

**Characterisation of Host-Bacterial Interaction in the Invasion
Process of *C. jejuni***

Author

King, Rebecca May

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**Characterisation of Host-Bacterial Interaction in the
Invasion Process of *C. jejuni*.**

Rebecca May King

B. Health Sc., B. Health Sc (Hons)

School of Medical Science

Griffith Health

Griffith University Gold Coast

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Abstract

Campylobacter jejuni is a major cause of human bacterial gastroenteritis worldwide, however despite its importance, relatively little is known about its mechanisms of pathogenesis or interaction with the host. Previous studies have identified flagella, motility, and other surface structures as essential requirements for *C. jejuni* pathogenesis, with exact interactions still to be elucidated. It has been hypothesised that *C. jejuni* interacts with cell surface glycoproteins known as mucins; these structures have previously been identified as chemoattractants for *C. jejuni*. Specifically, MUC1 is a membrane bound mucin expressed on the cell surface of the epithelium lining the gastric mucosa. This study proposed that *C. jejuni* interacts with MUC1 to facilitate attachment and penetration into host mucosal cells.

To investigate this hypothesis, *in vitro* co-cultures using an intestinal cell line expressing MUC1 were employed. Co-culture assays showed correlation with previous observations that MUC1 promotes more rapid adherence to the cell surface, and further demonstrated adherence and invasion of *C. jejuni* 81116 was significantly increased in the presence of MUC1. Additionally, an alternative co-culture assay that uses incubating conditions that resemble the gastrointestinal mucosa more closely was established. These assay conditions produced more reliable and consistent assay data for a microaerophilic organism.

To explore the precise interaction that occurs between the cs-mucin MUC1 and *C. jejuni*, manipulation of integrative and shuttle vectors to incorporate genes encoding for fluorescence and luminescence markers was performed. A range of integrative and shuttle vectors containing a *Campylobacter* specific promoter sequence coupled with

either a *gfp/rfp* gene or *lux* operon was generated, however it was not possible to successfully express these genes of interest in *C. jejuni*. Lack of expression was concluded to be contributed to specific codon usage and difficulties in foreign gene expression by *C. jejuni*.

This study also describes the development of an immunomagnetic separation (IMS) technique to isolate infecting *C. jejuni* from the gastrointestinal tract of avian and mammalian hosts, followed by the use of these *in vivo* isolated bacteria in carbohydrate binding and gene expression analysis. The use of the newly developed IMS technique resulted in successful recovery of 10^5 *C. jejuni* cells using three strains with varying colonising and invasive capabilities propagated *in vivo* in avian and mammalian hosts. The number of cells isolated was not affected by the presence of components of normal intestinal mucosa. *C. jejuni* 81116 cells isolated by IMS were subsequently used in the evaluation of carbohydrate binding and gene expression analysis.

Glycan array analysis of *in vivo* isolated *C. jejuni* 81116 from chicken caecal contents was compared to *in vitro* grown cells and revealed significant differences in binding of galactose, glucose and mannose containing structures. These sugars are known to be involved in the composition of cs-mucins like MUC1, thus indicating possible recognition sites for *C. jejuni* binding.

Exploration into differences in gene regulation between *in vivo* and *in vitro* cultured *C. jejuni* 81116 using microarray transcriptome analysis and Quantitative PCR identified approximately 100 regulated genes with 47 of known or hypothesised function. Significant up-regulation of *tlp* and *cdt* genes associated with chemotaxis and toxin

production was identified, in agreement with previously postulated hypothesis that these genes may be utilised by *C. jejuni* during pathogenesis *in vivo*.

This study demonstrated the influence cs-mucin MUC1 has on adherence and invasion of *C. jejuni* 81116 into intestinal cell lines and the difficulty in construction of *C. jejuni* reporter vectors to generate fluorescent and bioluminescent strains of *Campylobacter* spp. In addition, this study resulted in successful development of an IMS technique that allows analysis of the glycome and transcriptome of *C. jejuni* isolated directly from avian and mammalian animal models. This is a significant development that opens many opportunities for further analysis of *in vivo* gene expression during host-bacterial interactions, allowing further exploration into mechanisms of pathogenesis in *C. jejuni*.

Declaration

I solemnly declare that except where due acknowledgement has been made; the work in this thesis is original. This work has not previously been submitted for a degree or diploma at any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Rebecca May King

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Abbreviations

°C	Degrees Celsius
aa	amino acid
Amp	ampicillin
Amp ^r	ampicillin resistance marker
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CDT	cytotoxic distending toxin
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
cfu	colony forming units
cs-	cell surface
DAPI	4', 6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	deoxyribonucleotides
EDTA	ethylene-diamine-tetra-acetic acid
Fn	fibronectin
GAGs	glycoaminoglycans
GFP	green fluorescent protein
GI	gastrointestinal tract
HCT116	Human colon adenocarcinoma epithelial cell line
IMS	immunomagnetic separation
Km	kanamycin
Km ^r	kanamycin resistance marker
LOS	lipooligosaccharide
lux	luciferase
Mb	mega base pairs
mRNA	messenger RNA
Neu5Ac	N-acetylneuraminic acid
PBS	phosphate buffered saline
P _c	<i>Campylobacter</i> specific promoter

PCR	polymerase chain reaction
QPCR	quantitative PCR
RFP	red fluorescent protein
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	Reverse transcription
TAE	tris-acetate-ethylene-diamine-tetra-acetic acid
U	units
VBNC	viable but non-culturable

CHAPTER 1

Introduction

1.1 *Campylobacter*

Campylobacter jejuni is a slim (1.5-6.0 μm long and 0.2-0.5 μm wide), flagellated gram-negative rod with a spiral morphology (Ketley, 1997). *C. jejuni* is typically motile with a characteristic corkscrew-like motion facilitated by an unsheathed polar flagellum at one or both ends of the cell (Altekruse *et al.*, 1999; van Vliet & Ketley, 2001).

C. jejuni is a microaerophilic organism and thermophile requiring a restricted atmospheric concentration of 5% O_2 and optimum growth temperature of 42°C (Blaser, 1997; Davis & DiRita, 2008). It is considered to be a commensal organism in the intestinal tracts of a variety of hosts, especially birds and chickens, and colonises these asymptotically. However accidental colonisation of this pathogen can induce disease in a human host (Fearnley *et al.*, 2008; Ketley, 1997; Wassenaar & Blaser, 1999).



Figure 1.1 *Campylobacter jejuni*

Prof Julian Ketley: www.le.ac.uk/genetics/ket/camhome.htm

The genome of *Campylobacter* species is approximately 1.6-1.7Mbp of AT rich DNA; with a GC ratio of approximately 30.6% (Ketley, 1997; Konkel *et al.*, 2001). The genome encodes approximately 1,654 coding sequences, with function of all genes yet to be elucidated. The relatively small genome of *C. jejuni* however, is highly efficient with 94.3% encoding for proteins (Parkhill *et al.*, 2000). Extrachromosomal elements including plasmids and bacteriophages have also been identified in *Campylobacter* spp., which often encode transposable elements such as antibiotic resistance markers (Bacon *et al.*, 2000).

The complete genome sequences of *C. jejuni* 11168 and more recently *C. jejuni* 81116 have identified hypervariable regions within the genome. These regions are responsible for synthesising modifying surface structures, such as LOS, flagella and capsular polysaccharides allowing phase-variation of genes (Parkhill *et al.*, 2000; Pearson *et al.*, 2007). Furthermore, these genome sequences have provided instrumental information into gene organisation and highlighted *C. jejuni* 81116 as an isolate that is amenable to genetic manipulation, infective for chickens and reported as being relatively genetically stable (Manning *et al.*, 2001; Pearson *et al.*, 2007). It has been suggested that this strain of *C. jejuni* may prove to be a more stable and reliable strain suitable for *in vivo* experiments (Pearson *et al.*, 2007).

1.2 Epidemiology and clinical outcomes of *Campylobacter* spp. infection

Campylobacter species are recognised as one of the most important human pathogens to date, as it is the leading cause of acute human bacterial gastroenteritis worldwide (van Vliet & Ketley, 2001; Wallis, 1994). Of the *Campylobacter* genus, *Campylobacter jejuni* and *Campylobacter coli* are the most significant human pathogens and account for the majority of *Campylobacter* related infections (Ketley, 1997; Park, 2002; Penner,

1988). With *C. jejuni* the most frequently isolated in cases of human infection (Padungton & Kaneene, 2003).

The estimated annual incidence of campylobacteriosis in the global population varies from country to country, however in the United States alone *C. jejuni* causes an estimated incidence of 432 cases per 100 000 population, representing approximately 2 million cases annually (Ailes *et al.*, 2008). In recent years the incidence rate of culture confirmed *Campylobacter* spp. infection has declined to 12.7 cases per 100 000 in 2006, but still has the second highest incidence rate under surveillance in FoodNet (Ailes *et al.*, 2008). The incidence of *Campylobacter* spp. infection each year in Australia is estimated to be >120 cases per 100 000, involving nearly 1% of the population (Olson *et al.*, 2008; OzFoodNet, 2007).

Infection with *Campylobacter* spp. is considered to be a zoonotic infection due to the organisms' ability to be transmitted from animals or animal products to humans (McCarthy *et al.*, 2007; WHO, 2000). *Campylobacter* spp. is known to be part of the normal intestinal flora of a diverse range of domestic and wild animals and birds (Blaser, 1997; Ketley, 1997), this wide distribution in the environment adds to the complexity of infection. However, the majority of sporadic outbreaks are caused by transmission to humans by the consumption of food products including unpasteurised milk and the most recognised source being the ingestion of contaminated or undercooked poultry (Allos, 2001; Blaser, 1997; Butzler & Oosterom, 1991; Konkel *et al.*, 2001). It has been reported that *Campylobacter* spp. inhabits 98% of chicken and poultry flocks, therefore it is not surprising that chicken meat is the main source for transmission (Altekruse *et al.*, 1999). Other reported sources of *Campylobacter* spp. infection include the consumption of contaminated water, direct contact with infected

animals, especially pets, and travel to developing countries where *Campylobacter* spp. is prevalent (Blaser, 1997; Park, 2002).

The clinical features of *Campylobacter* enteritis can range from asymptomatic to acute gastrointestinal illness characterised by watery diarrhoea, fever and abdominal cramps, and as in most cases in industrialised nations to the more severe development of inflammatory diarrhoea that consists of mucoid, bloody diarrhoea accompanied by the presence of leucocytes in the stool (Blaser, 1997; Ketley, 1997; Wassenaar & Blaser, 1999). Infection with *Campylobacter* spp. is usually self-limiting; however, one important post-infection sequelae is the development of Guillain-Barré syndrome (Blaser & Engberg, 2008; Carvalho *et al.*, 2001). GBS is an autoimmune disorder of the peripheral nervous system, which is characterised by acute flaccid paralysis and can result in respiratory compromise and subsequent death (Bacon *et al.*, 2001; Hughes & Rees, 1997). This polyneuropathy is triggered by a preceding *C. jejuni* infection which results in *C. jejuni* expressing lipooligosaccharides (Freitas *et al.*, 2002) that mimic the carbohydrates of host gangliosides, antibodies against these carbohydrates cause acute demyelination of the nerves in the peripheral system by the host immune system (Nachamkin, 2002; van Doorn *et al.*, 2008; Winer, 2001).

1.3 Treatment and resistance

Campylobacter spp. infection is usually self-limiting and does not require therapy, apart from supporting therapy such as hydration and fluid replenishment (Moore *et al.*, 2006). There are certain instances however that do require antibiotic intervention, these include; systemic and post-infection manifestations, immunocompromised individuals, infection with HIV, pregnant women and patients with severe and prolonged display of symptoms (Allos, 2001; Altekruse *et al.*, 1999). Currently, macrolides are the

antimicrobial agents of choice when therapeutic intervention is warranted (de Saussure, 2009). With intravenous aminoglycoside therapy considered the treatment of choice for serious bacteraemia and other systemic infections due to *Campylobacter* spp. (Alfredson & Korolik, 2007).

The emergence of antimicrobial resistance has become a major public health concern worldwide (Lindow *et al.*, 2010; Nachamkin *et al.*, 2002), and as *Campylobacter* spp. are considered to be zoonotic pathogens, antimicrobial resistance among isolates in the animal reservoir has serious implications for the treatment of campylobacteriosis in humans (Moore *et al.*, 2006). To date *Campylobacter* spp. with resistance to ciprofloxacin or other fluoroquinolones, macrolides and lincosamides, chloramphenicol, aminoglycosides, tetracycline, ampicillin and other β -lactams, cotrimoxazole, and tylosin have been reported (Padungton & Kaneene, 2003). But despite the emergence of acquired resistance to antimicrobial agents, macrolides still remain an effective antibiotic for treatment. In addition, Erythromycin is still considered the drug of choice in the treatment of human *Campylobacter* spp. disease, with Tigecycline a new antimicrobial agent currently under clinical trials, possibly being highly effective against multi-drug resistant *Campylobacter* spp. strains (Allos, 2001; Engberg *et al.*, 2001; Lehtopolku *et al.*, 2010). The best treatment available is prevention. Current risk reduction strategies include efforts to increase public awareness of proper food handling practices to reduce the incidence of cross contamination between poultry meat and other food, improving hygiene and discouraging consumption of raw milk and undercooked meat (Allos, 2001; Skirrow, 1991). Other measures include implementing strategies to reduce the spread of *Campylobacter* spp. through livestock and poultry slaughter houses, with investigations currently underway for development of vaccines (Hillers *et al.*, 2003; Prokhorova *et al.*, 2006; Scott, 1997).

1.4 Pathogenicity

Bacterial pathogens have evolved specific mechanisms in order to exploit host defences. These attributes may be bacterial or species dependent, therefore pathogenic ability is not only influenced by the relative susceptibility of the host, but also by the relative virulence of the infecting bacterium (Kopecko *et al.*, 2001; Malik-Kale *et al.*, 2007; Sansonetti & Phalipon, 1999). Previous studies have identified flagella and motility, along with other surface structures as essential requirements for *C. jejuni* pathogenesis (Bacon *et al.*, 2001; Fry *et al.*, 2000; Karlyshev *et al.*, 2002; Kilcoyne *et al.*, 2006). With adherence of microbial pathogens to mucosal surfaces being a primary step in the pathogenesis of many intestinal bacteria that cause infections and appears to be a prerequisite for invasion (Biswas *et al.*, 2000).

The ability of *C. jejuni* to colonise the gastrointestinal tract of humans is proposed to be essential for disease (Monteville *et al.*, 2003), however little is still understood about the mechanisms of *Campylobacter* pathogenesis other than observations that motility and chemotaxis are required for host colonisation (Hickey *et al.*, 1999).

The role of motility in *Campylobacter* pathogenesis has been well established and is pivotal throughout *C. jejuni* infection (Yao *et al.*, 1997). Functional flagella is one of the most important aspects associated with adherence of *C. jejuni* and is involved in colonisation of the gastrointestinal tract (Alm *et al.*, 1993b; Grant *et al.*, 1993; Karlyshev *et al.*, 2002) as it provides motility for the bacteria to approach and penetrate the mucosal layer coating intestinal epithelial cells (Pei *et al.*, 1998). The flagellum is composed of a basal body, hook and filament (Konkel *et al.*, 2004). The flagella filament consists of two proteins, FlaA and FlaB which are transcribed concurrently; the *flaA* gene is regulated by σ^{28} and the *flaB* gene regulated by σ^{54} (Alm *et al.*, 1993b;

Hendrixson *et al.*, 2001). It was found that motility and expression of the *flaA* gene is necessary for the maximal invasion of *C. jejuni* into host mammalian cells and for the translocation of polarised cell monolayers (Grant *et al.*, 1993; Wassenaar *et al.*, 1991; Wassenaar & Blaser, 1999). The flagellum is also an important component of *C. jejuni* chemotaxis, as flagella rotation is controlled by chemotaxis and quorum sensing mechanisms (Yao *et al.*, 1997), subsequently providing chemotactic motility and contributing to efficient colonisation (Hendrixson & DiRita, 2004; Marchant *et al.*, 2002). *C. jejuni* is known to be highly motile in the mucus gel environment and therefore possesses the ability to penetrate the mucus gel barrier through its motive forces (Shigematsu *et al.*, 1998; Szymanski *et al.*, 1995). Colonisation of the GI tract of infant mice has been shown to differ between strains of *C. jejuni* according to flagella phenotypes (Diker *et al.*, 1992). Non-motile variants were swept along the tract, however, motile strains with bipolar flagella reached relatively constant population sizes within the GI tract and remained for long periods (4 weeks). Colonisation of the large intestine of mice with flagellated *Campylobacter* spp. can occur with as few as 1000 colony forming units (cfu) per mouse (Diker *et al.*, 1992).

More recently the role of flagella has been established to be involved in more than just motility and chemotaxis. It has been suggested to play a role in secretion of virulence proteins, autoagglutination (AAG), microcolony formation and avoidance of the innate immune response (Guerry, 2007). In addition, chemotactic motility was shown to be a prerequisite for *C. jejuni* pathogenesis and survival and the importance of chemotaxis in pathogenesis has been demonstrated via the investigation of phenotypic changes in non-chemotactic mutants *in vivo* (Hendrixson & DiRita, 2004; Takata *et al.*, 1992) and *in vitro* (de Melo & Pechere, 1988).

It has also been established that *C. jejuni* possess adhesins, such as lipopolysaccharides (LPS) and major outer membrane proteins (MOMP) as well as cell surface attachment factors that enable adherence and subsequent invasion of epithelial cells (Carvalho *et al.*, 2001). One outer membrane protein proposed to be an adhesin and suggested to play a direct role in bacterium-epithelial cell surface interactions is PEB1 (Pei & Blaser, 1993; Pei *et al.*, 1998). It has been observed that ablation of PEB1 affects adherence and invasion of epithelial cells, as well as colonisation of mice (Wassenaar & Blaser, 1999).

Another adhesin identified as promoting adherence of *Campylobacter* spp. is the outer membrane protein CadF (Fouts *et al.*, 2005). This 37kDa protein is thought to mediate the binding of the organism to the extracellular matrix component of mammalian cells, fibronectin (Fn), to promote adherence of the bacterium to the host cell surface (Jin *et al.*, 2001; Monteville *et al.*, 2003) and appears to be essential for preferential uptake at the basolateral host cell surface (Monteville & Konkel, 2002). The likely binding site of Fn to CadF has been identified as AA134-137, a four aa sequence consisting of phenylalanine-arginine-leucine-serine (Konkel *et al.*, 2005). It has been proposed that CadF may be responsible for promoting initial interaction of *C. jejuni* with the host cell receptors involved in uptake (Monteville *et al.*, 2003). More recently another *C. jejuni* adhesin, FlpA, has been identified. This 46kDa protein is proposed to act cooperatively with CadF to promote binding of *C. jejuni* to Fn (Konkel *et al.*, 2010).

A genetic marker that has been identified is a 1116bp open reading frame (ORF), *jlpA* gene that encodes the polypeptide, JlpA (Jin *et al.*, 2001). This polypeptide is known to be a surface exposed lipoprotein in *C. jejuni* and is loosely associated with the bacterial cell surface, therefore is suggestive of a role in binding of the bacterium to human epithelial cells (Jin *et al.*, 2003).

The ability of some *Campylobacter* spp. strains to penetrate a variety of cultured cells is assumed to reflect the ability of *C. jejuni* to invade host intestinal cells (Biswas *et al.*, 2000). Although *C. jejuni* strains were reported to invade intestinal epithelial cells, levels of invasion by different strains vary considerably and appear to be strain-dependent (Bacon *et al.*, 2000; Newell *et al.*, 1985b), consequently demonstrating bacterial invasiveness as an important virulence factor for *Campylobacter* spp. pathogenesis *in vitro* and *in vivo* (Kopecko *et al.*, 2001; Szymanski *et al.*, 1995). A plasmid termed pVir, has been associated with *C. jejuni*, and possesses four open reading frames encoding proteins with homology to the proteins of the type IV secretion systems. These are involved in formation of a channel or gate through which proteins may be transferred (Bacon *et al.*, 2000).

It has been suggested that upon adherence of the bacteria to the host cell, *C. jejuni* up-regulates invasion effector proteins, which are subsequently secreted into the host cell by a still unknown mechanism, potentially the flagella apparatus (Guerry, 2007; Konkel *et al.*, 2004); these secreted proteins are referred to as *Campylobacter* invasion antigen (Cia) proteins (Rivera-Amill *et al.*, 2001). One recently discovered gene for *C. jejuni* is the *ciaB* gene, which encodes a secreted protein necessary for the maximal invasion of *C. jejuni* into cultured epithelial cells (Konkel *et al.*, 1999; Ziprin *et al.*, 2001). It has been proposed that *C. jejuni* begins to synthesise the Cia proteins upon passage into the small intestine with secretion occurring only in the presence of a stimulating signal and upon contact with the cells lining the gastrointestinal tract (Rivera-Amill *et al.*, 2001). These effector proteins once secreted, stimulate host cell signalling cascades via protein phosphorylation, resulting in increased intracellular free Ca^{2+} and major cytoskeletal rearrangements near the site of bacterial attachment (Kopecko *et al.*, 2001). The interaction of the bacterium with the host cell is proposed to depolymerise localised

actin filaments and induce formation of a microtubule-based finger-like protrusion of the host membrane which is extended to the adjacent bacterium (Kopecko *et al.*, 2001). This pseudopod formation in the host cell has been implicated by Biswas *et al.* to be an important contributor to the adherence process (Biswas *et al.*, 2000). Bacterial endocytosis subsequently occurs through the host membrane protrusion and the internalised bacterium within a vacuole appears to move via dynein motors along microtubules and/or microfilaments (Monteville *et al.*, 2003) to the basolateral surface where exocytosis can occur. The endosome containing bacteria may be directed along microtubules to specific intracellular sites (Biswas *et al.*, 2003; Kopecko *et al.*, 2001; Oelschlaeger *et al.*, 1993) with the M cells possibly serving as a major portal of mucosal entry for *C. jejuni* (Kopecko *et al.*, 2001; Walker *et al.*, 1988). The secretion of the Cia proteins is required for maximal apoptosis induction (Siegesmund *et al.*, 2004).

It has also been suggested that entry of *C. jejuni* may be host cell cycle dependent (Hu & Kopecko, 1999), with the production of the cytolethal distending toxin of *C. jejuni* reported to block the eukaryotic target cell cycle in the G₂ phase and is suggested to play a role in diarrhoeal disease (Hickey *et al.*, 2000; Lee *et al.*, 2003; Whitehouse *et al.*, 1998).

The invasion of *C. jejuni* into gastrointestinal cells is associated with a local acute inflammatory response, which involves the secretion of IL-8, a pro-inflammatory cytokine. This factor is a chemoattractant for many immune effector cells (Kagnoff & Eckmann, 1997) with the host inflammatory response thought to mediate many of the clinical symptoms associated with *C. jejuni* infection (Hickey *et al.*, 1999).

Glycoproteins present on human epithelial cells, such as mucin, have also been proposed to act as specific adhesins for *C. jejuni* (Sylvester *et al.*, 1996a), as mucin

promotes invasion and is a prerequisite for colonisation (de Melo & Pechere, 1988).

And more recently studies have highlighted the importance of the cell surface mucin MUC1 in limiting bacterial infection in a murine model (McAuley *et al.*, 2007).

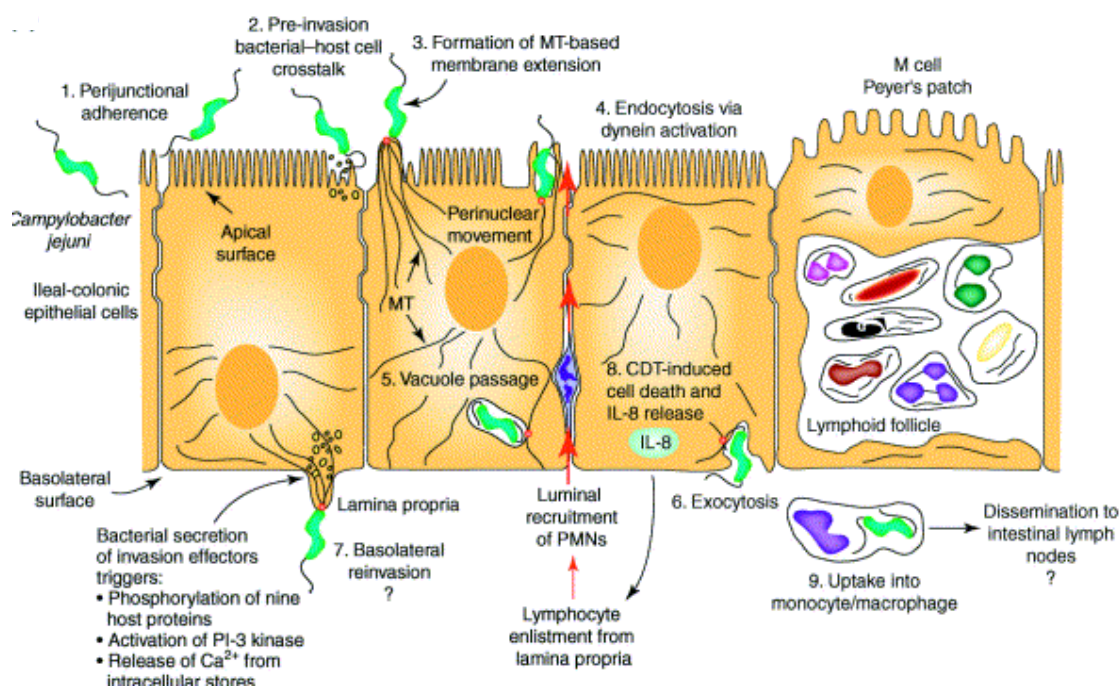


Figure 1.2 Proposed model of pathogenicity. Schematic diagram illustrating the overall steps in *C. jejuni* mucosal invasion and pathogenesis (Kopecko *et al.*, 2001).

1.5 Genetic manipulation

Advances in genetic tools in recent years have contributed to the elucidation of a variety of *Campylobacter* spp. virulence factors, however a majority of the mechanisms involved in *Campylobacter* spp. pathogenesis are still poorly understood (Blaser & Engberg, 2008; Ketley, 1997). Investigations into pathogenesis have been hampered by difficulty in manipulation of *Campylobacter* spp. DNA. *E. coli* broad host range

plasmids do not replicate in *Campylobacter* spp. and cloning of *Campylobacter* spp. DNA into *E. coli* has achieved limited success (Luo & Zhang, 2001). Generally genes from *Campylobacter* spp. are difficult to clone and analyse in *E. coli* due to the high AT content (Taylor, 1992). Contributing to these difficulties is variation in promoter sequences required for efficient expression of genes in *E. coli* and *Campylobacter* spp. Variation can sometimes lead to expression from normally non-functional promoter-like sequences or lack specific expression due to absence of other required accessory factors (Wösten *et al.*, 1998). Furthermore, there are differences in methylation of DNA between *E. coli* and *Campylobacter* spp. resulting in lack of stability and limitation of expression of *Campylobacter* spp. genes due to the presence of suboptimal codons for synthesis of proteins in *E. coli* (Taylor, 1992).

Native plasmids are routinely used as cloning and expression vectors for genetic manipulation of a range of pathogens. This is especially relevant for *Campylobacter* spp. as it has been shown to be most efficiently transformed by plasmid DNA from its own species (Taylor, 1992). Previously genetic manipulation has been dominated by the use of shuttle vectors constructed from pILL550 as it is able to function in *E. coli* as well as *Campylobacter* spp. (Labigne-Roussel *et al.*, 1987). The pILL550 vector was the basis of a range of improved vectors, namely the pUOA (Wang & Taylor, 1990) and pRY series (Yao *et al.*, 1993). More recently the shuttle vector series pMW was developed, again using pILL550 as the precursor (Wösten *et al.*, 1998). Modification of pMW10 resulted in shuttle vectors containing genes encoding for fluorescent proteins, pWM1000 series (Miller *et al.*, 2000) and pMEK (Mixer *et al.*, 2003).

Additionally development of integrative vectors have also been shown to be successful, with pUOA18 used as the basis for construction (Bleumink-Pluym *et al.*, 1999). More

recently an integrative vector system using a constitutively expressed promoter and integration into the spacer region of an rRNA cluster was successful in expression of a variety of genes in *Campylobacter* spp. (Karlyshev & Wren, 2005).

The pGU0202 vector system was also developed and enabled successful transformation and expression of genes in *E. coli* and *C. coli/C. jejuni*. This vector is one of the few shuttle vectors described that does not originate from pILL550 and is smaller than previously described cloning vectors (Labigne-Roussel *et al.*, 1987; Yao *et al.*, 1993). It has successfully demonstrated that cloning of *Campylobacter* spp. DNA sequences in *E. coli* and return back into *Campylobacter* spp. can be used for analysis of gene function and expression (Alfredson & Korolik, 2003), highlighting the potential for development of more shuttle vectors using replication regions from *Campylobacter* spp. cryptic plasmids. Generation of the pCC2228 series adopted this approach and developed vectors that may prove to be beneficial in *Campylobacter* spp. DNA manipulation with the potential to lead to the development of a generation of new shuttle vectors (Miller *et al.*, 2007).

Even with the developments of these genetic tools, there are still limitations in the versatility of vector systems, both shuttle and integrative.

Additionally, developments in transposon mutagenesis for *C. jejuni* has been combined with the availability of the entire nucleotide sequence of the *C. jejuni* genome to allow the application of specialised mutagenesis protocols to identify virulence genes as well as isolation of *in vivo* expressed genes (Colegio *et al.*, 2001). A transposon mutant library was constructed in a hyper-invasive clinical isolate of *C. jejuni* to investigate the molecular basis of invasion. Using this method, a number of previously uncharacterised genes with a potential role in host-cell invasion were identified (Javed *et al.*, 2010).

1.6 Animal models

The wide variety of factors that are likely to be involved to produce disease after *C. jejuni* infection in humans, and the fact that this organism does not normally produce disease in rodents, birds and other animals makes analysis of pathogenicity of *C. jejuni* difficult. A suitable animal diarrheal disease model is yet to be established, however animal models such as mice, chickens, rabbits, ferrets and to a lesser extent macaque monkeys have been used to mimic the course of infection of *C. jejuni* in a host (Newell, 2001). They have been instrumental in identifying and understanding *C. jejuni* pathogenicity factors such as chemotaxis, motility and adherence (Hendrixson & DiRita, 2004; Ziprin *et al.*, 2001).

As *C. jejuni* preferentially colonises the avian gut, this is the most important and accepted model for investigation of bacterial colonisation. The chick colonisation model has been useful in assessment of colonisation potential of defined mutants and variants of *C. jejuni* (Newell, 2001). Murine hosts have previously shown the importance of flagella (Diker *et al.*, 1992) and cell binding factor *peb1A* (Pei *et al.*, 1998). Recently a model for disease using NF- κ B-deficient mice demonstrated colonisation with one strain of *C. jejuni* that produces cytolethal distending toxin (CDT) causing gastroenteritis in mice (Fox *et al.*, 2004). Furthermore, the development of a murine model which demonstrated the importance of the cell surface mucin MUC1 could potentially be a clinically relevant animal model to study *C. jejuni* infection (McAuley *et al.*, 2007).

Newly developed immune knockout mouse models of infection have provided new information on host response to *C. jejuni* and could prove to be a useful tool for future studies on pathogenesis (Poly & Guerry, 2008).

1.7 Systems biology approach to analysis of virulence

The use of whole-genome microarrays based on sequenced strains of *C. jejuni* has identified virulence factors associated with pathogenesis (Leonard *et al.*, 2003). While these studies have been instrumental in investigating gene expression *in vitro*, the need for identifying genes involved in *in vivo* infection is paramount to understanding this diverse organism.

Majority of transcriptome profiling analysis use laboratory grown bacteria to mid log phase for RNA isolation and subsequent analysis, even though this can identify genes up/down regulated *in vitro*, changes in gene regulation and altered expression *in vivo* remain to be determined (Reid *et al.*, 2008). Using microarray based technology, a cluster of genes involved in stringent response pathways were identified as being up-regulated during infection *in vitro* (Gaynor *et al.*, 2005).

Similarly comparative proteomic studies have been performed that lead to the identification of favourable factors such as biofilm formation, which have been suggested to contribute to colonisation of GI tract in chickens and humans (Kalmokoff *et al.*, 2006). Even though this has been investigated *in vitro*, an *in vivo* role for biofilms has not yet been demonstrated for *C. jejuni* (McLennan *et al.*, 2008).

There is also a need for understanding the biological significance of *Campylobacter* spp. cell surface carbohydrates, warranting the need to assess the glycome in avian and mammalian host immune systems (Karlyshev *et al.*, 2005). The huge repertoire of genes encoding carbohydrates in the genome has fostered extensive glycomic studies in major surface carbohydrates (Poly & Guerry, 2008). And may benefit our understanding of both the commensal and pathogenic nature of *C. jejuni* (Karlyshev *et al.*, 2005). A recent study investigated the binding of *C. jejuni* to glycan structures highlighting the

importance of exploration of glycan-lectin interactions in the initiation and maintenance of *C. jejuni* colonisation and infection (Day *et al.*, 2009).

Identification of genes/carbohydrates that may be involved in bacterial adaptation, survival or host cell-bacterial interaction is essential to compare laboratory grown *C. jejuni* and *C. jejuni* directly isolated from *in vivo* host. This could provide information of *C. jejuni* pathogenesis in avian and mammalian hosts to get a comprehensive overview of processes involved with host structures at normal colonising temperature of 42°C and human core body temperature (37°C).

1.8 *In vivo* and *in vitro* isolation

Typically studies are conducted using microarray technology, glycoarrays, multilocus sequence typing and tissue culture to investigate genes possibly involved in *C. jejuni* pathogenesis with chick colonisation models used to reinforce phenotype (Flanagan *et al.*, 2009; Wösten *et al.*, 2010).

A recent study by Woodall *et al.*, 2005, used a newborn chick model of colonisation to assess the transcriptional response of *C. jejuni* within the chick caecum. Isolation of *C. jejuni* was performed at 12 hrs post infection and successfully evaluated gene expression (Woodall *et al.*, 2005). However, long term studies were not described. Likewise microarray technology was successfully used to assess *C. jejuni* NCTC 11168 lifestyle in the gut by transcriptome profiling during host colonisation and pathogenic development. This was performed using a mammalian model of gastroenteritis, the rabbit ileal loop (RIL) model. *C. jejuni* was assessed at 24 or 48 hrs post inoculation, with the *C. jejuni* strain passaged three times *in vitro* before inoculation in the ileal loops. There has been a report of microarray analysis being performed on blood samples

from chicks, but this was mainly used in studies on host immune response (Meade *et al.*, 2009).

Even though these studies provide insight into alterations in gene expression during initial colonisation, there is still the need to investigate difference in glycan binding and gene expression that occurs during *in vivo* colonisation and infection.

1.9 Mucins

Intestinal host cells produce protective proteins known as mucins. It is widely assumed that mucins may protect the host against bacterial colonisation by modifying or inhibiting bacterial associations with the mucosal surface. Conversely, it is possible that mucin acts as an ecological niche for both commensal and pathogenic micro-organisms (Rojas *et al.*, 2002) and therefore may promote pathogenesis of some bacteria (de Melo & Pechere, 1988).

The binding and trapping of micro-organisms by mucus is a fundamental component of innate immunity at mucosal surfaces, acting to limit the growth of micro-organisms and subsequent infection of mucosal epithelial cells (Corfield *et al.*, 1992). Mucins are large, abundant, filamentous glycoproteins that are present at the interface between many epithelia and their extracellular environments (Dekker *et al.*, 2002). Several of these mucins are known to form secreted mucus layers, whereas others form the glycocalyx on the intestinal enterocytes. These mucins may be directly implicated in the development as well as the integrity of the intestinal epithelium (Moniaux *et al.*, 2001). The expression level and diversity of mucins is greatest in those mucosal tissues with the most direct exposure to the external environment, predominantly the gastrointestinal tract. In addition to multiplicity of expression, there is considerable dynamism of cell-surface mucin expression within the gastrointestinal tract (Williams *et al.*, 2001).

The extensive O-glycosylation of mucins is a defining feature, with a total of seventeen human mucin (MUC) genes already identified (Packer *et al.*, 2004). Common structural features shared by all the identified transmembrane mucins include: C-terminal cytoplasmic domains with motifs suggestive of a role in signal transduction, a SEA module in the extracellular domain and a large mucin domain in the N-terminus of the extracellular subunit (Williams *et al.*, 2001). Mucin oligosaccharide chains that attach to the protein core comprise of the primary sugars; N-acetylglucosamine (GlcNAc), galactose, N-acetylgalactosamine (GalNAc), fucose (fuc) as well as sialic acid (NeuAc), mannose and glucose (Forstner *et al.*, 1995). The sugars are linked via an alpha-glycosidic bond between N-acetylgalactosamine and either serine or threonine (Freitas *et al.*, 2002). Oligosaccharide chains often terminate with sialic acid or sulphate groups. These sulphate moieties are usually attached by ester linkages to GlcNAc, galactose and sometimes GalNAc residues. The amount and type of carbohydrates present denotes the charge of the O-glycan chain (Deplancke & Gaskins, 2001; Olson *et al.*, 2005).

1.9.1 Classification

Epithelial mucins are a family of both cell surface and secreted glycoproteins produced by glandular and ductal epithelial tissues with clear and distinct differences in structure and function (Hollingsworth & Swanson, 2004). They are characterised by a tandem repeat structure, which comprises mostly of the protein backbone and is the scaffold for a large number of complex O-linked carbohydrate side chains. A number of epithelial mucins have been identified to date which contain one or more extended carbohydrate rich domains, comprising of repeating amino acid sequences rich in serine and threonine residues (Carraway *et al.*, 2003).

1.9.2 Gel Forming Mucins

Gel forming mucins are the classical goblet or mucus-derived mucins largely responsible for the viscoelastic properties of mucus. They are packaged into granules and when stored together constitute the distinguishing thecae of goblet cells (Laboisse *et al.*, 1995; Williams *et al.*, 2001). Mucin secretion occurs by both simple and compound exocytosis and by compound apocrine-like secretion involving concurrent loss of cytoplasmic components. The goblet cells secrete mucins in response to neuroendocrine and inflammatory stimuli. These processes are accompanied by hydration, causing rapid and dramatic swelling of granules, resulting in the formation of a mucus gel (Verdugo, 1991). The resultant mucus acts to lubricate epithelial surfaces to enhance protection from both chemical and physical damage, to bind bacteria and aid in the passage of materials through epithelial tracts (Perez-Vilar & Hill, 1999).

1.9.3 Cell Surface Mucins

Membrane-bound mucins are organised into domains, and this rearrangement is suspected to provide certain adhesive properties (Moniaux *et al.*, 2001). All cell surface mucins contain large variable numbers of tandem repeat (VNTR) domains that lie extracellularly and are predicted to form rigid structures, extending high above most other molecules in the glycocalyx. Their rod-like shape and glycosylated nature also suggest that these molecules occupy a large amount of space. Together with their expression, these molecules are likely to be a prominent and dominant constituent of the inner and outer glycocalyx (Carraway *et al.*, 2003; Williams *et al.*, 2001).

Cell surface mucins comprise two subunits: a β subunit containing the cytoplasmic tail, a transmembrane region and a short extracellular domain, which is the point of attachment to the α subunit containing the large, complex, heavily O-glycosylated

mucin domain (Seregini *et al.*, 1997). A key feature of cell surface mucins is the ability to release the α subunit containing the mucin domain. Interaction with microbial adhesins may trigger the release of the α subunit thereby coating the micro-organism and preventing access to the cell surface via the cell surface mucin (McAuley *et al.*, 2007; McGuckin, 2001; Singh & Hollingsworth, 2006).

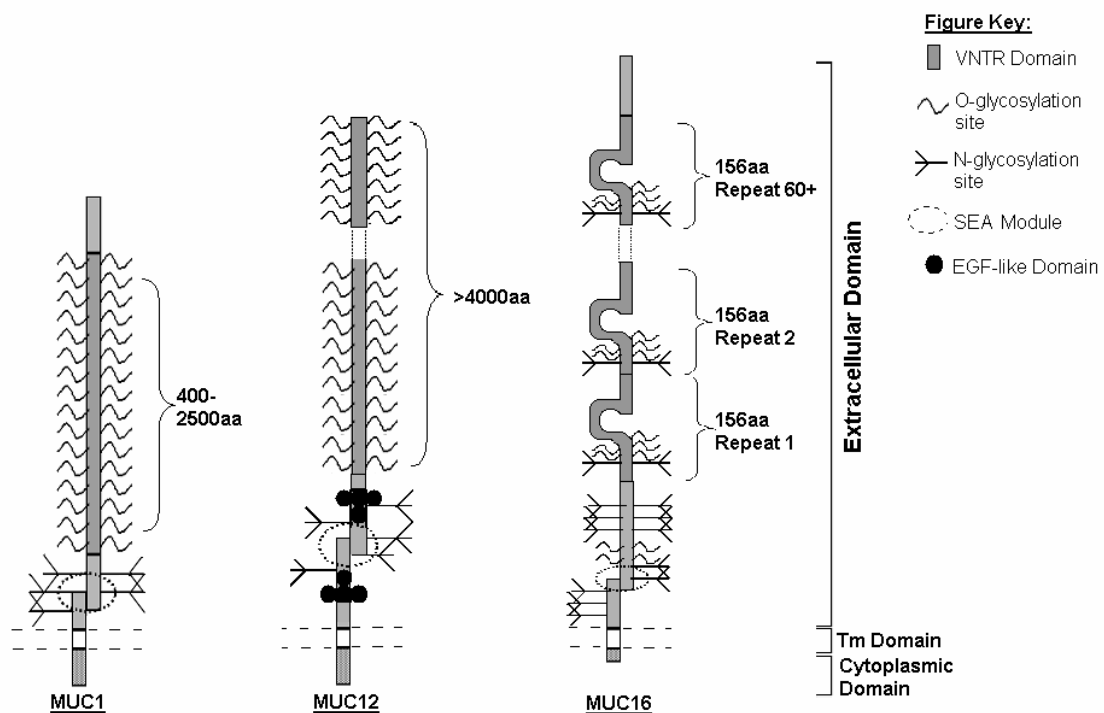


Figure 1.3 Members of the human cs-mucin family. Depiction of the cs-mucins MUC1, MUC12 and MUC16 showing extensive O-glycosylation and the number of amino acids (aa) in the entire VNTR domain, potential N-glycosylation sites, the SEA module (MUC16 also contains SEA modules within the VNTR domain, not shown), EGF-like domains (MUC12 only), transmembrane (Tm) domain and the cytoplasmic domain of each (figure is not to scale).

Emerging data suggests that the cell surface mucins are involved in intracellular signalling due to the highly conserved cytoplasmic tail sequence, which undergoes tyrosine phosphorylation. In fact bacterial binding has been shown to trigger signalling

in epithelial cells (Winterford *et al.*, 1999). The strategic position of mucins places these structures at the centre of many disease processes in which the interactions of epithelial cells and their surroundings have gone astray (Dekker *et al.*, 2002). This results in glycosylation processes frequently being perturbed in disease conditions, with alterations of this protective mucus coat and mucin expression already noted in various diseases such as colon cancers (Shekels *et al.*, 1998a). Thus, mucin degradation may be an important facet of the pathogenicity of some micro-organisms. Altered mucin expression has been associated specifically with colon adenocarcinomas and it has been suggested that expression of MUC 12, 13 and more significantly 11 is down-regulated in colon cancers. There is also variable expression of MUC1 in this disease phenotype (Williams *et al.*, 1999).

1.10 Mucin-bacterial interaction

Bacterium-mucin interactions have previously been identified as a process that occurs *in vivo*. Observations have been made that suggest cell surface mucins limit bacterial colonisation of epithelial surfaces (Dekker *et al.*, 2002; McAuley *et al.*, 2007). This interaction may have the potential to influence intestinal colonisation and mucosal injury (Sylvester *et al.*, 1996a), with the possibility that interaction between mucins and bacteria are an important part of normal cell surface mucin function (McGuckin, 2001).

It has also been proposed that specific adherence to mucin receptors during infection by pathogenic organisms, may actually enhance penetration of the mucin barrier by allowing the bacteria to harbour on the cell surface prior to breaching the intestinal mucosa in order to reach the underlying epithelial cells (Sylvester *et al.*, 1996a). *S. enterica* ser. Typhimurium has been demonstrated *in vitro* to bind to and metabolise mucin carbohydrate structures such as mannose, glucose, galactose, glucosamine,

galactosamine and sialic acid (Vimal *et al.*, 2000). *H. pylori* has the ability to weaken mucus by alteration of synthesis and glycosylation of mucins and has been demonstrated by several *in vitro* studies to decrease the mRNA expression levels of *MUC1* (Byrd & Bresalier, 2000; Byrd *et al.*, 2000; Corfield *et al.*, 1992).

Research focus has recently become more concentrated on bacterial biofilms. Microbial biofilms are surface-associated microorganisms encased within an extracellular matrix and embedded within gel mucus. Extracellular polysaccharides are key components of the matrix, critical for building and maintaining biofilm structure (Irie *et al.*, 2010; Kalmokoff *et al.*, 2006; Macfarlane & Dillon, 2007). They enable differentiation and growth of bacteria and are thought to provide protection from any adverse effects of the mucosal environment forming part of pathogenic strategies by organisms such as *Pseudomonas aeruginosa* (Ramsey & Whiteley, 2004). Observations have suggested *C. jejuni* autoagglutinates, however, it is unclear if autoagglutination represents a biofilm mode of growth in this species (Joshua *et al.*, 2006).

C. jejuni is highly motile in the mucus gel environment due to its typical corkscrew motility, with motility directed by chemotactic stimuli increasing the effectiveness of mucosal colonisation (Shigematsu *et al.*, 1998; Walker *et al.*, 1986). It has been suggested by Wooldridge and Ketley, through a study in 1997 (Wooldridge & Ketley, 1997), that a carbohydrate moiety may mediate invasion of *Campylobacter* spp. in intestinal cells. In addition it has been demonstrated that components of mucins such as L-fucose (a carbohydrate commonly found in intestinal mucin oligosaccharides) and the amino acids L-aspartate, L-cysteine, L-glutamine and L-serine (McSweegan *et al.*, 1987), act as chemoattractants for *C. jejuni* chemotaxis (Marchant *et al.*, 2002). Thus it

would be feasible to assume that individual mucin molecules such as MUC1 be utilised by bacteria to facilitate invasion and hence pathogenesis.

The aim of this work was to characterise the interaction between *C. jejuni* and the host cell during pathogenesis. Analysis of *C. jejuni* interaction with the epithelial cell surface structure MUC1 was undertaken to determine the influence of this glycoprotein in attachment and penetration. Development of a suite of *Campylobacter* specific vectors was undertaken to generate fluorescent and bioluminescent *C. jejuni* to investigate interaction during the invasion process. And an IMS technique was optimised to allow *in vivo* isolation of *C. jejuni* in order to assess gene expression and glycan binding to potentially elucidate *C. jejuni* virulence factors associated with pathogenesis.

Specific aims and objectives:

This study was designed to investigate the pathogenesis of *C. jejuni* using several different approaches:

1. To investigate the influence the cs-mucin MUC1 has on *C. jejuni* during adherence and invasion.
2. To develop an integrative and shuttle vector to incorporate GFP/RFP into the *C. jejuni* genome to allow efficient manipulation of *C. jejuni* and assess interaction with intestinal cells *in vitro*.
3. To develop a shuttle vector to generate bioluminescent *C. jejuni* for use *in vivo* to develop a biological representation of the course of *C. jejuni* infection in avian and mammalian hosts.
4. To optimise an IMS protocol to allow rapid isolation of *C. jejuni* from an infecting avian/mammalian host for use in downstream applications.
5. To investigate the differences in glycan binding and gene expression between *C. jejuni* grown *in vitro* and isolated from *in vivo* to identify potential virulence factors associated with pathogenesis.

CHAPTER 2

Materials and Methods

2.1 General procedures

Sterilisation of glassware, instruments, pipette tips, media and appropriate solutions was achieved by autoclaving at 121°C for 15-20 min. All solutions were prepared using analytical grade chemicals and MilliQ purified water (Millipore). Solutions that were sterilised by filtration were passed through a sterile 0.2µM syringe filter disc (PALL Life Sciences). Eppendorf pipettes were used to measure volumes ranging from 0.1µL to 10mL.

Sterilised broth and media containing agar, were cooled to 55°C prior to addition of growth supplements and/or antibiotics. Agar solutions were aliquoted into sterile petri dishes (Sarstedt) and stored at 4°C.

Chemicals and reagents used in this study were of analytical grade and were purchased from Sigma Chemical Co., Chem-Supply, Oxoid, Merck, Amresco, Fluka, Bio-Rad, Lancaster, APS Finechem or Applichem. Enzymes used in this study were routinely purchased from NEB, Promega, Roche or Finnzymes. Centrifuges used were: Beckman Coulter Allegra 25R with rotors TA-14-50, TA 10.250, Sigma 1-15 bench top microcentrifuge and Sigma 3-16 refrigerated bench top centrifuge. Plastic ware and tips required for RNA experiments were purchased from Axygen.

2.2 Media and antibiotics

Antibiotic Stock Solutions

Ampicillin and kanamycin were dissolved in MilliQ water to give a final concentration of 100 mg/mL, filter sterilised and stored at -20°C. Ampicillin and kanamycin were used where appropriate in agar or broth media.

Brucella Broth (BB): Brucella broth powder (2.8% w/v) was dissolved in MilliQ water and sterilised by autoclaving under standard conditions. Once cooled was stored at 4°C.

Columbia Agar (HBA): 19.5 g Columbia Agar base in 500 mL MilliQ water. Once cooled defibrinated horse blood (5% v/v) (IMVS) and 1 vial of Skirrow's selective supplement (Oxoid) was added. Skirrow's supplement contains 5 mg vancomycin, 2.5 mg trimethoprim and 1250 i.u Polymyxin B. Antibiotics were added where appropriate. For 2% HBA plates, an additional 1% (w/v) Agar Bacteriological was added prior to autoclaving.

Tryptone Soy Agar (TSA): 20 g Tryptone Soy Agar base in 500 mL MilliQ water, 0.5% (w/v) yeast extract, supplemented with defibrinated horse blood (5% v/v) (IMVS), Skirrow's selective supplement (Oxoid) and antibiotics when appropriate. For 2% TSA plates, an additional 1% (w/v) Agar Bacteriological was added prior to autoclaving.

2% Heart Infusion (HI) Agar: 1 g HI broth and 0.8 g (2% w/v) Agar Bacteriological in 40 mL MilliQ water.

Luria-Bertani (LB) broth: 25 g/L Luria Broth Base (Oxoid) in MilliQ water. If **LB Media** was required 7 g of Agar Bacteriological was added prior to autoclaving. If required, antibiotics or other supplements were added once cooled and stored at 4°C.

SOC media: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl and 1.5 mM KCl in MilliQ water. After autoclaving the media was supplemented with 10 mM Mg²⁺ stock and 20 mM glucose.

Storage medium: 10% skim milk powder, 1% (w/v) tryptone and 10 mM Tris-Cl pH 7.5, autoclaved at 109°C for 30 min.

2.3 Solutions and buffers

Blocking buffer: 1X TBS, 0.1% Tween 20, 1% non-fat dry milk powder

TBS/Tween wash buffer: 1X TBS + 0.1% Tween 20

6X Gel-loading sample buffer: 40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol FF

50X TAE: 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5M EDTA pH8.0 made up to 1L with MilliQ water. Stored at room temperature in a glass bottle.

TE buffer: 10 mM Tris-Cl and 1 mM EDTA, pH 8.0

2.4 Bacterial strains, plasmids and primers

2.4.1 Bacterial strains and growth conditions

The bacterial strains used in this study are detailed in Table 2.1. *C. jejuni* strain 81116, *C. jejuni* NCTC11168 genome sequenced strain (Parkhill *et al.*, 2000), *C. jejuni* 11168-O (the original clinical isolate), kindly provided by D. G Newell, Centre of Veterinary Laboratories, London, UK and *C. coli* 427 were cultured on selective agar consisting of Columbia Agar Base or Tryptone Soy Agar (Oxoid) supplemented with 5 % (v/v) defibrinated horse blood (IMVS) and 1% (v/v) Skirrow's supplement (Oxoid) for 18-48 hrs at either 42°C or 37°C under microaerobic conditions (5 % O₂, 10 % CO₂, 85 % N₂). *C. jejuni* were harvested in Brucella Broth and the concentration adjusted from 10⁶ to 10¹² cfu/mL estimated from optical density (OD_{600nm}) followed by viable counts.

E. coli and *S. enterica* ser. Typhimurium were cultured on either LB agar or in LB broth at 37°C in aerobic conditions for 24 hrs. When required, appropriate media contained kanamycin (50mg/mL) and/or ampicillin (100mg/mL) as a selective agent.

Table 2.1 Bacterial Strains

Strain	Isolate	Source	Reference
<i>C. jejuni</i>			
81116	Moderately invasive enteritis isolate	Human (outbreak)	(Parkhill <i>et al.</i> , 2000)
NCTC11168-GS	Enteritis genome strain	Human (outbreak)	(Parkhill <i>et al.</i> , 2000)
NCTC11168-O	Enteritis original isolate (low passage)	Human (outbreak)	(Skirrow, 1991)
<i>C. coli</i> 427	invasive	Clinical strain	Griffith University Culture Collection
<i>S. enterica</i> ser. Typhimurium 8216915	invasive	Mouse	RMIT Culture Collection Department of Applied Biology
<i>E. coli</i> Genotype			
DH5 α	F ⁺ /endA1 hsdR17(r _K ⁻ m _K ⁺) subE44 thi-1 recA1 gyrA (Nal ^r) relA1 Δ (lacZYA-argF)U169 (ϕ 80lacZ Δ M15)		(Hanahan, 1983)
HB101	thi-1, hsdS20 (r _B ⁻ , m _B ⁻), supE44, recA13, ara-14, leuB6, proA2, lacY1, rpsL20 (str ^r) xyl-5. Mtl-1		(Sambrook & Russell, 2001)

2.4.2 Storage of bacteria

Bacterial strains were preserved in cryovials containing storage medium and placed at -80°C for long-term storage, or were maintained in semi-solid (0.4%) agar at 37°C for up to 1 month.

2.4.3 Plasmids

The plasmids used in this study, commercial and constructed are detailed in Table 2.2.

Table 2.2 Plasmids used in this study

Plasmid	Description	Resistance phenotype	Reference/Source
pMW2	pBluescriptΩ <i>C. coli</i> Km ^r	Km ^r , Amp ^r	M. Wösten unpublished
pBF6A	pBluescriptΩ <i>C. jejuni</i> 81116 <i>flaA flaB</i>	Km ^r , Amp ^r	(Fry, 1999)
pWM1007	pMW10Δ <i>lacZ</i> Ω[(T1) ₄ -P _c - <i>gfp</i> - T1]	Km ^r	(Miller <i>et al.</i> , 2000)
pGU0608	pBF6AΩP _c - <i>gfp</i> same orientation as Km ^r	Km ^r , Amp ^r	This study
pDsRed-Express	Cloning vector	Amp ^r	Clontech
pGU0311	pDsRed-ExpressΩcj 197bp P _c	Amp ^r	This study
pGU0515	pGU0311Δ <i>rfp</i> Ω <i>gfp</i>	Amp ^r	This study
pGU0202	<i>E. coli</i> - <i>Campylobacter</i> shuttle vectorΩ <i>aph</i> (3;)-III	Km ^r	(Alfredson & Korolik, 2003)
pGU0610	pGU0202ΩP _c - <i>gfp</i>	Km ^r	This study
pGU0611	pGU0202ΩP _c - <i>rfp</i>	Km ^r	This study
pT7	pT7Ω <i>X. luminescens lux</i> operon	Km ^r , Amp ^r	(Meighen & Szittner, 1992)
pGU0701	pDsRed-ExpressΩcj 197bp P _c	Amp ^r	This study
pGU0703	pGU0701Ω <i>lux</i> operon	Amp ^r	This study
pGU0704	pGU0202Ω <i>lux</i> operon	Km ^r	This study

2.4.4 PCR primers and primer design

Primers were designed based on plasmid sequences, the nucleotide sequence of *C. jejuni* 81116 or the nucleotide sequence of *C. jejuni* NCTC11168 (Parkhill *et al.*, 2000) for amplification of target genes or *Campylobacter* specific promoter sequences. To facilitate the cloning of amplicons into vectors, restriction enzyme (RE) sites were incorporated in the design of primers where appropriate. The Therm 1 and Therm 2.1 primers amplify the 23S RNA gene (Eyers *et al.*, 1994) and were used for controls of QPCR. All oligonucleotide primers were synthesised by Invitrogen and are detailed in Tables 2.3 and 2.4.

Table 2.3 Primers used for standard PCR

Primer	Sequence (5'-3')
Cprom <i>Bam</i> HI-F	GAGGATCCAATTCTATCATAATTGTGGTTTC
Cprom <i>Nco</i> I-R	GACCATGGATTATTTCTTCCTCTTTTCTAC
Km <i>Bg</i> III-F	GAAGATCTTCATGGCTAAAATGAGAATATC
Km <i>Bg</i> III-R	GAAGATCTTCTAAAACAATTCATCCAGT
pWM1007gfp-F	TAAGCGTCATGAGTAAAGGAGAAGA
pWM1007gfp-R	CCGGAATTCCTATTTGTATAGTTCATCCATG
Cprom <i>Eco</i> RI-F	GGAATTC AATTCTATCATAATTGTGGTTTC
gfp <i>Bam</i> HI-R	CGGGATCCCTATTTGTATAGTTCATCCATG
rfp-F	ATGGCCTCCTCCGAGGACGTCATCAAGGAGT
rfp <i>Bam</i> HI-R	CGGGATCCCTACAGGAACAGGTGGTGG
Cprom <i>Not</i> I-R	ATAAGAATGCGGCCGCATTATTTCTTCCTCTTTTCTA
Lux <i>Not</i> I-F	ATAAGAATGCGGCCGCATGACTAAAAAATTTTCATTCATTATTA
Lux <i>Bam</i> HI-R	CGGGATCCCTTTCTATGCCTTCTTTGCCACAT
gfp <i>Mfe</i> I-R	CCGCCGCAATTGCTATTTGTATAGTTCATCCATG
luxC-F	ATGACTAAAAAATTTTCATTCATTATTA
luxC-R	TTATGGGACAAATACAAGGAACTTA
luxE-F	ATGACTTCATATGTTGATAACAAG
luxE-R	TTTCTATGCCTTCTTTGCCACAT

Table 2.4 Primers used for QPCR

Primer	Sequence (5' - 3')
Tlp1p-F	TTGTTATCGTTTACGCTGATG
Tlp1p-R	TGGAAGATCTTTATTATAAATTTTTTAAGGGTTTAA
Tlp2p-F	CATATGCAAGCAATTTTTTCATGAAGTTGTGA
Tlp2p-R	CTCGAGTTATTTATAAACTGGAGCTTCTATTTGTT
Tlp3p-F	CATATGACCTCACTATATGAAAGCACTCTT
Tlp3p-R	CTCGAGTTATGCAGCTTTATAAATAGGTTTATTTATA
Tlp4p-F	CTCGAGGATTCGAGAAACAATACATATGAATT
Tlp4p-R	CTCGAGTTATTGTTTCATTA AAAATAGAATTAACAGC
Tlp7p-F	CATAGTTTTAAAAATACTGCCAATAAAAATGAG
Tlp7p-R	CTCGAGTTAAGATTGACTGGTTTTGCTTATATC
Tlp10p-F	CATATGAACTATTCTTCATCTAAAGATAATAA
Tlp10p-R	CTCGAGTTATTTAAATAAATTAGATTGTTCTATAGT
CdtB-F	ATCTTTTAACCTTGCTTTTGCA
CdtB-R	TTGCGCTAGTTGGAAAAACCACT
Therm 1	TTATCCAATACCAACATTAGT
Therm 2.1	GAAGATACGGTGCTATTTTG
CdtA-F	ATGCAAAAAATTATAGTT
CdtA-R	TCATCGTACCTCTCCTTG
CdtC-F	GGAGATTTGAAAGATTTTAGGG
CdtC-R	CATCTTGACAAGATTTTGCTCC
Cj0803-F	GGTTTAGCAGGAGGCTTTGATAAAAATG
Cj0803-R	CAACTCTCTTAAATTTTTAGTATTAGGAG
Cj1133-F	GATATAGAAATTCATTGGTTTGTTAGATG
Cj1133-R	CTACGCTAAAAATATCTTGTTTAAAGGC

2.5 Molecular Techniques

2.5.1 Plasmid purification using alkaline lysis

Small scale purification of plasmid DNA was achieved using the Alkaline Lysis method described by Birnboim and Doly (Birnboim & Doly, 1979) with modifications: 5mL LB broth containing appropriate antibiotics was inoculated with a single colony of *E. coli* containing the recombinant plasmid and was grown overnight at 37°C on an orbital shaker. 1.5mL of culture was centrifuged for 1 min at 14 000rpm and the resulting pellet resuspended in 200µL of ice-cold Solution I (glucose buffer) containing 10µg RNase A and incubated 5 min at room temperature. 200µL of Solution II (0.2M NaOH, 1% [w/v]

SDS) was added to lyse the cells and the suspension inverted 6-8 times. This was incubated on ice for 5 min. 150 μ L of ice-cold Solution III (60mL 5M potassium acetate, 11.5mL glacial acetic acid, 28.5mL water) was added and the suspension vortexed and placed on ice for 10 min. The suspension was centrifuged at 14 000rpm for 10 min, with the supernatant transferred to a new tube and a 1:1 volume of isopropanol added. This suspension was again vortexed and centrifuged for 10 min at 14 000rpm. The supernatant was removed and remaining pellet washed with 100 μ L 70% ethanol. The washed pellet was centrifuged for 2 min at 14 000rpm and supernatant was carefully removed to allow the pellet to air dry for 5-10 min with the subsequent purified DNA resuspended in sterile distilled water.

2.5.2 Alternate plasmid purification

Alternatively plasmid DNA from *E. coli* was extracted using the Eppendorf fast plasmid mini kit (Eppendorf) or QIAGEN Plasmid midi prep kit (QIAGEN). Plasmids from *Campylobacter* spp. were prepared using the Nucleospin® Plasmid kit (Machery-Nagal) according to the manufacturer's instructions with no modifications.

2.5.3 Quantification of DNA

The quantification of DNA was performed using spectrophotometric analysis at Absorbances $A_{260/280nm}$. The ratio was calculated giving an indication of DNA purity in the preparation. This method was used as previously described by (Sambrook & Russell, 2001) without modification.

2.5.4 Restriction endonuclease digestion

Restriction endonuclease digests were performed as per the manufacturer's instructions. The general reaction conditions included 20U/ μ g DNA with appropriate buffer, sterile water and BSA when required. Reactions were incubated 1-3 hrs at 37°C unless

otherwise stated for optimal activity of the restriction enzyme and heat inactivation performed when necessary.

2.5.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate DNA fragments of endonuclease digests, PCR products and purified DNA based on size and migration through a 1% agarose gel made with 1 x TAE buffer consisting of ethidium bromide. The gel was connected to a Bio-Rad Power Pac 200, sub cell GT electrophoresis tank and run at 80V for approximately 1 hr. DNA was visualised on a UV transilluminator, then imaged using GrabIt95 software.

2.5.6 DNA extraction from an agarose gel

The desired DNA fragments were excised from an agarose gel and purified using the Eppendorf PerfectPrep Gel Clean-up kit (Eppendorf) or the Nucleospin Extract II kit (Macherey-Nagel) as per manufacturer's protocol.

2.5.7 DNA sequencing

DNA sequencing reactions involved 400-1000ng purified plasmid or 20-50ng of purified PCR Product mixed with 6.4 μ mol of sequencing primer. DNA was sequenced using the ABI Big Dye Terminator v3.1 and analysed by the Australian Genome Research Facility (AGRF). Sequence chromatograms were generated and investigated.

2.5.8 PCR conditions

PCR reactions were carried out using 1X Reaction Buffer, 300 μ M dNTPs (NEB), 20 μ mol forward primer, 20 μ mol reverse primer, 1U of proofreading Taq Polymerase. Thermocycling conditions were as follows; initial denaturation of 98°C for 1 min, followed by 35 cycles of 98°C denaturation for 30 s, annealing T_m 53-64°C for 1 min,

72°C extension for 30 s and a final extension of 72°C for 10 min. All PCR's were performed in an Eppendorf Mastercycler personal.

2.5.9 Crude DNA preparation

Crude cell lysates of bacterial DNA were prepared by suspending a bacterial colony in MilliQ water and extracting DNA from the cell by boiling for 8 min. The cellular debris was removed by centrifuging at 14 000rpm for 5 min with the supernatant either used immediately or stored at -20°C.

2.6 Cloning Techniques

2.6.1 Preparation of *E. coli* competent cells

E. coli competent cells were prepared according to the method previously described by Cohen (Cohen *et al.*, 1973), with modifications. Briefly, *E. coli* DH5 α or HB101 cells were revived from storage on LB media overnight at 37°C. A 5mL LB broth culture was prepared with a single bacterial colony and incubated overnight at 37°C with agitation. A 1:20 dilution of the primary culture was prepared into fresh LB broth and agitation continued until the OD_{600nm} reached 0.6. The bacterial culture was chilled on ice for 5 min then centrifuged for 5 min at 3000g (4°C). The cells were resuspended in 40mL ice cold Tbf1 solution; 30mM KAc, 100mM KCl, 10mM CaCl₂, 50mM MnCl₂ and 15% glycerol. The pH was adjusted to 5.8 with 0.2M acetic acid which had been sterilised via filtration (0.2 μ m filter). Cells were incubated on ice for 5 min and centrifuged at 3000g for 5 min (4°C). The supernatant was discarded and the pellet suspended in 4mL ice cold Tbf2 solution; 10mM MOPS 75mM CaCl₂, 10mM KCl and 15% glycerol. The pH was adjusted to 6.5 with 1M KOH and sterilised via filtration (0.2 μ m filter). The cells were left on ice for a further 15 min then used immediately or aliquoted into suitable volumes and stored at - 80°C.

2.6.2 Transformation of *E. coli* competent cells

Transformations were carried out as per the pGEMT-easy manual (Promega). *E. coli* competent cells were thawed on ice and gently mixed by flicking. 2µL of DNA was added to 50µL of cells, mixed gently and placed on ice for 20 min. The cell-DNA mix was heat-shocked for 45-60 s at 42°C and immediately returned to ice.

950µL of pre-warmed SOC media was added and incubated with agitation for 1.5 hrs at 37°C. 100µL of the transformation culture was plated onto LB agar containing the appropriate antibiotics and incubated for 18-24 hrs at 37°C.

2.6.3 Ligations

DNA ligation reactions routinely had a final volume of 10-20µL, with each reaction containing 1x ligation buffer, 20-50ng insert DNA to be cloned, 50ng vector DNA, 1µL T4 DNA ligase (3weiss U/µL) and sterile MilliQ water to make up to the final volume. Reactions were incubated overnight at 4°C. The amount of plasmid DNA and insert DNA added to the ligation reaction was determined by the following equation:

$$\text{Insert DNA (ng)} = \frac{\text{Vector DNA (ng)} \times \text{size of insert (kb)}}{\text{Size of vector (kb)}} \times \text{molar ratio of 1:1/4:1/1:4}$$

2.6.4 Dephosphorylation of plasmid DNA

This was employed to prevent plasmid re-ligation during cloning. The 5' phosphates were removed using either Shrimp Alkaline Phosphatase (Promega) or Antarctic Phosphatase (NEB). 1U/µg DNA of enzyme was used in the reaction and incubated for 10-15 min at 37°C. The enzyme was heat-killed by incubating at 65°C for 20 min.

2.6.5 Natural transformation of *Campylobacter* spp.

Campylobacter strains were cultured on selective agar consisting of Columbia Agar Base or Tryptone Soy Agar (Oxoid) supplemented with 5% (v/v) defibrinated horse blood (IMVS) and 1% (v/v) Skirrow's supplement (Oxoid) for 20 hrs at 37°C under microaerobic conditions. *Campylobacter* spp. was subcultured onto fresh plates and grown for 15-18 hrs at 37°C under microaerobic conditions. The bacteria were harvested with pre-warmed Brucella broth and OD_{600nm} adjusted to 0.6. 1mL of the bacterial suspension was added to 1.5 mL microcentrifuge tubes (Eppendorf) containing 500µL of 2% HI agar and incubated under microaerobic conditions at 37°C for 3 hrs. 1-2µg of plasmid DNA was added to the suspension and gently mixed by pipetting. The suspension was incubated for an additional 5 hrs at 37°C under microaerobic conditions to allow sufficient time for uptake of DNA by *Campylobacter* spp. cells. The bacterial suspension was then plated onto appropriate selective agar and incubated for 48 hrs under microaerobic conditions at 42°C to facilitate the growth of *Campylobacter* spp. cells transformed with the recombinant plasmid.

2.6.6 Electro-transformation of *Campylobacter* spp.

Campylobacter strains were cultured on selective agar consisting of Columbia Agar Base or Tryptone Soy Agar (Oxoid) supplemented with 5% (v/v) defibrinated horse blood (IMVS) and 1% (v/v) Skirrow's supplement (Oxoid) for 36 hrs at 37°C under microaerobic conditions. The bacteria was harvested in 1mL ice cold wash buffer (272mM sucrose and 15% glycerol) and kept on ice. The suspension was centrifuged at 14 000rpm for 8 min (4°C) and the pellet was resuspended with ice cold wash buffer and concentrated into a single tube. An equal volume of wash buffer was added and the concentrated pellet resuspended. Bacterial density was determined at OD_{600nm} and

adjusted to 10^{11} cfu/mL. Cells were either used immediately or aliquoted into suitable volumes and stored at -20°C for 1 month.

For electroporation 50 μL of cells were used for each transformation and kept on ice in an Eppendorf tube. 1 μg of DNA was added to the cells and the cell-DNA mixture transferred to an ice cold electroporation cuvette and pulsed at 2.5kV, 4.0ms, 200 Ω and 25 μF . After pulsing, 100 μL of SOC buffer or Brucella broth was added to the cuvette and the bacterial suspension cultured onto non-selective agar and incubated under microaerobic conditions overnight at 37°C . The subsequent bacterial colonies were harvested with Brucella broth and cultured onto the appropriate selective agar under microaerobic conditions at 37°C for 2-4 days.

2.7 *In vitro* techniques

Cell lines used in this study are detailed in Table 2.5.

Table 2.5 Cell lines

Cell line	Description	Reference
HCT116	epithelial	MMRI
EC1	HCT116 with pcDNA3.1	(King, 2002)
EM10	HCT116 with pcDNA3.1-MUC1	(King, 2002)

2.7.1 Cell culture and maintenance

Cell suspensions stored in liquid nitrogen were revived by addition of 9mL RPMI-1640 (Invitrogen) containing 10% Foetal Calf Serum (FCS, Invitrogen) and were centrifuged at 400g for 5 min. The supernatant was discarded and cells resuspended in an

appropriate volume of RPMI-1640 with the number of viable cells counted using the Trypan Blue exclusion method. Routinely, 10^6 viable cells were seeded in 10mL RPMI-1640 in a 25cm² or 75cm² vented flasks and incubated in a 5% CO₂ humidifier incubator at 37°C until cell growth reached a complete monolayer. Cells were washed with PBS and the monolayer was detached by addition of an appropriate volume of 0.05% Trypsin-EDTA. The cells were passaged every 2-3 days or when ~80% confluence is reached at a split ratio of 1:5. When required for experimentation, the cell suspension was subject to viability determination using the Trypan Blue exclusion method and appropriate cell density reseeded. For stably transfected cell lines 200ug/mL of G418 was added to keep selective pressure on the cells expressing MUC1.

To store cells in liquid nitrogen, cells were harvested with 0.05% trypsin-EDTA and resuspended to 10^6 cells/mL in ice-cold freezing media (RPMI-1640 containing 20% FCS and 10% Dimethyl Sulphoxide, Sigma) and aliquoted into cryovials. The cryovials were then stored at -80°C in a freezer overnight to allow gradual freezing of the cells and transferred to liquid nitrogen or stored at -80°C until required.

2.7.2 Determination of viable cells

A known volume of cells resuspended in RPMI-1640 was added at a ratio of 1:1 with Trypan Blue solution (Invitrogen). The cell-trypan blue solution was then put in a haemocytometer and viable cells enumerated.

2.7.3 Assessment of MUC1 expression

The Human Intestinal cancer cell line HCT116 and its previously transfected variants (King, 2002); pcDNA3.1 vector (Invitrogen) or pcDNA3.1-MUC1 (truncated form of MUC1 containing 22 amino acid VNTR repeats) were assessed to determine expression of the cs-mucin MUC1. The parent and stably transfected cell lines were subject to a

sensitive two-step fluorescent staining technique for analysis by flow cytometry. Approximately 10^6 cells were spun in a centrifuge for 3 min at 400g and the supernatant discarded, the remaining pellet of cells were resuspended in 50 μ L of 0.1U/mL of neuraminidase in PBS and Mg^{2+} , Ca^{2+} , incubated at 37°C for 30 min to allow the neuraminidase to bind and strip off the sialic acid residues on the MUC1 surface. The cells were spun again under the conditions previously stated with 200 μ L of immunofluorescence (IF) buffer (0.5% BSA in PBS) added to cells. The cell suspension was split in half and spun again under the conditions previously stated with the supernatant discarded. The cells were labelled with either 50 μ L of the isotype control 401.21 conjugated FITC MAb (40 μ g/mL) in IF buffer or with the BC2 conjugated FITC MAb (40 μ g/mL) for MUC1 detection, as this antibody recognises peptide epitopes in the VNTR domains of apomucin. Once the primary antibodies were added, the cells were incubated on ice in the dark for 30 min. Following incubation, the cells were washed twice with IF buffer by centrifugation to remove unbound MAb, and stained with a secondary antibody, fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody and incubated on ice in the dark for another 30 min to bind to the monoclonal mouse antibody. After incubation, the cells were again washed twice with IF buffer and fixed in 200 μ L of 1% paraformaldehyde (PF) and transferred to the appropriate tubes to be analysed by FACS. Controls used included unstained cells to assess autofluorescence and omission of the primary and/or secondary antibody. Data was acquired using a Becton Dickinson FACS calibur instrument and Cellquest data acquisition software version 3.1 software package and analysed using the FCS Express v2.0.

2.7.4 Bacterial inoculum

The inoculum to be used in both adherence and invasion assays was prepared according to the method described by (Konkel & Joens, 1989) with slight modifications. An

overnight culture of *S. enterica* ser. Typhimurium or *E. coli* HB101 were prepared in LB broth and incubated for 24 hrs in a 37°C with agitation. The inoculum for *C. jejuni* 81116 was prepared by cultivating for 18 hrs on selective agar under microaerophilic conditions at 42°C then harvesting into pre-warmed Brucella broth. The optical density (OD_{600nm}) was measured by spectrophotometry and bacterial densities diluted to 10⁶ cells/mL. The numbers of bacteria in the inoculum were subject to viability counts by serially diluting and estimating colony forming units (cfu).

2.7.5 *In vitro* co-cultures

The parent HCT116 and HCT116 cells stably transfected with either the pcDNA3.1-MUC1 (truncated form of MUC1 containing 22 amino acid VNTR repeats) or the pcDNA3.1 vector were seeded at 10⁵ cells/well in a 24 well plate with RPMI 1640 (Invitrogen) media containing 10% FCS (Invitrogen) at 37°C in a 5% CO₂ humidifier incubator until confluent. After reaching confluence the cells were either transferred to microaerobic conditions for 2 hrs at 37°C prior to bacterial challenge with *C. jejuni* 81116 (Linden *et al.*, 2007; McAuley *et al.*, 2007) or co-cultures were performed under standard CO₂ conditions.

Adherence/Invasion Assay

Adherence and invasion assays were performed based on published *in vitro* methods (Konkel & Joens, 1989; Linden *et al.*, 2007). *C. jejuni*, *S. enterica* ser. Typhimurium and non-invasive *E. coli* were co-cultured with stably transfected HCT116 (parent, MUC1 expressing and control) cells. Briefly, for the adherence assay human intestinal cells were washed with sterile PBS, and 100µL of the bacterial inoculum containing approximately 5 x 10⁶ bacteria per mL were added to the cell monolayer with additional RPMI-1640 and incubated for 1 hr at 37°C either in standard CO₂ conditions or under

microaerobic conditions. The monolayer of cells were subsequently washed three times with sterile PBS to remove the non-adherent bacteria and lysed with 200 μ L of 0.1% Triton-X-100 in PBS. The adherent bacteria were enumerated by viable counts and determination of cfu.

To measure invasion, after washing with sterile PBS to remove the non-adherent bacteria, monolayers were co-cultured in fresh RPMI-1640 medium containing 400 μ g/mL gentamicin for a further 2 hrs at 37°C either in standard CO₂ conditions or under microaerobic conditions. The cells were subsequently harvested and cfu determined.

2.7.6 Data analysis

All *in vitro* assays of bacterial adherence and invasion were conducted in triplicate. The values obtained are expressed as the mean percentage \pm the standard error of the mean (SEM). An analysis of variance (ANOVA) was performed on the data obtained for the adherence and invasion assays to examine the effect MUC1 had on the adhesion and invasion rates. If a significant difference was detected, appropriate Post Hoc tests were applied to determine where the significant difference in the data lies. A $p < 0.05$ was considered significant. Statistical analysis of viable bacterial counts was performed with Microsoft Excel using independent sample T-test.

2.7.7 Staining of *C. jejuni* 81116 infection of HCT116 MUC1 transfected cell line

HCT116 cells expressing MUC1 were grown on cover slips, and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilised with 0.5% saponin in PBS for 10 min at room temperature. Non specific binding was blocked with 0.5% bovine serum and 0.5% saponin in 2.5% BSA PBS for 15 min. The MUC1 extracellular domain antibody BC2 was added at a concentration of 5 μ g/mL in 0.5%

bovine serum and 0.5% saponin in 2.5% BSA PBS and incubated for 1 hr. The cover slips coated in cells were washed 3 times for 5 min in PBS and saponin. The secondary antibody anti-mouse Alexa 633 or goat anti-mouse 594 (Invitrogen #A11032) was added at a concentration of 5 μ g/mL in PBS and saponin and incubated for 40 min. The cover slips were washed again 3 times for 5 min in PBS and saponin. DAPI was added to each well and incubated for 10 min. The wells were rinsed with PBS and cover slips were placed onto glass slides with 10 μ L of ProLong Gold antifade (Invitrogen P36430). Slides were stored in the dark at 37°C for 1 hr and sealed, then stored long term in the dark at 4°C. Relevant controls were included (no primary antibody and no antibodies).

2.8 Dynabead[®] M-280 procedures

2.8.1 Coating of Dynabeads[®] M-280 with *Campylobacter jejuni* specific antibody

Dynabead[®] M-280 Sheep anti-Rabbit IgG 2.8 μ m beads (Dyna) were prepared according to the manufacturer's protocol. An aliquot of dynabeads[®] M-280 containing 6 x 10⁸ beads/mL were washed with excess isotonic PBS pH 7.4 and separated using a Dynal MPC (magnetic particle separator). The washed Dynabeads[®] M-280 were resuspended in a round bottom tube and the rabbit anti-*Campylobacter jejuni* polyclonal antibody (Fitzgerald Cat# 20-CR89) added at a concentration of 60 μ g (5 mg/mL) per 6 x 10⁸ beads. This bead-antibody complex was mixed for 30 min in a MACSmix tube rotator (MACS) allowing tilt rotation at room temperature followed by overnight tilt rotation at 4°C to ensure complete coating of the beads with the antibody. The bead-antibody complex was then washed three times with excess PBS containing 0.1 % BSA using the MPC and suspended at a final concentration of 6 x 10⁸ beads/mL and stored in this buffer at 4°C. The bead-antibody complex was washed immediately prior to use with this buffer.

2.8.2 Immunomagnetic separation (IMS) optimisation

C. jejuni 81116, *C. jejuni* 11168 and 11168-O were diluted to variable densities with Brucella broth and subjected to Immunomagnetic Separation (IMS) with previously coated dynabeads[®] M-280. The bacterial densities assessed ranged from 10^6 to 10^{12} cfu/mL. 40 μ L of immunocoated dynabeads[®] M-280 were added to 1mL of bacterial suspension and mixed by tilt rotation for a time course of 15-60 min at room temperature to allow sufficient time for the beads to bind the bacteria. The bead-*C. jejuni* complex was washed three times with PBS pH 7.4 containing 0.1% tween 20 and suspended in 100 μ L Brucella broth. Serial dilutions of each suspension was performed and plated onto selective agar for determination of recovery from IMS in triplicate for each bacterial concentration.

2.8.3 Elution optimisation

Elution of *C. jejuni* from the dynabeads[®] M-280 was optimised using 0.05% trypsin-0.02% EDTA in Hanks balanced salt solution (Invitrogen), 0.1M citrate pH 3 (Sigma) and dTT in DEPC treated H₂O (A. G. Scientific). These solutions were assessed for their ability to elute the bacteria from the beads as well as their effect on *C. jejuni* viability. The Dynabead[®] M-280-*C. jejuni* complex was subject to treatment with 500 μ L of eluting solution and mixed using tilt rotation for 3 min. After IMS the solution containing the eluted *C. jejuni* was transferred to a new tube and this step repeated 3 more times. The eluted *C. jejuni* was concentrated by a 5 min centrifugation step at 10 000g (room temperature) and serially diluted to determine the viable count (cfu/mL). Viable counts were also determined for the remaining beads to establish the efficiency of the elution of *C. jejuni* from the beads and viability of the organism using the various elution solutions.

2.9 *In vivo* techniques

2.9.1 Inoculation of *C. jejuni* into chicken host

Newly hatched male Ross breed chickens (Barthers, Rochedale, QLD) were used to assess the efficiency of recovery using IMS of *C. jejuni* from the contents removed from the caecum following colonisation. All animal experiments were approved by the Griffith University Animal Ethics Committee (approval number: MSC/04/08/AEC) and performed as described previously (Ringoir & Korolik, 2003; Ringoir *et al.*, 2007). One day after hatching pre-inoculation cloacal samples were obtained and cultured to ensure absence of *C. jejuni*. The following day the chickens were orally inoculated with 30µL of Brucella broth containing approximately 10^8 *C. jejuni* 81116 cells. Cloacal samples were then taken and cultured on days 1, 2, 3 and 4 post-inoculation to confirm *C. jejuni* colonisation. The chickens were sacrificed 5 days post-inoculation and the content of the caeca removed and weighed aseptically. Enumeration of bacteria was performed by viable counts of *C. jejuni* as viable cells per gram of caecal content. Control groups were confirmed as negative for *C. jejuni* colonisation.

2.9.2 Inoculation of *C. jejuni* into 129/SvJ mice

129/SvJ background male mice (Animal Resource Centre, Western Australia) aged between 6-8 weeks, were housed under clean conventional conditions with free access to sterilised food and water. All experiments were approved by the Griffith University Animal Ethics Committee (Approval number:BDD/01/07). Mice were orally challenged with 30µL of Brucella broth containing approximately 10^8 *C. jejuni* 81116 cells. The mice were euthanised by cervical dislocation 5 days post-inoculation and the gastrointestinal tissues; small and large intestine and caecal contents, were collected

aseptically in Brucella broth. Enumeration of bacteria was performed by viable counts of *C. jejuni* as viable cells per gram of tissue and/or caecal content.

2.9.3 Isolation of *C. jejuni* from *in vivo* by IMS

IMS of the *C. jejuni* cells from the caecal content was performed as previously described with the following modifications: due to the viscosity of the caecal content Brucella broth was used to dilute the content to a volume of 2mL to allow diffusion of the beads and maximum efficiency of binding of the *C. jejuni* cells to immunocoated dynabeads. A 5 s centrifugation at 10 000g (4°C) step was introduced to remove large particulate material that could interfere with the movement of the beads, and the supernatant was transferred to a clean tube. IMS was performed using 80µL beads for 2mL diluted caecal content and once isolated the bead-*C. jejuni* complex was washed 4-5 times with PBS containing 0.1% Tween 20 to remove mucous and other viscous components present within the caecal content. Bacteria were eluted with 0.05% trypsin; the suspension was concentrated by centrifugation for 10 000g 5 min at 4°C. All IMS steps requiring RNA isolation were performed on ice or at 4°C. The bacterial pellet was either stored at -80°C for RNA extraction or used immediately for downstream applications.

2.9.4 Dot blot assay

To determine the specificity of the rabbit anti-*Campylobacter jejuni* polyclonal antibody for isolation of *C. jejuni* from an *in vivo* animal host, a dot blot assay was performed. 50µL of bacterial suspension at a concentration of 10^8 cells/mL was loaded into a 96 well plate. The bacterial suspension was blotted onto PVDF membrane transferring approximately 10^7 bacteria and allowed to dry for 15 min. The membrane was blocked for 1 hr in 5% skim milk powder PBS with 0.05% tween 20. This membrane was

washed three times for 5 min in PBS with 0.05% tween 20. The primary antibody; rabbit anti-*C. jejuni* polyclonal (Fitzgerald) was added at a concentration of 5 μ g (2 μ L) in 10mL of PBS with 5% skim milk powder and 0.05% tween 20 and incubated with agitation at room temperature for 1-1.5 hrs. The membrane was washed three times with PBS containing 0.05% tween 20 for 5 min and the secondary antibody; Anti Rabbit IgG, HRP-linked (Cell Signalling Technology) added at the same concentration as the primary antibody. This was incubated for 1 hr with agitation. Again the membrane was washed three times and transferred to X-ray film as per standard methods.

2.10 Arrays

2.10.1 Glycan array

C. jejuni grown either on selective media at 37°C or 42°C for 18-24 hrs under microaerobic conditions or were isolated from chicken/mouse caecal content by IMS after infection. Colonies from agar cultures were harvested into Brucella broth and centrifuged for 1 min at 10 000g and processed using the IMS protocol previously described.

Fluorescent labelling of *C. jejuni*: The isolated bacteria were pelleted, washed twice in PBS pH 7.4 before resuspension in 500 μ L of PBS pH 7.4 containing 25 μ M carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; Molecular Probes). Fluorescent labelling was allowed to proceed for 30 min at 42°C, following which cells were collected by centrifugation, washed three times with PBS pH 7.4 and suspended in a final volume of 500 μ L. Hybridisation, data acquisition and analysis of glycan binding was performed as previously described (Day *et al.*, 2009).

2.10.2 RNA purification and labelling for microarray

Independent purification of RNA from *in vivo* isolated *C. jejuni* 81116 and laboratory grown *C. jejuni* 81116 was performed using the Qiagen RNeasy mini isolation kit. Briefly RNase Inhibitor (Invitrogen) was added directly to the bacterial pellet which was resuspended in 100 μ L TE buffer containing 400 μ g lysozyme/mL by vortexing. After 5 min incubation the manufacturer's instructions were followed including the 'on-column' DNase treatment with 50 μ L RNase-free water added to the membrane, and the spin column immediately centrifuged at 8000g for 1 min. RNA from *C. jejuni* was labelled for microarray analysis with Alexa Fluor 555 using the SuperscriptTM Plus Indirect cDNA labelling System (Invitrogen) as per the manufacturer's protocol.

2.10.3 Oligonucleotide design for microarray

The arrays used for transcriptome analysis are based on the genomes of *C. jejuni* 11168, *C. jejuni* RM1221, *C. coli* RM2228 along with any miscellaneous genes and plasmids from other strains available in the database. They are composed of synthetic oligonucleotide 70-mers: T_m 71°C \pm 5°C, location from 3' end of gene >70bp, poly (N) tract <8bp, stem length in potential hairpin <8bp contiguous bases common to any non-self ORF <20bp, cross-hyb identity to all other genes <70%. Kindly provided by Ian Connerton (School of Biosciences, Faculty of Science, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE125RD, United Kingdom).

2.10.4 Microarray hybridisations

The slides prepared for microarray were γ APS coated, A⁺ bar-coded Schott nexterion slides. The slides were printed according to *Campylobacter* dense array gal by *Microgrid* II GOD robot using TAS software version V2.4.03. This had 2500 pins with a 26 x 26 pin grid configuration. The spacing between the spots was 0.165 mm and the

slides were rehydrated by incubation at room temperature in a humidity chamber for 30 min followed by a 30 min incubation at 60°C to dry. The slides were blocked in a solution containing 100mM ethanolamine, 1M Tris (pH 9.0), and 0.1% SDS for 15 min at 50°C, then thoroughly rinsed with water and spun dry. The slides contain duplicate sets of oligonucleotides representing the genomes of *C. jejuni* NCTC11168, RM1221 and *C. coli* RM2228.

Microarray slides were pre-scanned at 532nm before being washed and pre-blocked for hybridisation. Washes were performed in 0.2% SDS PBS and followed by a 1 hr blocking step in 5 x saline sodium citrate (SSC), 0.2% SDS, 0.1% BSA. The slides were then rinsed twice with water, once with ethanol and centrifuged dry (100g for 3 min). The slides were then ready for hybridisation. Microarray hybridisations were performed using cDNA derived from both *in vitro* and *in vivo* isolated *C. jejuni*.

2.10.5 Data acquisition and analysis

Fluorescence intensities of array spots were measured using the ProScanArray Microarray 4-Laser Scanner (PerkinElmer). Microarray slides were scanned using the software setting for Alexa 555, green HeNe 543.5nm excitation laser set to 555 excitation and 565 emission. Glycan array slides were scanned on the FITC setting using the blue argon 488 excitation laser set to 494 excitation and 518 emission. Both sets of arrays were further analysed using the ProScanArray imaging software ScanArray Express (PerkinElmer). An arbitrary five-fold regulation was used as a minimum fold change to be considered regulated due to insufficient array-wide data.

2.11 Real-Time PCR

2.11.1 RNA purification and cDNA synthesis

Extraction of RNA was performed using the Qiagen RNeasy mini isolation kit with 'on-column' DNase treatment. 40ng of total RNA was processed into cDNA using the Promega Improm-II reverse transcriptase system using 3mM of MgCl₂, random primers and dNTPs. All samples were transcribed using universal conditions, 42°C for 1 hr and the same reverse transcriptase mastermix, to minimise differences in RT efficiency.

2.11.2 Quantitative real-time PCR

The QPCR reactions were set up to a final volume of 20µL with 2µL of cDNA, 5µmol of forward and reverse gene specific primers (Table 2.4) and 1x SensiMix SYBR green PCR master mix (Quantace, UK). The PCR were performed using the Bio-Rad iQ5 system and analysed using the iQ5 software package.

All QPCR reactions were carried out using the same thermal profile conditions, an initial step of 94°C for 5 min, followed by 45 cycles of 30 s at 94°C, then 30 s at 48°C and 72°C for 1.5 min with fluorescence measured during the 72°C extension phase. Melt curves were produced for each amplification product and these were measured 80 times over the incremental increases in temperature. Amplification plots and melt curves were analysed by the Bio-Rad iQ5 optical system software program. Products were reconfirmed by performing agarose gel electrophoresis. The cycle threshold C(t) of gene expression in different samples was used to analyse the relative expression corrected to 16S rRNA levels present in each sample (fold regulation). To calculate the fold difference in gene expression, the difference in C(t) in each sample is calculated using the comparative C(t) method (Schmittgen & Livak, 2008).

CHAPTER 3

Interaction of *Campylobacter jejuni* with a human colon adenocarcinoma cell line expressing MUC1

The content of this chapter has partially contributed toward the publication “MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. 2007. *J Clin Invest* **117**, 2313-2324.” Manuscript in appendix A.

3.1 Introduction

In vitro studies are an important tool used to highlight and investigate pathogenesis at the molecular and cellular level of many pathogenic organisms (Cottet *et al.*, 2002). The widespread use of tissue culture assays provides an alternative to investigating interactions between bacteria such as *Campylobacter* spp. and the human host epithelium during infection (Friis *et al.*, 2005). Like other human enteric pathogens, *C. jejuni* infects humans by colonising the mucus layer of the intestine followed by adherence and subsequent invasion of intestinal epithelial cells (Everest *et al.*, 1992; Harvey *et al.*, 1999; van Alphen *et al.*, 2008).

Previous studies have employed an *in vitro* cell invasion model to study *C. jejuni* pathogenesis successfully (MacCallum *et al.*, 2006). These include the elucidation of the role of flagella and importance of chemotaxis (Wassenaar *et al.*, 1994; Yao *et al.*, 1997). Motility is an important mechanism involved in the colonisation and pathogenicity of *C. jejuni* with observations that movement occurs toward mucin (Hugdahl *et al.*, 1988). Chemoattractants of *C. jejuni* include components of mucins such as L-fucose and L-serine (Hugdahl *et al.*, 1988). It has also been reported that *C. jejuni* can colonise the intestinal mucus itself, by actively swimming up and down the caecal crypts (Lee *et al.*, 1986) rather than adhering directly to epithelial cells. Therefore chemotaxis toward mucin and efficient motility in a viscous environment enables the bacteria to be localised within the mucus (Korolik & Ketley, 2008; Szymanski *et al.*, 1995).

In mice *C. jejuni* colonise the mucus layer and crypts of the intestinal mucosa, mainly on the colon and caecum, while in other animals different parts of the gastrointestinal tract may be colonised depending on the ecological niche (Lee *et al.*, 1983). *C. jejuni*

typically colonises the ileum and colon after passage through the stomach of a human (Hu & Kopecko, 2008), therefore for the purpose of *in vitro* studies, cell lines of human intestinal epithelial origin are considered to be most appropriate for studying invasion of human host cells (Friis *et al.*, 2005). Extensive investigation has suggested that *C. jejuni* have specific stress response mechanisms to cope with the toxic effects of oxygen outside the environment of the gut (van Vliet *et al.*, 2002) but the potential effects of aerobic incubation, as routinely performed in *in vitro* studies, on the bacterial survival and invasion is unknown (Friis *et al.*, 2005). As most *in vitro* invasion assays are based on the gentamicin protection assay utilising CO₂ conditions, it is logical to assume that these conditions may be suboptimal for microaerophilic bacteria such as *C. jejuni*. Current studies have explored the use of microaerobic conditions for *in vitro* studies (Cottet *et al.*, 2002; Grant *et al.*, 2006; Linden *et al.*, 2007; McAuley *et al.*, 2007) thus this chapter will explore the use of an alternative *in vitro* technique utilising microaerobic conditions for investigating the influence the cs-mucin MUC1 has on the pathogenesis of *C. jejuni*.

3.2 Results

3.2.1 Analysis of MUC1 expression in an *in vitro* culture model

A previously constructed plasmid containing the full length MUC1 recombinant cDNA insert (pcDNA3.1-MUC1)(MMRI, 2002) was transfected into the human colon adenocarcinoma cell line HCT116 deficient in MUC1 (King, 2002). The expression levels of MUC1 in HCT116 cells were determined in order to assess the influence MUC1 has on the ability of *C. jejuni* 81116 to adhere and invade human intestinal cells *in vitro*. A number of transfected clones were randomly selected and subjected to flow cytometric analysis to determine the fraction of cells expressing MUC1. Analysis of 2 such clones are shown in Figure 3.1.

Analysis of these clones indicates that control HCT116 (pcDNA3.1) cells did not express MUC1 (Figure 3.1A), as indicated by the lack of a shift in the cytometric curve of HCT116 cells labelled with the BC2 monoclonal antibody specific for MUC1 (dark line), compared to the control cells labelled with the non-specific monoclonal antibody 401.21, which provided a null control for MUC1 expression (grey curve). This cell line was designated EC1.

In contrast, HCT116 cells transfected with the gene encoding cell surface mucin MUC1 indicated a greater than 1 log shift for the BC2 labelled cells (dark line), compared to the control labelled (401.21) cells (grey area) (Figure 3.1B). This significant shift in fluorescence intensity indicates that the HCT116 (pcDNA3.1-MUC1) cells are expressing a moderate to high level of MUC1 and were subsequently designated EM10.

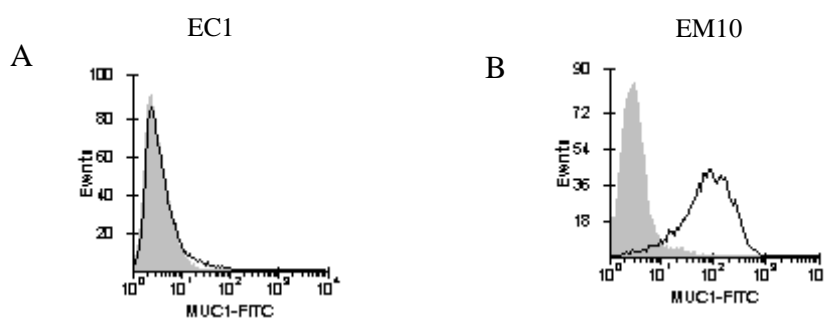


Figure 3.1 MUC1 expression of HCT116 transfected clones

Flow cytometric analysis of transfected HCT116 cells. Harvested pcDNA3.1 and pcDNA3.1-MUC1 transfected HCT116 cells were labelled with BC2 conjugated FITC MAb for MUC1 detection (dark line), or labelled with the isotype control 401.21 conjugated FITC MAb (grey area). (A) Control cells with pcDNA3.1 vector only. (B) pcDNA3.1-MUC1 transfected HCT116 cells.

3.2.2 Analysis of *in vitro* culture conditions using HCT116 cells

Standard *in vitro* culture conditions employ using CO₂ as the incubating atmospheric condition, however previous studies have shown that for microaerophilic organisms such as *Helicobacter* spp. and *Campylobacter* spp., using microaerobic conditions produces more consistent and reliable results as it allows the bacteria to interact with mammalian cells in an environment that mimics the gastrointestinal milieu more realistically (Linden *et al.*, 2007).

To determine if atmospheric culture conditions influence the *in vitro* adherence and invasion of *C. jejuni* 81116, co-culture with the cell line HCT116 was performed under standard CO₂ conditions and microaerobic conditions (5% O₂, 10% CO₂, 85% N₂). Adherence of *C. jejuni* 81116 to HCT116 cells was determined to be 1.8% for CO₂ incubating conditions and 0.195% for microaerobic incubating conditions (Figure 3.2A). This indicates that there is a significant difference ($p < 0.05$) in the ability of *C. jejuni* 81116 to adhere to HCT116 cells in the presence of standard 10% CO₂ and 90%

atmospheric O₂ concentrations compared to the restricted 5% O₂ concentration in microaerobic conditions. This data suggests that atmospheric culture conditions influence the ability of *C. jejuni* to adhere to the host cell.

Invasion of *C. jejuni* 81116 into the HCT116 cell line however did not show a significant difference between culture conditions (Figure 3.2B). The average invasion under CO₂ was determined to be $5.47 \pm 2.3\%$ compared to invasion under microaerobic conditions ($2.16 \pm 0.08\%$).

One important aspect that has been identified by comparing atmospheric culture conditions is the reproducibility of adherence and invasion rates under CO₂ versus microaerobic conditions (see error bars Figure 3.2). Previous studies have shown that cell lines are not adversely affected by microaerobic growth (Linden *et al.*, 2007; McAuley *et al.*, 2007), however it is evident by the range of standard error obtained that co-culture under microaerobic conditions produced more reliable and consistent data compared to culture under standard CO₂ conditions. Additionally the co-culture assays performed under microaerobic conditions promoted a higher and more consistent proportion of *C. jejuni* 81116 cells displaying high motility as observed in plate motility assays.

3.2.3 Analysis of MUC1 expression in an *in vitro* culture model

In order to investigate the effect MUC1 expression has on *C. jejuni* adherence and invasion, an *in vitro* bacterial–mammalian co-culture assay was performed under microaerobic conditions. Three cell lines were used; the parent HCT116 cells and EC1

(HCT116pc-DNA3.1) cells were used as the control cell lines, and EM10 (HCT116pcDNA3.1-MUC1) was used as the mucin expressing cell line.

Adherence of *C. jejuni* 81116 to the control cell lines HCT116 and EC1 was determined to be 0.19% and 0.75%, respectively. This corresponds to the 0.195% adherence rate determined previously for this cell line under microaerobic conditions. In contrast, adherence of *C. jejuni* 81116 to EM10 cells expressing MUC1 appears to be significantly higher (4.6%, $p < 0.001$, Figure 3.3A). Thus, suggesting that MUC1 may enhance binding and attachment of *C. jejuni* 81116 to the host cell surface.

Invasion of *C. jejuni* 81116 into EM10 cells was also significantly higher (4.8%; $p < 0.001$, Figure 3.3B) than invasion into the control cell lines; parent HCT116 (2.1%) and EC1 (2.8%). This enhanced invasion into MUC1 expressing cells illustrates that the presence of MUC1 on the epithelial cell surface has a significant impact on the ability of *C. jejuni* 81116 to not only attach to the cell surface but also to penetrate human intestinal cells.

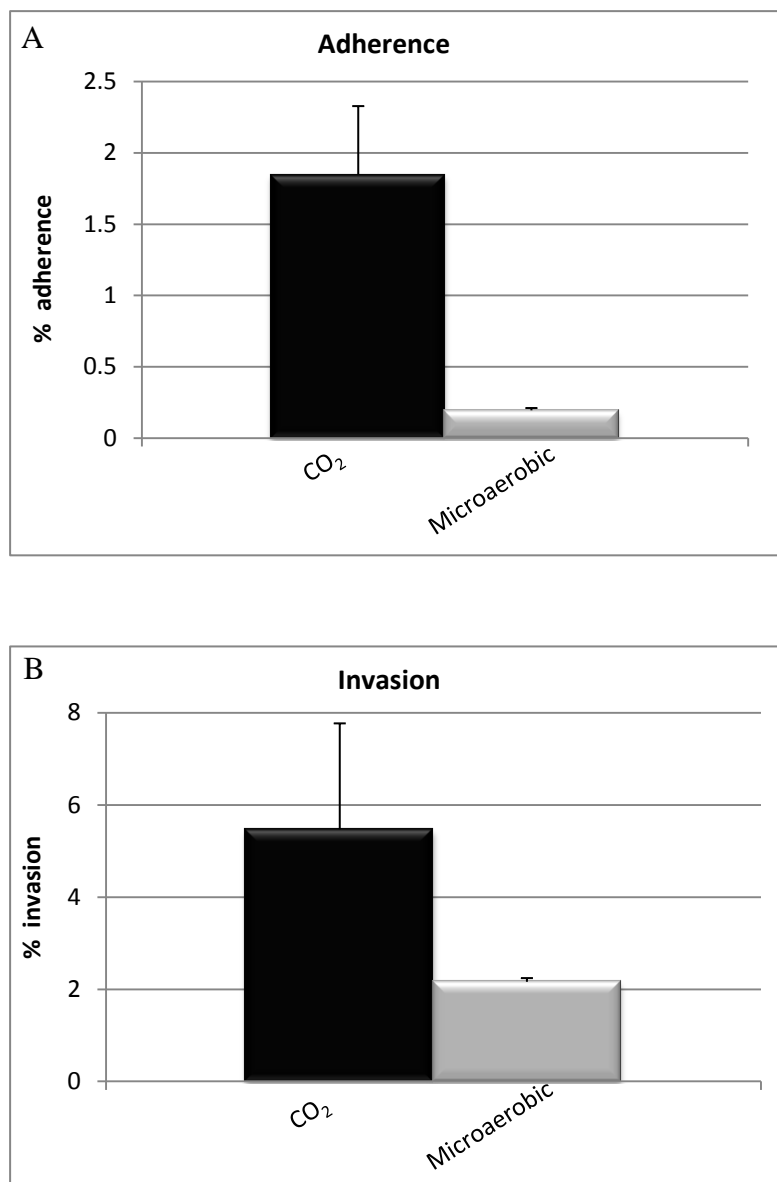


Figure 3.2 Adherence and invasion of *C. jejuni* 81116 into HCT116 cells. Adherence and invasion of *C. jejuni* *in vitro* cell culture model using HCT116 cells. (A) Adherence and (B) invasion analysis of *C. jejuni* 81116 on HCT116 parent cell line incubated in either CO₂ culture conditions (black bar) or microaerobic culture conditions (grey bar). Results are presented as mean adherence or invasion of six independent assays. Standard errors are indicated. Two tailed t-test showed significant difference in adherence between culture conditions $p < 0.05$ and no significant difference in invasion between culture conditions ($p = 0.35$).

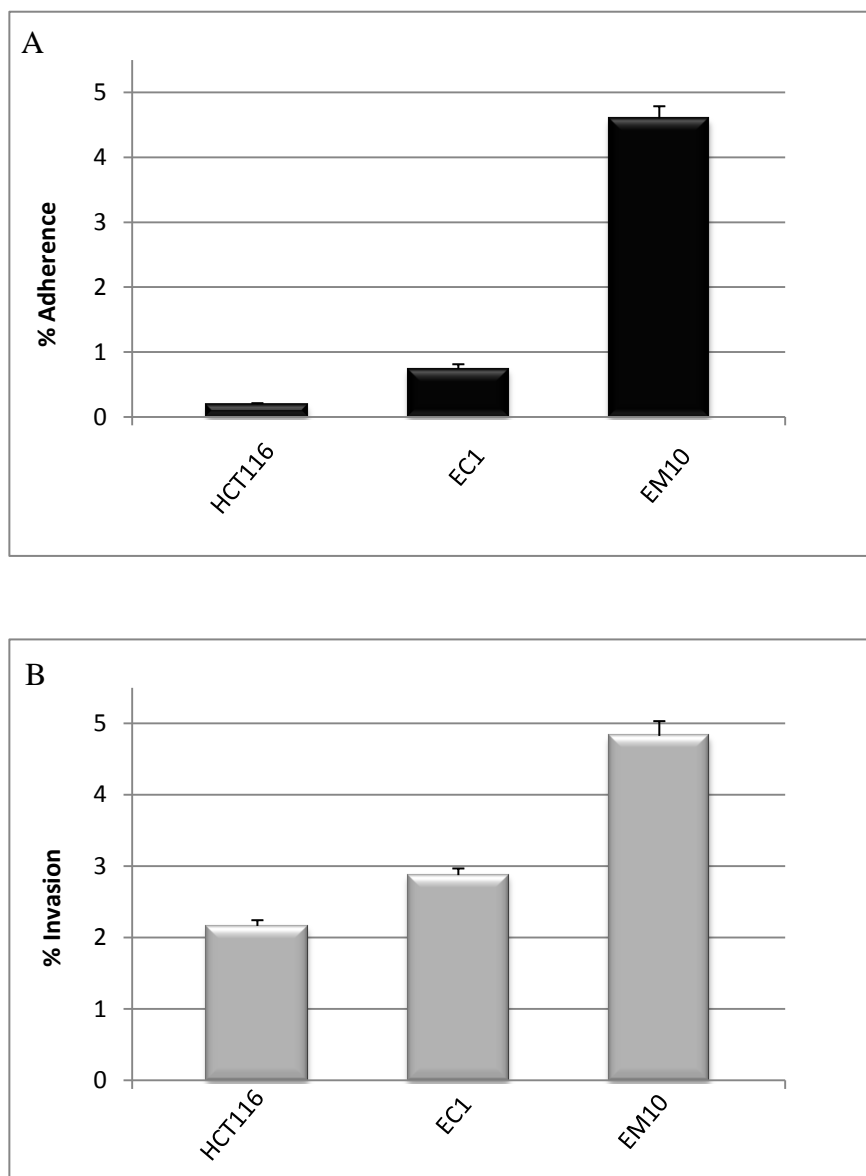


Figure 3.3 Adherence and invasion of *C. jejuni* 81116 into MUC1 expressing cells. Adherence and invasion of *C. jejuni* *in vitro* cell culture model using HCT116 cells. (A) Adherence analysis of *C. jejuni* 81116 on HCT116 parent cell line, HCT116 cells transfected with vector only, HCT116 cells expressing MUC1. Results are presented as mean adherence of four independent assays. Standard errors are indicated. Two tailed t-test showed a significant increase in adherence of HCT116-pcDNA3.1-MUC1 compared to the parent or vector only cell line $p < 0.001$. (B) Invasion analysis of *C. jejuni* 81116 on HCT116 parent cell line, HCT116 cells transfected with vector only, HCT116 cells expressing MUC1. Results are presented as mean invasion of four independent assays. Standard errors are indicated. Two tailed t-test showed a significant increase in invasion of HCT116-pcDNA3.1-MUC1 compared to the parent or vector only cell line $p < 0.001$.

3.2.4 Cell surface interaction of *C. jejuni* 81116 with HCT116 cells expressing MUC1

To investigate the cell surface interaction of *C. jejuni* with HCT116 cells expressing MUC1 an immunofluorescence detection assay was performed. The staining of both bacteria and cell line allowed visualisation of the interaction that occurs between *C. jejuni* and the cs-mucin MUC1 during the attachment process *in vitro*.

C. jejuni 81116 was fluorescently labelled with CFDA-SE to produce green fluorescing cells as per materials and methods section 2.10.1. EM10 cells expressing moderate to high levels of MUC1 were subject to a three-step staining technique. Initially EM10 cells were labelled with the BC2 antibody to bind the MUC1 extracellular domain and subsequently labelled with a secondary antibody (goat anti-mouse 594) to generate red fluorescence when MUC1 is expressed on the HCT116 cell surface. The final step involved labelling the HCT116 cells with DAPI to produce nuclei fluorescing blue as per materials and methods section 2.7.7.

Imaging of host cell-bacterial interaction was achieved using confocal microscopy. Figure 3.4 highlights the *C. jejuni* cells (green) migrating and concentrating toward the surface of intestinal cells expressing the higher concentration of MUC1 (red). Consequently, confirming the *in vitro* co-culture data which suggests that the presence of MUC1 on the apical cell surface significantly influences interaction of *C. jejuni* with the cell surface and facilitates subsequent adherence and invasion processes.

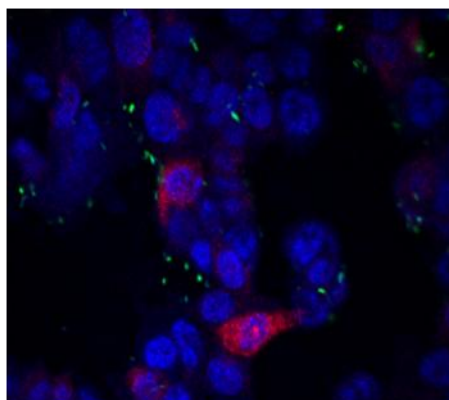


Figure 3.4 Interaction of *C. jejuni* with HCT116 cells expressing MUC1. Observed fluorescent images of interaction between *C. jejuni* (green fluorescence) and HCT116 intestinal cells (blue fluorescence) expressing MUC1 (red fluorescence) as captured by confocal microscopy.

3.3 Discussion

It has previously been established that cs-mucins and mucin carbohydrates influence the adherence of bacteria such as *H. pylori* and *P. aeruginosa* to mucosal cells (Lillehoj *et al.*, 2001; Linden *et al.*, 2004). We hypothesised that *C. jejuni* may also potentially use the cs-mucin MUC1 to exploit human mucosal epithelial cells and facilitate pathogenesis. This chapter investigated the influence MUC1 has on *C. jejuni* attachment and subsequent penetration of intestinal epithelial cells.

Co-culture between *C. jejuni* 81116 and HCT116 cells expressing MUC1, resulted in significantly greater adherence and invasion of *C. jejuni* to HCT116 cells expressing MUC1 compared to cells with no MUC 1 expression (Figure 3.3). Earlier studies have shown that *C. jejuni* can bind to mucin and mucin oligosaccharides (Hugdahl *et al.*, 1988; Ruiz-Palacios *et al.*, 2003) and have established that the presence of MUC1 on the epithelial surface promotes more rapid adherence of *C. jejuni* (McAuley *et al.*, 2007). In addition to this study, we have shown that the presence of MUC1 increases the number of *C. jejuni* that adhere to the cell surface subsequently leading to higher invasion.

The heightened level of invasion however may be directly related to the number of bacteria that adhered to the cell line. Invasion of *C. jejuni* into host epithelial cells is known to be dependent on adherence and motility (Newell *et al.*, 1985a). It had been previously theorised that high level of motility of *C. jejuni* cells in viscous environments increases the number of contacts or collisions of the bacteria with the host cell surface. In turn this results in increased attachment or binding, resulting in more organisms invading (Szymanski *et al.*, 1995). Our results support this theory with adherence and invasion rates of *C. jejuni* 81116 being 2-4% when interacting with cells

expressing MUC1 on the cell surface, implicating MUC1 as the influencing factor for this increase.

To confirm this finding, confocal microscopy was employed to visualise the interaction of *C. jejuni* with human intestinal cells expressing MUC1. Upon co-culture with cells expressing MUC1 on the surface it appears that *C. jejuni* migrate toward and concentrate around the surface of the cells with the most MUC1 present (Figure 3.4). This observation further supports the *in vitro* data which is suggestive of the cs-mucin MUC1 playing a significant role in the attachment and subsequent penetration of *C. jejuni* into human intestinal epithelial cells.

It has been suggested that MUC1 acts as a releasable decoy for bacterial adhesins thereby limiting attachment and subsequent invasion of *C. jejuni* to cells in the GI tract (McAuley *et al.*, 2007). In contrast we propose that *C. jejuni* may actually use MUC1 favourably to facilitate attachment and penetration of gastrointestinal cells. In order to initiate infection *in vivo* *C. jejuni* requires a low infective dose of approximately 500 organisms (Black *et al.*, 1988). This combined with observations that *C. jejuni* becomes hypermotile in highly viscous environments like that of the GI tract (Ferrero & Lee, 1988; Lee *et al.*, 1986; Wisessombat *et al.*, 2010) supports this assumption.

In vitro investigation of *C. jejuni* interaction with MUC1 yielded highly variable results, necessitating investigation of the effect of atmospheric incubating conditions has on adherence and invasion of microaerophilic bacteria. The gentamicin protection assay is established as a useful tool in studying host-bacteria interaction and cell binding, with the choice of cell line an important factor (Larson *et al.*, 2008). In order to assess interaction of *C. jejuni* to human host cells in an *in vivo* situation, cells must have the ability to form an adherent continuous polarised layer which is a prerequisite to bacteria

interacting with the apical cell surface (Friis *et al.*, 2005). Therefore the cell line HCT116 has been selected for the assessment of cs-mucin MUC1 which closely mimics the gastrointestinal environment in which the bacteria would normally interact with the epithelium of host cells.

Recent *in vitro* studies of microaerophilic bacteria have shown that host cell lines are not adversely affected by microaerobic culture conditions (Linden *et al.*, 2007). And that not only do *C. jejuni* proliferate better after microaerobic co-culture but also change virulence and gene expression that more closely resemble *in vivo* infection in the gastrointestinal tract (Klancnik *et al.*, 2006; Linden *et al.*, 2007). Recovery of *C. jejuni* displaying high motility from co-culture under microaerobic conditions correlates with these observations.

Furthermore, assessment of *in vitro* culture conditions in this study has shown that there is a significant difference in the ability of *C. jejuni* to adhere to cells under standard CO₂ conditions compared to incubation under microaerobic conditions (Figure 3.2). In the gastric and intestinal mucosa the O₂ concentration is 3-5% and CO₂ is 9% (Dawson *et al.*, 1965; Pruden *et al.*, 1986), indicating that the microaerobic conditions in which *C. jejuni* thrive are actually closer to the environmental conditions *in vivo* for the human gastrointestinal tract than standard culture conditions.

An interesting and important factor that has been identified from comparing CO₂ and microaerobic co-culture conditions is the reproducibility of assay results appears to be more consistent and reliable when performed under microaerobic conditions (Figure 3.2 error bars). This alternative assay technique may contribute to standardising adherence and invasion data between laboratories.

This chapter has demonstrated that culture conditions more conducive to *C. jejuni* can be used as an alternative to the standard CO₂ culture conditions practiced in most laboratories and these conditions actually produce more consistent and reliable data with reference to adherence and invasion rates of a microaerophilic organism. Furthermore, the use of *in vitro* assays has demonstrated the importance of the cs-mucin MUC1 in the adherence and invasion of *C. jejuni* into intestinal cells.

CHAPTER 4

**Construction and analysis of a *Campylobacter* specific integrative
expression vector containing Green Fluorescent Protein (GFP)**

4.1 Introduction

The production of *Campylobacter* spp. expressing fluorescence is a unique step in characterising the interaction that occurs during adherence and invasion of bacteria with the host epithelium. It will allow *in vitro* processes to be visualised via microscopy in order to develop a greater understanding of the pathogenic mechanisms utilised by bacteria to invade cultured cells. It has been demonstrated in the past that genetic manipulation of *Campylobacter* spp. is problematic (Taylor, 1992) with genes from *C. jejuni* often being difficult to clone or analyse in *E. coli* primarily due to the AT rich *C. jejuni* genome (Wösten *et al.*, 1998). Thus efficiency of expression may be affected due to the absence of required accessory factors (Ketley, 1997). The development of a *Campylobacter* expression vector containing Green Fluorescent Protein (GFP)/Red Fluorescent Protein (RFP) incorporating a *gfp/rfp* gene with its own promoter sequence would be beneficial for genetic studies and may enable more efficient genetic manipulation of *Campylobacter* spp. DNA.

The aim of the work undertaken for this chapter was to investigate the ability of an integrative expression vector to be used to introduce a gene that encodes for GFP or RFP into the genome of *C. jejuni* via homologous recombination. This can be achieved by inserting the *gfp/rfp* gene into the *flaB* region to generate *C. jejuni* stably expressing GFP/RFP for use in *in vitro* and *in vivo* investigations of pathogenesis.

Genetic variation in *Campylobacter* strains is well documented (Ahmed *et al.*, 2002; Leonard *et al.*, 2003; Poly *et al.*, 2005), therefore for efficient integration of DNA into the genome of *C. jejuni*, the length of homologous DNA to be integrated requires no less than 200bp (Wassenaar *et al.*, 1993). Additionally, efficiency and location of integration increases with homologous DNA length or size (Richardson & Park, 1997;

Wassenaar *et al.*, 1993). In *C. jejuni* 81116 a high species specificity of DNA is required for recombination and if flanking homologous sequences are sufficient in length, a double cross over event should occur (Bleumink-Pluym *et al.*, 1999). One prospective region for insertion of *gfp* into the genome is the *fla* genes. It is well documented that a functional *flaA* gene in *C. jejuni* is responsible for the production of flagella and subsequent motility (Wassenaar *et al.*, 1991), while *flaB* is not essential for motility but is thought to be a DNA donor to compensate for mutation in the *flaA* region or to increase the immunogenic repertoire (Alm *et al.*, 1993a). It has been suggested that *flaB* may potentially be more stable than *flaA* (Mellmann *et al.*, 2004) as insertional mutagenesis of *flaB* has previously been shown to produce cells with functional flagella (Meinersmann & Hiett, 2000). This is a factor that was considered during construction of the *gfp/rfp* containing integrative vector as the vector pBF6A contains sequence amplified from the *flaA* and *flaB* genes from *C. jejuni* 81116 (Bleumink-Pluym *et al.*, 1999). And as *C. jejuni* are most efficiently transformed by DNA from its own species (Taylor, 1992) selection of this vector was a logical choice.

Previous studies have shown that the Green Fluorescent Protein of *Aequorea victoria* and Red Fluorescent Protein of *Discosoma* coral, can be expressed in a diverse number of bacterial species (Valdivia *et al.*, 2006). The cDNA encoding GFP/RFP can be fused to the DNA of virtually any protein of interest and once expressed, folds independently of the protein to which it is fused, becoming a strong fluorescent label (Bonsma *et al.*, 2005; Lukyanov *et al.*, 2010). One advantage of using the reporter gene *gfp* or DsRed *rfp*, is its ability to fluoresce in the absence of any added cofactor or substrate, and requires only molecular oxygen for the formation of the fluorescent chromophore (Amoh *et al.*, 2008; Chalfie *et al.*, 1994; Lukyanov *et al.*, 2010). Furthermore, as GFP/RFP remains intracellular, interference between bacteria and host cell is minimal,

thus allowing assessment of host-bacterial interactions (Valdivia *et al.*, 2006). An extra advantage of RFP is it has reduced autofluorescence and photodamage at longer wavelength excitations (Mizuno *et al.*, 2001; Muller-Taubenberger & Anderson, 2007), thus is preferable for use in live cell microscopy. Both GFP and RFP are attractive reporters which can be easily distinguished from each other and are compatible (Dunn *et al.*, 2006; Sorensen *et al.*, 2003). Furthermore, DsRed does not overlap with green autofluorescence of cells (Bonsma *et al.*, 2005), therefore permitting the possibility of dual-colour applications.

Organisms with a high AT rich genome like *Helicobacter* spp. have also been shown not to pose a barrier to expression (Josenhans *et al.*, 1998). And single copy fusions with strong promoters like that demonstrated in *Pseudomonas* spp. have yielded fluorescent bacteria (Christensen *et al.*, 1996). A major benefit of incorporating a gene encoding for Green Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP) into the *C. jejuni* genome is the potentially stable expression of this protein using an intrinsic tag for studying adherence and invasion *in vitro*. It was reasonable to assume that the *gfp/rfp* reporter gene coupled with a *Campylobacter* promoter consensus sequence will function at maximal capacity in *C. jejuni*.

4.2 Results

4.2.1 Amplification and cloning of the *Campylobacter* promoter consensus (P_c) sequence

Previous work by Miller *et al.*, 2000, described the construction of a *Campylobacter* shuttle vector system that was successful in generating fluorescent *C. jejuni* strains, however use of this shuttle vector system in our laboratory did not produce any *Campylobacter* spp. cells expressing GFP. This lack of expression may be contributed to strain specificity of the vector system; therefore an alternative approach to generating *Campylobacter* spp. strains of interest expressing Green Fluorescent Protein (GFP) was developed.

In order to achieve expression of fluorescence in *C. jejuni*, a vector containing the gene encoding for Green Fluorescent Protein (GFP) and *Campylobacter* promoter needed to be constructed. Initially the promoter consensus sequence was amplified from the promoter region upstream of the *Campylobacter* kanamycin resistance cassette at nucleotide 62 to 309 from the pMW2 vector (Wösten, 1997). This promoter contains the -10, -16 and -35 nucleotide sequences, ensuring the distances between the ribosomal binding site and translational start codon were maintained, correlating with the previously identified *Campylobacter* consensus sequence (Wösten *et al.*, 1998). The 197bp *Campylobacter* promoter sequence (P_c) was amplified by PCR with primers CpromBamHI-F and CpromNcoI-R incorporating *Bam*HI and *Nco*I restriction sites at the 5' and 3' termini respectively. Using standard cloning techniques the PCR product was cleaved and inserted into the commercial cloning vector pDsRed-Express (Clontech), an expression vector that encodes the gene for Red Fluorescent Protein, a multiple cloning site for expression in *E. coli* and an Amp^r gene (description in Table

2.2 and vector map in Appendix B). The presence of promoter was verified by PCR amplification (Figure 4.1) and sequence analysis (data not shown). This newly generated vector was designated pGU0311.

Additionally, after insertion of the P_c upstream of the *rfp* gene in pDsRed-express, expression of RFP was still observed to be functional in *E. coli*.

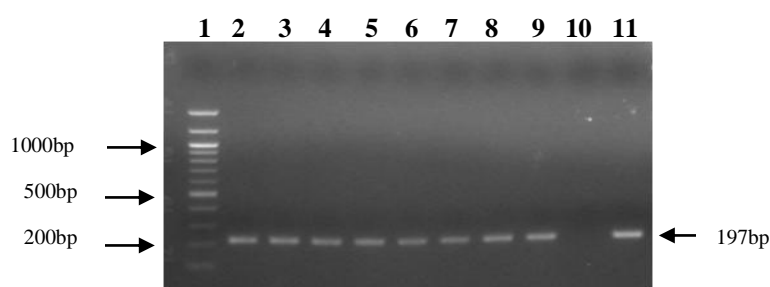


Figure 4.1 *Campylobacter* promoter amplicon resolved by gel electrophoresis. PCR amplification of the *Campylobacter* promoter consensus sequence (P_c). Lane 1: 100bp DNA ladder; Lane 2-9 amplified promoter sequence; Lane 10: H₂O control; Lane 11: DNA control.

4.2.2 Amplification and cloning of the Green Fluorescent Protein (*gfp*) gene

Due to a more complicated cloning strategy required to be implemented for generation of an *rfp* containing integrative vector, the integrative vector containing *gfp* was initially constructed. If integration and subsequent expression of GFP was successful in *C. jejuni*, construction of the *rfp* integrative vector would be undertaken.

To incorporate a gene encoding for GFP, a *gfp* gene previously used for development of vectors expressing fluorescent proteins was amplified from nucleotide 891 to 1607 in the vector pWM1007 (Miller *et al.*, 2000) using the primer set pWM1007gfp-F and

pWM1007gfp-R (Table 2.3). The amplified PCR fragment had the restriction sites *Bsp*HI and *Eco*RI incorporated at the 5' and 3' termini respectively to facilitate compatible insertion with the *gfp* gene downstream of the P_c sequence in pGU0311. The amplified *gfp* gene fragment was visualised by agarose gel electrophoresis (Figure 4.2), excised and purified as described in Chapter 2 section 2.5.6.

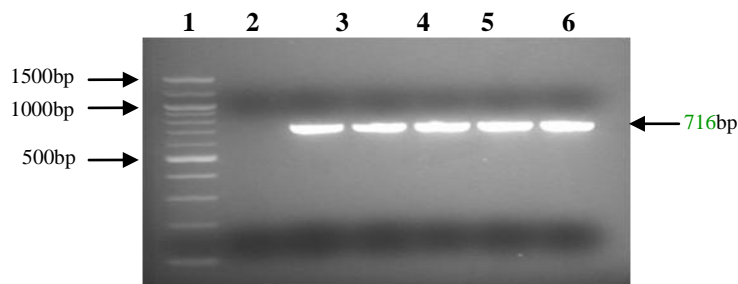


Figure 4.2 *gfp* amplicon resolved by gel electrophoresis
 PCR amplification of the *gfp* gene from pWM1007. Lane 1: 100bp DNA ladder;
 Lane 2: H₂O control; Lane 3-7: *gfp* amplicon.

To generate a cloning intermediate, the purified *gfp* gene was then used to replace the existing *rfp* gene in pGU0311 and subsequently the ligation product carrying the *Campylobacter* specific P_c and *gfp* gene was transformed into competent *E. coli* DH5 α cells. Putative transformants containing the generated construct were subject to selective pressure by ampicillin and screened for the presence of the plasmid by PCR using primers Cprom*Bam*HI-F and pWM1007gfp-R to amplify the P_c-*gfp* region (not shown). Restriction digest and sequence analysis was performed to confirm the integrity of the *gfp* gene and *Campylobacter* promoter sequence (P_c) to be without nucleotide deletions or substitutions (Figure 4.3 and 4.4 respectively). This cloning intermediate construct was designated pGU0515.

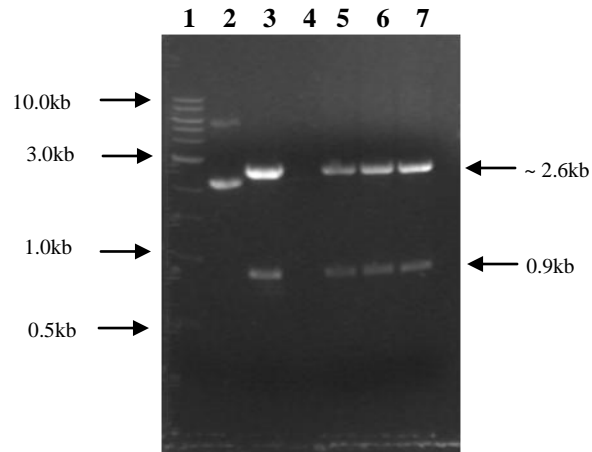


Figure 4.3 Restriction digest analysis of pGU0515

Restriction digest analysis of the constructed vector pGU0515 Lane 1: 1kb DNA ladder; Lane 2: Undigested pGU0311; Lane 3: Digested pGU0311 excising the P_c -*rfp* gene; Lane 4-7: digested potential pGU0515 showing the excision of the P_c -*gfp* fragment. Restriction digests were performed using *Bam*HI and *Eco*RI restriction enzymes.

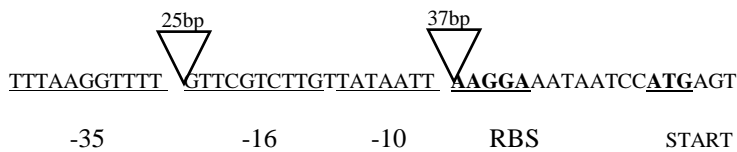


Figure 4.4 Schematic representation of P_c with the *gfp* gene

Schematic diagram depicting the incorporation of the P_c with the start codon of the *gfp* gene. Full sequence in appendix C.

4.2.3 Cloning of P_c -*gfp* into the integrative vector pBF6A

In order to generate an integrative vector for incorporating a *gfp* gene with *Campylobacter* P_c into the genome of *Campylobacter* spp., the cloning intermediate pGU0515 was purified and used as a template to amplify the P_c -*gfp* fragment. *Eco*RI and *Mfe*I restriction sites were incorporated at the 5' and 3' ends respectively, using primer set Cprom*Eco*RI-F and *gfpMfe*I-R. Concurrently, the pBF6A integrative vector was cleaved using the *Mfe*I restriction enzyme to allow compatible end ligation of the P_c -*gfp* fragment. The resulting recombinant plasmids were transformed into competent *E. coli* DH5 α cells, and putative recombinant transformants screened initially by PCR (not shown) followed by restriction digest analysis (Figure 4.5A). Sequence analysis was performed on purified plasmid DNA to ensure the integrity of the *gfp* integrative vector prior to transformation into *Campylobacter* spp. This analysis confirmed that the P_c -*gfp* gene is located in the *flaB* gene of the pBF6A vector in the same orientation as the Km^r cassette (Figure 4.5B). Modification of pBF6A created the construct pGU0608.

4.2.4 Transformation of pGU0608 into *Campylobacter* spp.

The pGU0608 *gfp* integrative vector was then transformed into *C. jejuni* 81116, *C. jejuni* 11168 and *C. coli* 427. Although electroporation was attempted to introduce pGU0608 into other *Campylobacter* spp. strains, no transformants were obtained. Therefore natural transformation was optimised and used for each strain of *Campylobacter* spp. to introduce the DNA into the genome. Natural transformation of *C. jejuni* 81116 was the most efficient with this vector construct, and the generation of GFP containing positive transformants exhibiting Km^r were verified by PCR (Figure 4.6A). The presence and location of the Km^r cassette and *gfp* gene in the chromosome of

each transformant was detected by using gene specific primers Km*Bgl*II-F and R; and pWM1007*gfp*-F and R (Table 2.3). However only one *C. jejuni* 81116 transformant was confirmed and verified to contain the integrated *gfp* gene (Figure 4.6B). It was observed that the *C. jejuni* 81116 successful transformant also displayed a highly motile phenotype.

DNA sequence analysis was attempted to investigate the integrity of the P_c-*gfp* gene in the genome of *C. jejuni* 81116; however due to the high sequence homology of the *flaA* and *flaB* genes only partial sequence of the *gfp* gene was obtained (data not shown). Despite multiple repeats and optimisation of the transformation parameters, no other *C. jejuni* 11168 or *C. coli* 427 successful transformants were detected.

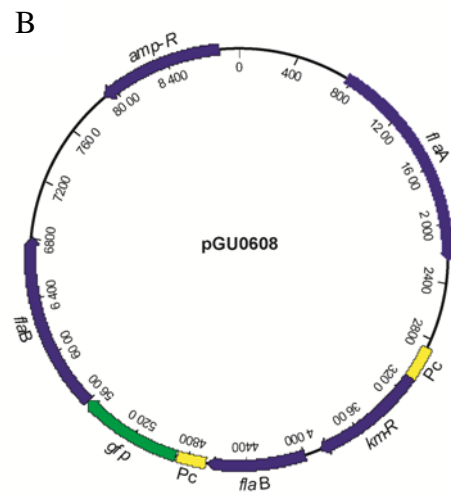
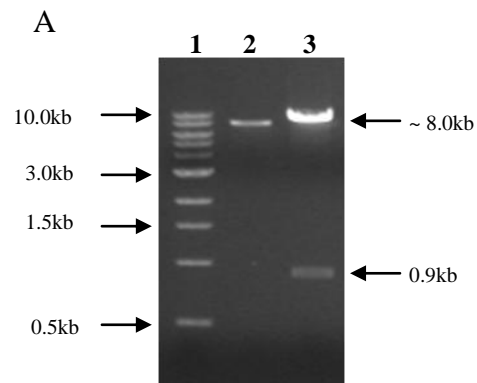


Figure 4.5 Confirmation of pGU0608 vector construct

(A) Restriction digest analysis confirming the presence of the P_c -*gfp* gene in pGU0608. Lane 1: 1kb DNA ladder; Lane 2: linearised parent vector pBF6A; Lane 3: digested pGU0608 vector indicating excision of the P_c -*gfp* gene from the vector backbone. (B) Schematic diagram representing pGU0608 using MacVector 9.0. The arrow heads indicate the confirmed orientation of genes, the yellow boxes represent the promoter sequences and the green arrow represents the *gfp* gene.

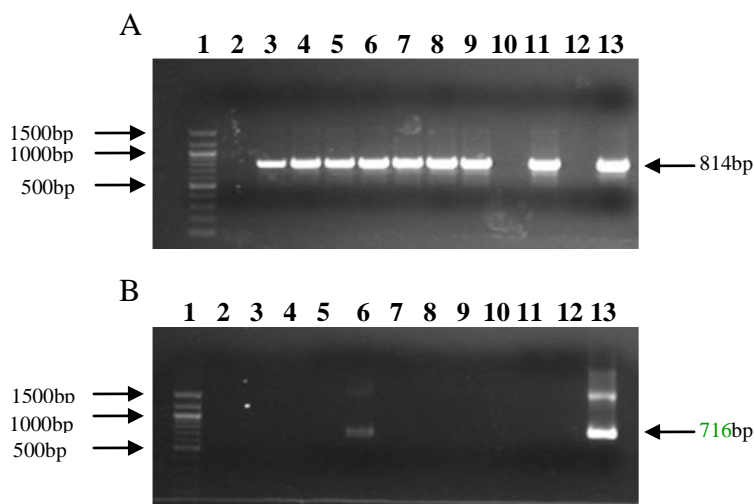


Figure 4.6 Amplicons of *Km^r* and *gfp* genes in *C. jejuni* 81116 transformants. (A) PCR analysis of *C. jejuni* 81116 transformants amplifying the *Km^r* cassette. (B) PCR analysis of *C. jejuni* transformants amplifying the *gfp* gene. Lane 1: 100bp DNA ladder; Lane 2: *C. jejuni* 81116; Lane 3: *C. jejuni* 81116 transformed with parent vector pBF6A; Lane 4-9: *C. jejuni* 81116 putative pGU0608 transformants; Lane 10: *C. jejuni* 11168; Lane 11: *C. jejuni* 11168 transformed with parent vector pBF6A; Lane 12: H₂O control; Lane 13: DNA control.

4.2.5 Assessment of GFP expression in *C. jejuni* 81116

Following confirmation of the introduction of the *P_c-gfp* gene by homologous recombination (section 4.2.4), expression and functionality of *C. jejuni* 81116 with the *gfp* gene in the genome was assessed.

Fluorescent Microscopy analysis was performed and resulted in the observation of green fluorescing *C. jejuni* 81116 cells under high magnification (1000x). These observations indicated that *C. jejuni* 81116 was expressing green fluorescence and there were on average 1% of fluorescent cells per field of view (data not shown). However unfortunately, the frequency of *C. jejuni* 81116 cells and subsequent overall intensity of fluorescence was at a level that was not able to be visualised by capture of fluorescent

images. Therefore a second strategy for assessing expression of fluorescence was employed.

Flow Cytometric Analysis was performed using a FACScalibur cytometer (BD Biosciences) and analysed using CellQuest software (BD BioSciences) to quantify the proportion of *C. jejuni* expressing detectable levels of green fluorescence. Again due to low level of fluorescence expression there was no significant shift in the cytometric curve of GFP compared to *C. jejuni* 81116 and *C. jejuni* 81116 cells containing the Km^r selective marker only (not shown). The third strategy implemented to determine expression was the measurement of fluorescence intensity in a Victor plate reader.

Fluorescence Intensity was measured by comparing expression of *C. jejuni* 81116 containing *gfp* as well as the selective marker Km^r cassette to *C. jejuni* 81116 containing the Km^r cassette only. Figure 4.7A indicates that there was more fluorescence detected in the cells containing the *gfp* gene. However the intensity of GFP expression in *C. jejuni* 81116 when compared to *E. coli* containing the pGU0608 vector was approximately 16-fold lower (Figure 4.7B). Higher level of expression in *E. coli* may be due to the pGU0608 vector performing as a replicative vector in *E. coli* and therefore producing more copies of the *gfp* gene to be detected.

The inability to quantify fluorescence illustrates that one copy of the *gfp* gene in *C. jejuni* 81116 genome may not be sufficient to get a level of GFP expression that is required to detect the bacterial cell and use in downstream applications.

4.2.6 Confirmation of integration of *gfp* into the *Campylobacter* spp. genomes during transformation

As only one fluorescent transformant was obtained for *C. jejuni* 81116 it was decided to investigate whether the overall integration of DNA into the genome of *C. jejuni* 81116, *C. jejuni* 11168 and *C. coli* 427 following transformation occurred. As successful integration of the gene was verified in *C. jejuni* 81116, this strain was included as the reference strain. During natural transformation the transformation (competent cells and DNA) mixture was subjected to treatment with DNase. This has been shown previously to perform efficiently in liquid culture and eliminate remnant DNA (Wilson *et al.*, 2003). Thus, the bacterial cells isolated could be assessed for *gfp* integration into the *Campylobacter* spp. genome.

Molecular Analysis of the transformation mixture was performed to confirm the integration of the Km^r cassette, which is the selective marker in the integrative control vector pBF6A and its *gfp* containing derivative pGU0608. This was achieved by amplification of Km^r cassette using a gene specific primer set, KmBgIII-F and R. Figure 4.8A shows that the Km^r cassette has been integrated into the genome of all 3 *Campylobacter* spp. strains for both the control vector pBF6A and pGU0608. The following PCR (Figure 4.8B) confirms the presence of the *gfp* gene within the genome of the 3 *Campylobacter* spp. strains using gene specific primers (pWM1007gfp-F and R). This analysis suggests that at the transformation stage, vector DNA was able to be introduced into the cells.

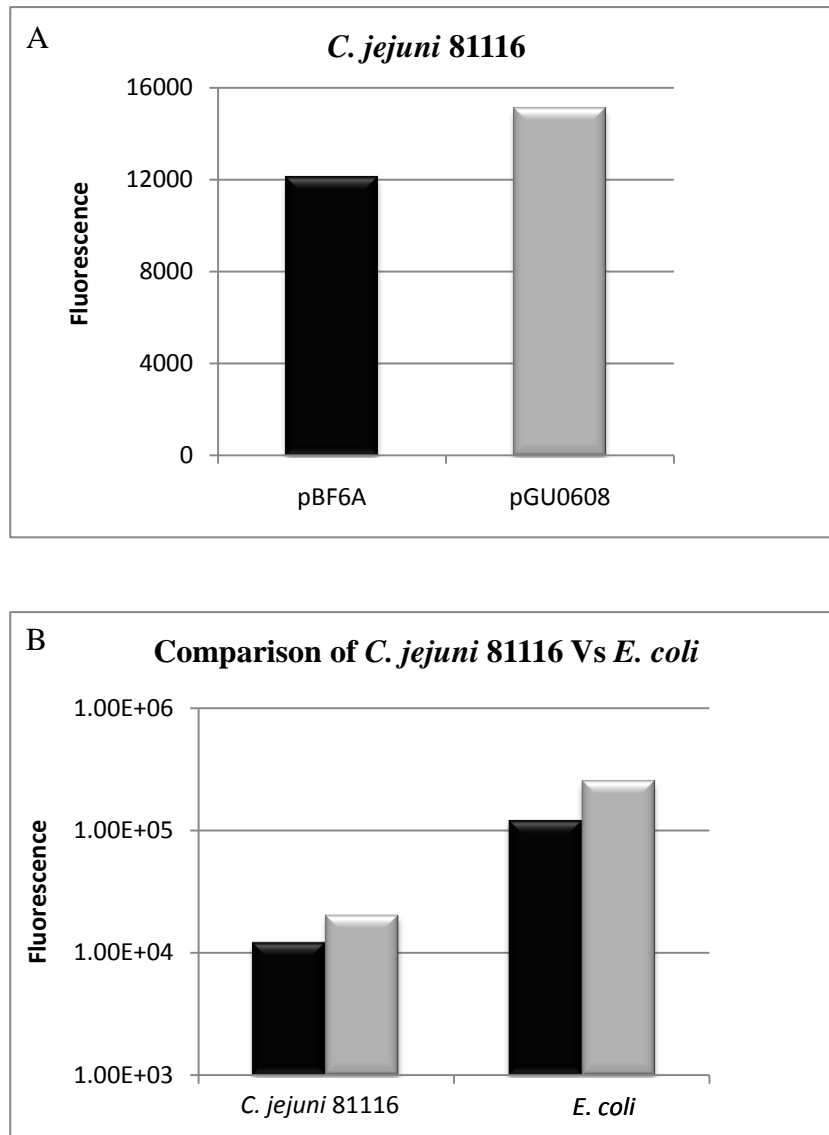


Figure 4.7 GFP expression of the integrated *gfp* gene in *C. jejuni* 81116. Intensity of GFP expression in *C. jejuni* 81116 as measured in a Victor plate reader. (A) Shows the difference in fluorescence detected between the genome of *C. jejuni* 81116 with the Km^r cassette only incorporated (represented by the black bar) compared to the Km^r and P_c -*gfp* gene incorporated into the *C. jejuni* 81116 genome (grey bar). (B) Indicates the level of fluorescence detected between *C. jejuni* 81116 containing one copy of the P_c -*gfp* gene in the genome compared to fluorescence detected in *E. coli* containing multiple copies of the *gfp* gene. The pBF6A control vector is represented by the black bar and *gfp* expression in pGU0608 is represented by the grey bar.

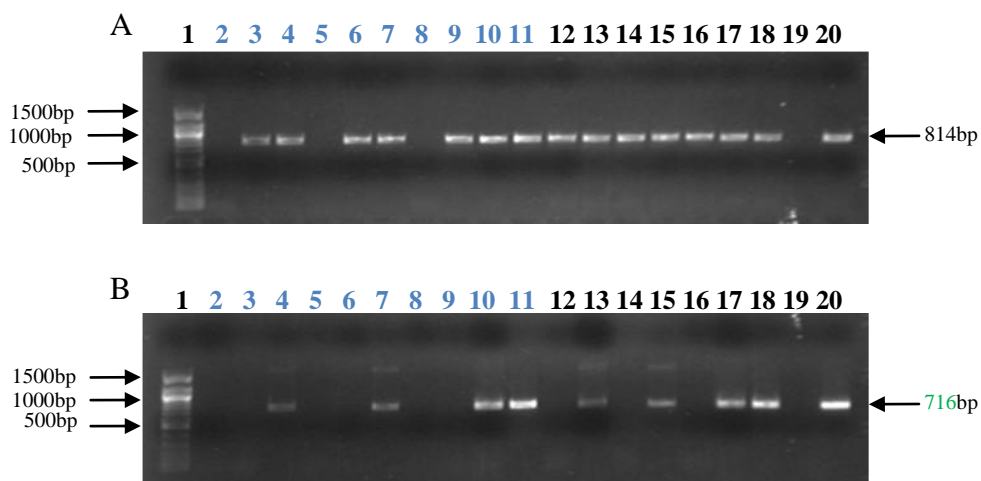


Figure 4.8 Amplicons of Km^r and *gfp* genes integrated into *Campylobacter* spp. genome during natural transformation. (A) PCR analysis of isolated *Campylobacter* strains amplifying the Km^r cassette from the transformation mixture. (B) PCR analysis of isolated *Campylobacter* strains amplifying the *gfp* gene from the transformation mixture. Lane 1: 100bp DNA ladder; Lane 2: *C. jejuni* 81116; Lane 3 & 12: *C. jejuni* 81116 pBF6A; Lane 4 & 13: *C. jejuni* 81116 pGU0608; Lane 5: *C. jejuni* 11168; Lane 6 & 14: *C. jejuni* 11168 pBF6A; Lane 7 & 15: *C. jejuni* 11168 pGU0608 Lane 8: *C. coli* 427; Lane 9 & 16: *C. coli* 427 pBF6A; Lane 10 & 17: *C. coli* 427 pGU0608 Lane 11 & 18: *E. coli* pGU0608; Lane 19: H₂O control; Lane 20: DNA control. The lanes highlighted blue (2-11) were subjected to treatment with DNase.

4.2.7 Assessment of fluorescence in transformation mixture

To confirm expression of GFP after integration of the *gfp* gene into *Campylobacter* spp. genome, fluorescence analysis was performed.

Fluorescence Microscopy analysis of transformation mixture identified green fluorescing *C. jejuni* 81116, *C. jejuni* 11168 and *C. coli* 427 cells under high magnification (1000x). The highest proportion of fluorescent bacterial cells was observed for *C. jejuni* 81116 with approximately 25% per field of view (data not shown). In contrast only 0.5% and 0.1% of fluorescent cells were observed for *C. jejuni*

11168 and *C. coli* 427, respectively. Even though fluorescent cells were observed, the intensity of fluorescence was at a level that was not able to be visualised by capture of fluorescent images. Unfortunately, these fluorescent bacteria could not be recovered as pure culture.

Flow Cytometric Analysis of the transformation mixture was performed using a FACScalibur cytometer (BD Biosciences) and analysed using CellQuest software (BD BioSciences) to assess the population of *C. jejuni* expressing detectable levels of green fluorescence. Expression of green fluorescence was at a level that did not produce a significant shift in the cytometric curves (data not shown).

Fluorescence Intensity was measured by a Victor plate reader. This allowed the comparison of intensity of fluorescence between the *Campylobacter* spp. genomes containing the Km^r cassette only and genome containing the Km^r and *gfp* genes. Detection of GFP in *C. jejuni* 81116 correlated with the previous observation that the *gfp* containing genome has a 1.6 fold higher level of fluorescence. Whereas *C. jejuni* 11168 and *C. coli* 427 did not appear to have any marked increase of fluorescence detected (Figure 4.9).

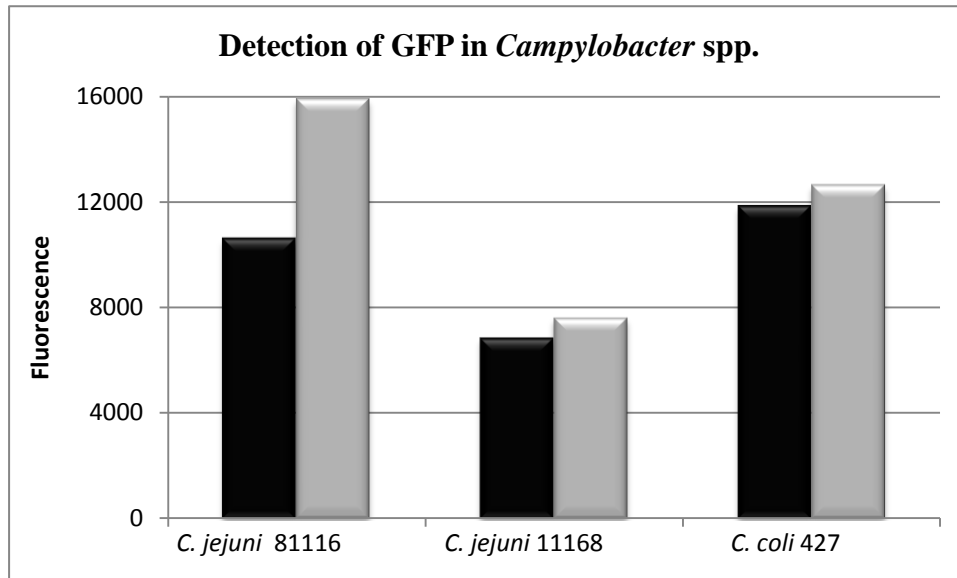


Figure 4.9 GFP expression of integrated *gfp* gene in *Campylobacter* spp. during natural transformation. Measurement of fluorescence intensity in *C. jejuni* 81116, *C. jejuni* 11168 and *C. coli* 427 during natural transformation. The black bar represents the level of background fluorescence by the integration of the Km^r cassette only of the control vector pBF6A and the grey bar represents the detection of fluorescence with the Km^r and P_c -*gfp* gene incorporated into the genome.

4.3 Discussion

This chapter describes the construction of an integrative *Campylobacter* vector containing *gfp* and subsequent analysis of GFP expression in *C. jejuni* 81116. Previous work by Miller *et al.* (2000) and more recently Mixer *et al.* (2003) described the construction of *Campylobacter* shuttle vector systems that were successful in generating fluorescent *C. jejuni* strains. However, use of the pWM1007 shuttle vector system by Miller *et al.* (2000) in our laboratory did not produce any *Campylobacter* spp. cells expressing GFP. Likewise, Mixer *et al.* (2003) also found that the expression of fluorescence was 10-fold lower compared to the vector system constructed in their laboratory. Additionally, stronger expression of fluorescent protein genes was documented by Mixer *et al.* (2003) when the *gfp* reporter gene was under the control of the *ompE* promoter (Mixer *et al.*, 2003).

One explanation for the inability to generate fluorescent *C. jejuni* using this vector system is the variation in *Campylobacter* spp. strains used. An ongoing issue with *Campylobacter* research is the lack of a universal vector system that will allow efficient delivery and expression of genes in a variety of *Campylobacter* spp. strains, regardless of use in different research laboratories. Therefore this study attempted to incorporate a previously used *gfp* gene (Miller *et al.*, 2000) with a known *Campylobacter* specific promoter sequence to enhance compatibility and facilitate expression in the *C. jejuni* genome. With the potential to provide an indiscriminate vector for introducing genes into various *Campylobacter* spp. strains, and enable more stable expression of GFP.

C. jejuni strains have very different promoter consensus sequences compared to numerous other prokaryotes and very few genes that function efficiently in other organisms. Therefore the introduction of a *Campylobacter* promoter sequence that

correlates with the consensus sequence previously identified for *C. jejuni* (Wösten *et al.*, 1998) was thought to be a strong promoter that would be able to initiate transcription and subsequent expression of the *gfp* gene.

To facilitate integration of the P_c-*gfp* gene into the genome of our target strain *C. jejuni* 81116, the integrative vector pBF6A was utilised. This particular strain is naturally highly competent and more receptive to DNA transfer via natural transformation (Bleumink-Pluym *et al.*, 1999; Wang & Taylor, 1990; Wassenaar *et al.*, 1993; Wiesner *et al.*, 2003). Additionally, it is able to undergo homologous DNA recombination with identical sequence between the DNA being introduced and genomic DNA (Bleumink-Pluym *et al.*, 1999; Wang & Taylor, 1990). With reference to pBF6A this vector contains the *flaA* and *flaB* genes from *C. jejuni* 81116 greatly enhancing efficiency as similar sequence is present (Bleumink-Pluym *et al.*, 1999). Insertion of the P_c-*gfp* gene into the *flaB* region of the pBF6A vector, in theory, should allow for homologous recombination to occur between the *flaA* and *flaB* genes (Nuijten *et al.*, 1990) due to presence of high sequence similarity. Furthermore, insertion into *flaB* should not disrupt the production of functional flagella and subsequent motility. The results obtained for transformation of pGU0608 into *C. jejuni* agrees with this finding, as natural transformation of *C. jejuni* 81116 was the most efficient with this vector construct and the transformant verified as containing the *gfp* gene was highly motile, suggestive of development of functional flagella. Observation of functional flagella indicates that introduction of *gfp* into genomic *flaB* is still able to produce a motile phenotype.

In contrast even though *C. jejuni* 81116 was transformed successfully with pBF6A, its *gfp* derivative pGU0608 only produced one transformant. This inability to recover

transformants may be associated with *C. jejuni* cells not being able to tolerate integration of the *gfp* gene into the genome, and as a result being toxic to the cell.

Analysis of GFP expression from this single recovered recombinant revealed that although the *gfp* gene was confirmed as being integrated into the genome, expression was at a level that was unable to be detected by methods other than fluorescence microscopy. And even then, fluorescence was low and images could not be generated to show expression above background autofluorescence. The GFP chromophore requires molecular oxygen, therefore level of fluorescence expressed decreases under reduced oxygen conditions. Perhaps incubation of *C. jejuni* under microaerophilic conditions affects the ability of the GFP chromophore to be expressed at maximal level. Additionally, expression depends on rate of synthesis and stability of a functional protein and if it is stable, rate of dilution as the cell divides (Valdivia *et al.*, 2006), therefore one copy of the gene integrated into the *C. jejuni* genome may not be sufficient for expression under microaerobic conditions.

Another explanation for loss of expression may be the phase variation in flagella. Phase variation can occur via strand slippage, recombination or gene inversion, resulting in activation/inactivation of genes that would affect the regulation of flagella assembly or motility (Grant *et al.*, 1993; Harris *et al.*, 1987; Neal-McKinney *et al.*, 2010). Consequently resulting in rearrangements of the inserted *gfp* gene and rendering it non-functional.

Alternatively perhaps lack of GFP expression in *C. jejuni* may be due to the low level of translation caused by different codon usage (Ketley, 1997). A relatively low fluorescence level in this case could be explained by a suboptimal codon usage in the *gfp* gene. Due to a low AT content in the *Campylobacter* spp. genome, its codon

preference is different from that in *E. coli* and eukaryotic cells. Codon adaptation more optimal for *C. jejuni* and the use of an additional promoter may allow for more efficient expression of GFP in *C. jejuni* even when the gene is present as just a single copy per cell, as was documented after the commencement of this project (Karlyshev & Wren, 2005).

Cloning strategies and difficulties experienced during construction were unexpected and to completely explain the cause of these difficulties requires further investigation. Due to time constraints mRNA and analysis of transcription was not performed. Therefore future work to investigate would include the analysis and adaptation of codon usage specific for *C. jejuni* and its environmental requirements.

CHAPTER 5

Construction of a shuttle vector expressing Green Fluorescent Protein

(GFP) and Red Fluorescent Protein (RFP) in *Campylobacter* spp.

5.1 Introduction

The integrative vector carrying *gfp*, described in Chapter 4.0, did not result in successful expression of GFP in levels required for downstream applications. The use of this vector resulted in one copy of the *gfp* gene integrated into the genome (Chapter 4.0). It was therefore considered that perhaps multiple copies of fluorescent protein genes may be required to enhance expression of GFP/RFP to detectable levels.

In order to introduce multiple copies of GFP or RFP into *Campylobacter* spp. strains, a shuttle expression vector, pGU0202 that was previously established in our laboratory was used. This vector has been previously shown to be efficient in expressing genes in both the *E. coli* transitional host and *Campylobacter* spp. strains, thus minimising the limitations associated with genetic manipulation, such as maintaining gene expression in both bacterial species (Alfredson & Korolik, 2003). Another benefit of using pGU0202 is it contains sequence from the cryptic plasmid pCJ419 from *C. jejuni* and plasmids originating in *Campylobacter* species have been shown to be transferable to related species (Alfredson & Korolik, 2003; Taylor *et al.*, 1981). A *Campylobacter* promoter consensus sequence that has been previously identified as a strong initiator (Wösten *et al.*, 1998) was chosen to drive the expression of *gfp* and *rfp* genes.

Fluorescence imaging technology is an invaluable tool for studying complex host-bacterial interactions. The evolution of fluorescent protein genes is ever increasing the possibilities for exploring *in vitro* and *in vivo* processes that occur between bacteria and the host. This chapter describes the development of a suite of *Campylobacter* shuttle vectors that will potentially enable more efficient genetic manipulation of *Campylobacter* spp. DNA and will aid in studies of host-*C. jejuni* interactions.

5.2 Results

5.2.1 Amplification of the P_c-Green Fluorescent Protein (*gfp*) gene and P_c-Red Fluorescent Protein (*rfp*) gene

To generate a shuttle vector containing genes that encode for GFP or RFP with expression driven by *C. jejuni* promoter, the P_c-*gfp* and P_c-*rfp* genes were amplified from the previously constructed vectors pGU0515 and pGU0311 (section 4.2.2 and 4.2.1), respectively. This was achieved using Cprom*EcoRI*-F and gene specific (*gfpBamHI*-R or *rfpBamHI*-R) primers. The P_c-*gfp* and P_c-*rfp* amplicons were visualised by agarose gel electrophoresis (Figure 5.1), excised and purified for further cloning. This approach incorporated an *EcoRI* restriction site at the 5' termini and *BamHI* at the 3' termini of the PCR amplicons to allow for compatible cloning into the replicative vector pGU0202.

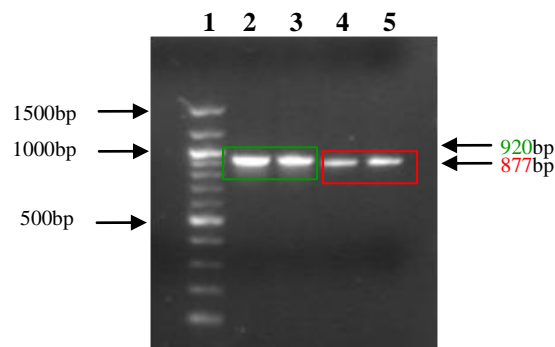


Figure 5.1 P_c-*gfp* and P_c-*rfp* amplicons resolved by gel electrophoresis. PCR amplification of the P_c-*gfp* gene from pGU0515 and P_c-*rfp* gene from pGU0311. Lane 1: 100bp DNA ladder; Lane 2-3: P_c-*gfp* amplicon (represented by green box); Lane 4-5: P_c-*rfp* amplicon (represented by red box).

5.2.2 Cloning of P_c -*gfp* and P_c -*rfp* into the replicative vector pGU0202

In order to generate a shuttle vector enabled for GFP and RFP expression, the existing vector pGU0202 was cleaved by restriction enzyme digest with *Eco*RI and *Bam*HI to allow compatible ligation between the vector and fluorescent protein gene amplicons (Figure 5.2).

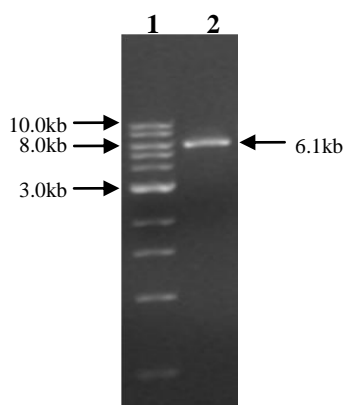


Figure 5.2 Linearisation of pGU0202 using restriction enzyme digest. Restriction digest of shuttle vector pGU0202. Lane 1: 1.0kb DNA ladder; Lane 2: linearised pGU0202 vector.

The ligation products containing the shuttle vector and fluorescent protein genes were transformed into competent *E. coli* DH5 α cells and potential transformants selected using kanamycin. The putative transformants that displayed Km^r were assessed by restriction digest profiles (Figure 5.3A), followed by DNA sequence analysis that confirmed the construct to be without nucleotide deletions or substitutions (data not shown). Insertion of the P_c -*gfp/rfp* genes was identified to be in the same orientation as the Km^r cassette (Figure 5.3B). The manipulated pGU0202 derivative containing the P_c -*gfp* gene was named pGU0610 and the vector containing the P_c -*rfp* gene was named pGU0611.

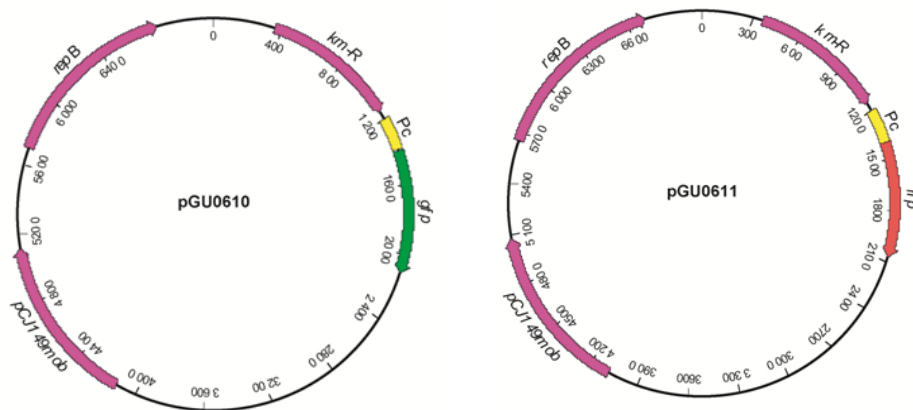


Figure 5.3 Confirmation of pGU0610 and pGU0611 vector constructs. (A) Restriction digest analysis to confirm the constructed shuttle vectors containing P_c -*gfp* and P_c -*rfp*. Lane 1: 1.0kb DNA ladder; Lane 2 & 6: Uncut positive pGU0610 transformants; Lane 3 & 7: pGU0610 transformants digested with *Eco*RI and *Bam*HI to excise the P_c -*gfp* gene; Lane 4 & 8: pGU0610 transformants linearised with *Eco*RI; Lane 5 & 9: pGU0202 parent vector linearised with *Eco*RI; Lane 10 & 14: Uncut positive pGU0611 transformants; Lane 11 & 15: pGU0611 transformants digested with *Eco*RI and *Bam*HI to excise the P_c -*rfp* gene; Lane 12 & 16: pGU0611 transformants linearised with *Eco*RI; Lane 13 & 17: pGU0202 parent vector linearised with *Eco*RI. (B) Schematic diagram representing the organisation of genes in the genetic map of constructs pGU0610 and pGU0611 using MacVector 9.0.

5.2.3 Transformation of pGU0610 and pGU0611 into *Campylobacter* spp.

As previously described (Chapter 4.0), *Campylobacter* spp. more readily uptakes DNA by natural transformation, therefore the introduction of *gfp* and *rfp* containing shuttle vector constructs pGU0610 and pGU0611 into *C. jejuni* 81116, 11168 and *C. coli* 427 was attempted using a previously optimised natural transformation protocol. After transformation into *Campylobacter* spp., the transformants exhibiting Km^r were investigated for the presence of the Km^r cassette and *gfp* or *rfp* genes using gene specific primer sets (Km*Bgl*III-F and R) or (pWM1007*gfp*-F and R; *rfp*-F and *rfpBam*HI-R). After numerous attempts only transformants for *C. coli* 427 were obtained.

The putative transformants obtained for *C. coli* 427 transformed with pGU0610 containing GFP were verified by PCR. The amplicon size for both the Km^r cassette and *gfp* gene were visualised using agarose gel electrophoresis (Figure 5.4A and B respectively).

Transformants exhibiting Km^r after transformation with pGU0611 containing RFP were similarly examined using PCR. The presence of Km^r and *rfp* amplicons was visualised by agarose gel electrophoresis (Figure 5.5).

Sequence analysis was performed on the purified plasmid DNA recovered from *C. coli* 427 transformants to assess if rearrangements or point mutations occurred during the transformation process. These constructs were confirmed to be without nucleotide deletions, substitutions or rearrangements (data not shown).

Despite multiple repeats, no transformants for *C. jejuni* 81116 or *C. jejuni* 11168 with either the control vector pGU0202 or the *gfp/rfp* derivatives were recovered.

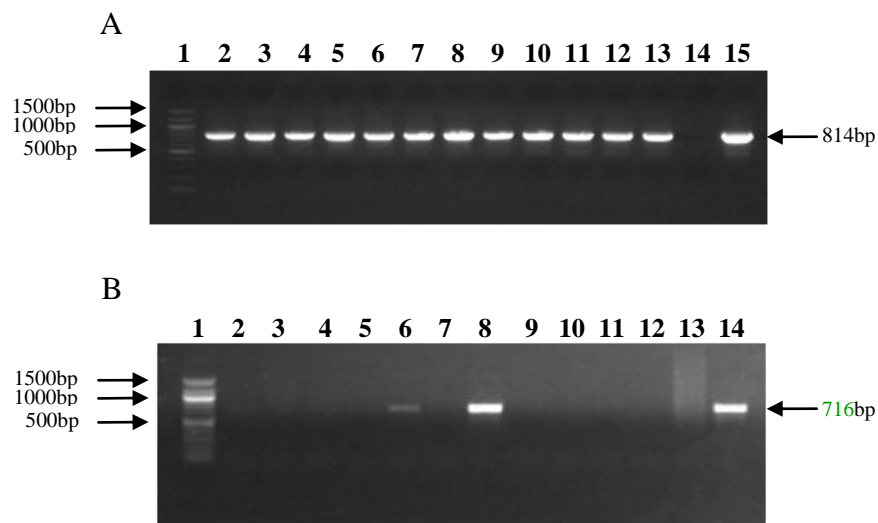


Figure 5.4 Amplicons of *Km^r* and *gfp* genes to confirm *C. coli* 427 (pGU0610) transformants. (A) Lane 1: 100bp DNA ladder; Lane 2-13: randomly selected *C. coli* 427 positive transformants; Lane 14: H₂O control; Lane 15: DNA control. (B) Lane 1: 100bp DNA ladder; Lane 2-11: *C. coli* 427 positive transformants with *gfp* gene amplicon; Lane 12: H₂O control; Lane 13: *C. coli* 427 pGU0202; Lane 14: DNA control.



Figure 5.5 Amplicons of *Km^r* and *rfp* genes to confirm *C. coli* 427 (pGU0611) transformants. PCR analysis of *C. coli* 427 positive transformants amplifying the *Km^r* gene and *rfp* gene Lane 1: 100bp DNA ladder; Lane 2: *C. coli* 427; Lane 3: *C. coli* 427 containing pGU0202; Lane 4-9: *C. coli* 427 pGU0611; Lane 10: H₂O control; Lane 11: DNA control; Lane 12: *C. coli* 427; Lane 13-18: *C. coli* 427 pGU0611; Lane 19: H₂O control; Lane 20: DNA control. The red box and numbers represent the generation of the *rfp* gene amplicon from the *C. coli* 427 transformants.

5.2.4 Assessment of GFP and RFP expression in *C. coli* 427

Following the introduction of the shuttle vectors containing *gfp* and *rfp* genes into *C. coli* 427, the level of GFP and RFP expression was assessed.

Fluorescent Microscopy analysis was initially performed in order to determine the presence of fluorescing *C. coli* 427 cells. Under high magnification (1000x) *C. coli* 427 expressing green or red fluorescence were observed. On average there were ~18-20% of fluorescent cells per field of view. It was also noted that the green fluorescing cells appeared to the naked eye to be more intense than the red fluorescing *C. coli* 427. In addition, it was also observed that the intensity of fluorescence was higher than the previously described integrative vector pGU0608 (Chapter 4.0). This appears to correlate with the number of copies of the fluorescent protein gene in the *Campylobacter* spp. cell. However, the frequency of fluorescing *C. coli* 427 cells was comparable to the integrated *gfp* gene in *C. jejuni* 81116 (Chapter 4.0 section 4.2.5), with the subsequent total intensity of fluorescence again at a level that was not able to be visualised by capture of fluorescent images.

Flow Cytometric Analysis was performed to determine the population of *C. coli* 427 cells expressing either GFP or RFP. Due to the low level of fluorescence expressed there was no significant shift in the cytometric curves for GFP or RFP compared to the parent strain *C. coli* 427 and *C. coli* 427 cells containing control vector pGU0202 (not shown).

Fluorescence Intensity was measured in a Victor plate reader to allow detection of fluorescence using appropriate settings for both GFP expression and RFP expression. There was a 2-fold increase of GFP expression by *C. coli* 427 cells transformed with the pGU0610 vector construct. And a 1.8-fold increase of RFP expression in *C. coli* 427

cells transformed with the pGU0611 vector construct (Figure 5.6) when compared to the *C. coli* 427 cells containing the control vector.

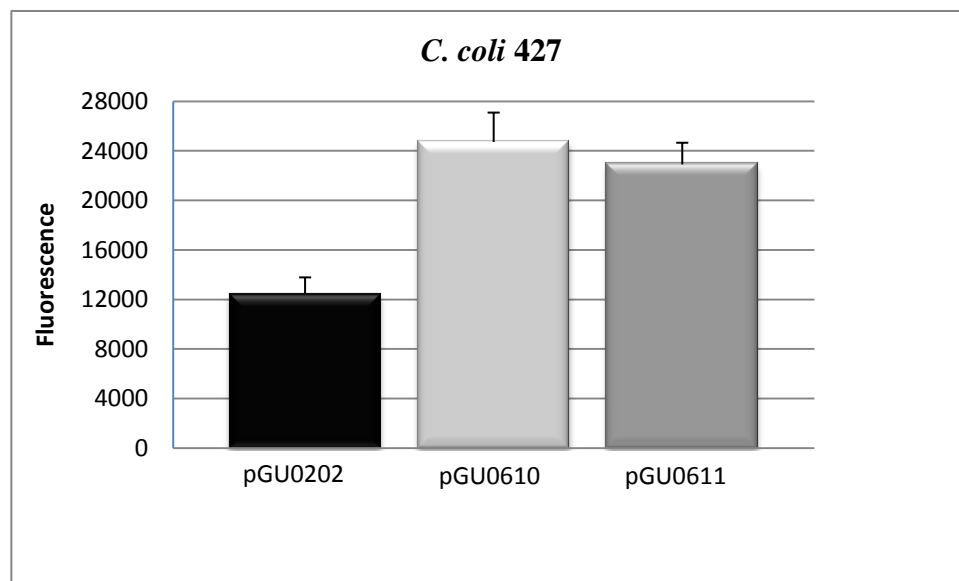


Figure 5.6 Expression of GFP and RFP in *C. coli* 427 transformants containing shuttle vectors pGU0610 and pGU0611. Intensity of fluorescence expressed in *C. coli* 427 transformants. The black bar represents the level of background fluorescence by the control vector pGU0202, the grey bar represents detection of fluorescence in *C. coli* 427 containing pGU0610 and the dark grey bar represents detection of fluorescence in *C. coli* 427 containing pGU0611.

5.2.5 Comparison of *C. coli* 427 and *E. coli* expressing GFP or RFP

In order to assess the relative level of intensity of GFP and RFP expression that is required to be detected using microscopy, flow cytometry and a victor plate reader, *C. coli* 427 transformants containing the vector constructs pGU0610 and pGU0611 were compared to expression of *E. coli* containing the same vectors. The results indicated that *E. coli* containing pGU0610 expresses GFP at a level 38-fold more intense than *C. coli* 427 (Figure 5.7A). Additionally RFP expression in *E. coli* containing pGU0611 expresses only a 2-fold more intensity than *C. coli* 427.

5.2.6 Confirmation of introduction of shuttle vectors into *Campylobacter* spp. during transformation

As only transformants for *C. coli* 427 were obtained, it was decided to investigate whether pGU0610 and pGU0611 were being introduced into *C. jejuni* 81116 and *C. jejuni* 11168. Due to the successful introduction of pGU0202 and its derivatives containing *gfp* and *rfp* in *C. coli* 427, this strain was included as a reference. During natural transformation, the transformation (competent cells and DNA) mixture was subject to treatment with DNase. This has been shown previously to perform efficiently in liquid culture and eliminate remnant DNA (Wilson *et al.*, 2003). Thus, the bacterial cells isolated could be assessed for the presence of pGU0610 and pGU0611 in the *Campylobacter* spp. cell.

Molecular Analysis was performed to confirm the presence of the control vector pGU0202 and its derivatives pGU0610 or pGU0611. This was achieved initially by using a gene specific primer set, KmBg/III-F and R, to amplify the Km^r cassette. Figures

5.8A and 5.9A show that the Km^r cassette has been amplified in the transformation mixture for all 3 *Campylobacter* spp. strains transformed with pGU0202, pGU0610 and pGU0611. To determine the presence of the *gfp* gene or *rfp* gene, gene specific primers; pWM1007gfp-F and R or rfp-F and rfpBamHI-R were used to amplify the fluorescent protein genes only (Figure 5.8B and 5.9B for *gfp* and *rfp* respectively). The fluorescent protein gene amplicon sizes were visualised by agarose gel electrophoresis. Analysis suggests that during transformation the vector DNA was able to be introduced into the bacterial cell using natural transformation.

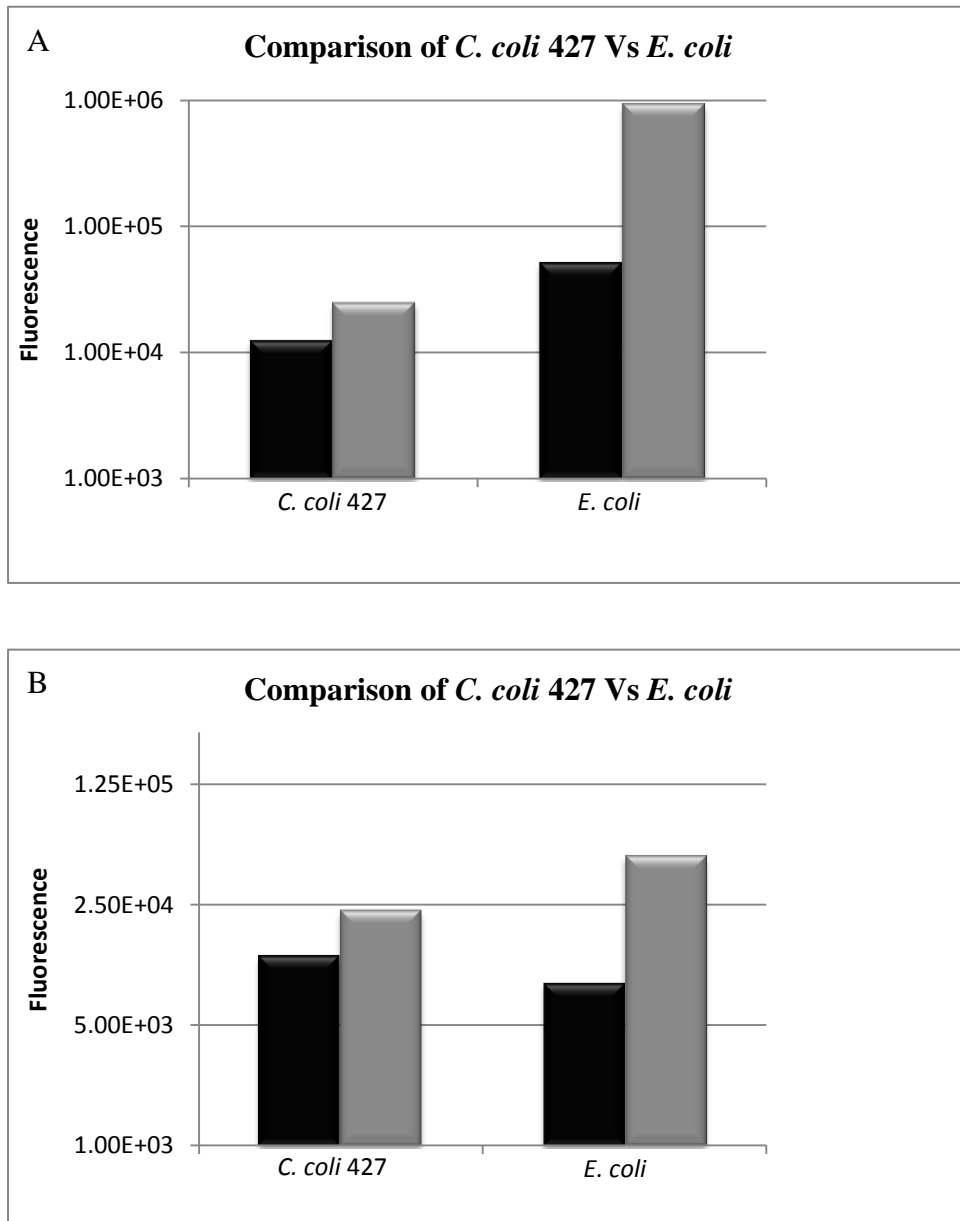


Figure 5.7 Expression of GFP and RFP in *C. coli* 427 compared to *E. coli*. Intensity of GFP expression as measured in a Victor plate reader. (A) Shows the difference in fluorescence detected between the control vector pGU0202 (represented by the black bar) and pGU0610 *gfp* expression vector (represented by the grey bar) in both *C. coli* 427 and *E. coli*. (B) Indicates the level of fluorescence detected between the control vector pGU0202 (represented by the black bar) and pGU0611 *rfp* expression vector (represented by the grey bar) in both *C. coli* 427 and *E. coli*.

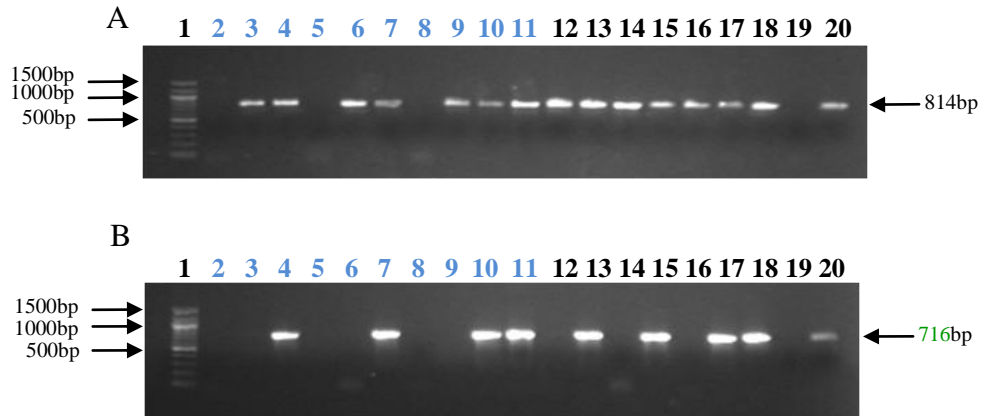


Figure 5.8 Amplicons of *Km^r* and *gfp* genes in *Campylobacter* spp. during natural transformation. (A) PCR analysis of isolated *Campylobacter* strains amplifying the *Km^r* gene from the transformation mixture. (B) PCR analysis of isolated *Campylobacter* strains amplifying the *gfp* gene from the transformation mixture. Lane 1: 100bp DNA ladder; Lane 2: *C. jejuni* 81116; Lane 3 & 12: *C. jejuni* 81116 pGU0202; Lane 4 & 13: *C. jejuni* 81116 pGU0610; Lane 5: *C. jejuni* 11168; Lane 6 & 14: *C. jejuni* 11168 pGU0202; Lane 7 & 15: *C. jejuni* 11168 pGU0610 Lane 8: *C. coli* 427; Lane 9 & 16: *C. coli* 427 pGU0202; Lane 10 & 17: *C. coli* 427 pGU0610 Lane 18: *E. coli* pGU0610; Lane 19: H₂O control; Lane 20: DNA control. The lanes highlighted blue (2-11) were subjected to treatment with DNase.

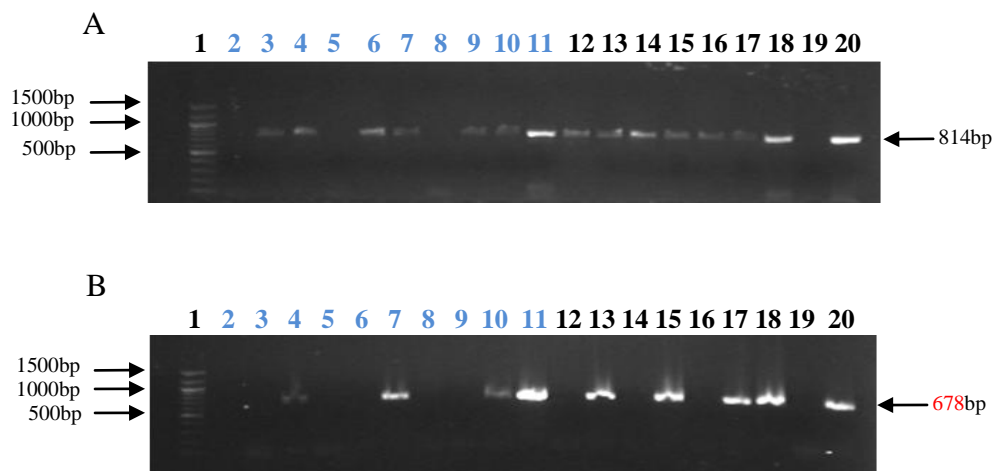


Figure 5.9 Amplicons of *Km^r* and *rfp* genes in *Campylobacter* spp. during natural transformation. (A) PCR analysis of isolated *Campylobacter* strains amplifying the *Km^r* gene from the transformation mixture. (B) PCR analysis of isolated *Campylobacter* strains amplifying the *rfp* gene from the transformation mixture. Lane 1: 100bp DNA ladder; Lane 2: *C. jejuni* 81116; Lane 3 & 12: *C. jejuni* 81116 pGU0202; Lane 4 & 13: *C. jejuni* 81116 pGU0611; Lane 5: *C. jejuni* 11168; Lane 6 & 14: *C. jejuni* 11168 pGU0202; Lane 7 & 15: *C. jejuni* 11168 pGU0611 Lane 8: *C. coli* 427; Lane 9 & 16: *C. coli* 427 pGU0202; Lane 10 & 17: *C. coli* 427 pGU0611 Lane 18: *E. coli* pGU0611; Lane 19: H₂O control; Lane 20: DNA control. The lanes highlighted blue (2-11) were subjected to treatment with DNase.

5.2.7 Assessment of fluorescence from transformation mixture

Fluorescence Microscopy analysis identified green or red fluorescing *C. jejuni* 81116, *C. jejuni* 11168 and *C. coli* 427 cells under high magnification (1000x). The highest proportion of fluorescent bacterial cells was observed for *C. coli* 427 with approximately 30% per field of view (data not shown). In contrast only 0.1% of fluorescent cells were observed for *C. jejuni* 81116 and *C. jejuni* 11168. Even though fluorescent cells were observed, the intensity of fluorescence was at a level that was not able to be visualised by capture of fluorescent images.

Flow Cytometric Analysis was performed using a FACScalibur cytometer (BD Biosciences) and analysed using CellQuest software (BD BioSciences) to assess the population of *C. jejuni* expressing detectable levels of green or red fluorescence. The level of GFP and RFP expression by *Campylobacter* spp. did not produce a significant shift in the cytometric curves (data not shown).

Fluorescence Intensity was measured by the use of a Victor plate reader. This allowed the comparison of intensity of fluorescence between *Campylobacter* spp. containing the control vector pGU0202 only and the shuttle vectors containing *gfp* and *rfp* genes. Detection of fluorescence indicates that all 3 *Campylobacter* spp. strains transformed with pGU0610 appear to express GFP at a higher intensity compared to the pGU0202 which provides the background fluorescence (Figure 5.10A). The *Campylobacter* spp. strains transformed with pGU0611 also show a marked increase of RFP expression compared to the control vector pGU0202 (Figure 5.10B).

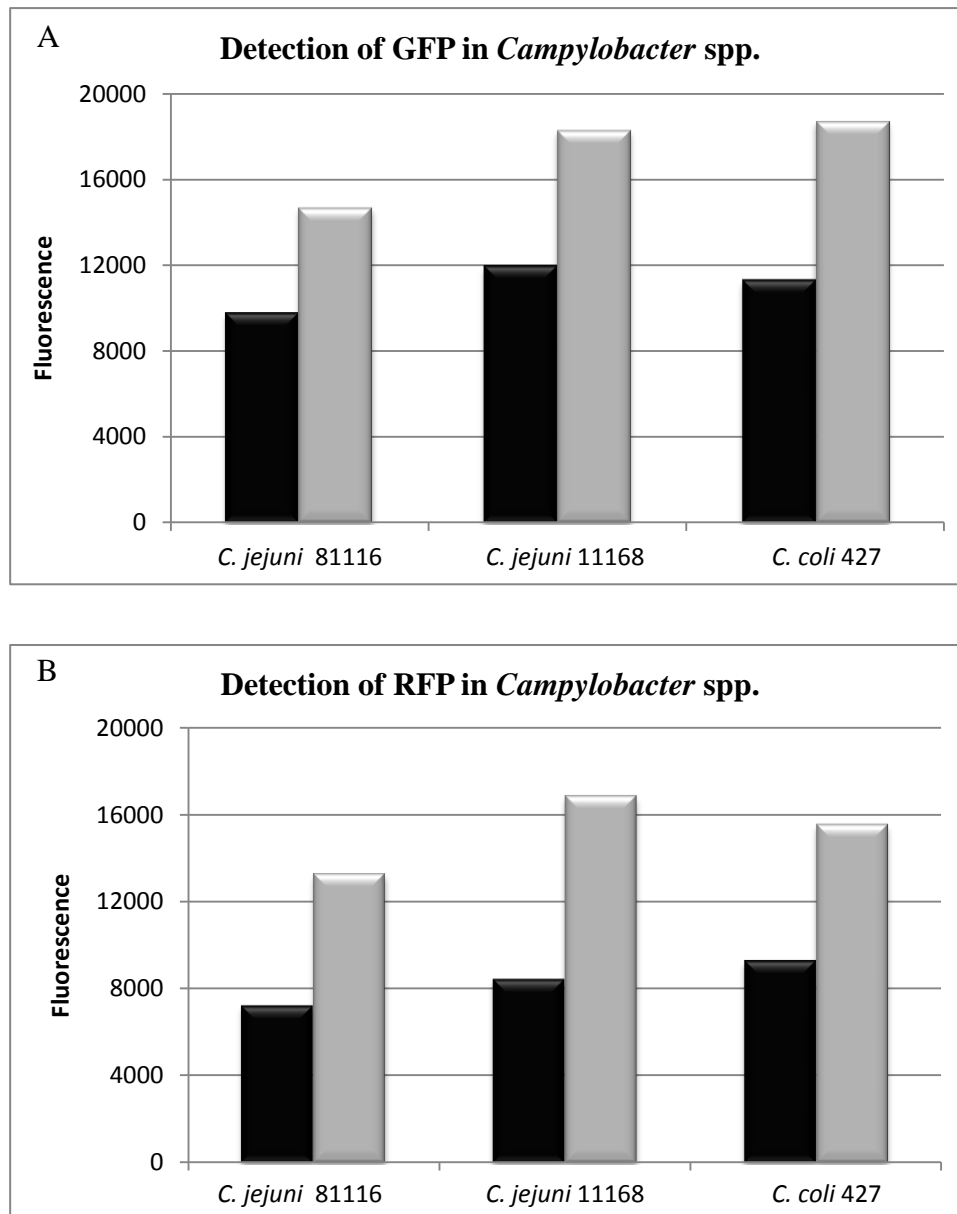


Figure 5.10 Expression of GFP and RFP in *Campylobacter* spp. via shuttle vectors during natural transformation. Measurement of fluorescence intensity in; *C. jejuni* 81116, *C. jejuni* 11168 and *C. coli* 427 during natural transformation. (A) Indicates expression of GFP, the black bar represents the level of background fluorescence of the control vector pGU0202 and the grey bar represents the detection of fluorescence with pGU0610. (B) Indicates expression of RFP, the black bar represents the level of background fluorescence of the control vector pGU0202 and the grey bar represents the detection of fluorescence with pGU0611.

5.3 Discussion

This chapter describes the construction of a shuttle vector system to enable more efficient manipulation of *Campylobacter* spp. by generating strains expressing GFP or RFP.

Transformation of *Campylobacter* spp. strains with shuttle vectors pGU0610 and pGU0611 only produced transformants for *C. coli* 427. This result correlates with previous findings that the pGU0202 shuttle vector is efficiently transformed into *C. coli* 427 (Alfredson, 2004) and as yet there is no documented reports that pGU0202 has successfully been directly transformed into *C. jejuni* 81116 or 11168 without prior passage through *C. coli* 427. Inability to initiate uptake of DNA into *C. jejuni* 81116 and *C. jejuni* 11168 may be directly related to restriction/modification systems between *Campylobacter* spp. strains (Fouts *et al.*, 2005). It is known that strain to strain diversity in the number and composition of restriction modification systems exist (Fouts *et al.*, 2005) and it is thought that prior passage of shuttle vectors into a *Campylobacter* spp. strain may be less likely to be subject to nuclease activity by other *Campylobacter* spp. strains and overcome host-specific restriction (Karlyshev & Wren, 2005).

Previous works by Miller *et al.* (2000) had first transformed their shuttle vector system into an Str^r derivative of *Campylobacter* spp. via conjugation. Only plasmids then extracted from *C. jejuni* could transform the same strain of *C. jejuni* (Miller *et al.*, 2000). For that reason it was thought that the introduction of vectors initially into *C. coli* 427 then into *C. jejuni* strains would allow transfer of the vector to any *C. jejuni* strain. Unfortunately due to the lack of detectable GFP and RFP expression in *C. coli* 427 this was not attempted.

The inefficient level of expression of GFP and RFP required for detection and subsequent *in vitro* use of *Campylobacter* spp. may be associated with differing codon usage. Due to an AT rich genome, it is reasonable to hypothesise that lack of detectable expression of GFP and RFP may be due to differences in codon preference between the cloning intermediate *E. coli* and *C. jejuni*. The nucleotide sequences analysed in *C. coli* 427 transformants appear to be without DNA rearrangements or nucleotide changes, therefore indicating expression is being hindered perhaps by reduced levels of transcription and subsequent translation of the fluorescent protein genes.

Alteration of codon usage in *gfp* has been shown to enhance expression once codons more optimal for *C. jejuni* have been introduced (Karlyshev & Wren, 2005). Furthermore, in the same study presence of two tandem promoters coupled with more efficient codon preference was shown to contribute to a higher level of *gfp* expression (Karlyshev & Wren, 2005). Other studies have also found that the *rfp* gene was not at detectable levels above background autofluorescence (Dunn *et al.*, 2006; Sorensen *et al.*, 2003). Adaptation of the red allele was necessary to obtain *rfp* expression (Dunn *et al.*, 2006). It is known that codon preference by *C. jejuni* is biased toward codons ending with either A or T. This was illustrated in a previous study that identified optimal and rare codons based on 67 genes of *C. jejuni* strain ATCC 11168 (Gray & Konkel, 1999). Differences were noted between *C. jejuni* 11168 and the *C. coli* subsets mainly in the optimal codon usage for glycine, valine, and arginine (Gray & Konkel, 1999).

Site-directed and random mutagenesis has been performed to adapt *gfp* and *rfp* genes to express more efficiently in target bacteria, with residue alterations in the *gfp* and DsRed sequences improving absorption and emission wavelengths (Shaner *et al.*, 2007).

Variants of *A. victoria* and *Discosoma* spp. have also been developed, altering emission peaks subsequently developing fluorescence ranging from blue (BFP) to yellow (YFP) (Nienhaus & Wiedenmann, 2009). Both cyan and yellow fluorescence has been established in *C. jejuni* (Miller *et al.*, 2000), however they have also produced varying low levels of fluorescence and are not versatile. These extensive efforts have made significant advances in fluorescent protein technology and highlight the possibility of adapting species specific fluorescent protein genes. Fluorescent protein variants were established after the construction portion of this research was complete, therefore were not investigated in this study.

This chapter has described an alternative strategy for introducing fluorescent genes into *Campylobacter* spp. As the desired outcome was not achieved, this study has emphasised the limitations that can be encountered with respect to expressing genes of interest universally in *C. jejuni* and *C. coli*. Future work that may be conducted is adaptation of codon use to develop fluorescent protein genes that will function maximally in *Campylobacter* spp., and the continual development of vector systems that can deliver genes and facilitate expression in both *C. jejuni* and *E. coli*.

CHAPTER 6

Construction and expression of shuttle vector pGU0202 containing *lux*

6.1 Introduction

Due to lack of success in generating *Campylobacter* spp. strains expressing fluorescence, a new strategy was developed to construct a shuttle vector to generate bioluminescent *Campylobacter* spp. strains.

Expression of luciferase has a higher magnitude of luminescence signal compared to GFP (Valdivia *et al.*, 2006), therefore one advantage of bioluminescence is the absence of significant background bioluminescent light from mammalian cells (Contag *et al.*, 1995). As limitations in expression of fluorescence have been experienced to date in this study, it was reasonable to speculate that the presence of a *lux* operon high in AT (63%) composition in a shuttle vector may be compatible with the AT rich genome of *C. jejuni*, and potentially use similar codons for translation of luciferase genes. As described in chapters 4.0 and 5.0, a *Campylobacter* promoter sequence was used to initiate expression of the genes comprised in the *lux* operon.

Luciferase expression is encoded by five essential genes that are organised in an operon, *luxCDABE* (Francis *et al.*, 2000). In the operon; *luxAB* genes encode for luciferase, which ultimately form the enzyme that emits light. The *luxCDE* genes encode for the polypeptides responsible for generation of the fatty acid reductase complex (Frackman *et al.*, 1990; Szittner & Meighen, 1990). These five genes are crucial for the production of light (Francis *et al.*, 2000).

Furthermore, *in vivo* detection of bioluminescent bacteria is non-invasive, detects only live/viable bacteria and allows rapid monitoring of the infective state of cells in an animal host (Francis *et al.*, 2000). Studies on *Salmonella* spp. have highlighted the advances that can be made in using bioluminescent bacteria (Contag *et al.*, 1995).

Previous studies have utilised bioluminescent *C. jejuni* to assess colonisation of fresh and retail eggshells (Allen & Griffiths, 2001), thus the potential for use in investigating biological processes in *C. jejuni* is plausible. The construction of a shuttle vector containing the *lux* operon will generate bioluminescent strains of *C. jejuni* which may be used in time course experiments to monitor the course of infection throughout the GI tract of an avian or mammalian host.

The prospect of developing bioluminescent *C. jejuni* could potentially shed more light on *C. jejuni* pathogenesis *in vivo*, by developing a real time representation of progression of disease (Contag *et al.*, 1995). Thus providing more information about how this pathogenic organism interacts with the host gastric mucosa and subsequently translocates the epithelial barrier.

This chapter describes the development of a prospective universal shuttle vector that could be used in *Campylobacter* spp. to generate bioluminescent strains. The use of which has potential to play a major role in the identification and elucidation of biological processes that may be used to study pathogenesis in *C. jejuni*.

6.2 Results

6.2.1 Amplification and cloning of the *Campylobacter* promoter consensus (P_c) sequence

In order to develop a construct that will allow luciferase expression, the commercial cloning vector pDsRed-Express (Clontech) was used as an intermediate to incorporate a *Campylobacter* promoter consensus sequence (P_c). The 197bp *Campylobacter* promoter sequence (P_c) was amplified from pMW2 by PCR using Cprom*EcoRI*-F and Cprom*NotI*-R primers to incorporate *EcoRI* and *NotI* restriction sites at the 5' and 3' termini respectively. The P_c amplicon was visualised by agarose gel electrophoresis (Figure 6.1), excised, purified and subsequently inserted into pDsRed-Express (as described previously in Chapter 4.0 section 4.2.1) using standard cloning techniques. The presence of promoter was verified by PCR and sequence analysis (data not shown). This newly generated vector was designated pGU0701.

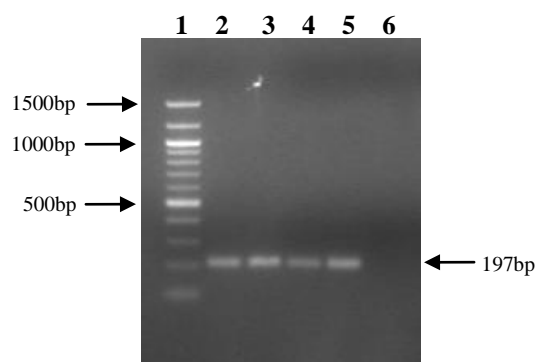


Figure 6.1 P_c amplicon resolved by gel electrophoresis
Lane 1: 100bp DNA ladder; Lane 2-5: P_c amplicon; Lane 6: H₂O control.

6.2.2 Amplification of the *lux* operon

To incorporate the genes that encode for luciferase, the *lux* operon in pBluescript[®] II SK; kindly provided by Professor Ifor Beacham; was used as template DNA. The *lux* operon, which comprises of 5 organised genes, was amplified from the pBluescript[®] II SK vector using the primer set Lux*Not*I-F and Lux*Bam*HI-R to amplify from nucleotide 510 to 6873 of the *X. luminescens* Hb strain (ATCC 29999). The incorporation of restriction sites *Not*I and *Bam*HI at the 5' and 3' termini allowed for compatible directional end cloning of the gene cluster downstream of the P_c in pGU0701. The *lux* operon was visualised as a ~7.0kb DNA fragment using agarose gel electrophoresis (Figure 6.2), excised and purified for further cloning.

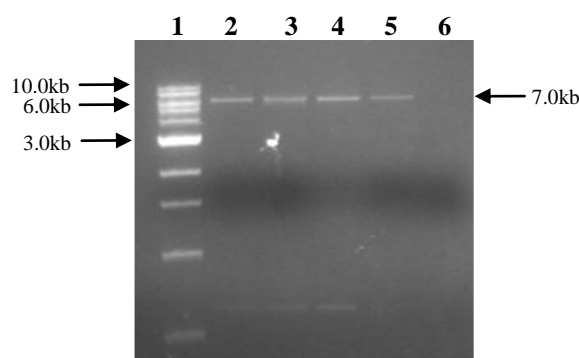


Figure 6.2 Amplicon of *lux* operon resolved by gel electrophoresis
Lane 1: 1.0kb DNA ladder; Lane 2-5: *lux* operon amplicon; Lane 6: H₂O control.

The purified *lux* operon DNA was ligated into pGU0701 downstream of the *Campylobacter* specific P_c and transformed into competent *E. coli* DH5α cells. The recombinant vector was isolated from *E. coli* DH5α and screened for the presence of the P_c and *lux* operon by restriction digest analysis (data not shown). Verification of the

constructed vector confirmed the presence of the P_c and *lux* operon, which was designated pGU0703.

6.2.3 Cloning of P_c -*lux* operon into replicating vector pGU0202

The cloning strategy implemented to develop a shuttle vector able to express luciferase in both *E. coli* and *C. jejuni* utilises the previously established vector pGU0202 (Alfredson & Korolik, 2003). As the P_c -*lux* operon is ~7.0kb, rather than generating a PCR product that has potential to introduce base mismatches and random mutations, it was decided to incorporate the P_c -*lux* fragment into the pGU0202 vector by compatible directional end cloning using restriction endonuclease digestion (section 2.5.4). In order to generate the desired vector construct, excision of the P_c -*lux* operon from pGU0703 was performed using the restriction enzymes *EcoRI* and *BamHI*. Concurrently, pGU0202 was cleaved using *EcoRI* and *BamHI* to enforce a compatible ligation event. The ligation product carrying the P_c -*lux* operon was transformed into competent *E. coli* DH5 α cells and putative clones subjected to selective pressure using kanamycin to promote the growth of transformants carrying the recombinant vector. The transformants displaying Km^r were purified and verified by restriction digest analysis (Figure 6.3A). Generation of restriction digest profiles confirmed the presence of the recombinant vector.

Partial DNA sequencing was performed on purified plasmid DNA to ensure the integrity of the P_c and start of the *luxC* and end of *luxE* genes, which also confirmed the *lux* operon to be in the same orientation as the Km^r cassette (Figure 6.3B). This new recombinant vector was designated pGU0704. The inclusion of the *lux* operon into an *E. coli*-*Campylobacter* shuttle vector should allow for expression of luciferase in both

bacterial species. Therefore the pGU0704 vector in *E. coli* DH5 α was assessed for luciferase expression using NightOwl Imaging technology (Figure 6.3C). This illustrates that *E. coli* is capable of transcribing and translating the proteins required for luciferase expression and as a result the bacterial cell is luminescent.

6.2.4 Transformation of pGU0704 into *Campylobacter* spp. and detection of bioluminescence

Due to the size of the constructed vector pGU0704 (~13.0kb) and previous observations that the *Campylobacter* strains used in this study are naturally competent, natural transformation was attempted. The protocol (as described in section 2.6.5) was adopted for introduction of pGU0704 and control vector pGU0202 into *C. jejuni* 81116, *C. jejuni* 11168 and *C. coli* 427.

After numerous attempts, uptake and subsequent replication of both pGU0202 and its *lux* containing derivative pGU0704 only produced transformants for *C. coli* 427. This was similar to observations for replicative vectors described in the previous Chapter 5.0 (section 5.2.3).

To determine the level of luciferase expression, parent *C. coli* 427, *C. coli* 427 transformant containing the control vector pGU0202, and 5 putative *lux* transformants were screened using NightOwl Imaging technology. As *E. coli* was previously determined to express luciferase from pGU0704, this was included as a reference. Initially transformants were screened for luciferase expression directly on the culture plate, and then further assessed by suspension in saline and transferred to a 96 well plate for standardisation of imaging. Expression of luciferase was detected for *E. coli*

containing pGU0704; however no luminescence was detected for the *C. coli* 427 transformants (Figure 6.4).

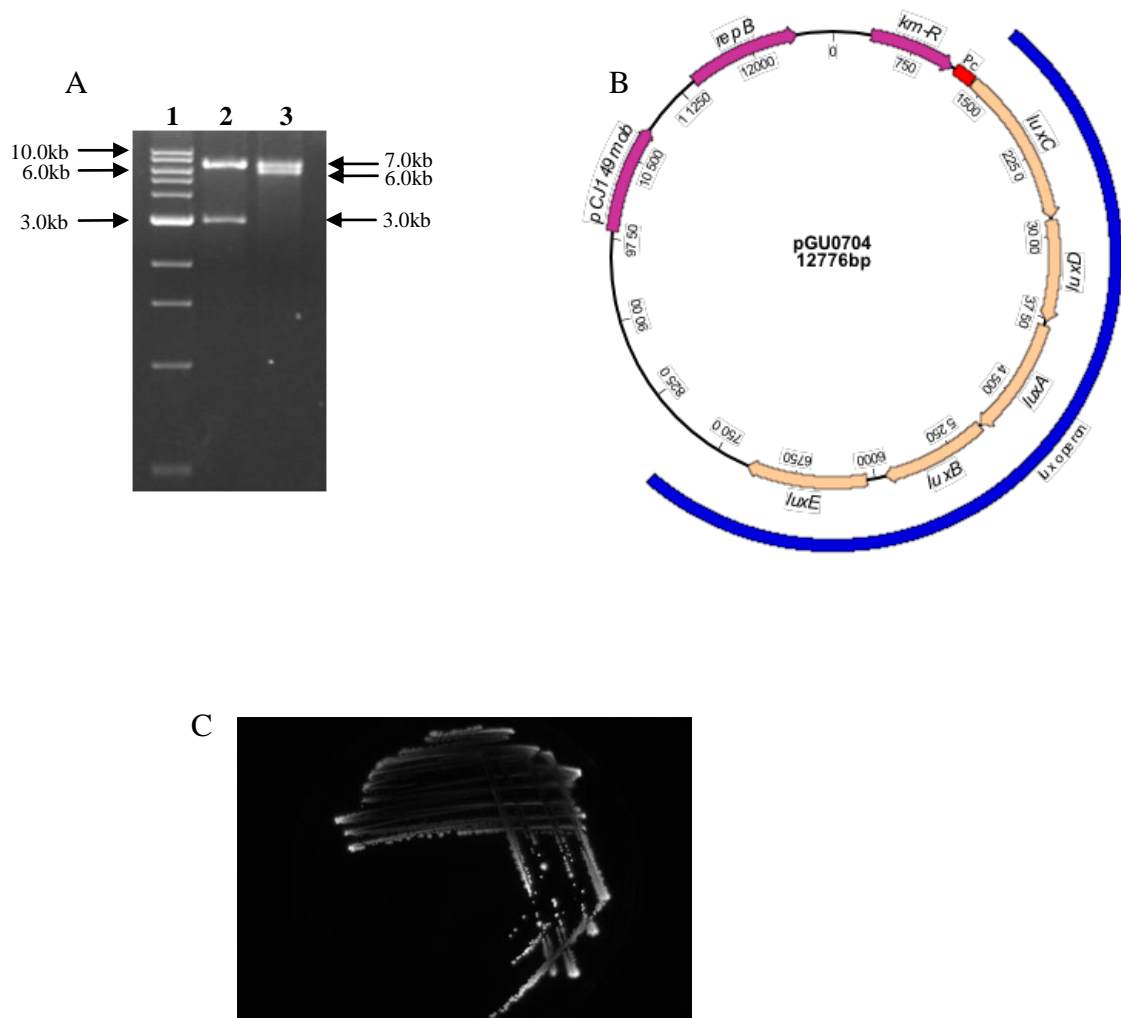


Figure 6.3 Confirmation of pGU0704 vector construct

(A) Restriction digest profile to confirm the pGU0704 construct. Lane 1: 1.0kb DNA ladder; Lane 2: pSK with *lux* operon excised; Lane 3: pGU0704 with *lux* operon excised from pGU0202 vector backbone. (B) Schematic diagram representing pGU0704 using MacVector 9.0. The arrow heads indicate the confirmed orientation of genes, the blue box represents the *lux* operon and the pink arrows represent the individual *lux* genes that comprise the *lux* operon. (C) Luminescent image of *E. coli* DH5 α expressing luciferase from the shuttle vector pGU0704 using NightOwl Imaging technology.

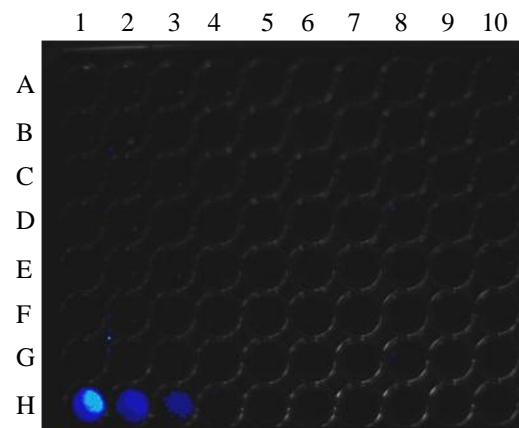


Figure 6.4 Detection of luminescence in *C. coli* 427 (pGU0704) transformants. 96 well plate showing detection of luminescence in colour-overlay using NightOwl Imaging technology. (A): *C. coli* 427 pGU0202; (B-F): *C. coli* 427 pGU0704 transformants; (G): *C. coli* 427; (H): *E. coli* pGU0704. The lane numbers correspond to 1:10 dilutions. Lane 1 to 9 (10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 & 10^1); Lane 10: PBS control.

6.2.5 Molecular analysis of *C. coli* 427 (pGU0704) putative transformants

To elucidate the reason for the inability to detect bioluminescence in *C. coli* 427, molecular analysis was undertaken to investigate the presence or absence of the *lux* operon and P_c .

Putative transformants exhibiting Km^r were investigated for the presence of the Km^r cassette and *lux* operon. Initially PCR analysis using gene specific primers $Km^{Bg}/III-F$ and R, to amplify the Km^r cassette was performed. Figure 6.5 illustrates that the Km^r marker was present in *C. coli* 427 containing pGU0202 (Lane 2) and putative *C. coli* 427 pGU0704 transformants. Gene specific primers (*luxC-F* and R) and (*luxE-F* and R) were unable to amplify the *luxC* and *luxE* genes, from any of the transformants (data not shown), indicating absence of intact pGU0704.

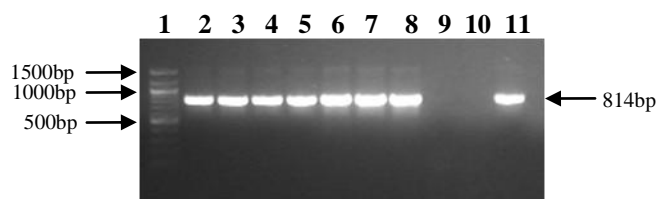


Figure 6.5 Amplicon of Km^r cassette in *C. coli* 427 (pGU0704) transformants. Lane 1: 100bp DNA ladder; Lane 2: *C. coli* 427 pGU0202; Lane 3-8: *C. coli* 427 pGU0704; Lane 9: *C. coli* 427; Lane 10: H₂O control; Lane 11: DNA control.

6.2.6 Confirmation of introduction of pGU0704 into *Campylobacter* spp. during transformation

As only transformants were obtained for *C. coli* 427 it was decided to investigate if the vector containing the *lux* operon was being introduced into *C. jejuni* 81116 and *C. jejuni* 11168. Due to the introduction and verification of Km^r cassette from pGU0202 and pGU0704 into *C. coli* 427, this strain was included as the reference. During natural transformation the transformation (competent cells and DNA) mixture was subject to treatment with DNase, as performed in Chapter 4.0 and Chapter 5.0 to remove any exogenous remnants of DNA. The transformation mixture was then assessed for ability to luminesce.

Luminescence Intensity of the transformation mixture was measured by the use of a Victor plate reader and NightOwl Imaging technology. Comparison of intensity of luminescent *Campylobacter* spp. containing the control vector pGU0202 and the pGU0704 shuttle vector was performed to determine if the *lux* operon was functional at the transformation stage. No luminescence was detected using either method (data not shown).

Molecular Analysis was performed to confirm the presence of the control vector pGU0202 and pGU0704 in the *Campylobacter* spp. strains. This was achieved initially by using a gene specific primer set, Km^rBg/III-F and R, to amplify the Km^r cassette. Figure 6.6 confirms the presence of the Km^r cassette in the transformation mixture of all 3 *Campylobacter* spp. strains for both the control vector pGU0202 and pGU0704.

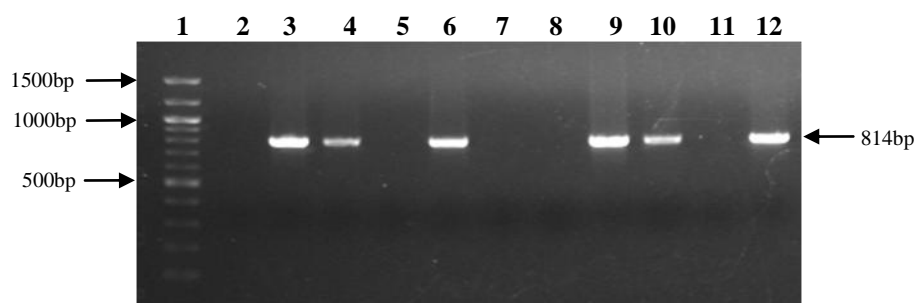


Figure 6.6 Amplicon of Km^r cassette in *Campylobacter* spp. during natural transformation. Lane 1: 100bp DNA ladder; Lane 2: *C. jejuni* 81116; Lane 3: *C. jejuni* 81116 pGU0202; Lane 4: *C. jejuni* 81116 pGU0704; Lane 5: *C. jejuni* 11168; Lane 6: *C. jejuni* 11168 pGU0202; Lane 7: *C. jejuni* 11168 pGU0704; Lane 8: *C. coli* 427; Lane 9: *C. coli* 427 pGU0202; Lane 10: *C. coli* 427 pGU0704; Lane 11: H₂O control; Lane 12: DNA control.

After DNase treatment to remove any untransformed DNA molecules, plasmid DNA from the transformation mixture was isolated and purified to determine the presence of the *lux* operon. Integrity of pGU0704 was verified using restriction digest analysis. These restriction profiles indicate that for *C. jejuni* 81116, *C. jejuni* 11168 and *C. coli* 427, the control vector pGU0202 and pGU0704 were present during natural transformation (Figure 6.7).

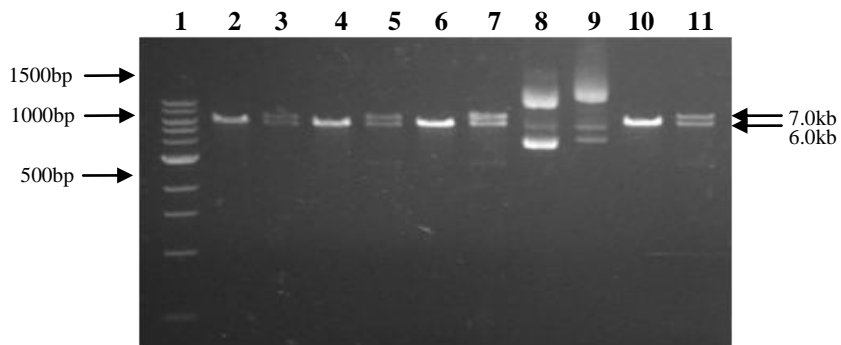


Figure 6.7 Restriction digest analysis of pGU0704 in *Campylobacter* spp. during natural transformation. Lane 1: 1.0kb DNA ladder; Lane 2: *C. jejuni* 81116 pGU0202; Lane 3: *C. jejuni* 81116 pGU0704; Lane 4: *C. jejuni* 11168 pGU0202; Lane 5: *C. jejuni* 11168 pGU0704; Lane 6: *C. coli* 427 pGU0202; Lane 7: *C. coli* 427 pGU0704; Lane 8: pGU0202 undigested; Lane 9: pGU0704 undigested; Lane 10: pGU0202 linearised; Lane 11: pGU0704 control vector with *lux* operon excised from vector backbone.

6.3 Discussion

This chapter described the construction of an *E. coli-Campylobacter* shuttle vector to introduce genes encoding for luciferase in *C. jejuni*. Expression of luciferase and subsequent imaging of bioluminescent *E. coli* suggested that the vector was functional and had the potential to produce bioluminescent *C. jejuni*.

Transformation of the control vector pGU0202 and *lux* containing vector pGU0704 produced transformants only for *C. coli* 427. This was previously observed for pGU0202 and its derivatives in Chapter 5.0 and similarly was postulated to be caused by strain to strain diversity in restriction modification systems corresponding to that described by (Fouts *et al.*, 2005). Assessment of luciferase activity indicated that none of the *C. coli* 427 transformants were capable of expressing the *lux* operon and further investigation revealed that only the Km^r cassette appeared to be present in *C. jejuni* cells. These results suggest transformation of pGU0704 was occurring however the intact plasmid could not be recovered from *C. coli* 427 transformants.

It is possible that absence of the *lux* operon may be due to plasmid rearrangement in *C. coli* 427 induced by either toxicity or inability to replicate the *lux* operon. *C. jejuni* and *C. coli* are well known for recombination and rearrangements of introduced DNA during natural transformation, thus the surviving cells may have been able to rescue the transforming plasmid by recombination with residential plasmids using mechanisms described by (Karlyshev & Wren, 2005) and (Wang & Taylor, 1990). This could result in rescue of part of pGU0704 including Km^r cassette, but not the *lux* operon.

Alternatively it is possible that the lack of a compatible restriction modification system may lead to elimination of introduced heterogenic DNA. Initially the *lux* operon was amplified from *X. luminescens* and passaged through *E. coli* for construction; it is

possible that the plasmid DNA being introduced into *C. jejuni* and *C. coli* was not protected from the action of nucleases (Richardson & Park, 1997; Wang & Taylor, 1990). Restriction-modification systems in *C. jejuni* ensure methylation of host cell DNA is protected against cleavage, however ‘foreign DNA’ is subject to restriction by endonucleases (Gaasbeek *et al.*, 2010; Miller *et al.*, 2005).

Problems encountered during construction of the shuttle vector containing the *lux* operon were unexpected and need to be fully understood in order to formulate an alternative strategy for introducing the *lux* operon into *C. jejuni* cells. This was, unfortunately, outside the scope of this study.

Even though the desired outcome was not achieved in this chapter, the potential use of bioluminescent *C. jejuni* to follow the course of infection through the gastrointestinal tract and systemic organs to generate a bioluminescent representation of *C. jejuni* infection *in vivo* is still an exciting prospect for future investigations.

CHAPTER 7

Development of an Immunomagnetic Separation technique for isolating *Campylobacter* spp. *in vivo*

The content of this chapter has fully contributed toward the publication “Analysis of carbohydrate binding and gene expression by *in vitro* and *in vivo* propagated *Campylobacter jejuni* isolated by Immunomagnetic Separation” Under revision to Microbiology, 2010. Manuscript in appendix D.

The content of this chapter has partially contributed toward the publication “Variation of chemosensory receptor content of *Campylobacter jejuni* strains and modulation of receptor gene expression under different *in vivo* and *in vitro* growth conditions” Under revision to Applied and Environmental Microbiology, 2010. Manuscript in appendix F.

7.1 Introduction

The majority of studies of *C. jejuni* pathogenicity have been conducted *in vitro* using bacterial/mammalian cell co-culture systems that are a good representation of bacterial-host interaction; however these do not replicate the intricate and complex *in vivo* mucosal environment that is present within the host GI tract. Previous studies have shown that culture of *C. jejuni in vitro* with purified human intestinal mucin revealed up-regulation of multiple pathogenicity genes, demonstrating the influence of this host factor (Tu *et al.*, 2008).

C. jejuni cells penetrate the mucus gel layer through use of their flagellum and chemotactic strategies, adhere to epithelial cells by adhesins/insertion structures and either colonise at the epithelial surface, or deep within the intestinal crypts (Fauchere *et al.*, 1985; Lee *et al.*, 1986; Wassenaar, 1997).

To investigate differences between *in vitro* and *in vivo* grown bacteria requires isolation techniques with minimal culture steps. Methodologies using immunomagnetic separation (IMS) have been used previously to isolate *H. pylori* from water and food products (Velazquez & Feirtag, 1999) and more recently have isolated *Campylobacter* spp. from poultry products including chicken carcasses and ground poultry meat (Mandrell & Wachtel, 1999; Yu *et al.*, 2001). Routine isolation of *C. jejuni* for identification in food products or gene expression profiling involves a recovery phase involving enrichment in selective broth or culture on selective agar which can take up to 48 hrs (Mandrell & Wachtel, 1999). Isolation using IMS will enable the recovery and subsequent assessment of bacteria within 1-2 hrs. The aim of the work undertaken in this chapter was to investigate the efficiency of isolating *Campylobacter* spp. cells using IMS and its potential use in *in vivo* experiments.

7.2 Results

7.2.1 Determination of efficiency of capture of *Campylobacter* spp. using IMS

In order to ensure this novel IMS technique will be a viable option for use with *C. jejuni*, the efficiency and sensitivity of *C. jejuni* 81116 capture using *Campylobacter* specific antibody coated dynabeads[®] M-280 was required to be analysed. 6×10^8 dynabeads[®] M-280 were coated with 60 μ g (5 mg/mL) rabbit anti-*Campylobacter jejuni* polyclonal antibody according to manufacturer's instructions. The parameters optimised for IMS included: bacterial concentration, dynabead concentration and incubation time, as described in materials and methods (section 2.8).

The optimal conditions for *C. jejuni* capture by immunocoated dynabeads[®] M-280 were determined to be 2.4×10^7 dynabeads for 30 min with a bacterial concentration of 10^9 /mL (Table 7.1). A lower concentration of dynabeads did not allow as many bacterial cells to be isolated and higher concentrations did not significantly increase the amount of cells recovered. Similarly, the time course experiment showed that there was a slight increase in the amount of bacteria recovered between 15 and 30 min, but no difference in recovery between 30 and 60 min. An inoculum size between 10^6 to 10^{12} cfu/mL did not alter the final recovered yield, however 10^9 cfu/mL was chosen as this most closely resembles the bacterial load found in the luminal content of an avian or mammalian host.

Table 7.1 Parameters for efficient *C. jejuni* recovery

Conc. Of beads	Incubation time (min)	Inoculum levels (cfu/mL)	Number of bead captured cells
1.2 x 10⁷	15	1 x 10 ¹²	3.9±0.1x10 ⁵
	15	1 x 10 ⁹	6.0±0.17x10 ⁵
	15	1 x 10 ⁶	2.1±0.26x10 ⁵
	30	1 x 10 ¹²	7.5±0.26x10 ⁵
	30	1 x 10 ⁹	8.3±0.17x10 ⁵
	30	1 x 10 ⁶	4.03±0.39x10 ⁵
	60	1 x 10 ¹²	5.5±0.11x10 ⁵
	60	1 x 10 ⁹	5.0±0.14x10 ⁵
	60	1 x 10 ⁶	3.7±0.1x10 ⁵
2.4 x 10⁷	15	1 x 10 ¹²	9.2±0.4x10 ⁵
	15	1 x 10 ⁹	1.1±0.14x10 ⁶
	15	1 x 10 ⁶	3.0±0.18x10 ⁵
	30	1 x 10 ¹²	2.3±0.15x10 ⁶
	30	1 x 10 ⁹	2.7±0.06x10 ⁶
	30	1 x 10 ⁶	9.0±0.3x10 ⁵
	60	1 x 10 ¹²	1.7±0.07x10 ⁶
	60	1 x 10 ⁹	2.5±0.09x10 ⁶
	60	1 x 10 ⁶	5.4±0.09x10 ⁵
6 x 10⁷	15	1 x 10 ¹²	1.1±0.06x10 ⁶
	15	1 x 10 ⁹	1.0±0.05x10 ⁶
	15	1 x 10 ⁶	5.0±0.08x10 ⁵
	30	1 x 10 ¹²	3.0±0.1x10 ⁶
	30	1 x 10 ⁹	2.9±0.1x10 ⁶
	30	1 x 10 ⁶	8.8±0.1x10 ⁵
	60	1 x 10 ¹²	3.3±0.07x10 ⁶
	60	1 x 10 ⁹	3.1±0.1x10 ⁶
	60	1 x 10 ⁶	9.0±0.4x10 ⁵

In vitro cultured *C. jejuni* 81116 at 3 concentrations were incubated with differing concentrations of anti-*C. jejuni* coated dynabeads[®] M-280 for 15-60 min and the number of bead captured bacteria determined by culture. (N=6 independent experiments; +/- 1 SD) and expressed as cfu/mL.

7.2.2 Optimisation of elution protocol to separate *C. jejuni* from antibody-coated dynabeads[®] M-280

Following bacterial isolation with dynabeads[®] M-280 an appropriate elution protocol needed to be established so that the isolated *C. jejuni* 81116 could be efficiently removed from the beads and used for downstream applications. Four solutions of varying concentrations (0.1 M citrate, 20 mM dTT, 50 mM dTT and 0.05% trypsin-EDTA) were included in this trial to determine not only the most efficient eluate, but also the solution that would leave *C. jejuni* 81116 viable for further applications. Table 7.2 shows that the solution trypsin-EDTA was optimal for elution with <0.02% of bacteria remaining, on average, attached to the beads. The other solutions tested (citrate and dTT) resulted in little or no elution of the bacteria from the beads. Furthermore, it was noted that *C. jejuni* 81116 eluted with trypsin-EDTA displayed a highly motile phenotype as observed by plate motility assays, compared to cells eluted with citrate (50%) and the few *C. jejuni* recovered using dTT all displayed no motility.

Table 7.2 Eluting solutions

Elution solution	No. of <i>C. jejuni</i> cells isolated	No. of <i>C. jejuni</i> cells eluted	No. of <i>C. jejuni</i> cells remaining on beads after elution
0.1 M citrate	$1.6 \pm 0.08 \times 10^6$	45 \pm 5.4	$1.0 \pm 0.12 \times 10^6$
20 mM dTT	$2.4 \pm 0.11 \times 10^6$	15 \pm 3.7	$5.7 \pm 0.06 \times 10^5$
50 mM dTT	$2.9 \pm 0.1 \times 10^6$	30 \pm 3.4	$1.1 \pm 0.11 \times 10^6$
0.05% trypsin-EDTA	$1.8 \pm 0.09 \times 10^6$	$6.3 \pm 0.14 \times 10^5$	100 \pm 14.7

C. jejuni 81116 bound to antibody-coated beads were exposed to elution solutions as per materials and methods section 2.8.3 and the number of bacteria eluted and remaining bound to the beads determined by culture (N=6 +/- 1SD) and expressed as cfu/mL.

7.2.3 Capture and elution of different strains of *C. jejuni*

In order to ensure that the capture and elution protocol established can be used universally for other *Campylobacter* spp. strains, two other commonly used variants with varying colonising and invasive capabilities were assessed. No significant difference ($p < 0.05$) was noted between the quantities of isolated cells of *C. jejuni* 11168-GS and *C. jejuni* 11168-O using the optimised conditions outlined in section 7.2.1 and 7.2.2. On average 4×10^5 cfu/mL were obtained for *C. jejuni* 11168-GS and 11168-O after isolation and elution (Table 7.3), which is similar to the established recovery of *C. jejuni* 81116 (8.5×10^5).

Table 7.3 Efficiency of *C. jejuni* capture and elution

<i>C. jejuni</i> strain	No. of <i>C. jejuni</i> cells isolated	No. of <i>C. jejuni</i> cells eluted	No. of <i>C. jejuni</i> cells remaining on beads after elution
81116	$2.2 \pm 0.1 \times 10^6$	$8.5 \pm 1.0 \times 10^5$	$9.3 \pm 0.1 \times 10^1$
11168-GS	$7.2 \pm 0.2 \times 10^5$	$4.0 \pm 0.7 \times 10^5$	$4.0 \pm 0.7 \times 10^2$
11168-O	$6.1 \pm 0.9 \times 10^5$	$4.6 \pm 1.0 \times 10^5$	$2.0 \pm 0.08 \times 10^2$

In vitro cultured *C. jejuni* strains were assessed for their ability to be isolated using the IMS technique developed. The number of bead captured bacteria, eluted bacteria and bacteria remaining on the beads were determined by culture. (N=4 +/- 1SD) and expressed as cfu/mL.

One trend that emerged from performance of biological repeats for this experiment over an extended period of time is that freshly coated beads; which is considered to be immunocoated beads stored for up to 1 month; were found to yield up to 1 log more bacteria than aged bead-antibody complexes with reduction of binding capacity of the beads observed over an extended period of time (data not shown).

7.2.4 Capture and elution of *C. jejuni* cells from artificially spiked chicken caecal content

The IMS isolation technique was assessed in artificially spiked chicken caecal material to evaluate the efficacy of isolation of *C. jejuni* from a chicken GI tract. This allowed assessment of the isolation protocol in the presence of components of the normal mucosa which could be a potential limiting factor for the binding of the bacteria to the immunocoated dynabeads and subsequently affect the amount of bacteria recovered. The IMS isolation of *C. jejuni* directly from artificially spiked chicken caecal material (10^9 *C. jejuni* cells/mL Brucella broth) indicated that, on average, 10^5 - 10^6 cfu/mL were recovered with approximately 10^5 *C. jejuni* cells eluted from the beads (Table 7.4), with no significant difference to cell numbers recovered from Brucella broth ($p < 0.05$, Table 7.3). These results strongly suggest that mucosal and other components present in the GI tract do not significantly affect the efficiency of *C. jejuni* recovery using the IMS technique. Further, this novel technique may potentially be used for *in vivo* studies regardless of host species.

Table 7.4 Efficiency of *C. jejuni* capture in caecal material

<i>C. jejuni</i> strain	No. of <i>C. jejuni</i> cells isolated	No. of <i>C. jejuni</i> cells eluted	No. of <i>C. jejuni</i> cells remaining on beads after elution
81116	$2.4 \pm 0.01 \times 10^6$	$5.2 \pm 0.06 \times 10^5$	$1.5 \pm 0.1 \times 10^2$
11168-GS	$8.5 \pm 0.3 \times 10^5$	$3.9 \pm 0.1 \times 10^5$	$3.0 \pm 0.1 \times 10^2$
11168-O	$6.3 \pm 0.1 \times 10^5$	$1.6 \pm 0.1 \times 10^5$	$2.2 \pm 0.1 \times 10^2$

In vitro cultured *C. jejuni* were mixed with chicken caecal homogenates and incubated with immunocoated beads and then eluted with trypsin-EDTA. The number of bead captured bacteria, eluted bacteria and bacteria remaining on the beads were determined by culture ($N=4 \pm 1$ SD) and expressed as cfu/mL.

7.2.5 Assessment of specificity of *C. jejuni* antibody

In order to determine if the rabbit anti-*C. jejuni* polyclonal antibody used to coat the dynabeads[®] M-280 is specific for *C. jejuni* alone and would not react with intestinal content of the host, it was necessary to assess for the antibody's binding capacity for normal flora and mucosal components that may be present in the GI tract of avian and mammalian hosts prior to *in vivo* experimentation. The dot blot immunoassay was performed and included a variety of organisms and solutions that may be encountered during *in vivo* infection. The assay indicated that the antibody is capable of binding *C. jejuni* 81116 and 11168-GS (Figure 7.1 A&B). No substantial reactivity was observed with normal flora or mucosal components in chicken caeca (Figure 7.1 C), and it was also evident that the antibody is capable of binding *C. jejuni* 81116 in the presence of normal mucosal components in the chicken caeca (Figure 7.1 D&E). The dynabeads[®] M-280 are also able to bind *C. jejuni* 81116 strongly in the presence of mouse GI tissue (Figure 7.1 F&G). However, weak cross-reactivity of the antibody with mucosal components of mouse GI tissue was detected. Other organisms and solutions that were included in this analysis were *Proteus* spp., isolated and known normal flora of the chicken GI tract, *E. coli* DH5 α and a Gram positive organism which was isolated and considered as normal flora from the mouse GI tract. Brucella broth and PBS were also included in the assay as these are the solutions routinely used in the preparation of bacterial inoculums and the IMS protocol to ensure there is no background binding. No binding to any of these components was observed (data not shown).

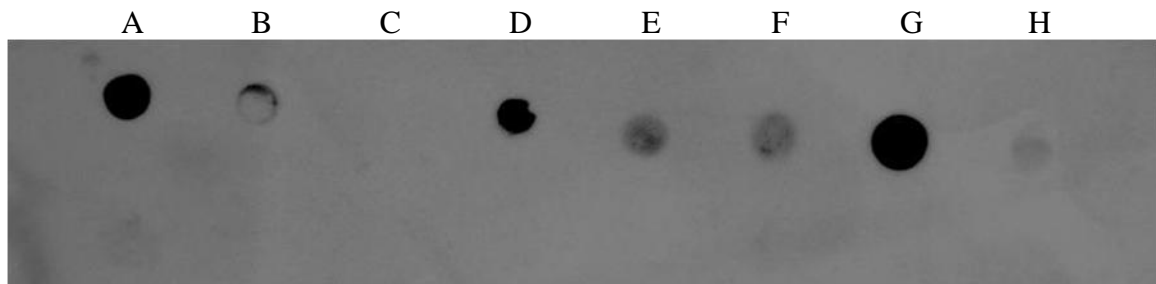


Figure 7.1 Dot Blot assay of chicken and mouse caecal contents with a *Campylobacter* specific antibody. (A) *C. jejuni* 81116, (B) *C. jejuni* 11168, (C) Chicken caecal content, (D) Chicken caecal content spiked with 10^7 *C. jejuni* 81116, (E) Chicken caecal content spiked with 10^5 *C. jejuni* 81116 (F) Mouse GI content spiked with 10^5 *C. jejuni* 81116, (G) Mouse GI content spiked with 10^7 *C. jejuni* 81116, (H) Mouse GI content.

7.2.6 Isolation of *C. jejuni* from an avian host

In order to assess the efficacy of the IMS protocol *in vivo* and potential use of recovered *C. jejuni* in further experiments, a previously established chicken colonisation model was utilised (Ringoir & Korolik, 2003). Colonisation of chickens by *C. jejuni* 81116 was established as 1.5×10^9 cfu/g of caecal content (Table 7.5), with the major normal flora evident in the caeca identified as *Proteus* spp. The normal flora was identified using culture phenotyping and Gram stain analysis, however further speciation was not performed. After IMS, the eluted *C. jejuni* cell number averaged at 10^5 cfu/g caeca. Approximately 10^1 *Proteus* spp. was co-eluted from immunocoated dynabeads[®] M-280. This level of isolation of *C. jejuni* was not significantly different to the maximum possible cell number isolated from Brucella broth or spiked caecal content (sections 7.2.1, 7.2.2 & 7.2.4). With each washing step the amount of *Proteus* spp. decreased (data not shown), suggesting that the *Proteus* spp. cells were not bound to the immunocoated dynabeads but captured and removed during the separation step.

Table 7.5 Isolation of infecting *C. jejuni* from an avian host

Organism recovered	cfu per g of content	No. of specific bacteria isolated from caeca	No. of specific bacteria eluted from dynabeads
<i>C. jejuni</i> 81116	1.5×10^9	1.9×10^5	9.8×10^4
<i>Proteus</i> spp.	1.0×10^7	8.6×10^1	1.3×10^1

Caecal homogenates from chickens infected with *C. jejuni* strain 81116 were incubated with anti-*C. jejuni* coated dynabeads and then eluted with trypsin-EDTA. The number of bead captured bacteria and eluted bacteria were determined by culture and expressed as cfu/g.

7.2.7 Isolation from a mammalian host

129/SvJ mice previously established to be a colonisation model for *C. jejuni* infection (McAuley *et al.*, 2007) were used to assess efficacy of the IMS system in a mammalian host. Colonisation of mice by *C. jejuni* 81116 was established as 4×10^5 cfu/g of GI content (Table 7.6), with the major normal flora evident in the GI tract identified by Gram staining as Gram positive bacilli. After IMS the recovered *C. jejuni* cell number averaged at 5×10^2 cfu/g GI content with approximately 4×10^3 cfu/g of the normal flora (Gram positive bacilli) isolated using the immunocoated dynabeads[®] M-280. A high proportion of captured bacteria were eluted for *C. jejuni* 81116 (2.5×10^2 cfu/g), however this was also evident for the contaminating normal flora (1.1×10^3 cfu/g). The level of isolation of *C. jejuni* is significantly lower compared to the isolation previously determined *in vitro* and in an avian host.

Table 7.6 Isolation of infecting *C. jejuni* from a murine model

Organism recovered	cfu per g of content	No. of specific bacteria isolated from GI contents	No. of specific bacteria eluted from dynabeads
<i>C. jejuni</i> 81116	4.79×10^5	5.0×10^2	2.5×10^2
Contaminant	1.1×10^6	4.0×10^3	1.13×10^3

Caecal homogenates from mice infected with *C. jejuni* strain 81116 were incubated with anti-*C. jejuni* coated dynabeads and then eluted with trypsin-EDTA. The number of bead captured bacteria and eluted bacteria were determined by culture and expressed as cfu/g.

7.3 Discussion

This chapter has described the development of an optimised IMS system for rapid isolation of *C. jejuni* from an *in vivo* host. An average of 10^5 viable *C. jejuni* cells were recovered that could be used for further gene profiling and binding assays.

The newly optimised IMS protocol allowed the successful isolation of $6-8 \times 10^5$ cfu/mL of three different strains of *C. jejuni* from the beads, indicating that this methodology is likely to be universally applicable for all *C. jejuni* strains displaying varying colonising potential.

An important factor observed by assessing IMS over a significant length of time, was if the bead-antibody complex had been stored for greater than 1 month, the isolation efficiency decreased by a minimum of 1 log. This was evident and a trend established that the older the bead-antibody complex got the less amount of bacteria were bound by the beads and isolated. Thus for IMS to perform at maximal capacity, bead-antibody complexes needed to be used fresh or within 1 month of coating.

Assessment of IMS showed no significant difference in recovery of *C. jejuni* cells from Brucella broth, artificially spiked caecal contents or from *in vivo* grown chicken caecal contents, indicating that the presence of gut flora and other mucosal components did not have a major influence on the ability to recover *C. jejuni* cells *ex vivo*.

The modified IMS protocol for recovery of viable cells developed in this study resulted in a 10-fold greater recovery of *C. jejuni* *in vitro* and *in vivo* compared to the IMS model by Yu *et al.* (2001) which was used to isolate *C. jejuni* from broth and ground poultry products. The number of *C. jejuni* required in the inoculum to yield the maximum isolated bacteria was similar to that reported by Yu *et al.* (2001), however,

appears to be 100-fold lower than that reported by Lamoureux *et al.* (1997). These data indicate that the optimised conditions described in this study compare extremely well with or exceed previous IMS conditions reported for *C. jejuni* (Lamoureux *et al.*, 1997; Mandrell & Wachtel, 1999; Yu *et al.*, 2001).

Specificity of the polyclonal antibody used to coat the dynabeads[®] M-280 was restricted to *C. jejuni* only since the dot blot immunoassay showed no cross-reactivity with the mucosa or mucosal components of an avian host (Figure 7.1). However minor cross reactivity was observed with mucosal components in murine GI tract. Considering that *Proteus* spp. is known to be normal flora in chicken caeca and develops in large numbers between days 4-7 post hatching (Coloe *et al.*, 1984), and that *Lactobacilli* spp., a large Gram positive bacilli often found as normal flora in mouse GI tract (He *et al.*, 2010), it was necessary to determine if the antibody specifically bound the normal flora found in avian and murine models, or was a by product of non-specific binding. The results of the dot blot immunoassay indicated that the *C. jejuni* antibody did not bind specifically to the normal flora representatives tested, indicating that it is feasible to assume that any weak binding observed was not species specific.

It is significant to note that lower *C. jejuni* cell numbers were isolated from a murine host as compared with that from an avian host. It is likely that the cell numbers isolated from mice are determined by the maximum cell number of *C. jejuni* that colonise the mouse GI tract. Previous work has shown that the on average 10^5 - 10^6 *C. jejuni* 81116 colonise mouse GI tract in the 129/SvJ murine model (McAuley *et al.*, 2007) as opposed to the 5×10^9 that are routinely found in chicken caeca (Ringoir *et al.*, 2007). Therefore the percentage of *C. jejuni* recovered from *in vivo* is comparable between an avian and murine host.

Interestingly low levels of *Proteus* spp. (0.01% of total isolated bacteria) were co-isolated with *C. jejuni* from chicken caeca. Contamination by *Proteus* spp. may be due to the abundance and size of this organism which leads to co-localisation with the M-280-*C. jejuni* complex, with a higher level of contaminating bacteria co-isolated from the mouse GI tract. Co-isolation of normal flora is likely to be caused by the bacterial cells becoming entrapped within the mucosal material and remain associated with the beads rather than due to specific binding. A similar issue was previously described for IMS of *Microsporidia* (Hoffman *et al.*, 2007). Therefore further optimisation to reduce the level of co-isolated normal flora would be required and may include the introduction of more washing steps in greater volumes to allow the dissociation of the contaminating bacteria from the beads. Or in the case for a murine host, IMS could be significantly improved with higher titre and/or specificity of the antibody.

Successful isolation of *C. jejuni* from an avian and murine host has demonstrated that further analysis of the bacteria without culture or passage can be performed. The presence of low numbers of contaminating bacteria is unlikely to interfere with isolation of *Campylobacter* spp. from food products, assays testing glycan binding or microarray studies (as described in Chapter 8.0). However if the desired application was proteomics, the IMS system would require further optimisation to ensure complete removal of the normal flora.

CHAPTER 8

Application of Immunomagnetic Separation to isolate *C. jejuni* directly from *in vivo* hosts

This work has fully contributed to the publication “Analysis of carbohydrate binding and gene expression by *in vitro* and *in vivo* propagated *Campylobacter jejuni* isolated by Immunomagnetic Separation.” Under revision to Microbiology, 2010. Manuscript in appendix D.

8.1 Introduction

Investigation of genome wide expression profiling using microarray technology is widely used to identify potential virulence factors (Stintzi *et al.*, 2005). And more recently, glycan arrays have become the preferential technique for identifying and elucidating protein-carbohydrate interactions (Day *et al.*, 2009). To investigate true differences between *in vitro* and *in vivo* grown bacteria requires techniques to isolate bacteria without culture steps.

Current bacterial isolation techniques involve enrichment in selective broth or culture on selective agar which can take up to 48 hrs (Mandrell & Wachtel, 1999). More specifically traditional methods for the detection of *C. jejuni* in food samples take 4-5 days (Che *et al.*, 2001). This study had previously demonstrated the use of immunomagnetic separation (IMS) to isolate *C. jejuni* from an *in vivo* host directly without culture (Chapter 7.0). IMS had also been proven to be successful in isolating *E. coli* and verifying use in PCR based identification tools (Bopp *et al.*, 2003). Protocols implementing IMS have been established to be more sensitive thus improving detection methods like that for *S. enterica* ser Typhimurium and *L. monocytogenes* (Amagliani *et al.*, 2006; Moreira *et al.*, 2008).

A major advantage of using IMS is that gene expression and bacterial characteristics can be evaluated immediately following isolation from the host rather than undergoing the routine 24 to 48 hr subculture, whereby variation in regulation of virulence factors or loss of gene expression may occur.

The work presented in this chapter describes the application of carbohydrate binding analysis and gene expression analysis to identify differences between laboratory grown bacteria and those isolated from an *in vivo* infection model using IMS. With changes in

both glycan binding and gene expression, including known pathogenicity genes, identified in *C. jejuni* within an avian/mammalian host.

8.2 Results

8.2.1 Glycan array

Bacteria-carbohydrate interactions were assessed using glycan arrays for *C. jejuni* 81116 cells recovered from chicken caeca using IMS and compared to that from *C. jejuni* 81116 grown under standard laboratory conditions at 42°C. The complete set of glycans used in the arrays are listed in Appendix G. Glycan binding found to be significantly different ($p < 0.05$) between *in vivo* and *in vitro* isolates are described in Table 8.1.

Table 8.1. *In vitro* and *in vivo* isolated *C. jejuni* with a significant difference in binding

ID	Glycan structure
Neutral glycans	
1A.	Gal β 1-3GlcNAc
1G	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc
1H	Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc
1J.	Gal β 1-4GlcNAc β 1-6(Gal β 1-3GlcNAc β 1-3)Gal β 1-4Glc
1P.	Gal α 1-3Gal β 1-4Glc
2B.	Gal β 1-6Gal
2E.	Gal α 1-4Gal β 1-4GlcNAc
4A.	GlcNAc β 1-4GlcNAc
4B.	GlcNAc β 1-4GlcNAc β 1-4GlcNAc
4E.	GlcNAc β 1-4MurNAc
5B.	GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man
5D.	Man α 1-3Man
5E.	Man α 1-4Man
5H.	Man α 1-6(Man α 1-3)Man α 1-6(Man α 1-3)Man
7H.	Gal β 1-4(Fuc α 1-3)Glc
7L.	Fuc α 1-2Gal β 1-4(Fuc α 1-3)Glc
7M.	Gal β 1-3(Fuc α 1-2)Gal
Negatively charged glycans	
8A.	SO ₃ -3Gal β 1-3(Fuc α 1-4)GlcNAc
8B.	SO ₃ -3Gal β 1-4(Fuc α 1-3)GlcNAc
10C.	Neu5Ac α 2-3Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc
10D.	Gal β 1-4(Fuc α 1-3)GlcNAc β 1-6(Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-3)Gal β 1-4Glc
10K.	Neu5Ac α 2-3Gal β 1-4GlcNAc
12A.	C ₂₄ H ₃₆ O ₂₅ S ₂ Na ₂
12E.	C ₄₈ H ₇₀ O ₄₉ S ₄ Na ₄
12H.	C ₁₂ H ₁₆ NO ₁₆ S ₂ Na ₃

12I.	C ₁₂ H ₁₆ NO ₁₆ S ₂ Na ₃
12N.	C ₁₄ H ₁₉ NO ₁₄ SNa ₂
12O.	C ₁₄ H ₁₉ NO ₁₄ SNa ₂
13G.	(GlcAβ1-3GlcNAcβ1-4) _n (n=8)
13M.	(GlcA/IdoAβ1-3(±6S)GalNAcβ1-4) _n (n<250)

Binding to terminal Galactose or Gal/GalNAc structures

In general, *in vivo* and *in vitro* isolated bacteria showed similar binding for all terminal galactose (Gal) structures with some notable exceptions (Figure 8.1). Binding to disaccharide Galβ1-3/4/6Gal structures was observed to be significantly higher in the laboratory grown strain (Figure 8.1, 2B; $p < 0.05$). In contrast, *C. jejuni* 81116 isolated from the chicken host appeared to bind a disaccharide Galβ1-3GlcNAc (1A) significantly greater than for the laboratory grown strain ($p < 0.05$). However the addition of any sugars that lengthen this disaccharide structure resulted in a loss of binding.

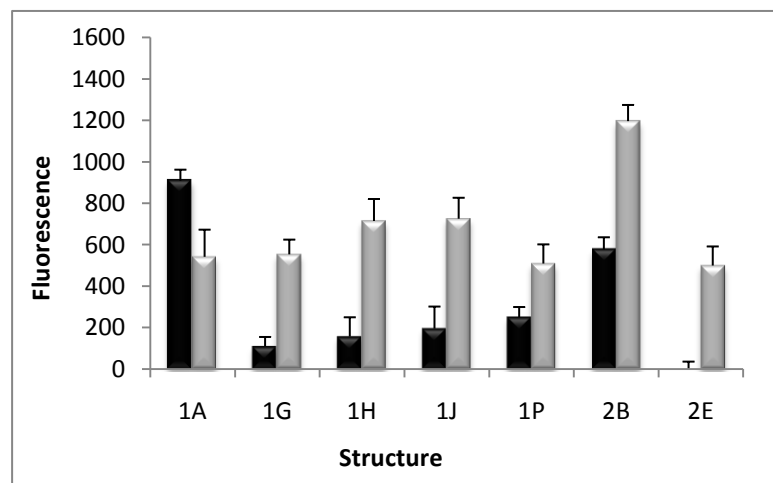


Figure 8.1 Glycan array binding of *C. jejuni* 81116 to terminal galactose structures. Binding of *C. jejuni* 81116 to uncapped terminal Gal structures. Fluorescence intensities associated with *in vivo* isolated *C. jejuni* 81116 (black bar) and laboratory grown *C. jejuni* 81116 (grey bar) at 42°C ($p < 0.05$).

Binding to glucose or GlcNAc structures

The binding of the laboratory grown strain 81116 to repeating N-acetylglucosamine (GlcNAc β 1-4GlcNAc units) containing structures was consistent across a range of repeat lengths. For the *in vivo* isolated strain, a general increase in binding was observed as the number of GlcNAc repeats increased to a maximum of 4 sugars (4B). Binding of *in vivo* isolated bacteria to GlcNAc structures longer than 4 repeats appeared to decrease (Figure 8.2).

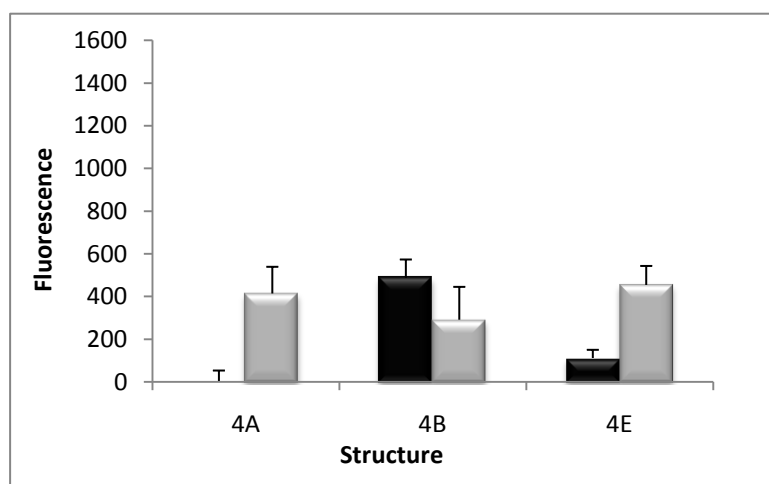


Figure 8.2 Glycan array binding of *C. jejuni* 81116 to GlcNAc structures. Binding of *C. jejuni* 81116 to GlcNAc structures. Fluorescence intensities associated with *in vivo* isolated *C. jejuni* 81116 (black bar) and laboratory grown *C. jejuni* 81116 (grey bar) at 42°C ($p < 0.05$).

Binding to mannose containing structures

The binding to mannose (Man) structures by *C. jejuni* 81116 was found to be different between the laboratory grown and *in vivo* isolated bacteria. The bacteria isolated from caecal content showed greater binding to α 1-3 linked di-Man (5D) when compared to the binding by the laboratory grown strain, however the laboratory strain favoured binding to α 1-4 linked di-Man (5H) (Figure 8.3).

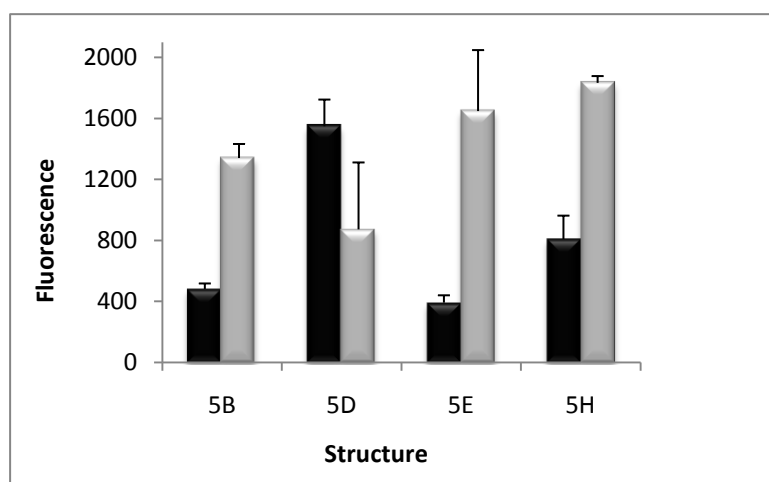


Figure 8.3 Glycan array binding of *C. jejuni* 81116 to mannose containing structures. Binding of *C. jejuni* 81116 to Man containing structures. Fluorescence intensities associated with *in vivo* isolated *C. jejuni* 81116 (black bar) and laboratory grown *C. jejuni* 81116 (grey bar) at 42°C (p<0.05).

Binding to fucosylated structures

The *in vivo* isolated *C. jejuni* 81116 also showed reduced binding to the shorter fucosylated compounds compared with laboratory grown bacteria (7H & M; Figure 8.4), and less binding was also noted to glycans with the branched fucose linked to a non-terminal Gal/Glc (7H, L & M). The binding to glycans with subterminal fucose by *C. jejuni* 81116 isolated from chicken host was not reduced in the presence of structures containing GlcNAc or GalNAc. Binding to negatively charged fucosylated structures such as sulfo-Lewis^a and sulfo-Lewis^x (8A & B) was also higher for the laboratory grown *C. jejuni* strain 81116 when compared to *in vivo* isolated bacteria ($p < 0.05$).

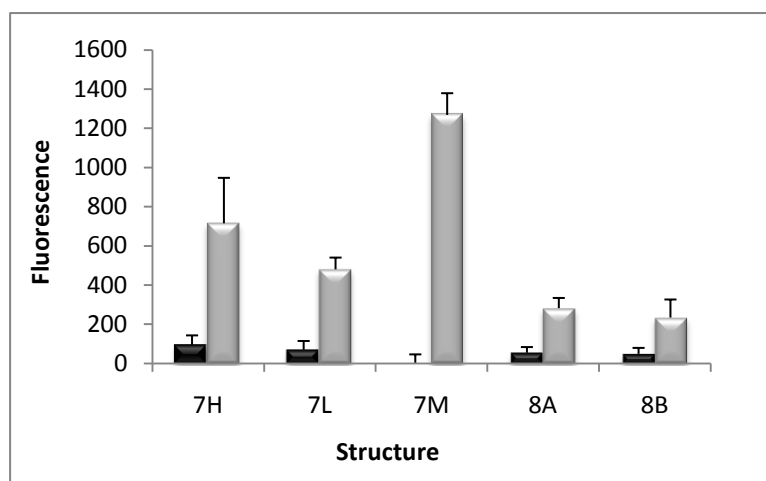


Figure 8.4 Glycan array binding of *C. jejuni* 81116 to fucosylated structures. Binding of *C. jejuni* 81116 to fucosylated structures. Fluorescence intensities associated with *in vivo* isolated *C. jejuni* 81116 (black bar) and laboratory grown *C. jejuni* 81116 (grey bar) at 42°C ($p < 0.05$).

Binding to sialylated or Neu5Ac structures

Differences were observed between laboratory grown and IMS isolated *C. jejuni* 81116 in the binding of negatively charged glycans such as sialylated and sulphated structures, with significantly more binding observed for laboratory grown bacteria. Although, in general, the binding observed for *C. jejuni* 81116 to sialic acid was lower than the other structures tested. Few structures show significant binding for either isolate, however the biantennary structure 10D (Gal β 1-4(Fuc α 1-3)GlcNAc β 1-6(Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-3)Gal β 1-4Glc) was strongly bound by the laboratory grown strain. Binding to the unbranched structures containing Neu5Ac α 2-3Gal β 1-3/4GlcNAc (10C & 10K) was also observed for the laboratory grown strain. No significant binding to sialylated structures was observed for *C. jejuni* 81116 that had been isolated from chicken caecal content.

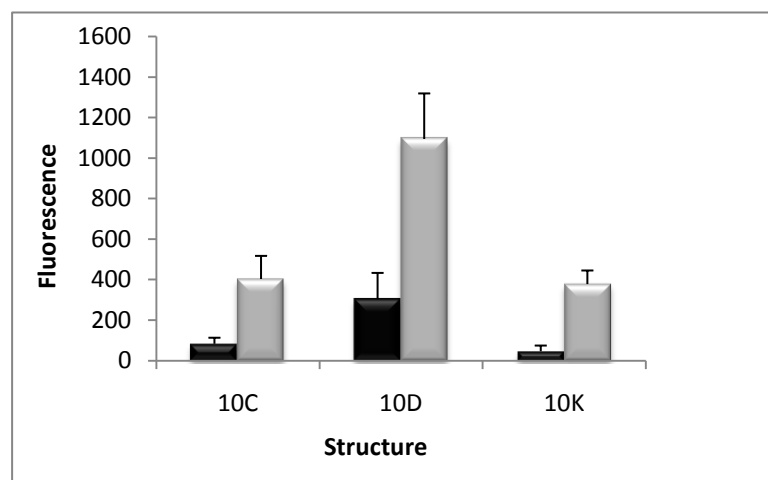


Figure 8.5 Glycan array binding of *C. jejuni* 81116 to sialylated structures. Binding of *C. jejuni* 81116 to Neu5Ac structures. Fluorescence intensities associated with *in vivo* isolated *C. jejuni* 81116 (black bar) and laboratory grown *C. jejuni* 81116 (grey bar) at 42°C (p<0.05).

Binding to carageenans and glycosaminoglycans (GAG)

With respect to the ability of *C. jejuni* 81116 to bind Carageenans (polysaccharide extracts from *Chondrus crispus*) and glycosaminoglycan (GAG) structures, binding of the laboratory grown bacteria to negatively charged glycans was greater than the *in vivo* isolated bacteria (Figure 8.6).

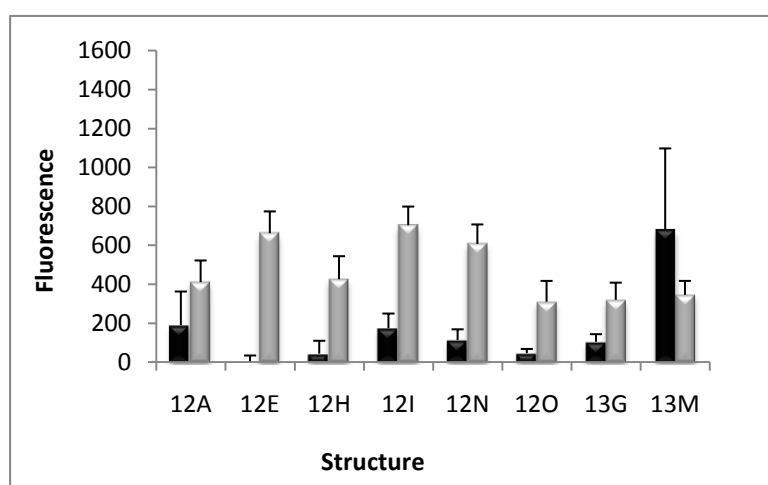


Figure 8.6 Glycan array binding of *C. jejuni* 81116 to carageenan and GAG structures. Binding of *C. jejuni* 81116 to carageenan and GAG structures. Fluorescence intensities associated with *in vivo* isolated *C. jejuni* 81116 (black bar) and laboratory grown *C. jejuni* 81116 (grey bar) at 42°C ($p < 0.05$).

Due to co-isolation of *Proteus* spp. during IMS (Chapter 7.0), this organism was also assessed for glycan binding. No significant binding of glycan structures above background was observed when using 10^4 - 10^6 bacteria.

Comparative glycan binding analysis on *C. jejuni* 81116 grown *in vitro* at 37°C and isolated from a murine host was attempted, however due to concurrent isolation of normal flora during IMS (Chapter 7.0); any results obtained for glycan binding appeared to be obscured by the normal flora contaminant.

8.2.2 Gene expression analysis of IMS isolated *C. jejuni* in an avian host

To evaluate the suitability of IMS isolated bacteria for analysis of gene expression, total RNA was isolated from *C. jejuni* obtained from chicken caeca, labelled and hybridised to an oligonucleotide based microarray, as described in Material and Methods (sections 2.10.2 to 2.10.5). The scan of the array showed that the labelled cDNA successfully hybridised to the chip with sufficient signal to perform a partial analysis (data not shown). The RNA isolated directly from the *in vivo* bacteria, without any amplification or other alterations was used to circumvent any bias that may occur due to AU rich RNA content during amplification. We were able to identify the regulation of more than 100 genes between *in vivo* isolated and laboratory grown *C. jejuni*. The majority of the 100+ genes found were hypothetical or putative proteins of unknown function (Appendix D, Table SM3); however those genes of known function are shown in Table 8.2.

8.2.3 Confirmation of expression by QPCR

Verification of microarray results was performed by testing 5 of the regulated genes; *tlp2*, *cdtA* *cdtC*, *waaC* and *Cj0803* with QPCR (Figure 8.7). While there were some differences in the increase/decrease values obtained through the two different methods, in each case the QPCR confirmed the altered regulation of the gene.

Table 8.2 Genes of known function with altered expression *in vivo* in an avian host as determined by microarray analysis

Up-regulated	Gene #	Fold Change
acid membrane antigen A	Cj1363	269
asps	Cj0640c	27
capsular biosynthesis sugar kinase, putative	CJE1610	199
cbpA	Cj1229	5
cdtA	Cj0079c	360
cdtC	Cj0077c	66
exbB1	Cj0179	13
fliE	Cj0526c	24
fliN	Cj0351	41
htpG	Cj0518	153
infA	Cj1590	77
leuC	Cj1717c	52
MdaB protein homolog	Cj1545c	53
methyl-accepting chemotaxis signal transduction Tlp2	Cj0144	68
mobA	Cj1350	201
modB	Cj0301c	323
neuA2	Cj1311	66
nifU protein homolog	Cj1639	8
Non-haem iron protein	Cj0012c	576
nusB	Cj0382c	53
Ogt	Cj0836	42
oorD	Cj0535	488
outer membrane protein	Cj1170c	86
oxidoreductase	Cj0833c	9
panB	Cj0298c	35
Pnp	Cj1253	16
prsA	Cj0918c	19
rplA	Cj0475	15
rpmF	Cj0330c	5
serC	Cj0326	187
signal-transduction regulatory protein	Cj1024c	18
Site-specific DNA-methyltransferase, putative	CJE0550	189
spot	Cj1272c	32
tail tape measure protein, TP901 family	CJE0222	15
transmembrane efflux protein	Cj0366c	30
trpA	Cj0349	55
trpB	Cj0348	43
waaF	Cj1148	54
wlaJ	Cj1122c	28

Down-regulated	Gene #	Fold Change
galU	Cj1536c	0.20
gatA	Cj1059c	0.06
haloacid dehalogenase-like hydrolase	CJE1606	0.03
msbA	Cj0803	0.16
napA	Cj0780	0.06
proA	Cj0558c	0.17
waaC	Cj1133	0.02
wlaM	Cj1119c	0.06

Gene expression of *in vitro* cultured *C. jejuni* strain 81116 was compared with the same strain recovered from chicken caeca using IMS. The microarray contained all 81116 genes and only genes of known function with significantly different gene expression determined by t-tests ($p < 0.05$) are shown.

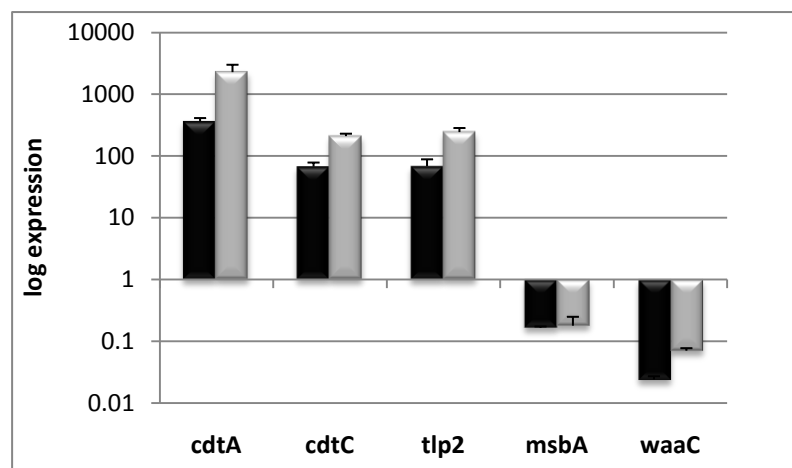


Figure 8.7 Relative expression of genes identified from *in vivo* by microarray and QPCR. Standard errors are shown as bars above fold differences between microarray and QPCR identified genes. N=3 independent repeats, difference is chicken/lab grown bacteria \pm 1 standard deviation. Black bar represent microarray data and grey bar represents QPCR data.

Microarray analysis also identified expression of potential virulence factors such as, toxin and chemotaxis gene expression, therefore additional analysis of related genes was performed using QPCR. As up-regulation of *cdtA* and *cdtC* genes were identified in

microarray analysis, investigation of *cdtB* was performed as the *cdt* genes are known to be co-regulated for toxin production. Similarly, up-regulation of the chemotaxis gene *tlp2*, lead to the investigation of other chemotaxis genes. Analysis of gene expression of *cdtB* and *tlp1*, 3, 4, 7 and 10, revealed up-regulation of these genes in *C. jejuni* 81116 isolated from chicken caecal content when comparing the two bacterial isolates using QPCR (Figure 8.8). The regulation of these related genes was similar to that observed for genes identified through microarray analysis with up-regulation observed in *in vivo* isolated *C. jejuni* 81116.

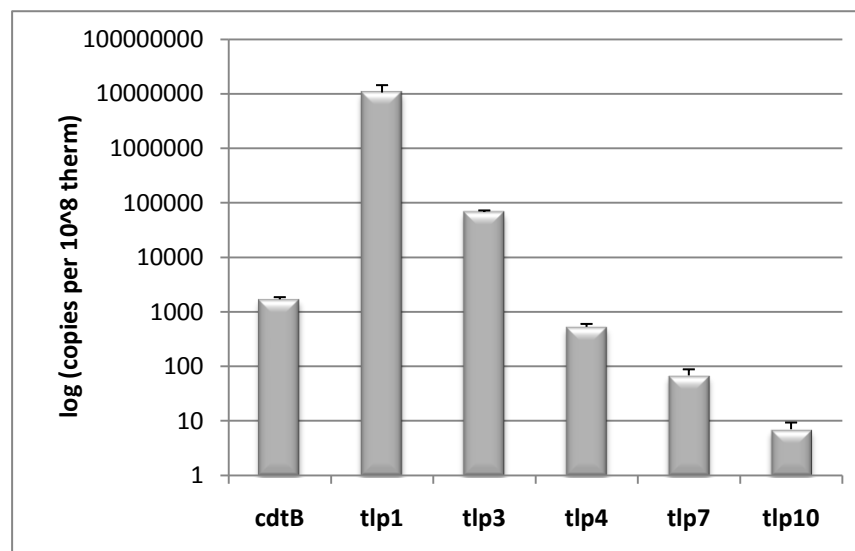


Figure 8.8 Relative expression of toxin and chemotaxis genes

Fold differences in *C. jejuni* gene expression *in vivo* of other toxin and chemotaxis genes determined by QPCR normalised to 16S rRNA expression (N=3 independent repeats, Difference is chicken/lab grown bacteria \pm 1 standard deviation).

8.2.4 Gene expression analysis of IMS isolated *C. jejuni* in mammalian host

To evaluate *C. jejuni* gene expression in a murine infection model, bacteria isolated from intestinal content using IMS were processed for isolation of total RNA and hybridised to an oligonucleotide based microarray. The scan of the array showed that the labelled cDNA successfully hybridised to the chip with sufficient signal to perform a partial analysis (data not shown). Regulation of more than 100 genes between *in vivo* isolated and laboratory grown *C. jejuni* 81116 were identified. The majority of the genes identified were hypothetical or putative proteins of unknown function (Appendix E), however the genes of known function are shown in Table 8.3.

Early preliminary data on microarray analysis identified regulation of potential virulence factors, most notably, expression of genes involved in chemotaxis. Due to time restrictions, QPCR and further analysis of gene expression was not able to be performed.

To determine possible differences in expression of genes between *in vivo* isolated *C. jejuni* from a chicken host and murine host, microarray data was assessed. 13 genes identified as either up or down-regulated from both *in vivo* hosts were compared (Figure 8.9).

Table 8.3 Genes of known function with altered expression *in vivo* in a murine host as determined by microarray analysis

Up-regulated	Gene #	Fold Change
acpS	Cj1409	12
alaS	Cj0506	16
acid membrane antigen A	Cj1363	8
aroE	Cj0405	41
aspS	Cj0640c	72
ceuB	Cj1352	56
ceuD	Cj1354	23
cheA	Cj0284c	16
cheR	Cj0923c	6
cheV	Cj0285c	11
cheY	Cj1118c	2253
cstA	Cj0917c	29
dnaB	Cj0562	20
dnaJ	Cj1260c	11
fabD	Cj0116	15
fliA	Cj0061c	81
fliM	Cj0060c	21
ftsK (putative cell division protein)	Cj0886c	53
galE	Cj1131c	126
galU	Cj1536c	23
groEL	Cj1221	5
hipO	Cj0985c	15
htrB	Cj1134	7
kpsD	Cj1444c	14
leuB	Cj1718c	18
lysS	Cj0401	27
metA	Cj1726c	22
mobA	Cj1350	314
murB	Cj1676	11
neuA2	Cj1311	7
nuoB	Cj1578c	263
nuoN	Cj1566c	30
pal (peptidoglycan associated lipoprotein omp18)	Cj0113	27
peb1A	Cj0921c	18
pheA	Cj0316	39
pheT	Cj0896c	22
porA	Cj1259	27
proA	Cj0558c	37
secD	Cj1093c	4
spoT	Cj1272c	31
SurE protein homolog	Cj0293	17
waaC	Cj1133	6

waaD	Cj1151c	10
waaE	Cj1150c	7
waaV	Cj1146c	7
wlaB	Cj1130c	20
wlaC	Cj1129c	163
wlaI (putative transferase)	Cj1123c	34
wlaM (putative integral membrane protein)	Cj1119c	5
Down-regulated	Gene #	Fold Change
hydA2 putative Ni/Fe-hydrogenase small subunit	Cj1399c	0.844991
ppa	Cj0638c	0.019

Gene expression of *in vitro* cultured *C. jejuni* strain 81116 was compared with the same strain recovered from mouse intestinal content using IMS. The microarray contained all 81116 genes and only genes of known function with significantly different gene expression determined by t-tests ($p < 0.05$) are shown.

Gene regulation appears to be consistent for the majority of the genes identified with a few exceptions. There appears to be an up-regulation of *galU*, *proA* and *waaC* from *C. jejuni* 81116 isolated from mouse intestinal content, in contrast these genes are down-regulated from *C. jejuni* 81116 isolated from the chicken caeca. *amaA*, *dnaJ* and *nusB* are up-regulated from both *in vivo* hosts, however there is 10-fold more expression of these genes from *C. jejuni* isolated from a chicken host. The most noteworthy difference is the regulation of the chemotaxis response regulator *cheY*, where expression was 1000-fold greater in *C. jejuni* isolated from mouse intestinal content compared to chicken caeca.

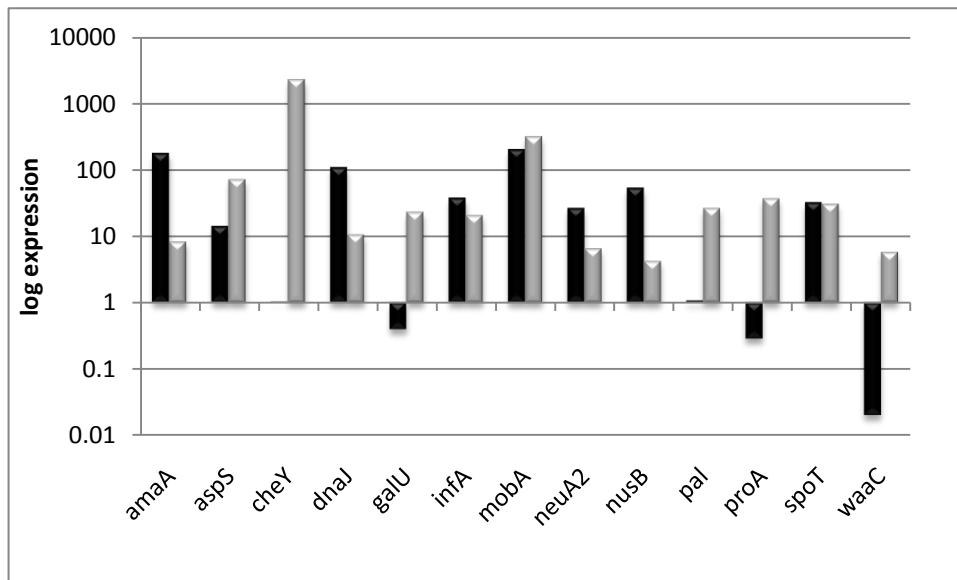


Figure 8.9 Relative expression of genes identified in avian and mammalian host by microarray. Fold differences between *in vivo* isolated *C. jejuni* 81116 from chicken caeca and mouse intestinal content. Black bar represents genes expressed in chicken host and grey bar represents genes expressed by murine model.

8.3 Discussion

This chapter describes the profiling of glycan binding and gene expression by DNA microarray and QPCR using *C. jejuni* 81116 isolated from an *in vivo* host using IMS.

Considering that glycans have been shown to be important structures for the adherence of *C. jejuni* to host cells (Day *et al.*, 2009), the glycan arrays assessing glycoconjugates potentially involved in host-bacteria interactions for *C. jejuni* 81116 were interrogated. Glycan array analysis in this study revealed a number of differences in overall binding between *in vivo* isolated *C. jejuni* from a chicken host and laboratory grown bacteria. The main observed differences were in the ability to bind extended glycan structures, and negatively charged glycan structures. With binding to negatively charged glycans reduced in *C. jejuni* 81116 isolated from the chicken caecal content when compared to laboratory grown bacteria.

It is also noteworthy that the laboratory grown strain 81116 bound negatively charged glycans containing groups such as sulphur or sialic acid residues whereas the bacteria recovered from chicken caeca did not. These observations are similar to that reported for *C. jejuni* NCTC 11168 and are consistent with the contention that the binding of sialic acid may be important for initial host-pathogen interaction but not for prolonged colonisation (Day *et al.*, 2009). That is, *C. jejuni* isolated from chicken caecal content was already present in and adapted to its preferred gastrointestinal niche and so is unlikely to require the ability to recognise sialic acid structures.

As previously described in Chapter 7.0, small numbers of bacteria belonging to *Proteus* spp. were co- isolated at low level along with *C. jejuni* 81116 from the chicken caeca and therefore was also subjected to glycan array analysis to investigate if this bacterium influenced *C. jejuni* binding to the glycan array. No significant binding of glycan

structures above background by *Proteus* spp. was observed when using 10^4 - 10^6 bacteria, a 3-5 log number in excess to those co-isolated following IMS.

IMS isolated *C. jejuni* was also successfully used to isolate whole cell RNA that enabled the identification of genes differentially expressed by *C. jejuni* when colonising chicken caeca and during infection in a murine model without amplification. Microarray analysis revealed the regulation of approximately 47 genes including toxin and chemotaxis genes. The method produced clear-cut data for over 100 of the genes present on the *Campylobacter* oligonucleotide arrays described in this study. Approximately one half of the genes identified by the array as regulated *in vivo* during the colonisation of chicken/mammalian hosts were of known function. Of these, 16 genes have been reported previously in array studies of *C. jejuni* gene regulation during infection or colonisation of animal hosts (Stintzi *et al.*, 2005; Woodall *et al.*, 2005). When compared to these reports, the direction of regulation was in agreement for *amaA*, *LeuC*, *panB*, *spoT* (Stintzi *et al.*, 2005) and *proA* (Woodall *et al.*, 2005) for *C. jejuni* isolated from chicken caeca only. However, previously published results for regulation of *Cj1170c* and *panB* were contradictory, being reported as up-regulated by Stintzi *et al.* (2005), and down-regulated by Woodall *et al.* (2005). These contradictory observations are likely to reflect the differences between hosts used in respective studies and bacterial isolation techniques. Our study showed that *C. jejuni* 81116 directly isolated from chicken host without any propagation, up-regulates *Cj1170c* and *panB*, in agreement with Stintzi *et al.* (2005). Also bacteria isolated from the luminal content of the chicken caeca had increased expression of the *cdt* genes in agreement to analysis performed by Tu *et al.* (2008), which showed higher *cdt* expression in the presence of MUC2.

CheY is known to be important in colonisation and pathogenesis of chickens and mice (Hendrixson & DiRita, 2004; Takata *et al.*, 1992). Comparison of gene expression for *in vivo* isolated *C. jejuni* 81116 revealed a significant difference in expression of the chemotaxis gene *cheY*. Up-regulation was observed in a murine model; conversely regulation was not significantly altered in a chicken model. One possible explanation for this phenomenon may be that greater control of directed bacterial motility may be required for chemotaxis in mammals.

The level of RNA isolated from IMS purified bacteria was sufficient for basic transcriptome analysis where only highly expressed genes were identified. In addition, this circumvents the likelihood of any bias that may occur during RNA amplification due to the use of random primer sets. This however, was at expense of sensitivity usually offered by standard microarray analysis using greater quantities of RNA isolated from larger numbers of cultured bacteria. The data for the chicken host showed excellent correlation with RNA levels determined using QPCR, which is exemplified by the correlation of the microarray data with the QPCR verifications for *cdtA*, *cdtC*, and *tlp2* genes. QPCR analysis of related genes also confirmed the up-regulation of *cdtB* and *tlp1*, 3, 4, 7 and 10 in IMS isolated bacteria from chicken caecal content compared to laboratory grown bacteria.

Preliminary data on gene expression from *in vivo* isolated *C. jejuni* using IMS has given some promising results with regards to differential expression in animal hosts. Even though experimentation is still in its infancy, there is definite plausibility for continued refinement of this technique. Currently in progress is the use of RNA amplification to ensure a full microarray data set is able to be obtained for *in vivo* isolated *C. jejuni*.

This chapter demonstrates the use of glycan array and gene expression studies on IMS isolated *C. jejuni* 81116 from animal hosts to identify potential glycan binding and virulence genes that may be responsible for colonisation and pathogenesis *in vivo*.

CHAPTER 9

General Discussion

The overall aim of work described in this dissertation was to characterise host-bacterial interactions involved in the invasion process of *C. jejuni*. To achieve this goal, the influence of the cell surface mucin MUC1 with regards to attachment and penetration was investigated and differences in ligand-binding and gene expression between *in vitro* grown and *in vivo* isolated *C. jejuni* 81116 analysed.

It is thought that mucins provide a protective barrier to the intestinal mucosa by facilitating the removal of bacteria from the intestinal epithelium (Shekels *et al.*, 1998b). It has been proposed that MUC1 acts as a releasable decoy for bacterial adhesins thereby limiting attachment and subsequent invasion of *C. jejuni* to cells in the GI tract (McAuley *et al.*, 2007). In contrast, it has also been suggested that mucins may act as cell surface receptors and facilitate the entry of certain bacterial species, such as *C. jejuni* into intestinal host cells (Sylvester *et al.*, 1996b). It has been hypothesised that the interaction of *C. jejuni* with the cell surface glycoprotein MUC1 may facilitate attachment and penetration into host mucosal cells.

To date, numerous interactions between mucins and other pathogens have been demonstrated, these include *H. pylori* which binds to Lewis blood group antigens present on epithelial mucins (Linden *et al.*, 2004), *H. influenzae* which binds respiratory mucins (Reddy *et al.*, 1996) and *S. enterica* ser. Typhimurium which has been shown to bind intestinal mucins (Vimal *et al.*, 2000). Studies have also highlighted that pathogenic organisms like *E. coli* O157:H7 have the ability to bind to glycolpeptides, such as the N-linked oligomannoside side chain of mucin (Sajjan & Forstner, 1990). The cs-mucin MUC1 has previously been shown to be an adhesion site for *P. aeruginosa*, with further studies indicating that the major bacterial component responsible for adhesion was flagellin (Lillehoj *et al.*, 2002). In addition *P. aeruginosa*

has been shown to bind the extracellular domain of MUC1 expressed in CHO cells and triggers phosphorylation of the MUC1 cytoplasmic tail (Lillehoj *et al.*, 2001).

Growth conditions influence the ability of bacteria to bind to host cells and this study has demonstrated that microaerobic incubating conditions for *in vitro* co-culture assays with respect to adherence and invasion of *C. jejuni* were more favourable than standard culture under CO₂. This alternative co-culture assay showed that MUC1 expressed on the surface of intestinal epithelial cells promoted adherence and subsequent invasion of *C. jejuni* into the cell, in agreement with a previous study (McAuley *et al.*, 2007). Microscopic observation of *C. jejuni* interacting with MUC1 expressed on the epithelial cell surface of an intestinal cell line *in vitro*, further supported this hypothesis.

To facilitate further analysis and microscopic visualisation of the cs-mucin MUC1 interaction with *C. jejuni*, it was decided to construct a suite of reporter vectors, both integrative and shuttle, capable of generating fluorescent and bioluminescent *C. jejuni*. Additionally, it was thought that generation of bioluminescent *C. jejuni* would be assessed in an animal model to develop a time course for *Campylobacter* spp. infection. The incorporation of genes encoding fluorescent proteins and luciferase into pathogens has been used widely to assess interaction with host cells, with exceptional advances in *Salmonella* spp. virulence (Contag *et al.*, 1995; Valdivia & Falkow, 1996). Therefore it was feasible to assume that development of fluorescent and bioluminescent *C. jejuni* would facilitate studies of interaction with the host and possibly identify virulence factors important for *C. jejuni* pathogenesis. However, all attempts to design integrative and shuttle vectors for incorporation and expression of genes encoding for fluorescence and luciferase into *C. jejuni* were not successful. Although in one of the transformants GFP was integrated into the *C. jejuni* genome, the level of expression was too low for

use in microscopy. This was most likely due to suboptimal codon usage between the fluorescent protein genes and *C. jejuni* genome. Due to a high AT composition in the *C. jejuni* genome, codon preference may need to be manipulated to enhance translation of fluorescence and luciferase encoding mRNA. The same issue has been described previously, with alterations in codon usage and secondary promoters required for efficient expression of fluorescent protein genes in *Campylobacter* spp. (Karlyshev & Wren, 2005; Miller *et al.*, 2000; Mixer *et al.*, 2003).

Due to lack of success with generation of fluorescent or luminescent *C. jejuni*, an alternative strategy for assessment of host-bacterial interaction needed to be developed. Therefore an improved technique for isolation of *C. jejuni* directly from an *in vivo* host without prior passage was developed and implemented to facilitate the comparison of glycan binding and gene expression between *C. jejuni* isolated from animal hosts.

Immunomagnetic separation (IMS) techniques have been used in diagnostic microbiology for isolation of pathogenic organisms in food products with great success (Olsvik *et al.*, 1994). An advantage of using IMS is that the target bacteria are separated from the environment and can be concentrated for accurate detection and cultivation for identification by molecular typing and PCR techniques. Such applications have been performed for *M. paratuberculosis*, a slow growing pathogenic organism that can contaminate milk from livestock (Grant *et al.*, 1998), *E. coli* O157:H7 which was isolated from a waterborne outbreak in the US and bovine faeces (Bopp *et al.*, 2003; Parham *et al.*, 2003) and *S. enterica* ser. Typhimurium which has been isolated from contaminated food products and bovine faecal samples (Moreira *et al.*, 2008; Salehi *et al.*, 2007). IMS of fastidious organisms like *H. pylori* and *Campylobacter* spp. has allowed isolation from the environment and food products not only of viable cells but

also bacteria in the VBNC state, therefore providing added sensitivity for detection (Velazquez & Feirtag, 1999). More recently an application of IMS for *Campylobacter* spp. has been successful in isolating contaminating bacteria from poultry products (Che *et al.*, 2001; Lamoureux *et al.*, 1997; Mandrell & Wachtel, 1999; Yu *et al.*, 2001). The numbers of bacterial cells isolated by IMS were sufficient for PCR and other diagnostic applications, however a greater number of *C. jejuni* are required for downstream analysis such as transcriptomics or glycomics. It was therefore necessary to develop an improved method which would increase the yield of IMS separated bacteria. The IMS technique described in this study enabled isolation of 10^5 *C. jejuni* cells which not only compares exceptionally well or surpasses this previous work, but is the first report of successful isolation of *C. jejuni* from the GI tract of both avian and mammalian hosts.

C. jejuni cells isolated using this IMS technique allowed the investigation of specific interactions between the bacteria and host epithelial cells using glycan binding and differential gene expression analysis between *in vitro* and *in vivo* isolated *C. jejuni*. As previously described, mucin is a known chemoattractant of *C. jejuni* (Hugdahl *et al.*, 1988), and furthermore sugars involved in the composition of mucins, like fucose, mannose and N-acetylglucosamine (GlcNAc) have recently been demonstrated to influence *C. jejuni* binding to glycans (Day *et al.*, 2009; Hartley, 2009).

Implementation of a glycan array allowed the investigation of differences in binding between *C. jejuni* 81116 grown *in vitro* compared to *C. jejuni* isolated from chicken caecal content. The most notable differences in *in vivo* isolated *C. jejuni* 81116 was an increase in binding on direct non-extended GlcNAc structures and α 1-3Man glycans. Binding of *C. jejuni* 81116 to mannose containing structures corresponds with *C. jejuni* 11168-O which was determined to have glycan specific binding to certain mannose

structures, however in general, binding of *C. jejuni* 11168-O and *C. jejuni* 11168-GS was variable between strains grown at 42°C. There appears to be distinct differences in Man-binding potential between *C. jejuni* 81116, *C. jejuni* 11168-O and *C. jejuni* 11168-GS at avian *in vivo* conditions (Day *et al.*, 2009), which may reflect the colonising potential of respective strains.

The glycan data accumulated in this study suggests that upon interaction of *C. jejuni* 81116 with the GI tract in an avian host, it is likely to preferentially bind sugars that may be in abundance and may be possible recognition sites for initial adherence to host tissue. Furthermore, previous studies have shown that binding of pathogenic organisms to lectin structures on mucins appears to be species specific. For example GlcNAc was able to strongly inhibit *E. coli* binding to Caco-2 cells *in vitro*, while binding of *S. enterica* ser. Typhimurium to Caco-2 in the presence of GlcNAc was increased (Parker *et al.*, 2010). Thus, the role of individual lectins may play a specific function in colonisation of the host epithelium depending on bacterial species and its repertoire of virulence factors.

Direct isolation of *C. jejuni* 81116 from avian caecal contents following infection allowed subsequent analysis of genes differentially expressed between *in vitro* grown and *in vivo* isolated bacteria. Of the 47 differentially expressed genes of known function, the most interesting was the expression of toxin and chemotaxis genes, which were highly up-regulated. This finding also correlated with previously reported gene regulation during infection/colonisation of animal hosts (Stintzi *et al.*, 2005; Woodall *et al.*, 2005). The most significant differences occurred in expression of *tlp* and *cdt* genes. *C. jejuni* 81116 isolated from the caecal content of the chicken was determined to have an increased expression of *cdtA*, *cdtB* and *cdtC*, correlating with a previous study by Tu

et al. (2008). This study demonstrated that *C. jejuni* utilises MUC2 for the modulation of expression of virulence genes such as *cdt*, *cia* and putative mucin-degrading enzymes. In addition a study by Wisessombat *et al.* (2010), showed that the optimal temperature for chemotaxis of *Campylobacter* spp. to mucin was 42°C (Wisessombat *et al.*, 2010). Therefore it is feasible that *C. jejuni* utilises MUC1 in a similar fashion, warranting further investigation into the role MUC1 has on gene expression *in vivo*.

Studies on gene regulation have previously shown differential modulation of *tlp* genes *in vivo* (Konkel *et al.*, 1998; Reid *et al.*, 2008; Stintzi, 2003). Investigations into expression of *tlp* genes have revealed a role in colonisation of the host GI tract. Up-regulation of *tlp1* and *tlp2* has been demonstrated *in vivo* (Hartley, 2009; Reid *et al.*, 2008), along with *tlp10* which has been shown to be a determinant in colonisation of the chicken intestinal tract (Hendrixson & DiRita, 2004), corresponding to the findings in this study.

Assessment of gene regulation in a murine host indicated that a number of genes were differentially expressed *in vivo* compared to *in vitro* grown *C. jejuni*. A variety of genes involved in a range of *C. jejuni* biological processes were identified with majority of these genes corresponding to previously identified regulation (Reid *et al.*, 2008; Stintzi, 2003; Woodall *et al.*, 2005). These included the up-regulation of *ceuBD* genes encoding the components of the ferric-enterobactin uptake permease; *galE*, *waaE* and *neuA2* which encode proteins involved in surface structure biosynthesis and modification. With the most significant change in regulation identified in *cheY* and *fli* genes that are known or presumed to be involved in chemotaxis and flagella biosynthesis. Gene regulation identified in IMS isolated *C. jejuni* from mouse GI tract, shows consistent regulation to that observed in the rabbit ileal model of infection (Stintzi *et al.*, 2005).

The glycan and microarray data presented in this study requires further experimentation; however preliminary data suggests that genes involved in the chemotaxis pathway appear to be significantly up-regulated *in vivo* compared to *in vitro*. Differential expression of *tlp* genes in the chicken colonisation model and *cheY* in a murine model for infection, supports existing data that chemotaxis is involved in colonisation. And further indicates that the method of IMS may prove to be an invaluable tool in the elucidation of potential host specific virulence genes for *C. jejuni*.

The research presented in this thesis utilised glycome and transcriptome analysis, which has become an increasingly important part of *Campylobacter* spp. research. These techniques have been instrumental in the elucidation of many virulence factors involved in *C. jejuni* pathogenesis. Development of an IMS protocol to isolate infecting *C. jejuni* directly from an *in vivo* host and investigation of glycan binding and gene expression has the potential to contribute to identification and understanding of processes that occur to establish *C. jejuni* commensalism or pathogenesis.

In conclusion, this study has revealed possible involvement of the cs-mucin MUC1 in *C. jejuni* adherence and penetration of host mucosal cells. Future directions include investigation into the role MUC1 has on *C. jejuni* carbohydrate binding and gene modulation *in vivo* using refined IMS technique and continued development of *Campylobacter* reporter vectors to enhance incorporation and expression of genes encoding for fluorescence or bioluminescence.

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