraw, Veterinary laboratories agency, Webridge helped in conducting the chick colonisation assay, data analysis and interpretation.

7.3.2.8 Lipooligosaccharide analysis

Lipooligosaccharide (LOS) structures are generally important for invasion in many pathogenic bacteria (Kanipes *et al.*, 2004; Lambotin *et al.*, 2005; Marsden *et al.*, 2009; Preston *et al.*, 1996; Swords *et al.*, 2000). LOS biosynthesis genes are clustered in a locus (*Cj1131c – Cj1152c*) in *C. jejuni* NCTC11168 (Parkhill *et al.*, 2000). Sequencing and microarray analysis of the LOS biosynthesis locus in the *C. jejuni* genome have shown that this locus is highly variable (Gilbert *et al.*, 2002; Parker *et al.*, 2008), which may contribute to the phenotypic variation in *C. jejuni*.

In this study, a transposon insertion in a gene (*Cj1136* homologue) encoding for a putative glycosyltransferase resulted in reduced invasion in Caco-2 cells compared to *C. jejuni* 01/51 wildtype strain. LOS is important for invasion in *C. jejuni* (Marsden *et al.*, 2009). The *Cj1136* is part of the LOS biosynthesis locus in *C. jejuni* NCTC11168 and *Cj1136* has yet not been characterised and its role in LOS biosynthesis is not known. Our hypothesis was that *Cj1136* might be involved in LOS biosynthesis and inactivation of *Cj1136* would have altered the LOS structure that consequently resulted in reduced invasion in cultured Caco-2 cells and chick colonisation in the mutant. So LOS was extracted from *C. jejuni* 01/51 wildtype, its mutant in *Cj1136* and complemented *Cj1136* mutant and analysed by SDS-Tricin polyacrylamide gel electrophoresis and silver staining. LOS extracted from the mutant gave a band with higher electrophoretic mobility compared to that extracted from the 01/51 wildtype stain, LOS from the complemented mutant showed an electrophoretic mobility similar to that from the wildtype strain (Figure 7.10). It confirmed that *Cj1136* is involved in LOS biosynthesis in *C. jejuni* 01/51 as the *Cj1136* mutant appeared to have generated a truncated LOS, it also confirmed that normal LOS biosynthesis was restored on complementation of the mutation.

Lipopolysaccharide (LPS) and lipooligosaccharide (LOS) are the major surface antigens of Gram-negative bacteria and play an important role in the interaction of these bacteria with their host and/or the environment (Caroff *et al.*, 2002). These surface polysaccharide molecules in *C. jejuni* also serve as host mucosal adherence factors. LOS is made up of two covalently linked domains: a hydrophobic lipid A moiety and non-repeating unit of inner and outer core oligosaccharide (Aspinall *et al.*, 1995). The LOS core oligosaccharides are synthesised by adding sugar moieties by

glycosyltransferases and modifying enzymes. The LOS is important for virulence of *C. jejuni.*

Inactivation of *cgtB* encoding for a putative galactosyltransferase in *C. jejuni* 81-176 reduced the invasion in INT-407 cells and chick colonisation (Gilbert *et al.*, 2002) while a site-specific insertional mutant of *C. jejuni* strain 81-176 in *cgtA* gene which encodes for an *N*-acetylgalactosaminyltransferase, showed a significant, more than twofold, increase in invasion of INT-407 cells compared to the wildtype (Guerry *et al.*, 2002). Previously in an attempt to correlate the colonisation and invasion ability of *C. jejuni* strains with PCR-based presence or absence of selected LOS biosynthesis genes it was found that *cgtB* and *wlaN* were present in 68% of *C. jejuni* strains with strong colonisation and invasive ability compared to 20% in weak or non-invasive strains (Muller *et al.*, 2007). In our laboratory, the presence of *Cj1136* was checked in *C. jejuni* strains with hyper and low invasion potentials (Fearnley *et al.*, 2008) and some evidence of a correlation between *Cj1136* presence and invasion potential was found as the gene was present in 67% (n = 4/6) hyperinvasive and 25% (n = 1/4) low-invasive *C. jejuni* strains (Appendix 5), however, the screen of a large number of hyperinvasive and low-invasive strains is needed. This data is in contrast to a previous report in which PCR detection of *Cj1136* and other genes encoding for putative galactosyltransferases (except *wlaN* and *cgtB*) in 40 *C. jejuni* isolates indicated no correlation between the occurrence of the genes and the colonisation and invasion ability of the strains (Muller *et al.*, 2007).

There are other genes as well as *Cj1136* that encode for putative galactosyltransferase including *cgtB* in *C. jejuni* 81-176 (Gilbert *et al.*, 2002), and eight genes encode putative galactosyltransferases (*wlaH*, *wlaG*, *Cj1136*, Cj1138, *wlaN* (*Cj1139c*), *Cj1434c, Cj1438c* and *Cj1440c*) in *C. jejuni* NCTC11168 (Parkhill *et al.*, 2000). Sequencing of *C. jejuni* RM1221 resulted in the annotation of two putative galactosyltransferases (*CJE1278* and *CJE1280*) with no similarity to genes of NCTC11168 (Fouts *et al.*, 2005). Apart from *Cj1136*, those genes encoding for putative galactosyltransferases in *C. jejuni* 01/51 are not known but if there are any others they were unable to compensate for the lack of *Cj1136* gene products in *C. jejuni* 01/51Δ*Cj1136*.

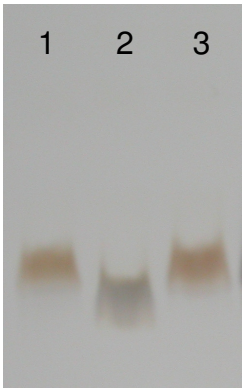

Figure 7.10 Comparison of LOS extracted from *C. jejuni* 01/51 with that from *Cj1136* mutant and complemented mutant.

LOS extracted from *C. jejuni* 01/51, *Cj1136* mutant and complemented mutant were electrophoresed on 15% SDS-PAGE gel and silver stained. LOS from the *Cj1136* mutant showed a higher electrophoretic mobility compared to that extracted from the 01/51 wildtype or complemented mutant. Lane 1: 01/51 wildtype; lane 2: *Cj1136* mutant; lane 3: complemented *Cj1136* mutant.

7.4 Summary and conclusions

After confirming the virulence-associated role of selected genes identified in *C. jejuni* 01/51 transposon insertion mutants with reduced invasion phenotype by generating targeted mutagenesis and the mutated genes were complemented. The adhesion and invasion ability of *C. jejuni* 01/51 wildtype, selected mutants and complemented mutants were tested to verify the involvement of the mutated genes in virulence. Complementation of the mutated genes partially but significantly restored the invasion in Caco-2 cells. *C. jejuni* 01/51 and mutants showed similar growth in MH broth and there was no significant difference in motility, resistance to atmospheric oxygen, H_2O_2 and triton X-100 stress between the 01/51 wildtype strain and mutants which confirmed that the reduced invasion phenotype in the mutants was not affected by growth, motility or the survival of mutants in different stresses encountered during the gentamicin protection assays.

C. jejuni 01/51 and mutants in *cipA*, *Cj1245c* and *Cj1136* were tested for their ability to colonise the avian gut in a chick colonisation model. The *cipA* mutant showed colonisation levels similar to the wildtype strain and *Cj1245c* mutant showed some reduction in colonisation but it was not statistically significant suggesting that these

genes were not involved in chick colonisation by *C. jejuni* 01/51. The *Cj1136* mutant showed significantly reduced colonisation in the chick gut compared to the wildtype strain highlighting a role for this gene in chick colonisation.

Analysis of lipooligosaccharides extracted from *C. jejuni* 01/51, the *Cj1136* mutant and complemented mutant showed that *Cj1136* was involved in LOS biosynthesis in 01/51, and the altered LOS biosynthesis in the mutant was most probably responsible for the reduced invasion and chick colonisation phenotypes in the *Cj1136* mutant.

Chapter Eight

GENERAL DISCUSSION AND FUTURE DIRECTIONS

8 GENERAL DISCUSSION AND FUTURE DIRECTIONS

C. jejuni is the most common cause of bacterial food-borne diarrhoea worldwide, but its pathogenic mechanisms are not clear. However, previous studies showed that toxin production, invasion and motility are important for *Campylobacter* pathogenesis (Ketley, 1997). Invasion of host epithelial cells is believed to be an important virulence mechanism of *C. jejuni* and not all strains have the same virulence potential (Everest *et al.*, 1992). *C. jejuni* strain dependent variability in invasion in eukaryotic cells has been reported by many researchers (Everest *et al.*, 1992; Konkel and Joens, 1989; Malik-Kale *et al.*, 2007; Newell *et al.*, 1985). The differences in invasiveness in *C. jejuni* isolates of human, poultry and environmental origin and the identification of hyperinvasive strains of *C. jejuni* were recently reported (Fearnley *et al.*, 2008). This research project aimed to investigate the molecular basis of this hyperinvasiveness in *Campylobacter jejuni* by identifying and characterising invasion-associated genes in the hyperinvasive strain 01/51.

The variability in invasiveness in *C. jejuni* strains might be due to differences in their genetic content and the presence of novel virulence-associated genes might be responsible for the hyperinvasive phenotype observed in some *C. jejuni* strains. Genetic differences in bacterial strains have been determined by whole genome sequencing, comparative genomic microarrays and/or subtractive hybridisation techniques (Ahmed *et al.*, 2002; Dorrell *et al.*, 2001; Fouts *et al.*, 2005; Hinchliffe *et al.*, 2003; Hofreuter *et al.*, 2006; Howard *et al.*, 2006; Poly *et al.*, 2007b). These methods are powerful tools to determine the similarity or variation in genetic content among strains and may provide the basis for some of the phenotypic variations observed. However, the differences in genomic content do not account for all the phenotypic diversity observed. Different phenotypes could be exhibited by genetically matched strains and the phenotypic variation might be contributed by small changes in nucleotide sequence of the gene including point mutation and/or nucleotide deletions or insertions leading to frame shifts (Malik-Kale *et al.*, 2007). Whole genome sequencing can reveal these minor changes in the genes but it is an expensive and demanding procedure, moreover, whole genome sequence is less likely to reveal the phenotypic variations that are due to gene regulation/expression differences. Transposon mutagenesis is an excellent and relatively

cost effective tool to determine the phenotypic variation and functions of the genes. Transposon mutagenesis has successfully been used to identify virulence associated genes in many bacterial species (Autret *et al.*, 2001; Shah *et al.*, 2005); this technique has also previously been used in *C. jejuni* to determine the bacterial factors that contribute to chicken colonisation ability, motility and autoagglutination in the organism (Golden and Acheson, 2002; Grant *et al.*, 2005; Hendrixson *et al.*, 2001; Hendrixson and DiRita, 2004). A transposon mutant library was constructed in a hyperinvasive, clinical isolate of *C. jejuni* strain 01/51 in order to investigate at the molecular level the hyperinvasive phenotype observed in this organism. The hyperinvasive strains exhibit a significant difference in invasion potential from poor invasive strains (Fearnley *et al.*, 2008). Thus generation of a mutagenesis library in a hyperinvasive strain was expected to provide a good opportunity to identify the genes that promote the ability of the organism to invade the host cells. The mutant library was screened to find the mutants with reduced invasion in the human intestinal epithelial cell line, INT-407 cells and results were also confirmed using an alternative human colonic cell line, Caco-2. A screen of 768 mutants in an *in vitro* assay of invasiveness revealed 174 mutants with a reduction in invasion compared to the parent strain and these were subsequently checked for their motility. Motility is an important virulence factor in *Campylobacter jejuni* and previous studies with nonmotile mutants demonstrated that motility is essential for *C. jejuni* invasion in intestinal epithelial cells (Grant *et al.*, 1993; Wassenaar *et al.*, 1991; Yao *et al.*, 1994). Location of the transposon insertions was determined in only those mutants that were more than 75% motile as compared to the parent strain 01/51 to focus the effort on identifying those genes with a role in invasion rather than motility. Eleven genes that were potentially involved in the invasion phenotype in *C. jejuni* were identified in this study. All but two of the mutants had transposon insertions in genes shared with the genome sequenced strain NCTC11168; one of the genes did not show match to available genome sequence databases and it might be unique to strain 01/51. None of the transposons had inserted into orthologues of any of known invasion-related genes found in other bacteria, such as in *Salmonella* or *Yersinia*. This is not too surprising, as from the numerous genome sequences that are now available it is apparent that *C. jejuni* does not contain any such invasion-related genes (Champion *et al.*, 2008), however this does not preclude there being novel invasion-related genes present in strain 01/51. Genes previously recognised for invasion in cultured epithelial cells by other *C. jejuni* strains, including *cadF*, *ciaB*, *galE* and

csrA (Fields and Thompson, 2008; Fry *et al.*, 2000; Konkel *et al.*, 1999; Monteville *et al.*, 2003) were not identified in this study indicating that our screen was not exhaustive.

This study identified 11 genes that are potentially important for the invasion of *C. jejuni* into intestinal epithelial cells. Most of the genes have previously not been characterised and their role in invasion was not known. Two genes, *Cj1020c* encoding for a cytochrome C and *Cj1503c* encoding for a putative proline dehydrogenase, identified in this study are potentially involved in energy generation and metabolism. It is possible that the virulence-associated role of these factors is as yet not known. Moreover, invasion of *C. jejuni* in host cells requires the synthesis of nascent proteins (Konkel and Cieplak, 1992), an energy dependent process, and as energy is generated from metabolic activity these two genes may have an indirect role in the invasion process. Metabolically active *C. jejuni* were capable of invading cultured eukaryotic cells. *C. jejuni* fixed with bacteriostatic chemical sodium azide, or when these bacteria were tested in the presence of chloramphenicol which blocks protein synthesis, *C. jejuni* lost their ability to invade the cultured epithelial cells (Konkel *et al.*, 1992a). So metabolically efficient strains should show higher invasiveness compared to metabolically impaired strains. Further characterisation of these genes especially in terms of the growth characteristics of the mutants may give more details about the role of these genes.

The mutants selected for transposon localisation exhibited very low levels of invasion in INT-407 and Caco-2 cells (Section 4.3.3). The role of six potentially invasion-associated genes (*cipA*, *Cj0690c*, *Cj1136*, *Cj1245c*, *Cj1305c* and *Cj1539c*) mutated by transposon insertion was reconfirmed by targeted mutagenesis. All the mutants showed a reduction in invasion in cultured human intestinal epithelial cells but the levels of adherence to host cells were variable in mutants; however, inactivation of *Cj1539c* encoding for a putative anion-uptake ABC-transport system permease protein abolished both the invasion and adhesion potential of the mutant (Section 6.2). Adherence to host cells is considered a prerequisite for invasion but the adherence and invasion processes may involve different proteins (Konkel *et al.*, 1999). It seems that the genes i.e. *Cj0690c*, *Cj1136*, *Cj1245c* and *Cj1305c* identified in this study are important in invasion in INT-407 and Caco-2 cells but they are not crucial for adhesion to the host cells used. A reduction in invasion without adhesion has also previously been reported by others. Inactivation of *ciaB* in *C. jejuni* resulted in reduced invasion in INT-407 cells

but not adhesion (Konkel *et al.*, 1999); a mutation in a putative DNA methyltransferase (*Cj1461*) showed higher adhesion to INT-407 cells but only a 50-fold reduced invasion compared to wildtype *C. jejuni* strain 81-176 (Kim *et al.*, 2008).

In aerobic growing organisms, reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide are formed during oxidative metabolism (Alvarez-Peral *et al.*, 2002). The toxic effects of ROS are crucial in the destruction of intracellular pathogens by macrophages and reflect the ability to damage essential cellular components such as nucleic acids, lipids and proteins (Jamieson, 1998; Storz *et al.*, 1987). To circumvent the harmful nature of ROS, many organisms have evolved specific defence mechanisms, which involve the synthesis and/or activation of protective molecules or enzymes including catalase and superoxide dismutase (Jamieson, 1998), and methionine sulfoxide reductase (Mintz *et al.*, 2002). Ferritin in *C. jejuni* is involved in iron storage and protection from intracellular oxidative stress, a *C. jejuni* mutant of ferritin-encoding gene (*cft*) was more sensitive to H_2O_2 killing than parent strain (Wai *et al.*, 1996). The mutants in *cipA*, *Cj0690c*, *Cj1136*, *Cj1245c* and *Cj1539c* were tested for sensitivity to hydrogen peroxide to determine if the reduced invasion potential of the mutants was due to intracellular killing by ROS. Results confirmed that the reduced invasiveness in the mutants was not due to increased sensitivity to hydrogen peroxide which is generated in the eukaryotic cells. The catalase activity was determined in these mutants and all of them possessed a catalase activity which might be responsible for their resistance to H_2O_2.

Most of the genes identified in this study are also present in the first *C. jejuni* genome sequenced strain NCTC11168. The prevalence of six invasion-associated genes (*cipA*, *Cj0690c*, *Cj1136*, *Cj1245c*, *Cj1305c* and *Cj1539c*) that were further characterised by targeted mutagenesis in this study, was screened in six hyperinvasive and four poor invasive *C. jejuni* strains using PCR. All the genes showed random distribution but *Cj1245c* and *Cj1539c* were present in all the hyperinvasive and poor invasive strains tested (Appendix 5). The distribution of *cipA*, *Cj0690c* and *Cj1136* was higher among the hyperinvasive strains. *Cj1305* encoding for a hypothetical protein was found only in hyperinvasive *C. jejuni* strains (5/6) which suggest that this gene might be important for the hyperinvasive phenotype however a screening of a higher number of hyperinvasive and poor invasive strains is needed to strengthen the evidence. *Cj1305c* was identified as part of *Campylobacter jejuni O*-linked glycosylation locus (*Cj1293 - Cj1342*);

however, a previous study reported that flagellin glycosylation was not affected in a mutant of *Cj1305c* in *C. jejuni* 81-176 (Guerry *et al.*, 2006). A strong correlation between the presence or absence of known virulence-associated genes of *C. jejuni* with virulence potential of the strains could not be established in previous PCR based studies (Fearnley *et al.*, 2008; Muller *et al.*, 2006) which suggests that many molecules play a role in this phenotype and the absence of one bacterial determinant might be complemented by another in a different strain. Alternatively more than one mechanism of invasion might be used by *C. jejuni* strains and different invasion-related determinants might be involved in these different mechanisms.

It has been suggested that genetic content and diversity is dictated by the environment in which an organism lives (Konstantinidis *et al.*, 2006). It has also been observed that phenotype including invasiveness and ability of the same *C. jejuni* strain to colonise the chicken gut changes depending upon the environments in which they had been prior to the experiments. A poor invasive strain may become hyperinvasive on repeated passage through host cells (van Alphen *et al.*, 2008); on the other hand, the same strain might lose invasion potential on repeated passage through laboratory culture media (Konkel *et al.*, 1990). Host colonisation ability of the *C. jejuni* strains has also been shown to increase on passage *in vivo* (Cawthraw *et al.*, 1996b). The increased invasiveness or ability to colonise the host gut by passage *in vivo* is more likely due to changes in bacterial gene expression levels (Konkel *et al.*, 1990). Thus the strain dependent hyperinvasive phenotype in *C. jejuni* may also be due to differences in expression of invasion-associated genes. Further studies of expression of the virulence-associated genes identified in this study along with previously known virulence genes may help understand the contribution of gene expression to hyperinvasiveness. However, a previous study found that the relative chick colonisation ability of *C. jejuni* strains NCTC11168 and 81116 that were passaged *in vivo* was still different (Ahmed *et al.*, 2002); this relative difference among the strains is more likely to be dictated by genetic content of the strains rather than gene expression levels. The difference in genetic content and its contribution to differences in strain dependent phenotypes therefore can not be ignored.

This study showed that the putative galactosyltransferase encoded by *Cj1136* is involved in lipooligosaccharides (LOS) biosynthesis in *C. jejuni*. Inactivation of *Cj1136* in *C. jejuni* 01/51 resulted in a significant reduction in invasion in INT-407 and Caco-2

cell and production of truncated LOS. The reduced invasion phenotype observed in the *Cj1136* mutant is most probably an indirect effect due to the abolished biosynthesis of intact and functional LOS, however, a more direct role of this determinant in the invasion process other than LOS biosynthesis can not be ruled out. LOS might be involved in the activation of some host cell bacterial uptake processes. Further gentamicin protection assays using the wildtype strain 01/51 and the *Cj1136* mutant in the presence of purified LOS from a wildtype strain may reveal whether the reduced invasion phenotype was due to altered LOS or some other reasons. Many previous studies have shown the implication of bacterial LOS in invasion of host cells by the organism (Guerry *et al.*, 2002; Kanipes *et al.*, 2004; Lambotin *et al.*, 2005; Muller *et al.*, 2007; Perera *et al.*, 2007; Swords *et al.*, 2000). The structure of LOS in strain 01/51 and the polysaccharide units introduced by the galactosyltransferase (*Cj1136*) are not yet known and mass spectrometry and nuclear magnetic resonance analysis of purified LOS would help our understanding of the structure. After genome sequencing of *C. jejuni* NCTC11168, eight genes encoding putative galactosyltransferases (*wlaN, wlaH*, *wlaG*, *Cj1136*, *Cj1138*, *Cj1434c*, *Cj1438c* and *Cj1440c*) were annotated (Parkhill *et al.*, 2000). Besides *Cj1136*, the genes encoding for a putative galactosyltransferase in *C. jejuni* 01/51 are not known; it will be interesting to know the virulence-related roles of other galactosyltransferases, if any, in strain 01/51. Furthermore, in addition to LOS biosynthesis, a putative galactosyltransferase encoded by *Cj1136* might also be involved in the production of other surface polysaccharides or protein glycosylation in *C. jejuni.* A previous report suggested that the mutation of *wlaK,* a member of the LOS biosynthesis genes cluster, resulted in the production of altered LOS as well as impaired *O*-linked glycosylation in *C. jejuni* 81116 (Wood *et al.*, 1999).

The molecular mechanisms through which the genes identified in this study contribute to hyperinvasiveness in *C. jejuni* are not known and further characterisation of other genes which have not been characterised in this study by mutagenesis and complementation and functional analysis, is needed to understand their particular role in this phenotype.

In this study transposon insertion sites were localised in only those mutants that were highly motile and showed less than 20% invasion compared to wildtype strain 01/51. It does not mean that other mutants with reduced invasion potential in which the transposon insertion site was not determined were unlikely to reveal the genes involved

in hyperinvasiveness; identification and characterisation of the genes mutated in other transposon insertion mutants with reduced invasion and good levels of motility would likely reveal other genes that contribute to invasion potential in *C. jejuni*.

In summary, eleven invasion-associated genes were identified in this study using transposon mutagenesis (Table 5.1). The virulence-associated role of all the genes identified, apart from *cipA*, was previously unknown and this study revealed their importance in the invasion process. Six selected genes were mutated by targeted mutagenesis to reconfirm their contribution to invasiveness in *C. jejuni* and four of them were successfully complemented which partially restored invasion potential of the mutants. Also confirmed was the fact that the reduced invasion observed in *C. jejuni* mutants in *cipA*, *Cj0690c*, *Cj1136*, *Cj1245c* and *Cj1539c* was not due to reduced growth or motility, atmospheric air stress, killing in complete cell culture media, intracellular survival or killing of the mutants with detergent used in the gentamicin protection assays. A putative galactosyltransferase encoded by *Cj1136* was found crucial for chick gut colonisation by *C. jejuni*. Chick colonisation potential of the mutant in *cipA* was similar to the wildtype strain 01/51. The mutant in *Cj1245c* showed a reduction in chick colonisation but this was not statistically significant. This study also showed that the translation product of *Cj1136* is involved in LOS biosynthesis in *C. jejuni* which was previously not confirmed.

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APPENDICES

APPENDICES

Appendix 1. The levels and efficiency of invasion of *C. jejuni* strain 01/51 into Caco-2 cells with different bacterial multiplicities of infection (MOI).

MOI[a]	Inoculum[b]	Invasion[c]	Invasion efficiency
25	8.25E+06	2.79E+04	0.34
50	1.72E+07	5.30E+04	0.31
100	3.52E+07	9.55E+04	0.27
150	5.05E+07	1.36E+05	0.27
200	7.50E+07	2.34E+05	0.31
300	9.75E+07	2.29E+05	0.23
500	1.55E+08	2.24E+05	0.14
650	2.00E+08	2.40E+05	0.12
850	2.55E+08	2.28E+05	0.09

[a] The number of bacteria per eukaryotic cell that were added to the cell monolayer.

[b] The number of bacteria in the initial inoculum added to each well of cell monolayer.

[c] The bacterial CFU count that were recovered from each well after invasion assay.

Appendix 2. Results of transposon mutant library screening for invasion in INT-407 cells.

	1	2	3	4	5	6	7	8	9	10	11	12
3A	+++	++	+++	+++	+++	++	+++	+	+++	2	+	NG
3B	+++	+++	+++	+++	++	+++	++	+++	++	3	NG	NG
3C	X	+	+	++	+	+++	++	++	++	X	NG	NG
3D	+++	++	++	+++	++	++	+++	X	++	++++	NG	NG
3E	+	+++	+	++	+++	++	X	++	+	+++	+++	NG
3F	+++	+	+++	+++	X	+++	+++	++	X	NG	NG	NG
3G	++	+	++	X	++	++	+	+++	+	NG	NG	NG
3H	++	+++	X	X	X	2	X	X	X	NG	+++	NG
4A	+++	+++	3	+++	++	++	++	+++	+++	+	++	++
4B	+	++	+++	+++	+++	+++	+++	++	+++	++	+++	+++
4C	+	+++	+++	+	+++	++	++	++	+++	+++	+++	++
4D	++	++	++	+++	++	+++	++	++	++	+++	++	+++
4E	+++	3	+++	+	+++	+++	+++	+++	+	+++	++	+++
4F	+++	++	++	+++	++	+	+++	+	+	+++	+++	++
4G	++	++	+	+	+++	+++	+++	+++	++	+	3	+++
4H	X	+	++	+++	+++	+	+	++	+++	+++	++	+
5A	+	++	+++	+++	+	+++	+++	+++	++	+++	+	NG
5B	3	+++	+++	+	X	++	+	+++	+++	X	+++	NG
5C	+++	+	2	++	+++	+++	+	++	+++	+	NG	NG
5D	+++	++	+++	+++	+++	+++	+++	+++	+++	++	X	NG
5E	++	+++	+++	+++	++	+++	++	++	X	+	+	X
5F	++	+++	++	+++	+++	+++	+++	++	+++	X	+++	X
5G	++	++	++	+++	+++	+++	+++	++	+++	+++	++	+++
5H	+++	+++	+++	+	+++	+++	+++	++	+	X	+++	X
6A	X	X	X	+++	NG	+	X	X	X	+	+++	+
6B	+	+++	X	+++	+++	3	3	+++	2	2	X	X
6C	++	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
6D	+++	+	++	++	++	+++	+	+++	+++	+++	+++	X
6E	+	+++	++	+++	+++	NG	++	++	+	+	NG	X
6F	+	+	+++	++	++	+	+++	+++	+	+	+++	3
6G	+	++	X	+++	+	++	+++	++	+++	++	+++	X
6H	+++	++	X	+	+++	+++	+++	+++	++	X	+	+
7A	+++	+++	++	+++	+++	+++	++	++	+++	+++	+++	+++
7B	+++	++	+++	++	++	+++	+++	+	+++	+++	+++	+++
7C	+++	+++	++	+++	+++	++	+++	+++	+++	+++	++	++
7D	+++	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++
7E	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
7F	+++	+++	+++	+++	++	+++	+	+++	+++	+++	+++	+++
7G	+++	+++	+++	+++	+	++	+++	++	++	+++	2	+
7H	+	++	+++	++	+	3	++	+++	+++	+++	+++	+
8A	+++	+++	++	+++	+++	+++	++	+	++	+++	++	++
8B	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
8C	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
8D	+++	++	+++	+++	+++	+++	+++	+++	++	++	++	+++
8E	++	+++	+++	+++	++	+++	+++	++	++	+++	+++	++
8F	+++	+++	++	++	+++	+++	+++	++	+++	+++	+++	+++
8G	+++	+++	+++	+++	+++	+++	++	+++	++	+++	+++	+++
8H	+++	+++	+++	+++	++	++	++	+++	+++	+	+++	+++
9A	NG	+++	NG	+++	NG	+++	++	+++				
9B	NG	X	+++	NG	NG	++	+++	+++				
9C	NG	NG	+++	+++	+++	NG	+++	+++				
9D	X	X	+++	+	+++	NG	+++	NG				
9E	NG	X	NG	X	NG	+++	X	NG				
9F	NG	+++	+++	+++	NG	NG	NG	NG				
9G	+++	+++	NG	+++	++	+++	+++	+++				
9H	NG	+++	+++	NG	++	+++	3	NG				
10A	+++	++	+++	X	+	+	+++	2	+	+++	+++	NG

There was no bacterial growth in these wells.

10B	++	+++	+++	++	2	+++	+	3	2	+++	+	2
10C	+	++	++	++	+++	++	X	NG	+	+++	+++	+++
10D	++	+	+	2	+	NG	+	NG	++	2	3	2
10E	3	+++	+++	2	+++	+++	++	NG	1	2	3	3
10F	+++	+	+	X	+	++	++	++	+++	+++	+++	+++
10G	NG	NG	++	+	+++	++	+++	+++	++	+	NG	+
10H	X	3	1	+++	++	++	+++	+++	+++	NG	++	X
	01/51: +++								N/C: X			

A random transposon insertion library generated in *C. jejuni* 01/51 was screened to identify mutants in which their ability to invade INT-407 cells was reduced. Screening assays were repeated three times independently and results were combined from three sets of invasion screening assays. The results show the average levels of invasion of a mutant in three assays, for example if a certain mutant had shown ++ invasion in two assays and + in the third assay, it was reported as ++. Key: NG: no growth in inoculum; X: no colony seen in undiluted lysate; 1, 2 or 3: exact number of colonies counted; **+:** 4 to 6 colonies counted; **++:** 7 to 9 colonies counted; **+++:** more than 9 colonies per spot.

Appendix 3. Combined motility and invasion results of selected transposon mutants.

Mutant	Invsn[a]	Dia(cm)[b]	%motile[c]	Mutant	Invsn	Dia(cm)	%motile	Mutant	Invsn	Dia(cm)	%motile
3A8	+	0.5	10.2	**5C7**	+	5.4	110.2	**7F7**	+	3.4	69.4
3A10	2	4.3	87.8	**5C10**	+	6	122.4	**7G5**	+	4.6	93.9
3A11	+	NA	NA[d]	**5D11**	X	3.8	77.6	**7G11**	2	3.5	71.4
3B10	2	3.6	73.5	**5E9**	X	4.4	89.8	**7G12**	+	4.5	91.8
3C1	X	3.5	71.4	**5E10**	+	6	122.4	**7H1**	+	2.8	57.1
3C2	+	3	61.2	**5E11**	+	5.4	110.2	**7H5**	+	4.5	91.8
3C3	+	NA	NA	**5E12**	X	4.9	100	**7H6**	3	3.6	73.5
3C5	+	4.4	89.8	**5F10**	X	2.6	53.1	**7H12**	+	3.8	77.6
3C10	X	2.8	57.1	**5F12**	X	4.6	93.9	**8A8**	+	5.1	104.1
3D8	X	3.4	69.4	**5H4**	+	4	81.6	**8B12**	+	NA	NA
3E1	+	NA	NA	**5H9**	+	4.8	98	**8H10**	+	4.2	85.7
3E3	+	4.2	85.7	**5H10**	X	2.9	59.2	**9B2**	X	1	20.4
3E7	X	2.5	51	**5H12**	X	3.7	75.5	**9D1**	X	NA	NA
3E9	+	4.1	83.7	**6A1**	X	2.5	51	**9D2**	X	4.5	91.8
3F2	+	NA	NA	**6A2**	X	1.7	34.7	**9D4**	+	3.7	75.5
3F5	X	1.8	36.7	**6A3**	X	3	61.2	**9E2**	X	3.5	71.4
3F9	X	NA	NA	**6A6**	+	5.5	112.2	**9E4**	X	NA	NA
3G2	+	3.7	75.5	**6A7**	X	4.2	85.7	**9E7**	X	4.7	95.9
3G4	X	0.5	10.2	**6A8**	X	4.2	85.7	**9H7**	3	5	102
3G7	+	5.1	104.1	**6A9**	X	4	81.6	**10A4**	X	4.8	98
3G9	+	3.7	75.5	**6A10**	+	NA	NA	**10A5**	+	1.2	24.5
3H3	X	3.5	71.4	**6A12**	+	4.2	85.7	**10A6**	+	3	61.2
3H4	X	NA	NA	**6B1**	+	3.5	71.4	**10A8**	2	4.1	83.7
3H5	X	3.3	67.3	**6B3**	X	NA	NA	**10A9**	+	3.5	71.4
3H6	2	3.6	73.5	**6B6**	3	4.3	87.8	**10B5**	2	2.5	51
3H7	X	4	81.6	**6B7**	3	4.2	85.7	**10B7**	+	3.7	75.5
3H8	X	4	81.6	**6B9**	2	4.2	85.7	**10B8**	3	3.2	65.3
3H9	X	0.8	16.3	**6B10**	2	NA	NA	**10B9**	2	3.1	63.3
4A3	3	4.5	91.8	**6B11**	X	4.5	91.8	**10B11**	+	4	81.6
4A10	+	4	81.6	**6B12**	X	1.3	26.5	**10B12**	2	3.4	69.4
4B1	+	3.8	77.6	**6C2**	+	NA	NA	**10C1**	+	3.2	65.3
4C1	+	4.7	95.9	**6C3**	+	NA	NA	**10C7**	X	2.9	59.2
4C4	+	5	102	**6D2**	+	NA	NA	**10C9**	+	3	61.2
4E2	3	5	102	**6D7**	+	4.5	91.8	**10D2**	+	3.7	75.5
4E4	+	4.3	87.8	**6D12**	X	4.5	91.8	**10D3**	+	3.4	69.4
4E9	+	4.5	91.8	**6E1**	+	4.6	93.9	**10D4**	2	NA	NA
4F6	+	4.5	91.8	**6E9**	+	1.5	30.6	**10D5**	+	2.6	53.1
4F8	+	5.1	104.1	**6E10**	+	NA	NA	**10D7**	+	3.7	75.5
4F9	+	5	102	**6E12**	X	5.2	106.2	**10D10**	2	3.7	75.5
4G3	+	4.2	85.7	**6F1**	+	1.9	38.8	**10D11**	3	3.5	71.4
4G4	+	4.1	83.7	**6F2**	+	NA	NA	**10D12**	2	3.7	75.5
4G10	+	5	102	**6F6**	+	4.3	87.8	**10E1**	3	3.6	73.5
4G11	3	NA	NA	**6F9**	+	4	81.6	**10E4**	2	3.3	67.3
4H1	X	4.9	100	**6F10**	+	4.7	95.9	**10E9**	1	4.3	87.8
4H2	+	3.8	77.6	**6F12**	3	4.9	100	**10E10**	2	NA	NA
4H6	+	1.7	34.7	**6G1**	+	0.5	10.2	**10E11**	3	4.6	93.9
4H7	+	3	61.2	**6G3**	X	1	20.4	**10E12**	3	4.6	93.9
4H12	+	4.6	93.9	**6G5**	+	4.6	93.9	**10F2**	+	4.9	100
5A1	+	5.2	106.1	**6G12**	X	5	102	**10F3**	+	5	102
5A5	+	5.2	106.1	**6H3**	X	2.2	44.9	**10F4**	X	NA	NA
5A11	+	NA	NA	**6H4**	+	4.3	87.8	**10F5**	+	5.2	106.1

5B1	3	5.8	118.4	**6H10**	X	4.4	89.8	**10G4**	+	4.3	87.8
5B4	+	4.3	87.8	**6H11**	+	3	61.2	**10G10**	+	NA	NA
5B5	X	5.5	112.2	**6H12**	+	2.2	44.9	**10G12**	+	5.2	106.1
5B7	+	6	122.4	**7B8**	+	NA	NA	**10H1**	X	4.3	87.8
5B10	X	2	40.8	**7D2**	+	1.5	30.6	**10H2**	3	5.5	112.2
5C2	+	5	102	**7D3**	+	0.8	16.3	**10H3**	1	4.3	87.8
5C3	2	5.5	112.2	**7D4**	+	2	40.8	**10H12**	X	4.7	95.9
01/51	+++	4.9	100	**NegCon**[e]	X	0.5	10.2				

a: invasion in INT-407 cells observed in invasion screening assays.

b: diameter of growth measured in centimetres in motility assay.

c: percentage of motility shown by the mutants as compared to that shown by *C .jejuni* 01/51.

d: result not available.

e: results of *C. jejuni* 81116Δ*fla*A/*fla*B used as negative control in the assays.

One hundred and seventy four transposon mutants that showed very low invasion in the invasion screening assay were tested for motility. Forty mutants that showed up to three colonies in the invasion screening assay and more than 75% motility as compared to wildtype strain were selected (shaded) for confirmation of the reduced invasion phenotype in INT-407 and Caco-2 cells. Key: X: no colonies; 1, 2 or 3: exact number of colonies counted; +: 4 to 6 colonies; ++: 7 to 9 colonies; +++: more than 9 colonies counted in 3 µl spot of undiluted sample after invasion.

Appendix 4. Invasion efficiencies and percentage of invasion shown by the selected mutants in the standard invasion assays.

Mutant	Invasion efficiency	% invasion	% invasion reduction	Mutant	Invasion efficiency	% invasion	% invasion reduction
01/51	0.078	100	-	**4H1**	0.114	146.3*	*Nil (-46.3)
3A10	0.005	6.7	93.3	**5B5**	0.032	40.7	59.3
3G4	0.000	.04	99.9	**5C3**	0.037	47.5	52.5
3H7	0.008	10.6	89.4	**5D11**	0.091	117.8*	*Nil(-17.8)
3H8	0.014	17.9	82.1	**5E9**	0.053	68	32
3H9	0.001	1.04	99	**5E12**	0.083	106.7*	*Nil (-6.7)
4A3	.070	90.8	9.2	**5F12**	0.123	158.5*	*Nil (-58.5)
4E2	0.018	23.4	76.6	**5H12**	0.095	122.1*	*Nil (-22.1)
01/51	0.248	100	-	**6B11**	0.194	78.6	21.4
5B1	0.082	32.9	67.1	**6D12**	0.165	66.6	33.4
6A7	0.016	6.3	93.7	**6E12**	0.206	83.4	16.6
6A8	0.191	77.1	22.9	**6F12**	0.162	65.3	34.7
6A9	0.049	19.6	80.4	**6G1**	0.001	0.2	99.8
6B6	0.106	42.8	57.2	**6G3**	0.000	0.1	99.9
6B7	0.200	80.8	19.2	**6G12**	0.150	60.7	39.3
6B9	0.129	52.2	47.8	**6H10**	0.168	68	32
01/51	0.403	100	-	**10D12**	0.003	0.8	99.2
9B2	0.001	0.2	99.8	**10E9**	0.007	1.8	98.2
9D2	0.026	6.4	93.6	**10E11**	0.021	5.1	94.9
9E7	0.145	36	64	**10E12**	0.009	2.2	97.8
9H7	0.140	34.7	65.3	**10H1**	0.019	4.7	95.3
10A4	0.003	0.8	99.2	**10H2**	0.003	0.7	99.3
10A8	0.151	37.5	62.5	**10H3**	0.016	4	94
10D10	0.004	1	99	**10H12**	0.002	0.6	99.4

*Mutant showed higher invasion (value shown in bracket) in INT-407 cells than parent strain in standard gentamicin protection assays.

Invasiveness of the selected mutants that showed low invasion in INT-407 cells but maintained good motility in preliminary screening assays was checked in standard gentamicin protection assays. Invasion efficiencies shown in the table were the mean values of triplicate wells for each mutant and percent invasion and percent invasion reduction are the percentage of invasion and percentage of reduction in invasiveness, respectively, shown by the mutants compared to the wildtype strain *C. jejuni* 01/51. The selected mutants were tested for invasion in INT-407 cells in three independent assays which are shown by separated blocks in the above table. Mutants 3G4, 3H9, 9B2, 6G1 and 6G3 were motility mutants which were used as negative controls in the assays. Mutants that showed less than a 20% invasion *i.e.* greater than an 80% reduction in invasion as compared to wildtype strain were selected (shaded) for reconfirmation of the reduced invasion in INT-407 cells and testing their invasiveness in Caco-2 cells.

Appendix 5. Prevalence of invasion-related genes amongst *C. jejuni* isolates with hyper- and low-invasion potentials.

A:

Strain	Source	Invasion phenotype	*cipA*	*Cj0690c*	*Cj1136*	*Cj1245c*	*Cj1305c*	*Cj1539c*
01/04	Human	Hyper	+	+	-	+	+	+
01/10	Human	Hyper	+	+	+	+	+	+
01/35	Human	Hyper	+	-	-	+	+	+
01/41	Human	Hyper	+	+	+	+	+	+
01/51	Human	Hyper	+	+	+	+	+	+
EX114	Puddle	Hyper	-	-	+	+	-	+
01/39	Human	Low	+	+	-	+	-	+
01/40	Human	Low	+	-	+	+	-	+
01/42	Human	Low	+	+	-	+	-	+
81116	Human	Low	-	-	-	+	-	+

B:

Phenotype	*cipA*	*Cj0690c*	*Cj1136*	*Cj1245c*	*Cj1305c*	*Cj1539c*
Hyperinvasive (n = 6)	5 (83%)	5 (83%)	4 (67%)	6 (100%)	5 (83%)	6 (100%)
Low invasive (n = 4)	3 (75%)	2 (50%)	1 (25%)	4 (100%)	0 (0%)	4 (100%)
Total (n = 10)	8 (80%)	7 (70%)	5 (50%)	10 (100%)	5 (50%)	10 (100%)

A: The presence of invasion-related genes identified in this study was determined by PCR in hyper- and low-invasive *C. jejuni* isolates reported previously (Fearnley *et al.*, 2008). The primers used for PCR amplification of *cipA* (685-F and 685-R), *Cj0690c* (690-F and 690-R), *Cj1136* (1136-F and 1136-R), *Cj1245c* (aj1-FOR and aj1-REV), *Cj1305c* (1305-F and 1305-R) and *Cj1539c* (1539-F and 1539-R) are given in brackets with each gene and the primer details are described in Table 2.3.

B: The percentage of hyer- and low-invasive strains carrying invasion-associated genes identified in this study. The prevalence of the genes was tested by Mr. G K Wilson, a final year research project student under my supervision.