The development and application of an *in vitro* model of *Campylobacter jejuni* interaction with host cell receptors

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This thesis is being submitted in partial fulfilment of the requirements of Kingston University for the award of Doctor of Philosophy.

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Declaration

I declare that the work reported in this thesis is entirely my own and has been carried out at Kingston University, UK.

This thesis has not been submitted, in whole or in part, for any other degree at this or any other University.

Sona Rubinchik

Abstract

Campylobacter jejuni is the most common cause of acute bacterial gastroenteritis in humans worldwide. Adhesion to the host cells is an important stage in pathogenesis of *C. jejuni* infection. It was hypothesised that some N-linked glycosylated proteins might be involved in bacterial attachment via interaction with lectins located on the surface of host cells. Glycan moieties of such glycoproteins contain terminal *N*-acetylgalactoseamine (GalNAc) residues that are specific to soybean agglutinin (SBA) lectins that are analogues of host cell receptors. *C. jejuni* is also known to express capsule, the role of which in adhesion still remains unknown. The main aims of this project were to investigate the putative glycoprotein adhesins of *C. jejuni* and the involvement of capsule in bacterial adhesion.

In this study an *in vitro* assay was developed to assess bacterial adhesion to immobilised analogues of host cell receptors. This assay was used to verify the binding mechanism of *C. jejuni* through competitive inhibition studies, exoglycosidase treatment and site-directed mutagenesis, with the *C. jejuni* strain 11168H and its isogenic mutants. Results obtained in this study demonstrated that PEB3 is one of the cell surface glycoproteins involved in bacterial interaction with an analogue of a host cell receptor and that the presence of capsule reduces bacterial attachment. In addition, it was demonstrated that the presence of the glycoprotein adhesin JlpA is not required for this interaction. It was also shown that the genes involved in capsule and PEB3 adhesin biosynthesis are differentially regulated, suggesting that capsule and adhesins may be expressed at different stages of infection. Based on these results we hypothesise that capsule may be required for preventing the recognition of adhesins by the host immune system at the initial stages of infection. Following this initial evasion of the host immune response, the genes for capsule

production may be down-regulated, leading to reduction in capsule production and the resulting exposure of cell-surface adhesins and cell surface molecules required for attachment and/or modulation of the host immune response.

Additionally, a recombinant *Escherichia coli* strain expressing lipopolysaccharide (LPS) modified with a *Campylobacter* glycan was shown to inhibit binding of *C. jejuni* cells to SBA lectin. This finding may be used as the basis for the design of novel antibacterials using competitive inhibition as the mechanism of action.

Overall, the results of this study will contribute to a better understanding of the pathogenic mechanism of *C. jejuni* infection and will help in the development of inhibitors of bacterial attachment to host cells. This will help to reduce the number of infections caused by *C. jejuni* and therefore reduce healthcare costs worldwide. Prevention of bacterial attachment or competitive inhibition used as a curative measure would therefore be beneficial to those in both developed and developing nations.

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ABC transporter	ATP-binding cassette transporter
Abs	Antibodies
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
Bp	Base pairs
CBA	Columbia blood agar
CCV	Campylobacter-containing vacuoles
CDT	Cytolethal distending toxin
c.f.u.	Colony-forming unit
CPS	Capsular polysaccharide
CRDs	Carbohydrate recognition domains
CT	Cycle threshold
DAB	3,3' Diaminobenzidine
DC	Dendritic cell
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
Fn	Fibronectin
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-regulated kinases
FSBA	Fluorescein-labelled SBA
ETEC	Enterotoxigenic *E. coli*
GalNAc	*N*-acetylgalactoseamine
GBS	Guillian-Barre syndrome
GTPases	Guanosine triphosphatases
His-tag	Histidine-tag
HIV	Human immunodeficiency virus
IFN	Interferon
Ig	Immunoglobulin

kDa	KiloDalton
Kb	Kilobase pair
LB	Luria Bertani/Luria broth
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MeOPN	6-O-methyl phosphoramidate
MH	Mueller-Hinton
MGL	Macrophage galactose-type lectins
MOMP	Major outer membrane protein
MUC2	Mucin 2
NF-κB	Nuclear factor-kappa B
NOD1	Nucleotide-binding oligomerisation domain-containing protein 1
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PRR	Pattern-recognition receptor
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SBA	Soybean agglutinin
SD	Standard deviation
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOC	Super optimal broth with catabolite repress
Spp.	Species
T4SS	Type IV secretion system
TNF	Tumour necrosis factor
TLR	Toll-like receptor
TMB	3,3',5,5' Tetramethylbenzidine

TTBS	Tris-buffered saline with Tween® 20
UDP	Uridine diphosphate
WT	Wild type

CHAPTER 1: Introduction

A comprehensive background on *Campylobacter jejuni* and a literature review on the topics relevant to this study are presented here.

1.1 An overview of *Campylobacter jejuni*

C. jejuni is the leading bacterial food-borne pathogen worldwide (Olson *et al.*, 2008; Zilbauer *et al.*, 2008; Young *et al.*, 2007; Allos, 2001). Consumption of contaminated poultry products is considered to be the main source of human infection, which usually manifests as acute gastroenteritis (Young *et al.*, 2007). Even though the majority of cases are self-limited, the infection can be associated with complications such as Guillain-Barre syndrome (GBS) or Miller Fisher syndrome (Allos, 1997; Zilbauer *et al.*, 2007) that may become life-threatening. *C. jejuni* are slow growing, Gram-negative, microaerophilic, spiral bacilli (1.5–6.0μm by 0.2–0.5 μm) with uni- or bi-polar flagellae and with an optimal growth temperature ranging from 37°C to 42°C, while cell death occurs between 56°C and 57°C (Altekruse *et al.*, 1999; Allos, 2001; Ketley, 1997; Keener *et al.*, 2004). This microorganism is very fastidious and sensitive to unfavourable growth conditions, such as changes in pH, temperature, the level of oxygen (optimal conditions are 5% O_2, 10% CO_2, and 85% N_2) and dehydration (Jackson *et al.*, 2009; He and Chen, 2010; Mihaljevic *et al.*, 2007). Under unfavourable growth conditions (lack of nutrients, suboptimal growth temperature, osmolarity changes and pH), *C. jejuni* enters a viable, but non-culturable state (Rollins and Colwell, 1986; Jones *et al.*, 1991; Jackson *et al.* 2009).

1.2 The genus *Campylobacter*

In 1906, McFadyean and Stockman described new bacteria that were frequently

isolated from the aborted foetuses of ewes (Skirrow, 2006). Smith and Taylor isolated a similar microorganism several years later (Smith and Taylor, 1919). Due to the spiral shape, *Campylobacter* were initially classified as *Vibrio*, however in 1963 the name *Campylobacter* was proposed by Sebald and Veron due to fundamental differences between these and known species of *Vibrio*, such as DNA base composition and fermentative metabolism (On, 2001).

C. jejuni was first isolated from the stool sample of a patient with diarrhoea in 1968 by Butzler and colleagues (Dekeyser *et al.*, 1972). In the late 1970s selective media for *Campylobacter* were developed that allowed to test stool samples for the presence of the pathogen (Skirrow, 1977). Since that time *Campylobacter* has been recognised as the most common cause of acute bacterial gastroenteritis in both children and adults (Altekruse *et al.*, 1999; Butzler, 2004; Stafford *et al.*, 2007).

At present the genus *Campylobacter* contains 16 species and 6 subspecies, and belongs to the epsilon class of proteobacteria in the order *Campylobacteriales*. This order also includes the genera *Helicobacter* and *Wolinella* (On, 2001; Young *et al.*, 2007).

1.3 *C. jejuni* genetics and diversity

The genome of *C. jejuni* NCTC11168 was sequenced in 2000 by Parkhill *et al.*, 2000). It has one circular chromosome of 1,641,481 base pairs (bp) with 30.6% G + C content, encoding 1,643 proteins. The genome of *Campylobacter* is also known to have 29 homopolymeric G tracts, which are multiple repetitions of consecutive G residues (Parkhill *et al.*, 2000; Gundoglu *et al.*, 2007). The presence of homopolymeric tracts has the potential to cause phase variation, which may be responsible for the ability of the bacterium to evade immune

response and persist in the host (Young *et al.*, 2007; Karlyshev *et al.*, 2005; Guerry *et al.*, 2002). Genome-wide analysis has revealed that the *C. jejuni* genome has hypervariable regions, most of which encode proteins required for the synthesis or modification of cell surface structures such as lipooligosaccharides (LOS), capsule and flagellum (Parkhill *et al.*, 2000). The genome-based analysis of different *C. jejuni* isolates suggests that there is a considerable genetic diversity between strains (Pearson *et al.*, 2003; Poly *et al.*, 2005, Hofreuter *et al.*, 2006). *C. jejuni* is naturally competent. It is able to uptake naked DNA from the environment and incorporate it into its genome by recombination (Wang and Taylor, 1990). This mechanism allows for the generation of genetic diversity in *Campylobacter* (Young *et al.*, 2007). Natural transformation is one of the mechanisms of horizontal gene transfer in *C. jejuni*. It plays an important role in the spread of new factors, such as antibiotic resistance (Jeon *et al.*, 2010). Components of the type II secretion system, LOS biosynthesis as well as N-linked glycosylation are required for natural transformation (Avrain *et al.*, 2004; Bacon *et al.*, 2000; Fry *et al.*, 2000; Larsen *et al.*, 2004).

1.4 Epidemiology

Infection caused by *Campylobacter* is a considerable problem worldwide since it is a major cause of acute bacteria-mediated gastroenteritis (Olsen *et al.*, 2008; Zilbauer *et al.*, 2008; Young *et al.*, 2007). Over the last 25 years there has been an increase in the number of cases of campylobacteriosis in England and Wales, with 65,032 laboratory confirmed cases of *Campylobacter* infection in 2012 alone (Public Health England, 2013; Nichols *et al.*, 2012). It is the most common cause of diarrhoea in these regions. A UK study reported that *Campylobacter* species were the most common bacterial pathogens causing intestinal disease, with around

9.3 cases per 1000 people in 2008–2009 (Tam *et al.*, 2011). According to the UK Food Standards Agency, *Campylobacter* infection affects 300,000 people each year in this country. In 2013, the estimated costs of campylobacteriosis were approximately £900 million (Wearne, 2013).

The number of *Campylobacter* infection cases is increasing in the European Unioin (EU) as well, with 9 million cases a year costing approximately €2.4 billion (EFSA, 2011). In the United States, incidence of *C. jejuni* infection in 2013 was substantially higher than in 2008, increasing from 5,825 to 6,621 laboratory-confirmed cases per 100,000 people (CDC, 2009; CDC, 2014). Incidence of campylobacteriosis has increased over the last few years in the USA, UK and EU, which adds urgency to the need to better understand the pathogenesis of *C. jejuni* infection.

Campylobacter is found in a wide range of domestic animals; in particular it colonises the gastrointestinal tract of chickens. Infected poultry is thus considered to be the greatest source of human infection (Friedman *et al.*, 2004; Sheppard *et al.*, 2009; Altekruse *et al.*, 2009; Young *et al.*, 2007). Consumption of undercooked meat (especially poultry), unpasteurised milk, untreated water or cross-contaminated food products are usually the key risk factors associated with campylobacteriosis (Silva *et al.*, 2011).

Although *Campylobacter* species are the most common causes of diarrhoea worldwide, there is a difference in the epidemiology of *Campylobacter* infections in developed and developing countries. In developed countries there are sporadic cases as well as outbreaks of *Campylobacter*, with the majority of cases occurring in summer. Young adults (15–24 years) are found to be more susceptible (Olson *et al.*, 2008; Altekruse *et al.*, 1999; Coker *et al.*, 2002; Nichols *et al.*, 2012; Friedman *et al.*, 2000). In developing countries, infection is more common in

young children due to poor sanitation and close human contact with animals, and tends to be asymptomatic (Ketley, 1997).

1.5 Clinical features and complications of campylobacteriosis

The majority of campylobacteriosis cases are caused by *C. jejuni* and *C. coli* (Lastovica and Skirrow, 2000; Ellis-Iversen *et al.*, 2009). Humans become infected through consumption of contaminated food or drink with symptoms starting after 24–72 hours of exposure to the pathogen. The common manifestation of the disease is acute gastroenteritis with non-specific symptoms such as diarrhoea, fever and abdominal cramps (Blaser, 1997; Allos, 2001). The symptoms last from three to seven days. The outcome of the disease depends on virulence of the strain and host immunity (Zilbauer *et al.*, 2007). Infection caused by the antibiotic-resistant strains leads to more severe clinical outcomes compared with antibiotic-sensitive strains of *C. jejuni* (Helms *et al.*, 2005). Even though in the majority of cases the disease is self-limiting, post-infection complications can be severe, especially in immunocompromised patients (Butzler, 2004).

The *C. jejuni* pathogen is also implicated in the development of musculoskeletal disorders such as reactive arthritis, in which knees and ankles are most affected, and GBS, which is associated with paralysis of cranial and peripheral nerves (Hannu *et al.*, 2004; Hannu *et al.*, 2002; Allos, 1997).

Several weeks after *Campylobacter* gastroenteritis the symptoms of GBS can develop with some cases requiring hospitalisation (Butzler, 2004). However, only approximately one in 1000 infected people is affected by GBS (Guerry and Szymanski *et al.*, 2008). The structural similarity (molecular mimicry) between sialylated surface carbohydrate structures (LOS) of certain *C. jejuni* strains and human ganglioside structures present on the peripheral nerves plays an important

role in the development of GBS (Yuki *et al.*, 2004). Molecular mimicry leads to the production of autoreactive antibodies followed by inflammation, tissue damage and temporary infection-associated paralysis (Godschalk *et al.*, 2004; Komagamine and Yuki, 2006; van Doorn *et al.*, 2008). Around 50 to 60% of *C. jejuni* isolates can express sialic acid-containing LOS and these isolates are more invasive compared to strains that express nonsialylated LOS (Godschalk *et al.*, 2004; Louwen *et al.*, 2008). Genes responsible for the synthesis, modification and transfer of sialic acid are found in several LOS classes (A, B, C, M and R), however development of GBS is associated only with LOS class A (Parker *et al.*, 2008). Molecular mimicry can also lead to Miller Fisher Syndrome, which is a milder form of GBS (Ang *et al.*, 2002; Mori *et al.*, 2001) and is associated with LOS class B (Godschalk *et al.*, 2004).

Other complications of campylobacteriosis include intestinal haemorrhages (Chamovitz *et al.*, 1983), toxic megacolon (McKinley *et al.*, 1980) and peritonitis (Lang *et al.*, 2009), although these complications are rare. In some cases irritable bowel syndrome has been reported as a consequence of *C. jejuni* infection (Gradel *et al.*, 2009). Chronic *C. jejuni* infection was also linked to the development of small bowel mucosa-associated lymphoid tissue (MALT) lymphomas, also known as marginal zone lymphomas (Lecuit *et al.*, 2004; Suarez *et al.*, 2006).

1.6 Pathogenesis

Despite being the most common cause of acute bacterial gastroenteritis, the pathogenic mechanism of *C. jejuni* infection is still not completely understood. The bacteria colonise the distal small intestine and colon and induce local acute inflammatory changes (Ketley, 1997). Similar to other intestinal pathogens, *C. jejuni* adherence and invasion of the gastrointestinal epithelium are considered to

be important events in establishment of the disease (Janssen *et al.*, 2008; Zilbauer *et al.*, 2008). There are several defences that the host uses, including saliva, a mucus layer and peristalsis that intestinal pathogens need to avoid. Thus, to establish the disease, *C. jejuni* resists the peristaltic forces of the small intestine and penetrates the mucus layer in order to reach the underlying epithelium (Figure 1.1).

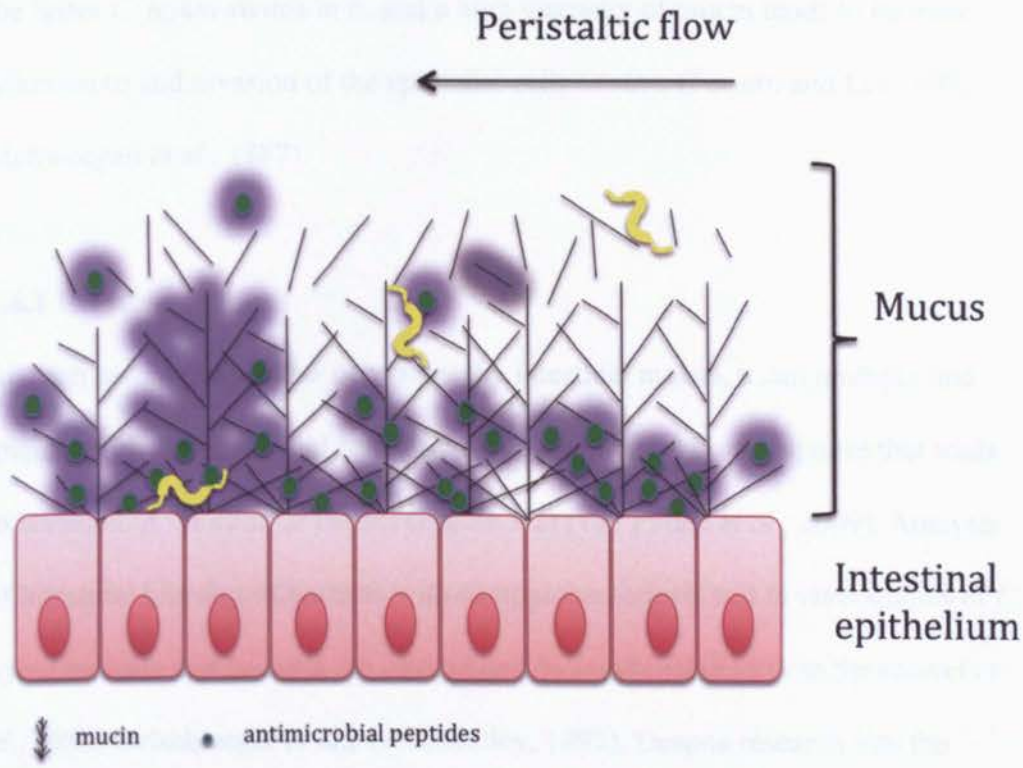


Figure 1.1. Schematic representation of human intestinal lumen. *Campylobacter* is able to actively swim through the mucus layer that covers the intestinal epithelium and survive in intestinal crypt. (Diagram adapted from Heikema, 2013)

In order to cross the mucus layer, *C. jejuni* is able to avoid entrapment in the lumenal mucins and the bactericidal effect of antimicrobial peptides (Hansson, 2012; Lievin-Le and Servin, 2006). Mucins are net-like polymers, the major functions of which are to limit bacterial contact with the intestinal epithelium via

entrapment of bacteria and to transport them out of the intestinal tract (Hansson, 2012). However, *Campylobacter* is not trapped in the mucin since it freely moves along the mucus strands, which might be due to its small size, spiral shape and flagella-mediated motility (Lee *et al.*, 1986: McSweegan *et al.*, 1987). Moreover, it was shown that mucin is a strong chemoattractant for *Campylobacter,* and the viscosity of mucin affects the speed and swimming pattern of this bacterium (Hugdahl *et al.*, 1988; Shigematsu *et al.*, 1998). The higher the viscosity of mucin, the faster *C. jejuni* swims in it, and a high viscosity of mucin tends to increase adhesion to and invasion of the epithelial cells *in vitro* (Ferrero and Lee 1988; McSweegan *et al.*, 1987).

1.6.1 Invasion

As soon as *Campylobacter* penetrates the intestinal mucus, it can multiply and interact with host epithelial cells provoking an inflammatory response that leads to invasion of the cellular barrier (Figure 1.2) (van Putten *et al.*, 2009). Analysis of intestinal biopsies of patients with campylobacteriosis and *in vitro* studies of *C. jejuni* indicate that bacteria are internalised by epithelial cells (van Spreeuwel *et al.*, 1985; Oelschlaeger *et al.*, 1993; Ketley, 1997). Despite research into the process of internalisation of pathogen by host cells, the mechanism remains unclear (Ó Croinin and Backert, 2012).

The majority of studies report that *C. jejuni* enters epithelial cells via local depolymerisation of cortical actin filaments and it forms microtubuli-based projections (Oelschlaeger *et al.*, 1993; Hu and Kopecko, 1999; Kopecko *et al.*, 2001; Monteville *et al.*, 2003; Russell *et al.*, 1994). During contact with the epithelial cell, *C. jejuni* triggers membrane ruffling and invaginations. This process requires involvement of cellular factors such as caveolin-1 and small Rho

guanosine triphosphatases (GTPases) Rac1 and Cdc42, which leads to cytoskeletal rearrangements (Eucker and Konkel, 2012; Krause-Gruszxzynska *et al.*, 2007; Hu *et al.*, 2008). In addition to the actin- and/or microtubule-dependent uptake process, which is very energy consuming, an invasion pathway called sub-invasion has been identified (van Alphen *et al.*, 2008). It involves active migration of cells towards the subcellular spaces followed by bacterial entry at the basal cell side. *C. jejuni* is able to disrupt tight junctions between polarised cells and can be actively translocated across the epithelial monolayer, gaining access to the subepithelial tissue (Konkel *et al.*, 1992c; Bras and Ketley, 1999; Grant *et al.*, 1993). In later studies it was identified that *C. jejuni* invades the polarised intestinal cells via the subcellular invasion pathway, thus *C. jejuni* uses an actin- and microtubule-independent mechanism in order to invade polarised intestinal epithelial cells and remain alive intracellularly for up to 48 hours (Bouwman *et al.*, 2013). This process is controlled by nutrients and requires functional flagella (van Putten *et al.*, 2009).

In order to penetrate the intestinal barrier via the basolateral membrane, *C. jejuni* may use mucosal M cells (van Alphen *et al.*, 2008), which are specialised epithelial cells that deliver the pathogen to the underlying immune cells (Walker *et al.*, 1988; Sansonetti and Phalipon, 1999). From infected M cells, *C. jejuni* spreads and penetrates the subepithelial layer, and after loss of tight junction integrity go on to invade villus epithelial cells (Kalischuk *et al.*, 2010; Chen *et al.*, 2006).

Following internalisation by epithelial cells, *C. jejuni* remains within a membrane-bound intracellular compartment and rolls along microtubules (Russel *et al.*, 1994; Hu and Kopecko, 1999; Konkel *et al.*, 1992c). *C. jejuni* are encapsulated within endosomal-like compartments called Campylobacter-

containing vacuoles (CCVs) that are able to avoid delivery to lysosomes (Watson and Galain, 2008). Once formed, CCV are transported along microtubules to the Golgi apparatus in the perinuclear region. Invasion from the basolateral surface results in the presence of *C. jejuni* in endolysosomal compartments (Bouwman *et al.*, 2013). Replication of internalised bacterial cells results in premature death of host epithelial cells due to the cytotoxic effects of *C. jejuni* (Konkel *et al.*, 1999; Russell *et al.*, 1993).

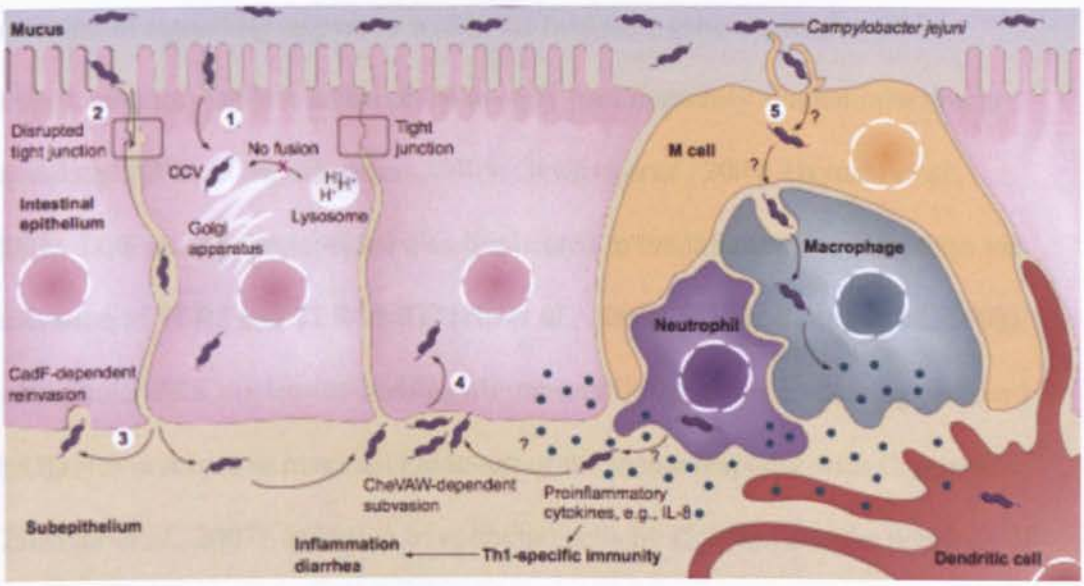


Figure 1.2. Current model of *C. jejuni* invasion strategies. Followed by initial adhesion, *C. jejuni* enters epithelial cells (1) at the apical side (actin- and/or microtubule-dependent uptake process), where it is survives inside of CCV. (2) Disruption of tight junction between epithelial cells enables migration (3) into subcellular space. (4) Sub-invasion, which is internalisation of the pathogen by epithelial cells at the cell basolateral side. (5) Transcytosis, which is uptake and transport of the pathogen across M cells. CheVAW: chemotaxis proteins CheA, CheV and CheW (van Alphen *et al.*, 2008). (Figure adapted from Jagusztyn-Krynicka *et al.*, 2009).

1.6.2 Host response to infection

Interaction of *Campylobacter* with host epithelial lining of the gut initiates appropriate innate immune responses such as the induction of cytokines, chemokines and antimicrobial peptides (β-defensins) (Eckmann, 2005; Zilbauer *et al.*, 2005; Hickey *et al.*, 2000).

Pattern recognition receptors (PRRs) on the surface of epithelial cells recognise pathogen-associated molecular patterns (PAMPs) on the surface of the pathogen. This interaction leads to initiation of the innate immune response, which is crucial for subsequent adaptive immune activation and the outcome of the infection (Akira *et al.*, 2006; Sanderson and Walker, 2007; Eckmann, 2005; Boyd *et al.*, 2007). The first PRRs that *C. jejuni* comes into contact with are Toll-like receptors (TLRs) that play a role in pathogen recognition and detection (Takeda *et al.*, 2003; Young *et al.*, 2007). TLR-5 is involved in flagella recognition and upon initiation of signalling responses within the host cell; gene expression of proinflammatory molecules involved in early host immunity against infection is stimulated (O'Hara and Shanahan, 2006; Gewirtz *et al.*, 2001; Hayashi *et al.*, 2001). LOS and lipoproteins are also implicated in the inflammatory response via activation of TLR2 and TLR4/MD2 (Hu *et al.*, 2006a, 2006b; Zheng *et al.*, 2008). Intracellular PRR nucleotide-binding oligomerisation domain-containing protein 1 (NOD1) is involved in maximal induction of the innate response with *C. jejuni* (Zilbauer *et al.*, 2007). Infection of epithelial cells by *C. jejuni* and the presence of cytolethal-distending toxin (CDT) activates the transcription factors nuclear factor-kappa B (NFκB) and activator protein 1 (AP-1), which results in phosphorylation of extracellular signal-regulated kinases (ERK) and p38 mitogen-activated protein (MAP) kinases, which in turn induce interleukin 8 (IL-8) production (MacCallum *et al.*, 2005a; Watson & Galan, 2005; Whitehouse *et al.*, 1998; Lara-Tajero and Galan, 2001, Hickey *et al.*, 2000; Zheng *et al.*, 2008). *C. jejuni* activation of the innate immune response leads to production of pro-inflammatory cytokines and chemokines such as IL-1α, IL-1ß, IL-2, IL-6 and tumour necrosis factor (TNF) α, interferon (IFN) γ and neutrophil chemoattractant IL-8 (Hickey *et al.*, 1999; Hickey *et al.*, 2000; Johanesen and Dwinell, 2006; Hu

and Hickey, 2005; Bakheit *et al.*, 2004; Jones,M. *et al.*, 2003). These factors are crucial in promoting tissue damage through recruitment of monocytes and neutrophils, the end result of which is the development of diarrhoea (Al-Banna *et al.*, 2008; MacCallum *et al.*, 2005b; Zilbauer *et al.*, 2008).

1.6.3 Interaction of *C. jejuni* with professional phagocytes

IL-8 production attracts immune cells such as lymphocytes and neutrophils to the site of inflammation. Interaction of *C. jejuni* with neutrophils results in the release of reactive oxygen species and, depending on the strain, may lead to bacterial killing (Wooldridge and Ketley, 1997). It is still not clear if *C. jejuni* is able to survive within professional phagocytes, with some studies reporting internalisation and killing of *C. jejuni* by monocytes and macrophages (Al-Banna *et al.*, 2008; Iovine *et al.*, 2008; Wassenaar *et al.*, 1997; Wooldridge and Ketley, 1997), whereas other studies report survival and replication of *C. jejuni* inside monocytes and macrophages, even though CDT-dependent apoptosis can occur (Hickey *et al.*, 2005; Siegesmund *et al.*, 2004). The differences between these results might be attributed to the use of different human macrophages or monocyte-derived macrophages. Another reason for survival of the pathogen inside the monocyte-derived macrophages might be the origin of the cells as monocytes derived from around 10% of donors were not able to kill *C. jejuni* (Wassenaar *et al.*, 1997). Survival of *C. jejuni* in murine macrophages for several days was attributed to the production of catalase by the bacterium (Day *et al.*, 2000). Human dendritic cells (DCs) internalise *C. jejuni,* which induces maturation and cytokine production in these cells as well as a specific cell-mediated immune response (Johanesen and Dwinell, 2006; Rathinam *et al.*, 2008; Hu *et al.*, 2006a). DCs are the major antigen-presenting cells in intestinal mucosa

that are involved in both the innate and adaptive immune response, and, being the source of proinflammatory cytokines, they assist in the activation of macrophages and T cells (Medzhitov and Janeway, 1997). It was shown that CD103+ DCs migrate to the intestinal draining lymph nodes where they present antigens to naïve T cells, inducing the primary immune response and differentiation (Schulz *et al.*, 2009).

1.7 Virulence determinants

Each step in the pathogenesis of *C. jejuni* infection appears to depend upon the expression of a combination of several virulence determinants. Bacterial virulence factors involved in epithelial cell invasion include flagella motility and chemotaxis, surface proteins, protein glycosylation, the presence of capsule and LOS (van Vliet and Ketley, 2001; Young *et al.*, 2007; van Putten *et al.*, 2009).

1.7.1 Flagella and motility

C. jejuni has a single flagellum at one or both poles, which plays an important role in virulence of this microorganism. Flagella-mediated motility is very important for colonisation as it helps to overcome peristalsis and enter the mucous layer of intestinal cells (Black *et al.*, 1988; Szymanski *et al.*, 1995; Wassenaar *et al.*, 1993b). The flagella of *C. jejuni* are composed of two flagellin subunits, FlaA and FlaB, which are approximately 59 kDa each and are highly homologous (Guerry *et al.*, 1991; Nuijten, *et al.*, 1990). Different promoters, sigma^{28} and sigma^{54} regulate the *flaA* and *flaB* expression, though the advantage of differential regulation of expression of these genes is unknown (Hendrixson and DiRita, 2003). Phase variation is observed in the expression of *Campylobacter* flagella, resulting in unstable motility phenotype *in vitro* and *in vivo* (Caldwell *et al.*, 1985;

Hendrixon, 2006; Park *et al.*, 2000). Phase variation occurs through slipped-strand mismatch in homopolymeric tracts, which leads to mutations in the genes involved in regulation and biosynthesis of flagella (*flhA, flgS, flgR* and *maf*) (Park *et al.*, 2000; Karlyshev *et al.*, 2002; Hendrixon, 2008). The *flaA* and *flaB* genes are both involved in proper flagella function; mutation of the *flaA* gene affects motility more than mutations in *flaB* (Guerry *et al.*, 1991; Wassenaar *et al.*, 1993b). In addition, the *flaA* mutant is not able to invade epithelial cells *in vitro* due to a truncated flagellum (Wassenaar *et al.*, 1991b; Yao *et al.*, 1994). Both *flhA* and *flhB* are involved in biofilm formation, whereas mutation of only *flhA* affects auto-agglutination (Golden and Acheson, 2002; Kalmakoff *et al.*, 2006). Glycosylation of the flagellum is required for flagellum formation (Linton *et al.*, 2000; Karlyshev *et al.*, 2002), and glycans of flagella affect flagella-mediated auto-agglutination and micro-colony formation (Guerry *et al.*, 2006).

Flagella are also involved in secretion of virulence-associated proteins, such as FlaC, FspA and several invasion antigens. Secreted via the flagella apparatus, FlaC binds to HEp-2 cells and mutation of the *flaC* gene reduces invasion (Song *et al.*, 2004). FspA 2 (which is the variant of FspA) was shown to be able to bind to eukaryotic cells and cause apoptosis of INT 407 cells (Poly *et al.*, 2007). In addition, the synthesis of a set of proteins termed *Campylobacter* invasive proteins (Cia proteins) might be crucial for internalisation (Konkel *et al.*, 1992a; Konkel *et al.*, 1999). Cia proteins are associated with *C. jejuni* invasion of INT 407 cells (Konkel, *et al.*, 1999) and are secreted only during the contact of bacterial cells with host cells or in the presence of serum or mucin (Rivera-Amill *et al*, 2001). Cia proteins were shown to cause host cell membrane ruffling, with subsequent intracellular signal events that promote bacterial uptake (Euker and Konkel, 2012). The involvement of flagella in adhesion is discussed in section

1.8.1.

Motility and chemotaxis have been recognised to play a role in pathogenesis of *C. jejuni* infection. Chemotaxis is the movement of an organism towards or away from a chemical stimulus and it has been shown that non-chemotactic mutants are unable to cause an infection (Takata *et al.*, 1992; Yao *et al.*, 1997), thus chemotaxis is required for the intestinal colonisation of animals. In the study by Hugdahl *et al.* (1988) it was shown that the major chemoattractants for *C. jejuni* are mucin and the amino acids serine, proline, aspartate and glutamate. Genome analysis of *C. jejuni* identified several orthologues of the chemotaxis genes, including *cheA, cheW, cheV, cheY, cheR,* and *cheB* (Dasti *et al.*, 2010). A mutant of *cheY* was shown to be hyperinvasive in cultured cells and CheY was required for directional motility in soft agar (Golden and Acheson, 2002; Yao *et al.*, 1997). The *C. jejuni cheY* mutant was found to be non-chemotactic but with a motile phenotype, and was unable to colonise mice or cause symptoms in infected ferrets (Yao *et al.*, 1997). Additionally, *C. jejuni* motility was found to be dependent on two energy-taxis proteins CetA and CetB (Hendrixon *et al.*, 2001) that provided energy for motility. However, it was established that only CetA rather than CetB was involved in invasion of human epithelial cells (Elliott and DiRita, 2008).

1.7.2 Cytolethal-distending toxin (CDT)

Similar to other bacterial enteropathogens such as *E. coli, Shigella* spp. and *Helicobacter* spp. (Johnson and Lior, 1987; Ocuda *et al.*, 1995; Young *et al.*, 2000), *C. jejuni* secretes CDT, a major virulence factor (Smith and Bayles, 2006). It has three subunits (CdtA, CdtB and CdtC), all of which are required for toxin activity (Lara-Tejero and Galan, 2001). CdtA and CdtC are necessary for binding to host cells (Lee *et al.*, 2003), whereas CdtB affects the nucleus of the cell and

induces arrest in G2 phase causing cell death through DNase activity (Whitehouse *et al.*, 1998; Jain *et al.*, 2008). It was shown that CDT subunits are secreted through outer membrane vesicles into the surrounding environment (Lindmark *et al.*, 2009). Being a membrane-associated protein, CDT stimulates production of IL-8 and activates the pro-inflammatory pathway, which can contribute to inflammatory diarrhoea (Hickey *et al.*, 2000; Zheng *et al.*, 2008). Several studies reported the importance of CDT for intestinal pathogenesis, as CDT affects the invasiveness of *Campylobacter* (Purdy *et al.*, 2000; Jain *et al.*, 2008).

1.7.3 Capsule

C. jejuni expresses surface-located glycolipids, including capsular polysaccharide (CPS) and lipooligosaccharide (LOS) (Linton *et al.*, 2001), both of which are considered to play a role in epithelial invasion (Bacon *et al.*, 2001; Kanipes *et al.*, 2004). The structures of CPS and LOS are highly variable in different strains as the genes associated with the biosynthesis of both are located in the most variable regions of the genome of *C. jejuni* (Parkhill *et al.*, 2000; Penner and Aspinall, 1997; Parker *et al.*, 2008).

Capsule plays an important role in the pathogenesis of the disease, and the presence of capsule is known to be important for persistence of the organism in the environment (Roberts, 1996). The existence of a capsule in *C. jejuni* was predicted following genome analysis performed by Parkhill and colleagues in 2000 and was confirmed by Karlyshev and colleagues in 2001 using the electron microscope (Parkhill *et al.*, 2000; Karlyshev *et al.*, 2001).

The structure of the capsule of *C. jejuni* 11168 strain contains variable 6-O-methyl phosphoramidate (MeOPN), methyl, ethanolamine and N-glycerol groups (Figure 1.3) (Michael *et al.*, 2002; McNally *et al.*, 2007).

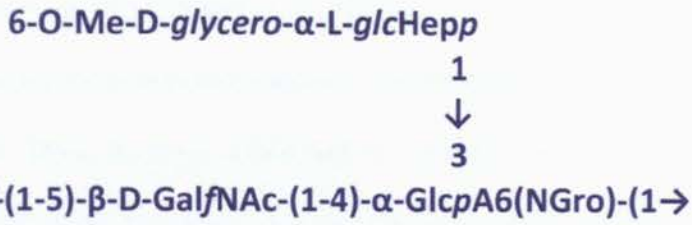


Figure 1.3. *C. jejuni* CPS structure of NCTC 11168. CPS structure is composed of ß -D-Ribose, ß -D-Gal*f*NAc,α-D-Glc*p*A6(NGro), a glucuronic acid modified with 2-amino-2-deoxyglycerol at C-6, and 6-O-methyl-D-glycero-α-L-gluco-heptopyranose as a side-branch. P, phosphate; Gal, galactose; Gro, glycerol; Me, methyl; Hep, heptose; Rib, ribose; GalNAc, *N*-acetylgalactoseamine; GlcA6, glucuronic acid; NGro, aminoglycerol (Figure adapted from Michael *et al.*, 2002).

Capsule is the major serodeterminant in the Penner serotyping and structural variations of CPSs are attributed to phase variation of expression of capsule and structural genes due to slip strand mispairing and MeOPN modification (Linton *et al.*, 2000; Karlyshev *et al.*, 2000, Karlyshev *et al.*, 2005; Maue *et al.*, 2013). Phase variation results in a mixed population of bacterial cells, some of which do not express capsule at all (Bacon *et al.*, 2001). This has a biological role, as the pathogen can regulate expression of capsule (on or off) during its life cycle. The role of *C. jejuni* capsule was suggested to enable resistance to hyperosmotic stress, in a study showing that absence of capsule may decrease colonisation under hyperosmotic stress (Cameron *et al.*, 2012). Studies using acapsulated mutants demonstrated the importance of capsule in serum resistance, adhesion and invasion as well as chicken colonisation, and virulence *in vivo* using a ferret model (Bacon *et al*, 2001; Jones *et al.*, 2004; Bachtiar *et al.*, 2007). It has been suggested that one of the functions of capsule in other pathogens is resistance to the non-specific and specific host immune response (Finlay and McFadden, 2006; Merino and Tomas, 2001). It was shown that *C. jejuni* capsule modulates the

immune response, including complement resistance and cytokine induction via NFκB signalling (Keo *et al.*, 2011; Maue *et al.*, 2013).

Capsule may also provide some resistance against ß defensins and lysozyme (Zilbauer *et al.*, 2008). These findings would help to explain how *C. jejuni* is able to colonise the intestinal tracts of animals and humans by evading the host immune response. Overall, capsule plays an important role ensuring *C. jejuni* survival in the environment outside its natural host (Zilbauer *et al.*, 2007; Young *et al*, 2007). The role of capsule in bacterial adhesion is discussed in section 1.8.3.

1.7.4 LOS

As a part of the outer membrane of Gram-negative bacteria, LOS is an important factor in the survival of microorganisms and pathogenesis of the infection (Preston *et al.*, 1996). LOS from *C. jejuni* consists of covalently bound lipid A and surface-exposed oligosaccharides (Moran, 1997). The structure of LOS is highly variable and capable of mimicking human neuronal gangliosides. Sialylation of *C. jejuni* LOS is associated with ganglioside mimicry (Heikema *et al.*, 2010; Gilbert *et al.*, 2002). This can induce the production of autoantibodies that cross-react with host ganglioside structures present on human peripheral nerves causing autoimmune diseases (Hughes *et al.*, 1999; Godschalk *et al.*, 2007). This may elicit pathogenic disorders such as GBS, which is characterised by acute neuromuscular paralysis (Ang *et al.*, 2002; Yuki *et al.*, 2004). The phase variation in LOS genes due to presence of homopolymeric tracts was linked to changes in LOS ganglioside mimicry (Guerry *et al.*, 2002; Linton *et al.*, 2000). Genes involved in ganglioside mimicry are highly diverse and interchangeable between strains (Gilbert *et al.*, 2004; Gilbert *et al.*, 2002). Recently, it was identified that *C. jejuni* with sialylated LOS binds a sialoadhesin, which results in

production of autoantibodies (Louwen *et al.*, 2008; Mortensen *et al.*, 2009; Heikema *et al.*, 2010).

Mutation of genes that are involved in the biosynthesis of LOS results in the decreased ability of cells to adhere and invade INT 407 cells (Fry *et al.*, 2000; Guerry *et al.*, 2002). It was also shown that LOS protects *C. jejuni* from mucosal antimicrobial peptides (Keo *et al.*, 2011).

1.7.5 Protein glycosylation

Glycosylation is the most common post-translational modification of proteins, responsible for ensuring the amino acids fold into the correct tertiary structure, resulting in proper function of proteins (Drickamer and Taylor, 1998).

There are two types of glycosylation, O-linked and N-linked, both of which are present in *C. jejuni*. *Campylobacter* O-linked glycans are highly diverse and characterised by attachment of glycans to the hydroxyl group of serine or threonine residues (Thibault *et al.*, 2001; Logan *et al.*, 2009; Szymanski *et al.*, 2002). In contrast, N-linked glycans are highly conserved and glycans are covalently linked to the amino acid group of asparagine. It was shown that O-glycosylation modifies flagella with pseudaminic acid or legionaminic acid derivatives, rather than sialic acid as it was previously thought (Thibault *et al.*, 2001; Logan *et al.*, 2009; Nothaft *et al.*, 2010). The O-glycosylation locus is highly variable between different strains and consists of around 50 genes in strain NCTC 11168 (Karlyshev *et al.*, 2005). Glycosylation of *C. jejuni* flagella is an important virulence factor, as mutation of the genes responsible for O-glycosylation leads to reduced adherence to and invasion of epithelial cells and decreased auto-agglutination (Guerry *et al.*, 2006). Recently it was shown that in addition to flagella, major outer membrane protein (MOMP) is also an O-

glycosylated protein, which is considered to be involved in biofilm formation and plays a role in the colonisation of chickens (Mahdavi *et al.*, 2014). These findings confirm the importance of O-linked glycosyation in the pathogenesis of *Campylobacter* infection.

There are around 38 identified N-linked glycoproteins in *C. jejuni* (Young *et al.*, 2002). The N-linked protein glycosylation system is encoded by the *pgl* locus consisting of 11 genes (*pglA-F*, *pglH-K* and *gne*), which is conserved among different *C. jejuni* strains and all N-linked glycoproteins contain the same terminal GalNAc residues (Figure 1.4) (Szymanski *et al.*, 1999; Young *et al.*, 2002).

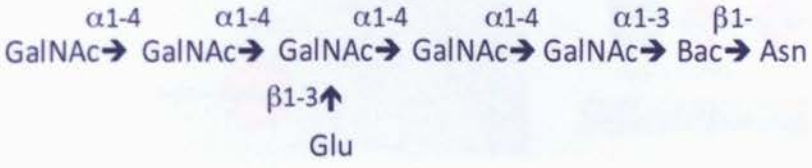


Figure 1.4. *C. jejuni* N-linked glycan structure. N-linked glycoproteins contain the same terminal GalNAc residues. Bac: bacillosamine, 2,4-diacetamido-2,4,6-trideoxyglucose linked to the asparagine residue in the acceptor sequon. GalNAc, *N*-acetylgalactoseamine; Glu, glucose; Asn, asparagine.

The assembly of heptasaccharide takes place at the cytoplasmic side of the membrane where UDP-bacillosamine is synthesised from UDP- *N*-acetylglucosamine (GlcNAc) by the modifying enzymes PglD, PglE and PglF (Figure 1.5). This is followed by a sequential addition of GalNAc residues by a series of biochemical reactions catalysed by the glycosyltransferases, PglC, PglA, PglJ, PglH and PglI (Glover *et al.*, 2005; Linton *et al.*, 2005). The ABC transporter PglK translocates the entire heptasaccharide across the membrane to the periplasm (Alaimo *et al.*, 2006; Kelly *et al.*, 2006). At the final stage,

oligosaccharyltransferase PglB catalyses the transfer of the heptasaccharide from the lipid to Asn residues of the D/E-X1- N-X2-S/T, where X1 and X2 are any amino acid except proline (Kowarik *et al.*, 2006).

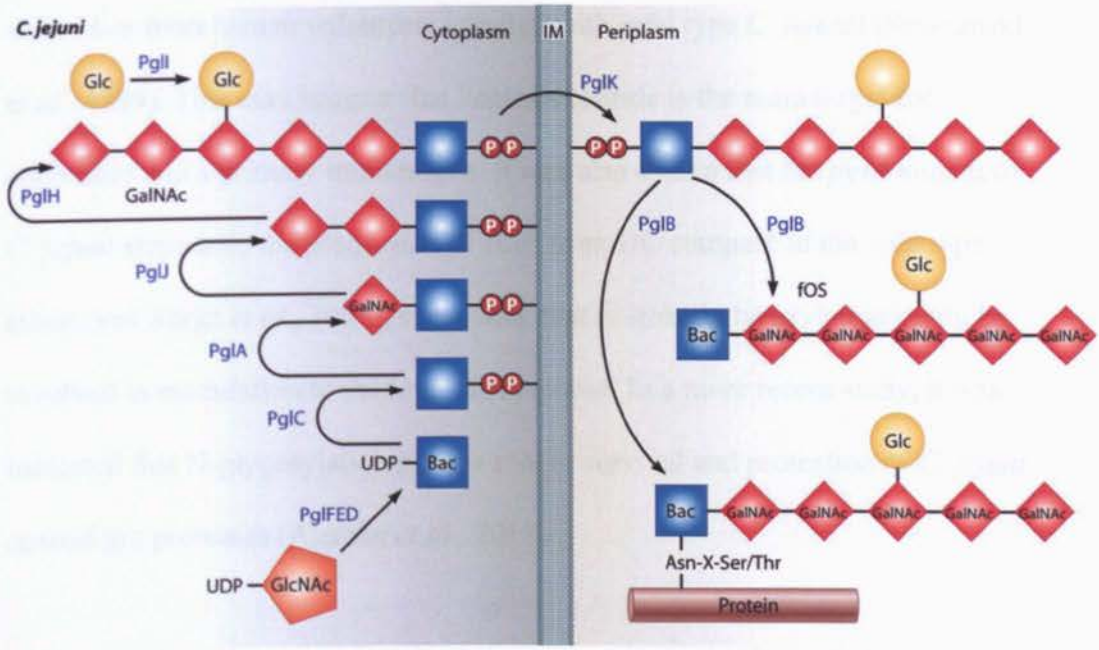


Figure 1.5. N-linked protein glycosylation system in *C. jejuni*. N-linked glycosylation modifies asparagine residues on many proteins. Glc, glucose; GlcNAc, *N*-acetylglucosamine; Bac, bacillosamine; fOS, free oligosaccharide. (Figure adapted from Gilbreath *et al.*, 2011).

N-linked glycosylation is confined to a small number of species, among them some *Helicobacter* and *Wolinella* spp (Nothaft and Szymanski, 2010).

The function of the N-linked glycosylation system is not completely understood. It plays a role in pathogenesis, as mutations in the *pgl* locus lead to a reduced ability of bacterial cells to bind and invade host epithelial cells, as well as impairment of the successful colonisation of the intestines of chicks (Hendrixson and DiRita, 2004; Karlyshev *et al.*, 2004; Szymanski *et al.*, 2002). Furthermore, loss of N-glycosylation affects transport of iron (Palyada *et al.*, 2004) and DNA uptake by the type IV secretion system (T4SS) (Scott *et al.*, 2011). It was shown that a component of a putative T4SS is modified by N-linked glycosylation and

plays a role in protein stability, as unglycosylated proteins are not able to produce stable structures (Larsen *et al.*, 2004).

In addition, the loss of a carbohydrate moiety from glycoproteins due to mutation of the *pgl* genes changes the immunoreactivity (no interaction with antisera or antibodies from human volunteers infected with wild type *C. jejuni*) (Szymanski *et al.*, 1999). This may suggest that heptasaccharide is the main target for antibodies and a primary immunogen. It was also shown that the *pglA* mutant of *C. jejuni* stimulates the production of IL-6 from DC compare to the wild type strain (van Sorge *et al.*, 2009), suggesting that N-linked glycosylation might be involved in modulation of the immune response. In a more recent study, it was indicated that N-glycosylation plays a role in survival and protection of *C. jejuni* against gut proteases (Alemka *et al.*, 2013).

1.8 Factors involved in *Campylobacter* host cell adhesion

Bacterial adhesion to the surface of host cells is an important step in the pathogenesis of many bacterial infections, which affects bacterial uptake (invasion) and innate immune responses (Kline *et al.*, 2009; Klemm and Schembri, 2000; Pizzaro-Cerda and Cossart, 2006). Adhesion is mediated by specialised surface proteins (adhesins), which recognise and bind to receptors on the host cell surfaces (Niemann *et al.*, 2004). There is a wide range of host cell receptors that adhesins recognise, including membrane-associated proteins and extracellular matrix proteins (such as fibronectin and collagen), surface immunoglobulins, glycoproteins and glycolipids (Wilson *et al.*, 2002). Many bacteria express more than one adhesin on their surface, helping pathogens to adapt to changes in environmental conditions, such as different hosts and host tissues (Gerlach and Hensel, 2007). Following adhesion of the microorganism to

the surface of the host cell, it usually starts multiplying, producing toxins and invading the host cells. This leads to initiation of specific biochemical processes such as activation of host cell signalling cascades (Wilson *et al.*, 2002).

In pathogenic bacteria the various types of adhesins have different structures, ranging from simple monomeric proteins to structured polymeric macromolecules (Gerlach and Hensel, 2007). Microbial adhesins can be separated into fimbrial and non-fimbrial. Fimbrial adhesins are hair-like structures protruding from the surface of the bacteria, whereas non-fimbrial adhesins remain close to the surface, forming structures that do not protrude (Wilson *et al.*, 2002; Gerlach and Hensel, 2007). The best-studied fimbria are P pili, type I pili and Afa/Dr adhesins, which are exported by the chaperone/usher pathway, in which a periplasmic chaperone and OM assembly platform (usher) are strictly required for pilus assembly (Sauer *et al.*, 2000). Fimbrial adhesins, in which the fimbrial tip binds to the host cell receptors, are present in *E. coli, Vibrio cholerae, Pseudomonas aeruginosa* and *Neisseria* species (Donnenberg, 2000; Merz and So, 2000; Hahn, 1997). Non-fimbrial adhesins usually generate closer contact with the host cell and are present in both Gram-positive and Gram-negative pathogens such as *Staphylococcus* spp., *Streptococcus* spp., *Yersinia* spp., enteropathogenic *E. coli, Neisseria* spp. and mycobacterial pathogens (Joh *et al.*, 1999; Novak and Tuomanen, 1999; Cleary and Cue, 2000; Bermudez and Sangari, 2000; Donnenberg, 2000; Merz and So, 2000). Among non-fimbrial adhesins are autotransporters and outer membrane associated proteins (OMPs). Autotransporter proteins are short monomeric or oligomeric proteins that are exported on the surface of Gram-negative bacteria via a Type V secretion system (Hori and Matsumoto, 2010). An example of monomeric autotransporters is ShdA of *Salmonella*, which has ability to bind to fibronectin *in vitro* (Kingsley *et al.*, 2002). Several OMPs have ß-barrel-

containing structures that function as adhesins (Neiman *et al.*, 2004). For example, *E.coli* OmpA is involved in adhesion to the Ecgp receptor on the surface of the human brain microvascular endothelial cells causing meningitides in newborn babies (Prasadarao, 2002; Kim, 2000). Both fimbrial and non-fimbrial adhesins may recognise and bind to proteins such as fibronectin, collagen, elastin, laminin, proteoglyans and fibrinogen on the surface of the host cell (Pizarro-Cerda and Cossart, 2006).

As mentioned previously, the host organism has a number of defence mechanisms such as the presence of saliva, mucous flow and peristalsis that can interfere with adhesion of pathogenic bacteria to host cells. The human intestinal mucous layer is the first barrier that *C. jejuni* must successfully penetrate before adhesion and invasion of host cells. Adhesion of *Campylobacter* is not mediated by fimbria or pili, like in other Gram-negative bacteria such as *E. coli* and *Salmonella* (van der Velden *et al.*, 1998; Nougayrede *et al.*, 2003), and instead there are a number of proteins that serve as adhesion factors.

Identification of the bacterial factors involved in *Campylobacter* interaction with host cells is the subject of much research (Flanagan *et al.*, 2009; Novik *et al.*, 2010; Dasti *et al.*, 2010). Currently, there are several *in vitro* methods available to study adhesion of *C. jejuni* to host cells. Cell association methods are based on the interaction of *C. jejuni* with eukaryotic cell lines *in vitro*. These experiments include the c.f.u.-based adherence assay and experiments using radioactively-labelled *C. jejuni* cells to infect cultured cells (Backert *et al.*, 2013). However, these methods do not allow direct quantification of extracellular-attached bacteria only, as all cell-associated bacteria including intracellular bacteria are recovered after lysis of cultured cells. The determination of extracellular-attached bacteria is only possible in combination with a gentamicin protection assay (which

determines the amount of intracellular bacteria) (Friis *et al.*, 2005). Another way to identify the number of bacterial cells on the surface of cultured epithelial cells is the use of immunofluorescence microscopy (IFM) (Backert et al, 2013).

A review of the literature has shown that use of c.f.u.-based adherence assay combined with an invasion assay is a useful method that characterises adhesion-defective mutants. For example, adhesins such as CadF, FlpA and JlpA were identified or confirmed using this approach (Monteiville *et al.*, 2003; Flanagan *et al.*, 2009; Jin *et al.*, 2001). In addition, the involvement of surface glycoconjugates such as CPS, LOS and N-linked glycoproteins in adhesion was identified using adhesion-defective mutants (using *kpsE* and *kpsM* mutants, *galE* mutant and *pglB* and *pglE* mutants) (Bacon *et al.*, 2007; Fry *et al.*, 2000; Szymanski *et al.*, 2002).

1.8.1 Confirmed protein adhesins

At present several confirmed adhesins in *C. jejuni* such as CadF, FlpA, JlpA, MOMP and FlaA have been described, the data of which are summarised in Table 1.1.

CadF is a 37 kDa outer membrane protein that binds to fibronectin and belongs to the OmpA-like protein family (Konkel *et al.*, 1997; Konkel *et al.*, 2005). In a study by Montewille *et al.* (2003), it was shown that the presence of anti-CadF antibodies inhibits the binding of *C. jejuni* mutant cells to human INT 407 cells by around 50% compared to wild type. Additionally, using a competitive inhibition binding assay with a *C. jejuni* F38011 wild type and F38011cadF mutant, only the F38011 wild type was able to inhibit binding of other wild type strain 81-176 to INT 407 cells (Montewille *et al.*, 2003).

Table 1.1. *Campylobacter* protein adhesins with identified host cell receptors[1] (modified from Rubinchik *et al.*, 2012).

Gene name	Product Name	Summary of experimental data	Host cell receptors	Conclusion
cj1478	CadF	Adhesion of F38011/*cadF* mutant INT 407 cells was reduced (Monteville *et al.*, 2003) Purified CadF from strain F38011adhered to INT 407 cells (Monteville *et al.*, 2003)	Fn	Both mutation and adhesion studies confirm specific interaction of CadF with host cell receptors
cj1279	FlpA	Reduced adhesion of F38011/*flpA* mutant to LMH cell line (Flanagan *et al.*, 2009) Reduced adhesion of of F38011/*flpA* mutant to INT 407 cells (Flanagan *et al.*, 2009) Phenotypic changes were confirmed by complementation studies (Konkel *et al.*, 2010)	Fn	Specific interaction with host cell receptors present on cells of human and chicken origin
cj0983	JlpA	Reduced adhesion of TGH9011/*jlpA* mutant in strain to HEp-2 cells (Jin *et al.*, 2001) Adhesion of TGH9011 to HEp-2 cells was reduced in the presence of purified *JlpA* in a dose-dependent manner (Jin *et al.*, 2001) No effect of *jlpA* mutation on adhesion of strain F38011 chicken LMH cells (Flanagan *et al.*, 2009) No difference in adhesion in strain 81-176 *jlpA* mutant to T84 cells (Novik *et al.*, 2010)	Hsp90α	Confirmed adhesin with TGH9011 cells but not with other cells
Cj1339	FlaA	Purified flagella from strain 81116 adhered to INT 407 cells (McSweegan *et al.*, 1986) Purified flagella did not prevent binding of strain 81116 to INT 407 cells (Wassenaar *et al.*, 1991b) No difference in adhesion of 81116 mutant to INT 407 cells (Grant *et al.*, 1993) Reduced adhesion of mutant in strain 81-176/flaA mutant to INT 407 cells (Yao *et al.*, 1994) Deletion of *flaA* in strain NCTC 11168 led to significant reduction in binding to BgAg (Mahdavi *et al.*, 2014)	BgAg	Confirmed interaction of FlaA with BgAg
cj1259	MOMP	Free MOMP inhibited *C. jejuni* adhesion to INT 407 cells (Moser *et al.*, 1997) Purified MOMP inhibited binding of NCTC 11168 to H-II glycoconjugate (Mahdavi *et al.*, 2014) Substitution of MOMP lead to significant reduction in binding to BgAg as well as inhibited binding of *C. jejuni* cells to Caco-2 cells (Mahdavi *et al.*, 2014)	BgAg	Confirmed interaction of MOMP with BgAg. Specific interaction with host cell receptors present on cells of human origin and chicken colonisation

[1]This table lists the genes and products involved in direct interaction with host cells receptors

Fibronectin is a component of the extracellular matrix and has a molecular mass of 250 kDa (Pankov and Yamada, 2002). It has been shown that adhesion of *Campylobacter* to fibronectin is required for maximal adherence and invasion of

intestinal epithelial cells (Ziprin *et al.*, 1999; Monteville *et al.*, 2003; Konkel *et al.*, 2005). Fibronectin is also a receptor for another confirmed adhesin, FlpA. Since it is a fibronectin-binding protein, it contains Fn type III domains (Flanagan *et al.*, 2009; Konkel *et al.*, 2010). Mutation of the *flpA* gene affects bacterial ability to colonise chicken intestine and reduces bacterial binding to chicken LMH hepatocellular carcinoma epithelial cells and to human INT 407 cells (Flanagan *et al.*, 2009). In addition, it has been shown that the presence of anti-FlpA specific antibodies inhibits binding of *C. jejuni* cells to host cells in a dose-dependent manner (Konkel *et al.*, 2010). Supported by complementation, these findings confirmed the role of FlpA in adhesion to fibronectin (Flanagan *et al.*, 2009; Konkel *et al.*, 2010).

Another confirmed adhesin is JlpA, which is associated with the outer membrane lipoprotein and has a molecular mass of 42 kDa (Jin *et al.*, 2001). The crystal structure of JlpA reveals protruding flexible surface loops with a wide hydrophobic concave face that is orientated toward the extracellular space (Kawai *et al.*, 2012). Mutation of *jlpA* gene resulted in reduced adherence of *Campylobacter* to HEp-2 epithelial cells (Jin *et al.*, 2001). Preincubation of HEp-2 epithelial cells with purified JlpA protein has been shown to inhibit the adhesion of bacterial cells to HEp-2 epithelial cells, and in addition, the presence of JlpA-specific antibodies was demonstrated to inhibit the binding in a dose-dependent manner (Jin *et al.*, 2001). Later it was shown that JlpA binds to heat shock protein 90α (Hsp90α) on the surface of HEp-2 epithelial cells and this binding is inhibited by the presence of geldamicin and/or anti-human Hsp90α antibody (Jin *et al.*, 2003). Interaction of JlpA and Hsp90α triggers the intracellular activation of NFκB and p38 MAP kinase, which suggests JlpA is involved not only in adhesion, but also in the pro-inflammatory immune response of the host (Jin *et*

al., 2003). However, these results contradict other studies, which have shown that a mutation of the *jlpA* gene did not result in diminished adherence to T84 cells (colonic adenocarcinoma cell line) and the *jlpA* mutant was still able to bind to chicken LMH cells (chicken hepatoma cell line) and subsequently colonise the intestinal tract of chickens (Novik *et al.*, 2010; Flanagan *et al.*, 2009). These conflicting results may suggest that the choice of host cell line is very important in adhesion studies, since only certain host cells are able to produce receptors for this adhesin.

As previously mentioned, several studies have suggested that the flagellum plays a role in the attachment of *C. jejuni* to host cells (McSweegan and Walker, 1986; Newell *et al.*, 1985; Song *et al.*, 2004). In particular, it was shown that purified flagella specifically bind to INT 407 cells and the presence of intact flagella is important for binding (McSweegan and Walker, 1986). Non-motile flagella adhere better to human epithelial cells *in vitro,* suggesting that motility might interfere with adhesion (McSweegan and Walker, 1986; Newell *et al.*, 1985). Conversely, incubation of INT 407 cells with purified flagella prior to infection with *C. jejuni* did not inhibit binding of bacterial cells to human epithelial cells, suggesting that flagella may not have specific adhesive properties (Wassenaar *et al.*, 1991b). In addition, it was also shown that both flagellated and aflagellated *C. jejuni* 81116 adhere to INT 407 cells (Grant *et al.*, 1993). These data contradict subsequent studies demonstrating that inactivation of a flagellin-encoding gene *flaA* resulted in a reduction of *C. jejuni* adhesion to the INT 407 cells (Yao *et al.*, 1994, Yanagawa *et al.*, 1994). Further to this, in a more recent study, it was suggested that flagella are required for adhesion and biofilm formation, as aflagellated mutants could not adhere to coverslips with the same efficiency as wild type cells (Svensson *et al.*, 2014). Flagella may be involved in the initial

steps of adhesion of bacteria to epithelial cells. However, further studies are required to confirm the validity of this finding.

Recently it has been shown that both FlaA and MOMP are BgAg-binding adhesins (Mahdavi *et al.*, 2014). BgAgs are common ABO histo-blood group antigens that are made up of a complex of fucosylated carbohydrates expressed on the surfaces of erythrocytes and gastrointestinal epithelium. BgAgs have also shown to be present in milk, saliva and tears (Hakomori, 1984; Lloyd, 1987). MOMP encoded by the *porA* gene consists of nine antigenically variable external loops and 16–18 membrane strands connected by short periplasmic turns (Zhang *et al.*, 2000). The MOMP is produced in large quantities and is involved in transport of ions across the bacterial cell wall (Goulhen *et al.*, 2004). It was shown that in the presence of free MOMP the adhesion of *C. jejuni* cells to INT 407 cells was partly inhibited, suggesting that MOMP plays a role in adhesion (Moser *et al.*, 1997).

As it was not possible to generate a *porA* mutant previously, the role of MOMP in interaction with host cells could not be confirmed (Flanagan *et al.*, 2009). However, in a very recent study by Mahdavi *et al.* (2014) it was confirmed that MOMP plays a role in adhesion. Furthermore, it was shown that MOMP is an O-linked glycoprotein. Glycosylation of MOMP affects conformation of the protein as well as its ability to bind to BgAg. The involvement of MOMP in adhesion to Caco-2 cells and in chicken intestinal colonisation was demonstrated in addition to its role in the formation of a biofilm and auto-aggregation (Mahdavi *et al.*, 2014).

1.8.2 Putative adhesins

It was suggested that other *C. jejuni* proteins could be involved in adhesion

(Flanagan *et al.*, 2009; Novik et al., 2010). However the limited number of studies and their contradictory results do not allow final conclusions to be drawn regarding the function of these proteins. They are therefore referred to as 'putative' and are listed in Table 1.2.

Table 1.2. *Campylobacter* adhesion-related proteins[1] (modified from Rubinchik *et al.*, 2012).

Gene name	Product Name	Summary of experimental data	Pfam domains	Conclusion
cj1349	Cj1349	Reduced adhesion of F38011/*cj1349* mutant to LMH cells (Flanagan *et al.*, 2009) Contains fibronectin-binding domain (Flanagan *et al.*, 2009)	Fn-binding domain (FbpA)	Studies with purified protein were not performed. Fibronectin predicted as a receptor (by similarity, not confirmed)
cj0628/ cj0629	CapA	Reduced adhesion of NCTC11168H/*capA* mutant to Caco-2 cells (Ashgar, *et al.*, 2007) Reduced adhesion of capA mutant in strain F38011 using LMH line (Flanagan *et al.*, 2009)	None	No complementation studies to confirm the results. Studies with purified protein were not performed
cj0091	Cj0091	Reduced adhesion of NCTC 11168/*cj0091* mutant to INT 407 cells (Oakland *et al.*, 2011)	None	Studies with purified protein were not performed
cj0268c	Cj0268c	Reduced adhesion of cj0268c mutant to Caco-2 cells (Tareen *et al.*, 2013) Phenotypic changes were confirmed by complementation studies (Tareen *et al.*, 2013)	None	Studies with purified protein were not performed and no information regarding receptors
cj0588	TlyA	Reduced adhesion of *cj0588* mutant in strains 81-176 and 81116 to Caco-2 cells (Salamaszynska-Guz *et al.*, 2008) Purified TlyA protein from 81-176 confirmed interaction of it with Caco-2 cells (Salamaszynska-Guz *et al.*, 2008)	FtsJ-like methyltransferase	No complementation studies to confirm the results
cj0921	PEB1	Purified PEB1 from strain 85H binds to HeLa cells (Kervella *et al.*, 1993) Reduced adhesion of *peb1A* mutant in strain 81-176 using HeLa cells (Pei *et al.*, 1998) No difference in adhesion of peb1A mutant of strain F38011 mutant to LMH line (Flanagan *et al.*, 2009) No difference in adhesion in strain 81-176 peb1A mutant to T84 line (Novik *et al.*, 2010)	Bacterial extra-cellular solute-binding proteins, family 3	Contradictory data
cj0289	PEB3	Major antigenic protein (Pei et al, 1991) Transporter protein (Min et al, 2009) Cell surface located glycoprotein (Linton *et al.*, 2002)	Bacterial extra-cellular solute-binding protein	Original data on its role as an adhesin not confirmed

cj0596	PEB4	Purified PEB4 from strain 85H did not bind to HeLa cells (Kervella *et al.*, 1993) Reduced adhesion of *peb4* mutant of strain NCTC11168 to INT 407 cells (Asakura *et al.*, 2007) Pleiotropic effect of mutation (severe changes in outer membrane profile – possible affect on adhesins) (Rathbun *et al.*, 2009)	Peptidyl prolyl isomerase	Contradictory data/ Complementation studies did not confirm changes in phenotype. Possible periplasmic chaperone
cj1677/ cj1678	CapB	Expression not detected (Ashgar *et al.*, 2007)	None	No experimental data available (no cell binding assays were performed). Putative adhesin (high level of similarity to CapA is expressed as a single polypeptide)
cj0737	P95	After incubation of *C. jejuni* 85H with Caco-2 cells, significant fluorescent signal was obtained, indicating that this strain is able to efficiently bind cultured cells (Kelle *et al.*, 1998)	Haemagglutination activity domain	No mutagenesis or adhesion studies were performed to confirm a proposed function of P95 as an adhesin

[1] This table lists proteins either involved in interaction with host cell receptors indirectly or those whose function as adhesins is not conclusive (e.g. insufficient experimental evidence or contradictory data). Host cell receptors for these proteins are not known.

Among identified putative adhesins is Cj1349, which is a potential Fn/fibrinogen-binding protein. Mutation of *cj1349c* resulted in reduced adhesion of bacterial cells to chicken LMH cells by 14%, however this mutation did not affect chicken colonisation by *C. jejuni* (Flanagan *et al.*, 2009). Due to the absence of complementation studies and studies with purified protein this protein is considered to be putative adhesin.

CapA is a cell surface lipoprotein autotransporter reported to be involved in *C. jejuni* adherence. A mutation of the *capA* gene resulted in a reduced ability to adhere to and invade human Caco-2 and chicken intestinal cells, and also caused low colonisation efficiency in a chick model (Ashgar *et al.*, 2007). In another study, mutation of *capA* did not reduce the capacity of mutant to colonise chicks, but showed decreased adherence to chicken epithelial cells *in vitro* (Flanagan *et al.*, 2009).

In addition to *capA*, another gene (*capB*) coding for a putative autotransporter was identified in the genome of *C. jejuni*. CapB protein might also be considered a putative adhesin as it has high amino acid sequence similarity to CapA and also contains two pairs of open reading frames (ORFs). However, while CapA was shown to interact with host cells, expression of CapB was not detected (Ashgar *et al.*, 2007).

Another protein found to be involved in *Campylobacter* adhesion is lipoprotein Cj0091, which mediates binding of *C. jejuni* to INT 407 cells and plays a role in chicken colonisation (Oakland *et al.*, 2011). As *cj0091* mutation affects colonisation in the early stages of the infection, there is a possibility that Cj0091 may only be involved in initial adherence.

Cj0268c is annotated as a putative transmembrane protein and shown to be involved in adhesion to and invasion of host cell (Novik *et al.*, 2010; Tareen *et al.*, 2013). *Cj0268c* mutants had a reduced ability to adhere to Caco-2 cells by 40%, and complementation of this gene restored binding ability to wild type adhesion levels (Tareen *et al.*, 2013). Since detailed characterisation of the protein was not performed and there is no information available regarding receptors on host cells, this protein is regarded as a putative adhesin.

Another protein that may be involved in *C. jejuni* adhesion is homologous to bacterial haemolysin TlyA (Salamaszynska-Guz and Klimuszko, 2008). In *H. pylori*, TlyA is a bifunctional protein; mutation of *tlyA* results in low haemolytic activity and decreased adhesion to gastric epithelial cells (Martino *et al.*, 2001). Recombinant TlyA expressed in *C. jejuni* 81-176, was found to be responsible for the interaction with Caco-2 cells. In addition, the ability of the *tlyA* mutant to adhere to Caco-2 cells was two-fold lower when compared to wild type (Salamaszynska-Guz and Klimuszko, 2008).

In 1989 Fauchere *et al.* characterised two proteins that bind to HeLa cells, which were later identified as major antigenic proteins of *C. jejuni*; PEB1 and PEB4 (Fauchere *et al.*, 1989; Pei *et al.*, 1991; Kervella *et al.*, 1993). Using acidic glycine extracts of whole *C. jejuni* cells and gel filtration chromatography, Pei *et al.* identified four cell-binding major antigenic proteins (PEB1 to PEB4) that might be suitable for vaccine development and serological detection (Pei *et al.*, 1991). PEB1, also known as PEB1a, is encoded by the *peb1A* gene and plays an important role in adherence and host colonisation (Pei *et al.*, 1993; Pei *et al.*, 1998). PEB1 is a surface exposed and periplasmic (partially exposed and partially periplamic) component of an aspartate/glutamate ABC transporter (Leon-Kempis *et al.*, 2006). PEB1 is a major periplasmic protein required for the uptake of important *in vivo* carbon sources such as aspartate and glutamate (Leon-Kempis *et al.*, 2006). Mutation of *peb1A* resulted in 50- to 100-fold reduction of *C. jejuni* adhesion to HeLa cells and also showed a reduction in the ability of bacterial cells to colonise intestinal cells of rats (Pei *et al.*, 1998). However, *peb1A* mutation showed no effect on the ability of *C. jejuni* to adhere to cultured epithelial cells and to chicken LMH cells, as was reported in more recent studies by Novik *et al.* (2010) and Flanagan *et al.* (2009). Although the results on the role of PEB1 in adhesion were contradictory, it was found that it is involved in binding and transport of L-aspartate and L-glutamate (Leon-Kempis *et al.*, 2006). Further studies are required to verify if this protein has a dual function.

The role of PEB4 (another major antigenic protein of *C. jejuni)* in adhesion was studied using a *peb4* mutant, which demonstrated reduced binding of bacterial cells to INT 407 cells, as well as a reduction in biofilm formation and colonisation of mice (Asakura *et al.*, 2007). It was also shown that *peb4* expression is induced at 37°C, demonstrating that it would be possible for it to be expressed in humans

(Rathbun *et al.*, 2009). However, no complementation studies were conducted to confirm the adhesin function of PEB4, thus it is still unclear whether this protein is an adhesin.

Another putative adhesin that was identified at the same time as PEB1 and PEB4 is PEB3 protein (Pei *et al.*, 1991). PEB3 is highly immunogenic and reactive with convalescent sera from people suffering with *C. jejuni* infection (Pei *et al.*, 1991). In a study designed to identify putative glycoproteins of *C. jejuni*, Linton *et al.* identified that soybean agglutinin (SBA) binds multiple *C. jejuni* proteins on Western blot using whole cell lysates (Linton *et al.*, 2002). In the same study, SBA was used to selectively purify putative glycoproteins that were identified as PEB3 and CgpA. Proteins were purified using a lectin-based affinity purification method and identified by mass spectrometry (Linton *et al.*, 2002). Later, Young *et al.*, using mass spectrometry and nuclear magnetic resonance spectroscopy, identified more than 30 proteins that are also N-linked glycosylated (Young *et al.*, 2002).

PEB3 is considered to be one of the most abundant glycoproteins that is glycosylated at Asn 90, and interacts with soybean agglutinin (SBA) *in vitro* due to the presence of α-linked GalNAc residues (Linton *et al.*, 2002; Rangarajan *et al.*, 2007). Rangarajan *et al.* (2007) reported that PEB3 is an adhesin based on personal communication, however there is no published data available supporting this suggestion. The crystal structure of PEB3 was described as a class II periplasmic binding protein with two domains and a citrate ligand-binding site between them (Figure 1. 6). Additionally, the findings of Min *et al.* (2009) indicate that one of the biological roles of PEB3 is to transport 3-phosphoglycerate, suggesting a possible dual function of this protein.

The amino acid sequence of PEB3 protein revealed 54% identity with AcfC (99%

coverage), an accessory colonisation factor of *V. cholera* (Peterson and Mekalanos, 1988) and 56% sequence identity with the Paa protein (97% coverage) of *E.coli* (Batisson *et al.*, 2003). Although the function of AcfC protein still remains unknown, it was shown it is necessary for efficient colonisation by *V. cholera* (Peterson and Mekalanos, 1988). Paa protein was suggested to play the role of an adhesin based on its cell surface location and the presence of a sulphite-binding domain (Batisson *et al.*, 2003). However, this hypothesis has not been supported by any experimental data. The *paa* mutant of *E.coli* was shown to be unable to bind to microvilli of intestinal epithelial cells (Batisson *et al.*, 2003).

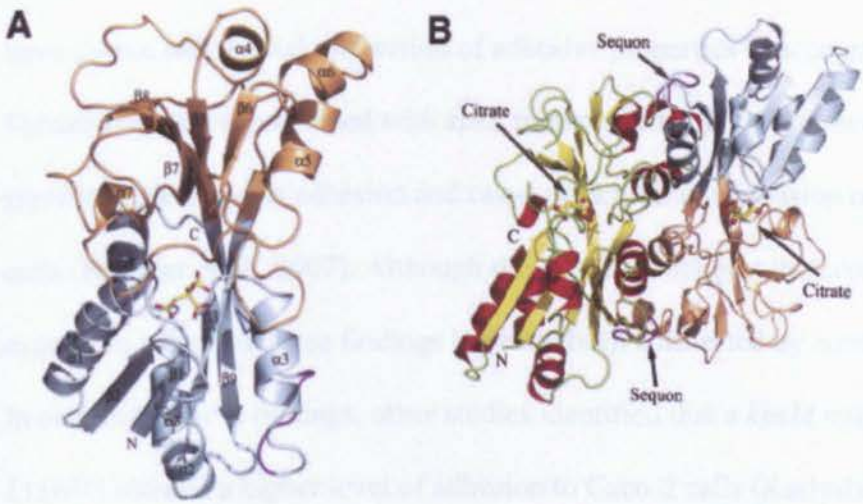


Figure 1.6. Crystal structure of *Campylobacter jejuni* PEB3 protein.
The PEB3 monomer (A) and PEB3 dimer (B) with citrate ligands and the sequons to which N-linked glycans can be attached shown with arrows. (Adapted from Rangarajan *et al.*, 2007).

1.8.3 The role of capsule in *Campylobacter* adhesion

The role of the capsule in bacterial adhesion depends on the nature of the bacterial pathogen, as well as structural features of adhesins. For example, the F1 capsule of *Yersinia pestis* prevents fimbrial adhesins from interaction with host cell

receptors (Runco *et al.*, 2008). Additionally, it was shown that capsule of *E. coli* and *Klebsiella pneumonia* shields the function of short bacterial adhesins, such as Ag43 and AIDA-I (Schembri *et al.*, 2004). While the production of a capsule by *Neisseria meningitides* does not have any effect on bacterial attachment mediated by PilC1 adhesin (Deghmane *et al.*, 2002), the Opc adhesin was reported to be active only in acapsulated strains (Virji *et al.*, 1995).

CPS of *C. jejuni* is a known virulence factor that may be involved in attachment, since the adhesion of capsule-deficient mutants to host cells *in vitro* was reduced (Bacon *et al.*, 2001; Bachtiar *et al.*, 2007). Inactivation of *kpsM* gene in strains 81-176 led to a two-fold decrease in adhesion to INT 407 cells and 10-fold less invasive ability when compared to the wild type strain. Complementation studies have shown only partial restoration of adhesive properties (Bacon *et al.*, 2001). Similar results were obtained with *kpsE* mutants of strain 81116, demonstrating a significant decrease in adhesion and two-fold decrease in invasion of INT 407 cells (Bachtiar *et al.*, 2007). Although this data may suggest the involvement of capsule in adhesion, these findings have not been confirmed by complementation. In contrast to these findings, other studies identified that a *kpsM* mutant in strain 11168H showed a higher level of adhesion to Caco-2 cells (Karlyshev *et al.*, 2008), suggesting a masking effect of capsule. The difference between the bacterial strains and tissue cell lines, as well as a discrepancy in assay conditions may explain contradictory results.

1.8.4 *Campylobacter* adhesion involving lectin-glycan interaction

As noted earlier, in the process of adhesion, specific structures on the bacterial cell surface interact with host cell receptors. Evidence shows that glycans of different pathogens such as viruses, bacteria, parasites and fungi can bind host

lectins and initiate the stimulation of an immune response (van Kooyk and Rabinovich, 2008; Vasta *et al.*, 2009). Glycans expressed on the surface of pathogens can be either similar to host glycans or can be completely unique and not expressed in the host (Garcia-Vallejo and van Kooyk, 2009).

C. jejuni has the ability to recognise and interact with multiple carbohydrate structures, such as mannose, N-acetylneuramic acid, galactose and fucose (Day *et al.*, 2009). Until recently, it was believed that *C. jejuni* could not metabolise carbohydrates, and only uses amino acids as a source of energy (Guccione *et al.*, 2008). However, several strains of *C. jejuni* use L-fucose for growth due to the presence of a gene operon that is upregulated in the presence of fucose or mucin (Muraoka and Zhang, 2011; Stahl *et al.*, 2011). However, due to the lack of 6-phosphofructokinase, which is a key enzyme in energy metabolism, the ability of *Campylobacter* to use carbohydrates as a source of energy for growth is limited (Velayudhan and Kelly, 2002). Observed interaction of *Campylobacter* with host carbohydrates may be important in adhesion as well as in host defence mechanisms. For example, the presence of fucosylated glycans in human milk is considered to prevent infection in infants (Newburg *et al.*, 2005). Additionally, binding of *C. jejuni* to mucin and Lewis antigen present in human breast milk was previously reported (McAuley *et al.*, 2007; Morrow *et al.*, 2004; Morrow *et al.*, 2005).

Mannose derived from the cell wall of *Candida albicans* inhibited the *in vitro* binding of *Salmonella typhimurium* to intestinal cells of day-old chickens (Oyofo *et al.*, 1989). Inhibition of binding of *C. jejuni* to Caco-2 cells in the presence of free mannose was later reported (Russell and Blake, 1994). Direct binding of *C. jejuni* to mannose was confirmed in a study by Day *et al.* (2009). This interaction was specific, as binding of *C. jejuni* to Caco-2 cells was inhibited in the presence

of the Man-specific lectin ConA. Being a component of N-glycan structures of host cells, mannose is used by many bacteria for adherence (Parry *et al.*, 2006). For example, uropathogenic *E. coli* binds to host tissues using Man-specific lectin FimH (Thomas *et al.*, 2004; Thomas *et al.*, 2002).

C. jejuni was shown to be attracted to mucin in the mucous layer of the intestine (Hugdahl *et al.*, 1988; McAuley *et al.*, 2007; Tu *et al.*, 2008). One of the mannosylated and sialylated structures that *C. jejuni* initially interacts with in the intestinal tissue is the cell surface-bound mucin receptor MUC1 (McAuley *et al.*, 2007; Linden *et al.*, 2008). *In vivo* studies have demonstrated that *C. jejuni* gastric colonisation was partially decreased by MUC1. Furthermore, membrane-bound MUC1 was shown to protect epithelial cells from CDT *in vitro* (Linden *et al.*, 2008a,b).

Another glycan that is recognised by *C. jejuni* is Le antigen, which is a fucosylated glycan present on mucins of the human intestine (Hanisch, 2001). *C. jejuni* binding to intestinal cells was inhibited in the presence of free fucose, indicating the interaction of this oligosaccharide with the bacterium is specific (Cinco *et al.*, 1984). Different pathogens bind to fucosylated glycans of the host cells. For example, *P. aeruginosa* Fuc-specific lectin binds to the fucosylated mucosal layer of airways, facilitating colonisation (Rhim *et al.*, 2001). Fucose, a prominent component of mucin, was shown to act as a chemoattractant for *C. jejuni* (Hugdahl *et al.*, 1988; Tu *et al.*, 2008).

Human milk was reported to prevent infection of HEp-2 cells, due to the presence of fucosylated oligosaccharide fractions (Ruiz-Palacios *et al.*, 2003). In the same study, fucosylated oligosaccharide inhibited colonisation of mice by *Campylobacter*. This interaction was due to the binding of *C. jejuni* to difucosylated terminal Le^b structures and 2' fucosyllactose that is present in

human milk glycoproteins (Morrow *et al.*, 2004). It was shown that human breast milk fucosylated glycoproteins inhibit colonisation by *C. jejuni*, indicating that *C. jejuni* interacts with the fucosylated glycoporoteins of host tissues (Morrow *et al.*, 2005). *C. jejuni* may recognise fucosylated and terminal galactose structures of another mucin MUC2, which is the most abundant gel-forming mucin (Day *et al.*, 2012). Day *et al.* (2009) confirmed the specificity of binding to fucose and galactose by inhibition of attachment of *C. jejuni* to Caco-2 cells using Fuc-specific lectin ulex europaeus agglutinin 1 (UEA-1; terminal alfa 1-2 Fuc binding lectin) and galactose-recognising lectin erythrina cristagalli agglutinin (ECA). Additionally, the interaction of *C. jejuni* with BgAgs, which are fucosylated carbohydrates expressed on the surfaces of erythrocytes and gastrointestinal epithelium, was recently reported (Mahdavi *et al.*, 2014). Day *et al.* (2009) hypothesised that interaction of *Campylobacter* with mannose and/or N-acetylneuraminic acid may be important in initial contact with host cells, followed by interaction with fucose and terminal galactose resulting in persistent infection. These findings highlight the importance of *C. jejuni* interaction with glycans of the host cells. However, the nature of recognition of the host glycans by *C. jejuni* is still not completely understood.

In contrast, as noted earlier, *C. jejuni* has its own surface-exposed glycan structures, such as N-linked glycoproteins and LOS, which may be involved in the recognition and interaction of glycan binding to receptors of host cells. The involvement of LOS in adhesion was shown in other pathogens (Abu-Lail and Camesano, 2003). Thus, this interaction may mediate binding of bacterial carbohydrates to host cell protein receptors, such as integrins (Makihira *et al.*, 2002; Isberg and Leong, 1990; Wong and Isberg, 2005).

Carbohydrate recognition is important for the proper function of the innate

immune system of the host. Various classes of glycan-binding receptors (lectins) are expressed on the surface of immune cells (Vasta, 2009; van Kooyk and Rabinovich, 2008). Glycan-binding lectins include C-type lectins, galectins, sialic acid-binding immunoglobulin-like lectins (siglecs), surfactant proteins A and D, nonintegrins (DC-SING and L-SING) and ficolin, and they possess carbohydrate recognition domains (CRDs) specific to oligosaccharide moieties present on CPS, LPS and glycoproteins of microbial pathogens (Mitchell *et al.*, 2001; Holmskov *et al.*, 2003; Alvarez *et al.*, 2002). N-linked glycans found on viruses such as the Nipah virus, which causes severe encephalitis, and HIV-1, interact with galectins (Levroney *et al.*, 2005; Rabinovitch and Toscano, 2009). An interaction of galectins with the surface glycans of *Trypanosoma cruzi* and *Leishmania* parasites has also been reported (Vasta, 2009). Another glycan structure that interacts with galectins is LacdiNAc (GalNac β1-4 GlcNAc) of *Shistasoma mansoni* (van der Berg *et al.*, 2004).

A human lectin intelectin has been shown to interact with D-galactofuranosyl residues of pathogenic Gram-positive bacterial species *Nocardia rubra* (Tsuji *et al.*, 2001). Interaction of human lectins with surface glycans of pathogenic microorganisms such as *K. pneumoniae*, *Helicobacter pylori*, *Streptococcus pneumoniae,* and *Neisseria gonorrhoeae* was earlier summarised (Vasta, 2009). It is possible that similar lectin-glycan interactions may take place during interaction of *Campylobacter* with host cells, as these bacteria are known to express a diverse range of cell-surface glycans (LOS, LPS, CPS and O- and N-linked glycoproteins) (Guerry and Szymanski, 2008). *C. jejuni* glycan structures are important virulence factors that are involved in adhesion and invasion of host cells, as well as in evasion of the immune system (Guerry *et al.*, 2002; Bacon *et al.*, 2001; Logan *et al.*, 2002, Guerry *et al.*, 2007). O-linked and N-linked

glycosylation of proteins have been identified as important processes affecting *Campylobacter* virulence (Logan *et al.*, 2002, Guerry *et al.*, 2007). Although it remains unknown whether the N-linked glycoproteins are required for adhesion, there is some evidence suggesting that they may modulate the host's immune response (Iovine *et al.*, 2008, van Sorge *et al.*, 2009). The interaction between *Campylobacter* glycans and carbohydrate-binding domains of the host may be important in immune evasion of *C. jejuni* (Jarvis and Vedros, 1987; Khatua *et al.*, 2010). The interaction of *C. jejuni* N-linked glycoproteins with C-type lectins of macrophage galactose-type lectins (MGL) specific to GalNAc-terminated glycans was reported by van Sorge *et al.* (2009).

C-type lectins are calcium-dependent carbohydrate-binding proteins that can be specific either to mannose (Man)-specific or galactose (Gal) (Weis *et al.*, 1998). The majority of C-type lectins have more than one CRD and are membrane-associated receptors that are recognised by different pathogens (Drickamer, 1999). C-type lectins are also involved in antigen uptake, signalling and adhesion (Geijtenbeek *et al.*, 2002).

Many C-type lectins are expressed on macrophages and DC, and it was shown that maturation of DC results in a loss of C-type lectin expression (Figdor *et al.*, 2002). Pathogens that target C-type lectins modulate DC response, which is the mechanism responsible for the evasion of host immune response. For example, interaction of cell-surface glycans of *Mycobacterium tuberculosis* with C-type lectins expressed on DC induces signalling that leads to the production of anti-inflammatory cytokines such as IL-10, which in turn inhibits maturation of DC, further assisting bacteria to evade the host immune response (Lugo-Villarino *et al.*, 2011).

Surface-exposed siglecs are a family of type I membrane proteins that are

expressed on immune cells and involved in immune recognition of bacteria and viruses (Crocker *et al.*, 2007; Jones, C. *et al.*, 2003; Rempel *et al.*, 2008; Vanserheijden *et al.*, 2003). More than 20 types of pathogenic microorganism are able to incorporate host sialic acids into their own glycoconjugates, among them *Neisseria meningitides, T. cruzi* and *Streptococcus* group B (Dam and Brewer, 2010). It is apparent that sialylation is important in the survival of the pathogen within the host, as it is involved in molecular mimicry, important in evasion of an immune response. Sialic acid-containing LOS of *Campylobacter* HS-19 strain has been shown to interact specifically with Siglec-7 on monocytes and natural killer cells (Avril *et al.*, 2006). Additionally, specific binding of GBS-related *C. jejuni* strains to Siglec-1 has been demonstrated (Heikema *et al.*, 2010). *Campylobacter* may benefit from expression of sialyated LOS as it may modulate the host innate immune response (Bax *et al.*, 2011). Similarly, Gram-negative pathogens such as *N. meningitidis* and *P. aeruginosa* demonstrate a greater ability to evade the immune system due to the variability of their surface antigenic structures such as LOS and glycoproteins (Jarvis and Vedros, 1987; Khatua *et al.*, 2010).

1.9 Management and control of *Campylobacter* infection

Control measures are important for the reduction of transmission of *Campylobacter* infection. Since *Campylobacter* is considered to be a food-borne pathogen of animal origin and humans become infected through consumption of contaminated food, it is suggested that strategies should focus on public education, personal hygiene and knowledge of potential cross-contamination in the kitchen and the importance of proper cooking of meat (Allos, 2001; Crushell *et al.*, 2004; Havelaar *et al.*, 2007).

1.9.1 Reduction strategies

As contaminated poultry meat represents the main source of *Campylobacter* infections in humans, the reduction of this pathogen in broiler chickens should be one of the strategies to reduce incidence. Considerable risk reduction may be obtained by freezing carcasses for 2–3 weeks (Sting *et al.*, 2012). However, the increase in demand for fresh meat enforces implementation of other strategies. The use of *Campylobacter*-specific bacteriophages may potentially decrease bacterial contamination in poultry (Connerton *et al.*, 2011; Carrillo *et al.*, 2005). However, this treatment has shown to be effective only for a short period of time due to the development of resistance to bacteriophages (Loc *et al.*, 2005; Wagenaar *et al.*, 2005). In order to be effective, the use of bacteriophage treatment should commence several days prior to slaughter (Jeon *et al.*, 2010).

One of the control strategies to reduce incidences of *Campylobacter* infection is called scheduled slaughter, which aims to identify colonised flocks before slaughter and keep them separate from non-colonised flocks, thus reducing the risk of contamination during slaughter as well as implementing decontamination treatment of colonised batches (Wagenaar *et al.*, 2005).

Another approach for controlling incidences of *Campylobacter* infection is the competitive expulsion concept, which is based on inhibiting *Campylobacter* colonisation by immediate feeding of freshly hatched chicks with products that contain microbiota from adult chickens (Barrow and Page, 2000; Zhang *et al.*, 2007). *Salmonella* infection has been successfully prevented using this method, however due to the lack of standardisation and safety this method has not been effective in *Campylobacter* (Laisney *et al.*, 2004; Wagner, 2006).

It is known that contamination of meat occurs during the slaughtering process, thus prevention of faecal contamination from the poultry itself should be

considered in reducing the spread of *Campylobacter* to food (Rosenquist *et al.*, 2006). One of the approaches, which have already been implemented, is a short-term feed withdrawal prior to slaughter in order to reduce the content of the intestine (Warris *et al.*, 2004). In general, prevention of faecal contamination during slaughter process should be one of the main strategies to reduce contamination of broiler carcasses by *Campylobacter* (Sting *et al.*, 2012).

1.9.2 Chemotherapy

As *Campylobacter* gastroenteritis is usually a self-limiting disease, antibiotics are rarely prescribed, and so maintenance of electrolyte balance and replacement of fluid are the common therapies (Altekruse *et al.*, 1999). However, in immunocompromised patients or in patients with severe enteritis, bacteraemia or extraintestinal complications, the use of antibiotics is necessary (Zilbauer *et al.*, 2008; Blaser and Engberg, 2008).

The first choice of antimicrobials in confirmed cases of *Campylobacter* infection is erythromycin, which is a macrolide antibiotic (Zilbauer *et al.*, 2007). For cases of enteritis of unknown origin or travellers' diarrhoea, fluoroquinolone (e.g. ciprofloxacin) is used (Zilbauer *et al.*, 2007). However, an increase in resistance of *Campylobacter* to both erythromycin and ciprofloxacin has been reported (Engberg *et al.*, 2001; Blaser and Engberg, 2008). Chloramphenicol can be considered when antibiotic resistance arises. In the case of serious systemic infection patients should be treated with aminoglycosides (Blaser and Engberg, 2008). Due to the increase in antibiotic resistance, current research should focus on development of alternative and novel therapeutics for both treatment and prophylaxis, such as development of vaccines and inhibitors of attachment.

1.9.3 Vaccines

The use of vaccines in poultry would prevent or reduce colonisation in chickens and thereby reduce the number of infections in humans (Neal-McKinney *et al.*, 2014). In addition to this, it may be used as a prophylaxis measure for travellers or immunocompromised patients. However there is currently no effective *Campylobacter* vaccine available due to challenges in vaccine development. One attempt using a viable, non-colonising strain of *C. jejuni* for vaccination of chickens did not provide any protection (Ziprin *et al.*, 2002). Additionally, it was demonstrated that the use of whole-cell vaccines only provided partial protection in chickens (de Zoete *et al.*, 2007; Noor *et al.*, 1995; Rice *et al.*, 1997). The use of an inactivated bacterial preparation on a ferret model produced similar results (Burr *et al.*, 2005). Due to the high level of genetic and antigenic diversity of *Campylobacter* strains, implementation of such a vaccine for poultry stock is challenging, as poultry becomes colonised with a number of different strains of *Campylobacter* during their lifetime (Jagusztyn-Krynicka *et al.*, 2009).

In humans, the use of inactivated or attenuated *Campylobacter* vaccines causes the potential risk of complications such as GBS or Miller Fisher syndrome (Moran *et al.*, 2005; Prokhorova *et al.*, 2006). Therefore, only strains of *Campylobacter* with LOS that does not undergo phase variation and cannot induce GBS through ganglioside mimicry should be used in the development of whole cell vaccines (van der Woude and Baumler, 2004). The phenomenon of phase variability in humans was demonstrated using inactivated vaccine preparations or by infecting volunteers with the virulent 81-176 strain (Prendergast *et al.*, 2004). Anti-ganglioside antibodies were detected, however they were transient and did not persist. Due to the gaps in the knowledge of GBS the development of a human whole cell vaccine is problematic.

Another approach to vaccine development, which is safer than a whole cell vaccine, is development of subunit vaccines that contain specific antigens from *C. jejuni*. Potential target proteins should be present on the surface of the cell, expressed at high concentrations, be recognised by the immune system and be conserved among different serotypes of the pathogen (Jagusztyn-Krynicka *et al.*, 2009). Flagellin, which is known to be recognised during infection, is one of the vaccine candidates (Martin *et al.*, 1989). Using a FlaA-based subunit vaccine on mice led to partial immune protection, inducing short-term immunity (Lee *et al.*, 1999). However, vaccine development remains challenging due to genetic diversity among strains and glycosylation of flagella (Wang and Taylor, 1990; Scott, 1997; Karlyshev *et al.*, 2005; Logan *et al.*, 2002).

The alternative approach to prevent *Campylobacter* infection is the use of glycoconjugated vaccine, where immunogenic protein is covalently linked to polysaccharide to induce both humoral and cellular immune responses (Trotter *et al.*, 2008). Among widely used glycoconjugated vaccines are vaccines against *Haemophilus influenzae* type b (Hib), *N. meningitides* and *S. pneumonia* (Trotter *et al.*, 2008). The CPS of *Campylobacter* can be used for development of a subunit vaccine, as some common structures of CPS (heptosyl and phosphoramid residues) have been already identified (Kanipes *et al.*, 2006; McNally *et al.*, 2005; McNally *et al.*, 2006; Szymanski *et al.*, 2003). Although the *Campylobacter* polysaccharide alone has low immunogenicity, CPS conjugate vaccine (conjugated to an inactive diphtheria toxin as a carrier protein) induced significant serum response and protection in mice and New World monkeys (Monteiro *et al.*, 2009). In this study diphteria toxin mutant was used as a carrier protein, which was CPS conjugated. Even though the use of CPS as a vaccine candidate looks promising, there may be a number of issues including safety, cross-reactivity,

structural variability of CPS and capsular switching phenomenon that require further development in order to make an effective CPS vaccine (Jagusztyn-Krynicka *et al.*, 2009).

At present, proteomics allows a quick search of potential targets for vaccine development. Prokhorova *et al.* (2006) has identified eight *Campylobacter* surface proteins that were cloned and expressed in *E. coli* and used for the immunisation of mice. Induction of a specific immune response and the partial protective effect in mice was observed and one of the proteins was tested for development of a human vaccine (Jagusztyn-Krynicka *et al.*, 2009). In another study, 200 membrane-associated proteins were identified, which included CadF, FlaA, Omp18, Omp50, Peb1, Peb2 and Peb4 that were recognised by convalescent patient sera (Cordwell *et al.*, 2008).

Using specific vectors, such as attenuated microbial strains, antigens of infectious bacteria can be delivered to the immune system. This approach was used for the development of a Salmonella vaccine expressing *Campylobacter* PEB1. Attenuated *Salmonella enterica* sv. Typhimurium was used as a vector carrying the *peb1* gene. This vaccine was used for oral immunisation of mice. However, despite inducing humoral immunity, IgG antibodies did not show a protective effect (Sizemore *et al.*, 2006).

Another antigen that was used in *Salmonella* vaccine was CjaA, which is the component of the ABC transport system, a solute-binding protein (Muller *et al.*, 2005). It was suggested that CjaA plays a role in *in vivo* colonisation (Holmes *et al.*, 2005). In the work by Wyszynska *et al.* (2004) it was demonstrated that vaccination of chickens by *S. enterica* sv. Typhimurium expressing *Campylobacter* CjaA induced an immune response and resulted in reduced colonisation. However, in a later study with the new generation of *Salmonella*

vaccines, no reduction in colonisation of the chicken intestine was observed, even though there was a strong immune response present (Laniewski *et al.*, 2014). According to the authors (Laniewski *et al.*, 2014) in order to develop an efficient anti-*Campylobacter* subunit vaccine based on attenuated *Salmonella*, this vaccine should be multicomponent. Another approach to increase the efficacy of subunit vaccines is the use of an adjuvant, such as the modified heat labile toxin (LT) or cholera toxin (Jagusztyn-Krynicka *et al.*, 2009).

1.9.4 Inhibitors of adhesion

The process of adhesion involves a variety of bacterial cell surface structures interacting with host cell receptors (as described above), thus the possibility of interfering with such interactions may provide a new focus in the development of antimicrobial strategies (Shoaf-Sweeney and Hutkins, 2009; Thomas, 2010). Use of anti-adhesive therapy, based on the prevention or reduction of contact between bacteria and the host cell, may be another method to control *Campylobacter* infection (Figure 1.7). This approach may provide a safer alternative to antimicrobial therapy and may also reduce the level of colonisation in poultry. Another advantage of this is its low level of activity on normal intestinal flora, due to targeting of pathogen-specific adhesins/receptors. The method also carries no risk of bacterial lysis that would result in the release of endotoxins, and has a low risk of developing resistance due to the conserved nature of host cell receptors.

Several approaches can be used to prevent pathogen colonisation by targeting specific adhesin-receptor interactions. One approach is the use of anti-adhesion compounds that either block or interferes with adhesion of pathogens to specific host cell receptors (Lane *et al.*, 2010). Another approach is to immunise the host

with bacterial surface epitopes in order to stimulate antibody production within the host (Krachler and Orth, 2013).

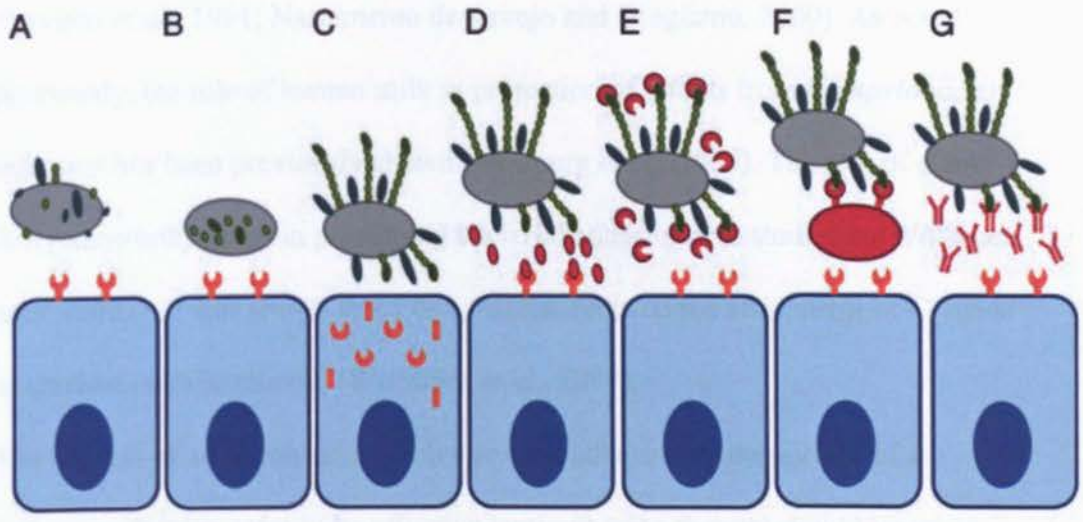


Figure 1.7. Illustration of anti-adhesion therapy strategies. Inhibition of bacterial attachment by affecting biosynthesis and expression of adhesin (A and B) or host cell receptors (C). Competitive replacement using soluble inhibitors of attachment (D, E and F) or using anti-adhesive antibodies (G). (Adapted from Krachler and Orth, 2013).

In most cases, specific interaction between host cell receptors and bacterial adhesins involves carbohydrates (Odenbreit, 2005). Carbohydrates are present in large quantities on the surface of both host cells and bacterial cells, thus the use of carbohydrate-binding antagonists may be an attractive approach for anti-adhesion therapy. Successful inhibition of attachment using oligosaccharides was shown in *H. pylori, P. aeruginosa* and *Legionella pneumophila* (Simon *et al.*, 1997; Gustafsson *et al.*, 2006; Ramphal *et al.*, 1991; Plotkowski *et al.*, 2001; Thomas *et al.*, 2004; Ader *et al.*, 2008).

Several studies have focused on the inhibitory effects of 'decoy' carbohydrates on *Campylobacter* attachment (Ruiz-Palacios *et al.*, 2003; Newburg *et al.*, 2005; Wittscher *et al.*, 2007). The majority of anti-adhesion carbohydrates are dietary

based, for example human and animal milk oligosaccharides, egg yolk and honey oligosaccharides, as well as oligosaccharides derived from plants (Sharon and Ofek, 2000; Lane *et al.*, 2010; Wittscher *et al.*, 2007). Milk oligosaccharides are proven to effectively inhibit *E.coli* binding to host cells (Martin-Sosa *et al.*, 2002, Cravioto *et al.*, 1991; Nascimento de Araujo and Giugliano, 2000). As noted previously, the role of human milk in protection of infants from *Campylobacter* infection has been previously shown (Newburg *et al.*, 2005). The role of plant-derived carbohydrates in preventing bacterial adhesion was studied by Wittscher *et al.* (2007). It was shown that Okra extracts reduced the attachment of *C. jejuni* to chicken colonic mucosa (Wittscher *et al.*, 2007).

The process of adhesion may involve several adhesins on the surface of a pathogen, thus, in order to be effective, anti-adhesion therapy should target as many different adhesins as possible. Therefore, identification of receptors to each adhesin, and the contribution of each adhesin to the process of adhesion and colonisation should be established. In spite of several adhesins of *Campylobacter* having already been described, a greater understanding of the adhesion process of *Campylobacter* to host cells may help in the further development of anti-adhesins for this pathogen.

1.9.5 Probiotics and bacteriocins

Probiotics, such as *Bifidobacterium* and *Lactobacillus* may be beneficial for the host as, when appropriately administered, they may prevent invasion of intestinal tissues by enteropathogens (Ventura *et al.*, 2009, Fooks and Gibson, 2002). The mechanism of action of probiotics is complex, and may include competition for host-surface receptors and nutrients, direct probiotic-pathogen interaction, production of specific antibacterials (bacteriocins and hydrogen peroxide) and

increase of host barrier protection due to increased mucin production (Sherman *et al.*, 2009; Mack *et al.*, 1999, Sherman *et al.*, 2005; Gueimonde *et al.*, 2006). Probiotics may work indirectly through utilisation of anti-adhesive carbohydrates as a food source, which, in turn, results in an increase in the number of probiotics (Sela *et al.*, 2008). Thus, the use of anti-adhesive carbohydrates may have a dual action, as they may also work as prebiotics (Lane, 2010).

The use of probiotics shortens the duration of viral-induced diarrhoea and prevents *Clostridium difficile*-associated diarrhoea (Szajewska *et al.*, 2007; Parkes *et al.*, 2009). Additionally, probiotics were shown to inhibit binding of *H. pylori, Salmonella cholerasuis* sv. Typhimurium and enterotoxigenic *E. coli* (ETEC) to human epithelial cells (Myllyluoma *et al.*, 2008; Candela *et al.*, 2008). The use of probiotics to treat a *Campylobacter* infection has not been studied excessively. However, selected *Lactobacillus* strains were able to inhibit *C. jejuni* invasion of human epithelial cells (Wine *et al.*, 2009). In addition, in a later study, it was shown that colonisation of chicken intestinal mucous by particular probiotic strains reduced the adhesion of *Campylobacter* cells *in vitro* (Ganan *et al.*, 2013). These strains may be useful in preventing infection in poultry. However, more research needs to be conducted before these probiotic strains will be used to control this food-borne pathogen.

Some probiotic strains can produce peptides called bacteriocins, which inhibit the growth of pathogenic bacteria (Cotter *et al.*, 2005). Several bacteriocins, such as OR-7 from *Lactobacillus salivarius* and E-760 from *Enterococcus* may significantly control the level of chicken gut colonisation by *Campylobacter*, as bacteriocin-treated chickens had a reduced level of colonisation by *Campylobacter* (Stern *et al.*, 2006; Line *et al.*, 2008). In a later study the use of bacteriocin E-760 for treatment of chickens through drinking water resulted in a

significant reduction of colonisation by *Campylobacter* (Svetoch and Stern, 2010). Additionally, treatment of *Campylobacter*-colonised chickens for more than three days before slaughter resulted in a five to six log reduction of caecal colonisation (Svetoch and Stern, 2010). These findings indicate that bacteriocin treatment prior to slaughter may be an effective strategy for controlling *Campylobacter* infection and colonisation.

1.10 Aims and objectives of study

The various methods of bacterial interaction with cellular molecules of the host are paramount in the pathogenesis of many bacterial infections (Casadevall and Pirofski, 2001; Patti *et al.*, 1994). *Campylobacter* attachment to host cells is an important stage in the pathogenesis of *C. jejuni* (Janssen *et al.*, 2008; Zilbauer *et al.*, 2008). With the increase in antibiotic resistance in *Campylobacter*, there is a greater need to further understand the molecular basis of host-pathogen interaction in order to control *Campylobacter* infection. The key to this lies in the inhibition of initial adhesion and colonisation. This, in turn, requires extensive understanding of the adhesins involved in this process. Although we know about the adhesins of *C. jejuni*, our understanding of the molecular basis of the adhesion process is still behind that of other enteropathogens (Poly and Guerry, 2008; Zilbauer *et al.*, 2008).

C. jejuni has an extensive glycan repertoire, including highly conserved glycans present in N-linked glycoproteins. N-linked glycoprotein PEB3 may be involved in bacterial attachment, but its role in adhesion has not yet been confirmed. The aim of this study was to develop an *in vitro* assay to evaluate the role of PEB3 and JlpA glycoproteins in adhesion of *C. jejuni* to an analogue of host cell receptors, SBA. The role of capsule in bacterial adhesion was also investigated.

The specific objectives of this work were:

1. Development and evaluation of an *in vitro* ELISA-like assay for the investigation of *C. jejuni* interaction with an analogue of host cell receptors, SBA.

2. Construction and confirmation of *peb3* and *jlpA* mutants in order to investigate the mutants' ability to bind to SBA.

3. Purification of PEB3 after overexpression in *E. coli* in order to identify putative host cell receptors.

4. Investigation of the role of the capsule in adhesion using an acapsulated (*kpsM*) mutant in an ELISA-like assay.

5. Assessment of gene expression (quantification and comparison of the levels of *peb3* and *kpsM* mRNA expression over time using RT-PCR).

6. Investigation of the inhibitory effect of *E. coli* expressing a recombinant adhesin on binding of *C. jejuni* to an analogue of host cell receptors.

CHAPTER 2: Materials and Methods

2.1 Bacterial strains and plasmids

Two *C. jejuni* strains were used in this study: 11168H, which is a hypermotile strain of *C. jejuni* NCTC 11168 (Karlyshev *et al.*, 2002), and an acapsulate mutant of this strain, 11168H/*kpsM* (Karlyshev *et al.*, 2000). *E. coli* XL1 Blue Supercompetent and *E. coli* XL2 Blue Ultracompetent cells (Stratagene) were used according to the manufacturer's instructions for cloning and/or protein expression experiments. The plasmids used in this study are listed in Table 2.1.

Table 2.1. Plasmids used in this study.

Plasmids	Description	Source /Reference
pGEM-T Easy	Cloning vector	Promega
pJMK30	Source of *kan*^{r} cassette	Van Vliet *et al.*, 1998
pAV35	Source of *cam*^{r} cassette	Van Vliet *et al.*, 1998
pBAD33	Contains pBAD promoter	Guzman *et al.*, 1995
pPGL1	*C. jejuni* 16 kb fragment (*pgl* locus) cloned into the *Bam*HI site of pBR322.	Fry *et al.*, 1998
pRRC	*cam*^{r} cassette cloned into pRR (plasmid with fragments of rRNA gene cluster)	Karlyshev and Wren, 2005
pRRBC	pBAD promoter from pBAD33 cloned into pRRC vector.	Ikeda, 2014

2.2 Bacterial growth media and supplements

All culture media made in the laboratory were autoclaved at 121°C for 15 min prior to use.

Columbia Blood Agar (CBA)

Columbia Agar powder (Oxoid) was suspended in distilled water according to the manufacturer's instructions and used for plate cultures of *C. jejuni.* After sterilisation, media was cooled to 50°C and sterile defibrinated horse blood (Oxoid) was added to final concentration of 6%.

Brain Heart Infusion Broth (BHI)

BHI (Oxoid) was used to support the growth of *C. jejuni* in liquid culture. Prior to sterilisation the appropriate amount of powder was suspended in distilled water according to manufacturer's instructions.

Super Optimal Broth (SOC) Medium

Pre-made SOC media (Invitrogen) was used in the final step of transformation in order to obtain maximal transformation efficiency of *C. jejuni* or *E. coli.* Unopened bottles were stored at 4°C. After opening, SOC medium was stored at -20°C.

Luria-Bertani Medium (LB)

LB (Sigma) powder was used for preparation of LB broth according to manufacturer's instructions and autoclaved as appropriate. LB agar (LBA) was prepared similarly except for the addition of 1% (w/v) agar to LB medium. Any necessary antibiotics were added once an appropriate temperature was reached following autoclaving.

Mueller-Hinton Broth

Mueller-Hinton (MH) Broth powder (Oxoid) was suspended in distilled water

according to the manufacturer's instructions and autoclaved. It was used for storage of *C. jejuni* stocks at -80°C.

Antibiotic Supplements

Ampicillin, kanamycin, chloramphenicol and Skirrow III supplement were obtained from Sigma-Aldrich. Ampicillin was ready to use at stock concentration of 100 mg/ml. 50 mg/ml stock solutions of kanamycin were prepared using distilled water. 34 mg/ml stock solutions of chloramphenicol were prepared using ethanol. Aliquots of antibiotic stocks were stored at -20°C. Skirrow supplement III was prepared using distilled water according to the manufacturer's instructions. Aliquots were stored at -20°C. Final concentration of the components of Skirrow supplement III: Polymyxin B, 2.5 IU/ml; Vancomycin, 10 μg/ml; and Trimethaprim, 5 μg/ml.

2.3 Growth and storage of bacterial strains

Campylobacter strains were grown under microaerophilic (5% O_2, 10% CO_2, 85% N_2) conditions in a controlled atmosphere incubator (Don Whitley) at 37°C on CBA supplemented with 6% defibrinated horse blood and Skirrow supplement. When necessary, the medium was supplemented with chloramphenicol (10 μg/ml) and/or kanamycin (50 μg/ml). An aliquot of the resulting bacterial culture was stored at -80°C in MH broth supplemented with 15% glycerol. For liquid cultures, bacterial cells were transferred from the solid media to BHI with Skirrow supplement and incubated shaking (90 rpm) at 37°C.

Based on viable count data, *C. jejuni* grown on BHI at 42°C has following growth stages: exponential or lag phase (0–16 h), retardation or log phase (16–20 h), stationary (20-40 h) and decline phase (40–66 h) (Wright *et al.*, 2009).

Two-day old plate culture grown on CBA at 37 °C has around 10% coccoid forms and only from day 3 around 90% of the population becomes coccoid (Ikeda, 2014). Compared to solid media there are around 40% coccoid forms present in two-day liquid culture grown in BHI at 37 °C (Ikeda, 2014).

In order to determine viable c.f.u, bacteria from two-day *C. jejuni* culture plates were suspended in PBS to OD_{600} 1. This was followed by a 10-fold dilution of suspension in PBS and plating 50 µl onto CBA supplemented with Skirrow. Plates were incubated microaerobically at 37°C for 2 days before colonies were counted. OD_{600} 1 corresponded to to $4x10^9$ c.f.u. of *C. jejuni.*

E. coli was grown on LB agar at 37°C or in LB broth under shaking conditions (180–200 rpm). Where necessary, media were supplemented with antibiotics to the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; or chloramphenicol, 10 µg/ml. Stocks of *E. coli* were stored at -80°C in LB with 15% glycerol, prepared from fresh one-day-old cultures.

2.4 Chemicals, buffers and solutions

Standard laboratory chemicals were purchased from Fisher Scientific and Sigma Aldrich as AnalaR or Molecular grade, unless otherwise stated.

All buffers and solutions were made using distilled water and stored at room temperature, at 4°C or -20°C, according to storage requirements.

2.5 Restriction endonucleases and DNA modification enzymes

Restriction endonucleases and Antarctic Phosphatase (AP) were purchased from

New England Biolabs (NEB). T4 DNA Polymerase and T4 DNA Ligase were purchased from Promega. All restriction endonucleases/enzymes were used to digest plasmids and DNA according to the manufacturer's instructions. Each restriction mixture had the appropriate NEB buffer at 1X concentration, and if required it was supplemented with 100 μg/ml bovine serum albumin (BSA). Antarctic Phosphatase (AP) (NEB) was used to catalyse the removal of the 5' phosphate groups from linear DNA fragments, thus preventing self-ligation. AP was used according to the manufacturer's guidelines.

T4 DNA polymerase (Promega) was used to form DNA blunt ends: filling-in of 5'-overhangs in the presence of dNTPs (NEB) and removal of 3'-overhangs. The enzyme was used according to protocol with either T4 DNA polymerase reaction buffer or restriction enzyme buffer. These were both appropriate for the reaction. The mixture was incubated according to protocol, followed by inactivation of the reaction mixture at 75°C for 10 min.

T4 DNA ligase was used for ligation of chromosomal DNA fragments with linearised vector, by catalysing the formation of a phosphodiester bond between 5'-phosphate and 3'-hydroxyl termini in DNA. Ligation reactions were set up with the amounts of vector and insert DNA recommended by manufacturer's instruction.

CloneChecker™ System (Invitrogen) was used in order to determine the presence of target plasmid DNA in recombinant bacterial colonies. "*The Supercoiled Plasmid*" protocol was used for assessing plasmids for the presence of an insert according to manufacturers' specifications.

2.6 General Cloning Techniques

2.6.1 Crude lysate preparation

Template DNA was prepared by the crude lysis boiling method, in which a single *C. jejuni* colony was boiled using heat block for 30 sec in 4 µl of Green solution (CloneChecker™ System). The lysate was subsequently diluted in 10 µl of TE buffer. For PCR and restriction digestion, 2 µl of diluted lysate was used. Distilled water was used for preparation of lysates for *E. coli.* A single colony was re-suspended in 20 µl of water and heated at 100°C for 30 sec. Two µl of lysate were used for PCR or restriction digestion.

2.6.2 DNA isolation

Genomic DNA was isolated using Gentra® Puregene® Yeast/Bacteria Kit (Qiagen) according to the manufacturer's protocol. A two-day-old culture of *C. jejuni* and a one-day-old culture of *E. coli* were used for extraction of chromosomal DNA. Cells were resuspended in 300 µl of PBS, centrifuged for 1 min at 11,337 x *g* (13,000 rpm) and then used for DNA extraction. DNA was eluted from the membrane using an elution buffer or nuclease-free deionised water.
Plasmid DNA was purified with Qiagen Plasmid Purification Kit (Qiagen) according to the manufacturer's protocol. Bacterial cells were cultured for one day on agar containing appropriate antibiotics. Cells were resuspended in 300 µl PBS, centrifuged for 1 min at 11,337 x *g* (13,000 rpm) and then used for plasmid purification. Plasmid DNA was eluted from membrane using the elution buffer.

2.6.3 Agarose gel DNA extraction and purification methods

Agarose Gel DNA Extraction Kit (Roche) was used for extracting DNA. Restriction digests were run on 1% agarose gel in order to separate the fragments.

The gel segment with the DNA fragment was cut out using a scalpel and a long wavelength (365 nm) UV source. The gel with DNA fragment was weighed and the appropriate amount of agarose solubilisation buffer was added. The remainder of the procedure was performed in line with the manufacturer's instructions. PCR products and restriction digests were purified using Qiagen's PCR Purification Kit (Qiagen) according to manufacturer's protocol. Purified products were run on agarose gel for confirmation of the product.

2.7 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used for a variety of purposes during this study. PCR was performed using a Multigene Gradient Optimax, Labnet 96-well thermocycler using either Phusion® High-Fidelity DNA Polymerase (NEB), One*Taq*® DNA Polymerase (NEB) or GoTaq® DNA Polymerase (Promega). All thermal cycler conditions for each of the DNA polymerases used are listed in Table 2.2. Each PCR mixture contained the DNA template, DNA polymerases and the appropriate buffer, dNTPs (Promega), appropriate forward and reverse primers and nucleotide-free water.

2.8 Confirmation and Quantification of DNA

2.8.1 Agarose Gel Electrophoresis

DNA was separated using gel electrophoresis in 1x Tris-Borate EDTA Buffer (TBE) (pH 8.3) (Fisher) at 120V in a Bio-Rad electrophoresis unit. 1% agarose gels (Sigma) and the tank with TBE buffer contained 0.5 µg/ml ethidium bromide (Fisher) for visualisation by ethidium bromide fluorescence. Samples were loaded into the gel with bromophenol blue-based loading buffer (NEB). 1 kb molecular weight ladder (NEB) was used as a standard to estimate the DNA fragment sizes.

Gels were visualised under a UV transilluminator, G: BOX (Syngene), and images captured and analysed using GeneSnap software (Syngene). When necessary, DNA samples were quantified using a NanoVue spectrophotometer.

Table 2.2. Thermal cycler conditions for PCR using different types of DNA polymerase.

PCR step	Temperature (°C)	Duration of step	Number of cycles
Phusion® High-Fidelity DNA Polymerase with Standard Phusion HF Buffer containing 1.5 mM $MgCl_2$ at 1x reaction concentration.			
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	30
Annealing	45	30 sec	
Extension	72	30 sec per kb	
Final extension	72	10 min	1
One*Taq*® DNA Polymerase with One*Taq*® Standard Reaction Buffer containing 1.8 mM $MgCl_2$ at 1x reaction concentration.			
Initial denaturation	94	30 sec	1
Denaturation	94	30 sec	30
Annealing	45	30 sec	
Extension	68	1 min per kb	
Final extension	68	5 min	1
GoTaq® DNA Polymerase with Colourless GoTaq® Reaction Buffer containing 1.5mM $MgCl_2$ at 1x reaction concentration			
Initial denaturation	95	2 min	1
Denaturation	95	30 sec	30
Annealing	50	30 sec	
Extension	72	1 min per kb	
Final extension	72	5 min	1

2.9 Sequencing data analysis

In order to verify that there was no mutation in target genes and plasmids, plasmids with insert were sent for sequencing to Cogenics (Beckman Coulter). Sequence chromatograms were analysed using Chromas 2 software.

2.10 Primer design

Primers used in this study were synthesised by Sigma Genosys, UK.

For amplification of *C. jejuni,* gene primers were designed using the published nucleotide sequence of the *C. jejuni* strain NCTC 11168 (www.sanger.ac.uk). When designing primers, the following parameters were taken into consideration: G+C content 30–40%, length and melting temperature (Tm). Normally the length of the primers should be about 18–24 bases (Dieffenbach *et al.*, 1993). However, because of the low G+C content of the *Campylobacter* genome, primer length was extended.

2.11 Preparation of competent cells

C. jejuni were grown as a lawn on CBA overnight under microaerophilic conditions at 37°C. Bacteria were suspended in ice-cold buffer containing 272 mM sucrose and 15% glycerol and washed three times with the same buffer. The bacterial suspension was centrifuged for 20 min at 3000 x *g* (3861 rpm) at 4°C, supernatant removed and pellet resuspended in fresh ice-cold wash buffer. The process was repeated 4 times followed by resuspension of the pellet in 1 ml of ice-cold wash buffer. Aliquots of competent cells were stored at -80°C.

E. coli culture was grown as a lawn on LBA overnight at 37°C. Bacteria were inoculated into 10 ml LB with an initial OD_{600} of 0.1 and left at 37°C with gentle shaking. When the culture reached OD_{600} 0.6, the bacterial suspension was

centrifuged for 5 min at 4000 x *g* (4458 rpm) at 4°C. The supernatant was then removed and the pellet resuspended in 5 ml ultra-purified water. The process was repeated 4 times and finally resuspended in 1 ml of water. Aliquots of competent cells were stored at -80°C.

2.12 Electroporation

The electroporation procedure was performed as previously described by Wassenaar *et al.* (1993a). After addition of plasmid DNA to competent cells the mixture was transferred to an ice-cold electroporation cuvette (MBP-Molecular Bioproducts). Electroporation was performed using a BioRad MicroPulser (BioRad) at 2.5 kV, 200 Ω, and 25 μF.

For electroporation of *C. jejuni* the content of the electroporation cuvette was rinsed with 100 μl of SOC buffer, and the bacterial suspension was transferred onto a non-selective CBA plate. Following overnight incubation at 37°C under microaerophilic conditions, bacteria were spread onto a CBA plate supplemented with selective antibiotic and incubated for two days. Colonies were re-streaked onto selective CBA plates and incubated for two days. PCR was then used to confirm the presence of the insert.

For *E. coli* electroporation, the contents of the electroporation cuvette were rinsed with 200 μl of SOC buffer, and the bacterial suspension was transferred onto a non-selective LBA plate for 1 h at 37°C. Following incubation for 1 h at 37°C, bacteria were spread onto an LBA plate supplemented with selective antibiotic and incubated overnight at 37°C. Colonies were re-streaked onto selective LBA plates and incubated for one day. PCR was then used to confirm presence of insert.

2.13 *E. coli* transformation using commercial competent cells

Transformations were performed according to the manufacturer's protocol with the following adjustments: 50 µl of competent cells, 5 µl of ligation mixture and 450 µl of SOC medium. Approximately 200 µl of transformation mixture was spread onto LB agar plates supplemented with appropriate antibiotics and plates were incubated overnight at 37°C.

XL1 Blue Supercompetent cells (Stratagene), according to the manufacturer, have an average transformation efficiency of $\geq 5 \times 10^8$ transformants/µg of supercoiled DNA and were resistant to tetracycline. XL2 BLue Ultracompetent (Stratagene) cells have an average transformation efficiency of $\geq 5 \times 10^9$ transformants/µg of supercoiled DNA and were resistant to tetracycline and chloramphenicol.

2.14 PEB3 protein expression

2.14.1 Construction of *peb3* knocking-in construct using pRRBC

A pRRBC-peb3 construct was designed for expression of PEB3 protein in *E. coli* and *C. jejuni.* Table 2.3 lists the primer sequences used for expression of PEB3 protein. Primers for pRRBC plasmid contained SD (Shine Dalgarno region), restriction site (*Kpn*I) and histidine (6xHis) tag-encoding sequence (Figure 2.1). A target gene was amplified using High Fidelity Phusion DNA polymerase. PCR conditions are shown in Table 2.2. PCR-amplified product was digested with *Kpn*I and inserted into digested pRRBC plasmid via ligation. Ligation product was used for transformation of *E. coli* XL1cells as described earlier. The transformation mixture was spread on LB agar plates supplemented with chloramphenicol. Selected clones were re-streaked and used for plasmid purification and restriction digestion (*Kpn*I and *Hind*III) in order to confirm the presence of the insert, and then were sent for sequencing using primers pBAD-for

and CamR1.

The construct (pRRBC-peb3) with correct (forward) orientation was used for electroporation into *C. jejuni.* In order to check the site of insertion into one of three RNA clusters (Karlyshev and Wren, 2005) the following primers were used: ak233 and pBAD-for, ak 234 and pBAD-up, ak235 and pBAD-up (Table 2.3).

peb3-for
ataa GGTACC aaggaaatact ATGAAAAAAATTATTACTTTATTTGGTGC
*Kpn*I SD

peb3-rev
aggt GGTACC TTA atg atg atg atg atg atg TTCTCTCCAGCCGTATTTTTTAAAAATTTC
*Kpn*I ter 6xHis tag

Figure 2.1. Composition of primers used for *peb3* gene for pRRBC plasmid. Both primers incorporate *Kpn*I sites.

Table 2.3. Primers used for PEB3 expression studies.

Primer Name	Primer sequence (5' to 3')
peb3_for	ATAAGGTACCAAGGAAATACTATGAAAAAAATTATTACTTTATTTGGTGC
peb3_rev	AGGTGGTACCTTAATGATGATGATGATGATGTTCTCTCCAGCCGTATTT TTTAAAAATTTC
peb3_pBAD-for	ATAATCTAGAAAGGAAATACT ATGAAAAAAATTATTACTTTATTTGGTGC
peb3_pBAD-rev	AGGTCTGCAGTTAATGATGATGATGATGTTCTCTCCAGCCGTATTTTTTA AAAATTTC
peb3_pRRC-for	ATAA TCTAGAAAGGAAATACT ATGAAAAAAATTATTACTTTATTTGGTGC
peb3_pRRC-rev	AGGTTCTAGATTA ATGATGATGATGATGATG TTCTCTCCAGCCGTATTTTTTAAAAATTTC
ak233	GCAAGAGTTTTGCTTATGTTAGCAC
ak234	GAAATGGGCAGAGTGTATTCTCCG
ak235	GTGCGGATAATGTTGTTTCTG
CamR1	CGTATTGCCAAAATAGTGGTCGAAATACTC
ak237	TCCTGAACTCTTCATGTCGATTG
pBAD-for	GATTAGCGGATCCTACCTGAC
pBAD-up	GCCGTCAAGTTGTCATAATTGGTAACG

2.14.2 Generation of a recombinant *E. coli* strain expressing *pgl* of *C. jejuni*

In order to assess if the *pgl* locus of *C. jejuni* could be used to generate a recombinant *E. coli* strain that could express glycosylated glycoproteins, pPGL1 plasmid carrying the entire *pgl* locus of *C. jejuni* 81116 was introduced into *E. coli* strain XL2. Recombinant *E. coli* was grown on LBA plates supplemented with ampicillin, as pPGL1 has an ampicillin resistant cassette.

2.14.3 Construction of recombinant *E. coli* strain expressing non-glycosylated PEB3

pBAD33 plasmid was used for overexpression of non-glycosylated PEB3 in *E. coli*. *Peb3* primers were designed with *Xba*I and *Pst*I restriction sites and for purification purposes the reverse primer encoded sequence for 6xHis residues (Figure 2.2). The *peb3* gene of *C. jejuni* 11168H was amplified by PCR using High Fidelity Phusion DNA polymerase and primers designed for pBAD33 plasmid (Table 2.3).

peb3_pBAD-for
ataa TCTAGA aaggaaatact ATGAAAAAAATTATTACTTTATTTGGTGC
*Xba*I SD

peb3-pBAD-rev
aggt CTGCAG TTA atg atg atg atg atg atg TTCTCTCCAGCCGTATTTTTTAAAAATTTC
*Pst*I ter 6xHis tag

Figure 2.2. Composition of primers used for amplification of *peb3* gene for pBAD33 plasmid.

PCR product was digested with *Xba*I and *Pst*I enzymes and cloned into pBAD33 vector yielding pBAD_peb3. Prior to ligation, pBAD33 was treated with the same restriction enzymes as the PCR product. Ligation mixture was used for

transformation into *E. coli* XL1 cells. Transformation mixture was plated onto LB agar supplemented with chloramphenicol. The presence of insert was confirmed with *Pfl*MI enzyme digestion. Plasmids with an insert (size of insert 0.8 kb) were sent for sequencing with peb3_pBAD_for and peb3_pBAD_rev primers. A plasmid with no PCR error in *peb3* was used for further experiments.

2.14.4 Construction of a recombinant *E. coli* strain expressing glycosylated PEB3

Glycosylated PEB3 was produced by *E. coli* strain containing pBAD33_peb3 and pPGL1. pBAD_peb3 construct was transformed into an *E. coli* strain harbouring pPGL1 plasmid. First competent cells were prepared from *E. coli*/pPGL1 as described in section 2.12.2. Recombinant *E. coli* was grown on LBA plates or in LB broth supplemented with chloramphenicol and ampicillin.

Glycosylated PEB3 was produced by *E. coli* strain containing pBAD33_peb3 and pPGL1. pPGL1 plasmid was transformed into an *E. coli* strain harbouring pBAD_peb3 plasmid. First competent cells were prepared from *E. coli*/ pBAD33_peb3 as described in section 2.12.2. Recombinant *E. coli* was grown on LBA plates or in LB broth supplemented with chloramphenicol and ampicillin.

2.14.5 Expression of recombinant PEB3 in *C. jejuni*

pRRC plasmid was used for constitutive expression of *peb3* in *C. jejuni,* and for complementation of *peb3* mutation. Primer sequences used for PCR contained *Xba*I restriction sites (Figure 2.3).

The target gene was amplified using High Fidelity Phusion DNA polymerase with the PCR conditions stated in Table 2.2. PCR product (*peb3*) was digested with *Xba*I and inserted into pRRC plasmid via ligation. pRRC plasmid was digested

with *Xba*I and treated with Antarctic Phosphatase prior to ligation. Ligation mixture was used for transformation into *E. coli* XL1 cells as previously described. The transformation mixture was spread on LB agar plates supplemented with chloramphenicol. Selected clones were re-streaked and used for plasmid purification and restriction digestion (*Eco*RV and *Pfl*MI) to confirm the presence of the insert and its orientation, and then sent for sequencing using primers peb3-XbaI-for and peb3-XbaI-rev.

peb3-XbaI-for
ataa TCTAGA aaggaaatact ATGAAAAAAATTATTACTTTATTTGGTGC
*Xba*I SD

peb3-XbaI-rev
aggt TCTAGA TTA atgatgatgatgatgatg TTCTCTCCAGCCGTATTTTTTAAAAATTTC
*Xba*I ter 6xHis tag

Figure 2.3. Composition of primers used for amplification of *peb3* gene for pRRC plasmid. Primers contained *Xba*I restriction sites for insertion into pRRC vector.

The construct with the correct orientation and confirmed sequence was used for electroporation into *C. jejuni.* In order to check the site of insertion into one of three RNA clusters the following primers were used: ak233 and ak237, ak234 and ak237, ak235 and ak237 (Table 2.3). Ak237 and pBAD-up primers were used to check the presence of *cam*^{r}. *C. jejuni* with pRRC_peb3 plasmid was grown on CBA or BHI supplemented with chloramphenicol and Skirrow supplement.

2.14.6 PEB3 protein purification

The recombinant *E. coli* strains: pBAD33_peb3/pPGL and pBAD_peb3 (with *peb3* 6xHis) were grown in LB broth overnight at 37°C under shaking conditions

and used to inoculate 100 ml LB broth to OD_{600} 0.05–0.1. The cultures were grown under the same conditions until an OD_{600} of 0.5–0.6 was achieved. Protein expression was induced by the addition of 0.1% L-arabinose (Acros Organics) to exponentially growing cultures which were subsequently incubated for 1 hour at 37°C. The cells were harvested by centrifugation at 4000 x *g* (4458 rpm) for 10 min at 4°C. Small aliquots were taken before and after induction for preparation of lysates (pellet from a 1 ml culture was suspended in 2X NuPAGE LDS Sample Buffer (Life Technologies)) and incubated for 5 min at 100°C using heat block.

The recombinant *C. jejuni* pRRC_peb3 (with *peb3* 6xHis) was grown overnight in BHI at 37°C under shaking conditions (180 rpm). Bacterial pellets were collected by centrifugation for 10 min at 4000 x *g* (4458 rpm) at 4°C and either used for protein purification immediately or stored at -80°C.

Recombinant 6xHis-tagged PEB3 was purified from *E. coli* cell lysates using Ni-NTA Fast Start Kit (Qiagen). *E. coli* lacking pPGL1 served as control.

Large-scale protein purification (60–100 ml culture volumes) was performed using Qiaexpress Ni-NTA Fast Start Kit (Qiagen) according to the manufacturer's "*Purification of 6xHis-tagged proteins under native conditions*" protocol.

Small-scale protein purification (10–20 ml culture volumes) was performed using Ni-NTA Agarose Magnetic Beads in line with the manufacturer's kit (Qiagen) using "*Purification in single reaction tubes*" protocol. The volume of the final elution was 10–20 µl. The lysates were analysed at the same time as protein fractions using SDS-PAGE and Western blotting (see section 2.14.7).

In order to determine protein concentrations, Pierce™ BCA Protein Assay Kit (ThermoScientific) was used according to the manufacturer's instructions. A standard bovine serum albumin (BSA) was used as reference.

2.14.7 SDS-PAGE and Coomassie Blue staining

Sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using standard procedure (120V for 1h 30min) with pre-cast polyacrylamide NuPAGE® Novex® 12.5% Bis-Tris Gels (Life technologies). For the SDS-PAGE procedure, samples of lysates and fractions (flow-through, wash and eluates) of purified protein were mixed with NuPAGE LDS Sample Buffer and heated for 2 min at 100°C. The samples and the protein molecular weight marker (Colourplus protein molecular weight marker (NEB) or protein size marker (FisherScientific) were loaded onto gels and run at 120V for approximately 90 min in 1X MOPS Buffer (pH 7.7) (Invitrogen). If necessary, two gels were run, one for Coomassie Blue (Life technologies) staining and another one for Western blotting. Gels were analysed via staining with SimplyBlue™ SafeStain (Invitrogen) for 1h followed by de-staining with distilled water for minimum of 2 h or overnight. Gels were visualised with GeneSnap software and photographed using a G:BOX SynGene camera.

2.14.8 Western blotting (WB)

After separating proteins on SDS-PAGE, the gel was incubated in transfer buffer for 15 min. Proteins were transferred onto 0.45 μm polyvinylidene difluoride membrane (PVDF) (Millipore) using a semi-dry transfer unit at 15V for 90 min. The PVDF membrane was treated with 100% methanol for 15 sec, washed with ultra-pure water for 2 min and incubated in transfer buffer for 5 min. Following the transfer the membranes were treated using two different protocols described below (depending on an application).

2.14.8.1 WB using anti-His antibody

For His-tag detection, membranes were blocked with 5% milk in Tris Buffered Saline with Tween® 20 (TTBS) for 1 h and then rinsed with TTBS three times. They were then treated (with gentle shaking overnight at 4°C) with mouse monoclonal anti-His antibody (NEB) (1:1000 dilution) in TTBS. Following an overnight incubation, the membrane was washed three times and a secondary anti-mouse IgG horseradish peroxidase (HRP)-linked antibody (NEB) (1:10000 dilution) in TTBS was added and incubated with gentle shaking for 2.5 h. After washing the membrane three times for 5 min in TTBS, blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The membrane was visualised using a GeneGnome imaging system (Syngene) with GeneSnap software.

2.14.8.2 WB using SBA lectin

For lectin probing and for detection of glycosylated proteins, blots were blocked in 0.5% PBS with 0.5% Tween® 20 (PBST) for 30 min. These were then incubated for 1 h at room temperature with biotinylated SBA (Vector Laboratories) at a concentration of 10 µg/ml in 0.2% PBST (PBS with 0.2% Tween® 20). Following three brief washes in 0.2% PBST, blots were incubated in HRP-conjugated streptavidin (Sigma) diluted 1 in 1000 in 0.05% PBST at room temperature for 30 min. Following three brief washes in 0.5% PBST, blots were developed using a diaminobenzidine (DAB) HRP substraite staining kit (Vector Laboratories). with nickel enhancement according to the manufacturer's protocol.

2.15 Construction of *C. jejuni* mutants

2.15.1 Construction of *peb3* and *jlpA* mutants

Fragments of the genes *peb3* and *jlpA* were PCR-amplified using the primers listed in Table 2.4 and cloned into pGEM-T Easy vector to produce plasmids pGEM_peb3 and pGEM_jlpA respectively. The kanamycin cassette was obtained from vector pJMK30 by digestion with *Sma*I. The chloramphenicol cassette was obtained from vector pAV35 by digestion with *Xba*I. In order to disrupt the *peb3* gene, the pGEM_peb3 plasmid was digested with *Pfl*MI, blunt ended and ligated with the *kan*^{r} cassette producing pGEM_peb3_*kan*^{r} construct. The *jlpA* gene was disrupted by insertion of the *cam*^{r} cassette into the *Bsa*BI restriction site of pGEM_jlpA plasmid, yielding pGEM_jlpA_*cam*^{r}. In both cases, the orientation of the antibiotic resistance cassette was the same as that of the target gene to avoid a negative polar effect in the mutants. The derivatives were selected using media supplemented with appropriate antibiotics.

Allelic replacement was confirmed by PCR using gene-specific primers and chromosomal DNA extracted from mutants (Table 2.4).

Table 2.4. Primers used for mutation of *peb3* and *jlpA*, for complementation of *peb3 and* confirmation of *kpsM* mutant.

Primer	Sequence (5'–3')	Used for
peb3_for	ATGAAAAAAATTATTACTTTATTTGGTGCATG	Mutation of *peb3* gene
peb3 _rev	TTATTCTCTCCAGCCGTATTTTTTAAAAATTTC	
jlpA_for	ATGAAAAAAGGTATTTTTCTCTCTATTGG	Mutation of *jlpA* gene
jlpA_rev	TTAAAATGACGCTCCGCCCATTAACATAG	
peb3_*Xba*I_for	ATAATCTAGAAAGGAAATACTATGAAAAAAA TTATTACTTTATTTGGTGC	Complementation of *peb3* mutation
peb3_*Xba*I_rev	AGGTTCTAGATTAATGATGATGATGATGATGT TCTCTCCAGCCGTATTTTTTAAAAATTTC	
ak55-f	CCCCATCAAACCTATGCTAC	Mutation of *kpsM* gene
ak59-r	GCCTATAAACCTGTAAAGCCTATAC	

Insertion of the antibiotic cassette into a target gene was confirmed by the expected increase in size of the PCR product. If the whole plasmid was integrated into the locus this would result in two gene copies: a mutated one producing a large PCR product, and an original one without inserted antibiotic cassette producing a smaller PCR product. The absence of the latter in the mutants confirms that allelic replacement had occurred.

The mutants were designated 11168H/*peb3::kan*^{r} and 11168H/*jlpA::cam*^{r}.

2.15.2 Construction of the *kpsM* mutant

11168H/*kpsM::kan*^{r} strain (Karlyshev *et al.*, 2000) was confirmed using PCR and *kpsM*-specific primers (Table 2.4). In order to get more clonal isolates, the chromosomal DNA of the 11168H/*kpsM::kan*^{r} strain was purified and used for transformation into *C. jejuni* 11168H. Allelic replacement was confirmed by chromosomal DNA extraction and PCR using gene-specific primers (Table 2.4).

2.15.3 Complementation of *peb3* mutant

Peb3 gene was PCR-amplified using the primers described in section 2.14.5 (Figure 2.3). The product was digested with *Xba*I enzyme and cloned into *Xba*I-digested pRRC plasmid to produce pRRC_peb3 (Karlyshev and Wren, 2005). The pRRC construct was previously designed for use in complementation studies and gene expression in *C. jejuni*, including expression of genes of non-*Campylobacter* origin (Karlyshev and Wren, 2005). Restriction analysis verified that the gene was transcribed in the same orientation as the *cam*^{r} gene. After transformation of the 11168H/*peb3::kan*^{r} mutant with plasmid pRRC_peb3, Kan^r Cam^r clones were selected. PCR analysis confirmed integration of *peb3* gene into

one of the rRNA gene clusters. The complementation derivative was designated 11168H/*peb3::kan*^{r}/*peb3*$^+$.

2.16 Binding assay

An enzyme-linked immunosorbent assay (ELISA)-like technique was designed and used for the investigation of adhesins containing glycan moieties.

2.16.1 Coating of plates with SBA lectin

SBA lectin was obtained from Sigma. A solution of the SBA lectin was made up in ELISA buffer to a final concentration of 10 µg/ml. Stock solution was stored at -20 °C until used.

A 96-well MaxiSorp microtitre plate (Nunc, Fisher) was used as the solid phase for immobilising the ligand. 100 µl aliquots of the SBA lectin were immobilised in wells of the ELISA plate at 4°C overnight. Simultaneously, some wells were coated with 100 µl of 1% BSA at 4°C overnight to reduce non-specific binding. The following day, wells were washed with 0.2% PBST and then blocked using 250 µl blocking buffer (1% BSA in 0.2% PBST). Wells were incubated with BSA at 4°C overnight. Bacteria harvested from two-day *C. jejuni* or one-day *E. coli* culture plates were suspended in PBS to OD_{600} 1; 100 µl aliquots of bacterial suspension (corresponding to $4x10^8$ c.f.u. of *C. jejuni* and $5x10^8$ c.f.u. *E. coli*) were applied to the wells and the plate was incubated for 40 min at room temperature. *C. jejuni* grown at 37°C on CBA supplemented with Skirrow were used for all binding assays. When necessary, the medium was also supplemented with kanamycin (50 µg/ml) or chloramphenicol (10 µg/ml).

2.16.2 Detection of bacterial binding to immobilised SBA lectin

After washing with 0.2% PBST, the plate was incubated with 100 µl aliquots of biotinylated SBA (10 µg/ml) (Vector Laboratories) diluted in 0.2% PBST for 1 h at room temperature. Adhered bacteria were detected with HRP-conjugated streptavidin. 100 µl of HRP-conjugated streptavidin at a concentration of 0.05 µg/ml in 0.05% PBST was added to the each well and incubated at room temperature for 30 min. The wells were washed and incubated with 100 µl TMB (Sigma) substrate solution at room temperature for 10 min. Absorbance was measured at 450 nm after stopping the reaction with 50 µl stop solution (1M sulphuric acid) using a FLUOstar OPTIME plate reader (BMG LABTECH). All washing steps between the incubation stages were performed with 0.2% PBST four times. In order to prove specificity of the assay, bacterial cells were incubated in the presence of free lectin or GalNac for 40 min at room temperature.

2.16.3 Preparation of acid-glycine extracts

Glycine extraction was performed using the method described by McCoy *et al.*, 1975. Cells of *C. jejuni* grown for two days were harvested from CBA plates and weighed. Cells were then washed twice with 0.7% NaCl and centrifuged at 10,000 x *g* (12,210 rpm) for 5 min. The pellet was suspended in 0.2M glycine hydrochloride pH 2.2 (Sigma) (0.3g of cells per 10 ml) and stirred at room temperature for 15 min. The suspension was centrifuged at 11,000 x *g* (12,806 rpm) for 15 min in order to remove cell particles.

The supernatant was dialysed in PBS using 3.5 kDa dialysis tubes (Sigma Aldrich). The acid-glycin extracts were treated with exo-glycosidase then blotted and probed with SBA lectin as described in section 2.14.8.2.

2.16.4 Exo-glycosidase treatment

In order to remove terminal GalNAc residues, bacterial cells ($4x10^8$ c.f.u.) and acid-glycin extracts were treated with 20 U/ml of *N*-acetylgalactosaminidase (NEB) for 60 min in 10 µl total reaction volume at 37°C according to the manufacturer's protocol. Following incubation bacterial cells were diluted in PBS to a total volume of 100 µl and used for binding experiments.

2.16.5 Fluorescein SBA labelling of *C. jejuni* and *E. coli* cells

Fluorescent labelling of cells was performed as described previously (Cole *et al.*, 1984). Fluorescein-labelled SBA (FSBA) (Vector Laboratories) (100 µg/ml in PBS) was mixed with an equal volume of bacterial suspension and incubated for 40 min at room temperature. Bacteria were pelleted, washed twice in PBS and centrifuged at maximum speed to remove any unbound lectin. According to manufacturer's specification, FSBA has an appropriate number of fluorochromes bound to provide the optimum staining.

For visualisation, 5 µl of the solution with cells was trapped between the slide and the glass coverslip. Samples were observed using a laser scanning confocal microscope (Leica TCS SP2 AOBS) with a 63X oil immersion objective. Higher resolution and increased magnification was obtained using Leica software with digital zoom.

2.17 Inhibition of *C. jejuni* binding to immobilised SBA lectin

A SBA lectin-coated 96-well microtiter plate was blocked with 1% BSA (as previously described in section 2.16.1). Following the wash step the wells were incubated with a 1-day culture *E. coli*/pPGL suspended in PBS to OD_{600} 1 for 40 min at room temperature. After washing with 0.2% PBST the plate was incubated

with *C. jejuni* suspended in PBS (OD_{600} 1) for 40 min at room temperature. After 3 washes with 0.05% PBST the presence of *C. jejuni* was checked using antibodies specific to *C. jejuni.*

2.17.1 *C. jejuni* detection using anti-*Campylobacter* antibodies

Two different anti-*Campylobacter* antibodies (Abs) were used:

1) Mouse anti-*Campylobacter* Abs (1:10000) with secondary goat anti-mouse HRP (1:5000) (AbD Serotec antibodies) and 2) rabbit anti-PEB1 *Campylobacter* Abs (1:10000) with secondary anti-rabbit HRP (1:1000) (Antibodies Online).

SBA lectin-coated plates were used for incubation of whole cells as described in section 2.16.1, and then the following protocol was used:

100 µl of anti-*Campylobacter* Abs was added to the wells and incubated for 60 min at 37°C. After 3 washes, 100 µl of secondary Abs was added and incubated for 60 min at 37°C. Following 3 washes, 100 µl of TMB was added and incubated for 10 min. The reaction was stopped by addition of 50 µl stop solution. Washing of plates and dilution of all antibodies were performed using 0.05% PBST.

2.17.2 DNA quantification of bound *C. jejuni* using qPCR

2.17.2.1 DNA extraction

DNA samples were purified from microtitre plate wells after incubation with *E. coli*/pPGL and/or *C. jejuni* (as described in section 2.17.1) using QIAGEN kit for DNA purification from small volumes (QIAamp UCP Pathogen Mini Kit). Purification was performed according to "*Sample Prep Spin Protocol*". The only protocol modification was that all the reagents used were scaled down by a factor of four. After washing and drying the wells, 100 µl of buffer ATL (from the kit) was added. The rest of the procedure was carried out according to the protocol.

Purified DNA samples were stored at -20°C. DNA purity using the A260/280 ratio was found using a spectrophotometer (NanoVue).

2.17.2.2 qPCR

Microbial DNA qPCR assay kit for *flhA* gene (one of the *C. jejuni* virulence genes) (Qiagen) was used to detect the presence of *C. jejuni* cells. All procedures were performed according to the manufacturer's protocol: "*Real-Time PCR Using Microbial DNA qPCR*".

The reaction was conducted in a total volume of 25 µl, with 12.75 µl master mix (HotStart DNA Polymerase) 1 µl DNA samples, 1 µl microbial DNA qPCR mixture with pre-dispensed *flhA* gene specific primers and 10.75 µl water. Each qPCR run contained a 'no template' control (RNase-free water added instead of DNA), a DNA positive control included in the kit, as well as control DNA purified from $4x10^8$ c.f.u. of *C. jejuni* cells. Purified DNA was then diluted several times to correspond to the different number of cells. DNA dilutions corresponding to $1x10^6$, $1x10^5$,$1x10^4$, $1x10^3$, $1x10^2$ cells were used. Each control sample was analysed in duplicate. The PCR was run using a CFX96 Real Time Thermocycler System C1000 (Bio-Rad).

The cycle parameters were as follows: an initial PCR activation step for ten min at 90°C was followed by a 2-step cycle: denaturation at 95°C for 15 sec and annealing/extension at 60°C for 2 min. The number of cycles was 40.

2.17.2.3 Data Analysis

An absolute quantification method with a standard curve was used to determine the number of DNA copies in samples, where the number of DNA copies corresponded to the number of cells. Standard curves were plotted using cycle

threshold (CT) values vs number of cells (DNA copies).

2.18 RNA isolation and one-step RT-qPCR

2.18.1 qPCR primers

RT-qPCR was used for gene expression studies of *peb3* and *kpsM* using the primers listed in Table 2.5.

Table 2.5. qPCR primers used for gene expression studies.

Primer name	Primer sequence (5'-3')	Used for
q-16s-for	ACAGGTGCTGCACGGCTGTC	Control
q-16s-rev	TGCTCGGCCGAACCGTTAGC	
Peb3_qpcr_for	GCATCGGCTTGATCTTGTGCG	*peb3* expression studies
Peb3_qpcr_rev	CGTTGTGCCTGAAGGTGCTGG	
kpsM_qpcr_for	GCCTGAGTTCCATTCATAAGCTGGG	*kpsM* expression studies
kpsM_qpcr_rev	TGGGTAGTTGGGGAGCCTATGAG	

Primers were designed from *C. jejuni* NCTC 11168 DNA sequences using NCBI web server (http://www.ncbi.nlm.nih.gov/nuccore/AL111168.1). In addition, potential secondary structures and primer-dimer formation were verified using an online tool, Sigma-Genosys DNA calculator. Primers were purchased from Sigma Genosys Ltd. The sequences of the genes of interest were put into Primer BLAST (NCBI) to produce primers. The length of primers designed was between 18 and 21 bp. The amplicon length using these primers should be between 85 and 250 bp. Once the primers were verified with these programs, they were checked through the NCBI BLAST nucleotide tool to ensure the specificity with *C. jejuni*.

2.18.2 RNA isolation

For RNA isolation, *C. jejuni* cells were grown for 48 h under microaerophilic conditions at 37°C in three separate flasks (biological replicates) in BHI (40 ml). Samples were taken at 14 h, 24 h, 38 h and 48 h intervals. Immediately after removing samples from the flasks RNAprotect Bacteria Reagent (Qiagen) was added to the cultures to stabilise mRNA. The culture and RNAprotect Bacteria Reagent were mixed and incubated for 5 min at room temperature, then centrifuged for 10 min at 5000 x *g* (4985 rpm). The supernatant was removed and the pellet was either processed immediately or stored at -80°C. The total RNA from each sample was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol (Protocol 7). The purified RNA samples were treated with On-Column DNase Digestion Kit (Qiagen) in order to remove residual DNA contamination. Isolated RNA samples were stored at -80°C or used immediately for quantitative real time PCR (RT-qPCR).

2.18.3 Quantification of total RNA

RNA concentration was estimated using a NanoVue spectrophotometer. RNA samples were considered pure with an A260/280 ratio between 1.7 and 2.0 and A260/230 ratio above 2.0. The quality and integrity of total RNA was monitored using the Agilent 2100 Bioanalyzer (Agilent Technologies). RNase-free water was used as a control.

2.18.4 RT-qPCR using QuantiFast SYBR® Green

One-step RT-qPCRs were performed using QuantiFast SYBR Green RT-PCR Kit (Qiagen) in a total volume of 12.5 µl, containing master mix (QuantiFast SYBR Green RT-PCR Buffer with $MgCl_2$, Hot start DNA Polymerase, dNTP mix and

ROX™ passive reference dye) and QuantiFast RT Mix (containing Omniscript® Reverse Transcriptase and Sensiscript® Reverse Transcriptase), forward and reverse primers, diluted RNA (50 ng) and RNase-free water.

Primers were added to 100 µM final concentration. Each sample was analysed in technical duplicates and biological triplicates. Every run contained a no template control (RNase-free water added instead of RNA), no primer control (RNase-free water added instead of primers) and a water control, and performed in duplicate. The PCR was run in a CFX96 Real time System C1000 Thermocycler (Bio-Rad). The cycle parameters were as follows: an initial reverse transcription step for ten min at 50°C, and then PCR initial activation step for five min at 95°C followed by two-step cycling: denaturation at 95°C for 10 sec and combined annealing at 60°C for 30 sec. The number of cycles was 35. The 16S rRNA was used as the internal control. The SYBR fluorescence was measured after each cycle and CT values produced from qPCR runs were compared against the control gene. The changes in gene expression (*n*-fold) were calculated from the qRT-PCR data.

2.18.5 Analysis using $2^{-\Delta\Delta CT}$ method

The relative change in gene expression was calculated using the Livak and Schmittgen (2001) $2^{-\Delta\Delta Ct}$ method. Statistical significance was calculated using one-way ANOVA (analysis of variance).

2.19 PEB3 probing of Caco-2 cell, THP-1 cell lysates and normal intestine cell lysates

Purified PEB3 protein was used to probe blots with Caco-2 cell lysate (Santa Cruz Biotechnology), THP-1 cell lysate (Santa Cruz Biotechnology) and normal intestine cell lysate (Abcam). 5 µl of each cell lysate was loaded onto SDS-PAGE

gels. After transfer, blots were either incubated with glycosylated PEB3 or non-glycosylated protein for 2 h at room temperature. Control blots were not treated with PEB3 protein. The rest of the procedure was carried out as described earlier (section 2.14.8.1 and/or 2.14.8.2).

2.20 Bioinformatics

Bioinformatics tools were used for restriction mapping and functional analysis. NEBcutter V2.0 tool was used to perform restriction mapping in order to ensure the correct orientation (http://tools.neb.com/NEBcutter2/).

C. jejuni nucleotide and amino acid sequences were obtained from databases at the Welcome Trust Sanger Institute website (http://www.genedb.org/Homepage/Cjejuni).

The National Centre for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search proteins similar to Cj0289c using BLASTP (protein vs protein) and SWISS-PROT database. The BLAST program was also used to compare the sequences of two different proteins for overlap.

The Artemis program downloaded from the Sanger Institute website (www.sanger.ac.uk) was used to check if the *C. jejuni* genes encoding glycoproteins were known to be adhesins.

PSORT (http://www.psort.org/psortb/) and Softberry (http://morissardjerome.free.fr/infobiogen/www.softberry.com/berry.html) programs were used for prediction of the subcellular location of putative adhesins.

2.21 Statistical analysis

All experiments were repeated a minimum of three times (biological replicates), and data were expressed as mean ± SD. Differences were considered significant for $P < 0.05$ (*, P=0.05–0.01; **, P <0.01–0.001, ***, P <0.001). Comparison of two groups was made with an unpaired, two-tailed student's t-test. Comparison of multiple groups was made with one way ANOVA (http://vassarstats.net/anova1u.html).

CHAPTER 3: Results

3.1. Interaction of *C. jejuni* cells with SBA lectin

In order to investigate the mechanisms and factors involved in *C. jejuni* interaction with host cells, a binding assay based on monitoring bacterial attachment to immobilised analogues of host cell receptors was developed. In this model it is suggested that some N-linked glycosylated proteins may be expressed on the bacterial cell surface, and play the role of an adhesin. As glycan moieties in these glycoproteins contain terminal GalNAc residues recognised by SBA (Linton *et al.*, 2002), the latter could be considered as an analogue of a host cell receptor. Although the *in vitro* interaction of glycoproteins with SBA lectin has been demonstrated previously, the interaction of SBA lectin with whole cells has not.

3.1.1 Validation of the binding assay

Bacterial adhesion was measured using an ELISA-like technique. This technique developed in this study. This *in vitro* model was used to measure bacterial attachment to SBA lectin. Adherent bacteria were detected using biotinylated SBA, which preferentially binds to oligosaccharide structures with terminal GalNAc residues. For detection of biotinylated SBA, the streptavidin-peroxidase polymer was used. Activated streptavidin, conjugated to horseradish peroxidase, is covalently conjugated to a polymer backbone, which increases the biotin binding capacity and amplifies the signal. BSA coated wells were used as a negative control.

A 96-well microtitre plate with immobilised SBA lectin was used for the investigation of bacterial cells producing adhesins containing N-linked glycan moieties. In order to get reproducible data, several experiments were carried out

confirming the specificity and sensitivity of binding (Figure 3.1). Omission of the BSA blocking step lead to false-positive results. No signal was present without bacterial cells or without incubation with biotinylated SBA (Figure 3.1 and Figure 3.2). Immediately after completion, the absorbance was measured and results were analysed using Excel for graphical comparison.

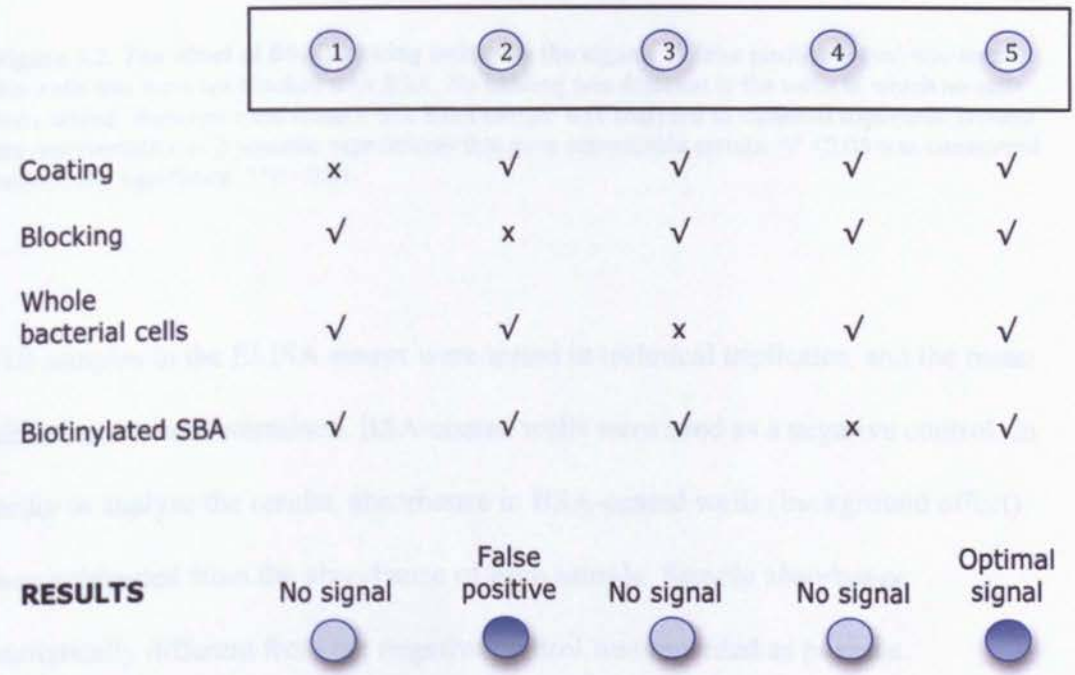


Figure 3.1. Validation of ELISA-like method. After coating a 96-well microtitre plate with immobilised SBA lectin, uncoated sites were blocked by incubation with 1% BSA to reduce non-specific binding. This was followed by incubation with whole bacterial cells. Negative and false-positive controls were obtained by omitting one of the components of the system during the process.
√, added; x, not added.

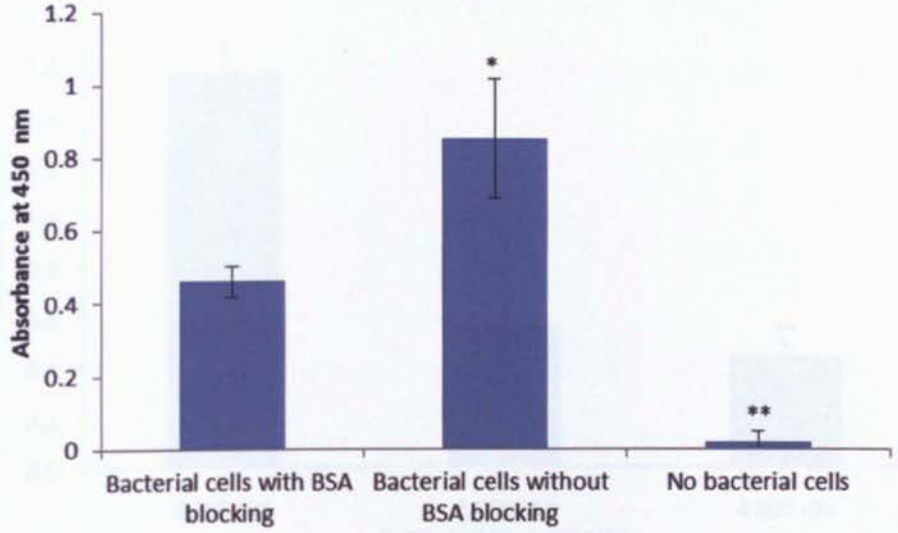


Figure 3.2. The effect of BSA blocking buffer on the signal. A false positive signal was seen in the wells that were not blocked with BSA. No binding was detected in the wells in which no cells were added. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. *P <0.05 was considered statistically significant. **P <0.01.

All samples in the ELISA assays were tested in technical triplicates, and the mean absorbance was determined. BSA-coated wells were used as a negative control. In order to analyse the results, absorbance in BSA-coated wells (background effect) was subtracted from the absorbance of each sample. Sample absorbance statistically different from the negative control was regarded as positive.

3.1.2 Dose-dependent binding of *C. jejuni* cells to immobilised SBA lectin

After validation of the method the system was successfully tested using *C. jejuni* strain 11168H. The results confirmed specific interaction of *C. jejuni* with immobilised SBA lectin. Incubation of *C. jejuni* cells ($4x10^8$ cells) produced an absorbance of 1.563 +/- 0.12, whereas at $4x10^6$ cells the absorbance was 0.440 +/- 0.05 (Figure 3.3). Incubation of cells in the presence of soluble SBA lectin reduced the absorbance from 1.563 +/- 0.12 to 0.867 +/- 0.16 (Figure 3.4).

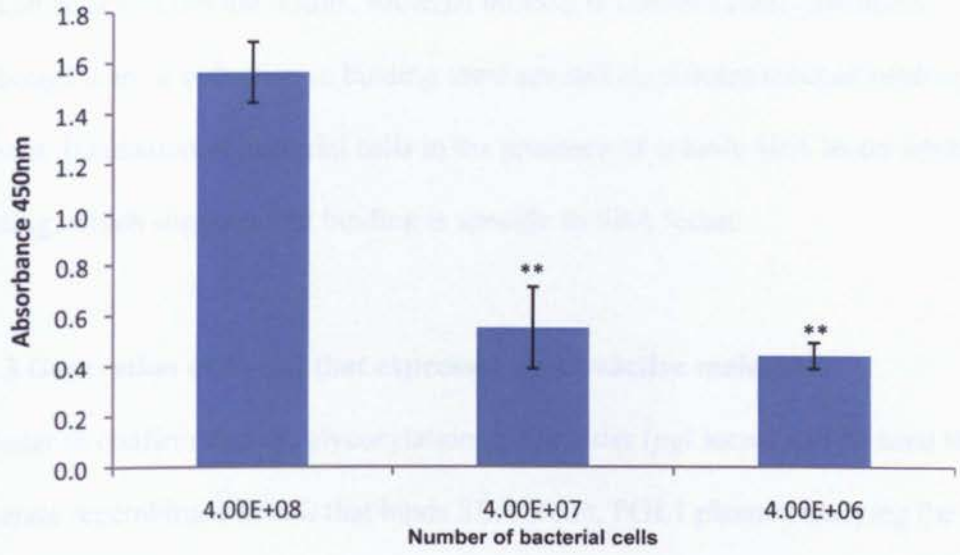


Figure 3.3. Interaction of *C. jejuni* 11168H with immobilised SBA lectin. Binding of whole cells with SBA lectin was concentration-dependent. The figures below the bars indicate the numbers of cells per well. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. *** P <0.001 was considered statistically significant.

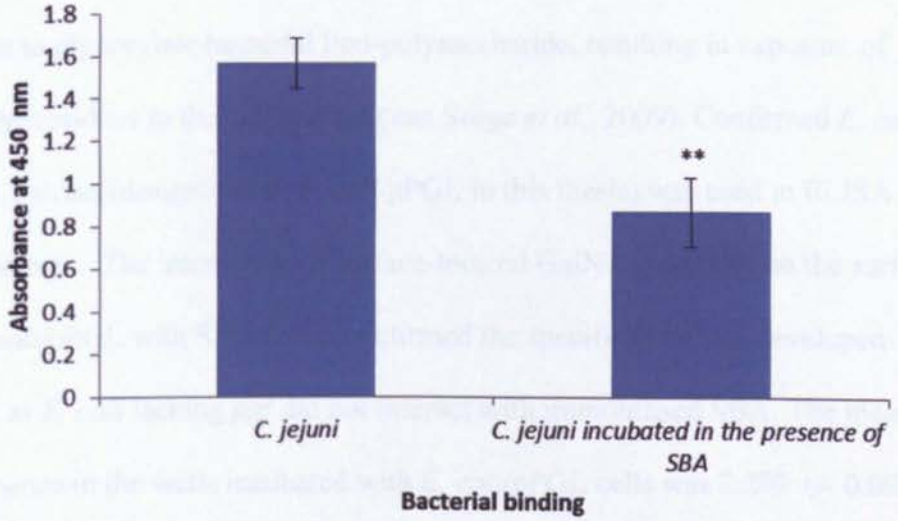


Figure 3.4. Interaction of *C. jejuni* 11168H with immobilised SBA in the presence of soluble SBA lectin. Free soluble SBA lectin at the concentration of 0.1 μM inhibits binding of whole *C. jejuni* 11168H cells. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. ** P <0.01 was considered statistically significant.

As can be seen from the results, bacterial binding is concentration-dependent. Although there is reduction in binding there are still significant residual binding present. Incubation of bacterial cells in the presence of soluble SBA lectin inhibits binding, which suggests that binding is specific to SBA lectin.

3.1.3 Generation of *E. coli* that expresses SBA-reactive molecules

In order to confirm that the glycosylation gene cluster (*pgl* locus) can be used to generate recombinant *E. coli* that binds SBA lectin, PGL1 plasmid carrying the entire N-linked protein glycosylation apparatus (*pgl* gene cluster) of *C. jejuni* 81116 (Fry *et al.*, 1998) was introduced into *E. coli* XL2 strain. Restriction analysis confirmed the presence of pPGL1 plasmid in *E. coli* (Figure 3.5). In order to further confirm that the developed model of attachment was specific and was based on the surface-located GalNAc moieties, binding experiments were repeated using *E. coli* carrying pPGL1 plasmid. Due to the absence of glycosylation acceptor proteins in strain *E. coli* XL2, the *pgl* system was found to be able to glycosylate bacterial lipo-polysaccharide, resulting in exposure of GalNAc residues to the cell surface (van Sorge *et al.*, 2009). Confirmed *E. coli* /pPGL1 strain (designated as *E. coli*/pPGL in this thesis) was used in ELISA experiments. The interaction of surface-located GalNAc moieties on the surface of *E. coli*/pPGL with SBA lectin confirmed the specificity of this developed assay, as *E. coli* lacking *pgl* did not interact with immobilised SBA. The mean absorbance in the wells incubated with *E. coli*/pPGL cells was 2.293 +/- 0.09, whereas in the presence of free GalNAc absorbance was 0.859 +/- 0.04 (Figure 3.6). The results confirmed that *E. coli*/pPGL cells are capable of binding to immobilised SBA lectin in a GalNAc-dependent fashion.

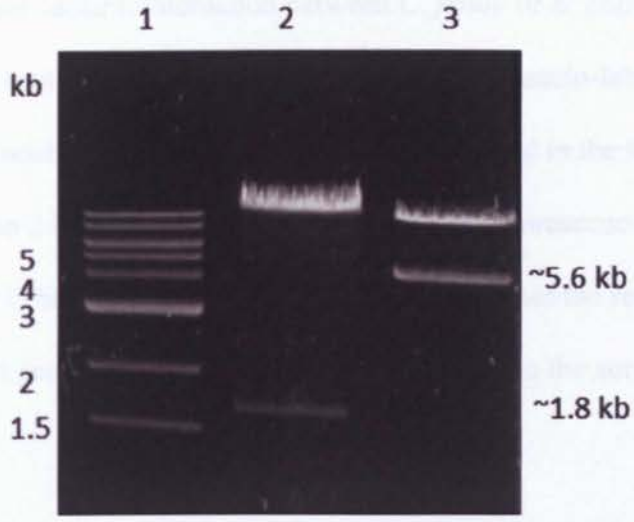


Figure 3.5. Restriction analysis of pPGL1 plasmid. Digestion of plasmid was performed using and *Sph*I (Lane 2) and *Eco*RV (Lane 3). The expected sizes of fragments using with *Eco*RV– 15.0 kb and 5.6 kb. Digestion with *Sph*I produces two fragments of 18.7 kb and 1.8 kb size. Lane1: 1 kb DNA ladder.

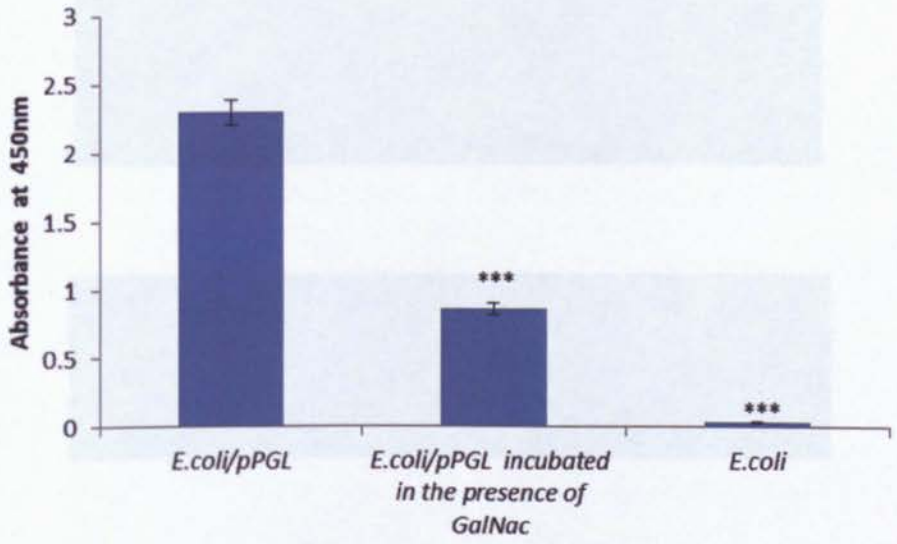


Figure 3.6. Attachment of *E. coli*/pPGL to immobilised SBA lectin. *E. coli* with the pPGL insert binds SBA lectin. Binding is inhibited by incubation with GalNAc at 25 μM. No binding of the recipient strain *E. coli* XL2 was detected. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. *** $P < 0.001$ was considered statistically significant.

In order to further confirm interaction between *C. jejuni* or *E. coli*/pPGL with SBA lectin, the bacterial cells were incubated with fluorescein-labelled SBA (FSBA). After incubation of cells with FSBA as described in the Materials and Methods (section 2.16.5), the cells were examined by fluorescence microscopy. Both *C. jejuni* 11168H and *E. coli* XL2/pPGL cells (but not the recipient *E. coli* XL2) fluoresced green, indicating that SBA lectin binds to the surface of the cells (Figure 3.7).

A

B

C

Figure 3.7. Confirmation of SBA binding to the surface *C. jejuni* and *E. coli*/pPGL cells. Confocal microscopy of *C. jejuni* 11168H (A) and *E. coli*/pPGL (B and C) cells after treatment with FSBA. Representative of several images taken of cells from 2 slides (x630 magnification). Resolution of *C. jejuni* was difficult due to the small size of bacteria.

Fluorescence microscopy with FSBA as a probe clearly demonstrated fluorescein-labelling of the cell surface of *E. coli*/pPGL, but not in *E. coli* lacking the *pgl* locus (data not shown), confirming the presence of the SBA ligand GalNAc on the surface of the recombinant *E. coli* strain. The results also confirmed that *C. jejuni* cell surface glycoproteins interacted with the SBA lectin and that the detection of N-linked glycans is possible using a confocal microscope. Together, these results indicated that *E. coli* XL2/pPGL express GalNAc residues on the surface of the cells.

3.1.4 Specificity of binding of *C. jejuni* to SBA lectin

Incubation of a suspension of *C. jejuni* 11168H cells with immobilised SBA resulted in bacterial attachment. This binding was found to be specific, as demonstrated by the inhibitory effects of both soluble GalNAc and a soluble form of SBA, in a dose-dependent manner. Inhibition was detected in the presence of 0.1 μM soluble SBA lectin, the lowest concentration used (Figure 3.8). Absorbance of the control (without soluble SBA lectin) was 1.388 +/- 0.09, whereas cells incubated in the presence of soluble SBA lectin at 0.1 μM concentration produced an absorbance of 0.706 +/- 0.01. The presence of soluble GalNAc resulted in an inhibitory effect with concentrations of 75 μM (the minimum inhibitory concentration) and above, giving a mean absorbance in the wells of 1.241 +/- 0.07 compared to 1.476 +/- 0.04 in the control (Figure 3.9). A decrease in absorbance indicates fewer bound cells to the immobilised SBA lectin, compared with control values.

Moreover, the bound cells could be detached in the presence of soluble forms of SBA lectin or GalNAc. The absorbance in wells that were first incubated with bacterial cells and then treated with 5 mM SBA lectin was 0.195 +/- 0.04, which

was lower compared to the absorbance in the wells that were treated with 5 mM GalNAc (absorbance was 0.596 +/- 0.01. Both of these were far reduced compared with the control value of 1.676 +/- 0.21. SBA lectin detached bound cells more efficiently (by 8.5 times) compared to the same molar concentration of GalNAc (2.8 times) (Figure 3.10).

Together, the results from the inhibition of attachment and detachment experiments suggest that competitive inhibition of binding is more pronounced with SBA lectin than with GalNAc. Further confirmation of specificity of binding was obtained by treatment of bacterial cells with an exoglycosidase. *C. jejuni* glycoproteins bind to SBA lectin due to GalNAc residues in the glycan component, thus whole bacterial cells were treated with N-acetylgalactosaminidase to remove terminal GalNAc. This resulted in a remarkable reduction of the ability of bacterial cells to attach (Figure 3.11), suggesting that the GalNAc moieties are a requirement for bacterial binding.

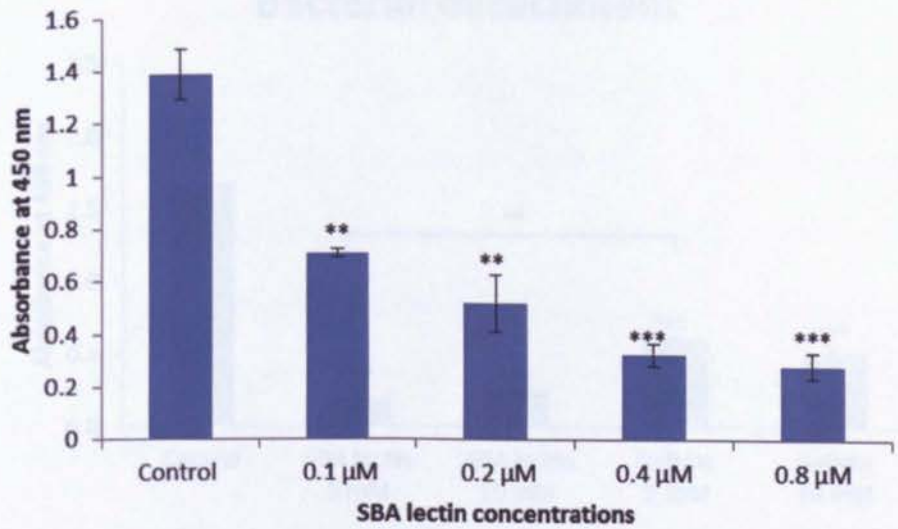


Figure 3.8. Effect of different concentrations of soluble SBA lectin on binding of *C. jejuni* 11168H. Cells were incubated with different concentrations of free (soluble) SBA lectin. No free SBA lectin was added to control samples. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. ** $P < 0.01$ was considered statistically significant. *** $P < 0.001$

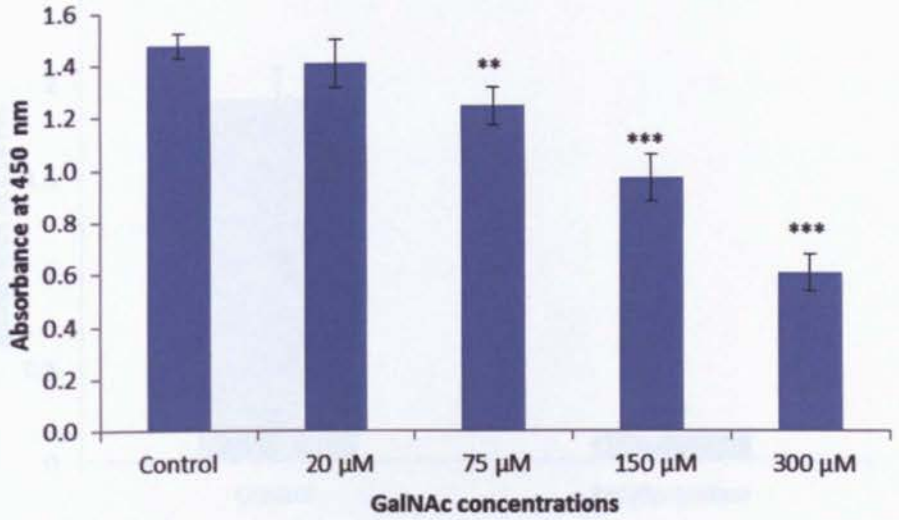


Figure 3.9. Effect of different concentrations of soluble GalNAc on binding of *C. jejuni* 11168H. Cells were incubated with different concentrations of free (soluble) GalNAc. No GalNAc was added to control samples. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. ** P <0.01 was considered statistically significant. *** P <0.001

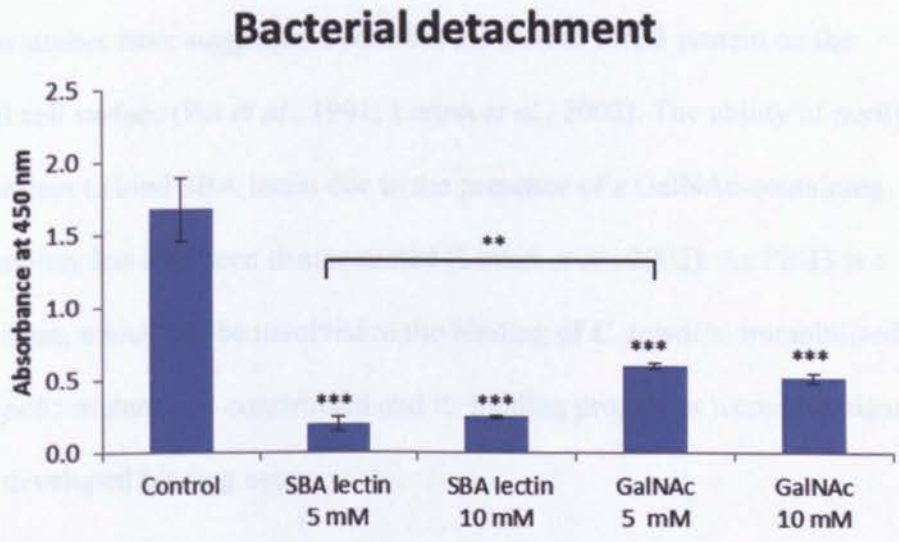


Figure 3.10. Detachment of *C. jejuni* 11168H cells in the presence of soluble SBA and GalNAc. After incubation of cells according to standard protocol, wells were washed and soluble SBA lectin or GalNAc were added to separate wells for 40 min at room temperature. The rest of the procedure was performed according to protocol. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. ** P <0.01 was considered statistically significant. *** P <0.001.

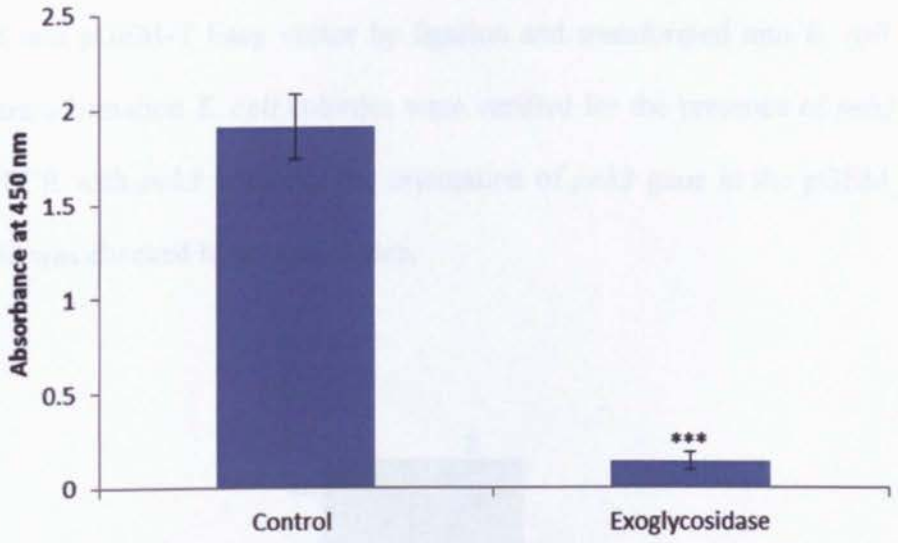


Figure 3.11. The effect of exoglycosidase on binding. Reduction of binding upon treatment of bacteria with GalNAc-specific exoglycosidase. Bacterial cells were incubated with exoglycosidase (20 U/ml) for 1h under microaerophilic conditions at 37°C in 10 µl total reaction volume. Following incubation cells were diluted in PBS and used in binding experiment. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. *** $P < 0.001$ was considered statistically significant.

3.2 *Peb3* gene is involved in the attachment of whole cells to SBA lectin

Previous studies have suggested a possible location of PEB3 protein on the bacterial cell surface (Pei *et al.*, 1991; Linton *et al.*, 2002). The ability of purified PEB3 protein to bind SBA lectin due to the presence of a GalNAc-containing glycan moiety has also been demonstrated (Linton *et al.*, 2002). As PEB3 is a glycoprotein, which can be involved in the binding of *C. jejuni* to immobilised SBA, a *peb3* mutant was constructed and its binding properties were investigated using a developed binding assay.

3.2.1 Construction and confirmation of *peb3* mutant

A PCR was used to amplify the gene of interest, *peb3,* using primers peb3_for and peb3_rev and *C. jejuni* 11168H genomic DNA. After amplification, the PCR

product was purified and run on an agarose gel (Figure 3.12). PCR product was cloned into pGEM-T Easy vector by ligation and transformed into *E. coli* cells. After transformation *E. coli* colonies were verified for the presence of *peb3* gene using PCR with *peb3* primers. The orientation of *peb3* gene in the pGEM_peb3 plasmid was checked in several clones.

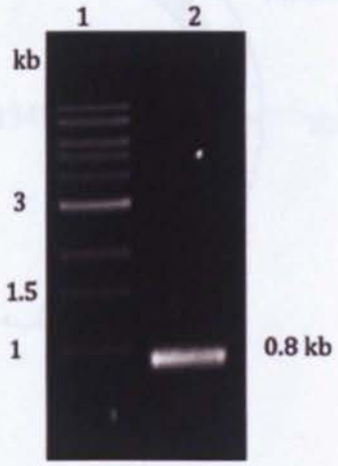
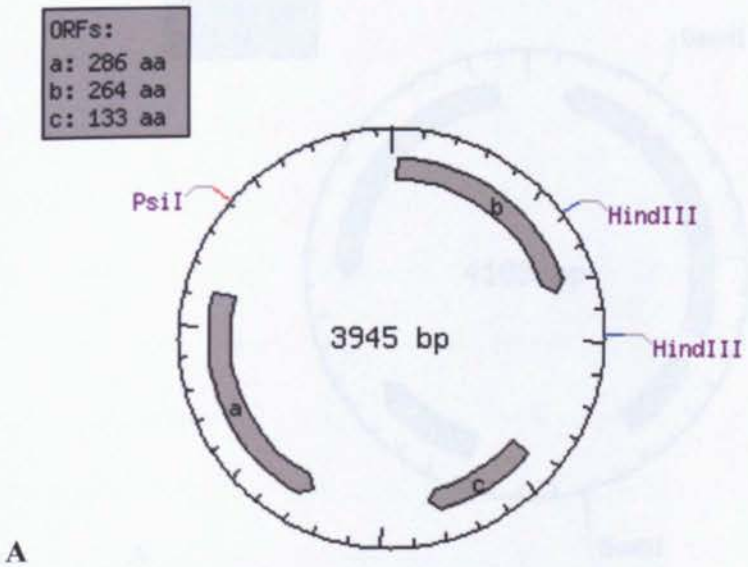


Figure 3.12. Purified PCR product (*peb3*). Gel electrophoresis analysis of purified PCR product. Lane1: 1kb DNA ladder; Lane 2: PCR product of ~0.8 kb.

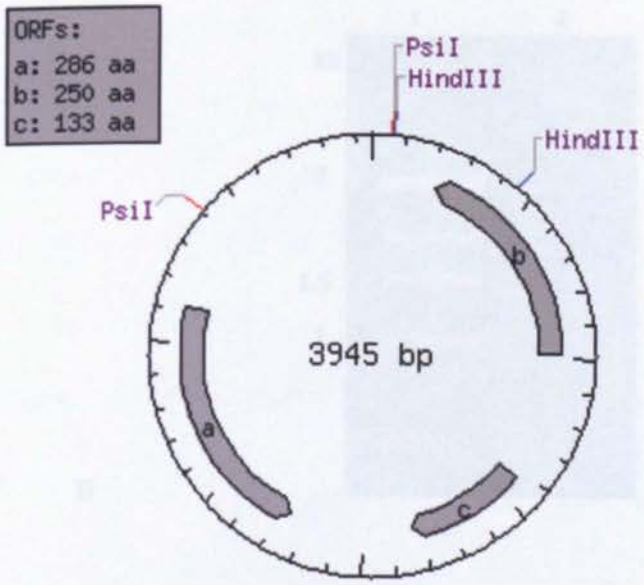
Two restriction maps were constructed using NEBcutter program using *Hind*III and *Psi*I enzymes (Figure 3.13, A and B). In forward orientation the sizes of generated fragments were 1130 bp, 388 bp and 2427 bp, whereas in the reverse orientation the sizes of fragments were 603 bp, 388 bp and 2954 bp. Restriction analysis was conducted to confirm the forward orientation. Double digestion generated three fragments of expected sizes (1.1 kb, ~0.4 kb and ~2.4 kb). The clone in forward orientation (with the same orientation as *peb3* gene) was used for the insertion of the *kan*^{r} gene.

The pJMK30 vector was the source of the *kan*^{r} cassette. Figure 3.14A shows the restriction map of pJMK30. Expected sizes of fragments after digestion with

*Bam*HI should be around 2684 bp and 1499 bp. Fragment sizes after digestion were verified using gel electrophoresis (Figure 3.14 B).

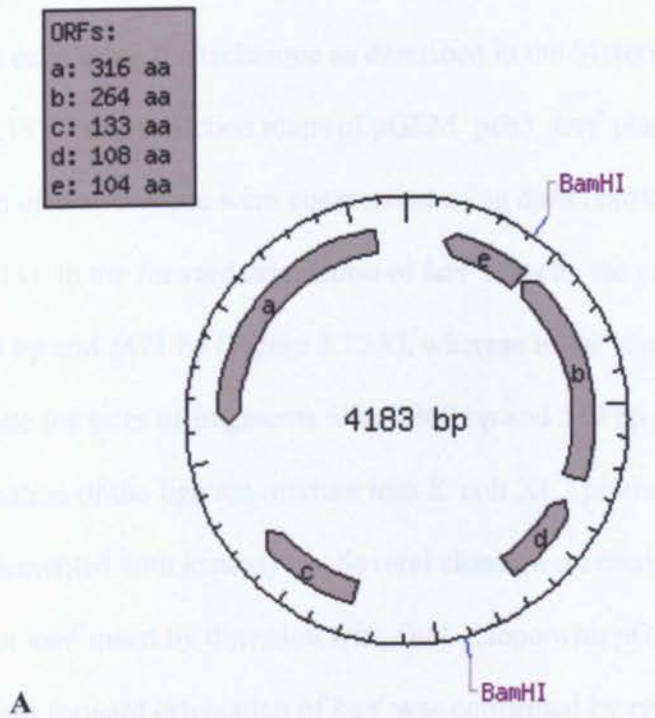


A



B

Figure 3.13. Restriction maps of pGMT_peb3. pGEM_peb3 digested with *Hind*III/*Psi*I in forward orientation (A) and reverse orientation (B). "b" fragment: the gene of interest– *peb3*. Image produced by NEBCutter v2.



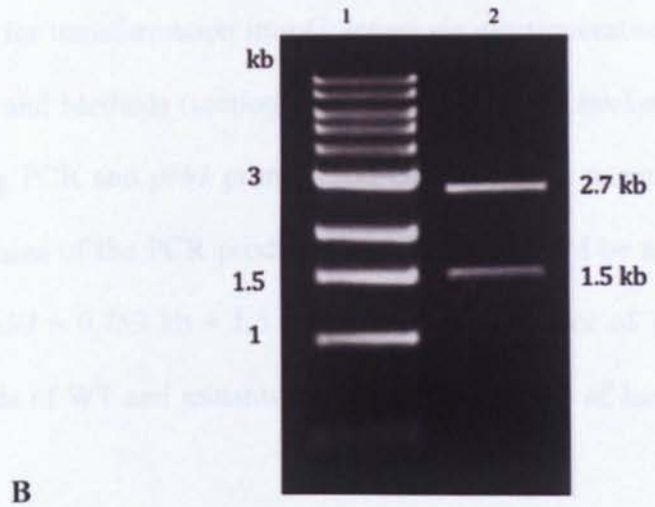
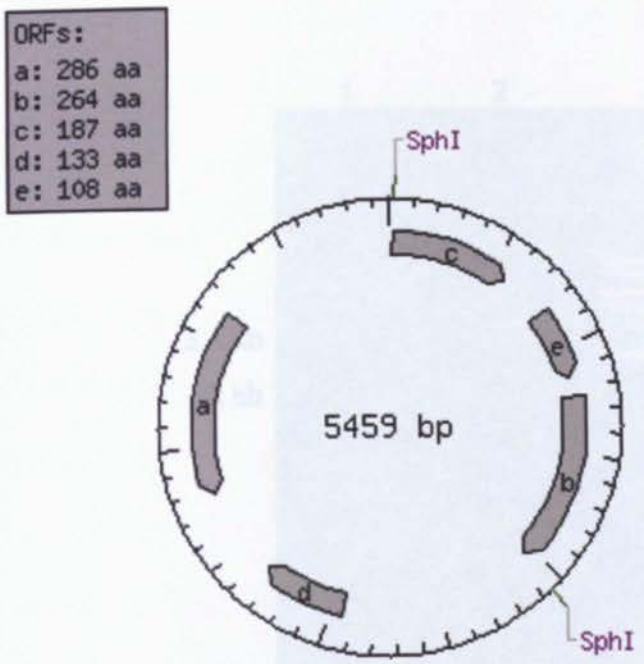


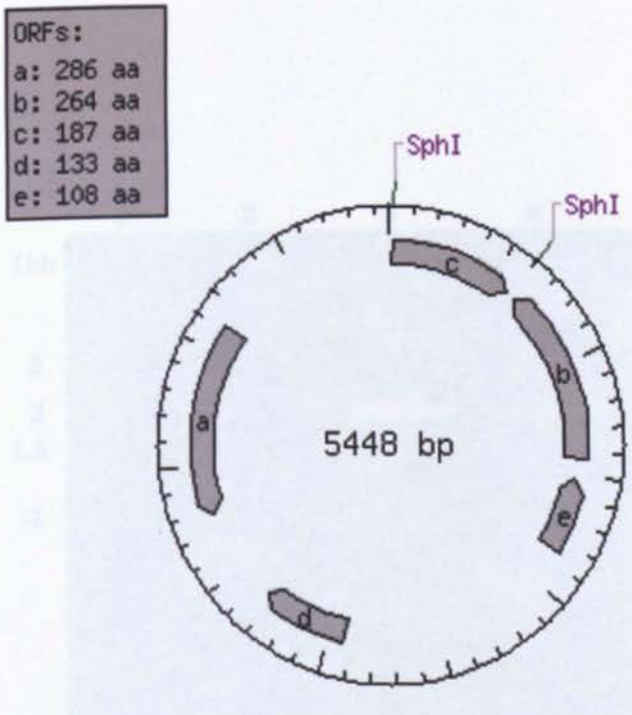
Figure 3.14. Verification of pJMK30 plasmid. A. pJMK30 restriction map with 2 cutters showing the *Bam*HI restriction sites flanking the kanamycin cassette. Image produced by NEBCutter v2. B. Gel electrophoresis analysis of pJMK30 digested with *Bam*HI. The approximate sizes of the bands were identified by comparison with the DNA ladder. Lane 1: 1 kb DNA ladder; Lane 2: pJMK30 digested with *Bam*HI.

*Pfl*MI-digested pGEM_peb3 plasmid was blunt-ended and ligated with *Bam*HI blunt ended *kan*^{r} fragment. The pGEM_peb3_*kan*^{r} was transformed into *E. coli* competent cells using the technique as described in the Materials and Methods (section 2.15). Two restriction maps of pGEM_peb3_*kan*^{r} plasmid with a different orientation of *kan*^{r} cassette were constructed using *Sph*I restriction enzyme (Figure 3.15). In the forward orientation of *kan*^{r} cassette the sizes of fragments were 2038 bp and 3421 bp (Figure 3.15A), whereas in the reverse orientation of *kan*^{r} cassette the sizes of fragments were 4869 bp and 579 bp (Figure 3.15B). Transformation of the ligation mixture into *E. coli* XL2 produced colonies on LB agar supplemented with kanamycin. Several clones were confirmed for the presence of *kan*^{r} insert by digestion with *Sph*I. Clone with pGEM_peb3_*kan*^{r} plasmid with forward orientation of *kan*^{r} was confirmed by restriction analysis (Figure 3.16).

The pGEM_peb3_*kan*^{r} plasmid with confirmed forward orientation of the *kan*^{r} was used for transformation into *C. jejuni* via electroporation as described in the Materials and Methods (section 2.12). Colonies were checked for the presence of *kan*^{r} using PCR and *peb3* primers and DNA of three mutants and the WT. The expected size of the PCR product in the mutant would be around 2.3 kb (actual size of *peb3* = 0.753 kb + 1.5 kb *kan*^{r}). The difference of 1.5 kb between *peb3* gene bands of WT and mutants indicates the presence of *kan*^{r} in mutants (Figure 3.17).



A



B

Figure 3.15. Restriction maps of pGEM_peb3_*kan*^{r} with two possible orientation of *kan*^{r} cassette. A. Forward orientation of *kan*^{r}. B. Reverse orientation of *kan*^{r}. Image produced by NEBCutter v2.

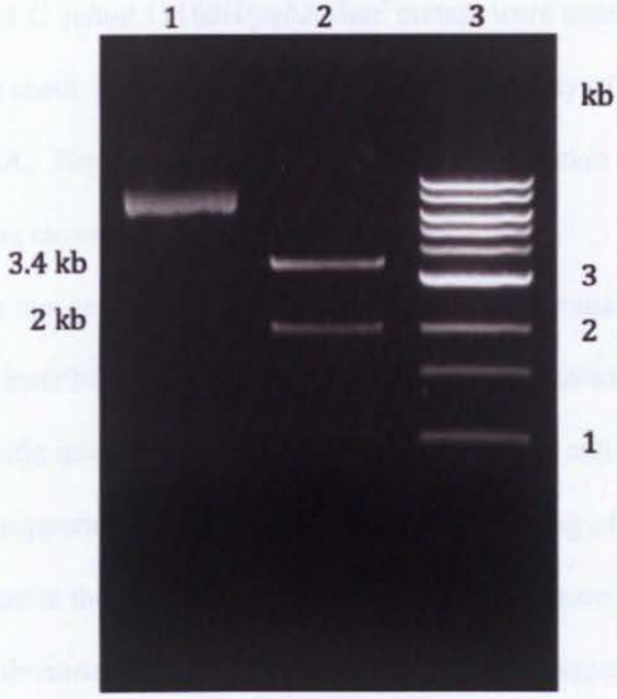


Figure 3.16. Gel electrophoresis analysis of pGEM_peb3_*kan*^{r}. pGEM_ peb3_*kan*^{r} construct digested with *Sph*I. Lane 1: uncut plasmid; Lane 2: digested plasmid; Lane 3: 1 kb DNA ladder.

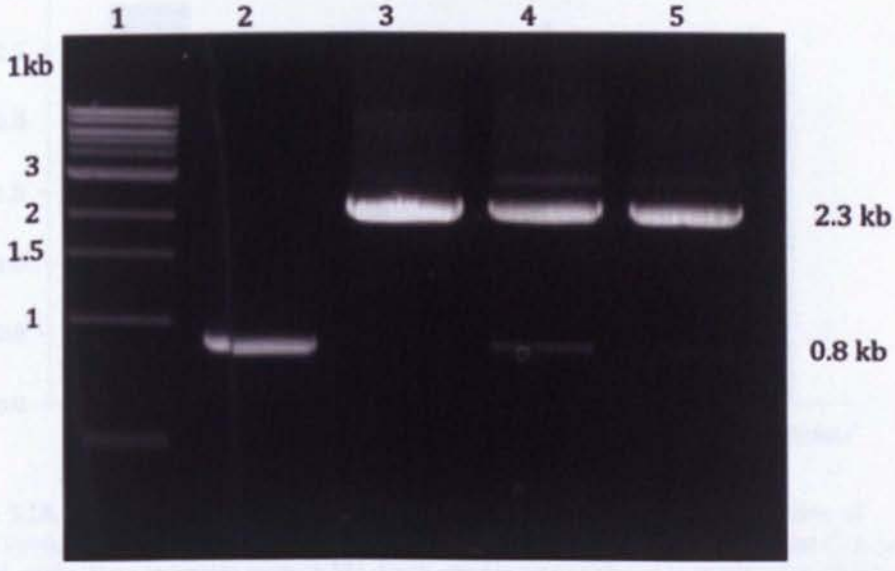


Figure 3.17. Confirmation of *peb3* mutant (11168H/*peb3::kan*^{r}). Gel electrophoresis analysis of three clonal isolates. Lane 1: 1 kb DNA ladder; Lane 2: wild type 11168H; Lanes 3–5 – three clonal isolates of 11168H/*peb3::kan*^{r}.

3.2.2 Mutation of *peb3* resulted in a reduced ability of cells to bind to SBA

Clonal isolates of *C. jejuni* 11168H/*peb3::kan*^{r} mutant were tested using the binding assay to check if mutation of *peb3* affected the ability of cells to bind to immobilised SBA. The mutation in *peb3* resulted in a reduction of binding in all three independent clonal isolates (Figure 3.18).

The results from this experiment also showed that the *peb3* mutation did not completely eliminate binding, suggesting that other glycoprotein(s) may be involved in specific interactions with this analogue of a host cell receptor. This hypothesis was supported by the reduction of residual binding of the 11168H/ *peb3::kan*^{r} mutant in the presence of soluble SBA lectin (Figure 3.19). In order to confirm the involvement of the *peb3* gene in binding, complementation of *peb3* mutation was performed next.

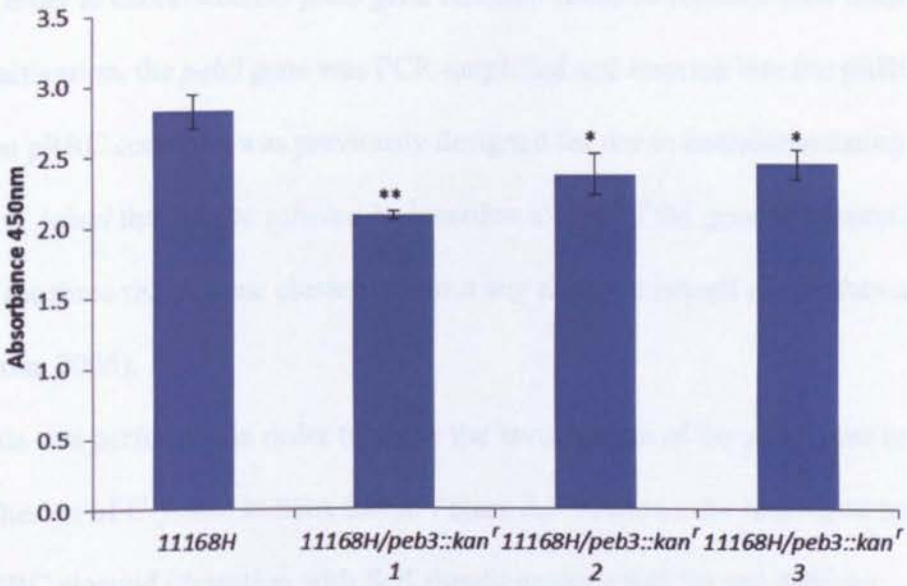


Figure 3.18. Binding of 11168H/*peb3::kan*^{r} mutants to SBA lectin. Reduced ability of 11168H/*peb3::kan*^{r} mutant to bind immobilised SBA lectin compared to the original *C. jejuni* 11168H strain. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. * $P < 0.05$ was considered statistically significant. ** $P < 0.01$. No significant difference between clone 1 and 2 in this experiment (P value 0.08). No significant difference in binding between 3 clonal isolates was found in other experiments.

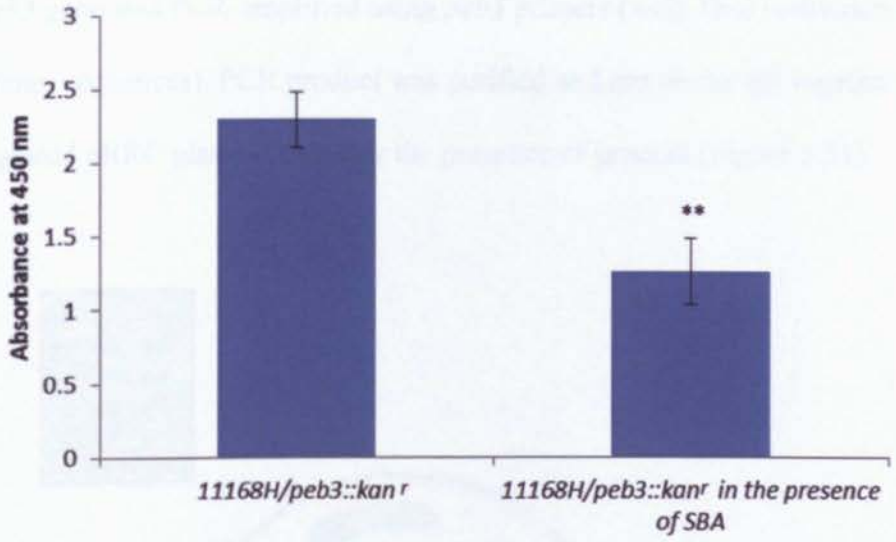


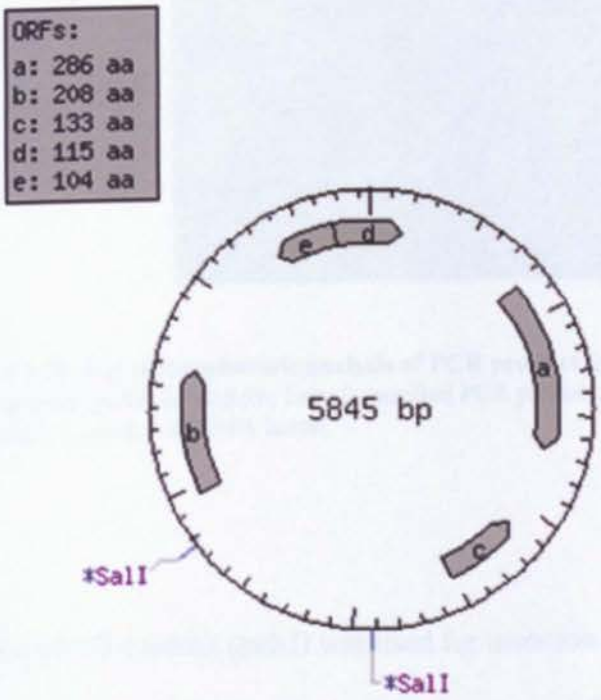
Figure 3.19. Effect of soluble SBA lectin on binding of 11168H/*peb3::kan*^{r} mutants. Comparison of binding of three clonal isolates of 11168H/*peb3::kan*^{r} mutant cells to immobilised SBA lectin in the presence and absence of soluble SBA lectin. Bars represent mean ± SD. Results are representative of 3 separate experiments that gave comparable results. ** P <0.01 was considered statistically significant.

3.2.3 Complementation of *peb3* restored binding ability of *C. jejuni*

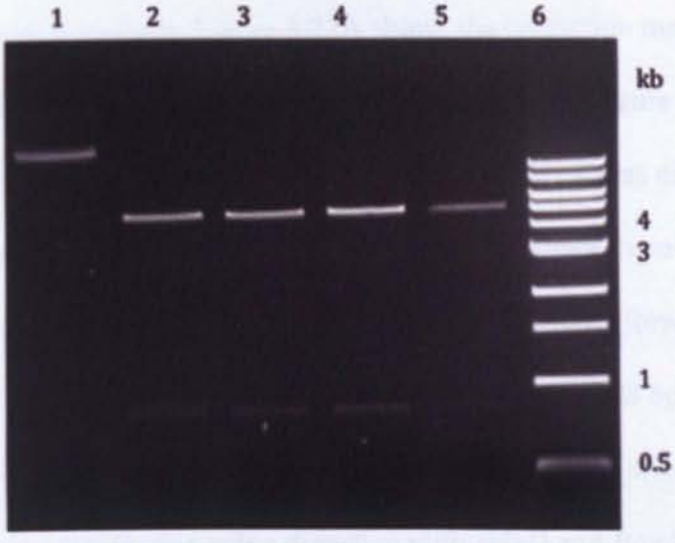
In order to check whether *peb3* gene function could be restored after insertional inactivation, the *peb3* gene was PCR-amplified and inserted into the pRRC vector. The pRRC construct was previously designed for use in complementation studies in *C. jejuni* that can be achived by insertion a copy of the gene of interest into one of the three rRNA gene clusters without any negative impact (Karlyshev and Wren, 2005).

This was performed in order to prove the involvement of the *peb3* gene in adhesion of *C. jejuni* to SBA lectin. Figure 3.20A shows the restriction map of pRRC plasmid (digestion with *Sal*I should produce 846 bp and 4999 bp fragments). Purified and digested plasmid was verified using gel electrophoresis and compared with the expected sizes of fragments (Figure 3.20B).

Peb3 gene was PCR-amplified using *peb3* primers (with *Xba*I restriction site in primer sequences). PCR product was purified and run on the gel together with digested pRRC plasmid to verify the presence of product (Figure 3.21).



A



B

Figure 3.20. Verification of pRRC plasmid. A. pRRC restriction map with 2 cutters showing the *Sal*I restriction sites. B. Gel electrophoresis analysis of pRRC digested with *Sal*I enzyme. Lane 1: uncut pRRC; Lane 2–5: different samples of pRRC digested with *Sal*I; Lane 6: 1 kb DNA ladder.

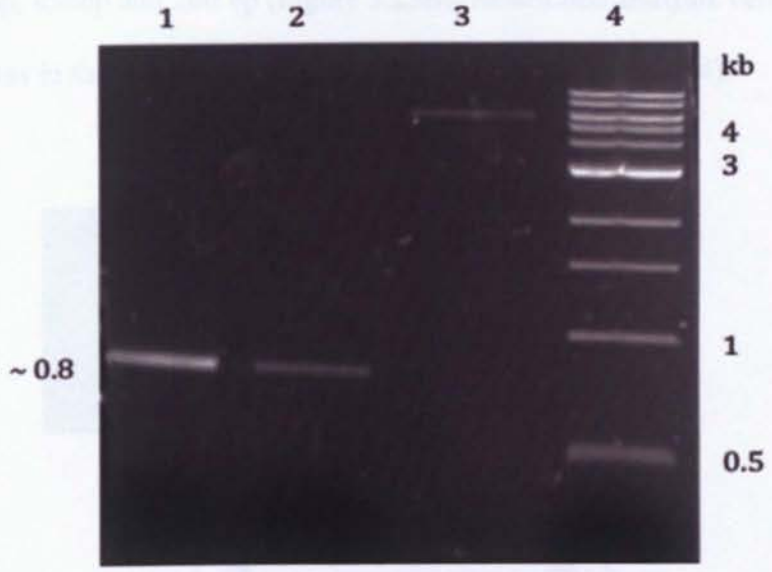


Figure 3.21. Gel electrophoresis analysis of PCR product (*peb3*) and pRRC plasmid. Lane 1: PCR product (*peb3*) of ~0.8 kb; Lane 2: purified PCR product; Lane 3: pRRC plasmid digested with *Xba*I; Lane 4: 1 kb DNA ladder.

Purified PCR product (*peb3*) was used for insertion into *Xba*I-digested pRRC vector by ligation. The product of ligation was used for transformation into *E. coli* competent cells. Recombinant *E. coli* colonies were verified for the presence of *peb3* using restriction analysis. Figure 3.22A shows the restriction map of pRRC_peb3. The pRRC_peb3 plasmid with confirmed insert (Figure 3.22B, Lane 7) was used for checking the orientation of the *peb3* gene, as it was difficult to identify the orientation using *Pflm*I and *Eco*RV. Digestion with these enzymes gave relatively similar fragments on the gel in both orientations (forward orientation 4653 bp, 1415 bp and 577 bp; reverse orientation 4908 bp, 1160 bp and 577 bp). Two restriction maps of pRRC_peb3 with different orientations of the *peb3* gene were constructed using digestion with *Pfl*MI and *Bam*HI enzymes. In forward orientation of *peb3* the sizes of fragments are 5244 bp, 855 bp and 539 bp (Figure 3.23A). In reverse orientation of the *peb3* gene the sizes of fragments

are 5503 bp, 850bp and 280 bp (Figure 3.23B). Restriction analysis verified that the gene was in the same orientation as the *cam*^{r} gene (Figure 3.24).

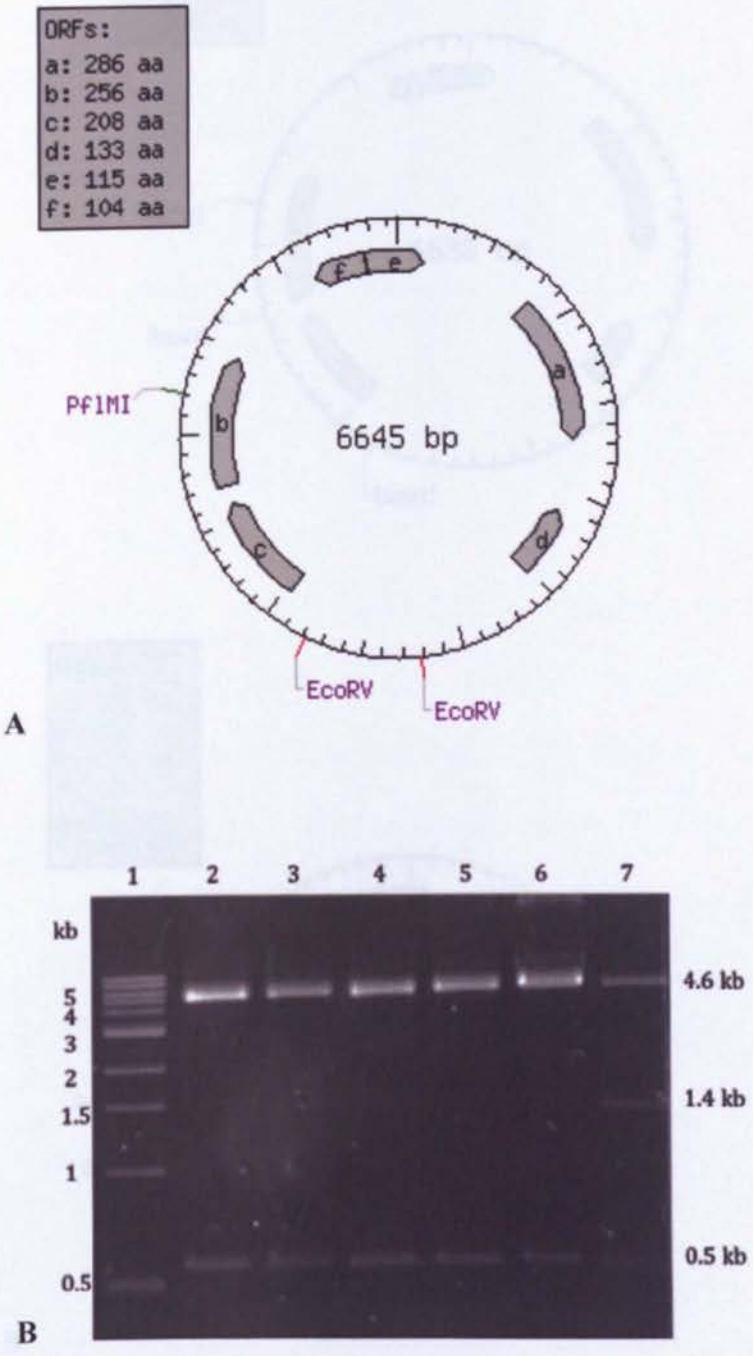
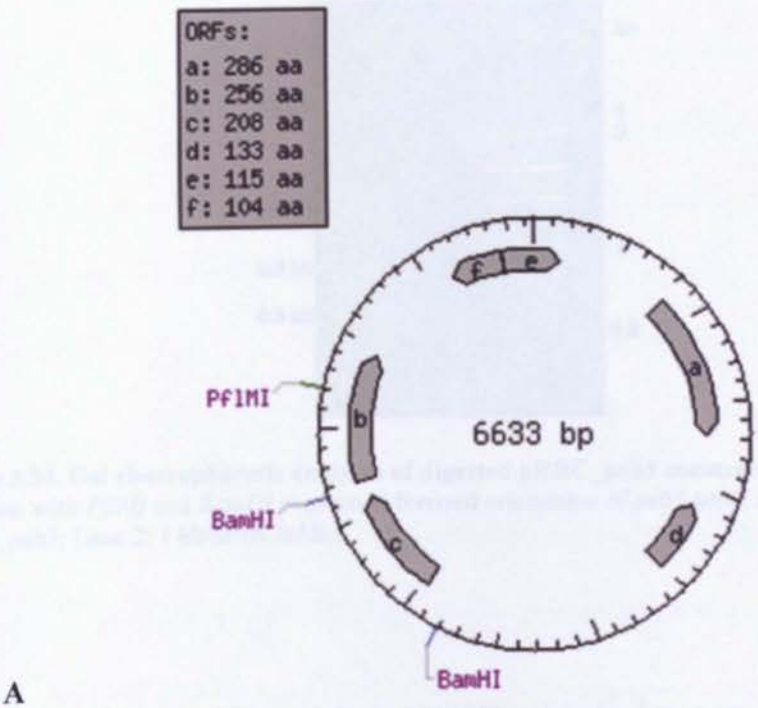
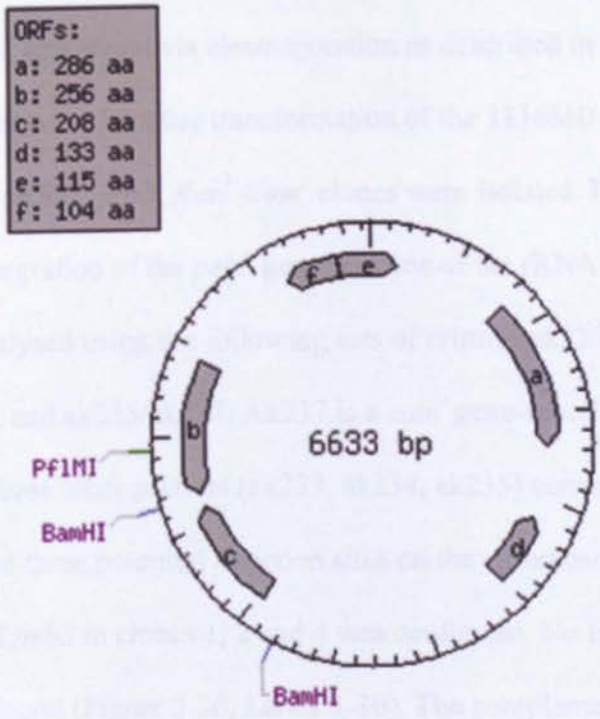


Figure 3.22. Restriction analysis of clonal isolates of pRRC_peb3 digested with *Pflm*I and *Eco*RV. A. Map in forward orientation of *peb3*. "b" fragment: the gene of interest– *peb3*. B. Restriction digestion of different clonal isolates. Expected sizes of fragments without insert: 5.2 kb and 0.5 kb; with insert: 4.6 kb, 1.4 kb and 0.5 kb. Lane 1: 1 kb DNA ladder; Lane2: empty pRRC; Lane 3–7: pRRC_peb3 plasmids purified from different clonal isolates. Plasmid in Lane 7 has an insert.



A



B

Figure 3.23. Restriction maps of pRRC_peb3 construct. Restriction enzymes used are *PflMI* and *BamHI*. A. Forward orientation of *peb3*. B. Reverse orientation of *peb3*. "b" fragment: the gene of interest– *peb3*. Image produced by NEBCutter v2.

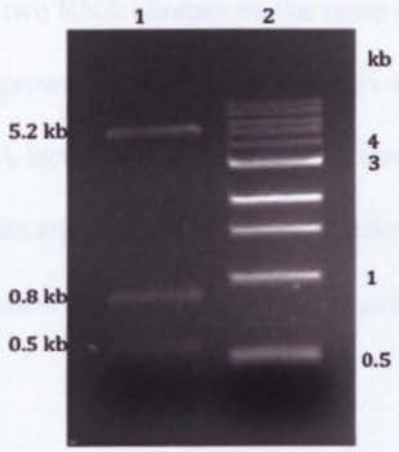


Figure 3.24. Gel electrophoresis analyses of digested pRRC_peb3 construct. Restriction digestion with *PflMI* and *BamHI* confirmed forward orientation of *peb3* gene. Lane 1: digested pRRC_peb3; Lane 2: 1 kb DNA ladder.

Competent cells were prepared from 11168H/*peb3::kan*^{r} mutant cells (from clonal isolate 1). The pRRC_peb3 plasmid was used for transformation into 11168H/*peb3::kan*^{r} strain via electroporation as described in the Materials and Methods (section 2.12). After transformation of the 11168H/ *peb3::kan*^{r} mutant with plasmid pRRC_peb3, *Kan*^{r} *Cam*^{r} clones were isolated. PCR analysis confirmed integration of the *peb3* gene into one of the rRNA gene clusters. Each clone was analysed using the following sets of primers ak233/ak237, ak234/ak237, and ak235/ak237. Ak237 is a *cam*^{r} gene-specific PCR primer, whereas the three other primers (ak233, ak234, ak235) correspond to the regions adjacent to the three potential insertion sites on the chromosome (Figure 3.25). Integration of *peb3* in clones 1, 2 and 4 was confirmed. No integration of *peb3* in clone 3 was found (Figure 3.26, Lanes 8–10). The complementation derivative was designated 11168H/*peb3::kan*^{r}/*peb3*$^+$.

As can be seen in Figure 3.26, in some cases PCR products with more than one primer pair could be detected. It has been shown previously that sometimes *cam*^{r}

gene could integrate into two RNA clusters on the same chromosome. However this has no effect on cell growth or function (Karlyshev and Wren, 2005).

The ability to bind to SBA lectin in the complementation derivative was fully restored (Figure 3.27), indicating expression of a functionally active PEB3 protein. These results confirm the involvement of the *peb3* gene in bacterial binding to SBA lectin.

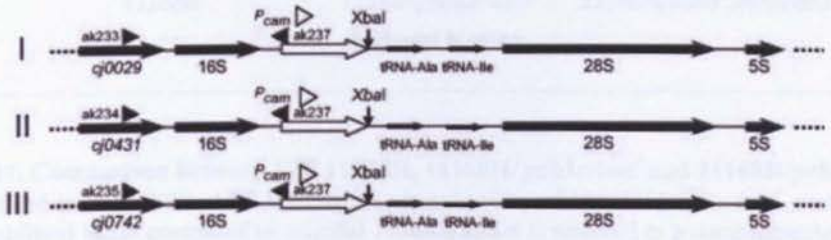


Figure 3.25. Organisation of the three rRNA gene clusters in strain NCTC 11168. Three possible products of allelic replacement resulting from recombination of pRRC with the genome (diagram taken from Karlyshev and Wren, 2005).

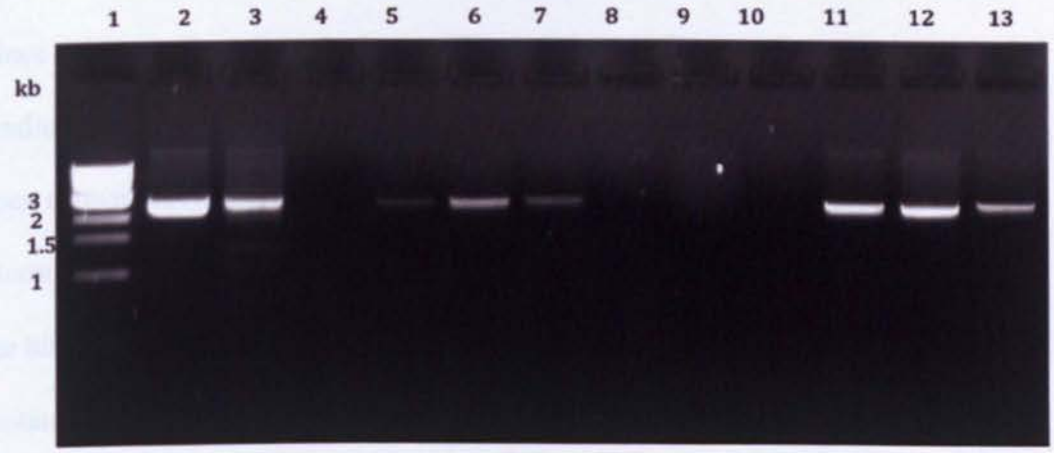


Figure 3.26. PCR analysis of four insertion derivatives (clones) resulting from transformation of strain 11168H with pRRC. Primers used were ak233/ak237, ak234/ak237, and ak235/ak237. Lane 1: 1 kb ladder; Lane 2: clone 1 (ak233/ak237); Lane 3: clone 1 (ak234/ak237); Lane 4: clone 1 (ak235/ak237); Lane 5: clone 2 (ak233/ak237); Lane 6: clone 2 (ak234/ak237); Lane 7: clone 2 (ak235/ak237); Lane 8: clone 3 (ak233/ak237); Lane 9: clone 3 (ak234/ak237); Lane 10: clone 3 (ak235/ak237); Lane 11: clone 4 (ak233/ak237); Lane 12: clone 4 (ak234/ak237); Lane 13: clone 4 (ak235/ak237).

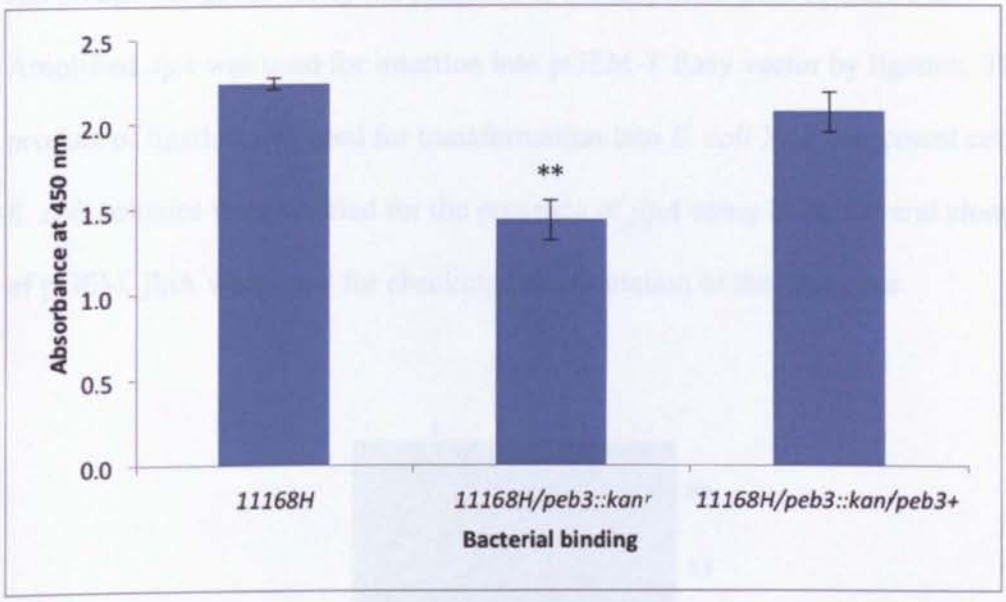


Figure 3.27. Comparison between WT 11168H, 11168H/*peb3::kan*^{r} and 11168H/*peb3:: kan*^{r} */peb3+* to bind to immobilised SBA lectin. Reduced ability of 11168H/*peb3:: kan*^{r} mutant to bind immobilised lectin compared to original 11168H strain is restored in a complemented derivative 11168H/*peb3:: kan*^{r} */peb3+*. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. ** P <0.01 was considered statistically significant.

3.3 Mutation of *jlpA* doesn't affect the ability of cells to bind SBA lectin

Since deletion of *peb3* resulted in a reduction, but not the complete elimination of binding, the involvement of other glycoprotein adhesins was suggested. It has been shown previously that putative adhesin JlpA is an N-linked glycoprotein (Scott *et al.*, 2009). Therefore it was expected that mutation of *jlpA* would reduce the binding ability of *C. jejuni*. To verify this hypothesis, we constructed a *jlpA* mutant and tested the effect of this mutation on attachment.

3.3.1 Construction and confirmation of *jlpA* mutant

PCR was used to amplify *jlpA* using primers jlpA_for and jlpA_rev and *C. jejuni* 11168H genomic DNA. After amplification, the PCR product was purified and

run on agarose gel to verify the presence of the amplified gene (Figure 3.28). Amplified *jlpA* was used for insertion into pGEM-T Easy vector by ligation. The product of ligation was used for transformation into *E. coli* XL2 competent cells. *E. coli* colonies were verified for the presence of *jlpA* using PCR. Several clones of pGEM_jlpA were used for checking the orientation of the *jlpA* gene.

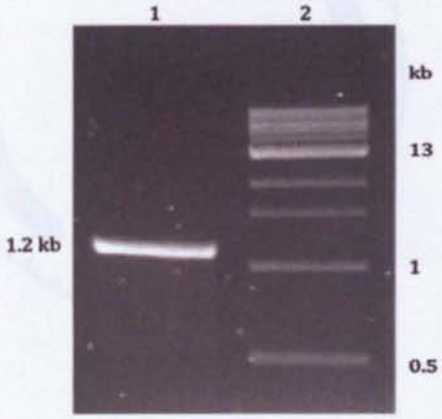
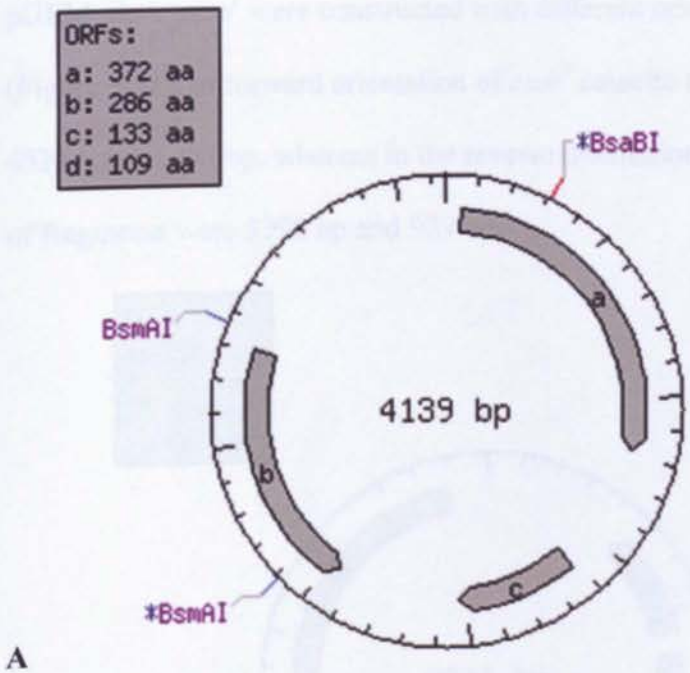
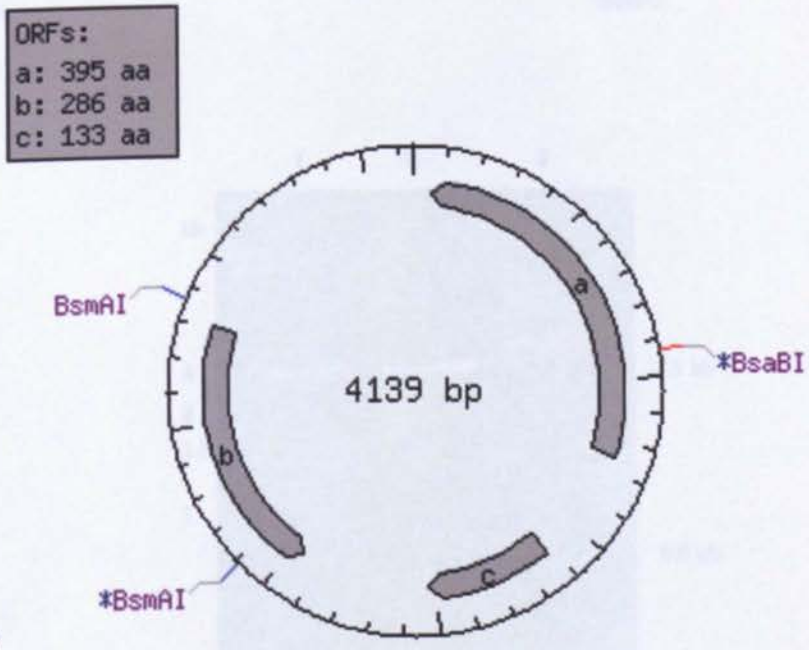


Figure 3.28. Purified PCR product (*jlpA*). Gel electrophoresis analysis of purified PCR product. Lane1: PCR product *(jlpA)* of ~1.2 kb; Lane 2: 1 kb DNA ladder.

Two restriction maps were constructed using the NEBcutter program (Figure 3.29, A and B). Enzymes used were *Bsm*AI and *Bsa*BI. In forward orientation the generated sizes of fragments were 2280 bp, 1083 bp and 776 bp, whereas in reverse orientation the sizes of generated fragments were 1700 bp, 1663 bp and 776 bp. Restriction digests were run on agarose gel to confirm the forward orientation. The clone in forward orientation was used for the insertion of the *cam*^{r} cassette. The pAV35 vector was the source of the *cam*^{r} cassette. Figure 3.30A shows the restriction map of pAV35. Expected sizes of fragments after digestion with *Bam*HI were 2961 bp and 850 bp, which were verified using gel electrophoresis (Figure 3.30B). The pGEM_jlpA plasmid was blunt-ended using *Bsa*BI and ligated with blunt-ended *cam*^{r} cassette.



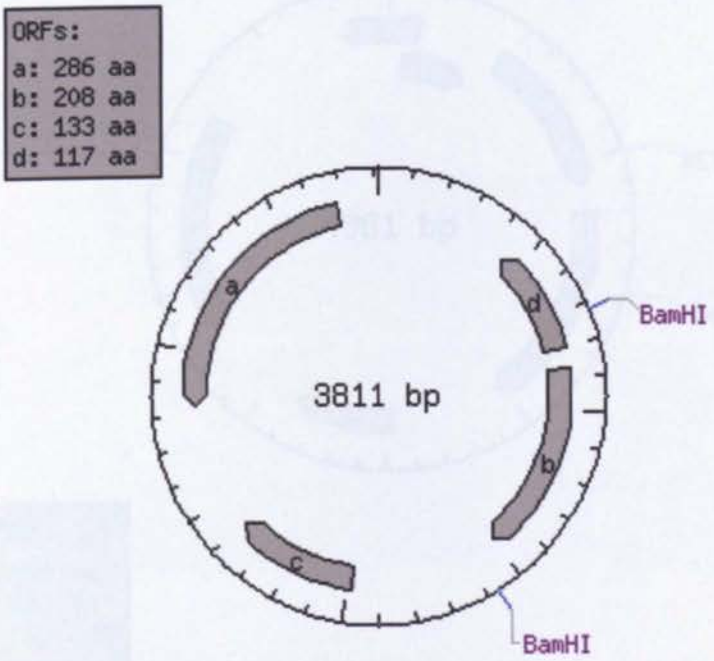
A



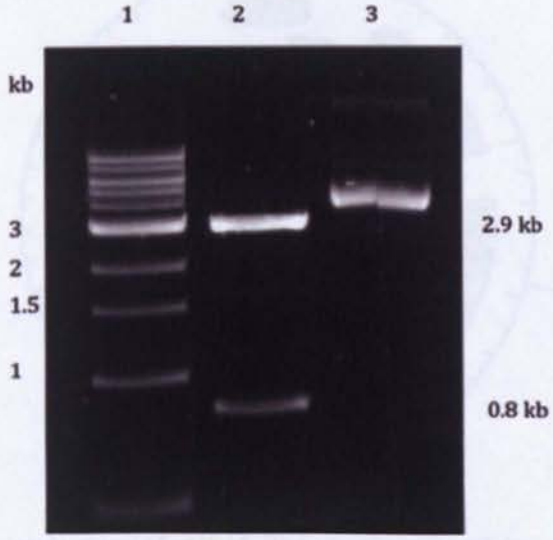
B

Figure 3.29. Restriction map of pGEM_jlpA. pGEM_jlpA digested with *Bsm*AI and *Bsa*BI in forward orientation (A) and reverse orientation (B). "a" fragment: gene of interest– *jlpA*. Image produced by NEBCutter v2.

Using *Cla*I and *Xba*I restriction enzymes, two restriction maps of pGEM_jlpA_*cam*^{r} were constructed with different orientation of *cam*^{r} cassette (Figure 3.31). In forward orientation of *cam*^{r} cassette the sizes of fragments were 4524 bp and 427 bp, whereas in the reverse orientation of *cam*^{r} cassette the sizes of fragments were 3992 bp and 989 bp.



A



B

Figure 3.30. Verification of pAV35 plasmid. A. pAV35 restriction map with 2 cutters showing the *Bam*HI restriction sites flanking the chloramphenicol cassette (fragment "b"). B. Gel electrophoresis analysis of pAV35. Lane 1: 1 kb DNA ladder; Lane 2: pAV35 digested with *Bam*HI; Lane 3: uncut pAV35

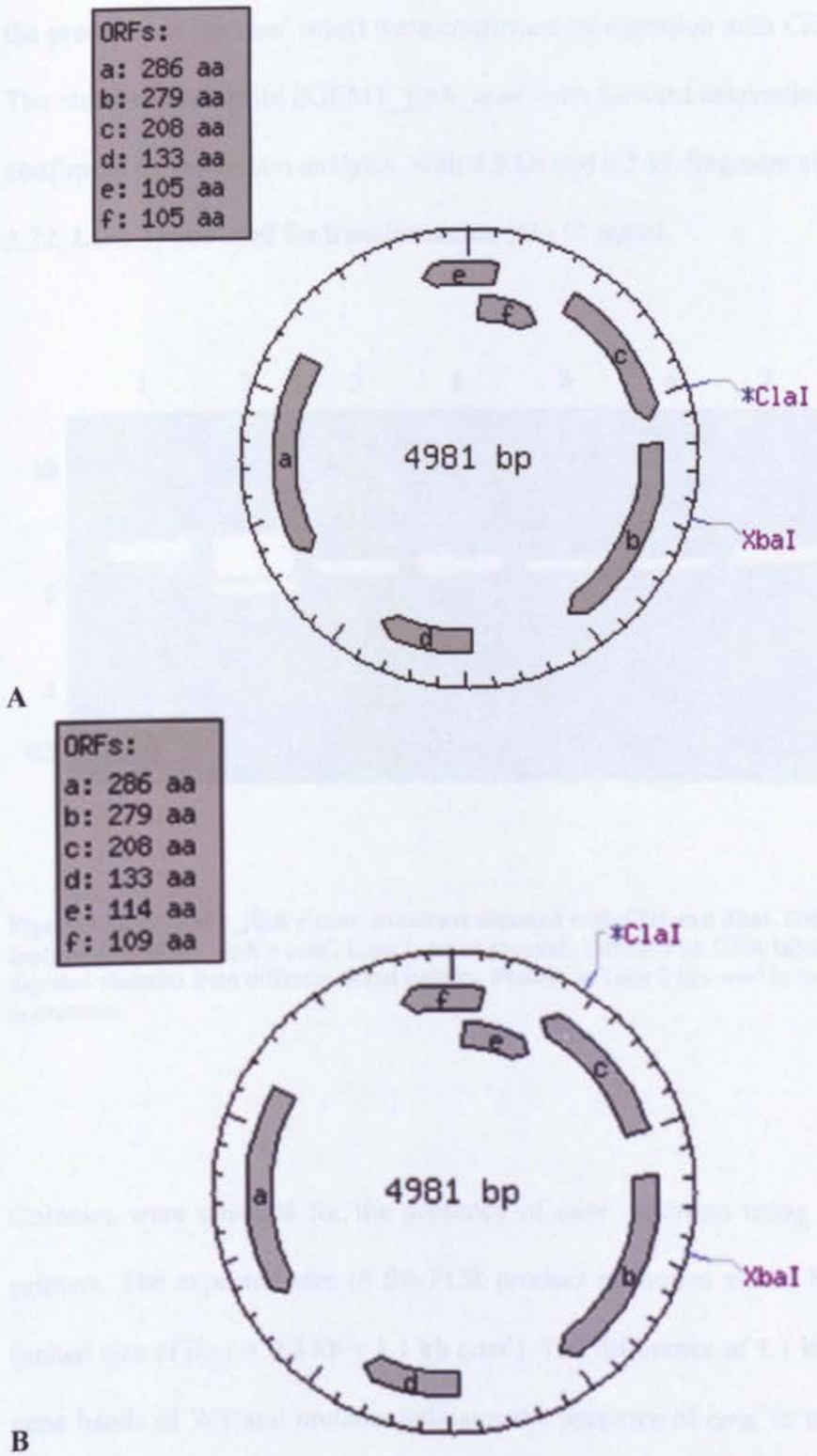


Figure 3.31. Restriction maps of pGEM_jlpA_*cam*^{r} construct digested with *Cla*I and *Xba*I. A. Forward orientation of *cam*^{r}; B. Reverse orientation of *cam*^{r}. Chloramphenicol cassette fragment "c" and *jlpA* fragment "b". Image produced by NEBCutter v2.

Transformation of ligated pGEMT_jlpA + *cam*^{r} into *E. coli* XL1 led to the growth of colonies on LB agar (supplemented with chloramphenicol). Several clones with the presence of the *cam*^{r} insert were confirmed by digestion with *Cla*I and *Xba*I. The clone with plasmid pGEMT_jlpA_*cam*^{r} with forward orientation of *cam*^{r} was confirmed by restriction analysis, with 4.5 kb and 0.5 kb fragment sizes (Figure 3.32, Lane 7) and used for transformation into *C. jejuni*.

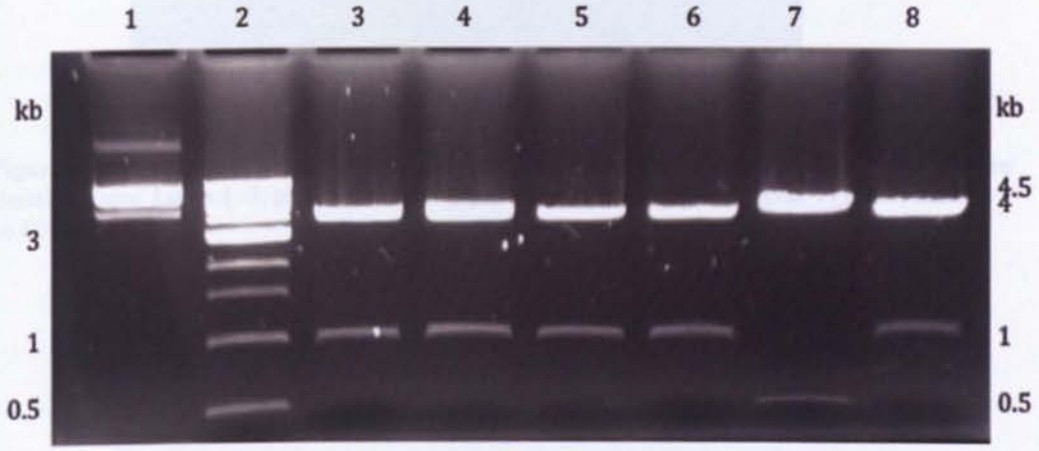


Figure 3.32. pGEMT_jlpA + *cam*^{r} construct digested with *Cla*I and *Xba*I. Gel electrophoresis analysis of pGEMT_jlpA + *cam*^{r}. Lane 1: uncut plasmid; Lane 2: 1 kb DNA ladder; Lanes 3–8: digested plasmids from different clonal isolates. Plasmid in Lane 7 has *cam*^{r} in forward orientation.

Colonies were checked for the presence of *cam*^{r} insertion using PCR and *jlpA* primers. The expected size of the PCR product in mutant would be around 2 kb (actual size of *jlpA* = 0.8 kb + 1.1 kb *cam*^{r}). The difference of 1.1 kb between *jlpA* gene bands of WT and mutants indicates the presence of *cam*^{r} in mutants (Figure 3.33).

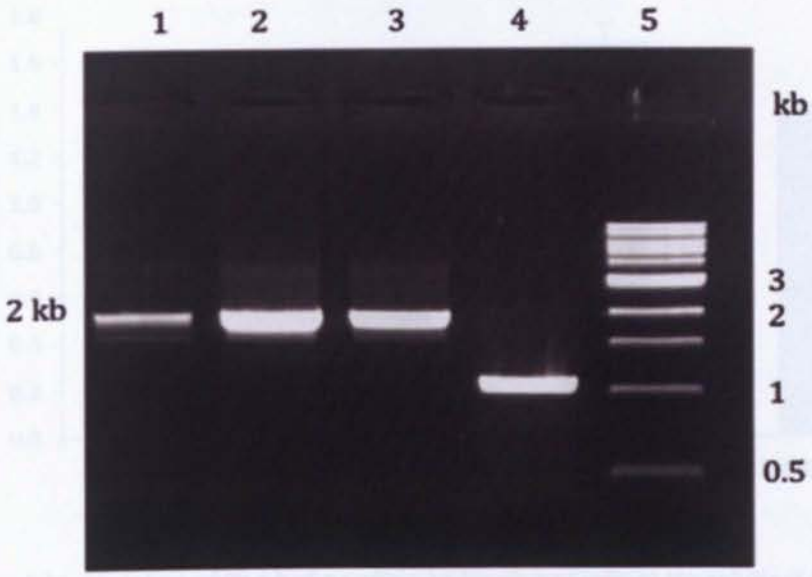


Figure 3.33. Confirmation of 11168H/*jlpA::cam*^{r} mutant. Gel electrophoresis analysis of three clonal isolates. Lanes 1–3: mutants 11168H/*jlpA::cam*^{r}; Lane 4: WT *C. jejuni* 11168H; Lane 5: 1 kb DNA ladder.

3.3.2 Effect of *jlpA* mutation on binding ability of cells to SBA lectin

Three clonal isolates of the *jlpA* mutant were checked using the binding assay (Figure 3.34). Mutation of the *jlpA* gene did not affect the ability of *C. jejuni* cells to bind to immobilised SBA lectin. Surprisingly, none of the three independent clonal isolates showed any difference when compared with the control recipient strain 11168H. This indicates that JlpA is not involved in SBA lectin binding (this is consistent with specificity of this protein for Hsp90α). Reduction of the residual binding of 11168H/*jlpA::cam*^{r} mutant in the presence of soluble SBA lectin confirmed the specificity of the mutant binding (Figure 3.35).

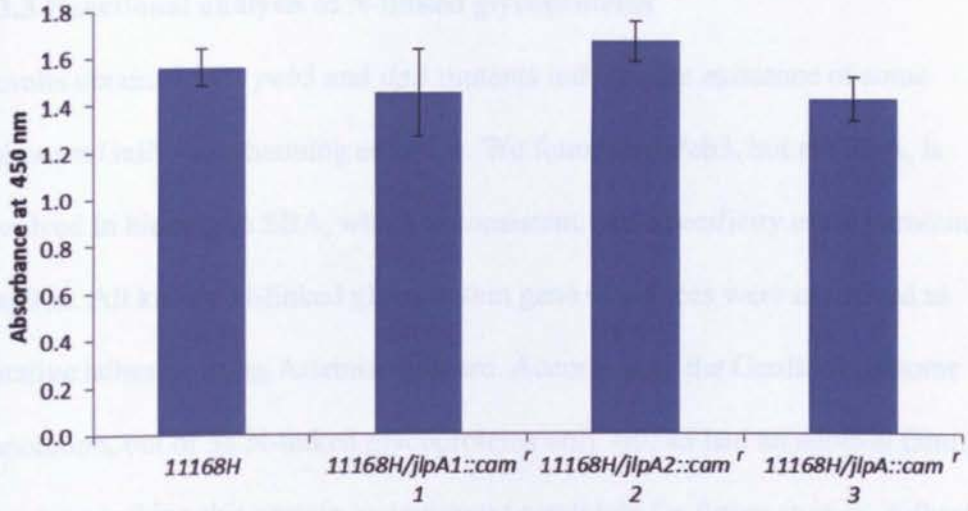


Figure 3.34. Mutation of *jlpA* had no effect on binding of cells to immobilised SBA lectin. Three clonal isolates of the jlpA mutant (11168H/*jlpA::cam*^{r})were checked for the ability to bind SBA lectin using ELISA. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results.

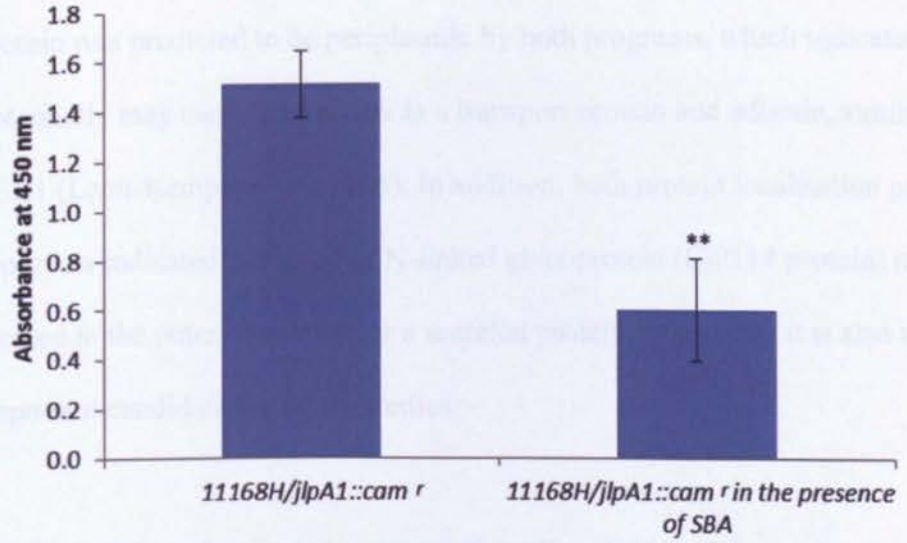


Figure 3.35. Effect of soluble SBA lectin on binding of 11168H/*jlpA::cam*^{r} mutant. Comparison of binding of three clonal isolates of 11168H/*jlpA::cam*^{r} mutant cells to immobilised SBA lectin in the presence and absence of soluble SBA lectin. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. ** P <0.01 was considered statistically significant.

3.3.3 Functional analysis of N-linked glycoproteins

Results obtained with *peb3* and *jlpA* mutants indicate the existence of some unknown GalNAc-containing adhesins. We found that Peb3, but not JlpA, is involved in binding to SBA, which is consistent with specificity of this protein for Hsp90α. All known N-linked glycoprotein gene sequences were examined as putative adhesins using Artemis software. According to the GenBank genome annotation, out of 38 N-linked glycoproteins only *cj0143* had an adhesin family signature making this protein an important candidate for future studies. Adhesion family fingerprint provides a signature for the adhesins.

In addition, two bioinformatic prediction programs were used to predict the cell localisation of known N-linked glycoproteins. PSORT and Softberry were chosen as they are based on the different prediction methods that help to give a combined result. During analysis of the sequences of known N-linked glycoproteins, Cj0143 protein was predicted to be periplasmic by both programs, which indicates that it potentially may carry dual action as a transport protein and adhesin, similar to PEB1 (Leon-Kempis *et al.*, 2006). In addition, both protein localisation prediction programs indicated that another N-linked glycoprotein (Cj0114 protein) might be located to the outer membrane or a secreted protein, suggesting it is also an important candidate for future studies.

3.4 Production of capsule has a negative effect on binding

The role of the capsule in bacterial attachment depends on the nature of a bacterial pathogen, and on the structural features of the capsules and adhesins. In order to investigate the role of *Campylobacter* capsule on the interaction with analogues of host cell receptors, an acapsulated (*kpsM*) mutant was tested using the *in vitro* ELISA-like binding assay.

3.4.1 Confirmation of the *kpsM* mutant

A previously constructed non-polar *kpsM* mutant of *C. jejuni* was used in this study (Karlyshev *et al.*, 2000). The mutant was constructed by inserting a *kan*^{r} gene containing an internal promoter, but lacking a transcriptional terminator. The presence of the *kan*^{r} cassette in the gene was verified using PCR with primers that flank the insertion site (ak55-f and ak59-r). Two DNA templates were used, one being *C. jejuni* 11168H genomic DNA (control) and another DNA from 11168H/*kpsM::kan*^{r} mutant. The PCR products were run on a gel to verify the presence of the amplified genes (Figure 3.36). The size of *kan*^{r} cassette is 1.5 kb, the size of *kpsM* gene is 0.7 kb. In total the size of PCR product using 11168H/*kpsM::kan*^{r} mutant DNA should be around 2.2 kb, which is what we found in the mutant.

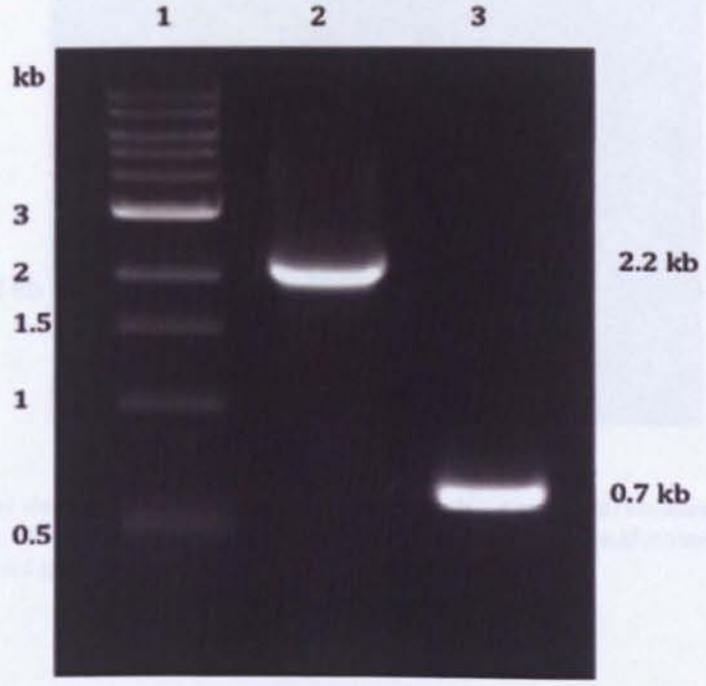


Figure 3.36. Confirmation of 11168H/*kpsM:: kan*^{r} mutant using PCR. PCR product was analysed by gel electrophoresis. Lane 1: 1 kb DNA ladder; Lane 2: 11168H/*kpsM:: kan*^{r} mutant, Lane 3: 11168H.

In order to get more *kpsM* clonal isolates, chromosomal DNA of 11168H/*kpsM::kan*^{r} was used for transformation into *C. jejuni* 11168H. Transformation involved plating onto plates with kanamicyn selecting only those transformants that acquired a mutated copy of the target gene and any potential 'spontaneous' mutations in *kpsM* would still retain the gene inactive (due to insertional gene inactivation).

After transformation, clonal isolates were checked for the presence of *kan*^{r} cassette using PCR (Figure 3.37). Confirmed clonal isolates were later used in a binding assay. In order to exclude the chance of spontaneous mutations occurring in the mutant, three independent clonal isolates were analysed.

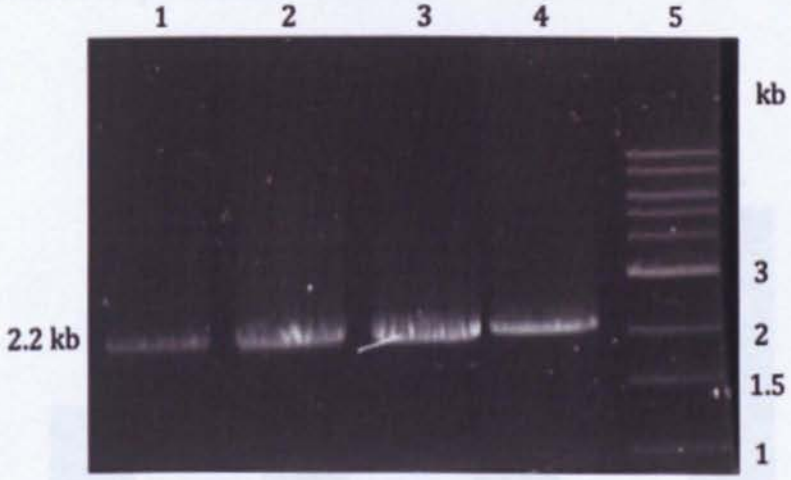


Figure 3.37. Gel electrophoresis analysis of 11168H/*kpsM:: kan*^{r} clonal isolates. PCR was used for confirmation of insertion of *kan*^{r} cassette in 3 clonal isolates of the *kpsM* mutant. Lane 1: control (confirmed *kpsM* mutant); Lanes 2– 4: three clonal isolates.

3.4.2 Mutation of *kpsM* resulted in an increased ability of cells to bind to SBA

Clonal isolates of 11168H/*kpsM::kan*^{r} were used in the binding assay together with the recipient strain 11168H in order to evaluate whether there was any

difference in binding between WT and mutants. The results shown in Figure 3.38 demonstrate a significantly higher affinity of binding of the non-capsular mutant of strain 11168H. This result, confirmed by the analysis of three independent clonal isolates of this mutant, suggests an interference effect of the *C. jejuni* capsule on bacterial interaction with host cell receptors. The fact that the phenotypic change was confirmed in all three of them suggests that the change observed is attributed to that particular mutation. Furthermore, confirmation of the phenotypic change in several mutants is a common practice used in the investigation of other bacteria, including *Campylobacter*. This is often considered to be an alternative to complementation, which is notoriously difficult in *Campylobacter* due to the lack of universal shuttle vectors. Results of binding assay provide strong and convincing evidence that the observed changes are indeed attributed to *kpsM* mutation.

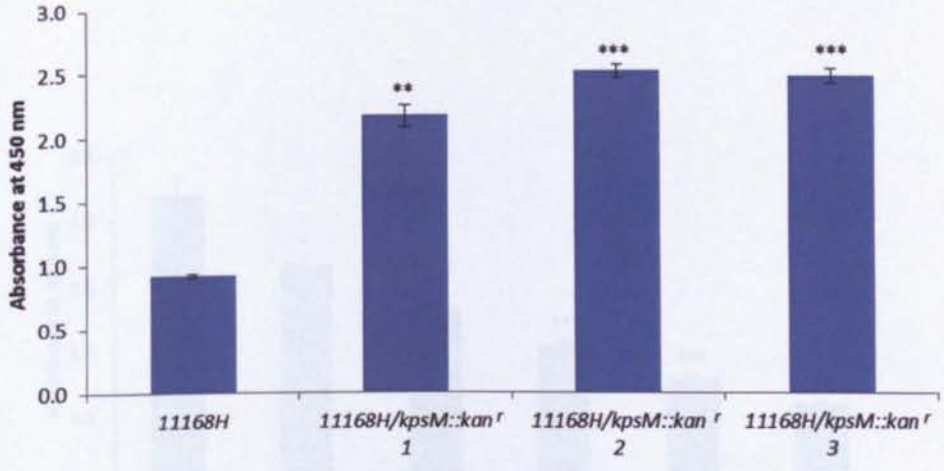


Figure 3.38. A negative effect of capsule on bacterial binding. WT *C. jejuni* 11168H and 11168H/*kpsM: kan*^{r} strains were analysed for interaction with immobilised SBA using ELISA. Bars represent binding of cells to immobilised SBA lectin (mean ± SD). Results are representative of 3 separate experiments that gave comparable results. ** $P < 0.01$ was considered statistically significant. *** $P < 0.001$. In this experiment *kpsM* mutants were compared to the WT, which was used as control. The difference in absorbance values of WT compared to previous results could be attributed to different batches of reagents and it is not critical for interpretation of the current set of experiments, where exactly the same batches of reagents were used and the results were reproducible. Confirmation of the phenotypic change in three clonal isolates makes complementation experiments unnecessary.

One clonal isolate was also used for inhibition studies using soluble SBA lectin and GalNAc. Inhibition of binding was detected from 0.01 μM SBA lectin, the lowest concentration used. Absorbance of the control was 2.202 +/- 0.10, whereas cells incubated in the presence of SBA lectin at 0.01 μM concentration produced an absorbance of 1.647 +/- 0.06 (Figure 3.39). Soluble GalNAc also showed an inhibitory effect, with concentrations of 20 μM and above yielding an absorbance of 2.054 +/- 0.11. The absorbance of the control (*C. jejuni* 11168H/*kpsM::kan*^{r}) sample was 2.398 +/- 0.11 (Figure 3.40).

Further confirmation of specific binding was obtained by treatment of bacterial cells with an exoglycosidase. *C. jejuni* glycoproteins bind to SBA lectin due to GalNAc residues in the glycan component. Whole bacterial cells (*C. jejuni* 11168H/*kpsM::kan*^{r}) were treated with *N*-acetylgalactosaminidase to remove terminal GalNAc. This resulted in a remarkable reduction in the ability of bacterial cells to attach (Figure 3.41).

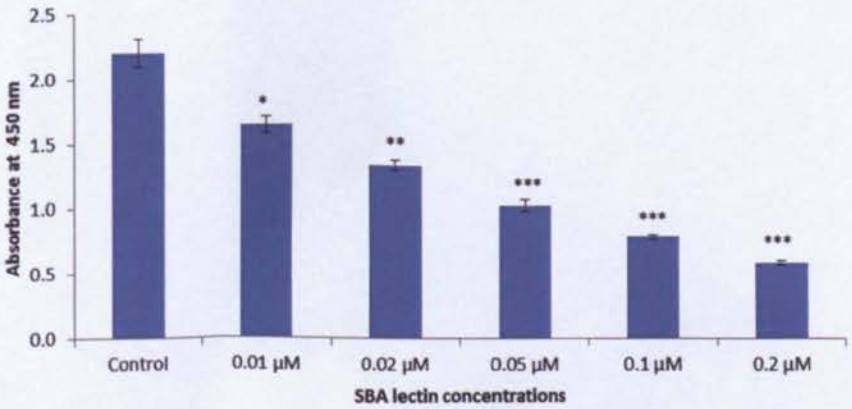


Figure 3.39. Effect of different concentrations of soluble SBA lectin on binding of *C. jejuni* 11168H/*kpsM::kan*^{r}. Cells were incubated with different concentrations of soluble SBA lectin. No SBA lectin was added to the control sample. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. * $P < 0.05$ was considered statistically significant. ** $P < 0.01$. *** $P < 0.001$.

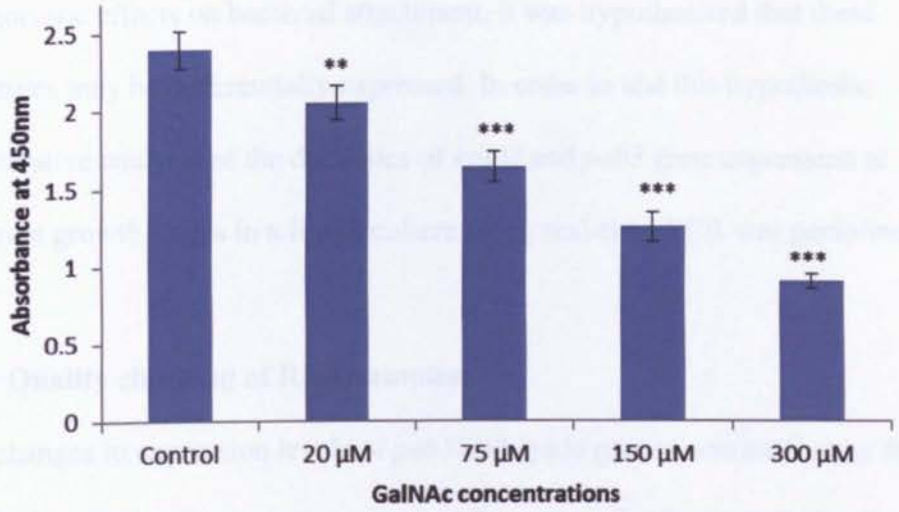


Figure 3.40. Effect of different concentrations of soluble GalNAc on binding of *C. jejuni* 11168H/*kpsM::kan*^{r}. Cells were incubated with different concentrations of soluble GalNAc. No GalNAc was added to the control sample. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. ** P <0.01 was considered statistically significant. *** P <0.001.

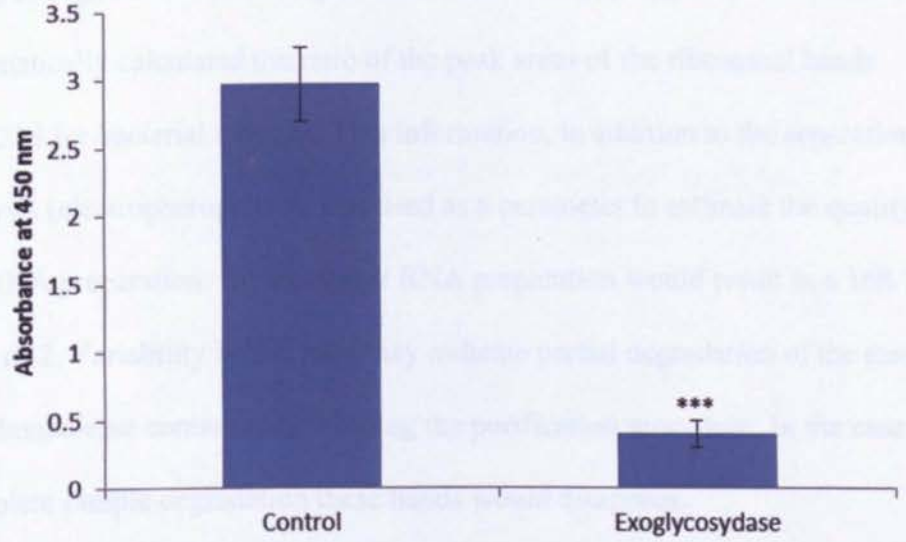


Figure 3.41. Reduction in binding of 11168H/*kpsM:: kan*^{r} mutant upon treatment of bacteria with GalNAc-specific exoglycosidase. Reduction of binding upon treatment of bacteria with GalNAc-specific exoglycosydase. Bacterial cells were incubated with exoglycosidase for 1h under microaerophilic conditions at 37°C. Bars represent mean ± SD. Each sample was analysed in technical triplicates. Results are representative of 3 separate experiments that gave comparable results. *** P <0.001 was considered statistically significant.

3.5 *Peb3* and capsule-related genes are differentially expressed

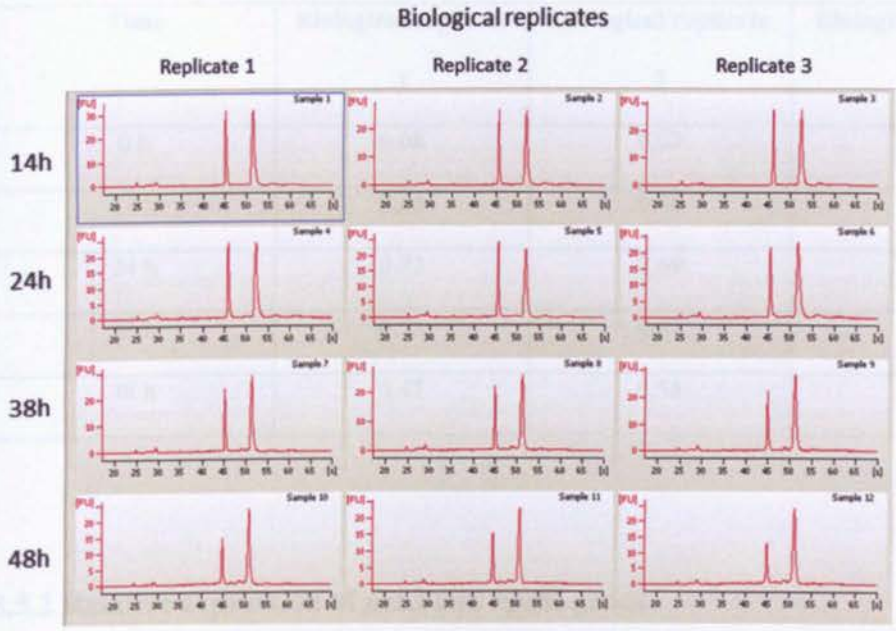
Since the results of these experiments show that capsule and PEB3 adhesin have antagonistic effects on bacterial attachment, it was hypothesised that these structures may be differentially expressed. In order to test this hypothesis, comparative analysis of the dynamics of *kpsM* and *peb3* gene expression at different growth stages in a liquid culture using real-time PCR was performed.

3.5.1 Quality checking of RNA samples

The changes in expression levels of *peb3* and *kpsM* genes were studied by RT-qPCR. In order to assess gene expression changes, cell cultures were incubated for 48 h. Samples were taken at 14 h, 24 h, 38 h and 48 h. Optical density was measured at each time point to assess the rate of cell growth (Table 3.1). RNA from each sample was purified as described in Materials and Methods (section 2.18.2). In order to assess the integrity/purity of RNA, samples were analysed using an Agilent 2100 bioanalyser and RNA 6000 LabChip kit. The software automatically calculated the ratio of the peak areas of the ribosomal bands 16S/23S for bacterial samples. This information, in addition to the separation analysis (electropherograms), was used as a parameter to estimate the quality of the RNA preparation. An ideal total RNA preparation would result in a 16S/23S ratio of 2. Variability in this ratio may indicate partial degradation of the sample by ribonuclease contamination during the purification procedure. In the case of complete sample degradation these bands would disappear.

The twelve samples (three biological replicates at each time point) were sequentially separated on the chip through a single separation channel and the resulting electropherograms are shown alongside the gel-like image (Figure 3.42).

A



B

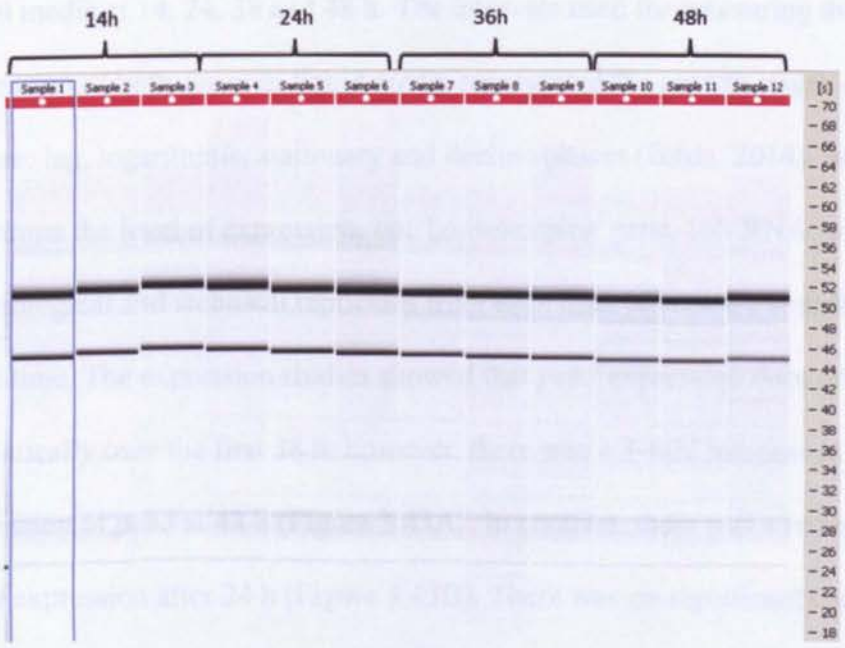


Figure 3.42. The integrity/purity of RNA samples analysed using the Agilent 2100 bioanalyser. Data displayed as electropherograms: (A)samples 1–12) as well as a gel-like image (B). The presence of distinct 16S and 23S peaks (A) and bands (B) can be observed in all samples. Total RNA samples were purified from 3 biological replicates at 14 h, 24 h, 38 h and 48 h.

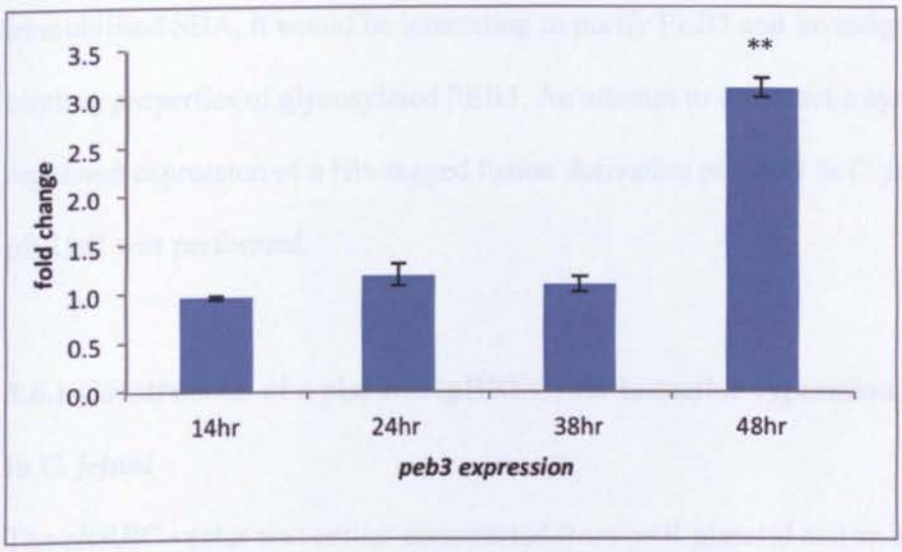
Table 3.1. *C. jejuni* optical density at 600 nm (OD_{600}) measured over 48 h. Cells were growing in BHI supplemented with Skirrow supplement (n=3). These data are representative of 3 separate experiments.

Time	Biological replicate 1	Biological replicate 2	Biological replicate 3
0 h	0.08	0.07	0.07
14 h	0.45	0.46	0.44
24 h	0.72	0.69	0.71
38 h	0.75	0.81	0.77
48 h	0.47	0.56	0.59

3.5.2 Relative expression of *peb3* and *kpsM* genes

The expression of *peb3* and *kpsM* of *C. jejuni* 11168H was measured (using qPCR) from samples cultured under microaerophilic growth conditions at 37°C in liquid media at 14, 24, 38 and 48 h. The intervals used for measuring the expression of both genes in liquid media represented the growth stages of the culture: lag, logarithmic, stationary and decline phases (Ikeda, 2014). In order to determine the level of expression, one housekeeping gene, 16S RNA, was used. All biological and technical replicates from each time point were analysed at the same time. The expression studies showed that *peb3* expression does not change dramatically over the first 38 h; however, there was a 3-fold increase in expression of *peb3* at 48 h (Figure 3.43A). In contrast, there was a reduction in *kpsM* expression after 24 h (Figure 3.43B). There was no significant change in *kpsM* gene expression after 24 h. It is suggested that the relative change in the expression of *peb3* and *kpsM* over the period of 48 h showed that *kpsM* gene expression decreased over time compared to expression of *peb3*.

A



B

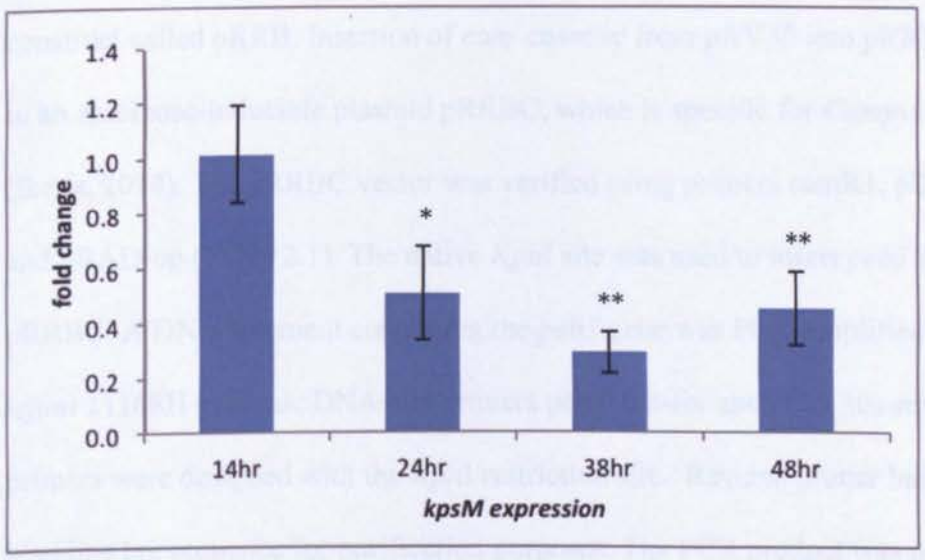


Figure 3.43. Increase of *C. jejuni peb3* gene expression (A) and decrease of *kpsM* gene expression (B) over time in a liquid culture. Gene expression levels relative to 16S rRNA were determined as described in Materials and Methods (section 2.18.5). Bars represent mean ± SD. Results are representative of three individual experiments. * $P < 0.05$. ** $P < 0.01$.

3.6 Expression and purification of His-tagged PEB3 from *E. coli* and *C. jejuni*

Since mutation in *peb3* reduces the binding ability of bacterial cells to immobilised SBA, it would be interesting to purify PEB3 and investigate the binding properties of glycosylated PEB3. An attempt to construct a system for regulated expression of a His-tagged fusion derivative of PEB3 in *C. jejuni* using pRRBC was performed.

3.6.1 Construction of a plasmid (pRRBC) for inducible expression of PEB3 in *C. jejuni*

The pRRBC vector was earlier constructed from pRR plasmid and an arabinose-induced promoter derived from pBAD33 plasmid (Ikeda, 2014). pRR plasmid was constructed via insertion of fragment of rRNA gene clusters into pGEM-T Easy vector (Karlyshev and Wren, 2005). *Xba*I-digested pRR and pBAD (isolated from pBAB33 by digestion with *Cla*I and *Sal*I) were ligated to form an intermediate construct called pRRB. Insertion of *cam*^{r} cassette from pAV35 into pRRB resulted in an arabinose-inducible plasmid pRRBC, which is specific for *Campylobacter* (Ikeda, 2014). The pRRBC vector was verified using primers camR1, pBAD-for and pBAD-up (Table 2.1). The native *Kpn*I site was used to insert *peb3* into pRRBC. A DNA fragment containing the *peb3* gene was PCR-amplified using *C. jejuni* 11168H genomic DNA and primers peb3_his-for and peb3_his-rev. *Peb3* primers were designed with the *Kpn*I restriction site. Reverse primer had a hexa-histidine tag sequence for purification purposes. The PCR product was purified and analysed by gel electrophoresis (Figure 3.44). Amplified DNA fragment was cloned into pRRBC. In order to assess the presence of insert in pRRBC-peb3 plasmid, restriction digestion was performed using *Kpn*I (to demonstrate

similarity of the insert size to that of the PCR product) (Figure 3.45, Lanes 2, 6 and 10).

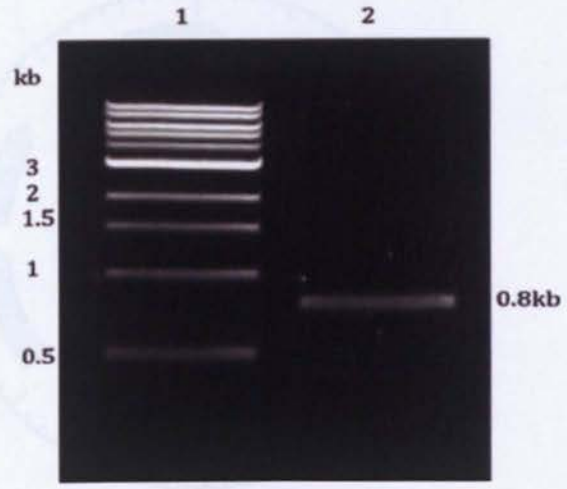
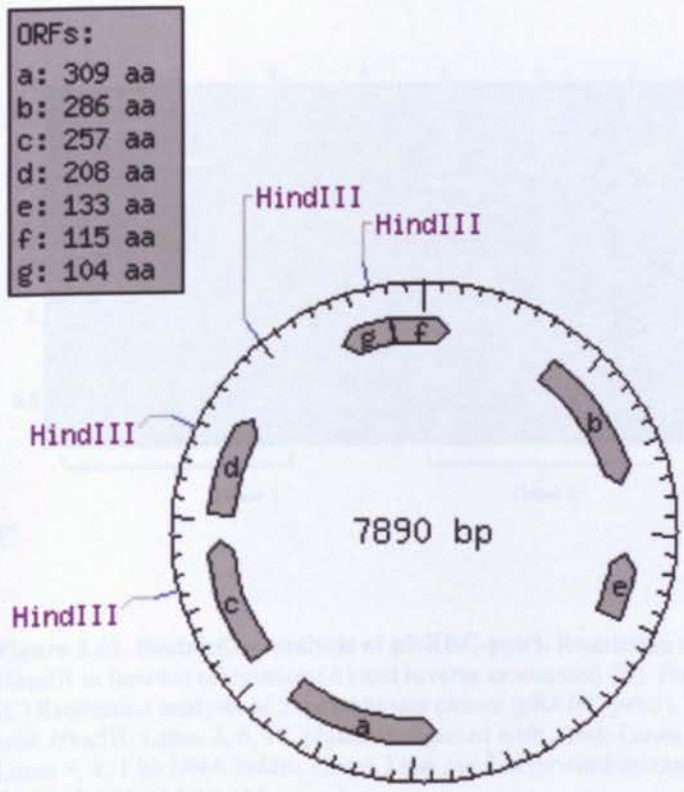
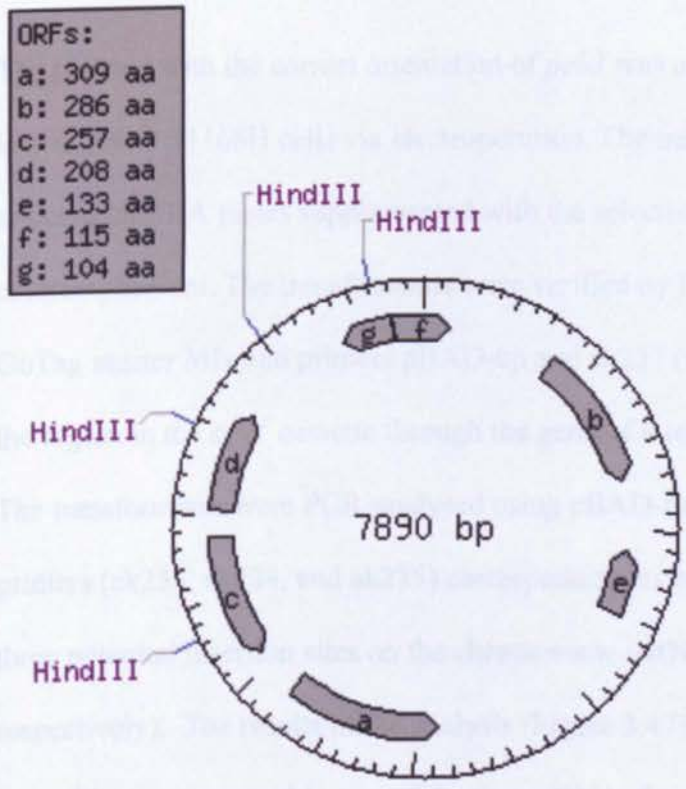


Figure 3.44. Gel electrophoresis of purified PCR product (*peb3*). Lane 1: 1 kb DNA ladder; Lane 2: amplified PCR product of expected size ~0.8 kb.

The orientation of pRRBC-peb3 was determined by restriction digestion using *Hind*III. Two restriction maps were constructed using the NEBcutter program (Figure 3.45A and B). Digestion with *Hind*III produced 4 fragments, two of which were the same size. Thus only three fragments could be observed on the gel. In forward orientation of *peb3* the generated sizes of fragments were 5890 bp, 822 bp, 550 bp and 609 bp, whereas in reverse orientation the sizes of generated fragments were 5597 bp, 1118 bp, 550 bp and 609 bp. The forward and correct orientation was detected in clone 3 (Figure 3.45C).


ORFs:
a: 309 aa
b: 286 aa
c: 257 aa
d: 208 aa
e: 133 aa
f: 115 aa
g: 104 aa
HindIII
HindIII
HindIII
HindIII
7890 bp
a
b
c
d
e
f
g

A


ORFs:
a: 309 aa
b: 286 aa
c: 257 aa
d: 208 aa
e: 133 aa
f: 115 aa
g: 104 aa
HindIII
HindIII
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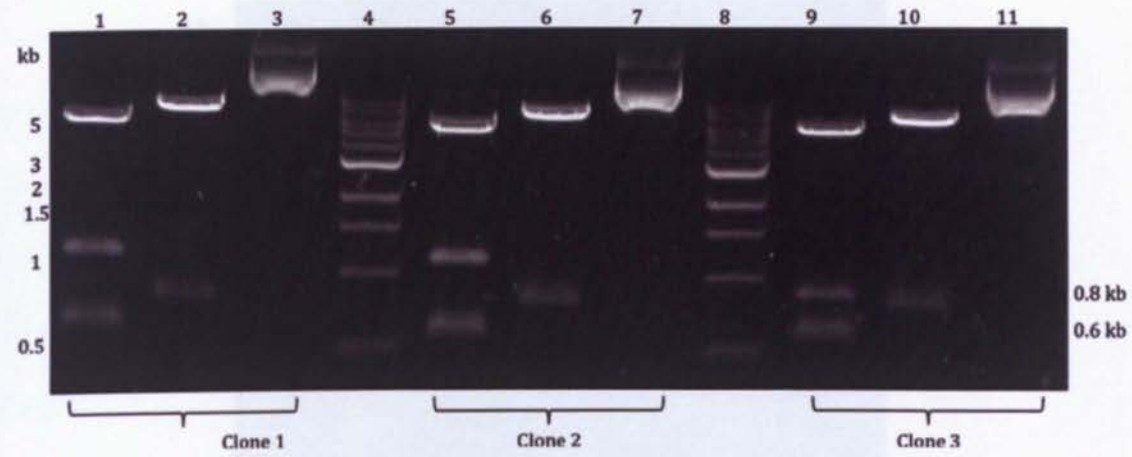
c
d
e
f
g

B



C

Figure 3.45. Restriction analysis of pRRBC-peb3. Restriction maps of pRRBC digested with *Hind*III in forward orientation (A) and reverse orientation (B). Fragment "c" is *peb3* gene. (C) Restriction analysis of 3 recombinant clones (pRRBC-peb3). Lanes 1, 5, 9: plasmids digested with *Hind*III; Lanes 2, 6, 10: plasmids digested with *Kpn*I; Lanes 3, 7, 11: undigested plasmids; Lanes 4, 8: 1 kb DNA ladder. Clone 3 has *peb3* in forward orientation (the sizes of fragments are 5.8 kb, 0.8 kb and 0.6 kb).

The plasmid with the correct orientation of *peb3* was used for the transformation into *C. jejuni* 11168H cells via electroporation. The transformants were then selected on CBA plates supplemented with the selective antibiotic, chloramphenicol. The transformants were verified by PCR (Figure 3.46) using GoTag master Mix and primers pBAD-up and ak237 (for cam^r), which amplify the region in the cam^r cassette through the gene of interest to the pBAD region. The transformants were PCR-analysed using pBAD-for primer and three other primers (ak233, ak234, and ak235) corresponding to the regions adjacent to the three potential insertion sites on the chromosome (rRNA clusters I, II and III respectively). The results of the analysis (Figure 3.47) demonstrated that recombination occurred in one of the three rRNA clusters (rRNA cluster I) in two clones.

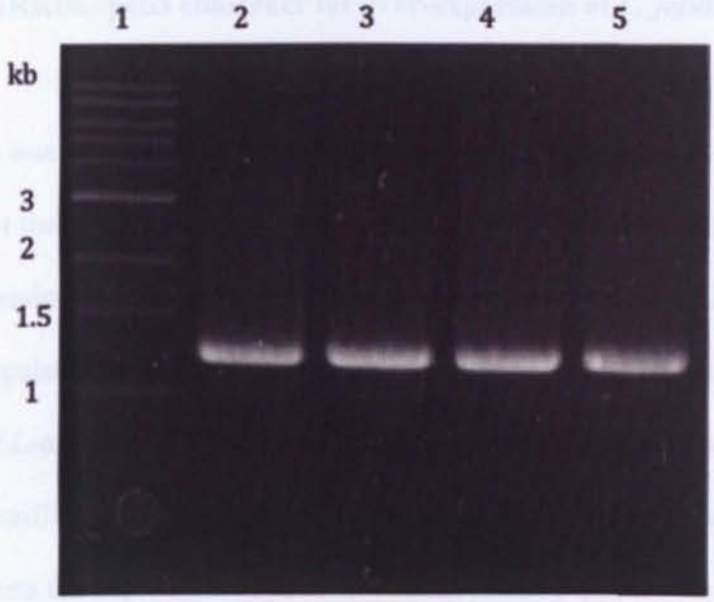


Figure 3.46. PCR analysis of 4 clones from transformation of 11168H with pRRBC-peb3. Verification of allelic replacement of pRRBC-peb3 using pBAD-up and ak237. The presence of the system creates an amplicon length of 1.3 kb. Lane 1: 1 kb DNA ladder; Lanes 2–5: 11168H/pRRBC-peb3 clones.

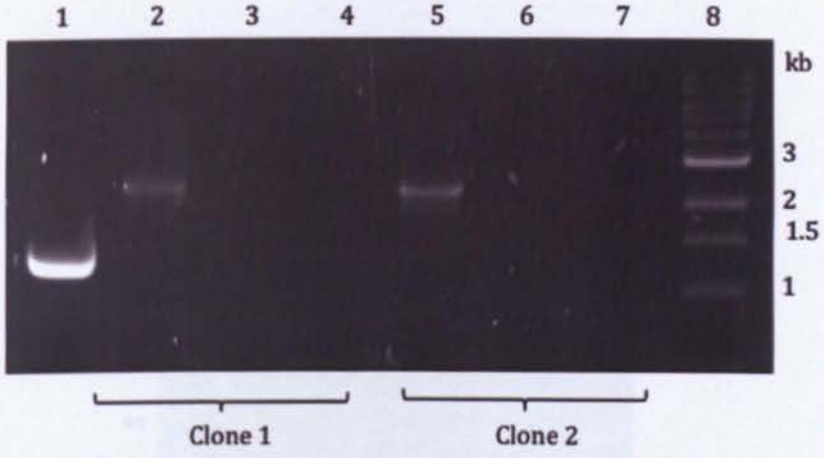


Figure 3.47. PCR analysis of two derivatives (clone 1 and 2) to assess the site of insertion in *C. jejuni* 11168H. Verification of allelic replacement using pBAD-for and ak233/ak234/ak235. The presence of pRRBC-peb3 was shown using primers ak233 and pBAD-for (cluster I) The 2.4 kb amplicon confirms the presence of the system. There were no products from using primers ak234/pBAD-for and ak235/pBAD-for. Lane 1: control (pBAD-up and ak237); Lanes 2 and 5: pRRBC-peb3 plasmid with ak233 and pBAD-for; Lanes 3 and 6: pRRBC-peb3 plasmid with ak234 and pBAD-for; Lanes 4 and 7: pRRBC-peb3 plasmid with ak235 and pBAD-for. Lane 8: 1 kb DNA ladder.

3.6.2 Use of pRRBC-peb3 construct for over-expression of *C. jejuni peb3* in *E. coli*

pRRBC-peb3 was constructed for a specific regulated expression for *C. jejuni*. In order to test the effect of the expression system, a pRRBC construct was used for over-expression of *peb3* in *E. coli*. The *peb3* gene was put under the control of a tightly-regulated promoter pBAD in vector pRRBC. The gene was induced by addition of L-arabinose in *E. coli* liquid culture and, after induction, fusion protein was purified and analysed using SDS-PAGE. In order to check whether arabinose affects the expression of *peb3* in *E. coli*, pRRBC-peb 3 was grown in liquid media in biological duplicates, one of which was used as a control (no arabinose was added). The rate of growth (assessed by optical density) was measured in both and was shown to decrease in the induced liquid culture compared to the non-induced control.

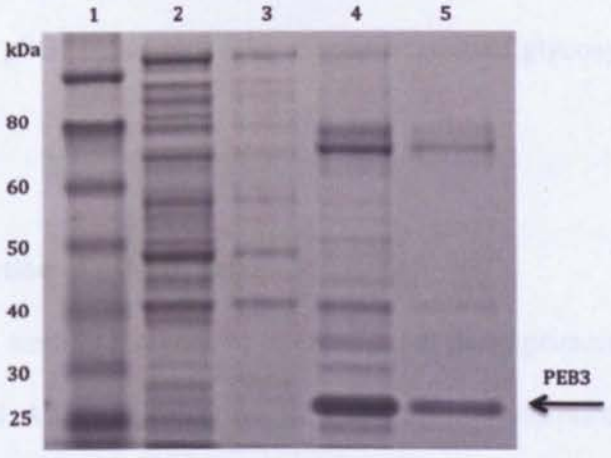


Figure 3.48. Coomassie-stained SDS-PAGE gel showing 6xHis-tagged PEB3 purified under native conditions. The fusion protein was present in both eluates, however in eluate 1 (Lane 4) there are some contaminant bands present. Lane 1: marker (P7709V); Lane 2: flow through; Lane 3: wash; Lane 4: eluate 1; Lane 5: eluate 2. Results are representative of 3 separate experiments.

The *E. coli* strain that harboured pRRBC-peb3 plasmid with correct orientation of *peb3* (Figure 3.45, clone 3) was used for PEB3 protein purification (Figure 3.48). However, it was not possible to purify glycosylated PEB3 from *C. jejuni* using pRRBC construct, as several attempts to purify PEB3 protein using *C. jejuni* liquid culture were unsuccessful.

3.6.3 Expression and purification of glycosylated and non-glycosylated His-tagged PEB3 from *E. coli* using pBAD33 and pPGL1 plasmids

The *E. coli* strain that harboured the pRRBC-peb3 plasmid with the correct orientation of *peb3* was used for PEB3 protein purification. However, it was only possible to purify non-glycosylated PEB3. In order to purify glycosylated PEB3 from *E. coli*, construction of the system containing two plasmids (pBAD33 and pPGL1) was considered. This system allowed the expression and purification of N-linked glycosylated proteins from *E. coli.* The pBAD_peb3 system allowed purification of non-glycosylated PEB3, whereas *E. coli* harbouring both pBAD_peb3 and pPGL1 plasmids allowed purification of glycosylated PEB3 from *E. coli.*

3.6.3.1 Construction of pBAD_peb3

PCR was used to amplify the gene of interest, *peb3,* using primers designed for pBAD33 plasmid. After amplification, the PCR product was purified and analysed by gel electrophoresis (Figure 3.49). PCR product was digested and used for ligation into pBAD33 plasmid, yielding pBAD_peb3. *E. coli* colonies (transformants) were verified for the presence of *peb3* using restriction analysis.

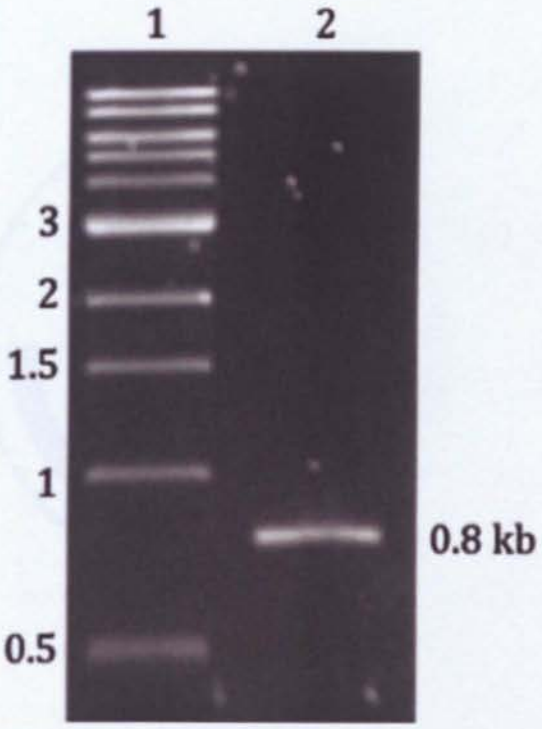
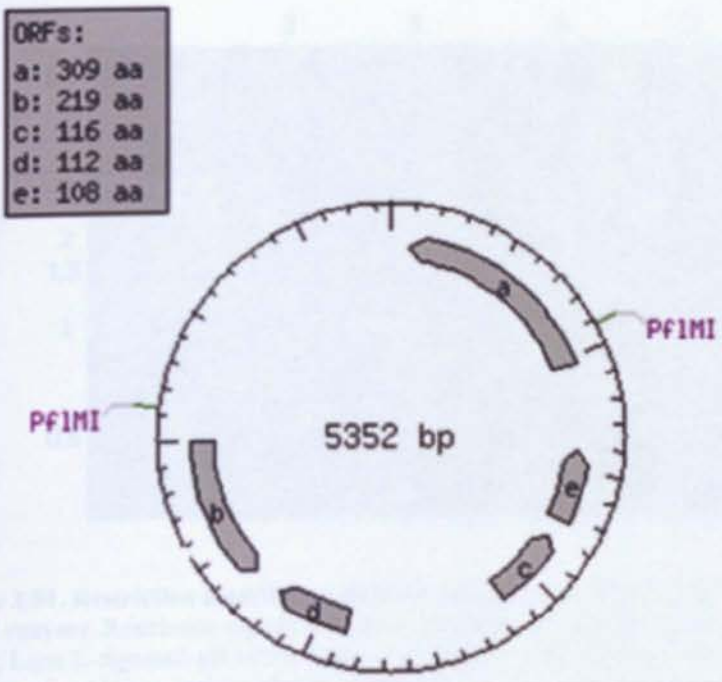
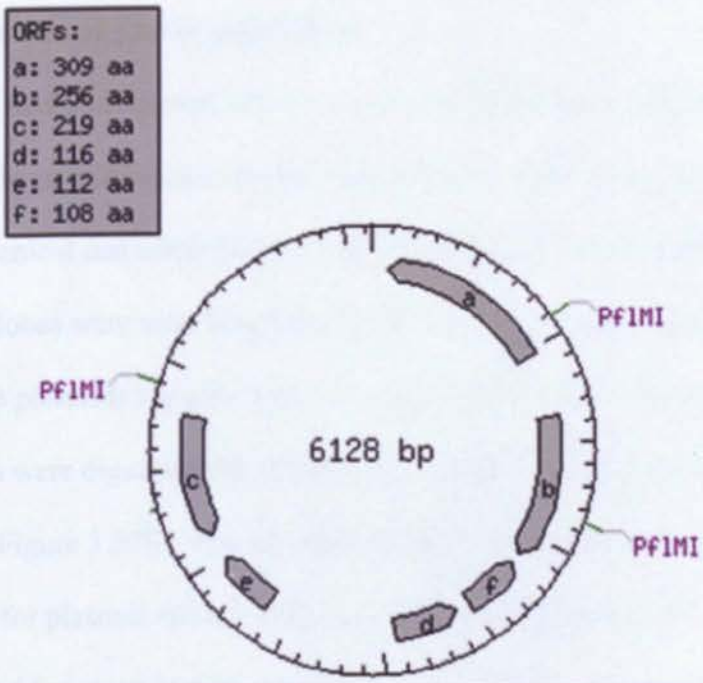


Figure 3.49. Purified PCR product *(peb3)*. PCR was run using *C. jejuni* DNA and specific primers. Gel electrophoresis of purified *peb3* PCR product. Lane1: 1 kb DNA ladder; Lane 2: PCR product (*peb3*) of ~0.8 kb.

Two restriction maps were constructed using NEBcutter program with and without insert using *Pfl*MI enzyme (Figure 3.50, A and B). Expected sizes of fragments with insert: 3.0 kb, 2.1 kb, 0.9 kb and without insert: 3.2 kb and 2.1 kb. Restriction digests were analysed by gel electrophoresis to confirm the presence of an insert. Digestion with *Pfl*MI generated three fragments of expected sizes (3.0 kb, ~2.1 kb and ~0.9 kb) (Figure 3.51). Clones with insert were sent for sequencing using primers peb3_pBAD-for and peb3_pBAD-rev. Clone 1 and 3 had mutations, but not clone 2. Thus, clone 2 was used for purification of non-glycosylated PEB3 and for further experiments.



A



B

Figure 3.50. Restriction maps of pBAD33 (A) and pBAD_peb3 (B) digested with *Pfl*MI. On map B, "b" fragment is the gene of interest – *peb3*.

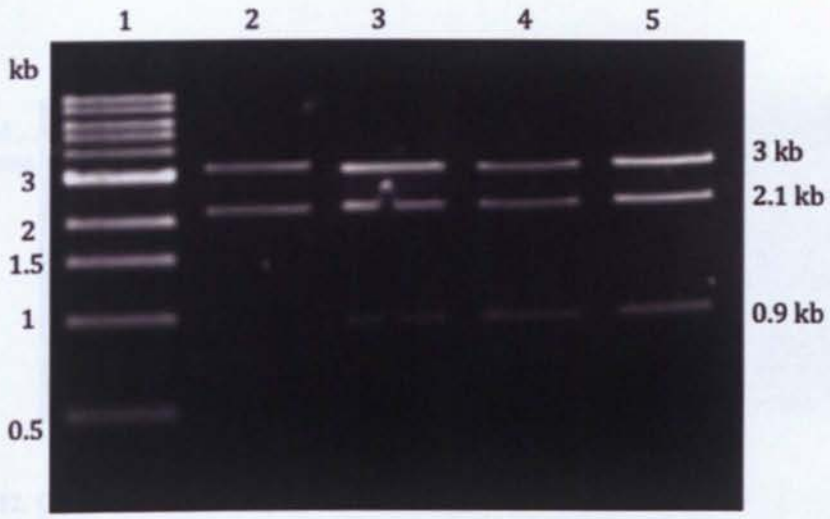


Figure 3.51. Restriction digestion analysis of pBAD33 and pBAD_peb3 construct using *Pfl*MI enzyme. Restriction digests were analysed using gel electrophoresis. Lane1: 1 kb DNA ladder; Lane 2: digested pBAD33; Lanes 3–5: pBAD_peb3 digested with *Pfl*MI confirming insertion of *peb3* gene in three clones (3.0 kb, ~2.1 kb and ~0.9 kb).

3.6.3.2 Construction of pBAD_peb3/pPGL

pPGL1 plasmid was transformed into an *E. coli* strain harbouring plasmid pBAD_peb3. The transformation mixture was plated on LB plates supplemented with chloramphenicol and ampicillin, in order to make sure that both plasmids were present. Clones were used for plasmid purification and assessed for the presence of both plasmids via gel electrophoresis (Figure 3.52A). The purified plasmid extracts were digested with *Pfl*MI enzyme and run on a gel along with the uncut samples (Figure 3.52B). The expected fragment size of the digested sample was as follows; for plasmid extract without pPGL1 three fragments of 3.0 kb, 2.1 kb and 0.9 kb and for plasmid extract with both plasmids five fragments of 11.7 kb, 8.8 kb, 3.0 kb, 2.1 kb and 0.9 kb. Clone 3 (Figure 3.52B, Lane 6) was used for PEB3 protein purification experiments, as it had the correct size and number of fragments.

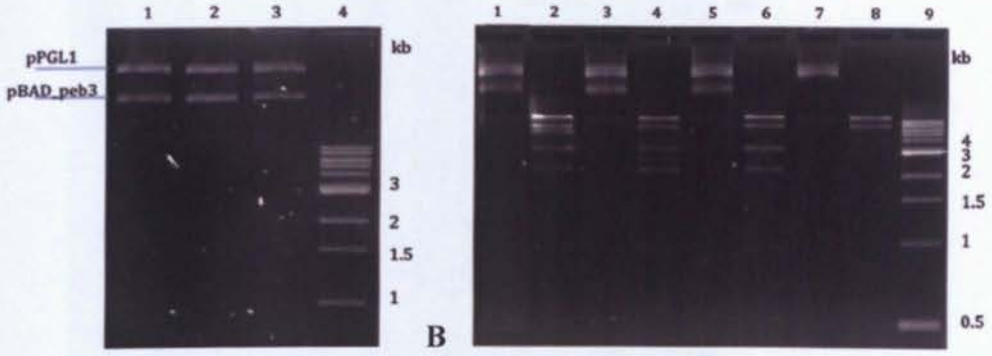


Figure 3.52. Confirmation of the presence of two plasmids (pBAD_peb3 and pPGL) in three clonal isolates after transformation of pBAD_peb3 into *E.coli*/pPGL strain using gel electrophoresis analysis. A– Lane 1–3: uncut purified plasmids from three clonal isolates; Lane 4: 1 kb DNA ladder. B. Restriction digestion analysis using *Pfl*MI of three clonal isolates with two plasmids. Sample in Lane 6 has both pBAD_peb3 and pPGL. Lanes 1, 3, 5: uncut plasmids; Lanes 2, 4, 6: digested plasmids; Lane 7: uncut pPGL1; Lane 8: digested pPGL1; Lane 9:1 kb DNA ladder.

3.6.3.3 Over-expression and purification of PEB3 using *E. coli* pBAD_peb3 and *E. coli* pBAD_peb3/pPGL

pBAD_peb3 *E. coli* strains with and without pPGL1 were used for liquid media cultures with a starting point of OD_{600} 0.1. PEB3 was over-expressed by addition of 0.1% arabinose and the proteins were purified under native conditions. The protein samples were run on a SDS-PAGE. As can be observed from Figure 3.53, two forms of PEB3 (glycosylated and non-glycosylated) were obtained using these strains. The size of glycosylated PEB3 (~27 kDa) was slightly larger than non-glycosylated PEB3 (~25.5 kDa).

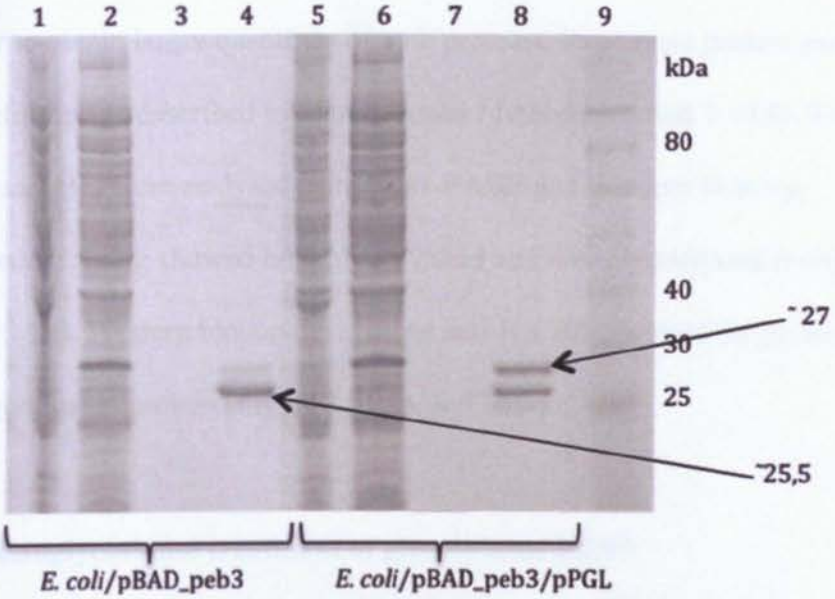


Figure 3.53. Small scale protein purification after over-expression of PEB3 using *E.coli* pBAD_peb3 and *E.coli* pBAD_peb3/pPGL strains. SDS-PAGE analysis of proteins purified from pBAD_peb3 *E. coli* strains with and without pPGL1. Lane 1: lysate after induction (pBAD_peb3); Lane 2: flow-through (pBAD_peb3); Lane 3: wash (pBAD_peb3); Lane 4: eluate (pBAD_peb3); Lane 5: lysate after induction (pBAD_peb3/pPGL); Lane 6: flow-through (pBAD_peb3/ pPGL); Lane 7: wash (pBAD_peb3/ pPGL); Lane 8: eluate (pBAD_peb3/pPGL); Lane 9: marker. Results are representative of 3 separate experiments.

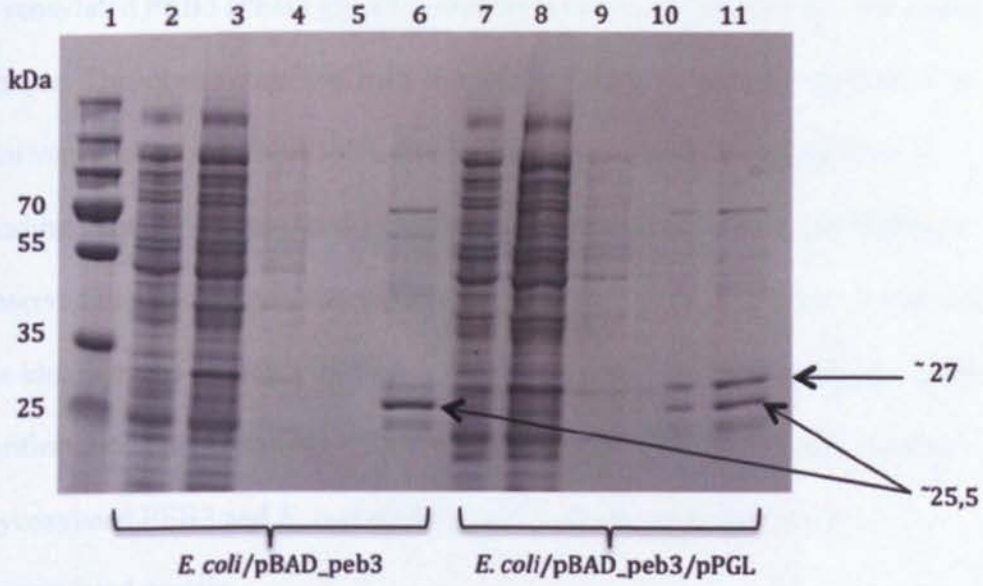


Figure 3.54. Large scale protein purification after over-expression of PEB3 using *E.coli* pBAD_peb3 and *E.coli* pBAD_peb3/pPGL strains. Identification of glycosylated and non-glycosylated proteins using SDS-PAGE. Lane 1: marker; Lane 2: lysate after induction (pBAD_peb3); Lane 3: flow-through (pBAD_peb3); Lane 4: wash 1 (pBAD_peb3); Lane 5: wash 2 (pBAD_peb3); Lane 6: eluate (non-glycosylated PEB3 with size ~25.5 kDa); Lane 7: lysate after induction (pBAD_peb3/pPGL); Lane 8: flow-through (pBAD_peb3/ pPGL); Lane 9: wash 1 (pBAD_peb3/ pPGL); Lane 10: wash 2 (pBAD_peb3/ pPGL); Lane11: eluate (pBAD_peb3/pPGL). Results are representative of 2 separate experiments.

In order to obtain larger quantities of both proteins, large scale protein purification was performed as described in Materials and Methods (section 2.14.6). The protein samples were analysed using SDS-PAGE and Western blotting. Coomassie staining showed both glycosylated and non-glycosylated proteins (Figure 3.54). Western blot analysis using anti-His Abs showed the presence of the tagged fusion protein (Figure 3.55, A and B).

3.6.3.4 Exoglycosidase treatment of glycosylated PEB3

In order to confirm the difference between the two forms of protein (one glycosylated and another non-glycosylated, used as control), both fractions were subject to treatment with exoglycosidase. Treated and untreated samples were subject to SDS-PAGE and Western blot analysis (Figure 3.55). On Figure 3.55A and 3.55B it can be observed that after treatment with exoglycosidase, the size of glycosylated PEB3 (PEB3 glyco) is slightly smaller compared to the non-treated sample. This observation confirms that this protein is indeed glycosylated. The blot was probed with SBA lectin (biotinylated for detection) to determine if binding between glycosylated PEB3 and SBA lectin occurred. It can be clearly observed that exoglycosidase digests glycosylated PEB3, as it is not observed on the blot that was probed with SBA lectin (Figure 3.55C, Lane 4). This is another confirmation that *E. coli* pBAD_peb3/pPGL can be used for the expression of glycosylated PEB3 and *E. coli* pBAD_pep3 for the expression of non-glycosylated protein.

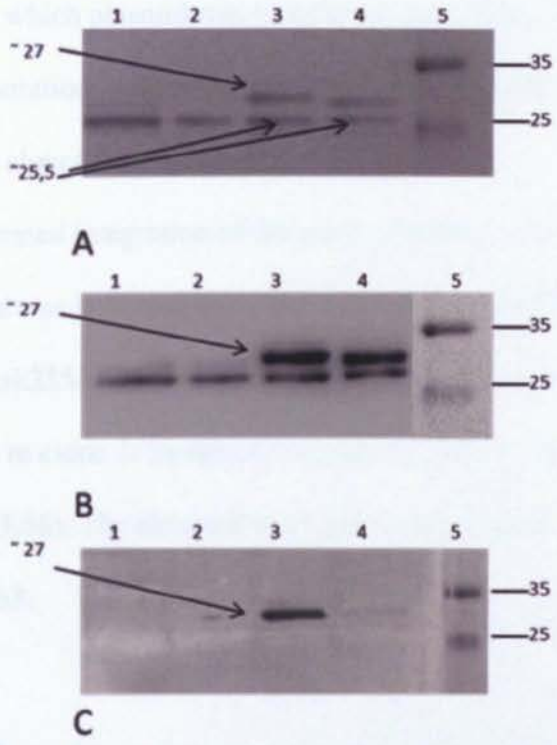


Figure 3.55. SDS-PAGE and Western blot analysis of glycosylated and non-glycosylated PEB3 before and after exoglycosiadase treatment. A. Coomassie-stained gel showing PEB3. B. Western blotting using Anti-His Abs. C. Western blotting using biotinylated SBA lectin for probing. Lane 1: undigested non-glycosylated PEB3; Lane 2: digested non-glycosylated PEB3; Lane 3: undigested glycosylated PEB3; Lane 4: digested glycosylated PEB3; Lane 5: marker. Results are representative of 2 separate experiments.

3.6.4 Over-expression of PEB3 in *C. jejuni*

Due to the lack of sugar uptake systems present in *C. jejuni*, it was not possible to obtain PEB3 using a pRRBC construct. Taking the above results into consideration, a slightly altered approach was used to obtain a construct using the pRRC plasmid, which allowed purification of PEB3 from *C. jejuni.*

3.6.4.1 Construction of pRRC_peb3 plasmid

pRRC plasmid was previously designed to be used as plasmid for gene expression studies in *C. jejuni* (Karlyshev and Wren, 2005). All procedures were performed as described in section 3.2.3 (complementation of the *peb3* gene), with exception

of the last stage, in which plasmid was transferred into WT *C. jejuni* competent cells using electroporation. After transformation of the 11168H with plasmid pRRC_peb3, Cam^r clones were selected.

PCR analysis confirmed integration of the *peb3* gene into one of the rRNA gene clusters. Each clone was analysed using the following set of primers ak233/ak237, ak234/ ak237, and ak235/ak237. Gel electrophoresis analysis confirmed integration of *peb3* in clone 2. Integration of *peb3* in clones 1 and 3 was not confirmed (Figure 3.56). The obtained derivative was designated 11168H/pRRC_peb3.

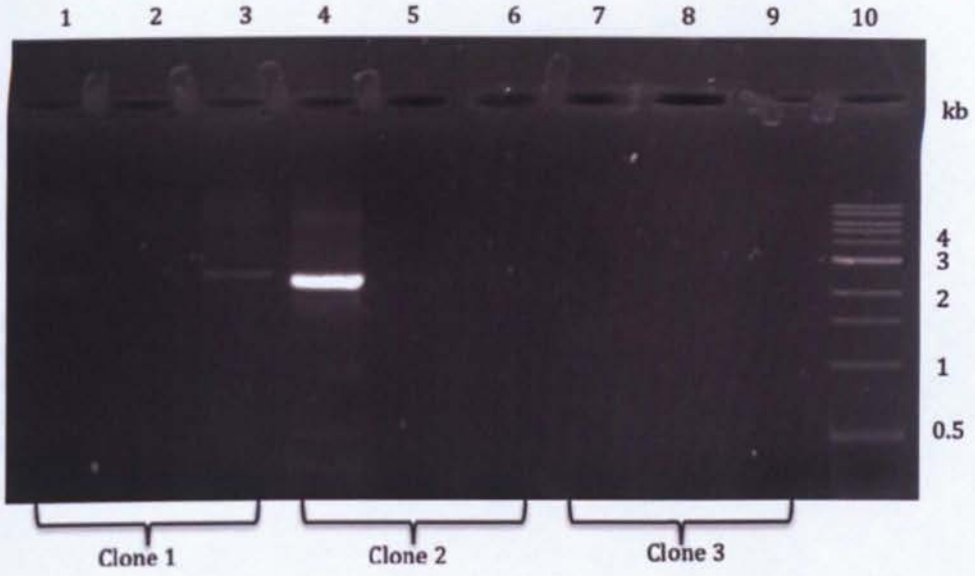


Figure 3.56. PCR analysis of three insertion derivatives to check the site of insertion in 11168H. Lanes1,4 and 7: primers ak233/ak237; Lanes 2, 5 and 8: ak234/ak237; Lanes 3, 6 and 9: ak235/ak237; Lane 8: 1 kb DNA ladder.

3.6.4.2 Expression and purification of glycosylated PEB3 from *C. jejuni* using pRRC_peb3 construct

The 11168H/pRRC_peb3 *C. jejuni* strain was grown overnight. The protein was purified from the pellet as described in Materials and Methods (section 2.14.6)

using small scale purification. Purified fractions were analysed using SDS-PAGE and Western blotting. Equal amounts of fractions were applied onto the gel (Figure 3.57).

Both proteins, one from *E. coli* pBAD_peb3/pgl and another from pRRC_peb3, were glycosylated and have the same molecular weight. Both proteins were detected using anti-His Abs and biotinylated SBA lectin (Figure 3.58).

These results show that *E. coli* pBAD_peb3/pPGL construct can be used for purification of larger quantities of glycosylated PEB3 protein. After large scale purification, the PEB3 protein was used for further experiments.

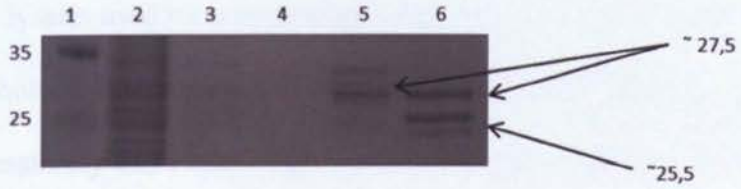


Figure 3.57. SDS-PAGE analysis of PEB3 purified from *C. jejuni*/pRRC_peb3 and *E. coli* pPAD_peb3 /pPGL Lane 1: marker; Lane 2: lysate from 11168H/pRRC_peb3; Lane 3: flow-through from 11168H/pRRC_peb3; Lane 4: wash from11168H/pRRC_peb3; Lane 5: eluate (PEB3) from11168H/pRRC_peb3; Lane 6: eluate (PEB3) from *E. coli* pBAD_peb3/pPGL. Results are representative of 2 separate experiments.

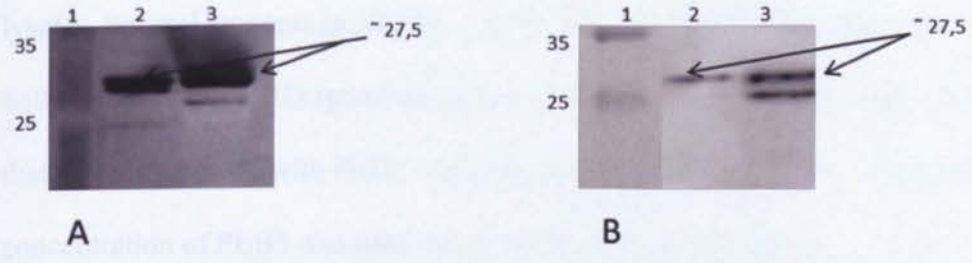


Figure 3.58. Western blot analysis of PEB3 purified from *C. jejuni*/pRRC_peb3 and *E. coli*/pPABD_peb3 /pPGL. A. Western blotting using SBA lectin. B. Western blotting using anti-His Abs. Lane 1: marker; Lane 2: PEB3 purified from *C. jejuni*/pRRC_peb3; Lane 3: PEB3 purified from *E.coli*/pBAD_peb3/pPGL. Results are representative of 2 separate experiments.

3.7. Interaction of PEB3 and *E. coli*/pPGL with the lysates of human cells

In order to determine the receptors to which PEB3 protein binds, Western blots using host whole cell lysates were probed with PEB3. Glycan moieties of PEB3 contain terminal GalNAc residues that can specifically bind to lectin-like host cell receptors. *Campylobacter* has the ability to interact with C-type lectins (including MGL receptors) produced by host cells (van Sorge *et al.*, 2009). MGL receptors specifically recognise terminal GalNAc residues and are expressed on macrophages and DC (Higashi *et al.*, 2002; van Vliet *et al.*, 2008). Other host cell receptors may be present on the surface of normal intestinal cells.

3.7.1 No interaction of PEB3 with proteins of human whole cell lysates

Human cell lysates used were normal intestinal cells, Caco-2 cells and THP-1 cells. The choice of these particular whole cell lysates was due to the fact that there is a possibility that PEB3 might interact with lectins present on either intestinal cells (normal intestinal cells and Caco-2 cells) or with lectins present on immune cells (THP-1 is a human monocytic cell line). Lysate samples were run on SDS-PAGE gels and transferred onto membranes according to manufacturers' instructions. After transfer, blots were probed with purified PEB3 and then with anti-His Abs, in order to identify binding of PEB3 to protein bands of whole cell lysates. Several attempts to identify any difference between blots that were treated with glycosylated PEB3 (purified from *E. coli* pBAD_peb3/pPGL) and controls that were not treated with PEB3 were not successful (Figure 3.59). The same concentration of PEB3 was used across all blotting experiments.

One of the reasons for no difference being observed between blots may be due to inactivation of binding sites of the host cells during denaturing the host cell

lysates. However, it is not possible to predict in advance if this may have an effect on binding sites.

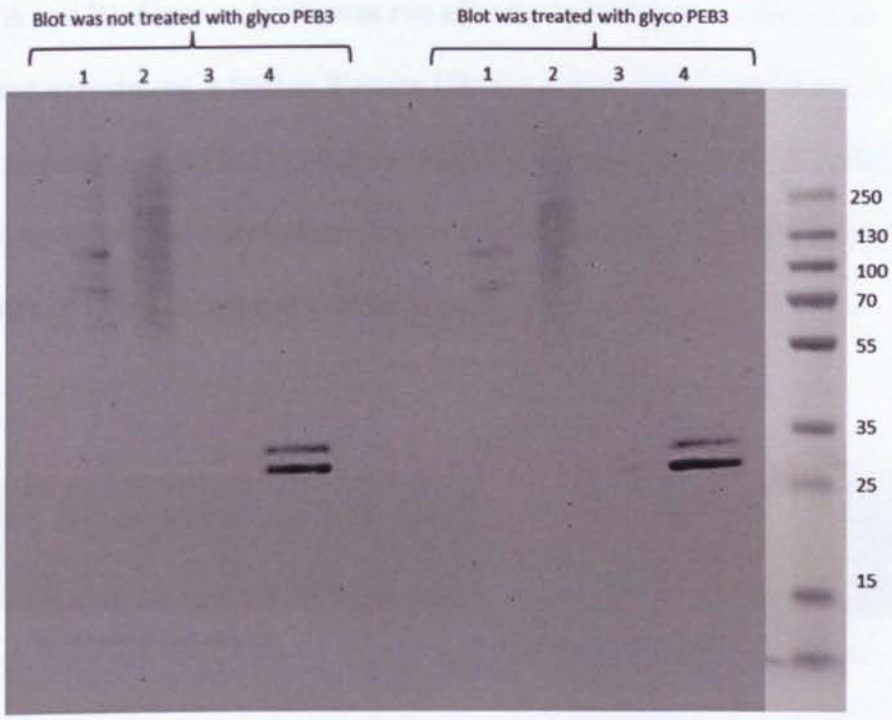


Figure 3.59. Comparison of blots treated and not treated with purified PEB3 protein. Samples were run on a 12.5% polyacrylamide gel before Western blot transfer. After incubation with PEB3, both blots were incubated with anti-His Abs in order to identify PEB3 attachment. Lane 1: THP-1 lysate; Lane 2: human intestinal cell lysate; Lane 3: SBA lectin; Lane 4: PEB3 glyco. Results are representative of 3 separate experiments.

Another reasons for no difference being observed between blots may be due to a low PEB3 protein yield used for probing the blots. In order to identify the reason for low PEB3 protein yield purified from *E. coli*, Western blot analysis of lysates before and after induction was performed (Figure 3.60). Induced and non-induced cultures were used for protein purification and Western blotting. It seems that the reason for obtaining a low PEB3 yield is due to low expression of the PEB3 protein. PEB3 can be observed in induced culture cell lysates, but not in flow-through, suggesting that PEB3 binds to the column. However there were no

problems present during the purification process itself, since there was no loss of protein during the purification process – the wash fractions were empty. Therefore the reason for low yield is thought to be due to low expression of PEB3 (Figure 3.60, A and B). Since no lysate was run after the induction procedure in the previous experiment, a further Western blotting experiment was performed in order to confirm that PEB3 protein was not lost during the purification process. Fractions before and after induction were also analysed in order to compare the presence of PEB3 in induced culture (Figure 3.61).

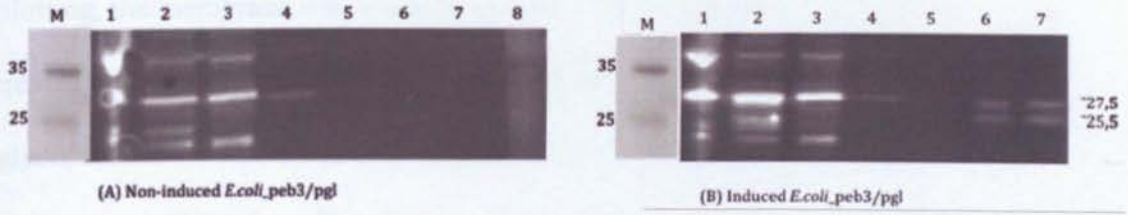


Figure 3.60. Western blot analysis of PEB3 protein expression using induced and non-induced *E.coli* pBAD_peb3/pPGL. Samples were run on 12.5% polyacrylamide gel before Western blot transfer. M: marker (picture taken before transfer); Lane 1: lysate before induction; Lane 2 on A: supernatant (without induction); Lane 2 on B: supernatant after induction (with induced protein); Lane 3: flow-through; Lane 4: wash 1; Lane 5: wash 2; Lane 6: eluate1; Lane 7: eluate 2; Lane 8: marker. Equal amounts of products were loaded on the gel in order to compare the intensity of the bands. Results are representative of 2 separate experiments

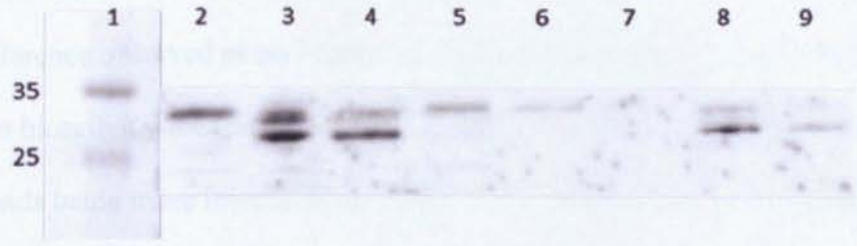


Figure 3.61. Analysis of PEB3 protein expression using Western blotting. Samples were run on a 12.5% polyacrylamide gel before Western blot transfer. Lane 1: marker; Lane 2: lysate before induction; Lane 3: lysate after induction; Lane 4: clear lysate (it has induced protein); Lane 5: flow-through; Lane 6: wash 1; Lane 7: wash 2; Lane 8: eluate 1; Lane 9: eluate 2. Equal amounts of products were loaded on the gel in order to compare the intensity of the bands. Band in Lane 2 is probably histidine rich protein of *E. coli.* Results are representative of 2 separate experiments. Band present in Lane 2 is probably histidine rich protein of *E.coli.*

3.7.2 *E. coli*/pPGL whole cells interact with human whole cell lysates

It was previously shown that *E. coli*/pPGL expresses glycosylated bacterial lipopolysaccharide, resulting in exposure of GalNAc residues on the cell surface (van Srorge *et al.*, 2009). The ELISA binding assay results, as well as confocal microscopy, confirmed that *E. coli*/pPGL is capable of binding to immobilised SBA lectin. Blots of human cell lysates were probed with whole *E. coli*/pPGL cells in order to check if surface GalNAc residues interact with human whole cell lysates.

The same amount of human whole cell lysates was used as in section 3.7.1. After blotting, the membrane was blocked, treated with *E.coli*/pPGL cells and then treated with biotinylated SBA lectin, in order to show the interaction of glycoproteins on whole bacterial cells with cell lysate proteins. Purified glycosylated PEB3 from *E. coli* was loaded onto the gel as control to confirm that the method works (Figure 3.62).

There are some differences in the intensity of bands between two blots, as well as the presence of extra bands in the blot that was treated with whole *E. coli*/pPGL cells. In order to check if the presence of pPGL plasmid affects the interaction of whole *E. coli* cells with lysates, both *E. coli* wild type strain and *E.coli*/pPGL were used to probe blots with normal intestinal cell lysates.

The difference observed as on Figure 3.62 was also observed in these results, between blots that were treated with the *E. coli* recipient strain and *E. coli*/pPGL, with bands being more intense in the blot that was incubated with *E. coli*/pPGL whole cells (Figure 3.63). This demonstrates the interaction of *E.coli*/pPGL cells with human cell lysates.

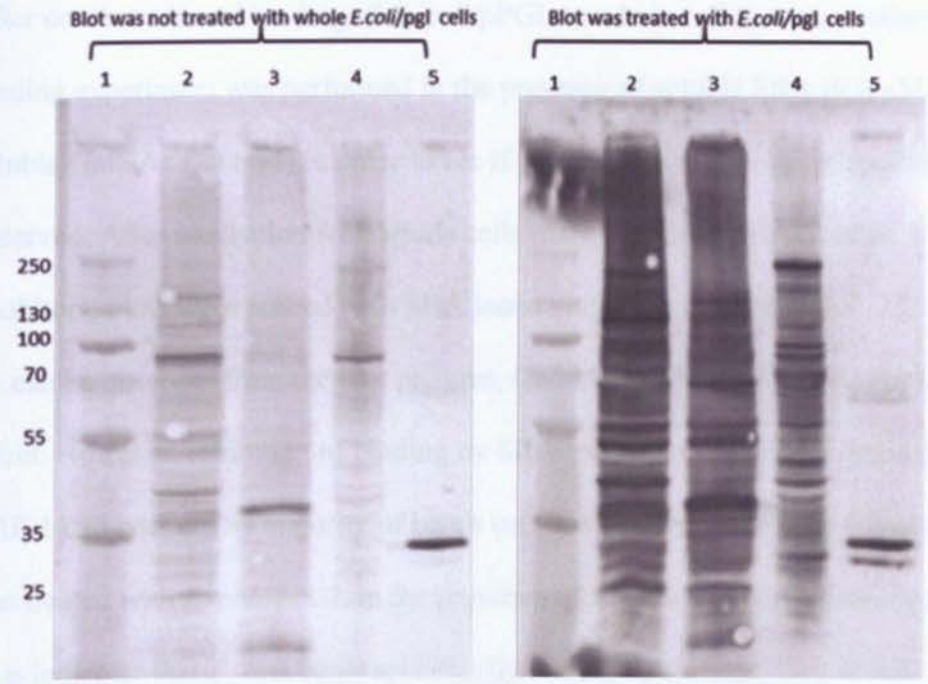


Figure 3.62. Comparison of blots treated and not treated with whole *E. coli*/pPGL cells. Samples were run on 12.5% polyacrylamide gel before Western blot transfer. After incubation of the blot with whole cells, both blots were incubated with biotinylated SBA lectin in order to identify any attachment of cells. Lane 1: marker; Lane 2: Caco-2 lysate; Lane 3: THP-1 lysate; Lane 4: normal intestinal cell lysate; Lane 5: purified PEB3 glyco. Results are representative of 2 separate experiments.

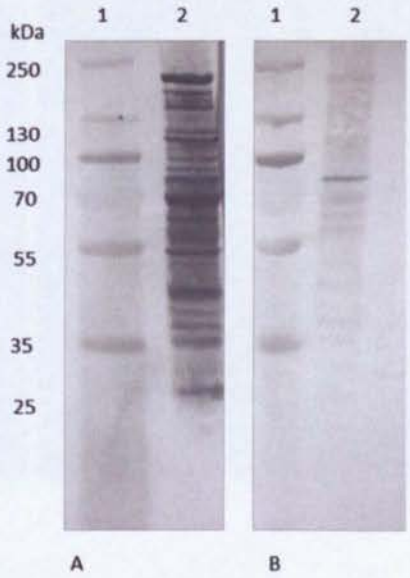


Figure 3.63. Comparison of blots treated with whole *E. coli*/pPGL cells (A) and *E. coli* recipient cells (B). Samples were run on 12.5% polyacrylamide gel before Western blot transfer. After incubation of blot with whole *E. coli* cells (with or without pPGL), both blots were incubated with biotinylated SBA lectin in order to identify any attached cells. Lane 1: marker; Lane 2: normal intestinal cell lysate.

After confirmation of binding of *E. coli*/pPGL to whole cell lysates, another binding experiment was performed in the presence of soluble SBA (0.2 μM) or soluble GalNAc (20 mM) in order to see if any inhibition of binding could be observed. After incubation with whole cells in the presence or absence of inhibitors, blots were probed with SBA lectin conjugate (Figure 3.64).

As can be observed from the blot pictures, GalNAc did not have an inhibitory effect. However, inhibition of binding by SBA lectin was detected in blots with THP-1 cell lysate. The majority of bands on the blot (lane with THP-1 lysate) that was treated with *E. coli*/pPGL in the presence of soluble SBA lectin are absent. This indicates that *E. coli* binds specifically to THP-1, through SBA lectin.

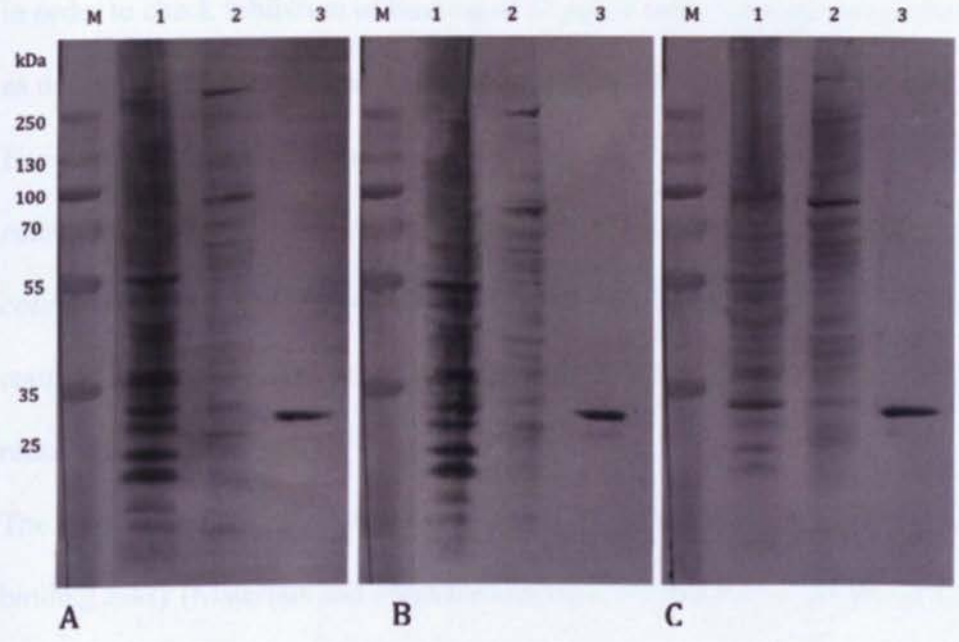


Figure 3.64. Western blot analysis of whole cell lysates. After transfer, blots were incubated with *E. coli*/pPGL cells only (A), incubated with *E. coli*/pPGL cells in the presence of GalNAc (B) and incubated with *E. coli*/pPGL cells in the presence of SBA lectin (C). Lane 1: THP-1 lysate; Lane 2: normal intestinal cell lysate; Lane 3: PEB3 protein (control). Blots represent those obtained in two different experiments.

3.8 *E. coli*/pPGL cells inhibit binding of *C. jejuni* to immobilised SBA

Since *E. coli*/pPGL whole cells were found to be able to bind SBA lectin, it was interesting to investigate if these cells could potentially compete with *C. jejuni* for attachment in our model of adhesion. If confirmed, this could be a useful model for the development of novel probiotics based on competition with pathogens for binding to specific host cell receptors. Thus, *E. coli*/pPGL was used for inhibition of binding of *C. jejuni* to SBA lectin. As the *C. jejuni* capsule may interfere with attachment, an acapsulated strain 11168H/*kpsM* was used for all inhibition studies.

3.8.1 Detection of inhibition of binding of *C. jejuni* cells using quantitative ELISA

The 96-well plate coated with SBA lectin and anti-*Campylobacter* Abs were used in order to check inhibition of binding of *C. jejuni* cells. Coating was performed as described in Materials and Methods (section 2.16.1). After blocking with BSA, *E. coli*/pPGL cells were incubated in the wells in order to bind to SBA. As *E. coli*/pPGL binds to SBA lectin in the same way as *C. jejuni*, incubation of *E. coli*/pPGL cells in the SBA-coated plate would block binding sites. This would result in an inability of *C. jejuni* cells to bind to immobilised SBA, or a very reduced amount of binding.

The detection of inhibition of binding was not possible using the earlier developed binding assay (Materials and Methods section 2.16) due to the fact that it detects the presence of both *Campylobacter* cells and *E. coli*/pPGL cells. In order to check whether *Campylobacter* cells bind to SBA after incubation with *E. coli*/pPGL, mouse anti-*Campylobacter* Abs were used. First the optimal dilution of primary and secondary Abs was determined. After coating of a 96-well

microtitre plate with immobilised SBA lectin, uncoated sites were blocked by incubation with 1% BSA to reduce non-specific binding. This was followed by incubation with whole bacterial cells. All samples in the ELISA assays were tested in triplicates, and the mean absorbance was determined. BSA-coated wells were used as negative controls.

After validation of the method the system was successfully tested using *C. jejuni* cells. The results confirmed specific interaction of *C. jejuni* with immobilised SBA lectin. It has been demonstrated that bacterial binding is concentration-dependent, and incubation in the presence of soluble SBA lectin inhibits binding, which proves that binding is specific for SBA and can be detected using mouse anti-Campylobacter Abs (Figure 3.65 and Figure 3.66).

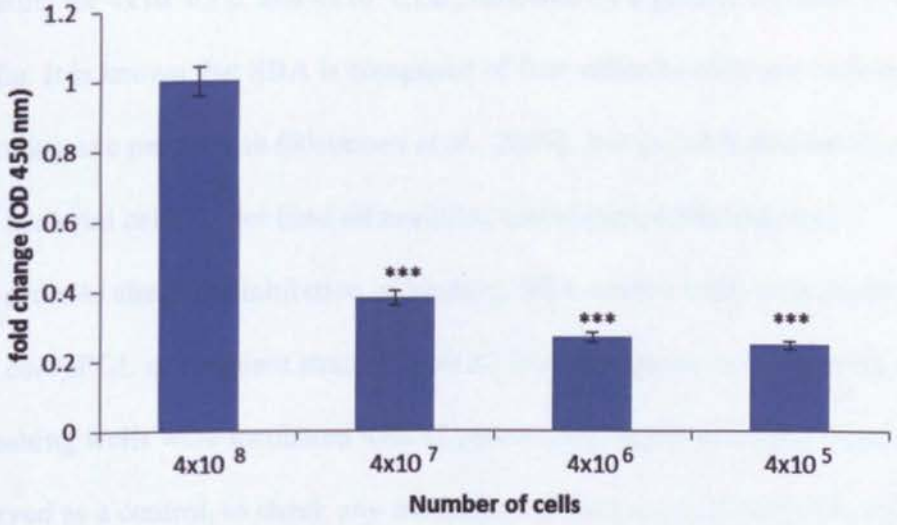


Figure 3.65. Interaction of *C. jejuni* 11168H with immobilised SBA lectin, detectable using mouse anti-*Campylobacter* Abs. Binding of whole cells with SBA lectin was concentration-dependent. The figures below the bars indicated the numbers of cells per well. Bars represent mean ± SD. Results are representative of 3 separate experiments that gave comparable results. *** $P < 0.001$ was considered statistically significant.

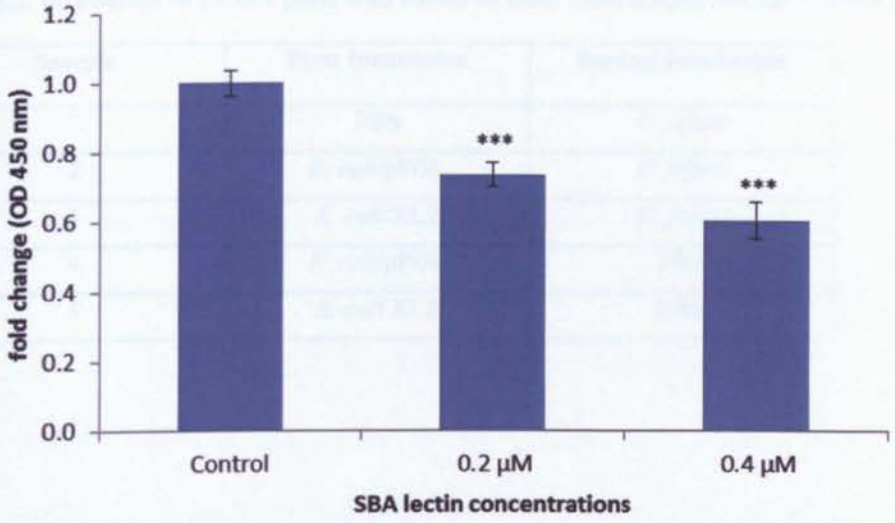


Figure 3.66. Binding in the presence of soluble SBA lectin using mouse anti-*Campylobacter* Abs. Soluble SBA lectin at a concentration of 0.1 µM inhibits binding of whole *C. jejuni* 11168H cells to immobilised SBA lectin. Bars represent mean ± SD. Results are representative of 3 separate experiments that gave comparable results. *** P <0.001 was considered statistically significant.

The observed dose-binding response is very similar in results described in Section 3.1.2. Both results (Figure 3.3 and Figure 3.65) show the same effect with similar results for $4x10^6$ c.f.u. and $4x10^7$ c.f.u., followed by a greater increase in $4x10^8$ c.f.u. It is known that SBA is composed of four subunits with one carbohydrate-binding site per subunit (Sletmoen *et al.*, 2009). It is possible that smaller number of bacterial cells do not bind all available carbohydrate-binding sites.

In order to check the inhibition of binding, SBA-coated wells were incubated with *E. coli*/pPGL or recipient strain *E. coli* XL2 (as a negative control) cells, and after washing wells were incubated with *C. jejuni* cells. Wells without *C. jejuni* cells served as a control, to check any interaction of mouse anti-Campylobacter Abs with *E. coli* and *E. coli*/pPGL cells. Table 3.2 shows the order in which the cells were added to the SBA-coated wells. Control wells (sample 1) were incubated with PBS and then with *C. jejuni*. Results from this experiment are shown in Figure 3.67.

Table 3.2. Incubation of ELISA plate with bacterial cells. Each sample was run in triplicate.

Sample	First Incubation	Second Incubation
1	PBS	*C. jejuni*
2	*E. coli*/pPGL	*C. jejuni*
3	*E. coli* XL2	*C. jejuni*
4	*E .coli*/pPGL	PBS
5	*E. coli* XL2	PBS

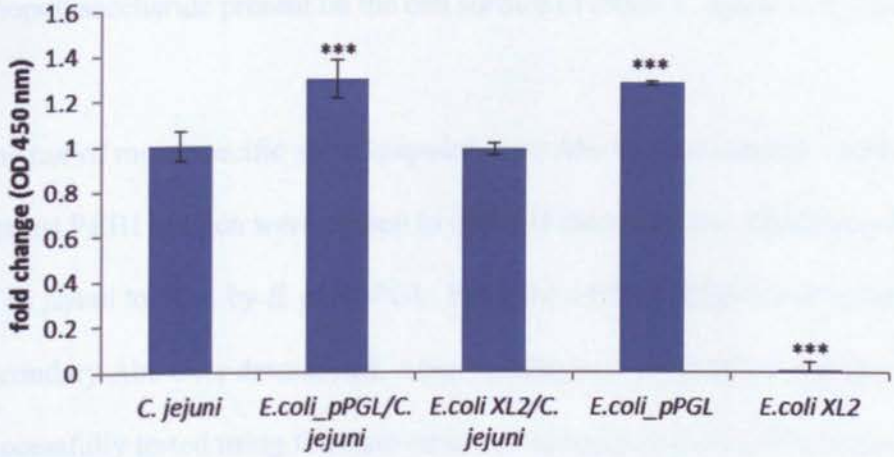


Figure 3.67 Mouse anti-*Campylobacter* Abs detect both *C. jejuni* and *E. coli*/pPGL. No signal in the wells that were incubated only with *E. coli* XL2. Bars represent fold change in absorbance (mean ± SD of n = 3). Results are representative of 3 separate experiments that gave comparable results. *** P <0.001 was considered statistically significant, compared to the control (in this case, the well with only *C. jejuni*).

The well with only *C. jejuni* cells was used as a positive control, and absorbance values from other wells were compared to this, with the fold change given. Wells in which *E. coli*/pPGL were added showed an unexpected increase in absorbance (and therefore increase in binding) than *C. jejuni*, showing that the anti-*Campylobacter* Ab was not specific to *C. jejuni* and cross-reacted with *E.*

coli/pPGL (Figure 3.67). Thus, it was not possible to detect inhibition of binding using this Ab. Several attempts to get information from the manufacturer about specificity of this antibody were unsuccessful. The manufacturer only provided the following description "recognises *Campylobacter jejuni* whole bacteria". The absorbance of the wells with *E. coli*/pPGL was 0.7 times higher than absorbance of the wells with *C. jejuni.* Since there was no signal detected in wells incubated with *E. coli* XL2 cells, it suggests that the Ab binds a common site of *C. jejuni* and *E. coli*/pPGL. It is proposed that the anti-*Campylobacter* Ab binds lipopolysaccharide present on the cell surface of either *C. jejuni* or *E. coli*/pPGL.

The use of more specific anti-*Campylobacter* Abs was considered. Antibodies against PEB1 antigen were chosen to check if there was any inhibition of binding of *C. jejuni* to SBA by *E. coli*/pPGL. First, the optimal dilutions of primary and secondary Abs were determined. After validation of the method, the system was successfully tested using *C. jejuni* cells. All samples in the ELISA assays were tested in triplicates, and the mean absorbance was determined. BSA-coated wells were used as negative control.

Before performing inhibition studies, first the cross reactivity of anti-PEB1 Abs with *E. coli* XL2 and *E. coli*/pPGL was checked. In the same type of experiment as above, incubation of cells in the presence of soluble SBA was performed to check the specificity of binding. Although a decrease in binding was demonstrated when *C. jejuni* was incubated with soluble SBA (compared to *C. jejuni* alone), an increase in binding was observed in wells with *E. coli*/pPGL, compared with the control *C. jejuni* cells (Figure 3.68). From these results it was concluded that anti-PEB1 Abs interacted with both *C. jejuni* and *E. coli*/pPGL cells, but not *E. coli* XL2 cells.

As both anti-*Campylobacter* Abs were interacting with *E. coli*/pPGL cells, it was not possible to detect if *E. coli*/pPGL could inhibit binding using an ELISA. There is a possibility that both Abs were raised against whole cells and not a specific antigen, which was not specified by the manufacturers. The lipopolysaccharide on the cell surface of *E. coli*/pPGL may have interacted with both anti-*Campylobacter* Abs.

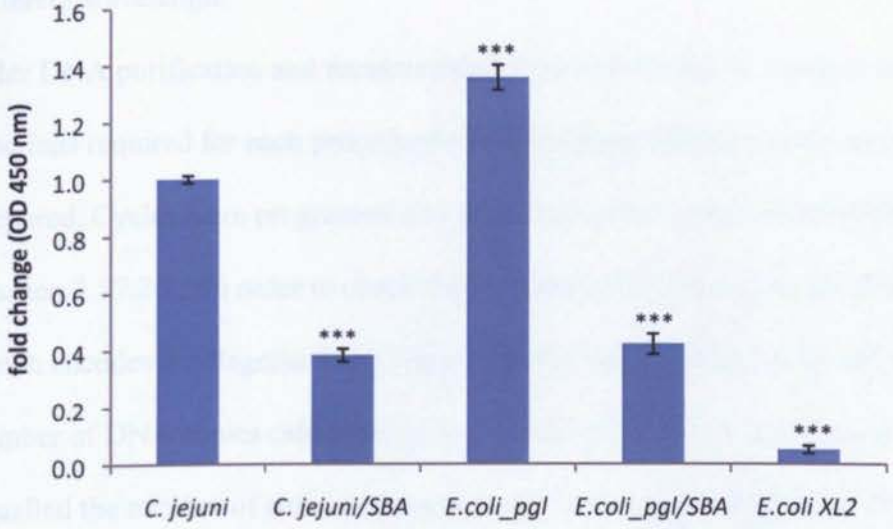


Figure 3.68. Anti-PEB1 Abs detect both *C. jejuni* and *E. coli*/pPGL cells. No signal was present in the wells that were incubated only with *E. coli* XL2. Soluble SBA inhibited binding of both cell types. Bars represent fold change in absorbance (mean ± SD of n = 3). *** $P < 0.001$ was considered statistically significant, compared to the control (in this case, the well with only *C. jejuni*).

3.8.2 Detection of inhibition of attachment of *C. jejuni* using qPCR

Since the use of ELISA was not successful to assess if *E. coli*/pPGL could inhibit the binding of *C. jejuni* to immobilised SBA lectin, the use of qPCR-based *C. jejuni* detection was considered. Incubation of ELISA wells with *E. coli*/pPGL and/or *C. jejuni* cells was performed as described in Table 3.2 above (Sample 1 and Sample 2). Following incubation, the DNA of attached cells was purified

from each well of a microtitre plate as described in the Materials and Methods (section 2.17.2). There were 4 replicates for each sample, making 8 samples in total. At the same time, DNA was purified from *C. jejuni* corresponding to 4×10^8 c.f.u. and used as control for PCR. The DNA concentration and quality of each sample was determined by measuring the absorbance at 260 nm using a NanoVue spectrophotometer. The ratio between the absorbance at 260 nm and 280 nm provided an estimate of DNA purity with respect to contaminants that absorb at a different wavelength.

After DNA purification and measurement of concentration, the number of reactions required for each procedure was calculated, and the master mix was prepared. Cycles were programmed as described in the Materials and Methods (section 2.17.2.2). In order to check the presence of *C. jejuni* cells the *flhA* gene, which encodes the flagellar biosynthesis protein was amplified using qPCR. The number of DNA copies calculated to be present at the start of the reaction equalled the number of cells, as there was only one copy of *flhA* gene present in the genome of *Campylobacter*. The amplified product was detected using target-specific fluorescent hydrolysis probes. Earlier purified control DNA from *C. jejuni* was diluted several times to correspond to the different number of cells ($1x10^6$, $1x10^5$,$1x10^4$, $1x10^3$, $1x10^2$). To determine the number of DNA copies in samples an absolute quantification method was used. CT values of diluted samples were used in a plot of the standard curve, with CT values vs the number of cells (DNA copies). The average of each control sample was used to plot the standard curve. Using this standard curve, the number of cells in each sample was determined (Table 3.3). In order to assess if *E. coli*/pPGL inhibited the binding of *C. jejuni* to immobilised SBA, the number of cells in both groups were compared and the difference was analysed (Figure 3.69).

Table 3.3. *E. coli*/pPGL inhibited binding of *C. jejuni* cells to immobilised SBA lectin. *C. jejuni* was incubated either with or without *E. coli*/pPGL bound to immobilised SBA lectin. The number of *C. jejuni* cells in each sample was determined using a standard curve.

Samples	Replicates	CT	Number of cells
C. jejuni cells only	1	29.4	1529
	2	29.19	1791
	3	29.80	1131
	4	29.54	1376
C. jejuni and incubation with *E. coli*/pPGL	1	28.52	2969
	2	30.32	764
	3	30.47	682
	4	30.16	862

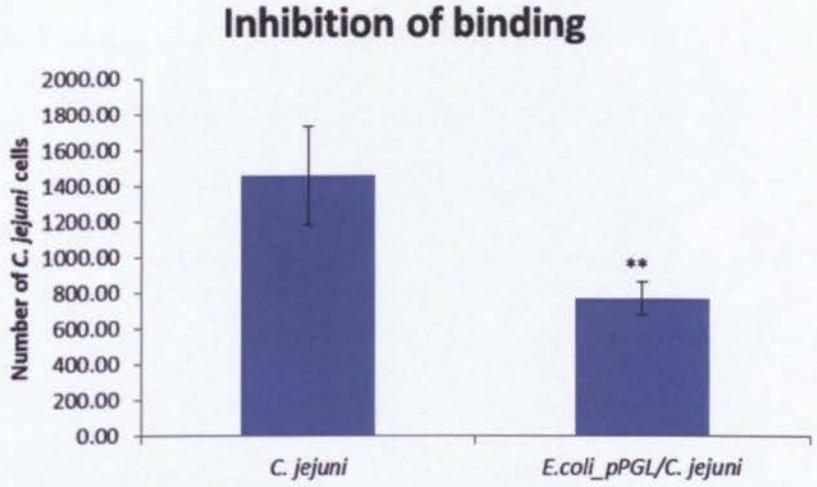


Figure 3.69. *E. coli*/pPGL pre-incubation affects binding of *C. jejuni* cells to immobilised SBA lectin. *E. coli*/pPGL incubation in SBA-coated ELISA plates were followed by incubation with *C. jejuni* cells. Bars represent the number of *C. jejuni* cells determined by qPCR (mean ± SD). Wells that were pre-incubated with *E. coli*/pPGL had a smaller number of *C. jejuni* cells compared with wells containing only *C. jejuni*. ** P <0.01 was considered statistically significant.

The average number of cells in four samples that were not pre-incubated with *E. coli*/pPGL was 1456, which was two times higher than the average number of cells in the wells that were pre-incubated with *E. coli*/pPGL (769).

'The CT value in replicate 1 was not similar to the CT values obtained from the other 3 replicates. It was thought that this sample produced an anomalous result

and therefore was discounted from the analysis. The high concentration found may have been due to contamination of the sample. From these preliminary results it is suggested that *E. coli*/pPGL was able to inhibit binding of *C. jejuni* cells to immobilised SBA. However, further studies would be need to be conducted to confirm these findings.

CHAPTER 4: Discussion

4.1 Interaction of *C. jejuni* cell surface glycans with analogues of host cell receptor

Pathogens may produce different types of adhesins that are differentially regulated and may be expressed at different stages of disease (Gerlach and Hensel, 2007). As stated earlier in Chapter 1, there are several *in vitro* cell culture-based methods available to study *Campylobacter* adhesion to host cells. Even though cell culture methods are commonly used for studying adhesion-defective mutants, they have some disadvantages. In addition to being time consuming, laborious and complicated, these techniques may give variation in results due to the use of different bacterial strains and cultured cell lines. Therefore, it may be useful to complement cell culture with non-cell culture methods, which are based on the binding of bacterial cells to inanimate surfaces. Studies involving *Campylobacter* binding to non-tissue surfaces have previously been carried out to identify the interaction of bacterial cells with specific receptors. For example Konkel *et al.* (1997) were able to observe binding of *C. jejuni* cells to immobilised fibronectin under the fluorescent microscope using acridine orange staining. This approach allows the identification of the occurrence of bacterial adhesion to proteins that are attached to a surface. Another approach used in the same study to assess bacterial interaction with the receptor was the radioactive labelling of fibronectin (Konkel *et al.*, 1997). Western blotting is another method used for the identification of adhesin receptors. For example, the JlpA receptor was identified using anti-Glutathione *S*-transferase (GST) antibodies to JlpA-GST (Jin *et al.*, 2003). ELISA may also be used to evaluate binding. In the study conducted by van Sorge *et al.* (2009) the bacterial cells were

first immobilised to the plate surface and then probed with the specific receptor, human macrophage galactose-type lectin (MGL). Successful binding of MGL was identified using anti-MGL antibodies. Each of the above methods when combined may complement each other and lead to better results.

In this study an *in vitro* model of bacterial attachment using a non-cell culture based method was developed. The assay enabled the assessment of bacterial binding to an immobilised analogue of putative host cell receptors, SBA lectin. *C. jejuni* expresses N-linked glycoproteins with a terminal α-linked GalNAc residue that may interact with SBA lectin, as well as the lectins present on the surface of host cells. The purpose of using SBA lectin was to demonstrate, if a positive interaction was found, that *C. jejuni* is likely to additionally bind animal cell lectins. The assay was also used to determine whether capsule was involved in bacterial binding to SBA lectin.

The two-day culture (mid-log growth stage) (Ikeda, 2014) was selected, as it was the phase in bacterial growth in which they are highly metabolically active (i.e. enzyme activity and gene synthesis), and thus it served to mimic bacterial dynamics inside a host. The *in vitro* infectious process depends on the growth phase of *Campylobacter*. Bacteria from mid-log growth culture were shown to have a higher invasion rate compared to bacteria in early stationary growth phase (Konkel *et al.*, 1992b; Hu and Kopeko, 1999). It was assumed that this growth phase should be optimal for the bacterial ability to adhere to SBA lectin. In addition, using the same growth stage for each binding experiment provided consistency in the physiological state of each *Campylobacter* strain.

Using the binding assay developed in this study it was demonstrated that bacterial attachment was dose-dependent, since a reduced number of bacterial cells led to a reduced signal. The interaction of SBA lectin with *C. jejuni* cells was also

confirmed using fluorescence microscopy. The specificity of interaction was confirmed by treatment of whole cells with exoglycosidase specific for terminal GalNAc residues, as well as by inhibition of binding in the presence of free soluble SBA lectin and soluble GalNAc. In addition, treatment of cells that were already bound to the immobilised SBA lectin with exoglycosidase resulted in detachment of cells. These experiments confirmed that bacterial binding was the result of interaction with the α-linked GalNAc of bacterial N-linked glycans.

It was previously shown that expression of the *C. jejuni pgl* cluster that encodes the bacterial N-glycosylation system in *E. coli* results in the modification of LPS molecules on the surface of *E. coli* (van Sorge *et al.*, 2009). The results confirmed that the introduction of the *pgl* locus into *E. coli* resulted in the generation of an SBA-reactive *E. coli* strain. This was confirmed with both the binding assay and with fluorescence microscopy. Bacterial cells were incubated with FSBA, which resulted in green fluorescing cells visible under a microscope, indicating that the SBA lectin binds to the surface of the cells. FSBA binding to glycoproteins of *Campylobacter* and recombinant *E. coli* provided a more direct indication of the interaction between these strains and SBA lectin. The main advantages of the use of FSBA were its simplicity and the quick confirmation of interaction between bacterial cells and SBA lectin. The presence of soluble GalNAc inhibited binding of recombinant *E. coli* cells to SBA lectin, confirming the specificity of binding. The results showing that *E. coli* lacking *pgl* did not interact with SBA lectin or FSBA further corroborate this.

The results obtained demonstrate the role of *C. jejuni* glycoprotein adhesins in the host-pathogen interaction. However, in order to develop a successful lectin-based *in vitro* assay for large-scale commercial application, further research with different *C. jejuni* strains and lectin types should be performed along with tests to

ensure durability of the assay after storage and within different laboratories. Although the developed system was used to test attachment of *C. jejuni* to SBA lectin, this method could also be used for the investigation of the interaction of other bacterial species and strains with purified host cell receptors or their analogues.

4.2 The roles of glycoproteins PEB3 and JlpA in adhesion to SBA lectin

In this study it was shown that some N-linked glycoproteins interact with immobilised SBA. However, it was still unclear which glycoprotein was interacting with the analogue of the host cell receptor. Among the N-linked glycoproteins that can act as an adhesin is PEB3, a highly immunogenic protein that was previously shown to have an affinity for SBA lectin (Pei *et al.*, 1991; Young *et al.*, 2002; Linton *et al.*, 2002). In this study, in order to investigate the involvement of PEB3 protein in adhesion the *peb3* gene was mutated. Any observed differences in binding between the wild type and *peb3* insertional knockout could indicate the involvement of PEB3 in *C. jejuni* binding to SBA lectin. In addition, the ability of the *peb3* mutant to grow on agar plates suggests that *peb3* is not an essential gene for survival *in vitro*, although this may be different *in vivo*.

Following inactivation of *peb3*, the mutant was tested using the developed binding assay. The results showed a difference in the amount of binding to immobilised SBA lectin between the mutant and wild type strains. Several experiments provided consistent results that demonstrated a reduced binding of the *peb3* mutant. Complementation of *peb3* mutation using the pRRC delivery plasmid resulted in restoration of binding and confirmation that a functional PEB3 is required for binding. However, mutation of *peb3* did not completely eliminate

binding, suggesting that the presence of other cell surface structures may compensate the role of inactivated PEB3 protein. This hypothesis was supported by inhibition of the residual binding of the *peb3* mutant in the presence of free soluble SBA lectin.

PEB3 was among the first identified glycosylated proteins of *Campylobacter* (Linton *et al.*, 2002), however the biological role of this protein is still not clear. PEB3 has 52% amino acid sequence identity with the putative adhesin Paa from *E. coli* (97% coverage with E-value 3e-83 ($3x10^{-83}$). Paa annotation suggests that the protein is involved in initial bacterial adhesion and may have specificity toward heparin sulfate receptors on the host cell surface (Batisson *et al.*, 2003). PEB3 is predicted to contain a sulphate-binding domain which may be associated with microbial adherence, however, purified wild type PEB3 did not show any interaction with sulphated oligosaccharides (Min *et al.*, 2009). PEB3 might carry a dual function as an adhesin and transport protein (Rangarajan *et al.*, 2007; Min *et al.*, 2009), similar to protein PEB1 (Leon-Kempis *et al.*, 2006). Despite knowledge that both of these proteins are major antigenic proteins of *C. jejuni* (Pei *et al.*, 1993), mutation of *peb3* in this study may not have completely abrogated cell binding but instead may have resulted in a deficiency of the transport functions of PEB3, which were not examined here.

Results from this study imply that PEB3 is involved in the interaction with GalNAc-specific lectin receptors on the host cells. It is known that there are many structurally different glycans present on the surface of human pathogens, which target glycan-binding protein lectins, such as C-type lectins. Some of them, including MGL, share binding specificity with SBA, used in this study. Thus the interaction of PEB3 with the lectins present on immune cells may assist in evasion of the host immune response, similar to other pathogens, where they specifically

target C-type lectin receptors to escape immune surveillance (Cambi *et al.*, 2005; Lugo-Villarino *et al.*, 2011).

Due to the presence of residual binding with the *peb3* mutant, insertional mutagenesis of *jlpA*, which encodes a confirmed glycoprotein adhesin, was performed. An isogenic *jlpA* mutant was constructed via insertion of the chloramphenicol resistance cassette instead of the kanamycin cassette, to allow the generation of a double (e.g. *peb3-jlpA*) mutant. However, mutation in the *jlpA* gene did not reduce *C. jejuni* binding to immobilised SBA lectin, thus the creation of a double mutant was not necessary. Among the possible reasons explaining these results was the absence of *jlpA* expression *in vitro*. It is also possible that JlpA did not interact with SBA due to instability of JlpA in the 11168H strain. Earlier it was reported that NCTC11168 JlpA is glycosylated only at one site compared with other strains, which may affect the stability of the glycoprotein (Scott *et al.*, 2009). Even though it was reported that *jlpA* mutation affects adhesion of *C. jejuni* to human epithelial cells, other studies have shown that the *jlpA* mutation had no effect on binding to host cells (Flanagan *et al.*, 2009; Novik *et al.*, 2010). This discrepancy might be the result of different cell lines, which were used for different experiments, suggesting that only certain host cells produce JlpA receptors.

Since the binding of *jlpA* mutants was not affected, and presence of residual binding in the 11168H/*peb3::kan*^{r} mutant strain was demonstrated, this suggests the presence of other unknown GalNAc-containing adhesins in *C. jejuni*. In this study, two further N-linked glycoproteins were identified as putative adhesins using bioinformatics tools. Out of 38 known N-linked glycoproteins one was predicted to be secreted and another one had an adhesin family signature. These

proteins may be involved in interaction with SBA lectin and it is suggested that this should be the target for future studies.

In addition, there was a possibility that LOS of *C. jejuni* could also be involved in the interaction with SBA lectin. LOS is known to be involved in adhesion, invasion and evasion of the host immune response (Louwen *et al.*, 2008; Guerry *et al.*, 2000). In addition, it was also shown that *C. jejuni* strains that have a terminal GalNAc residue in LOS interact with MGL (van Sorge *et al.*, 2009). However LOS of NCTC11168 does not have a terminal GalNAc structure, thus it is unlikely that the presence of residual binding was due to LOS.

4.3 Role of capsule in the host-pathogen interaction

Capsule is a well-known virulence factor in various pathogens, including *Campylobacter* spp. Although *C. jejuni* CPS is known to be an essential virulence factor, the role of capsule in pathogen-host interaction has not been extensively studied. In order to investigate whether the presence of the capsule affects binding of *C. jejuni* cells to the immobilised analogue of a host cell receptor, an acapsulated mutant of 11168H was tested using the same binding assay.

A previously constructed *kpsM* mutant was first confirmed for the presence of the kanamycin cassette in the *kpsM* gene using PCR with specific primers. The isogenic acapsulated mutant showed an increase in binding to immobilised SBA suggesting a negative effect of capsule on bacterial attachment. The inhibition of binding in the presence of free soluble SBA lectin and soluble GalNAc also confirmed specificity of binding. In addition, the specificity of the interaction was also confirmed by treatment of whole cells with exoglycosidase, indicating that binding occurs due to glycoproteins that have GalNAc residues.

We have found that PEB3 protein and capsule have different effects on binding of *C. jejuni* cells to SBA lectin. The presence of capsule in some pathogens may shield bacterial surface structures, such as adhesins (Schembri *et al.*, 2004), which is what may be occurring in our study. It has been shown that the F1 capsule of *Yersinia pestis* prevents interaction of fimbrial adhesins with receptors on the host cell (Runco *et al.*, 2008). Similarly, in another pathogen, *Klebsiella pneumonia,* capsule prevents adhesins from binding to epithelial cells (Sahly *et al.*, 2000). We therefore performed gene expression studies for *kpsM* and *peb3* genes using RT-PCR in order to see if these genes are differentially regulated.

Gene expression studies using samples obtained from liquid culture media exhibited some variation between biological replicates. However, after repeating the experiment a number of times it was possible to get consistency in the results. *Campylobacter* is able to turn CPS expression on and off (Bacon *et al.*, 2001), which may suggest that the presence or absence of capsule might be important at different stages of infection or in the bacterial life cycle. Results of RT-PCR studies showed an increase of *peb3* and a decrease of *kpsM* gene expression over time, suggesting that a shielding effect by the capsule may be essential at the initial stages of infection, hiding bacterial cell surface structures from the immune system. Subsequent down-regulation of CPS production during colonisation may lead to exposure of other bacterial cell surface structures required for the attachment and/or evasion of the host immune response. Reduction in expression of CPS was earlier reported in *C. jejuni* cells that were co-cultured with epithelial cells (Corcionivoschi *et al.*, 2009). Together these findings suggest variable modulation of CPS expression during infection.

The presence of capsule in some pathogens aids in immune response evasion (Raffatellu *et al.*, 2005; Unkmeir *et al.*, 2002). For example, in *Salmonella* spp.

the presence of capsule inhibits expression of host IL-8 via capsule-mediated inhibition of TLR signalling (Raffatellu *et al.*, 2005). A similar effect was present in *Neisseria meningitides,* where capsule inhibits TLR2 activation, and aids in adhesion to phagocytes (Unkmeir *et al.*, 2002). Similarly, in *C. jejuni,* it was shown that an acapsulated mutant or strain with modification of MeOPN induced expression of IL-6, IL-10 and TNF-α in mouse DCs when compared with the wild type strain (Rose *et al.*, 2012). A more recent study reported that the presence of CPS in the wild type resulted in reduced activation of both TLR2 and TLR4 (Maue *et al.*, 2013). These findings suggest that CPS helps *Campylobacter* to evade the host immune response during the initial stages of infection, which in turn helps to establish colonisation of the host.

4.4 The role of N-linked glycoproteins in binding to host cell receptors

Since the mutation of the *peb3* gene resulted in a reduction in bacterial binding, the binding abilities of purified PEB3 protein were investigated. In order to optimise purification of PEB3, His-tagged PEB3 was constructed. The use of the His-tag allowed the identification of the protein after purification using antibodies toward histidine. In addition, the presence of His-tag would also help to identify the PEB3 interaction with host cell receptors in the assay. The pRRBC plasmid was used to regulate *peb3* over-expression. This is the first regulated gene expression system for *C. jejuni* (Ikeda, 2014). The pRR was used as the basis in pRRBC to enable integration into the *C. jejuni* genome, as it encodes for several *Campylobacter*-specific non-coding rRNA gene clusters. As these sites are highly conserved and redundant, the insertion of the construct should therefore not have a major effect on the cell function (Karlyshev and Wren, 2005). It was assumed that the use of the pRRBC system would allow the purification of glycosylated

PEB3 from *C. jejuni*. In order to show if the system was functional, it was first successfully tested within *E. coli*. The disadvantage of this system was the inability to induce expression of the *peb3* gene in *C. jejuni* due to the absence of arabinose transporters within *C. jejuni* to induce the construct. *Campylobacter* metabolism depends mainly on amino acids rather than sugars, which are the main source of energy for *E. coli* (Guccione *et al.*, 2008; Stahl *et al.*, 2012). Thus, using the pRRBC system it was only possible to express non-glycosylated PEB3 from recombinant *E. coli*. To create a system that would allow arabinose induction in *C. jejuni* the use of the *E. coli* sugar transport gene *araE* was considered. The *araE* encodes a component of the high-affinity L-arabinose transport system and it is one of the operons of the L-arabinose regulon (Englesberg and Wilcox, 1974). In order to improve the pRRBC system, attempts to add an *araE* in pRRBC were performed (data not shown). Unfortunately, this work could not be completed due to time constraints.

Meanwhile, another approach was used to obtain glycosylated forms of PEB3. Taking into account the absence of a sugar transporter within *C. jejuni,* the use of two plasmids (pBAD33 and pPGL1) enabled purification of glycosylated PEB3 from *E. coli*. The pBAD33 plasmid (Guzman *et al.*, 1995) that contains the P_{BAD} promoter of the L-arabinose operon and its regulatory gene *araC* facilitated expression and subsequent purification of non-glycosylated PEB3 from *E. coli* using the pBAD_peb3 construct. Introduction of pBAD_peb3 into the recombinant *E. coli*/pPGL strain allowed regulatory expression and purification of glycosylated PEB3 from *E. coli*. Following induction with arabinose, the rate of growth (according to OD_{600}) of the induced culture was decreased compared to the uninduced culture. This was explained by divergence of energy to target protein synthesis. The advantage of this system was the ability to purify C-

terminal His-tagged glycosylated PEB3 from *E. coli* rather than from *C. jejuni*. To confirm that recombinant *E. coli* with the pBAD_peb3/pPGL construct expressed glycosylated PEB3 protein, it was compared to PEB3 purified from *C. jejuni*. Using the pRRC system the purification of His-tagged fusion PEB3 protein from *C. jejuni* was successfully performed. The pRRC plasmid was designed for constitutive expression and it had previously been suggested that it could be used for isolation of functional protein complexes (Karlyshev and Wren, 2005). Comparison of the two proteins using Western blotting, as well as exoglycosidase treatment, verified that the pBAD_peb3/pPGL system could be used for expression and purification of glycosylated PEB3 protein.

Purified glycosylated PEB3 was used in attempts to identify receptors in different types of human whole cell lysates. The fact that *Campylobacter* interacts with human intestinal cells was the reason for investigating the effect with normal intestinal cell lysates as well as Caco-2 cell lysates. In addition, the use of THP-1 cell lysate was considered. THP-1 cells are known to mimic monocytes and macrophages, resembling them in morphology and differentiation properties (Tsuchiya *et al.*, 1980). THP-1 cells were selected because N-linked glycoproteins interact with MGL receptors on macrophages. Unfortunately, it was not possible to identify any interaction of purified PEB3 with the receptors of host cells using Western blotting. This might be due to inactivation of binding sites of the host cells during denaturing the host cell lysates. However, some Western blotting experiments suggest that after denaturation proteins still retain active epitopes allowing their detection using specific antibodies (Jin *et al.*, 2003). Moreover, some proteins retain full enzymatic activity after denaturation and PAGE. The phenomenon is actually a basis of Zymography when many protein activities can still be detected after denaturing gel electrophoresis (Karlyshev *et al.*, 2014).

Furthermore, commercially available lysates of host cells, which were specifically designed for blotting, were used in these assays. Therefore, the experimental design used in this thesis was appropriate, as SDS gel electrophoresis was used as the first step in an attempt to detect host cell receptors, which is quite a challenging task. Future work may involve blotting with native proteins as well as other approaches.

Another reason explaining absence of interaction of purified PEB3 with the receptors of host cells might be due to production of a low yield of purified glycosylated PEB3. This might be caused either by low PEB3 expression, low binding in the column during large scale protein purification, or elution problems. Two purification methods were used, including column and beads, with various amendments to the methodology including increasing the volume of liquid culture as well as incubation time. However, all variations still produced low yields of the protein. As a means to check if there was a loss of protein during the purification procedure, a number of experiments were performed using recombinant and wild type *E. coli* (as wild type *E. coli* does not produce PEB3). These experiments confirmed that the low yield of protein was not caused by the loss of protein during the purification process. Among the possible reasons for low protein yield it is suggested that the protein was either unstable or toxic to the cells in higher concentrations. Another possible reason that prevented the detection of binding was the potential loss of the His-tag residue due to degradation during incubation of blots.

Previously, recombinant PEB3 protein was expressed and purified from *E. coli* using different constructs (pET*peb3* / pACYC*pgl* and pET*peb3* / pNBpgl) (Linton *et al.*, 2005; van Sorge *et al.*, 2009). In both cases PEB3 protein was designed to incorporate a 6-His residue tag in the C-terminal, to express the protein on the

surface of *E. coli* cells. In this study the same approach was applied for expression of the PEB3 on the surface of the *E. coli* cells (6-His residue tag incorporated onto the C-terminal) but using different constructs (pBAD_peb3/ pPGL1). Although it was possible to express glycosylated protein using *E. coli*, the yield of protein was unfortunately not high enough to identify its interaction with putative host cell receptors. It is not clear if other cited studies managed to express this protein at higher levels, thus comparison of efficiency of our construct was not possible. Identification of any potential host cell receptors using whole cell lysates was not possible due to the low yield of PEB3 protein. Therefore, recombinant *E. coli* cells harbouring pPGL plasmid were used to check the presence of any interaction between receptors of the host cell. *E. coli*/pPGL is able to glycosylate bacterial lipopolysaccharide, resulting in exposure of GalNAc residues on the cell surface. The developed binding assay results, as well as confocal microscopy, confirmed GalNAc-dependent binding of recombinant *E. coli* to immobilised SBA lectin. Thus, it was hypothesised that GalNAc residues on the surface of *E. coli*/pPGL cells may interact with host cell receptors. Blots with human whole cell lysates were probed with whole *E. coli*/pPGL cells to check whether glycosylated bacterial lipopolysaccharides would interact with human whole cell lysates. *E. coli*/pPGL expressing GalNAc residues were shown to interact with whole cell lysates immobilised on a PVDF membrane. No interaction was detected when a recipient *E. coli* strain was used. The interaction of *E. coli*/pPGL expressing surface GalNAc residues with THP-1 whole cell lysates was found to be specific, as it was inhibited in the presence of free SBA lectin.

SBA lectin has the same binding specificity as C-type lectins (including MGL receptors) produced by host cells. GalNAc residues are specifically recognised by SBA and human MGL receptors, which are expressed by macrophages and DCs

(Higashi *et al.*, 2002; van Vliet *et al.*, 2008a). MGL binding to GalNAc limits the migration of immature DCs (van Vliet *et al.*, 2008b), which may result in antigen presentation and uptake in MHC class II (Napoletano *et al.*, 2007; van Vliet *et al.*, 2007). It was previously shown that MGL is able to recognise a GalNAc-containing antigen of the helminth parasite *Schistosoma mansoni* and it was suggested that human DCs use MGL to internalise soluble egg antigen (SEA) of *S. mansoni* (van Vliet *et al.*, 2005). MGL receptors can also recognise members of the filoviridae family (Takada *et al.*, 2002). *N. gonorrhoeae* LOS phenotype C was the first described bacterial ligand to human MGL (van Vliet *et al.*, 2009). *N. gonorrhoeae* LOS phenotype C also carries terminal GalNAc residues (Yamasaki *et al.*, 1991a; Yamasaki *et al.*, 1991b). Targeting different C-type lectins (including MGL) enables *N. gonorrhoeae* to shift the immune response in favour of bacterial survival, via alteration in DC cytokine production (van Vliet *et al.*, 2008a).

Previously it was reported that *C. jejuni* N-linked glycans limit cytokine production, as the absence of a functional MGL ligand (GalNAc) induces secretion of IL-6 by DCs (van Sorge *et al.*, 2009). The role of human MGL receptors in *C. jejuni* infection is not clear. However, *C. jejuni* interaction with MGL is N-glycan-mediated and may subvert the host immune response. As stated earlier, interaction of *C. jejuni* cell surface structures with C-type lectins may result in evasion of host immune response like in other pathogens (Cambi *et al.*, 2005; Lugo-Villarino *et al.*, 2011). Results of blotting experiments with human whole cell lysates indirectly suggest that N-linked glycoproteins are involved in the interaction with host immune cells. However, further studies using cell line cultures need to be performed to support this hypothesis.

4.5 The use of *E. coli*/pPGL as an inhibitor of binding

The use of carbohydrate-binding antagonists may be an attractive approach for anti-adhesion therapy. In particular, the use of adhesin analogues as anti-adhesive agents may be beneficial for *Campylobacter* infection control. This strategy is based on the premise that some soluble molecules can mimic adhesins present on the bacterial cells and compete for adhesion with host cell receptors (Ofek *et al.*, 2003). In this study it is hypothesised that surface structures of *E. coli*/pPGL may inhibit binding of *C. jejuni* to immobilised SBA. Previously this system has been used for engineering diverse glycan structures that can be transferred onto recombinant proteins in *E. coli* through an N-linkage (Linton *et al.*, 2005).

It was earlier suggested that *pgl* gene locus-generated polysaccharides are incorporated into recombinant *E. coli* LPS or enterobacterial common antigen (ECA), which indicates that N-linked glycosylation and glycolipid biosynthesis have similar mechanisms (van Sorge *et al.*, 2009). In addition, similarity between *E. coli* and *C. jejuni* biosynthesis pathways were shown, when *E. coli* WecA protein, involved in glycolipid synthesis, substituted the activity of *C. jejuni* PglC in N-linked glycosylation (Linton *et al.*, 2005). As *E. coli*/pPGL has high affinity to SBA lectin, it may be used to block binding sites to prevent adhesion of *C. jejuni*. To test this hypothesis, *in vitro* inhibition experiments were conducted. In order to assess the anti-adhesive properties of recombinant *E. coli* strain against *C. jejuni*, *E. coli*/pPGL cells were pre-incubated with immobilised SBA, followed by incubation of *C. jejuni* cells.

The use of anti-*Campylobacter* antibodies to check inhibition proved ineffective due to the cross-reactivity with the *E. coli*/pPGL strain. However, preliminary results of qPCR confirmed that *E. coli*/pPGL strain indeed was able to inhibit *C. jejuni* binding to the analogue of host cell receptors, as the number of *C. jejuni*

cells was fewer in the wells that were pre-incubated with *E.coli*/pPGL compared with wells that only had *C. jejuni.*

These findings look promising, as they can be used for further development of inhibitors of *C. jejuni* attachment to host cell receptors and hence for the treatment or prevention of *Campylobacter* infection.

4.6 Conclusion

The results of this study demonstrate the feasibility of a new technique for measuring *C. jejuni* binding to SBA lectin. This novel technique was validated through microscopy, competitive inhibition studies and exoglycosidase treatment. Additionally, it was tested successfully using *C. jejuni* strain 11168H and its isogenic mutants: 11168H/*peb3,* 11168H/*jlpA* and 11168H/*kpsM.*

The study confirmed PEB3 as one of the cell surface glycoproteins required for bacterial interaction with an analogue of host cell receptors. In contrast, JlpA glycoprotein adhesin was shown not to be involved in this interaction. The study confirms the existence of GalNAc-containing cell surface structures able to interact with SBA lectin other than what is currently known in the published literature.

The results of the developed binding assay indirectly suggest that PEB3 plays a role in the interaction with receptors present on macrophages and DCs, which may lead to evasion of the host immune response. Additionally, it was shown that the presence of capsule has an interfering effect on bacterial attachment and that the genes involved in capsule and PEB3 adhesin biosynthesis are differentially regulated *in vitro.* It is likely that the developed methods could be applied to study *C. jejuni* interactions with other putative host cell receptors.

Additionally, in this study the *C. jejuni* N-linked glycosylation machinery was used to produce a glycosylated recombinant protein in *E. coli.* Using a recombinant *E. coli* strain producing LPS-like surface molecules it was shown that whole cells specifically interact with THP-1 cell lysate and are able to inhibit binding of *C. jejuni* cells to analogue of host cell receptors. The elucidation of the specific interactions of *C. jejuni* with analogues of host cell receptors could result in the development of inhibitors, which will prevent bacterial attachment, leading to the development of novel specific antibacterials.

4.7 Future Work

In the course of this study, other N-linked glycoproteins – Cj0143 and Cj0114 – that may act as an adhesins, were identified. It would therefore be interesting to study the *cj0143* and *cj0114* knock-out mutants similar to the *peb3* and *jlpA* mutants in order to establish their adhesive properties. Following confirmation of interaction between SBA lectin and these proteins and subsequent purification of both proteins one could study their interaction with host cell receptors using host cell lysates and/or cell culture.

Although in this study PEB3 was shown to play a role in adhesion, it has not been possible yet to demonstrate the interaction directly with any host cell receptors. Thus, further attempts at expression and purification of this protein should be considered.

Purification of PEB3 could be achieved by incorporation of a 6-His residue tag onto the N-terminus, instead of the C- terminus. A higher yield of PEB3 protein would allow investigation of its interaction with host cell receptors. It could also be used in adhesion and inhibition assays using cell culture methods. In order to get detailed structure crystalisation of recombinant protein might be considered.

Moreover, in order to confirm that PEB3 is indeed an adhesion factor, the use of a knock-out mutant and complemented derivative in binding experiments using cell line cultures may be performed. The production of antibodies against PEB3 protein would be the next logical step. The use of antibodies would allow examination of inhibition of PEB3 binding to epithelial or immune cells. Following confirmation of PEB3 binding to host cells, identification of PEB3 host cell receptors will be of interest.

The results of gene expression studies demonstrated that *peb3* and *kpsM* are differentially regulated in liquid culture. It would be interesting to investigate whether *peb3* and *kpsM* are also differentially regulated during the infection process. Based on these results and previous research in this field it is suggested that capsule and N-linked glycoproteins are involved in the interaction with immune cells. As *in vivo* studies of immune responses to *C. jejuni* are complex, *kpsM* and *peb3* mutants and the effects of wild type strains of *C. jejuni* could be studied on cell lines of macrophages or DCs first. This approach should help to develop a better understanding of the response generated by immune cells.

These results provide the first evidence that recombinant *E. coli* cells with a functional *pgl* locus may inhibit *C. jejuni* binding to an analogue of a host cell receptor. Further studies could test these results using other methods such as cell culture and microscopy.

Additionally, it would be interesting to test if the interaction between whole *E. coli* recombinant cells and DCs or THP-1 cells could be reproduced using cell culture methods. Also the ability of recombinant glycoproteins to detach already attached *C. jejuni* cells should be investigated.

The recombinant *E. coli* glycoproteins may act as analogues of adhesins by masking bacterial adhesins and blocking their access to host cell receptors.

Another approach in antimicrobial development would include further research to test if these recombinant glycoproteins successfully and continually mask bacterial adhesins, and can therefore be used as a basis for the development of novel antibacterials.

Improved understanding of bacterial adhesins is the key to the development of appropriate adhesion blockers. Effective production and delivery of these adhesion blockers to the host could lead to a major pharmaceutical breakthrough.

References

Abu-Lail, N. I. and Camesano, T. A. (2003) 'Role of lipopolysaccharides in the adhesion, retention, and transport of *Escherichia coli* JM109', *Environmental Science & Technology,* 37 (10), pp. 2173-2183.

Ader, F., Le Berre, R., Fackeure, R., Raze, D., Menozzi, F. D., Viget, N., Faure, K., Kipnis, E., Guery, B., Jarraud, S., Etienne, J. and Chidiac, C. (2008) '*In vivo* effect of adhesion inhibitor heparin on *Legionella pneumophila* pathogenesis in a murine pneumonia model', *Intensive Care Medicine,* 34 (8), pp. 1511-1519.

Alemka, A., Nothaft, H., Zheng, J. and Szymanski, C. M. (2013) 'N-glycosylation of *Campylobacter jejuni* surface proteins promotes bacterial fitness', *Infection and Immunity,* 81 (5), pp. 1674-1682.

Allos, B. M. (1997) 'Association between *Campylobacter* infection and Guillain-Barre syndrome', *The Journal of Infectious Diseases,* 176 Suppl 2 pp. S125-8.

Allos, B. M. (2001) '*Campylobacter jejuni* Infections: update on emerging issues and trends', *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America,* 32 (8), pp. 1201-1206.

Altekruse, S. F., Stern, N. J., Fields, P. I. and Swerdlow, D. L. (1999) '*Campylobacter jejuni*--an emerging foodborne pathogen', *Emerging Infectious Diseases,* 5 (1), pp. 28-35.

Alvarez, C. P., Lasala, F., Carrillo, J., Muniz, O., Corbi, A. L. and Delgado, R. (2002) 'C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans', *Journal of Virology,* 76 (13), pp. 6841-6844.

Ang, C. W., Laman, J. D., Willison, H. J., Wagner, E. R., Endtz, H. P., De Klerk, M. A., Tio-Gillen, A. P., Van den Braak, N., Jacobs, B. C. and Van Doorn, P. A. (2002) 'Structure of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity and clinical features of Guillain-Barre and Miller Fisher patients', *Infection and Immunity,* 70 (3), pp. 1202-1208.

Asakura, H., Yamasaki, M., Yamamoto, S. and Igimi, S. (2007) 'Deletion of *peb4* gene impairs cell adhesion and biofilm formation in *Campylobacter jejuni*', *FEMS Microbiology Letters,* 275 (2), pp. 278-285.

Ashgar, S. S., Oldfield, N. J., Wooldridge, K. G., Jones, M. A., Irving, G. J., Turner, D. P. and Ala'Aldeen, D. A. (2007) 'CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut', *Journal of Bacteriology,* 189 (5), pp. 1856-1865.

Avrain, L., Vernozy-Rozand, C. and Kempf, I. (2004) 'Evidence for natural horizontal transfer of tetO gene between *Campylobacter jejuni* strains in chickens', *Journal of Applied Microbiology,* 97 (1), pp. 134-140.

Avril, T., Wagner, E. R., Willison, H. J. and Crocker, P. R. (2006) 'Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated

glycans expressed on *Campylobacter jejuni* lipooligosaccharides', *Infection and Immunity,* 74 (7), pp. 4133-4141.

Babakhani, F. K., Bradley, G. A. and Joens, L. A. (1993) 'Newborn piglet model for campylobacteriosis', *Infection and Immunity,* 61 (8), pp. 3466-3475.

Bachtiar, B. M., Coloe, P. J. and Fry, B. N. (2007) 'Knockout mutagenesis of the *kpsE* gene of *Campylobacter jejuni* 81116 and its involvement in bacterium-host interactions', *FEMS Immunology and Medical Microbiology,* 49 (1), pp. 149-154.

Backert, S. and Hofreuter, D. (2013) 'Molecular methods to investigate adhesion, transmigration, invasion and intracellular survival of the foodborne pathogen *Campylobacter jejuni*', *Journal of Microbiological Methods,* 95 (1), pp. 8-23.

Bacon, D. J., Szymanski, C. M., Burr, D. H., Silver, R. P., Alm, R. A. and Guerry, P. (2001) 'A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176', *Molecular Microbiology,* 40 (3), pp. 769-777.

Barrow, P. A. and Page, K. (2000) 'Inhibition of colonisation of the alimentary tract in young chickens with *Campylobacter jejuni* by pre-colonisation with strains of *C. jejuni*', *FEMS Microbiology Letters,* 182 (1), pp. 87-91.

Batisson, I., Guimond, M. P., Girard, F., An, H., Zhu, C., Oswald, E., Fairbrother, J. M., Jacques, M. and Harel, J. (2003) 'Characterization of the novel factor paa involved in the early steps of the adhesion mechanism of attaching and effacing *Escherichia coli*', *Infection and Immunity,* 71 (8), pp. 4516-4525.

Bax, M., Kuijf, M. L., Heikema, A. P., van Rijs, W., Bruijns, S. C., Garcia-Vallejo, J. J., Crocker, P. R., Jacobs, B. C., van Vliet, S. J. and van Kooyk, Y. (2011) '*Campylobacter jejuni* lipooligosaccharides modulate dendritic cell-mediated T cell polarization in a sialic acid linkage-dependent manner', *Infection and Immunity,* 79 (7), pp. 2681-2689.

Bermudez, L. and Sangari, F.(2000) 'Mycobacterial invasion of epithelial cells', in Oelschlaeger, T. A. &Hacker, J. H. (eds.) *Bacterial Invasion into Eukaryotic Cells.* New York: Plenum Publishers, pp. 231-249.

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P. and Blaser, M. J. (1988) 'Experimental *Campylobacter jejuni* infection in humans', *The Journal of Infectious Diseases,* 157 (3), pp. 472-479.

Blaser, M. J. and Engberg, J.(2008) 'Clinical aspects of *Campylobacter jejuni* and *Campylobacter coli* infections', in Nachamkin, I., Szymanski, C. M. and Blaser, M. J. (eds.) *Campylobacter.* Washington, DC: ASM Press, pp. 99-121.

Blaser, M. J. (1997) 'Epidemiologic and clinical features of *Campylobacter jejuni* infections', *The Journal of Infectious Diseases,* 176 Suppl 2 pp. S103-5.

Bouwman, L. I., Niewold, P. and van Putten, J. P. (2013) 'Basolateral invasion and trafficking of *Campylobacter jejuni* in polarized epithelial cells', *PloS One,* 8 (1), pp. e54759.

Boyd, A., Philbin, V. J. and Smith, A. L. (2007) 'Conserved and distinct aspects of the avian Toll-like receptor (TLR) system: implications for transmission and control of bird-borne zoonoses', *Biochemical Society Transactions,* 35 (Pt 6), pp. 1504-1507.

Bras, A. M. and Ketley, J. M. (1999) 'Transcellular translocation of *Campylobacter jejuni* across human polarised epithelial monolayers', *FEMS Microbiology Letters,* 179 (2), pp. 209-215.

Burr, D. H., Rollins, D., Lee, L. H., Pattarini, D. L., Walz, S. S., Tian, J. H., Pace, J. L., Bourgeois, A. L. and Walker, R. I. (2005) 'Prevention of disease in ferrets fed an inactivated whole cell *Campylobacter jejuni* vaccine', *Vaccine,* 23 (34), pp. 4315-4321.

Butzler, J. (2004) '*Campylobacter*, from obscurity to celebrity', *Clinical Microbiology and Infection,* 10 (10), pp. 868-876.

Caldwell, M. B., Guerry, P., Lee, E. C., Burans, J. P. and Walker, R. I. (1985) 'Reversible expression of flagella in *Campylobacter jejuni*', *Infection and Immunity,* 50 (3), pp. 941-943.

Cambi, A., Koopman, M. and Figdor, C. G. (2005) 'How C-type lectins detect pathogens', *Cellular Microbiology,* 7 (4), pp. 481-488.

Cameron, A., Frirdich, E., Huynh, S., Parker, C. T. and Gaynor, E. C. (2012) 'Hyperosmotic stress response of *Campylobacter jejuni*', *Journal of Bacteriology,* 194 (22), pp. 6116-6130.

Carrillo, C.,Loc, Atterbury, R.,J., El-Shibiny, A., Connerton, P.,L., Dillon, E., Scott, A. and Connerton, I.,F. (2005) 'Bacteriophage Therapy To Reduce *Campylobacter jejuni* Colonization of Broiler Chickens', *Applied and Environmental Microbiology,* 71 (11), pp. 6554-6563.

Casadevall, A. and Pirofski, L. (2001) 'Host-pathogen interactions: the attributes of virulence', *The Journal of Infectious Diseases,* 184 (3), pp. 337-344.

Centers for Disease Control and Prevention (2014) *Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food — Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2006–2013.* edn. CDC. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6315a3.htm [Accessed March 2015]

Centers for Disease Control and Prevention (2009) *Preliminary FoodNet Data on the incidence of infection with pathogens transmitted commonly through food--10 States, 2008.* edn. CDC. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5813a2.htm [Accessed March 2015]

Chamovitz, B. N., Hartstein, A. I., Alexander, S. R., Terry, A. B., Short, P. and Katon, R. (1983) '*Campylobacter jejuni*-associated hemolytic-uremic syndrome in a mother and daughter', *Pediatrics,* 71 (2), pp. 253-256.

Chen, M. L., Ge, Z., Fox, J. G. and Schauer, D. B. (2006) 'Disruption of tight junctions and induction of proinflammatory cytokine responses in colonic epithelial cells by *Campylobacter jejuni*', *Infection and Immunity,* 74 (12), pp. 6581-6589.

Cinco, M., Banfi, E., Ruaro, E., Crevatin, D. and Crotti, D. (1984) 'Evidence for l-fucose (6-deoxy-l-galactopyranose)-mediated adherence of *Campylobacter* spp. to epithelial cells', *FEMS Microbiology Letters,* 21 (3), pp. 347-351.

Cleary, P. P. and Cue, D. (2000) 'High frequency invasion of mammalian cells by beta hemolytic streptococci', *Sub-Cellular Biochemistry,* 33 pp. 137-166.

Coker, A. O., Isokpehi, R. D., Thomas, B. N., Amisu, K. O. and Obi, C. L. (2002) 'Human campylobacteriosis in developing countries', *Emerging Infectious Diseases,* 8 (3), pp. 237-244.

Cole, H. B., Ezzell, J. W.,Jr, Keller, K. F. and Doyle, R. J. (1984) 'Differentiation of *Bacillus anthracis* and other *Bacillus* species by lectins', *Journal of Clinical Microbiology,* 19 (1), pp. 48-53.

Connerton, P. L., Timms, A. R. and Connerton, I. F. (2011) '*Campylobacter* bacteriophages and bacteriophage therapy', *Journal of Applied Microbiology,* 111 (2), pp. 255-265.

Corcionivoschi, N., Clyne, M., Lyons, A., Elmi, A., Gundogdu, O., Wren, B. W., Dorrell, N., Karlyshev, A. V. and Bourke, B. (2009) '*Campylobacter jejuni* cocultured with epithelial cells reduces surface capsular polysaccharide expression', *Infection and Immunity,* 77 (5), pp. 1959-1967.

Cordwell, S. J., Len, A. C., Touma, R. G., Scott, N. E., Falconer, L., Jones, D., Connolly, A., Crossett, B. and Djordjevic, S. P. (2008) 'Identification of membrane-associated proteins from *Campylobacter jejuni* strains using complementary proteomics technologies', *Proteomics,* 8 (1), pp. 122-139.

Cotter, P. D., Hill, C. and Ross, R. P. (2005) 'Bacteriocins: developing innate immunity for food', *Nature Reviews. Microbiology,* 3 (10), pp. 777-788.

Cravioto, A., Tello, A., Villafan, H., Ruiz, J., del Vedovo, S. and Neeser, J. R. (1991) 'Inhibition of localized adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk', *The Journal of Infectious Diseases,* 163 (6), pp. 1247-1255.

Crocker, P. R., Paulson, J. C. and Varki, A. (2007) 'Siglecs and their roles in the immune system', *Nature Reviews.Immunology,* 7 (4), pp. 255-266.

Crushell, E., Harty, S., Sharif, F. and Bourke, B. (2004) 'Enteric campylobacter: purging its secrets?', *Pediatric Research,* 55 (1), pp. 3-12.

Dam, T. K. and Brewer, C. F. (2010) 'Lectins as pattern recognition molecules: the effects of epitope density in innate immunity', *Glycobiology,* 20 (3), pp. 270-279.

Dasti, J. I., Tareen, A. M., Lugert, R., Zautner, A. E. and Gross, U. (2010) '*Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms', *International Journal of Medical Microbiology : IJMM,* 300 (4), pp. 205-211.

Day, C. J., Semchenko, E. A. and Korolik, V. (2012) 'Glycoconjugates play a key role in Campylobacter jejuni Infection: interactions between host and pathogen', *Frontiers in Cellular and Infection Microbiology,* 2 pp. 9

Day, C. J., Tiralongo, J., Hartnell, R. D., Logue, C. A., Wilson, J. C., von Itzstein, M. and Korolik, V. (2009) 'Differential Carbohydrate Recognition by *Campylobacter jejuni* Strain 11168: Influences of Temperature and Growth Conditions', *Plos One,* 4 (3), e4927.

de Zoete, M. R., van Putten, J. P. and Wagenaar, J. A. (2007) 'Vaccination of chickens against *Campylobacter*', *Vaccine,* 25 (30), pp. 5548-5557.

Deghmane, A. E., Giorgini, D., Larribe, M., Alonso, J. M. and Taha, M. K. (2002) 'Down-regulation of pili and capsule of *Neisseria meningitidis* upon contact with epithelial cells is mediated by CrgA regulatory protein', *Molecular Microbiology,* 43 (6), pp. 1555-1564.

Dekeyser, P., Gossuin-Detrain, M., Butzler, J. P. and Sternon, J. (1972) 'Acute enteritis due to related vibrio: first positive stool cultures', *The Journal of Infectious Diseases,* 125 (4), pp. 390-392.

Dieffenbach, C. W., Lowe, T. M. and Dveksler, G. S. (1993) 'General concepts for PCR primer design', *PCR Methods and Applications,* 3 (3), pp. S30-7.

Donnenberg, M. S. (2000) 'Pathogenic strategies of enteric bacteria', *Nature,* 406 (6797), pp. 768-774.

Drickamer, K. (1999) 'C-type lectin-like domains', *Current Opinion in Structural Biology,* 9 (5), pp. 585-590.

Drickamer, K. and Taylor, M. E. (1998) 'Evolving views of protein glycosylation', *Trends in Biochemical Sciences,* 23 (9), pp. 321-324.

Eckmann, L. (2005) 'Defence molecules in intestinal innate immunity against bacterial infections', *Current Opinion in Gastroenterology,* 21 (2), pp. 147-151.

Elliott, K. T. and Dirita, V. J. (2008) 'Characterization of CetA and CetB, a bipartite energy taxis system in *Campylobacter jejuni*', *Molecular Microbiology,* 69 (5), pp. 1091-1103.

Ellis-Iversen, J., Pritchard, G. C., Wooldridge, M. and Nielen, M. (2009) 'Risk factors for *Campylobacter jejuni* and *Campylobacter coli* in young cattle on English and Welsh farms', *Preventive Veterinary Medicine,* 88 (1), pp. 42-48.

Engberg, J., Aarestrup, F. M., Taylor, D. E., Gerner-Smidt, P. and Nachamkin, I. (2001) 'Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates', *Emerging Infectious Diseases,* 7 (1), pp. 24-34.

Englesberg, E. and Wilcox, G. (1974) 'Regulation: positive control', *Annual Review of Genetics,* 8 pp. 219-242.

Eucker, T. P. and Konkel, M. E. (2012) 'The cooperative action of bacterial fibronectin-binding proteins and secreted proteins promote maximal *Campylobacter jejuni* invasion of host cells by stimulating membrane ruffling', *Cellular Microbiology,* 14 (2), pp. 226-238.

European Food Safety Authority (2011) 'Scientific Opinion on *Campylobacter* in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain'.

Fauchere, J. L., Kervella, M., Rosenau, A., Mohanna, K. and Veron, M. (1989) 'Adhesion to HeLa cells of *Campylobacter jejuni* and *C. coli* outer membrane components', *Research in Microbiology,* 140 (6), pp. 379-392.

Ferrero, R. L. and Lee, A. (1988) 'Motility of *Campylobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria', *Journal of General Microbiology,* 134 (1), pp. 53-59.

Figdor, C. G., van Kooyk, Y. and Adema, G. J. (2002) 'C-type lectin receptors on dendritic cells and Langerhans cells', *Nature Reviews.Immunology,* 2 (2), pp. 77-84.

Finlay, B. B. and McFadden, G. (2006) 'Anti-immunology: evasion of the host immune system by bacterial and viral pathogens', *Cell,* 124 (4), pp. 767-782.

Flanagan, R. C., Neal-McKinney, J. M., Dhillon, A. S., Miller, W. G. and Konkel, M. E. (2009) 'Examination of *Campylobacter jejuni* putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization', *Infection and Immunity,* 77 (6), pp. 2399-2407.

Friedman, C. R., Hoekstra, R. M., Samuel, M., Marcus, R., Bender, J., Shiferaw, B., Reddy, S., Ahuja, S. D., Helfrick, D. L., Hardnett, F., Carter, M., Anderson, B., Tauxe, R. V. and Emerging Infections Program FoodNet Working Group (2004) 'Risk factors for sporadic *Campylobacter* infection in the United States: A case-control study in FoodNet sites', *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 38 Suppl 3 pp. S285-96.

Friis, L. M., Pin, C., Pearson, B. M. and Wells, J. M. (2005) '*In vitro* cell culture methods for investigating *Campylobacter* invasion mechanisms', *Journal of Microbiological Methods,* 61 (2), pp. 145-160.

Fry, B. N., Feng, S., Chen, Y. Y., Newell, D. G., Coloe, P. J. and Korolik, V. (2000) 'The *galE* gene of *Campylobacter jejuni* is involved in lipopolysaccharide synthesis and virulence', *Infection and Immunity,* 68 (5), pp. 2594-2601.

Fry, B. N., Korolik, V., ten Brinke, J. A., Pennings, M. T., Zalm, R., Teunis, B. J., Coloe, P. J. and van der Zeijst, B. A. (1998) 'The lipopolysaccharide biosynthesis locus of *Campylobacter jejuni* 81116', *Microbiology (Reading, England),* 144 (Pt 8) (Pt 8), pp. 2049-2061.

Ganan, M., Martinez-Rodriguez, A. J., Carrascosa, A. V., Vesterlund, S., Salminen, S. and Satokari, R. (2013) 'Interaction of *Campylobacter* spp. and human probiotics in chicken intestinal mucus', *Zoonoses and Public Health,* 60 (2), pp. 141-148.

Garcia-Vallejo, J. J. and van Kooyk, Y. (2009) 'Endogenous ligands for C-type lectin receptors: the true regulators of immune homeostasis', *Immunological Reviews,* 230 (1), pp. 22-37.

Geijtenbeek, T. B., Engering, A. and Van Kooyk, Y. (2002) 'DC-SIGN, a C-type lectin on dendritic cells that unveils many aspects of dendritic cell biology', *Journal of Leukocyte Biology,* 71 (6), pp. 921-931.

Gerlach, R. G. and Hensel, M. (2007) 'Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens', *International Journal of Medical Microbiology : IJMM,* 297 (6), pp. 401-415.

Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J. and Madara, J. L. (2001) 'Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression', *Journal of Immunology (Baltimore, Md.: 1950),* 167 (4), pp. 1882-1885.

Gilbert, M., Godschalk, P. C., Karwaski, M. F., Ang, C. W., van Belkum, A., Li, J., Wakarchuk, W. W. and Endtz, H. P. (2004) 'Evidence for acquisition of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a patient with Guillain-Barre syndrome, by horizontal exchange', *Infection and Immunity,* 72 (2), pp. 1162-1165.

Gilbert, M., Karwaski, M. F., Bernatchez, S., Young, N. M., Taboada, E., Michniewicz, J., Cunningham, A. M. and Wakarchuk, W. W. (2002) 'The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni.* Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide', *The Journal of Biological Chemistry,* 277 (1), pp. 327-337.

Glover, K. J., Weerapana, E. and Imperiali, B. (2005) '*In vitro* assembly of the undecaprenylpyrophosphate-linked heptasaccharide for prokaryotic N-linked glycosylation', *Proceedings of the National Academy of Sciences of the United States of America,* 102 (40), pp. 14255-14259.

Godschalk, P. C., Heikema, A. P., Gilbert, M., Komagamine, T., Ang, C. W., Glerum, J., Brochu, D., Li, J., Yuki, N., Jacobs, B. C., van Belkum, A. and Endtz, H. P. (2004) 'The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barre syndrome', *The Journal of Clinical Investigation,* 114 (11), pp. 1659-1665.

Golden, N. J. and Acheson, D. W. (2002) 'Identification of motility and autoagglutination *Campylobacter jejuni* mutants by random transposon mutagenesis', *Infection and Immunity,* 70 (4), pp. 1761-1771.

Goulhen, F., De, E., Pages, J. M. and Bolla, J. M. (2004) 'Functional refolding of the *Campylobacter jejuni* MOMP (major outer membrane protein) porin by GroEL from the same species', *The Biochemical Journal,* 378 (Pt 3), pp. 851-856.

Gradel, K. O., Nielsen, H. L., Schonheyder, H. C., Ejlertsen, T., Kristensen, B. and Nielsen, H. (2009) 'Increased short- and long-term risk of inflammatory bowel disease after salmonella or *Campylobacter* gastroenteritis', *Gastroenterology,* 137 (2), pp. 495-501.

Grant, C. C., Konkel, M. E., Cieplak, W.,Jr and Tompkins, L. S. (1993) 'Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures', *Infection and Immunity,* 61 (5), pp. 1764-1771.

Guccione, E., Leon-Kempis Mdel, R., Pearson, B. M., Hitchin, E., Mulholland, F., van Diemen, P. M., Stevens, M. P. and Kelly, D. J. (2008) 'Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate', *Molecular Microbiology,* 69 (1), pp. 77-93.

Gueimonde, M., Jalonen, L., He, F., Hiramatsu, M. and Salminen, S. (2006) 'Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli', *Food Research International,* 39 (4), pp. 467-471.

Guerry, P., Alm, R. A., Power, M. E., Logan, S. M. and Trust, T. J. (1991) 'Role of two flagellin genes in *Campylobacter* motility', *Journal of Bacteriology,* 173 (15), pp. 4757-4764.

Guerry, P., Ewing, C. P., Hickey, T. E., Prendergast, M. M. and Moran, A. P. (2000) 'Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*', *Infection and Immunity,* 68 (12), pp. 6656-6662.

Guerry, P., Ewing, C. P., Schirm, M., Lorenzo, M., Kelly, J., Pattarini, D., Majam, G., Thibault, P. and Logan, S. (2006) 'Changes in flagellin glycosylation affect *Campylobacter* autoagglutination and virulence', *Molecular Microbiology,* 60 (2), pp. 299-311.

Guerry, P., Ewing, C. P., Schoenhofen, I. C. and Logan, S. M. (2007) 'Protein glycosylation in *Campylobacter jejuni*: partial suppression of *pglF* by mutation of *pseC*', *Journal of Bacteriology,* 189 (18), pp. 6731-6733.

Guerry, P. and Szymanski, C. M. (2008) '*Campylobacter* sugars sticking out', *Trends in Microbiology,* 16 (9), pp. 428-435.

Guerry, P., Szymanski, C. M., Prendergast, M. M., Hickey, T. E., Ewing, C. P., Pattarini, D. L. and Moran, A. P. (2002) 'Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness *in vitro*', *Infection and Immunity,* 70 (2), pp. 787-793.

Gundogdu, O., Bentley, S. D., Holden, M. T., Parkhill, J., Dorrell, N. and Wren, B. W. (2007) 'Re-annotation and re-analysis of the *Campylobacter jejuni* NCTC11168 genome sequence', *BMC Genomics,* 8 pp. 162.

Gustafsson, A., Hultberg, A., Sjostrom, R., Kacskovics, I., Breimer, M. E., Boren, T., Hammarstrom, L. and Holgersson, J. (2006) 'Carbohydrate-dependent inhibition of *Helicobacter pylori* colonization using porcine milk', *Glycobiology,* 16 (1), pp. 1-10.

Guzman, L. M., Belin, D., Carson, M. J. and Beckwith, J. (1995) 'Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter', *Journal of Bacteriology,* 177 (14), pp. 4121-4130.

Hahn, H. P. (1997) 'The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa*--a review', *Gene,* 192 (1), pp. 99-108.

Hakomori, S. (1984) 'Tumor-associated carbohydrate antigens', *Annual Review of Immunology,* 2 pp. 103-126.

Hanisch, F. G. (2001) 'O-glycosylation of the mucin type', *Biological Chemistry,* 382 (2), pp. 143-149.

Hannu, T., Kauppi, M., Tuomala, M., Laaksonen, I., Klemets, P. and Kuusi, M. (2004) 'Reactive arthritis following an outbreak of *Campylobacter jejuni* infection', *The Journal of Rheumatology,* 31 (3), pp. 528-530.

Hannu, T., Mattila, L., Rautelin, H., Pelkonen, P., Lahdenne, P., Siitonen, A. and Leirisalo-Repo, M. (2002) '*Campylobacter*-triggered reactive arthritis: a population-based study', *Rheumatology (Oxford, England),* 41 (3), pp. 312-318.

Hansson, G. C. (2012) 'Role of mucus layers in gut infection and inflammation', *Current Opinion in Microbiology,* 15 (1), pp. 57-62.

Havelaar, A. H., Mangen, M. J., de Koeijer, A. A., Bogaardt, M. J., Evers, E. G., Jacobs-Reitsma, W. F., van Pelt, W., Wagenaar, J. A., de Wit, G. A., van der Zee, H. and Nauta, M. J. (2007) 'Effectiveness and efficiency of controlling *Campylobacter* on broiler chicken meat', *Risk Analysis : An Official Publication of the Society for Risk Analysis,* 27 (4), pp. 831-844.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M. and Aderem, A. (2001) 'The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5', *Nature,* 410 (6832), pp. 1099-1103.

He, Y. and Chen, C. Y. (2010) 'Quantitative analysis of viable, stressed and dead cells of *Campylobacter jejuni* strain 81-176', *Food Microbiology,* 27 (4), pp. 439-446.

Heikema, A. P. (2013) *Host-Pathogen interaction in Guillain-Barre syndrome: The role of Campylobacter jejuni lipooligosaccharide sialylation.* Erasmus University, Rotterdam.

Heikema, A. P., Bergman, M. P., Richards, H., Crocker, P. R., Gilbert, M., Samsom, J. N., van Wamel, W. J., Endtz, H. P. and van Belkum, A. (2010) 'Characterization of the specific interaction between sialoadhesin and sialylated *Campylobacter jejuni* lipooligosaccharides', *Infection and Immunity,* 78 (7), pp. 3237-3246.

Helms, M., Simonsen, J., Olsen, K. E. and Molbak, K. (2005) 'Adverse health events associated with antimicrobial drug resistance in *Campylobacter* species: a registry-based cohort study', *The Journal of Infectious Diseases,* 191 (7), pp. 1050-1055.

Hendrixson, D. R. (2008) 'Restoration of flagellar biosynthesis by varied mutational events in *Campylobacter jejuni*', *Molecular Microbiology,* 70 (2), pp. 519-536.

Hendrixson, D. R. (2006) 'A phase-variable mechanism controlling the *Campylobacter jejuni* FlgR response regulator influences commensalism', *Molecular Microbiology,* 61 (6), pp. 1646-1659.

Hendrixson, D. R., Akerley, B. J. and DiRita, V. J. (2001) 'Transposon mutagenesis of *Campylobacter jejuni* identifies a bipartite energy taxis system required for motility', *Molecular Microbiology,* 40 (1), pp. 214-224.

Hendrixson, D. R. and DiRita, V. J. (2004) 'Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract', *Molecular Microbiology,* 52 (2), pp. 471-484.

Hendrixson, D. R. and DiRita, V. J. (2003) 'Transcription of sigma54-dependent but not sigma28-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus', *Molecular Microbiology,* 50 (2), pp. 687-702.

Hickey, T. E., Baqar, S., Bourgeois, A. L., Ewing, C. P. and Guerry, P. (1999) '*Campylobacter jejuni*-stimulated secretion of interleukin-8 by INT 407 cells', *Infection and Immunity,* 67 (1), pp. 88-93.

Hickey, T. E., Majam, G. and Guerry, P. (2005) 'Intracellular survival of *Campylobacter jejuni* in human monocytic cells and induction of apoptotic death by cytholethal distending toxin', *Infection and Immunity,* 73 (8), pp. 5194-5197.

Hickey, T. E., McVeigh, A. L., Scott, D. A., Michielutti, R. E., Bixby, A., Carroll, S. A., Bourgeois, A. L. and Guerry, P. (2000) '*Campylobacter jejuni* cytolethal

distending toxin mediates release of interleukin-8 from intestinal epithelial cells', *Infection and Immunity,* 68 (12), pp. 6535-6541.

Higashi, N., Fujioka, K., Denda-Nagai, K., Hashimoto, S., Nagai, S., Sato, T., Fujita, Y., Morikawa, A., Tsuiji, M., Miyata-Takeuchi, M., Sano, Y., Suzuki, N., Yamamoto, K., Matsushima, K. and Irimura, T. (2002) 'The macrophage C-type lectin specific for galactose/N-acetylgalactosamine is an endocytic receptor expressed on monocyte-derived immature dendritic cells', *The Journal of Biological Chemistry,* 277 (23), pp. 20686-20693.

Hofreuter, D., Tsai, J., Watson, R. O., Novik, V., Altman, B., Benitez, M., Clark, C., Perbost, C., Jarvie, T., Du, L. and Galan, J. E. (2006) 'Unique features of a highly pathogenic *Campylobacter jejuni* strain', *Infection and Immunity,* 74 (8), pp. 4694-4707.

Holmes, K., Mulholland, F., Pearson, B. M., Pin, C., McNicholl-Kennedy, J., Ketley, J. M. and Wells, J. M. (2005) '*Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur', *Microbiology (Reading, England),* 151 (Pt 1), pp. 243-257.

Holmskov, U., Thiel, S. and Jensenius, J. C. (2003) 'Collections and ficolins: humoral lectins of the innate immune defense', *Annual Review of Immunology,* 21 pp. 547-578.

Hori, K. and Matsumoto, S. (2010) 'Bacterial adhesion: From mechanism to control', *Biochemical Engineering Journal,* 48, pp. 424–434.

Hu, L., Bray, M. D., Osorio, M. and Kopecko, D. J. (2006a) '*Campylobacter jejuni* induces maturation and cytokine production in human dendritic cells', *Infection and Immunity,* 74 (5), pp. 2697-2705.

Hu, L. and Hickey, T. E. (2005) '*Campylobacter jejuni* induces secretion of proinflammatory chemokines from human intestinal epithelial cells', *Infection and Immunity,* 73 (7), pp. 4437-4440.

Hu, L. and Kopecko, D. J. (1999) '*Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells', *Infection and Immunity,* 67 (8), pp. 4171-4182.

Hu, L., McDaniel, J. P. and Kopecko, D. J. (2006b) 'Signal transduction events involved in human epithelial cell invasion by *Campylobacter jejuni* 81-176', *Microbial Pathogenesis,* 40 (3), pp. 91-100.

Hu, L., Tall, B. D., Curtis, S. K. and Kopecko, D. J. (2008) 'Enhanced microscopic definition of *Campylobacter jejuni* 81-176 adherence to, invasion of, translocation across, and exocytosis from polarized human intestinal Caco-2 cells', *Infection and Immunity,* 76 (11), pp. 5294-5304.

Hugdahl, M. B., Beery, J. T. and Doyle, M. P. (1988) 'Chemotactic behavior of *Campylobacter jejuni*', *Infection and Immunity,* 56 (6), pp. 1560-1566.

Hughes, R. A., Hadden, R. D., Gregson, N. A. and Smith, K. J. (1999) 'Pathogenesis of Guillain-Barre syndrome', *Journal of Neuroimmunology,* 100 (1-2), pp. 74-97.

Ikeda, N. (2014) *Molecular mechanisms of coccoid form formation in Campylobacter jejuni.* Unpublished PhD thesis. Kingston University.

Iovine, N. M., Pursnani, S., Voldman, A., Wasserman, G., Blaser, M. J. and Weinrauch, Y. (2008) 'Reactive nitrogen species contribute to innate host defense against *Campylobacter jejuni*', *Infection and Immunity,* 76 (3), pp. 986-993.

Jackson, D. N., Davis, B., Tirado, S. M., Duggal, M., van Frankenhuyzen, J. K., Deaville, D., Wijesinghe, M. A., Tessaro, M. and Trevors, J. T. (2009) 'Survival mechanisms and culturability of *Campylobacter jejuni* under stress conditions', *Antonie Van Leeuwenhoek,* 96 (4), pp. 377-394.

Jagusztyn-Krynicka, E. K., Laniewski, P. and Wyszynska, A. (2009) 'Update on Campylobacter jejuni vaccine development for preventing human campylobacteriosis', *Expert Review of Vaccines,* 8 (5), pp. 625-645.

Jain, D., Prasad, K. N., Sinha, S. and Husain, N. (2008) 'Differences in virulence attributes between cytolethal distending toxin positive and negative *Campylobacter jejuni* strains', *Journal of Medical Microbiology,* 57 (Pt 3), pp. 267-272.

Janssen, R., Krogfelt, K. A., Cawthraw, S. A., van Pelt, W., Wagenaar, J. A. and Owen, R. J. (2008) 'Host-pathogen interactions in *Campylobacter* infections: the host perspective', *Clinical Microbiology Reviews,* 21 (3), pp. 505-518.

Jarvis, G. A. and Vedros, N. A. (1987) 'Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation', *Infection and Immunity,* 55 (1), pp. 174-180.

Jeon, B., Muraoka, W. T. and Zhang, Q. (2010) 'Advances in *Campylobacter* biology and implications for biotechnological applications', *Microbial Biotechnology,* 3 (3), pp. 242-258.

Jin, S., Joe, A., Lynett, J., Hani, E. K., Sherman, P. and Chan, V. L. (2001) 'JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells', *Molecular Microbiology,* 39 (5), pp. 1225-1236.

Jin, S., Song, Y. C., Emili, A., Sherman, P. M. and Chan, V. L. (2003) 'JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90alpha and triggers signalling pathways leading to the activation of NF-kappaB and p38 MAP kinase in epithelial cells', *Cellular Microbiology,* 5 (3), pp. 165-174.

Johanesen, P. A. and Dwinell, M. B. (2006) 'Flagellin-independent regulation of chemokine host defense in *Campylobacterj ejuni*-infected intestinal epithelium', *Infection and Immunity,* 74 (6), pp. 3437-3447.

Johnson, W. M. and Lior, H. (1988) 'A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp', *Microbial Pathogenesis,* 4 (2), pp. 115-126.

Jones, C., Virji, M. and Crocker, P. R. (2003) 'Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake', *Molecular Microbiology,* 49 (5), pp. 1213-1225.

Jones, D. M., Sutcliffe, E. M. and Curry, A. (1991) 'Recovery of viable but non-culturable *Campylobacter jejuni*', *Journal of General Microbiology,* 137 (10), pp. 2477-2482.

Jones, M. A., Marston, K. L., Woodall, C. A., Maskell, D. J., Linton, D., Karlyshev, A. V., Dorrell, N., Wren, B. W. and Barrow, P. A. (2004) 'Adaptation of *Campylobacter jejuni* NCTC11168 to high-level colonization of the avian gastrointestinal tract', *Infection and Immunity,* 72 (7), pp. 3769-3776.

Jones, M. A., Totemeyer, S., Maskell, D. J., Bryant, C. E. and Barrow, P. A. (2003) 'Induction of proinflammatory responses in the human monocytic cell line THP-1 by *Campylobacter jejuni*', *Infection and Immunity,* 71 (5), pp. 2626-2633.

Kalischuk, L. D., Leggett, F. and Inglis, G. D. (2010) '*Campylobacter jejuni* induces transcytosis of commensal bacteria across the intestinal epithelium through M-like cells', *Gut Pathogens,* 2 pp. 14-4749-2-14.

Kalmokoff, M., Lanthier, P., Tremblay, T. L., Foss, M., Lau, P. C., Sanders, G., Austin, J., Kelly, J. and Szymanski, C. M. (2006) 'Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation', *Journal of Bacteriology,* 188 (12), pp. 4312-4320.

Kanipes, M. I., Holder, L. C., Corcoran, A. T., Moran, A. P. and Guerry, P. (2004) 'A deep-rough mutant of *Campylobacter jejuni* 81-176 is noninvasive for intestinal epithelial cells', *Infection and Immunity,* 72 (4), pp. 2452-2455.

Karlyshev, A. V., Wren, B. W. and Moran, A. (2008) '*Campylobacter jejuni* capsular polysaccharide.', in Nachamkin I, Szymanski CM and Blaser MJ (ed.) *Campylobacter* Washington, DC, USA: ASM Press, pp. 505-521.

Karlyshev, A. V., Champion, O. L., Churcher, C., Brisson, J. R., Jarrell, H. C., Gilbert, M., Brochu, D., St Michael, F., Li, J., Wakarchuk, W. W., Goodhead, I., Sanders, M., Stevens, K., White, B., Parkhill, J., Wren, B. W. and Szymanski, C. M. (2005) 'Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses', *Molecular Microbiology,* 55 (1), pp. 90-103.

Karlyshev, A. V., Everest, P., Linton, D., Cawthraw, S., Newell, D. G. and Wren, B. W. (2004) 'The *Campylobacter jejuni* general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks', *Microbiology (Reading, England),* 150 (Pt 6), pp. 1957-1964.

Karlyshev, A. V., Ketley, J. M. and Wren, B. W. (2005) 'The *Campylobacter jejuni* glycome', *FEMS Microbiology Reviews,* 29 (2), pp. 377-390.

Karlyshev, A. V., Linton, D., Gregson, N. A., Lastovica, A. J. and Wren, B. W. (2000) 'Genetic and biochemical evidence of a *Campylobacter jejuni* capsular polysaccharide that accounts for Penner serotype specificity', *Molecular Microbiology,* 35 (3), pp. 529-541.

Karlyshev, A. V., Linton, D., Gregson, N. A. and Wren, B. W. (2002) 'A novel paralogous gene family involved in phase-variable flagella-mediated motility in *Campylobacter jejuni*', *Microbiology (Reading, England),* 148 (Pt 2), pp. 473-480.

Karlyshev, A. V., McCrossan, M. V. and Wren, B. W. (2001) 'Demonstration of polysaccharide capsule in *Campylobacter jejuni* using electron microscopy', *Infection and Immunity,* 69 (9), pp. 5921-5924.

Karlyshev A. V., Thacker G., Jones M. A., Clements M. O., Wren B. W. (2014) "*Campylobacter jejuni* gene cj0511 encodes a serine peptidase essential for colonization", FEBS Open Bio 4, pp. 468–472.

Karlyshev, A. V. and Wren, B. W. (2005) 'Development and application of an insertional system for gene delivery and expression in *Campylobacter jejuni*', *Applied and Environmental Microbiology,* 71 (7), pp. 4004-4013.

Kawai, F., Paek, S., Choi, K.J., Prouty, M., Kanipes, M.I., Guerry, P., Yeo, H.J. (2012) 'Crystal structure of JlpA, a surface-exposed lipoprotein adhesin of *Campylobacter* jejuni', *The Journal of Structural Bilogy,* 177 (2), pp. 583-8.

Keener, K. M., Bashor, M. P., Curtis, P. A., Sheldon, B. W. and Kathariou, S. (2004) 'Comprehensive Review of *Campylobacter* and Poultry Processing', *Comprehensive Reviews in Food Science and Food Safety,* 3 (2), pp. 105-116.

Kelly, C. G. and Younson, J. S. (2000) 'Anti-adhesive strategies in the prevention of infectious disease at mucosal surfaces', *Expert Opinion on Investigational Drugs,* 9 (8), pp. 1711-1721.

Kelly, C. G., Younson, J. S., Hikmat, B. Y., Todryk, S. M., Czisch, M., Haris, P. I., Flindall, I. R., Newby, C., Mallet, A. I., Ma, J. K. and Lehner, T. (1999) 'A synthetic peptide adhesion epitope as a novel antimicrobial agent', *Nature Biotechnology,* 17 (1), pp. 42-47.

Kelly, J., Jarrell, H., Millar, L., Tessier, L., Fiori, L. M., Lau, P. C., Allan, B. and Szymanski, C. M. (2006) 'Biosynthesis of the N-linked glycan in *Campylobacter jejuni* and addition onto protein through block transfer', *Journal of Bacteriology,* 188 (7), pp. 2427-2434.

Keo, T., Collins, J., Kunwar, P., Blaser, M. J. and Iovine, N. M. (2011) '*Campylobacter* capsule and lipooligosaccharide confer resistance to serum and cationic antimicrobials', *Virulence,* 2 (1), pp. 30-40.

Kervella, M., Pages, J. M., Pei, Z., Grollier, G., Blaser, M. J. and Fauchere, J. L. (1993) 'Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes', *Infection and Immunity,* 61 (8), pp. 3440-3448.

Ketley, J. M. (1997) 'Pathogenesis of enteric infection by *Campylobacter*', *Microbiology (Reading, England),* 143 (Pt 1), pp. 5-21.

Khatua, B., Ghoshal, A., Bhattacharya, K., Mandal, C., Saha, B., Crocker, P. R. and Mandal, C. (2010) 'Sialic acids acquired by *Pseudomonas aeruginosa* are involved in reduced complement deposition and siglec mediated host-cell recognition', *FEBS Letters,* 584 (3), pp. 555-561.

Kim, K.S. (2000) '*E. coli* invasion of brain microvascular endothelial cells as a pathogenetic basis of meningitis', Subcell Biochem. 33, pp. 47–59.

Klemm, P. and Schembri, M. A. (2000) 'Bacterial adhesins: function and structure', *International Journal of Medical Microbiology: IJMM,* 290 (1), pp. 27-35.

Kline, K. A., Falker, S., Dahlberg, S., Normark, S. and Henriques-Normark, B. (2009) 'Bacterial adhesins in host-microbe interactions', *Cell Host & Microbe,* 5 (6), pp. 580-592.

Komagamine, T. and Yuki, N. (2006) 'Ganglioside mimicry as a cause of Guillain-Barre syndrome', *CNS & Neurological Disorders Drug Targets,* 5 (4), pp. 391-400.

Konkel, M. E., Christensen, J. E., Keech, A. M., Monteville, M. R., Klena, J. D. and Garvis, S. G. (2005) 'Identification of a fibronectin-binding domain within the *Campylobacter jejuni* CadF protein', *Molecular Microbiology,* 57 (4), pp. 1022-1035.

Konkel, M. E. and Cieplak, W.,Jr (1992a) 'Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalization', *Infection and Immunity,* 60 (11), pp. 4945-4949.

Konkel, M. E., Corwin, M. D., Joens, L. A. and Cieplak, W. (1992b) 'Factors that influence the interaction of *Campylobacter jejuni* with cultured mammalian cells', *Journal of Medical Microbiology,* 37 (1), pp. 30-37.

Konkel, M. E., Mead, D. J., Hayes, S. F. and Cieplak, W.,Jr (1992c) 'Translocation of *Campylobacter jejuni* across human polarized epithelial cell monolayer cultures', *The Journal of Infectious Diseases,* 166 (2), pp. 308-315.

Konkel, M. E., Garvis, S. G., Tipton, S. L., Anderson, D. E.,Jr and Cieplak, W.,Jr (1997) 'Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*', *Molecular Microbiology,* 24 (5), pp. 953-963.

Konkel, M. E., Kim, B. J., Rivera-Amill, V. and Garvis, S. G. (1999) 'Bacterial secreted proteins are required for the internaliztion of *Campylobacter jejuni* into cultured mammalian cells', *Molecular Microbiology,* 32 (4), pp. 691-701.

Konkel, M. E., Klena, J. D., Rivera-Amill, V., Monteville, M. R., Biswas, D., Raphael, B. and Mickelson, J. (2004) 'Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus', *Journal of Bacteriology,* 186 (11), pp. 3296-3303.

Konkel, M. E., Larson, C. L. and Flanagan, R. C. (2010) '*Campylobacter jejuni* FlpA binds fibronectin and is required for maximal host cell adherence', *Journal of Bacteriology,* 192 (1), pp. 68-76.

Kopecko, D. J., Hu, L. and Zaal, K. J. (2001) '*Campylobacter jejuni*--microtubule-dependent invasion', *Trends in Microbiology,* 9 (8), pp. 389-396.

Kowarik, M., Young, N. M., Numao, S., Schulz, B. L., Hug, I., Callewaert, N., Mills, D. C., Watson, D. C., Hernandez, M., Kelly, J. F., Wacker, M. and Aebi, M. (2006) 'Definition of the bacterial N-glycosylation site consensus sequence', *The EMBO Journal,* 25 (9), pp. 1957-1966.

Krachler, A. M. and Orth, K. (2013) 'Targeting the bacteria-host interface: strategies in anti-adhesion therapy', *Virulence,* 4 (4), pp. 284-294.

Krause-Gruszczynska, M., Rohde, M., Hartig, R., Genth, H., Schmidt, G., Keo, T., Konig, W., Miller, W. G., Konkel, M. E. and Backert, S. (2007) 'Role of the small Rho GTPases Rac1 and Cdc42 in host cell invasion of *Campylobacter jejuni*', *Cellular Microbiology,* 9 (10), pp. 2431-2444.

Laisney, M. J., Gillard, M. O. and Salvat, G. (2004) 'Influence of bird strain on competitive exclusion of *Campylobacter jejuni* in young chicks', *British Poultry Science,* 45 (1), pp. 49-54.

Lang, C. L., Chiang, C. K., Hung, K. Y. and Wu, K. D. (2009) '*Campylobacter jejuni* peritonitis and bacteremia in a patient undergoing continuous ambulatory peritoneal dialysis', *Clinical Nephrology,* 71 (1), pp. 96-98.

Laniewski, P., Kuczkowski, M., Chrzastek, K., Wozniak, A., Wyszynska, A., Wieliczko, A. and Jagusztyn-Krynicka, E. K. (2014) 'Evaluation of the immunogenicity of *Campylobacter jejuni* CjaA protein delivered by *Salmonella enterica* sv. Typhimurium strain with regulated delayed attenuation in chickens', *World Journal of Microbiology & Biotechnology,* 30 (1), pp. 281-292.

Lara-Tejero, M. and Galan, J. E. (2001) 'CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity', *Infection and Immunity,* 69 (7), pp. 4358-4365.

Larsen, J. C., Szymanski, C. and Guerry, P. (2004) 'N-linked protein glycosylation is required for full competence in *Campylobacter jejuni* 81-176', *Journal of Bacteriology,* 186 (19), pp. 6508-6514.

Lastovica, A. J. and Allos, B. M.(2008) 'Clinical significance of *Campylobacter* and related species other thatn *Campylobacter jejuni* and *Campylobacter coli*', in Nachamkin, I., Szymanski, C. M. and Blaser, M. J. (eds.) *Campylobacter.* Washington, DC: ASM press, pp. 123-149.

Lecuit, M., Abachin, E., Martin, A., Poyart, C., Pochart, P., Suarez, F., Bengoufa, D., Feuillard, J., Lavergne, A., Gordon, J. I., Berche, P., Guillevin, L. and Lortholary, O. (2004) 'Immunoproliferative small intestinal disease associated with *Campylobacter jejuni*', *The New England Journal of Medicine,* 350 (3), pp. 239-248.

Lee, A., O'Rourke, J. L., Barrington, P. J. and Trust, T. J. (1986) 'Mucus colonization as a determinant of pathogenicity in intestinal infection by *Campylobacter jejuni*: a mouse cecal model', *Infection and Immunity,* 51 (2), pp. 536-546.

Lee, K. K., Wong, W. Y., Sheth, H. B., Hodges, R. S., Paranchych, W. and Irvin, R. T. (1995) 'Use of synthetic peptides in characterization of microbial adhesins', *Methods in Enzymology,* 253 pp. 115-131.

Lee, L. H., Burg, E.,3rd, Baqar, S., Bourgeois, A. L., Burr, D. H., Ewing, C. P., Trust, T. J. and Guerry, P. (1999) 'Evaluation of a truncated recombinant flagellin subunit vaccine against *Campylobacter jejuni*', *Infection and Immunity,* 67 (11), pp. 5799-5805.

Lee, R. B., Hassane, D. C., Cottle, D. L. and Pickett, C. L. (2003) 'Interactions of *Campylobacter jejuni* cytolethal distending toxin subunits CdtA and CdtC with HeLa cells', *Infection and Immunity,* 71 (9), pp. 4883-4890.

Leon-Kempis Mdel, R., Guccione, E., Mulholland, F., Williamson, M. P. and Kelly, D. J. (2006) 'The *Campylobacter jejuni* PEB1a adhesin is an aspartate/glutamate-binding protein of an ABC transporter essential for microaerobic growth on dicarboxylic amino acids', *Molecular Microbiology,* 60 (5), pp. 1262-1275.

Levroney, E. L., Aguilar, H. C., Fulcher, J. A., Kohatsu, L., Pace, K. E., Pang, M., Gurney, K. B., Baum, L. G. and Lee, B. (2005) 'Novel innate immune functions for galectin-1: galectin-1 inhibits cell fusion by Nipah virus envelope glycoproteins and augments dendritic cell secretion of proinflammatory cytokines', *Journal of Immunology (Baltimore, Md.: 1950),* 175 (1), pp. 413-420.

Lievin-Le Moal, V. and Servin, A. L. (2006) 'The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota', *Clinical Microbiology Reviews,* 19 (2), pp. 315-337.

Linden, S. K., Sutton, P., Karlsson, N. G., Korolik, V. and McGuckin, M. A. (2008) 'Mucins in the mucosal barrier to infection', *Mucosal Immunology,* 1 (3), pp. 183-197.

Linden, S. K., Wickstrom, C., Lindell, G., Gilshenan, K. and Carlstedt, I. (2008) 'Four modes of adhesion are used during Helicobacter pylori binding to human mucins in the oral and gastric niches', *Helicobacter,* 13 (2), pp. 81-93.

Lindmark, B., Rompikuntal, P., Vaitkevicius, K., Song, T., Mizunoe, Y., Uhlin, B., Guerry, P. and Wai, S. (2009) 'Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from *Campylobacter jejuni*', *BMC Microbiology,* 9 (1), pp. 220.

Line, J. E., Svetoch, E. A., Eruslanov, B. V., Perelygin, V. V., Mitsevich, E. V., Mitsevich, I. P., Levchuk, V. P., Svetoch, O. E., Seal, B. S., Siragusa, G. R. and Stern, N. J. (2008) 'Isolation and purification of enterocin E-760 with broad antimicrobial activity against gram-positive and gram-negative bacteria', *Antimicrobial Agents and Chemotherapy,* 52 (3), pp. 1094-1100.

Linton, D., Allan, E., Karlyshev, A. V., Cronshaw, A. D. and Wren, B. W. (2002) 'Identification of N-acetylgalactosamine-containing glycoproteins PEB3 and CgpA in *Campylobacter jejuni*', *Molecular Microbiology,* 43 (2), pp. 497-508.

Linton, D., Gilbert, M., Hitchen, P. G., Dell, A., Morris, H. R., Wakarchuk, W. W., Gregson, N. A. and Wren, B. W. (2000) 'Phase variation of a beta-1,3 galactosyltransferase involved in generation of the ganglioside GM1-like lipo-oligosaccharide of *Campylobacter jejuni*', *Molecular Microbiology,* 37 (3), pp. 501-514.

Linton, D., Karlyshev, A. V. and Wren, B. W. (2001) 'Deciphering *Campylobacter jejuni* cell surface interactions from the genome sequence', *Current Opinion in Microbiology,* 4 (1), pp. 35-40.

Linton, D., Dorrell, N., Hitchen, P. G., Amber, S., Karlyshev, A. V., Morris, H. R., Dell, A., Valvano, M. A., Aebi, M. and Wren, B. W. (2005) 'Functional analysis of the *Campylobacter jejuni* N-linked protein glycosylation pathway', *Molecular Microbiology,* 55 (6), pp. 1695-1703.

Livak, K. J. and Schmittgen, T. D. (2001) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method', *Methods (San Diego, Calif.),* 25 (4), pp. 402-408.

Lloyd, K. O. (1987) 'Philip Levine award lecture. Blood group antigens as markers for normal differentiation and malignant change in human tissues', *American Journal of Clinical Pathology,* 87 (1), pp. 129-139.

Logan, S. M., Hui, J. P. M., Vinogradov, E., Aubry, A. J., Melanson, J. E., Kelly, J. F., Nothaft, H. and Soo, E. C. (2009) 'Identification of novel carbohydrate modifications on *Campylobacter jejuni* 11168 flagellin using metabolomics-based approaches', *FEBS Journal,* 276 (4), pp. 1014-1023.

Logan, S. M., Kelly, J. F., Thibault, P., Ewing, C. P. and Guerry, P. (2002) 'Structural heterogeneity of carbohydrate modifications affects serospecificity of *Campylobacter* flagellins', *Molecular Microbiology,* 46 (2), pp. 587-597.

Louwen, R., Heikema, A., van Belkum, A., Ott, A., Gilbert, M., Ang, W., Endtz, H. P., Bergman, M. P. and Nieuwenhuis, E. E. (2008) 'The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion', *Infection and Immunity,* 76 (10), pp. 4431-4438.

Lugo-Villarino, G., Hudrisier, D., Tanne, A. and Neyrolles, O. (2011) 'C-type lectins with a sweet spot for *Mycobacterium tuberculosis*', *European Journal of Microbiology and Immunology,* 1 pp. 25-40.

MacCallum, A., Haddock, G. and Everest, P. H. (2005) '*Campylobacter jejuni* activates mitogen-activated protein kinases in Caco-2 cell monolayers and *in vitro* infected primary human colonic tissue', *Microbiology (Reading, England),* 151 (Pt 8), pp. 2765-2772.

MacCallum, A., Hardy, S. P. and Everest, P. H. (2005) '*Campylobacter jejuni* inhibits the absorptive transport functions of Caco-2 cells and disrupts cellular tight junctions', *Microbiology (Reading, England),* 151 (Pt 7), pp. 2451-2458.

Mahdavi, J., Pirinccioglu, N., Oldfield, N. J., Carlsohn, E., Stoof, J., Aslam, A., Self, T., Cawthraw, S. A., Petrovska, L., Colborne, N., Sihlbom, C., Boren, T., Wooldridge, K. G. and Ala'Aldeen, D. A. (2014) 'A novel O-linked glycan modulates *Campylobacter jejuni* major outer membrane protein-mediated adhesion to human histo-blood group antigens and chicken colonization', *Open Biology,* 4 pp. 130202.

Makihira, S., Nikawa, H., Tamagami, M., Hamada, T., Nishimura, H., Ishida, K. and Yamashiro, H. (2002) 'Bacterial and Candida adhesion to intact and denatured collagen *in vitro*', *Mycoses,* 45 (9-10), pp. 389-392.

Martin, P. M., Mathiot, J., Ipero, J., Kirimat, M., Georges, A. J. and Georges-Courbot, M. C. (1989) 'Immune response to *Campylobacter jejuni* and *Campylobacter coli* in a cohort of children from birth to 2 years of age', *Infection and Immunity,* 57 (8), pp. 2542-2546.

Martino, M. C., Stabler, R. A., Zhang, Z. W., Farthing, M. J., Wren, B. W. and Dorrell, N. (2001) '*Helicobacter pylori* pore-forming cytolysin orthologue TlyA possesses *in vitro* hemolytic activity and has a role in colonization of the gastric mucosa', *Infection and Immunity,* 69 (3), pp. 1697-1703.

Martin-Sosa, S., Martin, M. J. and Hueso, P. (2002) 'The sialylated fraction of milk oligosaccharides is partially responsible for binding to enterotoxigenic and uropathogenic *Escherichia coli* human strains', *The Journal of Nutrition,* 132 (10), pp. 3067-3072.

Maue, A. C., Mohawk, K. L., Giles, D. K., Poly, F., Ewing, C. P., Jiao, Y., Lee, G., Ma, Z., Monteiro, M. A., Hill, C. L., Ferderber, J. S., Porter, C. K., Trent, M. S. and Guerry, P. (2013) 'The polysaccharide capsule of *Campylobacter jejuni* modulates the host immune response', *Infection and Immunity,* 81 (3), pp. 665-672.

McAuley, J. L., Linden, S. K., Png, C. W., King, R. M., Pennington, H. L., Gendler, S. J., Florin, T. H., Hill, G. R., Korolik, V. and McGuckin, M. A. (2007) 'MUC1 cell surface mucin is a critical element of the mucosal barrier to infection', *The Journal of Clinical Investigation,* 117 (8), pp. 2313-2324.

McKinley, M. J., Taylor, M. and Sangree, M. H. (1980) 'Toxic megacolon with *Campylobacter* colitis', *Connecticut Medicine,* 44 (8), pp. 496-497.

McNally, D. J., Lamoureux, M. P., Karlyshev, A. V., Fiori, L. M., Li, J., Thacker, G., Coleman, R. A., Khieu, N. H., Wren, B. W., Brisson, J. R., Jarrell, H. C. and Szymanski, C. M. (2007) 'Commonality and biosynthesis of the O-methyl phosphoramidate capsule modification in *Campylobacter jejuni*', *The Journal of Biological Chemistry,* 282 (39), pp. 28566-28576.

McNally, D. J., Jarrell, H. C., Khieu, N. H., Li, J., Vinogradov, E., Whitfield, D. M., Szymanski, C. M. and Brisson, J. (2006) 'The HS:19 serostrain of *Campylobacter jejuni* has a hyaluronic acid-type capsular polysaccharide with a nonstoichiometric sorbose branch and O-methyl phosphoramidate group', *FEBS Journal,* 273 (17), pp. 3975-3989.

McNally, D. J., Jarrell, H. C., Li, J., Khieu, N. H., Vinogradov, E., Szymanski, C. M. and Brisson, J. (2005) 'The HS:1 serostrain of *Campylobacter jejuni* has a complex teichoic acid-like capsular polysaccharide with nonstoichiometric fructofuranose branches and O-methyl phosphoramidate groups', *FEBS Journal,* 272 (17), pp. 4407-4422.

McSweegan, E., Burr, D. H. and Walker, R. I. (1987) 'Intestinal mucus gel and secretory antibody are barriers to *Campylobacter jejuni* adherence to INT 407 cells', *Infection and Immunity,* 55 (6), pp. 1431-1435.

McSweegan, E. and Walker, R. I. (1986) 'Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates', *Infection and Immunity,* 53 (1), pp. 141-148.

Medzhitov, R. and Janeway, C. A.,Jr (1997) 'Innate immunity: the virtues of a nonclonal system of recognition', *Cell,* 91 (3), pp. 295-298.

Merino, S. and Tomas, J.(2001) 'Bacterial Capsules and Evasion of Immune Responses', in 'Bacterial Capsules and Evasion of Immune Responses', in *eLS.* John Wiley & Sons, Ltd,.

Merz, A. J. and So, M. (2000) 'Interactions of pathogenic neisseriae with epithelial cell membranes', *Annual Review of Cell and Developmental Biology,* 16 pp. 423-457.

Michael, F. S., Szymanski, C. M., Li, J., Chan, K. H., Khieu, N. H., Larocque, S., Wakarchuk, W. W., Brisson, J. and Monteiro, M. A. (2002) 'The structures of the lipooligosaccharide and capsule polysaccharide of *Campylobacter jejuni* genome sequenced strain NCTC 11168', *European Journal of Biochemistry,* 269 (21), pp. 5119-5136.

Mihaljevic, R. R., Sikic, M., Klancnik, A., Brumini, G., Mozina, S. S. and Abram, M. (2007) 'Environmental stress factors affecting survival and virulence of *Campylobacter jejuni*', *Microbial Pathogenesis,* 43 (2-3), pp. 120-125.

Min, T., Vedadi, M., Watson, D. C., Wasney, G. A., Munger, C., Cygler, M., Matte, A. and Young, N. M. (2009) 'Specificity of *Campylobacter jejuni* adhesin PEB3 for phosphates and structural differences among its ligand complexes', *Biochemistry,* 48 (14), pp. 3057-3067.

Mitchell, D. A., Fadden, A. J. and Drickamer, K. (2001) 'A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands', *The Journal of Biological Chemistry,* 276 (31), pp. 28939-28945.

Monteiro, M. A., Baqar, S., Hall, E. R., Chen, Y. H., Porter, C. K., Bentzel, D. E., Applebee, L. and Guerry, P. (2009) 'Capsule polysaccharide conjugate vaccine against diarrheal disease caused by *Campylobacter jejuni*', *Infection and Immunity,* 77 (3), pp. 1128-1136.

Monteville, M. R., Yoon, J. E. and Konkel, M. E. (2003) 'Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization', *Microbiology (Reading, England),* 149 (Pt 1), pp. 153-165.

Moran, A. P. (1997) 'Structure and conserved characteristics of *Campylobacter jejuni* lipopolysaccharides', *The Journal of Infectious Diseases,* 176 Suppl 2 pp. S115-21.

Moran, A. P., Annuk, H. and Prendergast, M. M. (2005) 'Antibodies induced by ganglioside-mimicking *Campylobacter jejuni* lipooligosaccharides recognise epitopes at the nodes of Ranvier', *Journal of Neuroimmunology,* 165 (1-2), pp. 179-185.

Mori, M., Kuwabara, S., Fukutake, T., Yuki, N. and Hattori, T. (2001) 'Clinical features and prognosis of Miller Fisher syndrome', *Neurology,* 56 (8), pp. 1104-1106.

Morrow, A. L., Ruiz-Palacios, G. M., Altaye, M., Jiang, X., Guerrero, M. L., Meinzen-Derr, J. K., Farkas, T., Chaturvedi, P., Pickering, L. K. and Newburg, D. S. (2004) 'Human milk oligosaccharide blood group epitopes and innate immune protection against *Campylobacter* and calicivirus diarrhea in breastfed infants', *Advances in Experimental Medicine and Biology,* 554 pp. 443-446.

Morrow, A. L., Ruiz-Palacios, G. M., Jiang, X. and Newburg, D. S. (2005) 'Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea', *The Journal of Nutrition,* 135 (5), pp. 1304-1307.

Mortensen, N. P., Kuijf, M. L., Ang, C. W., Schiellerup, P., Krogfelt, K. A., Jacobs, B. C., van Belkum, A., Endtz, H. P. and Bergman, M. P. (2009) 'Sialylation of *Campylobacter jejuni* lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis', *Microbes and Infection / Institut Pasteur,* 11 (12), pp. 988-994.

Moser, I., Schroeder, W. and Salnikow, J. (1997) '*Campylobacter jejuni* major outer membrane protein and a 59-kDa protein are involved in binding to fibronectin and INT 407 cell membranes', *FEMS Microbiology Letters,* 157 (2), pp. 233-238.

Muller, A., Thomas, G. H., Horler, R., Brannigan, J. A., Blagova, E., Levdikov, V. M., Fogg, M. J., Wilson, K. S. and Wilkinson, A. J. (2005) 'An ATP-binding cassette-type cysteine transporter in *Campylobacter jejuni* inferred from the structure of an extracytoplasmic solute receptor protein', *Molecular Microbiology,* 57 (1), pp. 143-155.

Muraoka, W. T. and Zhang, Q. (2011) 'Phenotypic and genotypic evidence for L-fucose utilization by *Campylobacter jejuni* ', *Journal of Bacteriology,* 193 (5), pp. 1065-1075.

Myllyluoma, E., Ahonen, A. M., Korpela, R., Vapaatalo, H. and Kankuri, E. (2008) 'Effects of multispecies probiotic combination on helicobacter pylori infection *in vitro*', *Clinical and Vaccine Immunology : CVI,* 15 (9), pp. 1472-1482.

Napoletano, C., Rughetti, A., Agervig Tarp, M. P., Coleman, J., Bennett, E. P., Picco, G., Sale, P., Denda-Nagai, K., Irimura, T., Mandel, U., Clausen, H., Frati, L., Taylor-Papadimitriou, J., Burchell, J. and Nuti, M. (2007) 'Tumor-associated Tn-MUC1 glycoform is internalized through the macrophage galactose-type C-type lectin and delivered to the HLA class I and II compartments in dendritic cells', *Cancer Research,* 67 (17), pp. 8358-8367.

Nascimento de Araujo, A. and Giugliano, L. G. (2000) 'Human milk fractions inhibit the adherence of diffusely adherent *Escherichia coli* (DAEC) and enteroaggregative *E. coli* (EAEC) to HeLa cells', *FEMS Microbiology Letters,* 184 (1), pp. 91-94.

Neal-McKinney, J. M., Samuelson, D. R., Eucker, T. P., Nissen, M. S., Crespo, R. and Konkel, M. E. (2014) 'Reducing *Campylobacter jejuni* colonization of poultry via vaccination', *PloS One,* 9 (12), pp. e114254.

Newburg, D. S., Ruiz-Palacios, G. M. and Morrow, A. L. (2005) 'Human milk glycans protect infants against enteric pathogens', *Annual Review of Nutrition,* 25 pp. 37-58.

Newell, D. G., McBride, H. and Dolby, J. M. (1985) 'Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines', *The Journal of Hygiene,* 95 (2), pp. 217-227.

Nichols, G. L., Richardson, J. F., Sheppard, S. K., Lane, C. and Sarran, C. (2012) '*Campylobacter* epidemiology: a descriptive study reviewing 1 million cases in England and Wales between 1989 and 2011', *BMJ Open,* 2 (4): e001179.

Niemann, H. H., Schubert, W. D. and Heinz, D. W. (2004) 'Adhesins and invasins of pathogenic bacteria: a structural view', *Microbes and Infection / Institut Pasteur,* 6 (1), pp. 101-112.

Noor, S. M., Husband, A. J. and Widders, P. R. (1995) '*In ovo* oral vaccination with *Campylobacter jejuni* establishes early development of intestinal immunity in chickens', *British Poultry Science,* 36 (4), pp. 563-573.

Nothaft, H. and Szymanski, C. M. (2010) 'Protein glycosylation in bacteria: sweeter than ever', *Nature Reviews.Microbiology,* 8 (11), pp. 765-778.

Nougayrede, J. P., Fernandes, P. J. and Donnenberg, M. S. (2003) 'Adhesion of enteropathogenic *Escherichia coli* to host cells', *Cellular Microbiology,* 5 (6), pp. 359-372.

Novak, R. and Tuomanen, E. (1999) 'Pathogenesis of pneumococcal pneumonia', *Seminars in Respiratory Infections,* 14 (3), pp. 209-217.

Novik, V., Hofreuter, D. and Galan, J. E. (2010) 'Identification of *Campylobacter jejuni* genes involved in its interaction with epithelial cells', *Infection and Immunity,* 78 (8), pp. 3540-3553.

Nuijten, P. J., van Asten, F. J., Gaastra, W. and van der Zeijst, B. A. (1990) 'Structural and functional analysis of two *Campylobacter jejuni* flagellin genes', *The Journal of Biological Chemistry,* 265 (29), pp. 17798-17804.

Oakland, M., Jeon, B., Sahin, O., Shen, Z. and Zhang, Q. (2011) 'Functional characterization of a lipoprotein-encoding operon in *Campylobacter jejuni*', *PloS One,* 6(5):e20084.

Ó Croinin, T. and Backert, S. (2012) 'Host epithelial cell invasion by *Campylobacter jejuni*: trigger or zipper mechanism?', *Frontiers in Cellular and Infection Microbiology,* 2 pp. 25.

Odenbreit, S. (2005) 'Adherence properties of *Helicobacter pylori*: impact on pathogenesis and adaptation to the host', *International Journal of Medical Microbiology : IJMM,* 295 (5), pp. 317-324.

Oelschlaeger, T. A. (2001) 'Adhesins as invasins', *International Journal of Medical Microbiology : IJMM,* 291 (1), pp. 7-14.

Oelschlaeger, T. A., Guerry, P. and Kopecko, D. J. (1993) 'Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*', *Proceedings of the National Academy of Sciences of the United States of America,* 90 (14), pp. 6884-6888.

Ofek, I., Hasty, D. L. and Sharon, N. (2003) 'Anti-adhesion therapy of bacterial diseases: prospects and problems', *FEMS Immunology and Medical Microbiology,* 38 (3), pp. 181-191.

O'Hara, A. M. and Shanahan, F. (2006) 'The gut flora as a forgotten organ', *EMBO Reports,* 7 (7), pp. 688-693.

Olson, C. K., Ethelberg, S., van Pelt, W. and Tauxe, R. V. (2008) 'Epidemiology of *Campylobacter jejuni* infections in industrialized nations', in Nachamkin, I.,

Szymanski, C. and Blaser, M. J. (eds.) *Campylobacter.* Washington, DC, USA: ASM Press, pp. 163-189.

Okuda, J., Kurazono, H. and Takeda, Y. (1995) 'Distribution of the cytolethal distending toxin A gene (*cdtA*) among species of *Shigella* and *Vibrio*, and cloning and sequencing of the *cdt* gene from *Shigella dysenteriae*', *Microbial Pathogenesis,* 18 (3), pp. 167-172.

On, S. L. W. (2001) 'Taxonomy of *Campylobacter, Arcobacter, Helicobacter* and related bacteria: current status, future prospects and immediate concerns', *Journal of Applied Microbiology,* 90 (S6), pp. 1S-15S.

Oyofo, B. A., Droleskey, R. E., Norman, J. O., Mollenhauer, H. H., Ziprin, R. L., Corrier, D. E. and DeLoach, J. R. (1989) 'Inhibition by mannose of *in vitro* colonization of chicken small intestine by *Salmonella typhimurium*', *Poultry Science,* 68 (10), pp. 1351-1356.

Palyada, K., Threadgill, D. and Stintzi, A. (2004) 'Iron acquisition and regulation in *Campylobacter jejuni*', *Journal of Bacteriology,* 186 (14), pp. 4714-4729.

Pankov, R. and Yamada, K. M. (2002) 'Fibronectin at a glance', *Journal of Cell Science,* 115 (Pt 20), pp. 3861-3863.

Park, S. F., Purdy, D. and Leach, S. (2000) 'Localized reversible frameshift mutation in the flhA gene confers phase variability to flagellin gene expression in *Campylobacter coli*', *Journal of Bacteriology,* 182 (1), pp. 207-210.

Parker, C. T., Gilbert, M., Yuki, N., Endtz, H. P. and Mandrell, R. E. (2008) 'Characterization of lipooligosaccharide-biosynthetic loci of *Campylobacter jejuni* reveals new lipooligosaccharide classes: evidence of mosaic organizations', *Journal of Bacteriology,* 190 (16), pp. 5681-5689.

Parkes, G. C., Sanderson, J. D. and Whelan, K. (2009) 'The mechanisms and efficacy of probiotics in the prevention of *Clostridium difficile*-associated diarrhoea', *The Lancet.Infectious Diseases,* 9 (4), pp. 237-244.

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M. A., Rutherford, K. M., van Vliet, A. H., Whitehead, S. and Barrell, B. G. (2000) 'The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences', *Nature,* 403 (6770), pp. 665-668.

Parry, S., Hanisch, F. G., Leir, S. H., Sutton-Smith, M., Morris, H. R., Dell, A. and Harris, A. (2006) 'N-Glycosylation of the MUC1 mucin in epithelial cells and secretions', *Glycobiology,* 16 (7), pp. 623-634.

Pearson, B. M., Gaskin, D. J., Segers, R. P., Wells, J. M., Nuijten, P. J. and van Vliet, A. H. (2007) 'The complete genome sequence of *Campylobacter jejuni* strain 81116 (NCTC11828)', *Journal of Bacteriology,* 189 (22), pp. 8402-8403.

Pei, Z. and Blaser, M. J. (1993) 'PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems', *The Journal of Biological Chemistry,* 268 (25), pp. 18717-18725.

Pei, Z., Burucoa, C., Grignon, B., Baqar, S., Huang, X. Z., Kopecko, D. J., Bourgeois, A. L., Fauchere, J. L. and Blaser, M. J. (1998) 'Mutation in the peb1A locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice', *Infection and Immunity,* 66 (3), pp. 938-943.

Pei, Z. H., Ellison, R. T.,3rd and Blaser, M. J. (1991) 'Identification, purification, and characterization of major antigenic proteins of *Campylobacter jejuni*', *The Journal of Biological Chemistry,* 266 (25), pp. 16363-16369.

Penner, J. L. and Aspinall, G. O. (1997) 'Diversity of lipopolysaccharide structures in *Campylobacter jejuni*', *The Journal of Infectious Diseases,* 176 Suppl 2 pp. S135-8.

Perera, V. N., Nachamkin, I., Ung, H., Patterson, J. H., McConville, M. J., Coloe, P. J. and Fry, B. N. (2007) 'Molecular mimicry in *Campylobacter jejuni*: role of the lipo-oligosaccharide core oligosaccharide in inducing anti-ganglioside antibodies', *FEMS Immunology and Medical Microbiology,* 50 (1), pp. 27-36.

Peterson, K. M. and Mekalanos, J. J. (1988) 'Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization', *Infection and Immunity,* 56 (11), pp. 2822-2829.

Pizarro-Cerda, J. and Cossart, P. (2006) 'Bacterial adhesion and entry into host cells', *Cell,* 124 (4), pp. 715-727.

Plotkowski, M. C., Costa, A. O., Morandi, V., Barbosa, H. S., Nader, H. B., de Bentzmann, S. and Puchelle, E. (2001) 'Role of heparan sulphate proteoglycans as potential receptors for non-piliated *Pseudomonas aeruginosa* adherence to non-polarised airway epithelial cells', *Journal of Medical Microbiology,* 50 (2), pp. 183-190.

Poly, F., Ewing, C., Goon, S., Hickey, T. E., Rockabrand, D., Majam, G., Lee, L., Phan, J., Savarino, N. J. and Guerry, P. (2007) 'Heterogeneity of a *Campylobacter jejuni* protein that is secreted through the flagellar filament', *Infection and Immunity,* 75 (8), pp. 3859-3867.

Prendergast, M. M., Tribble, D. R., Baqar, S., Scott, D. A., Ferris, J. A., Walker, R. I. and Moran, A. P. (2004) '*In vivo*phase variation and serologic response to lipooligosaccharide of *Campylobacter jejuni* in experimental human infection', *Infection and Immunity,* 72 (2), pp. 916-922.

Preston, A., Mandrell, R. E., Gibson, B. W. and Apicella, M. A. (1996) 'The lipooligosaccharides of pathogenic gram-negative bacteria', *Critical Reviews in Microbiology,* 22 (3), pp. 139-180.

Prokhorova, T. A., Nielsen, P. N., Petersen, J., Kofoed, T., Crawford, J. S., Morsczeck, C., Boysen, A. and Schrotz-King, P. (2006) 'Novel surface polypeptides of *Campylobacter jejuni* as traveller's diarrhoea vaccine candidates discovered by proteomics', *Vaccine,* 24 (40-41), pp. 6446-6455.

Prasadarao, N.V. (2002) 'Identification of E. coli outer membrane protein A receptor on human brain microvascular endothelial cells', *Infect. Immun.* 70, 4556–4563.

Public Health England (2013) *Zoonoses Report UK 2012.* edn. London: Department for Environment, Food and Rural Affairs. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/236983/pb13987-zoonoses-report-2012.pdf [Accessed March 2015]

Purdy, D., Buswell, C. M., Hodgson, A. E., McAlpine, K., Henderson, I. and Leach, S. A. (2000) 'Characterisation of cytolethal distending toxin (CDT) mutants of *Campylobacter jejuni*', *Journal of Medical Microbiology,* 49 (5), pp. 473-479.

Rabinovich, G. A. and Toscano, M. A. (2009) 'Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation', *Nature Reviews.Immunology,* 9 (5), pp. 338-352.

Raffatellu, M., Chessa, D., Wilson, R. P., Dusold, R., Rubino, S. and Baumler, A. J. (2005) 'The Vi capsular antigen of *Salmonella enterica* serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa', *Infection and Immunity,* 73 (6), pp. 3367-3374.

Ramphal, R., Carnoy, C., Fievre, S., Michalski, J. C., Houdret, N., Lamblin, G., Strecker, G. and Roussel, P. (1991) *'Pseudomonas aeruginosa* recognizes carbohydrate chains containing type 1 (Gal beta 1-3GlcNAc) or type 2 (Gal beta 1-4GlcNAc) disaccharide units', *Infection and Immunity,* 59 (2), pp. 700-704.

Rangarajan, E. S., Bhatia, S., Watson, D. C., Munger, C., Cygler, M., Matte, A. and Young, N. M. (2007) 'Structural context for protein N-glycosylation in bacteria: The structure of PEB3, an adhesin from *Campylobacter jejuni*', *Protein Science: A Publication of the Protein Society,* 16 (5), pp. 990-995.

Rathbun, K. M., Hall, J. E. and Thompson, S. A. (2009) 'Cj0596 is a periplasmic peptidyl prolyl cis-trans isomerase involved in *Campylobacter jejuni* motility, invasion, and colonization', *BMC Microbiology,* 9 pp. 160.

Rathinam, V. A., Hoag, K. A. and Mansfield, L. S. (2008) 'Dendritic cells from C57BL/6 mice undergo activation and induce Th1-effector cell responses against *Campylobacter jejuni*', *Microbes and Infection / Institut Pasteur,* 10 (12-13), pp. 1316-1324.

Rhim, A. D., Stoykova, L., Glick, M. C. and Scanlin, T. F. (2001) 'Terminal glycosylation in cystic fibrosis (CF): a review emphasizing the airway epithelial cell', *Glycoconjugate Journal,* 18 (9), pp. 649-659.

Rice, B. E., Rollins, D. M., Mallinson, E. T., Carr, L. and Joseph, S. W. (1997) '*Campylobacter jejuni* in broiler chickens: colonization and humoral immunity following oral vaccination and experimental infection', *Vaccine,* 15 (17-18), pp. 1922-1932.

Rivera-Amill, V., Kim, B. J., Seshu, J. and Konkel, M. E. (2001) 'Secretion of the virulence-associated *Campylobacter*invasion antigens from *Campylobacter jejuni* requires a stimulatory signal', *The Journal of Infectious Diseases,* 183 (11), pp. 1607-1616.

Roberts, I. S. (1996) 'The biochemistry and genetics of capsular polysaccharide production in bacteria', *Annual Review of Microbiology,* 50 pp. 285-315.

Rollins, D. M. and Colwell, R. R. (1986) 'Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment', *Applied and Environmental Microbiology,* 52 (3), pp. 531-538.

Rose, A., Kay, E., Wren, B. W. and Dallman, M. J. (2012) 'The *Campylobacter jejuni* NCTC11168 capsule prevents excessive cytokine production by dendritic cells', *Medical Microbiology and Immunology,* 201 (2), pp. 137-144.

Rosenquist, H., Sommer, H. M., Nielsen, N. L. and Christensen, B. B. (2006) 'The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*', *International Journal of Food Microbiology,* 108 (2), pp. 226-232.

Rubinchik, S., Seddon, A. and Karlyshev, A. V. (2012) 'Molecular mechanisms and biological role of *Campylobacter jejuni* attachment to host cells', *European Journal of Microbiology and Immunology,* 2 (1), pp. 32-40.

Ruiz-Palacios, G. M., Cervantes, L. E., Ramos, P., Chavez-Munguia, B. and Newburg, D. S. (2003) '*Campylobacter jejuni* binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection', *The Journal of Biological Chemistry,* 278 (16), pp. 14112-14120.

Runco, L. M., Myrczek, S., Bliska, J. B. and Thanassi, D. G. (2008) 'Biogenesis of the fraction 1 capsule and analysis of the ultrastructure of *Yersinia pestis*', *Journal of Bacteriology,* 190 (9), pp. 3381-3385.

Russell, R. G. and Blake, D. C.,Jr (1994) 'Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*', *Infection and Immunity,* 62 (9), pp. 3773-3779.

Sahly, H., Podschun, R., Oelschlaeger, T. A., Greiwe, M., Parolis, H., Hasty, D., Kekow, J., Ullmann, U., Ofek, I. and Sela, S. (2000) 'Capsule impedes adhesion to and invasion of epithelial cells by *Klebsiella pneumoniae*', *Infection and Immunity,* 68 (12), pp. 6744-6749.

Salamaszynska-Guz, A. and Klimuszko, D. (2008) 'Functional analysis of the *Campylobacter jejuni* cj0183 and cj0588 genes', *Current Microbiology,* 56 (6), pp. 592-596.

Sanderson, I. R. and Walker, W. A. (2007) 'TLRs in the Gut I. The role of TLRs/Nods in intestinal development and homeostasis', *American Journal of Physiology.Gastrointestinal and Liver Physiology,* 292 (1), pp. G6-10.

Sansonetti, P. J. and Phalipon, A. (1999) 'M cells as ports of entry for enteroinvasive pathogens: mechanisms of interaction, consequences for the disease process', *Seminars in Immunology,* 11 (3), pp. 193-203.

Sauer, F.G., Barnhart, M., Choudhury, D., Knight, S.D., Waksman, G., Hultgren, S.J.(2000) 'Chaperone-assisted pilus assembly and bacterial attachment', *Curr Opin Struct Biol,* 10, pp.548–556.

Schembri, M. A., Dalsgaard, D. and Klemm, P. (2004) 'Capsule shields the function of short bacterial adhesins', *Journal of Bacteriology,* 186 (5), pp. 1249-1257.

Schroder, W. and Moser, I. (1997) 'Primary structure analysis and adhesion studies on the major outer membrane protein of *Campylobacter jejuni*', *FEMS Microbiology Letters,* 150 (1), pp. 141-147.

Schulz, O., Jaensson, E., Persson, E. K., Liu, X., Worbs, T., Agace, W. W. and Pabst, O. (2009) 'Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions', *The Journal of Experimental Medicine,* 206 (13), pp. 3101-3114.

Scott, D. A. (1997) 'Vaccines against *Campylobacter jejuni*', *The Journal of Infectious Diseases,* 176 Suppl 2 pp. S183-8.

Scott, N. E., Bogema, D. R., Connolly, A. M., Falconer, L., Djordjevic, S. P. and Cordwell, S. J. (2009) 'Mass spectrometric characterization of the surface-associated 42 kDa lipoprotein JlpA as a glycosylated antigen in strains of *Campylobacter jejuni*', *Journal of Proteome Research,* 8 (10), pp. 4654-4664.

Scott, N. E., Parker, B. L., Connolly, A. M., Paulech, J., Edwards, A. V., Crossett, B., Falconer, L., Kolarich, D., Djordjevic, S. P., Hojrup, P., Packer, N. H., Larsen, M. R. and Cordwell, S. J. (2011) 'Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS applied to the N-linked glycoproteome of *Campylobacter jejuni* ', *Molecular & Cellular Proteomics: MCP,* 10 (2), pp. M000031-MCP201.

Sharon, N. (2006) 'Carbohydrates as future anti-adhesion drugs for infectious diseases', *Biochimica Et Biophysica Acta,* 1760 (4), pp. 527-537.

Sharon, N. and Ofek, I. (2000) 'Safe as mother's milk: carbohydrates as future anti-adhesion drugs for bacterial diseases', *Glycoconjugate Journal,* 17 (7-9), pp. 659-664.

Sheppard, S. K., Dallas, J. F., Strachan, N. J., MacRae, M., McCarthy, N. D., Wilson, D. J., Gormley, F. J., Falush, D., Ogden, I. D., Maiden, M. C. and Forbes,

K. J. (2009) '*Campylobacter*genotyping to determine the source of human infection', *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America,* 48 (8), pp. 1072-1078.

Sherman, P. M., Ossa, J. C. and Johnson-Henry, K. (2009) 'Unraveling mechanisms of action of probiotics', *Nutrition in Clinical Practice : Official Publication of the American Society for Parenteral and Enteral Nutrition,* 24 (1), pp. 10-14.

Shigematsu, M., Umeda, A., Fujimoto, S. and Amako, K. (1998) 'Spirochaete-like swimming mode of *Campylobacter jejuni* in a viscous environment', *Journal of Medical Microbiology,* 47 (6), pp. 521-526.

Shoaf-Sweeney, K. D. and Hutkins, R. W. (2009) 'Adherence, anti-adherence, and oligosaccharides preventing pathogens from sticking to the host', *Advances in Food and Nutrition Research,* 55 pp. 101-161.

Siegesmund, A. M., Konkel, M. E., Klena, J. D. and Mixter, P. F. (2004) '*Campylobacter jejuni* infection of differentiated THP-1 macrophages results in interleukin 1 beta release and caspase-1-independent apoptosis', *Microbiology (Reading, England),* 150 (Pt 3), pp. 561-569.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A. and Teixeira, P. (2011) '*Campylobacter*spp. as a Foodborne Pathogen: A Review', *Frontiers in Microbiology,* 2 pp. 200.

Simon, P. M., Goode, P. L., Mobasseri, A. and Zopf, D. (1997) 'Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides', *Infection and Immunity,* 65 (2), pp. 750-757.

Sizemore, D. R., Warner, B., Lawrence, J., Jones, A. and Killeen, K. P. (2006) 'Live, attenuated *Salmonella typhimurium* vectoring *Campylobacter*antigens', *Vaccine,* 24 (18), pp. 3793-3803.

Skirrow, M. B. (2006) 'John McFadyean and the centenary of the first isolation of Campylobacter species', *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America,* 43 (9), pp. 1213-1217.

Skirrow, M. B. (1977) '*Campylobacter*enteritis: a "new" disease', *British Medical Journal,* 2 (6078), pp. 9-11.

Sletmoen, M., Dam, T. K., Gerken, T. A., Stokke, B. T., abd Brewer, C. F. (2009) "Single-Molecule Pair Studies of the Interactions of the α-GalNAc (Tn-Antigen) Form of Porcine Submaxillary Mucin with Soybean Agglutinin", *Biopolymers*, 91 (9), pp. 719-728.

Smith, J. L. and Bayles, D. O. (2006) 'The contribution of cytolethal distending toxin to bacterial pathogenesis', *Critical Reviews in Microbiology,* 32 (4), pp. 227-248.

Smith, T. and Taylor, M. S. (1919) 'Some Morphological and Biological Characters of the Spirilla (Vibrio Fetus, N. Sp.) Associated with Disease of the Fetal Membranes in Cattle', *The Journal of Experimental Medicine,* 30 (4), pp. 299-311.

Song, Y. C., Jin, S., Louie, H., Ng, D., Lau, R., Zhang, Y., Weerasekera, R., Al Rashid, S., Ward, L. A., Der, S. D. and Chan, V. L. (2004) 'FlaC, a protein of *Campylobacter jejuni* TGH9011 (ATCC43431) secreted through the flagellar apparatus, binds epithelial cells and influences cell invasion', *Molecular Microbiology,* 53 (2), pp. 541-553.

Stafford, R. J., Schluter, P., Kirk, M., Wilson, A., Unicomb, L., Ashbolt, R., Gregory, J. and OzFoodNet Working Group (2007) 'A multi-centre prospective case-control study of *Campylobacter*infection in persons aged 5 years and older in Australia', *Epidemiology and Infection,* 135 (6), pp. 978-988.

Stahl, M., Friis, L. M., Nothaft, H., Liu, X., Li, J., Szymanski, C. M. and Stintzi, A. (2011) 'L-fucose utilization provides *Campylobacter jejuni* with a competitive advantage', *Proceedings of the National Academy of Sciences of the United States of America,* 108 (17), pp. 7194-7199.

Stern, N., Eruslanov, B., Pokhilenko, V., Kovalev, Y., Volodina, L., Perelygin, V., Mitsevich, E., Mitsevich, I., Borzenkov, V., Levchuk, V., Svetoch, O., Stepanshin, Y., Svetoch,E. (2008) 'Bacteriocins reduce *Campylobacter jejuni* colonization while bacteria producing bacteriocins are ineffective', *Microbial Ecology in Health and Disease,* 20 (2), pp. 74-79 .

Stern, N. J., Svetoch, E. A., Eruslanov, B. V., Perelygin, V. V., Mitsevich, E. V., Mitsevich, I. P., Pokhilenko, V. D., Levchuk, V. P., Svetoch, O. E. and Seal, B. S. (2006) 'Isolation of a Lactobacillus salivarius strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system', *Antimicrobial Agents and Chemotherapy,* 50 (9), pp. 3111-3116.

Stingl, K., Knuver, M. T., Vogt, P., Buhler, C., Kruger, N. J., Alt, K., Tenhagen, B. A., Hartung, M., Schroeter, A., Ellerbroek, L., Appel, B. and Kasbohrer, A. (2012) 'Quo vadis? - Monitoring *Campylobacter*in Germany', *European Journal of Microbiology & Immunology,* 2 (1), pp. 88-96.

Suarez, F., Lortholary, O., Hermine, O. and Lecuit, M. (2006) 'Infection-associated lymphomas derived from marginal zone B cells: a model of antigen-driven lymphoproliferation', *Blood,* 107 (8), pp. 3034-3044.

Svensson, S. L., Pryjma, M. and Gaynor, E. C. (2014) 'Flagella-mediated adhesion and extracellular DNA release contribute to biofilm formation and stress tolerance of *Campylobacter jejuni* ', *PloS One,* 9 (8), pp. e106063.

Svetoch, E. A. and Stern, N. J. (2010) 'Bacteriocins to control *Campylobacter* spp. in poultry--A review', *Poultry Science,* 89 (8), pp. 1763-1768.

Szajewska, H., Skorka, A., Ruszczynski, M. and Gieruszczak-Bialek, D. (2007) 'Meta-analysis: *Lactobacillus* GG for treating acute diarrhoea in children', *Alimentary Pharmacology & Therapeutics,* 25 (8), pp. 871-881.

Szymanski, C. M., Burr, D. H. and Guerry, P. (2002) '*Campylobacter* protein glycosylation affects host cell interactions', *Infection and Immunity,* 70 (4), pp. 2242-2244.

Szymanski, C. M., King, M., Haardt, M. and Armstrong, G. D. (1995) '*Campylobacter jejuni* motility and invasion of Caco-2 cells', *Infection and Immunity,* 63 (11), pp. 4295-4300.

Szymanski, C. M., Michael, F. S., Jarrell, H. C., Li, J., Gilbert, M., Larocque, S., Vinogradov, E. and Brisson, J. R. (2003) 'Detection of conserved N-linked glycans and phase-variable lipooligosaccharides and capsules from *Campylobacter* cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy', *The Journal of Biological Chemistry,* 278 (27), pp. 24509-24520.

Szymanski, C. M., Yao, R., Ewing, C. P., Trust, T. J. and Guerry, P. (1999) 'Evidence for a system of general protein glycosylation in *Campylobacter jejuni* ', *Molecular Microbiology,* 32 (5), pp. 1022-1030.

Takada, A., Fujioka, K., Tsuiji, M., Morikawa, A., Higashi, N., Ebihara, H., Kobasa, D., Feldmann, H., Irimura, T. and Kawaoka, Y. (2004) 'Human macrophage C-type lectin specific for galactose and N-acetylgalactosamine promotes filovirus entry', *Journal of Virology,* 78 (6), pp. 2943-2947.

Takata, T., Fujimoto, S. and Amako, K. (1992) 'Isolation of nonchemotactic mutants of *Campylobacter jejuni* and their colonization of the mouse intestinal tract', *Infection and Immunity,* 60 (9), pp. 3596-3600.

Takeda, K., Kaisho, T. and Akira, S. (2003) 'Toll-like receptors', *Annual Review of Immunology,* 21 pp. 335-376.

Tam, C. C., Rodrigues, L. C., Viviani, L., Dodds, J. P., Evans, M. R., Hunter, P. R., Gray, J. J., Letley, L. H., Rait, G., Tompkins, D. S., O'Brien, S. J. and IID2 Study Executive Committee (2012) 'Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice', *Gut,* 61 (1), pp. 69-77.

Tareen, A. M., Dasti, J. I., Zautner, A. E., Gross, U. and Lugert, R. (2010) '*Campylobacter jejuni* proteins Cj0952c and Cj0951c affect chemotactic behaviour towards formic acid and are important for invasion of host cells', *Microbiology (Reading, England),* 156 (Pt 10), pp. 3123-3135.

Tareen, A. M., Luder, C. G., Zautner, A. E., Grobeta, U., Heimesaat, M. M., Bereswill, S. and Lugert, R. (2013) 'The *Campylobacter jejuni* Cj0268c protein is required for adhesion and invasion *in vitro*', *PloS One,* 8 (11), pp. e81069.

Thibault, P., Logan, S. M., Kelly, J. F., Brisson, J. R., Ewing, C. P., Trust, T. J. and Guerry, P. (2001) 'Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin', *The Journal of Biological Chemistry,* 276 (37), pp. 34862-34870.

Thomas, R. J. (2010) 'Receptor mimicry as novel therapeutic treatment for biothreat agents', *Bioengineered Bugs,* 1 (1), pp. 17-30.

Thomas, R. J. and Brooks, T. J. (2004) 'Oligosaccharide receptor mimics inhibit *Legionella pneumophila* attachment to human respiratory epithelial cells', *Microbial Pathogenesis,* 36 (2), pp. 83-92.

Thomas, W. E., Nilsson, L. M., Forero, M., Sokurenko, E. V. and Vogel, V. (2004) 'Shear-dependent 'stick-and-roll' adhesion of type 1 fimbriated *Escherichia coli', Molecular Microbiology,* 53 (5), pp. 1545-1557.

Thomas, W. E., Trintchina, E., Forero, M., Vogel, V. and Sokurenko, E. V. (2002) 'Bacterial adhesion to target cells enhanced by shear force', *Cell,* 109 (7), pp. 913-923.

Thornton, D. J., Rousseau, K. and McGuckin, M. A. (2008) 'Structure and function of the polymeric mucins in airways mucus', *Annual Review of Physiology,* 70 pp. 459-486.

Trotter, C. L., McVernon, J., Ramsay, M. E., Whitney, C. G., Mulholland, E. K., Goldblatt, D., Hombach, J., Kieny, M. P. and SAGE subgroup (2008) 'Optimising the use of conjugate vaccines to prevent disease caused by *Haemophilus influenzae* type b, *Neisseria meningitidis* and *Streptococcus pneumoniae', Vaccine,* 26 (35), pp. 4434-4445.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. and Tada, K. (1980) 'Establishment and characterization of a human acute monocytic leukemia cell line (THP-1)', *International Journal of Cancer.Journal International Du Cancer,* 26 (2), pp. 171-176.

Tsuji, S., Uehori, J., Matsumoto, M., Suzuki, Y., Matsuhisa, A., Toyoshima, K. and Seya, T. (2001) 'Human intelectin is a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cell wall', *The Journal of Biological Chemistry,* 276 (26), pp. 23456-23463.

Tu, Q. V., McGuckin, M. A. and Mendz, G. L. (2008) '*Campylobacter jejuni* response to human mucin MUC2: modulation of colonization and pathogenicity determinants', *Journal of Medical Microbiology,* 57 (Pt 7), pp. 795-802.

Unkmeir, A., Kammerer, U., Stade, A., Hubner, C., Haller, S., Kolb-Maurer, A., Frosch, M. and Dietrich, G. (2002) 'Lipooligosaccharide and polysaccharide capsule: virulence factors of *Neisseria meningitidis* that determine meningococcal interaction with human dendritic cells', *Infection and Immunity,* 70 (5), pp. 2454-2462.

van Alphen, L. B., Bleumink-Pluym, N. M., Rochat, K. D., van Balkom, B. W., Wosten, M. M. and van Putten, J. P. (2008) 'Active migration into the subcellular space precedes *Campylobacter jejuni* invasion of epithelial cells', *Cellular Microbiology,* 10 (1), pp. 53-66.

van den Berg, T. K., Honing, H., Franke, N., van Remoortere, A., Schiphorst, W. E., Liu, F. T., Deelder, A. M., Cummings, R. D., Hokke, C. H. and van Die, I. (2004) 'LacdiNAc-glycans constitute a parasite pattern for galectin-3-mediated immune recognition', *Journal of Immunology (Baltimore, Md.: 1950),* 173 (3), pp. 1902-1907.

van der Velden, A. W., Baumler, A. J., Tsolis, R. M. and Heffron, F. (1998) 'Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice', *Infection and Immunity,* 66 (6), pp. 2803-2808.

van der Woude, M. W. and Baumler, A. J. (2004) 'Phase and antigenic variation in bacteria', *Clinical Microbiology Reviews,* 17 (3), pp. 581-611, table of contents.

van der Woude, M. W. and Baumler, A. J. (2004) 'Phase and antigenic variation in bacteria', *Clinical Microbiology Reviews,* 17 (3), pp. 581-611.

van Doorn, P. A., Ruts, L. and Jacobs, B. C. (2008) 'Clinical features, pathogenesis, and treatment of Guillain-Barre syndrome', *The Lancet.Neurology,* 7 (10), pp. 939-950.

van Kooyk, Y. and Rabinovich, G. A. (2008) 'Protein-glycan interactions in the control of innate and adaptive immune responses', *Nature Immunology,* 9 (6), pp. 593-601.

van Liempt, E., van Vliet, S. J., Engering, A., Garcia Vallejo, J. J., Bank, C. M., Sanchez-Hernandez, M., van Kooyk, Y. and van Die, I. (2007) *'Schistosoma mansoni* soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation', *Molecular Immunology,* 44 (10), pp. 2605-2615.

van Putten, J. P., van Alphen, L. B., Wosten, M. M. and de Zoete, M. R. (2009) 'Molecular mechanisms of *Campylobacter* infection', *Current Topics in Microbiology and Immunology,* 337 pp. 197-229.

van Sorge, N. M., Bleumink, N. M., van Vliet, S. J., Saeland, E., van der Pol, W. L., van Kooyk, Y. and van Putten, J. P. (2009) 'N-glycosylated proteins and distinct lipooligosaccharide glycoforms of *Campylobacter jejuni* target the human C-type lectin receptor MGL', *Cellular Microbiology,* 11 (12), pp. 1768-1781.

van Spreeuwel, J. P., Duursma, G. C., Meijer, C. J., Bax, R., Rosekrans, P. C. and Lindeman, J. (1985) '*Campylobacter* colitis: histological immunohistochemical and ultrastructural findings', *Gut,* 26 (9), pp. 945-951.

van Vliet, A. H., Wooldridge, K. G. and Ketley, J. M. (1998) 'Iron-responsive gene regulation in a *Campylobacter jejuni* fur mutant', *Journal of Bacteriology,* 180 (20), pp. 5291-5298.

van Vliet, A. H. M. and Ketley, J. M. (2001) 'Pathogenesis of enteric *Campylobacter* infection', *Journal of Applied Microbiology,* 90 (S6), pp. 45S-56S.

van Vliet, S. J., Paessens, L. C., Broks-van den Berg, V. C., Geijtenbeek, T. B. and van Kooyk, Y. (2008a) 'The C-type lectin macrophage galactose-type lectin impedes migration of immature APCs', *Journal of Immunology (Baltimore, Md.: 1950),* 181 (5), pp. 3148-3155.

van Vliet, S. J., Saeland, E. and van Kooyk, Y. (2008b) 'Sweet preferences of MGL: carbohydrate specificity and function', *Trends in Immunology,* 29 (2), pp. 83-90.

van Vliet, S. J., Steeghs, L., Bruijns, S. C., Vaezirad, M. M., Snijders Blok, C., Arenas Busto, J. A., Deken, M., van Putten, J. P. and van Kooyk, Y. (2009) 'Variation of *Neisseria gonorrhoeae* lipooligosaccharide directs dendritic cell-induced T helper responses', *PLoS Pathogens,* 5 (10), pp. e1000625.

van Vliet, S. J., van Liempt, E., Saeland, E., Aarnoudse, C. A., Appelmelk, B., Irimura, T., Geijtenbeek, T. B., Blixt, O., Alvarez, R., van Die, I. and van Kooyk, Y. (2005) 'Carbohydrate profiling reveals a distinctive role for the C-type lectin MGL in the recognition of helminth parasites and tumor antigens by dendritic cells', *International Immunology,* 17 (5), pp. 661-669.

van Vliet, S., Aarnoudse, C., Broks-van den Berg, V., Boks, M., Geijtenbeek, T. and van Kooyk, Y. (2007) 'MGL-mediated internalization and antigen presentation by dendritic cells: A role for tyrosine-5', *European Journal of Immunology,* 37 (8), pp. 2075-2081.

Vanderheijden, N., Delputte, P. L., Favoreel, H. W., Vandekerckhove, J., Van Damme, J., van Woensel, P. A. and Nauwynck, H. J. (2003) 'Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages', *Journal of Virology,* 77 (15), pp. 8207-8215.

Vasta, G. R. (2009) 'Roles of galectins in infection', *Nature Reviews.Microbiology,* 7 (6), pp. 424-438.

Velayudhan, J. and Kelly, D. J. (2002) 'Analysis of gluconeogenic and anaplerotic enzymes in *Campylobacter jejuni* : an essential role for phosphoenolpyruvate carboxykinase', *Microbiology (Reading, England),* 148 (Pt 3), pp. 685-694.

Ventura, M., O'Flaherty, S., Claesson, M. J., Turroni, F., Klaenhammer, T. R., van Sinderen, D. and O'Toole, P. W. (2009) 'Genome-scale analyses of health-promoting bacteria: probiogenomics', *Nature Reviews.Microbiology,* 7 (1), pp. 61-71.

Virji, M., Makepeace, K., Peak, I. R. A., Ferguson, D. J. P., Jennings, M. P. and Moxon, E. R. (1995) 'Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides', *Molecular Microbiology,* 18 (4), pp. 741-754.

Wagenaar, J. A., Van Bergen, M. A., Mueller, M. A., Wassenaar, T. M. and Carlton, R. M. (2005) 'Phage therapy reduces *Campylobacter jejuni* colonization in broilers', *Veterinary Microbiology,* 109 (3-4), pp. 275-283.

Wagner, R. D. (2006) 'Efficacy and food safety considerations of poultry competitive exclusion products', *Molecular Nutrition & Food Research,* 50 (11), pp. 1061-1071.

Walker, R. I., Schmauder-Chock, E. A., Parker, J. L. and Burr, D. (1988) 'Selective association and transport of *Campylobacter jejuni* through M cells of rabbit Peyer's patches', *Canadian Journal of Microbiology,* 34 (10), pp. 1142-1147.

Wang, Y. and Taylor, D. E. (1990) 'Natural transformation in *Campylobacter* species', *Journal of Bacteriology,* 172 (2), pp. 949-955.

Warriss, P. D., Wilkins, L. J., Brown, S. N., Phillips, A. J. and Allen, V. (2004) 'Defaecation and weight of the gastrointestinal tract contents after feed and water withdrawal in broilers', *British Poultry Science,* 45 (1), pp. 61-66.

Wassenaar, T. M., Bleumink-Pluym, N. M. and van der Zeijst, B. A. (1991) 'Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion', *The EMBO Journal,* 10 (8), pp. 2055-2061.

Wassenaar, T. M., Engelskirchen, M., Park, S. and Lastovica, A. (1997) 'Differential uptake and killing potential of *Campylobacter jejuni* by human peripheral monocytes/macrophages', *Medical Microbiology and Immunology,* 186 (2-3), pp. 139-144.

Wassenaar, T. M., Fry, B. N. and van der Zeijst, B. A. (1993a) 'Genetic manipulation of *Campylobacter*: evaluation of natural transformation and electro-transformation', *Gene,* 132 (1), pp. 131-135.

Wassenaar, T. M., van der Zeijst, B. A., Ayling, R. and Newell, D. G. (1993b) 'Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression', *Journal of General Microbiology,* 139 Pt 6 pp. 1171-1175.

Watson, R. O. and Galan, J. E. (2008) '*Campylobacter jejuni* survives within epithelial cells by avoiding delivery to lysosomes', *PLoS Pathogens,* 4 (1), pp. e14.

Watson, R. O. and Galan, J. E. (2005) 'Signal transduction in *Campylobacter jejuni* -induced cytokine production', *Cellular Microbiology,* 7 (5), pp. 655-665.

Wearne, S. (2013) *A refereshed strategy to reduce campylobacteriosis from poultry.* edn. Food Standard Agency. http://www.food.gov.uk/sites/default/files/multimedia/pdfs/board/board-papers-2013/fsa-130904.pdf [Accessed March 2015]

Weis, W. I., Taylor, M. E. and Drickamer, K. (1998) 'The C-type lectin superfamily in the immune system', *Immunological Reviews,* 163 pp. 19-34.

Whitehouse, C. A., Balbo, P. B., Pesci, E. C., Cottle, D. L., Mirabito, P. M. and Pickett, C. L. (1998) '*Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block', *Infection and Immunity,* 66 (5), pp. 1934-1940.

Wilson, J. W., Schurr, M. J., LeBlanc, C. L., Ramamurthy, R., Buchanan, K. L. and Nickerson, C. A. (2002) 'Mechanisms of bacterial pathogenicity', *Postgraduate Medical Journal,* 78 (918), pp. 216-224.

Wine, E., Gareau, M. G., Johnson-Henry, K. and Sherman, P. M. (2009) 'Strain-specific probiotic (Lactobacillus helveticus) inhibition of *Campylobacter jejuni* invasion of human intestinal epithelial cells', *FEMS Microbiology Letters,* 300 (1), pp. 146-152.

Wittschier, N., Lengsfeld, C., Vorthems, S., Stratmann, U., Ernst, J. F., Verspohl, E. J. and Hensel, A. (2007) 'Large molecules as anti-adhesive compounds against pathogens', *The Journal of Pharmacy and Pharmacology,* 59 (6), pp. 777-786.

Wong, K. W. and Isberg, R. R. (2005) 'Emerging views on integrin signaling via Rac1 during invasin-promoted bacterial uptake', *Current Opinion in Microbiology,* 8 (1), pp. 4-9.

Wooldridge, K. G. and Ketley, J. M. (1997) '*Campylobacter*-host cell interactions', *Trends in Microbiology,* 5 (3), pp. 96-102.

Wright J. A., Grant A. J., Hurd D., Harrison M., Guccione E. J., Kelly D. J. and Maskell D.J. (2009) "Metabolite and transcriptome analysis of *Campylobacter jejuni* in vitro growth reveals a stationary-phase physiological switch", *Microbiology* 155, pp. 80–94.

Wyszynska, A., Raczko, A., Lis, M. and Jagusztyn-Krynicka, E. K. (2004) 'Oral immunization of chickens with avirulent *Salmonella* vaccine strain carrying *C. jejuni* 72Dz/92 *cjaA* gene elicits specific humoral immune response associated with protection against challenge with wild-type Campylobacter', *Vaccine,* 22 (11-12), pp. 1379-1389.

Yamamoto, K., Ishida, C., Shinohara, Y., Hasegawa, Y., Konami, Y., Osawa, T. and Irimura, T. (1994) 'Interaction of immobilized recombinant mouse C-type macrophage lectin with glycopeptides and oligosaccharides', *Biochemistry,* 33 (26), pp. 8159-8166.

Yamasaki, R., Bacon, B. E., Nasholds, W., Schneider, H. and Griffiss, J. M. (1991) 'Structural determination of oligosaccharides derived from lipooligosaccharide of *Neisseria gonorrhoeae* F62 by chemical, enzymatic, and two-dimensional NMR methods', *Biochemistry,* 30 (43), pp. 10566-10575.

Yamasaki, R., Nasholds, W., Schneider, H. and Apicella, M. A. (1991) 'Epitope expression and partial structural characterization of F62 lipooligosaccharide (LOS) of *Neisseria gonorrhoeae*: IgM monoclonal antibodies (3F11 and 1-1-M)

recognize non-reducing termini of the LOS components', *Molecular Immunology,* 28 (11), pp. 1233-1242.

Yanagawa, Y., Takahashi, M. and Itoh, T. (1994) 'The role of flagella of *Campylobacter jejuni* in colonization in the intestinal tract in mice and the cultured-cell infectivity', *Nihon Saikingaku Zasshi.Japanese Journal of Bacteriology,* 49 (2), pp. 395-403.

Yao, R., Burr, D. H., Doig, P., Trust, T. J., Niu, H. and Guerry, P. (1994) 'Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni* : the role of motility in adherence and invasion of eukaryotic cells', *Molecular Microbiology,* 14 (5), pp. 883-893.

Yao, R., Burr, D. H. and Guerry, P. (1997) 'CheY-mediated modulation of *Campylobacter jejuni* virulence', *Molecular Microbiology,* 23 (5), pp. 1021-1031.

Young, K. T., Davis, L. M. and Dirita, V. J. (2007) '*Campylobacter jejuni* : molecular biology and pathogenesis', *Nature Reviews.Microbiology,* 5 (9), pp. 665-679.

Young, N. M., Brisson, J. R., Kelly, J., Watson, D. C., Tessier, L., Lanthier, P. H., Jarrell, H. C., Cadotte, N., St Michael, F., Aberg, E. and Szymanski, C. M. (2002) 'Structure of the N-linked glycan present on multiple glycoproteins in the Gram-negative bacterium, *Campylobacter jejuni* ', *The Journal of Biological Chemistry,* 277 (45), pp. 42530-42539.

Young, V. B., Chien, C. C., Knox, K. A., Taylor, N. S., Schauer, D. B. and Fox, J. G. (2000) 'Cytolethal distending toxin in avian and human isolates of *Helicobacter pullorum*', *The Journal of Infectious Diseases,* 182 (2), pp. 620-623.

Yuki, N., Susuki, K., Koga, M., Nishimoto, Y., Odaka, M., Hirata, K., Taguchi, K., Miyatake, T., Furukawa, K., Kobata, T. and Yamada, M. (2004) 'Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain-Barre syndrome', *Proceedings of the National Academy of Sciences of the United States of America,* 101 (31), pp. 11404-11409.

Zhang, G., Ma, L. and Doyle, M. P. (2007) 'Potential competitive exclusion bacteria from poultry inhibitory to *Campylobacter jejuni* and *Salmonella*', *Journal of Food Protection,* 70 (4), pp. 867-873.

Zhang, Q., Meitzler, J. C., Huang, S. and Morishita, T. (2000) 'Sequence polymorphism, predicted secondary structures, and surface-exposed conformational epitopes of *Campylobacter* major outer membrane protein', *Infection and Immunity,* 68 (10), pp. 5679-5689.

Zheng, J., Meng, J., Zhao, S., Singh, R. and Song, W. (2008) '*Campylobacter*-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires *Campylobacter*-secreted cytolethal distending toxin- and Toll-like receptor-mediated activation of NF-kappaB', *Infection and Immunity,* 76 (10), pp. 4498-4508.

Zilbauer, M., Dorrell, N., Boughan, P. K., Harris, A., Wren, B. W., Klein, N. J. and Bajaj-Elliott, M. (2005) 'Intestinal innate immunity to *Campylobacter jejuni* results in induction of bactericidal human beta-defensins 2 and 3', *Infection and Immunity,* 73 (11), pp. 7281-7289.

Zilbauer, M., Dorrell, N., Wren, B. W. and Bajaj-Elliott, M. (2008) '*Campylobacter jejuni* -mediated disease pathogenesis: an update', *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 102 (2), pp. 123-129.

Zilbauer, M., Dorrell, N., Elmi, A., Lindley, K. J., Schuller, S., Jones, H. E., Klein, N. J., Nunez, G., Wren, B. W. and Bajaj-Elliott, M. (2007) 'A major role for intestinal epithelial nucleotide oligomerization domain 1 (NOD1) in eliciting host bactericidal immune responses to *Campylobacter jejuni* ', *Cellular Microbiology,* 9 (10), pp. 2404-2416.

Ziprin, R. L., Hume, M. E., Young, C. R. and Harvey, R. B. (2002) 'Inoculation of chicks with viable non-colonizing strains of *Campylobacter jejuni*: evaluation of protection against a colonizing strain', *Current Microbiology,* 44 (3), pp. 221-223.

Ziprin, R. L., Young, C. R., Stanker, L. H., Hume, M. E. and Konkel, M. E. (1999) 'The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein', *Avian Diseases,* 43 (3), pp. 586-589.

Appendix

Published abstracts and articles

Abstracts

Rubinchik, S., Seddon, A. and Karlyshev, A.V. (2012)
"Development of a novel *in vitro* assay for investigation of *Campylobacter jejuni* attachment to host cell receptors" was presented at CampyUK 2012 Conference in London (UK), 6th January 2012.

Karlyshev, A.V., Seddon, A. and Rubinchik, S. (2012)
"The role of *Campylobacter* surface N-link glycosylated proteins and capsule in interaction with host cell receptors" was presented at II International Conference on Antimicrobial Research – ICAR2012 in Lisbon (Portugal), 21st–23rd November 2012.

Rubinchik, S., Seddon, A. and Karlyshev, A.V. (2013)
"Effect of capsule on interaction of *Campylobacter jejuni* cells with an analogue of a host cell receptor" was presented at the 17th International Workshop on Campylobacter, Helicobacter and related organisms (CHRO) 2013 in Aberdeen (UK), 15th–19th September 2013.

Published articles

Rubinchik, S., Seddon, A. and Karlyshev, A. V. (2012) 'Molecular mechanisms and biological role of *Campylobacter jejuni* attachment to host cells', *European Journal of Microbiology and Immunology*, 2 (1), pp.32–40.

Rubinchik, S., Seddon, A. and Karlyshev, A. V. (2014) 'A negative effect of *Campylobacter* capsule on bacterial interaction with an analogue of a host cell receptor', *BMC Microbiology*, 14:141.

Presentation

S. Rubinchik (2011) '*Campylobacter* adhesins as novel antibacterial targets: *in vitro* models and mechanisms of attachment'. PowerPoint presentation. Postgraduate Research Seminar Day, Kingston University London.