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Glycoconjugates in *Campylobacter jejuni*-mediated disease

A thesis submitted to the National University of Ireland, Galway

for the degree of

Doctor of Philosophy

by

Marcus Twomey BSc. Hons. (Microbiology)

Molecular Biochemistry Laboratory,

Department of Microbiology



September 2013

Head of Department

Prof. Jim O’Gara

Research Supervisor

Prof. Anthony Moran

Thesis Supervisor

Dr. Cyril Carroll

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This thesis is dedicated to my Mam and Dad

LIST OF ABBREVIATIONS

Units of measurement

Seconds	s	Molarity	M
Hours	h	Grams	g
Years	yr	Metres	m
Litres	l	Daltons	Da
Gravities	g	Voltage	V
Base pairs	bp	Degrees Celcius	°C
Minutes	min	Absorbance	A

Unit prefixes

mega-	10^6	milli-	10^{-3}
kilo-	10^3	micro-	10^{-6}
deci-	10^{-1}	nano-	10^{-9}
centi-	10^{-2}	pico-	10^{-12}

Abbreviations

AAG	auto-agglutination
AIDP	acute inflammatory demyelinating polyradiculoneuropathy
AMAN	acute motor axonal neuropathy
APS	ammonium persulphate
ATCC	American Type Culture Collection
BS-1	lectin form <i>Bondeiraea simplicifolia</i>
BSA	bovine serum albumin
CaCl ₂	calcium chloride
CadF	<i>Campylobacter</i> adhesin to fibronectin

CapA	<i>Campylobacter</i> adhesin protein A
CDT	cytolethal distending toxin
CE-ESI-MS	capillary-electrophoresis assisted electrospray ionization mass spectrometry
CEF	chicken embryonic fibroblast
cfu	colony forming units
CHO	Chinese hamster ovary cells
Cia	<i>Campylobacter</i> invasion antigen
CJT	<i>C. jejuni</i> enterotoxin
CO ₂	carbon dioxide
COSY	correlation spectroscopy
CPS	capsular polysaccharide
CRD	carbohydrate-recognition domain
CT	cholera toxin
CuSO ₄	copper sulphate
dHexN	deoxyhexosamine
ddHexN	dideoxyhexosamine
dH ₂ O	deionized water
DC	dendritic cell
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ECA	lectin from <i>Erythrina crystagalli</i>
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylene diamine tetraacetic acid
<i>et al.</i>	<i>et alia</i>
ESI-MS	electrospray ionization mass spectrometry
FlpA	fibronectin-like protein A
Fn	fibronectin
Fuc	fucose
<i>FUT</i>	fucosyltransferase
Gal	galactose
GalNAc	<i>N</i> -acetylgalactosamine
GBS	Guillain-Barré syndrome

GI	gastrointestinal
Glc	glucose
GlcN	glucosamine
GlcN3N	2,3-diamino-2,3-dideoxy-D-Glc
GlcNAc	<i>N</i> -acetylglucosamine
GPC	gel-permeation chromatography
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
H ₂ SO ₄	sulphuric acid
HCL	hydrochloric acid
Hsp90α	heat-shock protein 90α
Hep	heptose
Hex	hexose
HexNAc	<i>N</i> -acetylhexosamine
HMBC	heteronuclear multiple-bond correlation spectroscopy
HMO	human milk oligosaccharide
HPW	hot phenol water
Hrp	horseradish peroxidase
HS	heat stable
HSA	human serum albumin
HSQC	heteronuclear single-quantum correlation spectroscopy
IEC	intestinal epithelial cell
IBD	inflammatory bowel disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Jlp	<i>jejuni</i> lipoprotein (Jlp)-A
Kdo	3-deoxy-D- <i>manno</i> -oct-2-ulosonic acid
LAL	<i>Limulus</i> amoebocyte lysate
leg	legionaminic acid
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LTA	lectin from <i>Lotus tetragonolobus</i>

MAA	lectin from <i>Maackia amurensis</i>
mAb	monoclonal antibody
Man	mannose
MAS-NMR	magic angle spinning nuclear magnetic resonance spectroscopy
MeOPN	methyl phosphoramidate
MgCl ₂	magnesium chloride
Mol	mole
MOMP	major outer membrane protein
MPW	mini-phenol-water
M _r	molecular-mass
MS	mass spectrometry
MS-CRAMM	Microbial Surface Components Recognising Adhesive Matrix Molecules
MYD88	myeloid differentiation primary response gene-88
N ₂	nitrogen
Na	sodium
NaCl	sodium chloride
NaOH	sodium hydroxide
NCTC	National Culture Type Collection
Neu5Ac	<i>N</i> -acetylneuraminic acid/ sialic acid
NGC	neoglycoconjugate
NHS	<i>N</i> -hydroxysuccinimide
NMR	nuclear magnetic spectroscopy
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
O ₂	oxygen
OD	optical density
OM	outer membrane
OMP	outer membrane protein
OMV	outer membrane vesicle
ORF	open reading frame
OS	oligosaccharide

OTase	oligosaccharyltransferase
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline, pH 7.3
PEtn	phosphoethanolamine
PFGE	pulsed field gel electrophoresis
<i>pgl</i>	protein glycosylation gene family
pH	hydrogen ion concentration
PIBM	polyisobutyl methacrylate
PK	proteinase K
PKWCE	proteinase K whole cell extract
PMB	polymyxin B
PNA	lectin from <i>Arachis hypogaea</i>
PRR	pattern recognition receptor
PS	polysaccharide
pse	pseudaminic acid
QuiNAc	<i>N</i> -acetylquinosovamine
QuiNFo	<i>N</i> -formylquinovosamine
R _f	retention factor
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
Rpm	revolutions per minute
RT	room temperature (~20°C)
SBA	lectin from <i>Glycine max</i>
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNA	lectin from <i>Sambucus nigra</i>
STA	lectin from <i>Solanum tuberosum</i>
TBS	tris-buffered saline
TCRS	Two-component regulatory system
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin-layer chromatography
TLR	Toll-like receptor
TOCSY	total correlation spectroscopy

TRIF	TIR-domain-containing adaptor-inducing interferon- β
Tris	trizma base
UEA	lectin from <i>Ulex europaeus</i>
UV	ultra-violet
v/v	volume to volume
WGA	lectin from <i>Triticum vulgare</i>
w/v	mass to volume

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ABSTRACT

Gram-negative bacterium *Campylobacter jejuni* is a major cause of gastroenteritis in humans worldwide, and the most frequent infectious trigger in patients developing Guillain-Barré syndrome (GBS). While *C. jejuni* is pathogenic in humans, it is a commensal in avian hosts. The mechanisms leading to *C. jejuni* infection of humans or persistent colonisation in chickens are not well understood, but host temperature may provide an important trigger for specific adaptation. *C. jejuni* and host cell-surface glycoconjugates play important roles in infection, colonisation, and development of GBS. Initially, phenotypic characterisation of *C. jejuni* isolates from clinical and food sources in Ireland observed high rates of cell-surface glycoconjugate variability, as well as ganglioside mimicry, an important factor in GBS pathogenesis. Following this, comparison of glycan binding by a group of closely related *C. jejuni* GBS- and enteritis-related strains indicated that enteritis-related strains have higher rates of glycan-mediated adherence, potentially due to increased sialic acid recognition. Furthermore, *C. jejuni* strains 81116 and 81-176 were cultured at 37 and 42°C to simulate human and avian host conditions, respectively, and whole cells were profiled on neoglycoconjugate (NGC) and lectin microarrays. Analysis on the NGC array detected only minor differences between the strains and growth temperatures, while, on the lectin microarray the profiles of both strains varied considerably and were distinguishable at both temperatures, due to difference in cell-surface glycoconjugate expression. Subsequently, a differential pro-inflammatory response was observed in chicken cells stimulated with lipooligosaccharide (LOS) isolated from *C. jejuni* 81116 grown at 37 and 42°C. Chicken embryonic fibroblast (CEF) cells responded differently to the LOS types, in which 37°C LOS, but not 42°C LOS, induced pro-inflammatory gene expression. Mass spectrometry analysis determined that this was due to changes in the ratio of lipid A backbone disaccharide variants between the temperatures. Moreover, *C. jejuni* capsular polysaccharide (CPS) was not found to be immunogenic to chicken cells *in vitro*. Thus, CPS expression as well as temperature-related lipid A variation by *C. jejuni*, may be important in chicken immune evasion or subversion, and contribute to persistent colonisation. Finally, a proposed structure for *C. jejuni* 81116 LOS was elucidated, which contained a novel *N*-formyl modified hexose.

Chapter 1.
Introduction

1.1 *Campylobacter jejuni*

Campylobacter jejuni is the leading bacterial cause of gastroenteritis globally and is the primary antecedent infection associated with the debilitating autoimmune neuropathy, Guillain-Barré Syndrome (GBS) (Khanna *et al.*, 2006; Day *et al.*, 2009). Consequently, *C. jejuni* infection represents a significant health and economic burden worldwide (Young *et al.*, 2007).

1.1.1 Background

Theodor Escherich was first to describe a *Campylobacter*-like organism in 1881, isolating the bacterium from the stools of infants with diarrhoea. However, the first report of a causal relationship between infection with *Campylobacter* and enteric disease was not until 1931, when a curved-rod shaped bacterium classified as *Vibrio jejuni* was identified (Jones *et al.*, 1931). Later, this bacterium and related species were assigned to a new genus, *Campylobacter*, since most *Vibrio* spp. were determined to be facultative anaerobes whereas *Campylobacter* were microaerophilic, and had distinct genetic, biochemical and serological features (Smith *et al.*, 1974). Subsequently, another breakthrough in *Campylobacter* research came with successful isolation and cultivation of the bacterium from faeces (Cooper & Slee, 1971; Skirrow, 1977; Butzler & Skirrow, 1979; Jin *et al.*, 2001).

To date, thirteen species have been assigned to the species *Campylobacter*, which occur as commensals or parasites in humans and wild and domestic animals (Silva *et al.*, 2011). Two species, *Campylobacter jejuni* and *C. coli* are the primary causes of *Campylobacter* enteritis in humans (Munroe *et al.*, 1983; Allos, 2001; Hughes & Cornblath, 2005; Siemer *et al.*, 2005; Ruiz-Palacios, 2007; Zilbauer *et al.*, 2008), and indeed *C. jejuni* alone is identified as one of the primary bacterial causes of gastroenteritis globally (Buzby & Roberts, 1997; Allos, 2001; Ruiz-Palacios, 2007). Clinically, these infections are not distinguishable, and faecal isolation is required for identification of the offending species (Sebauld & Veron, 1963; Altekruse, 2008). Infection leads to acute inflammatory enterocolitis with symptoms including diarrhoea, bloody stool, abdominal pain, nausea, fever and vomiting (Cooper & Slee, 1971; Skirrow, 1977; Butzler & Skirrow, 1979; Blaser & Reller, 1981; Blaser *et al.*, 1983; Chowdhury, 1984; Allos & Blaser, 1995; Jin *et al.*, 2001; Bereswill & Kist, 2003; Moore *et al.*, 2005). Natural reservoirs for *Campylobacter* include many domestic and

wild animals, insects and protozoans (Sahin *et al.*, 2002; Crushell *et al.*, 2004; Axelsson-Olsson *et al.*, 2005; Strother *et al.*, 2005; Silva *et al.*, 2011). Avian species, particularly poultry are identified as primary sources of human infection, with high carriage rates of 50-100% reported (Wempe *et al.*, 1983; Neill *et al.*, 1984; Prescott & Gellner, 1984; Fields & Swerdlow, 1999; Jin *et al.*, 2003; Lee & Newell, 2006). Contamination rates of raw retail chicken with *C. jejuni*, of up to 98% have been reported (Marinescu *et al.*, 1987; Kawai *et al.*, 2012), with Ireland having one of the highest rates in Europe (Kawai *et al.*, 2012; Eurosurveillance editorial team, 2013). Therefore, the broiler carcass reservoir represents the primary source of infection, accounting for up to 80% of cases of campylobacteriosis (Del Rocio Leon Kempis & Guccione, 2006; Eurosurveillance editorial team, 2013), and improper handling and consumption of raw or undercooked chicken is the main risk factor for enteritis due to *C. jejuni* infection (Fauchere *et al.*, 1986; Pei *et al.*, 1998; Lee & Newell, 2006).

1.1.2 Fundamental Microbiology and Genetics

Campylobacter jejuni is a microaerophilic, Gram-negative, non-spore-forming bacterium, with spiral-rod shaped morphology. The cells are 0.2 to 0.8 µm wide and 0.5 to 5.0 µm long (Crushell *et al.*, 2004; Snelling *et al.*, 2005; Del Rocio Leon Kempis & Guccione, 2006). *C. jejuni* is highly motile with a single, polar unsheathed flagellum which powers a corkscrew-like motion (Ashgar *et al.*, 2007; Guerry, 2007; Flanagan *et al.*, 2009). *C. jejuni* can grow at temperatures ranging from 30 to 47°C, with optimal growth at 42°C (Stintzi, 2003; Ashgar *et al.*, 2007). Amino acids and citric acid cycle intermediates are the primary carbon sources for *C. jejuni* (Hofreuter *et al.*, 2008; Flanagan *et al.*, 2009). Long believed to be asaccharolytic (unable to utilise carbohydrates as a carbon source), recent evidence has identified virulence-associated metabolism of the carbohydrate fucose in certain *C. jejuni* strains (Bacon *et al.*, 2001; Bachtiar *et al.*, 2007; Muraoka *et al.*, 2011; Stahl *et al.*, 2011). Biochemically, *C. jejuni* is oxidase and catalase positive. The ability of *C. jejuni* to hydrolyse hippurate can be used to differentiate from *C. coli*, which is hippurate negative (Harvey, 1980; Bacon *et al.*, 2001). However, some *C. jejuni* strains are also hippurate negative, meaning genotypic methods are more favorable for differentiation of *C. jejuni* and *C. coli* (Fry *et al.*, 2000; Silva *et al.*, 2011).

C. jejuni has a small genome (1.7 mbp) as determined by *SalI* and *SnaI* digestion, compared to *E. coli* (4.5 mbp) (Fry *et al.*, 2000; Parkhill *et al.*, 2000). The publication of the first *C. jejuni* genome for National Culture Type Collection (NCTC) 11168 in 2000 facilitated the development of multiple genetic and genomic tools for an unprecedented level of genetic research into the species (Dorrell *et al.*, 2001; Gilbert *et al.*, 2002; Karlyshev *et al.*, 2004; 2005; Parker *et al.*, 2005). Since then, the genome sequences of four other *C. jejuni* strains (81-176, 81116, CC8486, RM1221), have been completed (Karlyshev *et al.*, 2004; Fouts Derrick *et al.*, 2005; Hofreuter *et al.*, 2006; Pearson *et al.*, 2007; Young *et al.*, 2007; Poly *et al.*, 2008), and comparative genetic analysis has revealed a remarkable level of genomic diversity (Dorrell *et al.*, 2001; Young, 2002; Hofreuter *et al.*, 2006; Pearson *et al.*, 2007; Poly *et al.*, 2008). Characteristically, the *C. jejuni* genome has a low G+C content of about 30% (Karlyshev *et al.*, 2001) and the 11168 genome is predicted to encode 1,643 proteins (Corcoran *et al.*, 2006). Analysis of the *C. jejuni* genome reveals numerous repeat sequences or polymeric tracts (e.g. homopolymeric tracts of repeat As, Cs, Gs and Ts), enabling phase variation of genes and surface structures (Prendergast *et al.*, 2001).

1.1.3 Pathogenesis

Human infection with *C. jejuni* frequently results from consumption of contaminated poultry products. *C. jejuni* is considered a commensal of avian species such as chicken. Despite over 20 years of intensive research, the interaction of *C. jejuni* with its human and chicken host is not well understood, not helped by the high-level of genetic diversity that exists between strains of *C. jejuni* (Prendergast *et al.*, 1998; Nachamkin *et al.*, 1999; Yuki, 2010). Progress in understanding has suffered due to a lack of standardization of research methods in the literature, and the use different strains and cell models throughout, leading to sometimes incomparable and conflicting results (O'Croinin & Backert, 2012). Nonetheless, a clearer picture is beginning to emerge, and a number of virulence factors have been identified which are involved in *C. jejuni* host interaction, including the flagella and other outer membrane structures, toxin production and host cell adhesion and invasion.

1.1.3.1 Flagella and secretion

The flagellum is an important virulence factor of *C. jejuni*, which enables motility and plays a key role in infection (Moran *et al.*, 2005). Newell *et al.* (1984) first characterised the *C. jejuni* flagellum, which was shown to consist of a major protein (62 kDa) and a minor protein (87 kDa), both of which were absent in an aflagellate variant. Further studies by Newell *et al.* (1985) found that an aflagellate variant was a poor coloniser of mice, in comparison to a flagellated, non-motile variant which was a successful mouse coloniser. The author suggested that active flagella were only weakly associated with the host cell (Moran *et al.*, 2005). Nachamkin *et al.* (1993) refuted this suggestion, and demonstrated that intact motile flagella are integral in chick colonisation. Evidence has also suggested a role for the flagella in bacterial internalisation (Nachamkin *et al.*, 1999). Decreases in internalisation of 30-40 fold, were observed in *flaA*⁻ *flaB*⁻ motility negative mutants, indicating that either the FlaA or motility itself are required for internalisation (Nachamkin *et al.*, 1999).

Another suggested role for the flagellar apparatus is in the secretion of certain protein factors from the bacterial cell, such as the *Campylobacter* invasion antigens, CiaB and CiaC (Prendergast *et al.*, 1998). Translocation of CiaB in the host cytoplasm following cell adherence, has been observed which indicates its involvement as an effector molecule (Moran *et al.*, 2002), and mutation of *ciaB* and *ciaC* results in impaired invasion of INT407 cells (Sack *et al.*, 1998). Synthesis of Cia proteins occurs in the presence of the bile component deoxycholate, however Cia secretion does not occur until *C. jejuni* associates with the epithelial cell surface (Sack *et al.*, 1998). The precise role of these secreted proteins in *C. jejuni* invasion is unknown, although CiaB is required for secretion of other potential invasion factors (Guerry *et al.*, 2002a). Other proteins potentially secreted by the *C. jejuni* flagellum include CiaI which may promote intracellular survival (Godschalk *et al.*, 2004), and FlaC, observed to mediate invasion to HEp-2 cells (Guerry *et al.*, 2000; 2002b; Song *et al.*, 2004). However the importance of *C. jejuni* flagellar secretion in invasion is controversial, and it was recently reported that mutation of *ciaB* did not significantly reduce host cell invasion by *C. jejuni* 81-176 (Novik *et al.*, 2010).

A recurring theme amongst *C. jejuni* cell surface components is the capacity for structural variation. Phase variation results in alternate expression of the *C. jejuni* flagella (Aspinall *et al.*, 1993; Guerry, 2007), a trait that may aid *C. jejuni* host and environmental adaptation. Following ingestion, the *C. jejuni* flagella plays a central role

in host colonisation and infection, by enabling the bacterium to come in contact with the host cell. Upon epithelial attachment the flagella may then play a similar role to the secretion systems of *Yersinia* and *Salmonella* in the delivery of invasion factors to the host cell.

1.1.3.2 Toxin production

Certain *C. jejuni* strains were shown to produce enterotoxin by Daikoku *et al.* (1990), who reported a *Campylobacter jejuni* enterotoxin (CJT) similar to cholera toxin. Further studies described a CJT that was heat-labile, pH-dependant and non-haemolytic (Saha & Sanyal, 1990; Aspinall *et al.*, 1994; Suzuki *et al.*, 1994; Prendergast & Moran, 2000; Moran & Prendergast, 2001; Godschalk *et al.*, 2004; Takahashi *et al.*, 2005; Godschalk *et al.*, 2007). However, the production of enterotoxin by *C. jejuni* is controversial since many laboratories have consistently failed to detect any *C. jejuni* enterotoxigenicity. Furthermore, genome sequencing has failed to identify genes encoding a cholera toxin-like enterotoxin (Aspinall *et al.*, 1993; Yuki *et al.*, 1993; Moran, 1995; Pickett & Whitehouse, 1999; Moran *et al.*, 2002).

The reported enterotoxigenicity attributed to CJT may in fact be a consequence of another, better-characterised toxin, known as cytolethal distending toxin (CDT). This toxin was first reported by Johnson & Lior (1988), and has since been shown to be present in 97.4 % of *C. jejuni* isolates. Three genes encode the CDT multimer, *cdtABC*, the transcription of which may be regulated by the *luxS* gene, which is involved in quorum sensing (Elvers & Park, 2002). This implies that CDT production may be under community level regulation. The cellular receptor for CDT is yet to be characterised.

The effects of CDT on the host cell are thought to be a result of stalling of the cell cycle taking place during the G2 phase of mitosis. Whitehouse *et al.* (1998) detected accumulation of inactivated tyrosine-phosphorylated CDC2, in cells exposed to CDT, causing cell cycle arrest. Cytokine production in cells exposed to CDT has been described (Hickey *et al.*, 2000; O'Leary *et al.*, 2011), but ultimately exposure to the toxin results in cell distention, nuclear lysis and death (Pickett & Whitehouse, 1999; Patricia Guerry, 2012). Death of gut epithelial cells from CDT exposure is a potential contributor to diarrhoea observed during *C. jejuni* infection.

1.1.3.3 Outer membrane structures

Apart from the flagellum, other outer membrane structures are produced by *C. jejuni* which can have important roles in pathogenesis such as the major outer membrane protein (MOMP), outer membrane protein (omp)-H and the Omp50 porin, CmeC (involved in multidrug resistance), and surface adhesins which will be discussed later (Meinersmann *et al.*, 1997; Konkel *et al.*, 1997a; Galindo *et al.*, 2001; Dedieu *et al.*, 2002; Prendergast *et al.*, 2004; Fakhr *et al.*, 2007). Furthermore the outer membrane profile is influenced by the iron status of the environment, indicating that outer membrane porins are involved in iron acquisition, a virulence factor critical in pathogenesis (Schwartz *et al.*, 1994; Jacobs *et al.*, 1998; Moran & Prendergast, 2001; Yuki *et al.*, 2007). However, the predominant and most significant outer membrane components in *C. jejuni* are glycoconjugates. *Campylobacter jejuni* displays on its cell-surface a diverse array of glycoconjugates, including lipooligosaccharide (LOS) and capsular polysaccharide (CPS), as well as glycoproteins, which play important roles in pathogenesis. These molecules have been observed to have diverse roles in the disease process such as host cell adhesion and invasion, as well as immune evasion and modulation.

Comprising the outermost part of the cell, *C. jejuni* cell surface glycoconjugates are highly variable due to multiple genetic mechanisms, such as phase variation (Karlyshev *et al.*, 2005b; Moran, 2010). The high levels of variation that occur in LOS and CPS contributes to immune system evasion, which can prolong infection (Young *et al.*, 2007; Ogawa *et al.*, 2009). Immune evasion is also attributed to mimicry of host gangliosides, through the sialylation of *C. jejuni* LOS (Sack *et al.*, 1998; Moran & Prendergast, 2001). Furthermore, modification of CPS with methyl phosphoramidate (MeOPN) is another mechanism by which *C. jejuni* can resist attack by the host immune system and is involved in modulation of the immune system (Wassenaar & Blaser, 1999; Maue *et al.*, 2012). Immune system modulation, which can also involve LOS, is an important virulence factor that would enable *C. jejuni* to influence the inflammatory response and enable successful infection (Gilbert *et al.*, 2001; Heikema *et al.*, 2010; Klaas *et al.*, 2012). Numerous studies have highlighted the roles *C. jejuni* LOS and CPS in host cell adherence and invasion, with potential role for certain glycoproteins. These topics will be discussed in more detail in the coming sections.

1.1.3.4 Adherence

For enteric bacterial pathogens such as *C. jejuni*, adherence to gastrointestinal (GI) epithelial cells is essential in enabling successful infection of the host (Finlay & Falkow, 1997; Guerry *et al.*, 2002a). The process of host cell adherence by *C. jejuni* is a complex and poorly understood phenomenon, although a variety of cell-surface structures have been identified that may contribute to adhesion, including flagella, OMPs and cell-surface glycans.

Adhesins are cell-surface macromolecules that mediate binding to host cell receptors (Guerry *et al.*, 2002; O'Croinin & Backert, 2012). Early research into *C. jejuni* pathogenesis identified the flagellum as a potential adhesin involved in *C. jejuni* interaction with the host cell (Newell *et al.*, 1984; McSweegan & Walker, 1986; Prendergast *et al.*, 2004). It was later indicated that the flagellum itself has no direct role in host cell adhesion, although flagellar-motility is involved in positioning the bacterium to enable interaction of cell-surface adhesins with the epithelium (Grant *et al.*, 1993; Gilbert *et al.*, 2001). Subsequent to adhesion, the *C. jejuni* flagellum may then be involved in host cell invasion, potentially through the secretion of invasion factors (Konkel *et al.*, 1999; Karlyshev *et al.*, 2005a).

Konkel *et al.* (1997) identified a 37 kDa OMP that was involved in *C. jejuni* binding to immobilized and soluble fibronectin (Fn), a component of the epithelial cell extracellular matrix. *Campylobacter* adhesin to fibronectin (CadF), is found in all *C. jejuni* strains examined so far, and mediates adherence to INT407 cells (Moser & Schroeder, 1997; Monteville *et al.*, 2003; Taylor & Roberts, 2005) and T84 cells (Bacon *et al.*, 2001) *in vitro*, as well as enabling successful chick colonisation (Ziprin *et al.*, 1999; Karlyshev *et al.*, 2004; Flanagan *et al.*, 2009). Fibronectin is a ~250 kDa glycoprotein located at regions of cell-to-cell contact in the GI epithelium, but is particularly concentrated at the basolateral surface of intestinal epithelial cells (IECs) (Karlyshev *et al.*, 2001; Monteville & Konkel, 2002). A study by Monteville & Konkel (2002) found that Fn-mediated adherence of *C. jejuni* occurs preferentially at the basolateral surface of polarized epithelial cells. Host cell receptor saturation by competing *C. jejuni* on the apical surface of polarized epithelial cells may encourage paracellular translocation of unbound *C. jejuni* to the basolateral surface, where CadF then enables Fn-mediated adherence (Monteville & Konkel, 2002; Corcoran *et al.*, 2006). While the CadF molecule is highly immunogenic (Konkel *et al.*, 1997b; Prendergast *et al.*, 2001), post-translational modification of *cadF* gene products results

in a number of structural variants that, while not immunoreactive, retain the Fn-binding sequence FRLS (Prendergast *et al.*, 1998; Nachamkin *et al.*, 1999; Konkel *et al.*, 2005; Scott *et al.*, 2010; Yuki, 2010). Therefore, CadF modification may represent a form of antigenic variation contributing to immune evasion, while simultaneously facilitating adherence through Fn-binding (Scott *et al.*, 2010).

Infection-mediated by ECM-binding factors called Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs), which include CadF of *C. jejuni*, is a common theme amongst bacterial pathogens (Konkel *et al.*, 2010). Another recently identified *C. jejuni* protein known as fibronectin-like protein (Flp)-A is involved in adhesion of epithelial cells, as well as chick colonisation (Flanagan *et al.*, 2009). Like CadF, this molecule is conserved across the species, has Fn-binding activity and is a member of the MSCRAMM family (Flanagan *et al.*, 2009; Konkel *et al.*, 2010). However, an extensive transposon mutagenesis screen for invasion-related genes in *C. jejuni* did not identify either CadF or FlpA as being required for adhesion/ invasion (Novik *et al.*, 2010b). Although this may be due to the different cell models used. Evidence suggests that *C. jejuni* adherence is mediated by more than one adhesion molecule, and the knockout of no single putative adhesin alone, completely abrogates adherence (Prendergast *et al.*, 1998; O'Croinin & Backert, 2012).

A 42.3 kDa *C. jejuni* lipoprotein termed *jejuni* lipoprotein (Jlp)-A was reported to mediate adhesion to *in vitro* HEp-2 cells, and was detected unbound in the culture medium during the growth cycle (Jin *et al.*, 2001). Moderate reductions in adherence were achieved by mutation of *jlpA*, as well as by pre-incubating HEp-2 cells with anti-JlpA antibodies or purified JlpA (Jin *et al.*, 2001). A later study identified heat-shock protein 90 α (Hsp90 α) as the cellular receptor for JlpA, and their molecular interaction resulted in cell signaling events involved in the initiation of the inflammatory response (Jin *et al.*, 2003). Interestingly, crystal structure analysis of JlpA indicates that the binding site is capable of accommodating multiple ligands (Kawai *et al.*, 2012). However, studies by Flanagan *et al.* (2009) and Novik *et al.* (2010) reported no involvement of JlpA in adherence of another strain to either human T84 colon carcinoma cells or chicken LMH hepatocellular carcinoma epithelial cells, respectively. Although JlpA binding to INT407 cells has recently been reported (Kawai *et al.*, 2012). So, JlpA mediated adherence may be only be specific for particular *C. jejuni* strains or cell culture models. Tu *et al.* (2008) reported upregulation of JlpA with exposure to the

mucin glycoprotein MUC2. Since JlpA is upregulated with mucin exposure, and is found in culture medium, it may primarily exist as a secreted factor with an indirect involvement in adhesion, or have an alternative role in pathogenesis, such as manipulation of the host immune system.

A distinct group of *C. jejuni* membrane proteins ranging from 26-30 kDa known as CBF (cell-binding fractions) proteins, proposed to have roles in adhesion, were first identified by Fauchère *et al.* (1989) and later described as PEB1, PEB2, PEB3 and PEB4 (Karlyshev *et al.*, 2005; Del Rocio *et al.*, 2006). Initial studies implied roles for the PEB proteins in adhesion (Fauchère *et al.*, 1986; Pei *et al.*, 1998), although further research indicated that like JlpA the PEB proteins have roles distinct from adhesion, but nonetheless important in pathogenesis. For example, PEB1 which shares homology with an ABC transporter, is involved in amino acid acquisition, and the perceived reduction of adherence due to knockout of this protein was likely due to defects in essential metabolic processes rather than adhesion in itself (Del Rocio *et al.*, 2006). Kervalla *et al.*, (1993) showed that PEB1 and PEB4 were identical to CBF1 and CBF2, respectively.

The *C. jejuni* lipoprotein, CapA (*Campylobacter* adhesin protein A), was originally identified as an autotransporter protein (Ashgar *et al.*, 2007; Flanagan *et al.*, 2009). Mutation of *capA* resulted in decreases in adherence to Caco-2 cells of 30% (Ashgar *et al.*, 2007), and a reduction in adherence to chicken LMH cells of 47% (Ashgar *et al.*, 2007), although the protein was detected only in a subset of strains tested, and therefore is likely a strain-specific adhesin.

As previously stated *C. jejuni* cell-surface glycans, may also play important roles in *C. jejuni* adhesion with host cells. *C. jejuni* genes involved in capsular polysaccharide biosynthesis have been identified as adhesion molecules (Bacon *et al.*, 2001; Bachtiar *et al.*, 2007). Mutation of *kpsM* in *C. jejuni* 81-176 results in the loss of a serodominant high-M_r polysaccharide, identified as a capsule, and a significant reduction in adherence and invasion of INT407 cells (Bacon *et al.*, 2001). Bachtiar *et al.* (2006) similarly demonstrated the role of CPS in adherence of *C. jejuni* 81116, by knockout of capsular biosynthesis gene *kpsE*. Additionally, a role for *C. jejuni* LOS in adherence has been suggested. Early work by McSweegan and Walker (1986) demonstrated radiolabelled *C. jejuni* LOS binding to INT407 cells, as well as dose-dependent LOS-mediated inhibition of *C. jejuni* adherence. Mutations causing structural modification of *C. jejuni* LOS caused a reduction in adhesion of *C. jejuni* to *in vitro* epithelial cells (Fry *et al.*,

2000; Allos, 2001). A *galE* mutant resulting in a truncated LOS had reduced ability to adhere to INT407 cells (Fry *et al.*, 2000). Louwen *et al.* (2008) indicated a role for sialylation of LOS in particular, in adherence, but especially in invasion of certain *C. jejuni* strains. Other work has highlighted the involvement of the *C. jejuni* general glycosylation system, coded by the *pgl* protein glycosylation (*pgl*) gene family, in *C. jejuni* adherence (Karlyshev *et al.*, 2004). Szymanski *et al.* (2002) documented reductions in adherence in *C. jejuni* 81-176 of 38% and 59%, with mutation of *pglB* and *pglE*, respectively. Later work by Karlyshev *et al.* (2004) demonstrated dramatic reductions in *C. jejuni* adherence with loss of *pglH*. It has been suggested that glycosylated proteins may mediate attachment to host cells via interaction with host receptors such as lectins (Karlyshev *et al.*, 2004). Numerous other *C. jejuni* genes have been identified which appear to have an indirect involvement in adhesion, including *sodB* (Novik *et al.*, 2010b) and *htrA* (Baek *et al.*, 2011), which may influence the expression of certain OMPS such as adhesins, or influence critical metabolic and physiological processes.

1.1.3.5 Invasion

Epithelial cell invasion is an important pathogenicity-related factor and internalised *C. jejuni* are readily observed in tissue biopsies (Van Spreeuwel 1985), and in cell culture models *in vitro* (Oelschlaeger *et al.*, 1993; Watson & Galán, 2008; Eucker & Konkel, 2012). Different strains vary in their invasion capacities (Newell *et al.*, 1985), and in the invasion mechanisms they employ, making complete understanding of invasion process difficult. *C. jejuni* lacks the classical pathogenicity-related factors of other enteropathogens such as *Yersinia* and *Salmonella* (Dasti *et al.*, 2009), although some research has suggested that the flagellum can act as a type-III secretion system (T3SS) for the secretion of invasion proteins (Konkel *et al.*, 1999; Wassenaar *et al.*, 2000; Christensen *et al.*, 2009; Neal-McKinney *et al.*, 2010).

Cell-surface carbohydrates may also be involved in the *C. jejuni* invasion process. *C. jejuni* CPS has a role in adherence but also invasion of intestinal cells *in vitro* (Bacon *et al.*, 2001; Bachtiar *et al.*, 2007). Furthermore, research indicates that the sialylated LOS of *C. jejuni* is important in epithelial cell invasion, and once internalised, LOS contributes to *C. jejuni* intra-epithelial survival (Naito *et al.*, 2010). A study by Muller *et al.* (2007) identified the gene *cgtB* that encodes a putative galactosyltransferase as a factor related to the hyper-invasive phenotype of certain *C. jejuni* strains. Indeed, other studies have reported decreased epithelial cell invasion with truncation of *C. jejuni* LOS

through mutation of genes *galeE* (Fry *et al.*, 2000; Gilbert *et al.*, 2004), *waaC* (Kanipes *et al.*, 2004; Young *et al.*, 2007; Naito *et al.*, 2010), *cj1116* (Young *et al.*, 2007; Javed *et al.*, 2012), *wlaTB* and *wlaTC* (Hänel *et al.*, 2004; Holden *et al.*, 2012), which encode putative glycosyltransferases, as well as *wlaRG* which encodes a putative aminotransferase (Holden *et al.* 2012). In contrast, a *cgtA* mutant of *C. jejuni* 81-176 was significantly more invasive than the wild type, although the mutant LOS was lacking only a terminal GalNAc (Guerry *et al.*, 2002), compared to the deep structural effects of *galeE*, *waaC* and *cj1116* LOS mutants. Natural phase variation in *C. jejuni* 81-176 is a mechanism by which *cgtA* expression can be turned-off, resulting in a more invasive phenotype (Guerry *et al.*, 2002).

The relative importance of specific cytoskeletal components in *C. jejuni* invasion is controversial, due to strain specific-differences in contribution of microtubules and microfilaments (Biswas *et al.*, 2000; Monteville *et al.*, 2003; O'Croinin & Backert, 2012). The data suggests that certain strains may require microtubules (Oelschlaeger *et al.*, 1993), whereas other strains require microfilaments (de Melo & Pechere, 1990), or both microtubules and microfilaments (Monteville *et al.*, 2003), for invasion. A proposed invasion mechanism involves dual interaction of *C. jejuni* adhesins CadF and FlpA with host cell fibronectin leading to activation of integrin receptors, and a cascade of cell signaling events following secretion of Cia proteins (Eucker & Konkel, 2012). Following this, activation the the cell signaling G-protein Rac1 (from the Rho-family of GTPases) results in changes in microtubule and microfilament dynamics leading to membrane ruffling and bacterial internalisation (Krause-Gruszczynska *et al.*, 2007).

C. jejuni has also been observed invading epithelial cells via plasma membrane invaginations called caveolae, and then surviving intracellularly in endosomal vesicles that enable bacterial transcytosis across the intestinal epithelial barrier (Watson & Galán, 2008). Intracellular survival of *C. jejuni* within endosomes is aided by actively avoiding fusing with lysosomes, which may be achieved to secretion of CiaI (Watson & Galán, 2008). *C. jejuni* internalisation into epithelial cells and activation of signal transduction events leads to a pro-inflammatory response with typical induction of pro-inflammatory cytokines such as interleukin (IL)-8 and recruitment of immune system components to the site of infection, leading to the symptoms of campylobacteriosis (Hickey *et al.*, 2000). Invasion by *C. jejuni* has also been shown to cause internalisation and translocation of epithelial cells by non-pathogenic commensals (Kalischuk *et al.*,

2009; 2010), a factor that may be involved in inflammatory bowel disease (IBD) (Kalischuk *et al.*, 2010).

1.1.4 Infection in Humans

Infection with *C. jejuni* typically manifests after 2-5 days as an acute gastroenteritis, from an inoculating dose as low as 500-800 bacterial cells (Robinson, 1981; Black *et al.*, 1988). The virulence of the infecting strain and individual host susceptibility are determinants of the severity and specific presentation of disease (Fauchere *et al.*, 1986; Mortensen *et al.*, 2009). In the developed world the disease primarily consists of self-limiting, bloody diarrhoea with mucus (Blaser *et al.*, 1982; Altekruse, 2008). However, in the developing world, watery diarrhoea is the predominant outcome, with infection of young children more common (Coker *et al.*, 2002). Repeated exposure at a young age in the developing world may lead to immunity in later life and protect against the more severe symptoms that are experienced in the developed world (Coker *et al.*, 2002). The rate of asymptomatic carriage of *C. jejuni* in children in the developing world may be as high as 15% (Megraud *et al.*, 1990).

After ingestion, *C. jejuni* must traverse low pH environment of the stomach and survive the detergent action of bile in the upper intestine. Subsequently, in order to establish infection, *C. jejuni* must penetrate the mucus layer overlaying the epithelial cells of the GI tract (Young *et al.*, 2007). This is aided by a corkscrew morphology, and a high rate of flagellar motility that is upregulated upon entering the viscous environment of the mucus layer (Szymanski *et al.*, 1995). Furthermore, structural studies have demonstrated an absence of O-chain containing LPS in *C. jejuni*, and the presence of a shorter LOS molecule, which may reduce non-specific binding of intestinal mucin glycoproteins (McSweeney & Walker, 1986), and enable passage to the underlying epithelium. Following adherence, disease progression may proceed due to a number of mechanisms including toxin production leading to watery diarrhoea, invasion and proliferation within cells lining the gut resulting in inflammatory diarrhoea, and mucosal translocation, leading to invasion of extraintestinal sites and potential complications including meningitis (Jones *et al.*, 2004; Siegesmund *et al.*, 2004; Hu *et al.*, 2008).

1.1.4.1 Human immune response to *C. jejuni* infection

Typically, *C. jejuni* infections are resolved before an adaptive immune response can be mounted (Kuijf *et al.*, 2010), indicating that the innate immune defense is integral in clearing infection. Initial detection of *C. jejuni* occurs through components of the innate immune system expressed in the gut epithelium, known as pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPS) on the *C. jejuni* cell surface. The most important of these PRRs are the Toll-like receptors (TLRs). Ten human TLRs have been identified, the most important in bacterial detection are TLR1/2/6, TLR4/MD-2, TLR5 and TLR9, which detect lipoproteins, LPS/ LOS, flagellin and DNA, respectively (de Zoete *et al.*, 2010a). Activation of a TLR results in a signaling cascade involving the adaptor proteins, myeloid differentiation primary response gene-88 (MYD88), or TIR-domain-containing adaptor-inducing interferon- β (TRIF), which results in activation of nuclear transcription factors NF- κ B or IRF3, leading to expression of effector molecules such as cytokines (Akira, 2006).

C. jejuni avoids detection by TLR5 through a specific modification of the primary structure of its flagellin (de Zoete *et al.*, 2010b). Activation of TLR9 with *C. jejuni* results only in low levels of IL8 secretion (de Zoete *et al.*, 2010a). However, TLR-2 and TLR4/ MD2-mediated sensing of *C. jejuni* lipoproteins and LOS, respectively, through the initiation of MyD88 and TRIF, results in secretion of inflammatory mediators and the recruitment of dendritic cells (DCs) (Rathinam *et al.*, 2009). Dendritic cells are an important first line defense to pathogens and form a bridge between the innate and adaptive immune systems (Steinman & Hemmi, 2006). *C. jejuni* internalisation by DCs results in cell maturation and production of cytokines such as IL1, IL6, IL8 and tumor necrosis-factor (TNF) α (Kuijf *et al.*, 2010), and activation of Th1 and Th2 cells (Bax *et al.*, 2011). Furthermore, DCs express their own PRRs including TLR4, and *C. jejuni* LOS is responsible for much of the DC maturation (Kuijf *et al.*, 2010), which may also be influenced by sialic acid-binding immunoglobulin-like lectins (Siglecs) on DCs, which specifically interact with sialylated LOS of *C. jejuni* (Bax *et al.*, 2011). However, *C. jejuni* expression of *N*-glycans may result in modulation of DCs and reduce pro-inflammatory effects (Van Sorge *et al.*, 2009).

Secretion of pro-inflammatory cytokines such as IL8 results in an influx of neutrophils and monocytes/ macrophages to the site of infection (Borrmann *et al.*, 2007). These phagocytes exert their antimicrobial effect by the production of

antimicrobial peptides, reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Fang, 1997; Tosi, 2005). *C. jejuni* sialylation has been shown to cause increased phagocytosis by macrophages (Huizinga *et al.*, 2012). Conversely, *C. jejuni* sialylation may decrease immunogenicity and increase serum resistance, which would prolong infection (Guerry *et al.*, 2000).

Components of the innate defense such as DCs are also responsible for the proliferation of B cells leading to the generation of antibodies (Kuijf *et al.*, 2010). Despite a high level of antigenic diversity on the *C. jejuni* cell-surface, antibodies are generated during infection against cell surface structures (Jeon *et al.*, 2009; Kuijf *et al.*, 2010). Furthermore, a CDT neutralizing antibody response has been described (Abuoun *et al.*, 2005).

1.1.5 Infection in Chickens

C. jejuni colonisation of chickens is typically without pathological symptoms and the interaction is considered commensal in nature (Dhillon *et al.*, 2006). Therefore, contrasting outcomes exist in the *C. jejuni* interaction with its human or chicken host, the basis of which is not well understood. The interaction of *C. jejuni* with chickens is highly complex and requires considerable interplay between multiple key effectors essential in enabling successful and persistent colonisation. It is hoped that a better understanding of the processes involved would lead to more effective strategies for the control of *C. jejuni* in the foodchain and reduce the risk to the public (Young *et al.*, 2007).

After ingestion, *C. jejuni* travels toward the intestinal epithelial border, mediated by flagellar motility and chemotaxis (Hendrixson & DiRita, 2004), and within 24h a stable population is established (Coward *et al.*, 2008; Smith *et al.*, 2008). *C. jejuni* colonises the chicken GI tract in large numbers, with 10^8 - 10^9 cfu/g typically found in the cecum, the primary site of colonisation (Van Deun *et al.*, 2008; Meade *et al.*, 2009). Response of *C. jejuni* to chemotactic signals occurs via methyl-accepting chemotaxis proteins, such as DocB and Tlp1, both of which have essential roles in chick colonisation (Hendrixson & DiRita, 2004). Chemotactic and environmental signals are responsible for flagellar promoter activity enabling motility of the bacterium (Allen & Griffiths, 2001). *C. jejuni* exposure to intestinal factors such as low-pH, bile, deoxycholate, mucus, L-fucose, high osmolarity and chemotactic effectors such as

glutamate, citrate, aspartate, fumarate, succinate and α -ketoglutarate, results in *flaA* upregulation, which plays a key role in the colonisation process (Jones *et al.*, 2004; Hermans *et al.*, 2011). Other factors involved in flagellin biosynthesis and flagellar regulation, observed to be important in chick colonisation, include motility accessory factor 5 (*maf5*), *flgK*, *fliA*, *rpoN*, as well the FlgS/FlgR two-component signal transduction system (Hendrixson & DiRita, 2004; Fernando *et al.*, 2007).

C. jejuni adherence to the cecal epithelium has not been observed *in vivo* (Beery *et al.*, 1988), although *C. jejuni* is often found in extraintestinal sites including the spleen, thymus, liver, gallbladder and bursa of Fabricius (Cox *et al.*, 2005; Van Deun *et al.*, 2008; Meade *et al.*, 2009), which implies penetration of the epithelial cell barrier. Systemic dissemination is correlated with *C. jejuni* invasiveness in chick primary cecal epithelial cells (Van Deun *et al.*, 2008), indicating that transcellular rather than paracellular *C. jejuni* translocation of the epithelium occurs, which would require adhesion to initiate. The observation of invasion and systemic dissemination by *C. jejuni* in the chicken is atypical for commensals since the host microbiota is usually confined to mucosal surfaces (Yu *et al.*, 2012).

As previously stated, adhesion of *C. jejuni* in chickens may be mediated by flagella, adhesins including CadF and FlpA, as well as cell-surface carbohydrates such as glycoproteins, LOS and CPS. However, the specific role of these factors *in vivo* is debated (Hendrixson & DiRita, 2004; Novik *et al.*, 2010). Exposure to chicken mucus results in increased flagellar secretion of Cia proteins, with potential roles in chick colonisation (Ziprin *et al.*, 2001; Konkel *et al.*, 2004; Biswas *et al.*, 2007). *C. jejuni* is not known to multiply intracellularly, but probably does so within the mucus layer which is nutrient dense (Van Deun *et al.*, 2008). However, epithelial cell invasion by *C. jejuni* may occur occasionally, followed by evasion back into the mucus layer, cell growth and re-invasion in order to avoid mucosal clearance (Van Deun *et al.*, 2008).

In addition to motility, chemotaxis, adhesion and invasion, numerous other mechanisms such as bile and antimicrobial resistance, iron transport and regulation, oxidative and nitrosative stress defense, and various other metabolic elements play essential roles in enabling the persistent colonisation of *C. jejuni* in chickens (Hermans *et al.*, 2011)

1.1.5.1 Chicken immune response and *C. jejuni* commensalism

Although characterised by long term high-level colonisation with lack of pathology, *C. jejuni* colonisation of the chicken GI tract results in a pro-inflammatory response which does not lead to clearance of the bacterium, or prevention of systemic dissemination. *C. jejuni* immune evasion and modulation, combined with host immune inefficiency and a degree of host tolerance, are among the myriad factors likely responsible for this coexistence (Hermans *et al.*, 2011).

The low levels of inflammation induced during mucosal and systemic infection suggests that *C. jejuni* may be able to manipulate the chicken immune system and prevent an antimicrobial response. Early in the colonisation process the chicken homolog of human IL8 is produced by epithelial cells in response to *C. jejuni*, which typically in an infection would result in an influx inflammatory cells to the infected site. However, unlike during human infection, inflammatory cells such as heterophils (equivalent to mammalian neutrophils) are not recruited to the site of colonisation (Hendrixson & DiRita, 2004; Van Deun *et al.*, 2008; Shaughnessy *et al.*, 2009). This indicates that IL8 is post-translationally modified, or that there exists mechanisms by which *C. jejuni* interrupts chemotactic signalling by this chemokine (Shaughnessy *et al.*, 2009). Furthermore, *C. jejuni* downregulation of chicken antimicrobial factors has been described (Meade *et al.*, 2009). Secretory IgA, the predominant immunoglobulin at mucosal sites, is produced against *C. jejuni*, and although it doesn't completely inhibit *C. jejuni* proliferation and dissemination, it may play a role in limiting the infection (Cawthraw *et al.*, 1996; Widders *et al.*, 1998), potentially by coating the bacteria and mitigating invasiveness (Macpherson *et al.*, 2005).

As in humans, TLRs are an important component of the avian innate immune system, and are expressed on a range of cells throughout the chicken GI mucosa (de Zoete *et al.*, 2010a). Although chicken TLR biology is not well characterised, recent work has shed some light on their significance in *C. jejuni* recognition. Like humans, the chicken TLR4/myeloid differentiation protein-2 (chTLR4/chMD-2) complex, and chTLR2, recognise *C. jejuni* LOS and lipoproteins, respectively (de Zoete *et al.*, 2010a). Furthermore, as is the case with human TLR5, the *C. jejuni* flagella fails to activate chTLR5 (de Zoete *et al.*, 2010b). However, there are differences in recognition of DNA by chicken and human TLRs. As noted previously, TLR9 recognises *C. jejuni* DNA in humans. Chickens lack a homologue to human TLR9, but possess TLR21, unique to avian, fish and amphibian species, which recognises unmethylated single-

stranded microbial 2'-deoxyribo (cytidine-phosphateguanosine [CpG]) DNA motifs with broad ligand specificity (de Zoete *et al.*, 2010a). Despite this, viable *C. jejuni* have been shown to be poor activators of chicken TLR ligands (de Zoete *et al.*, 2010a). Potentially, the nutrient-rich environment in the chicken mucosa would sustain a low-level of bacterial cell lysis and potentially the pro-inflammatory effects of colonisation would be minimal (de Zoete *et al.*, 2010a).

Interferon- β (IFN- β) is a potent inducer of systemic inflammation, and in humans is involved in natural killer T-cell activation and maturation of dendritic cells. Interferon- β production in humans is induced by *C. jejuni* LOS after TLR4 activation through the MyD88-independent TRIF pathway. In chickens, the lack of TRAM (which links the TLR4 signalling domain to TRIF) results in an absence of the MYD88-independent pathway and an altered response to *C. jejuni* LOS, resulting in much lower toxicity for these molecules, which may be a factor accounting for the lack of pathological symptoms during chicken colonisation (de Zoete *et al.*, 2010a). This property of the chicken immune system combined with the high levels of antigenic diversity seen in *C. jejuni* cell-surface carbohydrates and flagella, would contribute to effective immune system subversion (Karlyshev *et al.*, 2004). Noteworthy also is that certain factors which are pathogenic in humans such as the CDT, do not have any observed pathological effect in chickens, and may have alternative roles such as immune modulation (Abuoun *et al.*, 2005). Moreover, in comparison to pathogenic *Salmonella* infection, no necrosis or tissue damage is observed during *C. jejuni* colonisation, further demonstration of the benign nature of the relationship (Van Deun *et al.*, 2008)

There are reports of reduced *C. jejuni* invasion of primary cecal epithelial cells after exposure to chicken mucus (Byrne *et al.*, 2007; Alemka *et al.*, 2010). So specific factors within the mucus layer may play a role in mitigating the level of *C. jejuni* pathogenicity within the chicken, although this effect may be inefficient since some invasion does occur.

C. jejuni colonises the cecal mucosa which like the lower intestine in humans supports high numbers of resident microflora. The site of infection in humans, the upper intestine, has a low resident microflora, due to the fast transit time of gut luminal contents in this region of the GI tract (Nataro *et al.* 2005). Therefore the resident chicken gut microflora, might compete with *C. jejuni* for available host epithelial

receptors and reduce infectivity (Soerjadi-Liem *et al.*, 1984). In turkeys, another avian species frequently colonised by *C. jejuni*, the composition of the microbiota has been shown to influence *C. jejuni* colonisation efficiency (Scupham, 2009; Scupham *et al.*, 2010). Research by Bereswill *et al.* (2011) in a mouse model, further highlights the influence of the host-specific microflora in susceptibility to *C. jejuni* infection. It was observed that *C. jejuni* infection of gnotobiotic mice, as well as “humanized” mice (with a human microbiota), lasted six weeks, whereas mice with a natural microbiota had cleared infection after two days (Bereswill *et al.*, 2011).

1.2 *Campylobacter jejuni* glycome

Glycoconjugates represent the dominant surface structures of all bacteria, including *C. jejuni*, which has an extensive carbohydrate repertoire consisting of LOS, CPS, and both *O*- and *N*- glycoproteins.

1.2.1 Endotoxin-LPS and LOS

Gram-negative bacteria are enveloped in a bilayered, phospholipid-containing outer membrane (OM) that plays a vital role in maintaining the physical integrity and functioning of the cell (Fig. 1.1) (Moran, 1995). The primary constituents of the outer leaflet of the OM are phosphorylated glycolipids, such as LPS and LOS (Raetz & Whitfield, 2002; Holst, 2007). In general, LPS consists of three domains: the lipid A, which is embedded in the OM; the core oligosaccharide covalently linked to the lipid A; and the O-chain, which is attached to the core and extends outwards from the cell (Holst, 2007). In comparison, LOS lacks the O-chain and the core oligosaccharide is the outer most component of the molecule (Raetz & Whitfield, 2002).

With approximately 3.5×10^6 LPS molecules on a bacterial cell, it comprises the dominant surface antigen (Lugtenberg, 1981), accounting for 3-8% of the dry mass of cells (Hancock 1994). These glycolipids play important roles in the bacterial protection from harmful compounds in the extracellular environment such as bile salts, and are critical virulence factors in the host (Holst *et al.*, 2009). Paradoxically, these molecules are potent stimulators of immune system components such as B-lymphocytes, granulocytes and mononuclear cell, and contain binding sites for antibodies and serum complement (Rietschel *et al.*, 1990; Rietschel *et al.*, 1991). However LPS/LOS also enable *C. jejuni* evasion of the immune system, due to high-levels of structural variation

(Guerry *et al.*, 2000). Release of LPS/LOS molecules in the blood stream by multiplying or lysing bacteria represents a medical emergency, as it can lead to endotoxic effects such as pyrogenicity, hypotension, septic shock and death. Although, certain chronically infecting species including *Helicobacter pylori* may produce LPS of low toxicity and immunological activity, a factor which may contribute to the development of chronic infection (Moran, 2007).



Figure 1.1 Schematic diagram of canonical enterobacterial lipopolysaccharide. Adapted from Gronow & Brade (2001). Figure has been removed due to copyright restrictions.

1.2.1.1 The O-specific Chain

The architecture of the O-chain consists of a polymer of repeating oligosaccharide units, composed of one to eight residues of a wide variety of sugars. A large potential for O-chain heterogeneity is afforded by variation in the nature, sequence, ring form, anomeric configuration, substitution and linkage of these sugar residues (Gronow & Brade, 2001; Raetz & Whitfield, 2002; Brandenburg *et al.*, 2003). Consequently, the structure of the O-chain differs widely from strain to strain, and between species (Raetz & Whitfield, 2002). The most common O-chain constituents are sugars such as hexoses, pentoses, 6-deoxyhexoses, 2-amino-2 deoxyhexoses, and uronic acids (Nixon-Anderson *et al.*, 1992, Raetz & Whitfield, 2002), although more unusual sugars and derivatives, as well as non-carbohydrate constituents are also found (Raetz & Whitfield, 2002). Comprising the outermost part of the LPS molecule, the O-chain is highly antigenic, forming the basis of the heat-stable serotyping of many species, and is a receptor for bacteriophage. As a consequence of the high rates of immunogenicity, the O-chain is hugely variable and multiple mechanisms of structural and antigenic variation exist. As an important

virulence determinant, the O-chain enables bacterial immune evasion and resistance to the antimicrobial effects of complement, thereby prolonging infection (Whitfield *et al.*, 1995).

1.2.1.2 Core Region

The core oligosaccharide is generally subdivided into two distinct regions: the inner core which is proximal to the lipid A; the outer core that connects to the O-antigen. The subdivision is based on the fact that the inner core is generally made up of unusual sugars such as heptoses and 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), whereas the outer core is generally composed of hexoses (Hex) and hexosamines (HexNAc) (Holst, 2011). The core region is more conserved than the O-antigen, but compared to the lipid A it shows some variation in structure, with variability mainly limited to outer core region. Moreover, in bacteria that lack the O-antigen such as *Neisseria* and *C. jejuni*, the outer core region tends to be structurally diverse since it now represents the primary antigenic molecule of these bacteria. Although lipid A is the toxic principal of endotoxically active LPS, the core region also contributes to toxicity by influencing the overall molecular conformation of the molecule (Holst *et al.* 2009). The core region is negatively charged and contributes to OM stability due to cross-linking with divalent cations (Holst *et al.* 2009).

1.2.1.2.1 Inner Core

Structurally, the inner core of LPS tends to be highly conserved within a bacterial family, and the inner core of all bacteria share common features reflecting its primary role in maintaining OM integrity (Raetz *et al.*, 2002; Fridrich & Whitfield *et al.*, 2005). In the *Enterobacteriaceae*, the inner core typically consists of heptose sugars and one or more Kdo residues or derivatives thereof. The tetrasaccharide L,D-Hep- α (1,7)-L,D-Hep- α (1,3)-L,D-Hep- α (1,5)-[Kdo- α (2,4)]-Kdo, (Hep III, Hep II, Hep I, Kdo II and Kdo I, respectively) is a typical constituent of the enterobacterial inner core, with attachment of Kdo I to lipid A via ketosidic linkage (Holst 2007; 2011). Heterogeneity in the inner core is achieved through substitutions that can include other sugars, phosphate residues, phosphoethanolamine (PEtn), acetyl residues and amino acids (Holst 2007; 2011)

Common to all *C. jejuni* inner cores is the trisaccharide L,D-Hep-(1,3)-L,D-Hep- α (1,5)-Kdo, with the Hep adjacent to the Kdo (Hep I) usually substituted with D-Glc

(Moran, 1997). This tetrasaccharide is common to most *C. jejuni*, although some strains possess a D-Gal residue in place of the D-Glc on Hep I (Moran, 1997). Recently, it has been discovered that certain *C. jejuni* strains substitute Hep II with the amino acid glycine (Dzieciatkowska *et al.*, 2007), the role of which is unknown. Furthermore, as with other bacteria the *C. jejuni* inner core can be substituted with phosphate residues and PEtn (Aspinall *et al.*, 1992; van Mourik *et al.*, 2010).

1.2.1.2.1 Outer core

The outer core region of LPS provides resistance to detergents and stabilizes the OM (Rietschel *et al.*, 1990). The outer core is sequentially assembled on lipid A at the cytoplasmic surface of the inner membrane, in a process directed by a number of membrane-associated glycosyltransferases (Firdich & Whitfield *et al.*, 2005). Constituent sugars of the outer core include D-glucose (Glc), D-galactose (Gal), D-N-acetyl-glucosamines (GlcNAc) (Rietschel *et al.*, 1990). The *C. jejuni* outer core typically contains these sugars, but many strains also include Neu5Ac. Sialic acid is a constituent of the gangliosides- a family of glycosaminoglycans that decorate the surface of cells in the nervous system. The core OS of many *C. jejuni* strains is structurally similar to a range of gangliosides, representing a form of molecular mimicry. *C. jejuni* ganglioside mimicry is an important predisposing factor in the development of the autoimmune disease Guillain-Barré syndrome (GBS) (Moran & Prendergast, 2001).

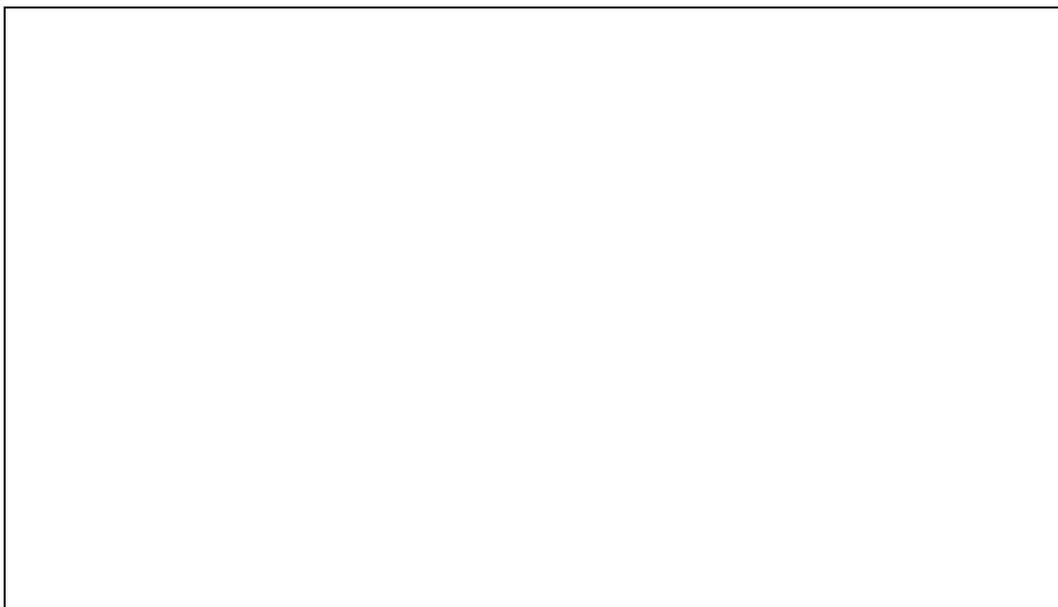


Figure 1.3 (A) Structure of GM₁ ganglioside (B) Schematic representation of gangliosides GM₁, GD_{1a}, GD_{1b} and GT_{1b}. Adapted from Prendergast & Moran (2000). Figure has been removed due to copyright restrictions.

Molecular mimicry by *C. jejuni* is not limited to that of gangliosides, with certain strains producing LOS structurally similar to a range of other host glycans (Houliston *et al.*, 2011). Indeed other *C. jejuni* strains do not have any mimicry in their LOS, including the HS:3 serostrain (Aspinall *et al.*, 1995), which has a core lacking Neu5Ac, but containing the unusual sugar residue 3-amino-3,6-dideoxy-D-glucose (*N*-acetylquinovosamine, QuiNAc), also found in *C. coli* LOS (Beer *et al.*, 1986).

1.2.1.3 Lipid A

Lipid A is the hydrophobic phospholipid that anchors LPS in the OM of Gram-negative bacteria, and is responsible for the endotoxic properties of the molecule. The general structure of lipid A is highly conserved and therefore it is readily detected by the ancient immune system receptor TLR4 (Raetz & Whitfield, 2002; van Mourik *et al.*, 2010). All lipid A examined so far consists of the *D*-*gluco*-configured pyranosidic hexosamine residues D-glucosamine [GlcN], or 2,3-diamino-2,3-dideoxy-D-Glc [GlcN3N], present in a β(1'-6)-linked homo- or heterodimer (Trent *et al.*, 2006). Classical, endotoxically active lipid A, typical of *E. coli* and *Salmonella enterica*, consists of a β(1'-6)-linked GlcN disaccharide backbone, phosphorylated at positions 1 and 4', with attached (R)-3-hydroxyacyl and (R)-3-hydroxyacyloxy chains at positions 2, 3, 2' and 3' (Trent *et al.*,

2006). Structural variation depends on the type of hexosamine disaccharide present, the amount of phosphorylation, the presence of phosphate substitutions, as well as the nature, chain length and position of acyl chains (Moran, 1997). Such modifications may occur in order to maintain OM stability in response to changing environmental conditions, as well as to protect from host antimicrobial factors (Friedrich & Whitfield, 2005). Modification of lipid A influences the immunoactivity of the LPS molecule within the host (Trent *et al.*, 2006).

Chemical analysis of *C. jejuni* HS:2 lipid A by Moran *et al.* 1991, revealed a lipid A structure deviating from the classical enterobacterial lipid A. The major molecular species is composed of a hybrid backbone of $\beta(1'-6)$ -linked GlcN3N-GlcN, with phosphate groups at positions 1 and 4', and compared to the 12 and 14 carbon acyl chains in *E. coli*, the *C. jejuni* lipid A consists of six fatty acid substitutions with 10 and 16 carbons (Moran *et al.*, 1991). Four hydroxytetradecanoic acid molecules are attached to the lipid A disaccharide at positions 2, 3, 2' and 3' and both position-3 acyl chains are substituted at their hydroxyl group with two hexadecanoic acid molecules, or one molecule of tetradecanoic and one of hexadecanoic acid (Moran *et al.*, 1991b). The other minor species vary according to the composition of the hexosamine backbone, and consist of either GlcN3N-GlcN3N (15%) or GlcN-GlcN (12%) disaccharides, with some interstrain variation in the ratios of these molecular species (Moran *et al.*, 1991b). The ratio of amide-linked acyl chains to ester-linked in *C. jejuni* is usually 3:1, compared to 2:2 for most *Enterobacteriaceae*. Esterification of the phosphate groups on *C. jejuni* lipid A with PEtn, offers resistance against cationic antimicrobial peptides such as polymyxin B (PMB) (Cullen & Trent, 2010). This is thought to reduce overall net negative charge of the LOS and prevent electrostatic interaction of the PMB with the OM (Cardoso *et al.*, 2007; Cullen & Trent, 2010).

1.2.1.4 Genetics

Investigation into the genetic basis of *C. jejuni* LOS biosynthesis began before the publication of the genome sequence, and the functions of certain genes were characterised by a number of research groups. Fry *et al.* (1998) reported *wla* biosynthesis genes thought to be involved in the production of the high- M_r PS in *C. jejuni* 81116. Gilbert *et al.* (2000) identified genes involved in generating ganglioside mimicry: a $\beta(1,4)$ -*N*-acetylgalactosaminyl-transferase (*cgtA*), a $\beta(1,3)$ -

galactosyltransferase (*cgtB*), and a bifunctional sialyltransferase (*cstII*), capable of transferring sialic acid to the O-3 position of galactose, as well as to O-8 position of a Neu5Ac α (2-3)-linked to gal. The publication of the complete genome sequence enabled a greater level of understanding of LOS biosynthesis (Parkhill *et al.*, 2000). Analysis of the *C. jejuni* 11168 genome identified a gene cluster spanning from *cj1131* (*galE*) to *cj1152* (*rfaD* or *gmhD*) with potential roles in inner and outer core biosynthesis (Parkhill *et al.*, 2000). The genes on one end of this cluster include *galE*, *waaC*, *htrB* (*waaM*) which code for uridine diphosphate- (UDP)-GlcNAc/Glc-4-epimerase, heptosyltransferase I, and a Kdo-lipid A acyltransferase, respectively (Gilbert *et al.*, 2008). Found at the opposite end of the cluster, *waaF*, *gmhA* and *gmhD*, encode a heptosyltransferase II, sedoheptulose-7-phosphate isomerase, and ADP-L-glycero-D-manno-heptose- 6 epimerase, respectively (Gilbert *et al.*, 2008). For 70 *C. jejuni* strains the sequences of the LOS biosynthesis genes between *cj1133* (*waaC*) and *cj1148* (*waaF*) are available, enabling the strains to be grouped into one to 19 classes (A-S), bases on gene content and organization (Gilbert *et al.*, 2008). Five LOS biosynthesis locus classes (A, B, C, M and R) are capable generating ganglioside-mimicking LOS, due to the presence of genes involved in LOS sialylation such as; *neuA*, a CMP-Neu5Ac synthase, *neuB*, a sialic acid synthase, *neuC*, an *N*-acetylglucosamine-6-phosphate 2-epimerase, and *cst-II*, a sialyltransferase. Guillain Barré syndrome-related strains most commonly belong to class A, whereas Miller Fisher syndrome is mostly associated with *C. jejuni* from class B (Parker *et al.*, 2005; Koga *et al.*, 2006; Godschalk *et al.*, 2006a; 2007). Recently, the gene *cj1136* was identified as a putative galactosyltransferase involved in LOS biosynthesis in *C. jejuni* 11168 (Javed *et al.*, 2012).

A number of genetic mechanisms exist which account for the high levels of variation observed in *C. jejuni* LOS. Horizontal transfer of entire LOS biosynthesis loci has been observed to occur between strains, which would account for the presence of an LOS biosynthesis locus in *C. jejuni* GBS isolate GB11, which is identical to the HS:19 serostrain, despite the genetic relatedness of GB11 to the NCTC 11168 genome strain (HS:2) (Gilbert *et al.*, 2004). Therefore, strain GB11 expresses LOS which mimics gangliosides GM_{1a} and GD_{1a}, similar to HS:19, compared to 11168 which produces GM_{1a} and GM₂-mimicking LOS (St Michael *et al.*, 2002a). Variability in LOS can also occur due to variation in expression of certain genes, such as *wlaN*, encoding a β (1,3)-

galactosyltransferase that converts the GM₂-mimicking LOS of 11168 to GM_{1a} (Linton *et al.*, 2000). Intragenomic homopolymeric G tracts present in *wlaN* enable the alternate on/off expression of the gene, due to slipped strand mismatching. This form of phase variation results in a population of 11168 cells expressing both GM_{1a} and GM₂-mimicking LOS. Furthermore, phase variation in the *C. jejuni* 81-176 *cgtA* gene results in variable expression of GM₂ and GM₃-mimicry, which influences the epithelial cell invasion (Guerry *et al.*, 2002). Prendergast *et al.* (2004) observed that phase variation of 81-176 LOS occurred in an *in vivo* experimental human volunteer challenge.

1.2.2 Capsular polysaccharide

The expression of a cell-surface polysaccharide capsule is a common feature of many bacteria. In general, CPS is composed of a polysaccharide (PS) chain, consisting of one or more sugar residues, attached to the bacterial outer membrane via a lipid anchor (Whitfield & Roberts, 1999). Diverse roles for CPSs include providing protection from the environment, as well as from the host immune system (Schembri *et al.*, 2004). There exists huge variation in the structure and composition of CPS, amongst bacterial strains and species (Whitfield & Roberts, 1999). Moreover, within a particular bacterial species CPS expression can be diverse, which combined with the LPS O-chain diversity, presents a dynamic and highly variable cell-surface (Whitfield & Roberts, 1999). High levels of CPS variation are afforded by genetic rearrangements and other genetic mechanisms such as phase variation.

Bacterial expression of hygroscopic extracellular PSs such as CPS in the *ex vivo* environment provide protection from cell desiccation (Potts, 1994). Within the host, the bacterial CPS can act as a cloak which masks antigenic components of the outer membrane, enabling immune evasion. Bacterial CPSs which mimic human glycolipids have been described. This includes the *E. coli* K1 CPS, which is composed of homopolymers of $\alpha(2-8)$ -linked sialic acid, identical to the terminal saccharide region of the neonatal neural cell adhesion molecule (N-CAM), found in neonates (Jann & Jann, 1987), as well the *E. coli* K5 CPS, which mimics an intermediate molecule in heparin biosynthesis (Jann & Jann, 1987). Due to biomimicry, these molecules are not recognised as “foreign” by the host immune system, and the bacteria are able to proceed with infection without detection by the immune system. Capsular polysaccharide antigenic variation and the inefficient humoral response to these surface antigens has

consequently hindered the development of efficient vaccines against CPS producing pathogens such as *Neisseria meningitidis* and *C. jejuni* (Harrison *et al.*, 2006; Guerry, 2012).

1.2.2.1 Capsular polysaccharide in *C. jejuni* serotyping

Penner and Hennessy (1980) introduced the heat-stable (HS)-antigen serotyping scheme for *Campylobacter*, based on passive hemagglutination. Capsular polysaccharide is now known to be the primary serodeterminant in the Penner serotyping scheme, but before the nature of high-M_r *C. jejuni* PS was found to be CPS, it was thought that the molecular basis of HS serotyping scheme was high-M_r LPS (Mills *et al.*, 1985). *Campylobacter jejuni* is now differentiated into 47 HS serotypes using this scheme, which reflects the diversity of CPS structures found in this species (Maue *et al.*, 2012).

Early investigators proposed that heat-extracted *C. jejuni* LPS was involved in serotyping by passive hemagglutination, whereas other antigens were not believed to be involved (Naess & Hofstad, 1985). Western blot analysis of purified *C. jejuni* LPSs with homologous antisera, identified high-M_r components with a banding pattern characteristic of high-M_r O-chain containing LPS (Kosunen *et al.*, 1984; Preston & Penner, 1987), and therefore, the *C. jejuni* HS-antigen was proclaimed to be LPS. However, while these molecules could be detected by typing antisera, these high-M_r molecules could not be visualized in electrophoresis gels by silver staining, whereas low-M_r components that were also detected by western blotting were readily stained. If the high-M_r polysaccharide were derived from LOS it too would be stainable with silver. Early researchers thought that *C. jejuni* produced two outer membrane glycolipids: a low-M_r LOS and a high-M_r LOS-derived, LPS-like molecule. However, certain studies had identified, and structurally characterised, high-M_r PS-independent of LOS, in HS:3 serostrain (Aspinall *et al.*, 1995) and a HS:41 clinical isolate (Hanniffy *et al.*, 1999). The publication of the first *C. jejuni* genome sequence (Parkhill *et al.*, 2000) provided the evidence that the genetic machinery for CPS biosynthesis was present in *C. jejuni*. Subsequently, Karlyshev *et al.* (2000) determined that the serodominant high-M_r molecule produced by *C. jejuni* was in fact capsular in origin, by carrying out mutational analysis of *kpsC*, *kpsM* and *kpsS* CPS biosynthesis genes. However, the precise involvement of the various *C. jejuni* cell-surface glycolipids in serotyping remains inconclusive, since there is evidence of involvement of LOS in the serotyping reactions (Moran & Penner, 1999). Furthermore, it is well characterised that cross-

reactive anti-ganglioside antibodies are generated against ganglioside-mimicking LOS (Prendergast & Moran, 2000), and while CPS may be the primary serodeterminant, it is clear that LOS plays a significant role in the serological reactions of *C. jejuni*.

1.2.2.2 Structural analysis of *C. jejuni* CPS

In the pre-capsule era, structural studies on *C. jejuni* high- M_r PSs described the molecules as LPS-associated (Aspinall *et al.*, 1992; 1994; Hanniffy *et al.*, 1999). A study carried out 1997, revealed that 16/ 38 strains tested by Western blot, reacted with antisera in the high- M_r region (Penner & Aspinall, 1997). However, certain early structural studies failed to identify high- M_r PS in *C. jejuni* strains which are now conclusively known to produce CPS, such as HS:1 (McNally *et al.*, 2005) and HS:2 (Karlyshev *et al.*, 2001; Szymanski *et al.*, 2002; Hendrixson & DiRita, 2004; Alemka *et al.*, 2013). Technical difficulties, combined with low concentration and the specific chemical properties of these molecule, likely contributed to their elusiveness in the early studies. Modern use of sensitive techniques such as magic angle spinning nuclear magnetic resonance spectroscopy (MAS-NMR), combined with conventional NMR, enabled the elucidation of CPS structure using low quantities of analyte (Szymanski *et al.*, 2003; Alemka *et al.*, 2013). MAS-NMR enabled the discovery of an unusual MeOPN modification in the CPS of certain strains (Szymanski *et al.*, 2003; McNally *et al.*, 2005; 2007; Nothaft *et al.*, 2010), a modification which is now found in 75% of *C. jejuni* CPSs (Maue *et al.*, 2012; Alemka *et al.*, 2013). Expression of both CPS (Bacon *et al.*, 2001; Kawasaki *et al.*, 2004) and the MeOPN modification (Erridge *et al.*, 2004; Wexler, 2007; Comstock, 2009; Sorensen *et al.*, 2011) is phase variable due to slipped-strand mismatch repair, a factor which is involved in immune and bacteriophage evasion (Karlyshev *et al.*, 2005; Yasuda *et al.*, 2008; Guerry & Szymanski, 2008; Sorensen *et al.*, 2011). Furthermore, both the CPS and the MeOPN modification confer serum resistance, and involvement in immune modulation has been documented (Wexler, 2007; Maue *et al.*, 2012).

1.2.2.3 CPS-independent *C. jejuni* polysaccharides

Adding to the complexity of the *C. jejuni* cell-surface carbohydrate repertoire, certain strains can produce additional PS molecules independent of CPS. *C. jejuni* 81116 was reported to produce two different PSs, one acidic, the other neutral (Muldoon *et al.*, 2002; Maue *et al.*, 2012). The acidic fraction was proposed to be capsular in nature,

while the neutral was potentially LPS-associated (Muldoon *et al.*, 2002; Kilcoyne *et al.*, 2006). Further chemical analysis revealed the structure of both PSs and the investigators agreed that the acidic PS was CPS, and the neutral PS was LPS-like (Kilcoyne *et al.*, 2006; Holst Sørensen *et al.*, 2012).

Bacon *et al.* (2001) reported two-independent high- M_r PS molecules in *C. jejuni* 81-176. Mutation of *kpsM* resulted in loss of one of these PSs and loss of reactivity with HS:23/ 36 antisera. Therefore this serodominant PS was deduced to be CPS in nature, whereas the nature of the other PS was not characterised (Bacon *et al.*, 2001; Muldoon *et al.*, 2002; Kilcoyne *et al.*, 2006; Holden *et al.*, 2012). Additional studies on *C. jejuni* 81-176 revealed another distinct surface PS, which was structurally characterised as a $\alpha(1,4)$ -linked glucan, and while not sero-reactive, was said to be capsular polysaccharide (Aspinall *et al.*, 1995; Papp-Szabo *et al.*, 2005; Parker *et al.*, 2008). Further PS production by *C. jejuni* 81-176 was described by McLennan *et al.* (2005), who detected a stress-response regulated, calcofluor white-reactive, CPS-independent PS.

1.2.2.4 Growth conditions influencing CPS structure and function

Production of CPS by *C. jejuni* has been observed to occur with growth in both liquid and solid media (Karlyshev *et al.*, 2001; Avril *et al.*, 2006; Corcoran & Moran, 2007; Heikema *et al.*, 2010; Bax *et al.*, 2011). Production of CPS in certain *C. jejuni* strains is growth phase-dependent (Corcoran & Moran, 2007), a factor which may be influenced by phase variation of CPS-biosynthesis genes, and which would effect antigenicity and virulence (Bacon *et al.*, 2001). Temperature-related transcriptional regulation of CPS expression in *E. coli* K5 is known, with production only occurring at 20°C or above (Rowe *et al.*, 2000). However, temperature was not observed to influence the expression of *kps* genes in *C. jejuni* using a microarray-based transcriptional analysis (Stintzi, 2003). Corcoran and Moran (2007) reported that production of CPS by *C. jejuni* NCTC 11168 was not influenced by temperature, but the production of another PS distinct from CPS was observed to be expressed at 37 but not 42°C. Temperature-related expression of a PS independent to CPS, was also observed in *C. jejuni* 81-176 (McLennan *et al.*, 2008). Increased production of this PS was observed after prolonged microaerobic incubation, and even greater production was observed with anaerobic incubation at 42°C (McLennan

et al., 2008). The structure and function of these temperature-influenced PSs is yet to be described.

1.2.3 Protein glycosylation

Protein glycosylation in eukaryotes is well characterised, and recognised as an important post-translational modification. Significant glycan diversity in prokaryotes means that in comparison, prokaryote protein glycosylation is poorly characterised. *C. jejuni* has one of the most extensive and best characterised capacities for protein glycosylation in bacteria (Szymanski & Wren, 2005). Many membrane and periplamic proteins in *C. jejuni* are post-translationally modified with glycans, which has diverse important biological roles. Two distinct protein glycosylation systems are expressed by *C. jejuni*: a general protein glycosylation pathway which modifies numerous proteins via an asparagine residue (*N*-glycosylation); and a pathway which modifies serine and threonine residues on flagella (*O*-glycosylation) (Guerry & Szymanski, 2008). The mechanism of *N*-glycosylation in *C. jejuni* is well characterised, although the biological roles of this modification are not (Nothaft *et al.*, 2010). Conversely, less is known about the mechanism underlying the *O*-glycosylation pathway, but its crucial role in flagellar assembly and as a virulence factor are well established (Guerry *et al.*, 2006).

1.2.3.1 Flagellar *O*-glycosylation

The *C. jejuni* flagella is modified with *O*-linked glycans, which in *C. jejuni* 81-176 constitutes 10% of the entire protein mass of the flagellar apparatus (Thibault *et al.*, 2001). Flagellar glycosylation is required for assembly of the flagellar filament (Lindenthal & Elsinghorst, 1999), and may be involved in the interaction between flagellar subunits with other components of the flagella (Young *et al.*, 2007). After assembly of the flagellar apparatus the *O*-linked glycans are surface exposed and may play important roles in surface associated interactions. Mutants deficient in flagellar glycosylation are not motile and have reduced levels of adherence and invasion of epithelial cells (Guerry & Szymanski, 2008). Flagellar glycosylation may play important roles in virulence of *C. jejuni*, such as in auto-agglutination (AAG), a recognised marker for virulence (Nothaft *et al.*, 2010).

Typically, *O*-glycans on *C. jejuni* flagella are derivatives of the 9-carbon sugars pseudaminic acid (Pse) and legionaminic acid (Leg), which are related to sialic acid.

The *C. jejuni* 81-176 flagella is glycosylated at up to 19 sites with Pse and Pse-derivatives only (Ewing *et al.*, 2009). Specific mutational loss of Pse acetamido from 81-176 results in loss of AAG activity, reduced adherence and invasion of intestinal epithelial cells *in vitro*, and impaired ferret colonisation (Guerry *et al.*, 2006). Autoagglutination in strain 81-176 is also influenced by loss of CPS and by the modification of LOS, indicating that interactions between flagellar glycans and other cell-surface glycoconjugates may be involved in AAG (Guerry *et al.*, 2006). By contrast, the *C. jejuni* NCTC 11168 flagella is modified with both Leg and Pse derivatives (Howard *et al.*, 2009; Nothaft *et al.*, 2010). Mutation of flagellar glycosylation in strain 11168 resulted in loss of AAG and impaired chicken colonisation, without influencing motility (Howard *et al.*, 2009). Interestingly, the related pathogen *H. pylori*, also modifies its flagella with Pse, a factor critical to the assembly of the *H. pylori* flagella, and consequently motility (Schirm *et al.*, 2003).

1.2.3.2 N-glycosylation

Originally thought unique to eukaryotes, the discovery of *N*-glycosylation in *C. jejuni* was the first example of this post-translational modification in prokaryotes. Although, the potential for *C. jejuni*-like *N*-glycosylation is being identified in other bacteria, *C. jejuni* remains the most well characterised. In contrast to the other forms of glycoconjugates produced by *C. jejuni* such as LOS, CPS and the *O*-glycan, *C. jejuni* *N*-glycosylation is highly conserved in the species (Szymanski & Wren, 2005). By a mechanism encoded by the *pgl* locus, the *N*-glycan heptasaccharide is assembled on cytoplasmic side of the inner membrane, on a lipid-linked precursor undecaprenyl phosphate (Nothaft *et al.*, 2010). This lipid-linked oligosaccharide (LLO) is flipped across the inner membrane to the periplasmic space, via PglK (Kelly *et al.*, 2006; Nothaft *et al.*, 2010), and then transferred to Asn residues of target proteins by PglB, a bacterial oligosaccharyltransferase (OTase) (Linton *et al.*, 2005). Unlike, the flagellar *O*-glycan a consensus sequence element consisting of Asp/Glu-X₁-Asn-X₂-Ser/Thr, where X₁ and X₂ represent any amino acid except Pro (Kowarik *et al.*, 2006).

Conservation of *C. jejuni* *N*-glycosylation suggests a more fundamental role in *C. jejuni* biology, compared to the diversity found in CPS and LOS, representing their role in antigenic variation. The influence of *N*-glycosylation on the immunoreactivity of certain proteins with both animal and human anti-sera has also been reported (Linton *et al.*, 2002). In strain 81-176, loss of *N*-glycosylation from the type-IV secretion system

protein VirB10 reduced the levels of natural competence (Larsen *et al.*, 2004). However, loss of *N*-glycosylation from putative adhesin Cj1496c did not effect chick colonisation compared to the wild type, whereas mutation of *cj1496c* significantly reduced colonisation (Kakuda & Dirita, 2006). Furthermore, *N*-glycan loss from the adhesin JlpA does not influence the immunoreactivity of the protein (Scott *et al.*, 2010). So, *N*-glycosylation has different potential roles for different proteins, and is probably vital to the functioning of these proteins (Nothaft *et al.*, 2010).

1.3 Guillain-Barré syndrome

Guillain-Barré Syndrome which is most common cause of acute neuromuscular paralysis, is preceded by some form of infectious disease in 50-70% of cases (Mishu & Blaser, 1993a; Mishu *et al.*, 1993; Allos & Blaser, 1995). A wide range of antecedent infections, both bacterial and viral have been associated with the development of the disease (Jacobs *et al.*, 1998). However, case-control studies have consistently identified *C. jejuni* as the most common preceding infection (Winer *et al.*, 1988; Mishu & Blaser, 1993b; Rees *et al.*, 1995; Sinha *et al.*, 2007). *C. jejuni* infection is also the most frequent proceeding infection associated with the development of Miller-Fisher syndrome (MFS) (Willison, 2005).

1.3.1 *C. jejuni* in GBS

In some parts of the world the development of GBS following *C. jejuni* infection has been linked with the presence of certain HS-serotypes rarely found in cases of enteritis (Moran *et al.*, 1996). In Japan, serotype HS:19 is strongly over-represented in cases of GBS (Fujimoto *et al.*, 1992; Yuki, 1997; Nachamkin *et al.*, 1999), associated only with 2% of Japanese enteritis cases (Nachamkin *et al.*, 1998). In South Africa, serotype HS:41 was found to be frequently associated with the development of GBS, despite only being isolated in 12/ 7119 of enteritis cases over a 19 year period (Goddard *et al.*, 1997; Lastovica *et al.*, 1997). Evidence indicates that both *C. jejuni* HS:19 and HS:41 represent globally distributed clonal populations and it has been suggested that they share unique virulence factors influencing their neuro-pathogenic potentials (Nachamkin *et al.*, 2001; Koga *et al.*, 2006). However, in other areas, the distribution of *C. jejuni* serotypes associated with GBS was found to be much more heterogenous (Endtz 2000; Moran 2001). This indicates that GBS-related virulence factors may not be

serotype restricted, and the strong association of certain serotypes such as HS:19 and HS:41 with GBS may be a geographic-related phenomenon (Moran & Prendergast, 2001). In Bangladesh, another serotype HS:23 was found to be overrepresented in case of GBS (Islam *et al.*, 2009).

Some studies suggest that preceding *C. jejuni* infection is more commonly associated with a more severe axonal form of GBS known as acute motor axonal neuropathy (AMAN), in comparison to classical form of GBS known as acute inflammatory demyelinating polyradiculoneuropathy (AIDP) (Rees *et al.*, 1995; Hughes & Cornblath, 2005). Indeed, some investigators conclude that preceding *C. jejuni* infection is a factor in AMAN exclusively (Yuki, 2010). However, evidence for *C. jejuni* involvement in AIDP has been reported (Ho *et al.*, 1995). Indeed, a recent study by Drenthen *et al.* (2011), demonstrated that while *C. jejuni* was more frequently involved in cases of AMAN in the Netherlands, it was also implicated in AIDP which is far more common in Western Europe, in comparison to Japan where AMAN is the most prevalent form of GBS. The investigators conclude that the geographic variation may be influenced by unexplained genetic or environmental factors (Drenthen *et al.*, 2011), and these should be considered before the precise role of *C. jejuni* in any of the variants of GBS can be defined.

1.3.2 Anti-ganglioside antibodies in *C. jejuni* GBS

The first reported involvement of anti-ganglioside antibodies in GBS was in 1988, when 20% of patients acute phase serum had anti-ganglioside reactivity (Ilyas *et al.*, 1988). Following this, a large body of literature has reported the presence of anti-glycolipid antibodies of a range of specificities, with around 60% of patients bearing these antibodies in the acute phase of disease (Kaida *et al.*, 2009). Anti-ganglioside antibody specificity varies widely between patients and reactivity with a wide range of gangliosides is reported, including gangliosides LM₁, GM₁, asialo-GM₁, GM_{1b}, GD_{1a}, GalNac-GD_{1a}, GD_{1b}, GD₂, GD₃, GT_{1b}, GQ_{1b} (Willison & Yuki, 2002; Yuki & Kuwabara, 2007). A correlation between GBS with anti-GM₁ antibodies, and preceding *C. jejuni* infection has frequently been reported (Oomes *et al.*, 1995; Yuki, 1999; Nores *et al.*, 2008). Approximately half of patients with preceding *C. jejuni* infection have anti-GM₁ reactivity (Moran, 2010). Although, there are also reports of anti-ganglioside antibodies in *C. jejuni* infected GBS patients reactive against gangliosides other than

GM₁, such as the GM₁-like structures GM_{1b}, GD_{1a} and GalNAc-GD_{1a} (Yuki *et al.*, 1996; Yuki, 1999; Moran, 2010). The issue is further complicated by the potential involvement of anti-ganglioside-complex antibodies in *C. jejuni* induced GBS (Kuijf *et al.*, 2007; Kaida *et al.*, 2009).

1.3.3 Cross-reactive antibodies

As previously noted the core OS of many *C. jejuni* strains is structurally similar to a range of gangliosides, representing a form of molecular mimicry. *C. jejuni* LOS's mimicking a wide range of human gangliosides including GM₁, GD_{1a}, GD_{1b}, GD_{1c}, GT_{1a}, GM₂, GD₂ and GD₃ has been described (Moran, 2010). The discovery of ganglioside molecular mimicry by *C. jejuni* motivated a number of studies investigating the reactivity of serum antibodies in GBS patients against *C. jejuni* LOS. Initial work by Yuki *et al.* (1992), showed that serum anti-GM₁ antibodies cross reacted with *C. jejuni* HS:19 LOS. Subsequently, several groups demonstrated the cross-reaction of anti-ganglioside antibodies with GBS-associated *C. jejuni* LOSs (Schwerer *et al.*, 1995; Yuki *et al.*, 1995; Neisser *et al.*, 1997), culminating in work by Prendergast *et al.* (1999) that definitively identified the oligosaccharide region of the LOS as the location of the cross-reactive epitope. These investigations led to the suggestion that the sialosyl-D-galactose structure α -Neu5Ac-(2-3)- β -Gal(1- as the minimal motif in the core oligosaccharide of *C. jejuni* LOS, required for GBS development (Moran *et al.*, 2002).

Cross-reaction between sera from MFS patients and *C. jejuni* as well as ganglioside GQ_{1b}, was also demonstrated in initial investigation (Jacobs *et al.*, 1995). However, none of the neuropathy-associated *C. jejuni* strains where the LOS has been structurally characterised have been shown to express a complete GQ_{1b} ganglioside mimic, and LOSs reactive with MFS sera have been shown to express GD₂/GD₃ mimicry or GT_{1a} and GD_{1c}-like mimics (Prendergast & Moran, 2000; Yuki, 2005). It has been proposed that for the development of MFS, the minimal structure motif required is a disialosyl-galactose structure α -Neu5Ac-(2-8)- α (2-3)- β -Gal-(1- (Moran *et al.*, 2002; Yuki, 2005). Interestingly, cross-reactivity of serum anti-ganglioside-complex antibodies with LOS from autologous *C. jejuni* isolates has also been demonstrated (Kuijf *et al.*, 2007), indicating that conformational epitopes can be formed between heterogenous LOS moieties on *C. jejuni* isolates, leading to the generation of anti-ganglioside complex antibodies which are increasingly being identified as important in

GBS neuropathogenesis (Kaida *et al.*, 2004; 2006).

1.3.4 Molecular mimicry hypothesis to describe the causation of *C. jejuni*-related GBS

Collectively, studies demonstrating reactivity of anti-ganglioside antibodies with *C. jejuni* LOS have confirmed the presence of ganglioside related epitopes in *C. jejuni* LOS (Schwerer *et al.*, 1995; Neisser *et al.*, 1997; 2000). The molecular mimicry hypothesis was proposed as a pathogenic mechanism for *C. jejuni*-related GBS, whereby an immune response is induced by infection resulting in the generation of cross-reactive anti-ganglioside antibodies that target neural gangliosides, leading to tissue damage and nerve dysfunction (Willison & Yuki, 2002). While ganglioside-mimicking LOS is more frequently found in GBS/MFS-related *C. jejuni* strains than enteritis-related controls (Ang *et al.*, 2002; Godschalk *et al.*, 2007), some GBS-related strains have been reported that lack ganglioside mimicry (Godschalk *et al.*, 2006b). Furthermore, ganglioside-mimicking *C. jejuni* is frequently isolated from cases of uncomplicated enteritis (Moran, 1995; Godschalk *et al.*, 2006a). However, coinfection with multiple strains of *C. jejuni* has been demonstrated in GBS patients (Godschalk *et al.*, 2006b), and the isolation of any one of these strains alone would be misleading as they may not be directly involved in the neuropathy. Moreover, the ability of phase variation and other genetic mechanisms to alter the nature of the ganglioside mimics (Guerry *et al.*, 2002), which may have been involved in neuro-pathogenesis, would contribute to the perceived obscurity. Nonetheless, despite the clear relevance of *C. jejuni* ganglioside mimicry in GBS pathogenesis, considering that only 30.4 *C. jejuni* infections per 100,000 result in GBS (McCarthy & Giesecke, 2001), it is clear that other bacterial or host factors, in addition to ganglioside mimicry, are involved in the etiology of the disease.

1.4 Scope of this thesis

Bacterial and host cell-surface glycoconjugates are fundamental to many aspects of *C. jejuni* pathogenesis and in its interaction with its various hosts. Therefore, the overall aim of this thesis was to investigate the involvement of *C. jejuni* cell-surface glycoconjugates, as well as *C. jejuni* binding of host glycoconjugates in *C. jejuni*-mediated disease, host interaction, and the development of GBS. Initially, cell-surface

glycoconjugates were isolated from 25 *C. jejuni* Irish clinical and food isolates, for phenotypic characterisation using a range of electrophoretic and serological techniques, and to reveal the extent of ganglioside mimicry, an important factor in GBS, in these strains. Following this, to investigate other bacterial factors in GBS pathogenesis, a group of closely related *C. jejuni* GBS- and enteritis-related strains were compared on adherence to an Australian glycan microarray, and to an *in vitro* cell monolayer. Subsequent investigations were carried out on *C. jejuni* grown at 37 and 42°C to investigate the influence of glycoconjugates in host adaptation. Successful development of glycan microarray technology in Galway enabled the analysis of growth temperature influences on glycan binding by two *C. jejuni* strains. These strains were then analysed on a lectin microarray, which determined temperature-related cell-surface glycoconjugate variation. Furthermore, the impact of this variation on the host innate immune system was investigated, and the molecular basis of temperature-related changes was determined.

Chapter 2.

Materials & Methods

2.1 BACTERIAL STRAINS USED IN THIS STUDY

In the course of this study, a wide range of *C. jejuni* strains and isolates were used, as well as *Escherichia coli*.

2.1.1 *C. jejuni* culture collection strains

The culture collection strains used in this study are presented in Table 2.1, and were obtained from the NCTC, London, U.K, and the American Type Culture Collection (ATCC), Manassas, VA, U.S.A.

Table 2.1 Culture collection strains used in this study.

Bacterial spp.	Serotype	Strain I.D.
<i>C. jejuni</i>	HS:2	^a NCTC 11168
<i>C. jejuni</i>	HS:6	NCTC 11828 (81116)
<i>E. coli</i>	Seattle 1946	^b ATCC 25922

^aNational Type Culture Collection

^bAmerican Type Culture Collection

2.1.2 *C. jejuni* clinical and food isolates

Clinical isolates of *C. jejuni* used in this study are presented in Table 2.2. Isolates were obtained from Dr. Patricia Guerry, Naval Medical Research Institute (Bethesda, Md. U.S.A.) and Dr. Albert J. Lastovica, Red Cross Children's Hospital (Rondebosch, Cape Town, South Africa).

Table 2.2 Serotyped clinical isolates of *C. jejuni* used in this study.

Bacterium	Serotype	Strain I.D.
<i>C. jejuni</i>	HS:23/36	^a 81-176
<i>C. jejuni</i>	HS:41	^b 238
<i>C. jejuni</i>	HS:41	^b 176.83
<i>C. jejuni</i>	HS:41	^b 233.95
<i>C. jejuni</i>	HS:41	^b 299.95
<i>C. jejuni</i>	HS:41	^b 260.94 RXH
<i>C. jejuni</i>	HS:41	^b 2813.94 GSH
<i>C. jejuni</i>	HS:41	^b 16971.94 GSH

Strain sources: ^aP. Guerry, ^bA. Lastovica

Clinical and food *C. jejuni* isolates of unknown serotype used in this study, were donated by Dr. Cyril Carroll, National University of Ireland Galway and are presented in Table 2.3. The *C. jejuni* were originally isolated from Cherry Orchard Hospital (Dublin, Ireland), Belfast City Hospital (Belfast, Northern Ireland), University College Hospital, Galway (Galway, Ireland), as well as retail sources in Galway, Dublin and Belfast.

Table 2.3 Clinical and food isolates of *C. jejuni* used in this study.

Bacterium	Strain I.D.
<i>C. jejuni</i>	32 BC
<i>C. jejuni</i>	250 BC
<i>C. jejuni</i>	260 BC
<i>C. jejuni</i>	300 BC
<i>C. jejuni</i>	301 BC
<i>C. jejuni</i>	2 DC
<i>C. jejuni</i>	16 DC
<i>C. jejuni</i>	18 DC
<i>C. jejuni</i>	26 DC
<i>C. jejuni</i>	28 DC
<i>C. jejuni</i>	180 DC
<i>C. jejuni</i>	38 GC
<i>C. jejuni</i>	248 DF
<i>C. jejuni</i>	462 BF
<i>C. jejuni</i>	591 BF
<i>C. jejuni</i>	592 BF
<i>C. jejuni</i>	1473 BF
<i>C. jejuni</i>	1474 BF
<i>C. jejuni</i>	1607 BF
<i>C. jejuni</i>	2325 BF
<i>C. jejuni</i>	1373 DF
<i>C. jejuni</i>	1644 DF
<i>C. jejuni</i>	2265 DF
<i>C. jejuni</i>	1450 GF
<i>C. jejuni</i>	2118 GF

Strain sources: ^aDC (Cherry Orchard Hospital); ^bBC (Belfast City Hospital); ^cGC (University College Hospital, Galway); ^dDF (Retail sources in Dublin); ^eBF (Retail sources in Belfast); ^fGF (Retail Sources in Galway).

2.1.3 Bacterial glycoconjugates used in this study

For use as positive controls in SDS-PAGE gels, pure LPS from a clinical *E. coli* isolate AM1 and *Salmonella enterica* that were available in the lab collection were used.

2.2 General cultivation of *C. jejuni*

C. jejuni was routinely cultured throughout this study using the following media, and was sterilized in an Astell AMA240 (Astell Scientific, Kent, UK) autoclave, for 20 min at 115°C.

2.2.1 Storage medium

C. jejuni was stored at -70°C in a glycerol-containing maintenance medium.

Tryptone soya broth (Oxoid, Hampshire, UK, CM0129)	3 g
Yeast extract (Oxoid, LP0021)	0.3 g
Bacteriological agar (Oxoid, LP0011)	50 mg
Glycerol	15% (v/v)

Maintenance medium was made up to 100 ml with dH₂O, and autoclaved before use.

2.2.2 Routine growth media for *C. jejuni*

C. jejuni was routinely grown on the following solid media, which were dissolved in 1 l of dH₂O. After autoclaving, the media was cooled to 55°C before pouring or addition of supplements. For blood agar, 5-7% defibrinated horse blood (Charles River Laboratories International Inc., Ballina, Co. Mayo, Ireland.) was aseptically added to Columbia agar base, and mixed well by inverting. Agar plates were prepared by pouring media into sterile Petri dishes, allowing to solidify, then dried in a laminar flow cabinet (SterileGUARD III Advance, The Baker Company, Sanford, ME, U.S.A).

Columbia agar

Columbia agar base (Oxoid, CM0331)	39 g
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Brucella agar

Brucella medium base (Oxoid, CM0169)	48 g
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As a nutrient enrichment for enhancement of *C. jejuni* growth, Brucella agar was supplemented with Isovitalax (Becton, Dickinson & Co., Franklin Lakes, NJ, U.S.A.), which was supplied as a dehydrated powder and a separate diluent containing 10% (w/v) dextrose. The supplement was combined and added at 0.35% (v/v) to the autoclaved and cooled Brucella agar. The rehydrated Isovitalax supplement contained the following constituents per litre:

Dextrose	100.0 g
L-Cysteine hydrochloride	25.9 g
L-Glutamine	10.0 g
L-Cystine	1.1 g
Adenine	1.0 g
Nicotinamide adenine	0.25 g
Thiamine pyrophosphate	0.1 g
Guanine hydrochloride	0.03 g
Ferric nitrate	0.02 g
<i>p</i> -Aminobenzoic acid	0.013 g
Vitamin _{B12}	0.01 g
Thiamine hydrochloride	0.003g

2.2.3 Selective growth media for *C. jejuni*

In order to prevent contamination of pure cultures, *C. jejuni* was grown on agar that contained Skirrow selective supplement (Oxoid, SR0069E). The rehydrated supplement was dissolved in 2 ml of sterile H₂O and aseptically added at 0.4% (v/v) to the autoclaved and cooled agar, before pouring. The Skirrow selective supplement consisted of the following per litre:

Vancomycin	10 mg
Trimethoprim lactate	5 mg
Polymyxin B	2500 mg

2.3 Incubation conditions

C. jejuni was cultured in anaerobic gas jars (Oxoid) at 37 or 42°C for 18-24 h. A microaerobic atmosphere (6% O₂, 10% CO₂ and 84% N) was created in the gas jars using disposable gas generating kits (Oxoid, BR0038), without a palladium catalyst.

2.4 Identification of *C. jejuni*

C. jejuni colony morphology consisted of small, grey colonies when grown on blood agar, and biomass had a skin color when transferred to the tip of a swab. To confirm the identity of *C. jejuni* cultures and to ensure the absence of contamination the following tests and examinations were used.

2.4.1 Gram staining

The modified Gram stain of Ogg 1962 was employed. A sterile loop was used to transfer a single colony to a loopful of sterile H₂O on a slide. The slide was passed through a Bunsen flame twice, to heat-fix the cells. The dried sample was then immersed in methyl violet for 1 min. After washing with water, iodine was added for 3 min. This allowed the formation of a water insoluble dye-iodine complex for stable retention in the cell membrane. The slide was washed again and 100% ethanol was added for 20 s, to remove the dye-iodine complex from Gram-negative cell membranes. The slide was subsequently washed and the sample was counterstained with carbol fuschin. The slide was then washed and dried, and examined microscopically. As a gram-negative bacterium *C. jejuni* appeared as a faint-pink color, and had characteristic spiral rod-shaped cell morphology.

2.4.2 Oxidase test

An oxidase diagnostic kit (Becton, Dickenson & Co.) was used to detect oxidase for the confirmation of *C. jejuni*. Briefly, an isolated colony was transferred to a piece of filter paper (Whatman paper Ltd., Maidstone, England). A drop of test kit reagent (consisting

of 1% N, N, N', N'-tetramethyl-*p*-phenylenediamine dihydrochloride) was added to the sample. Color change to purple indicated the presence of oxidase-positive bacteria (Smithbert *et al.*, 1981).

2.4.3 Catalase test

A loopful of bacteria was mixed with 3% (v/v) H₂O₂ (Sigma-Aldrich, St. Louis, MI, U.S.A) in a glass tube and was examined to the production of bubbles, indicating presence of a catalase-positive bacterium (Smithbert *et al.*, 1981).

2.5 General buffers and procedures

All chemicals were analytical grade and solutions were prepared using analytical grade water, purified using a Milli-Q (Millipore, Concord Rd., Bellerica, MA, U.S.A.) water purifier. All buffers were autoclaved at 115°C for 20 min before use and stored at 4°C.

2.5.1 Autoclaving

All materials were sterilized in a Astell AMA240 (Astell Scientific, Kent, UK) autoclave.

2.5.2 Phosphate buffered saline (PBS), pH 7.3

PBS was prepared by dissolving 1 PBS tablet (Oxoid) in 100 ml of dH₂O.

2.5.3 Tris-HCL buffer (0.1M)

A 0.1M Tris-HCL buffer was prepared by dissolving 1.2 g of Trizma Base (Sigma) to a total volume of 100 ml. Initially, Trizma base was dissolved in 50 ml of dH₂O and the pH was adjusted to 7.5 with 1 M HCL, in a graduated cylinder. The solution was then made up to 100 ml with dH₂O.

2.5.4 Spectrophotometry

The optical density (OD) of bacterial cultures was determined using a Thermo Scientific Helios ε spectrophotometer (Thermo Scientific, Madison, WI, U.S.A.). Scanning spectrophotometry was carried out on a SpectraMax M5e microplate reader (Molecular device, Inc., Berkshire, UK).

2.5.5 Centrifugation

A bench top Eppendorf 5417C centrifuge (Eppendorf AG, Hamburg, Germany) was used when centrifuging volumes < 1.5-2.0 ml. For larger volumes an Avanti J-20XP centrifuge (Beckman Coulter, Inc.) fitted with a JOLite JLA-16.250 rotor, was used.

2.5.6 Mass determination

Measuring materials > 1 g, was carried out with a Sartorius BL610 (Sartorius AG, Goettingen, Germany) top pan balance. The mass of materials < 1 g was measured using a Sartorius AC1205 (Sartorius AG) analytical balance.

2.5.7 pH measurement

To measure the pH of a solution an Orion 420 A+ pH meter (Orion Research Inc., Beverly, Ma, U.S.A.) was used. The pH of small volumes was estimated using pH paper (Whatman Paper Ltd.), using the color chart for comparison.

2.5.8 Sonication

Solubilisation of certain samples was assisted using a Branson sonication bath (model 2510, Branson Ultrasonics corp, Danbury, CT, U.S.A), with heating as necessary.

2.5.9 Sample desiccation

Samples were lyophilised using a Lyovac GT2 lyophiliser (Leybold Vacuum GmbH, Cologne, Germany). Large volumes in round-bottomed flasks were frozen in an acetone-dry-ice bath an attached directly to lyophiliser. Smaller volumes in tubes sealed with Parafilm (Parafilm M, Pechiney Plastic Packaging Comp., Chicago, IL, U.S.A.) were frozen in a ultra-low temperature freezer (Thermo Scientific), and lyophilised with a hole pierced in the Parafilm lid.

2.6 Isolation of *C. jejuni* glycoconjugates

Extraction and isolation of *C. jejuni* cell-surface glycoconjugates was carried out using a number of methods.

2.6.1 Whole cell extracts and proteinase-K whole cell extracts

Whole cell extractions (WCE) and proteinase-K whole cell extractions (PK) for SDS-PAGE analysis of *C. jejuni* proteins and cell surface glycoconjugates respectively, were prepared according to the method of Hitchcock & Brown (1983).

Biomass from one agar plate was harvested with 1.5 ml of PBS and washed once using PBS and centrifugation at $2600 \times g$ for 5 min. The OD₆₀₀ was adjusted to 0.3 nm using PBS and an aliquot of 1.5 ml was centrifuged as above. The pellet was resuspended in 200 μ l sample buffer (Table 2.4). The sample was boiled at 100°C for 10 min resulting in a WCE. For preparation of a PK, 40 μ l of 2 mg ml⁻¹ proteinase K (Sigma-Aldrich) was added to the cooled WCE and incubated at 60°C for 1 h, followed by incubation at 100°C for 5 min. All samples were stored at -20°C.

Table 2.4 Composition of sample buffer.

Reagent	Quantity
dH ₂ O	6.75 ml
0.5 M Tris-HCL buffer, pH 6.5	6.25 ml
20% SDS	4.5 ml
Glycerol	5.0 ml
β -mercaptoethanol	2.5 ml
Bromophenol blue	1 mg

2.6.2 Hot phenol-water (HPW) extraction

Large-scale isolation of *C. jejuni* cell-surface glycoconjugates was performed using the HPW method of Westphal and Jann (1965).

2.6.2.1 Preparation of crude HPW extract

C. jejuni biomass which had been washed and freeze-dried was resuspended in purified water to a concentration of 1 g per 10 ml and blended for 3 min using an Ultra Turrax

T25 homogenizer (Janke and Kunkel GmbH, Stauffel, Germany). An equal volume of 90% phenol (pre-heated to 65°C) was added to the resulting thick paste, followed by incubation of the mixture at 65°C for 10 min. The mixture was mixed by vortexing for 30 sec at 2 min intervals, followed by 2 min vortexing. Subsequently, the mixture was cooled on ice, transferred to glass centrifuge tubes and centrifuged at $5000 \times g$ at 10°C for 15 min. After centrifugation the following layers had formed (from tube bottom to top): a brown insoluble pellet; a brown phenol layer; a white interphasic layer consisting of insoluble proteins; an opaque aqueous layer; and a froth composed of denatured proteins. Carefully the aqueous layer was removed to a phenol resistant flask. An equal volume of water was added to the remaining phenol-layer and extracted once more as above. The aqueous phase were combined and dialysed (dialysis cassette, MWCO 2 kDa, Thermo Scientific) against 5 l of pure water for 3-4 days with two changes of water per day, until no residual phenol smell was detected.

2.6.2.2 Purification of crude HPW

Lyophilised crude HPW extract was dissolved to 10 mg ml^{-1} in sterile 0.1 M Tris-HCL buffer, pH 7.5, and 1 mg of RNase A (Sigma-Aldrich) and DNase II (Sigma-Aldrich) per 10 mg of extract was added, and the solution was incubated at 37°C for 18 h. Following this proteinase K was added at 0.1mg per 10 mg of extract, and incubated at 37°C overnight (~16 h), followed by 4 h at 65°C, and finally 100°C for 10 min. Samples were dialysed against dH₂O for 3 days, with 2 changes of dH₂O per day and then lyophilised and tested for contamination.

Purified HPW extracts were dissolved with sonication at 2 mg ml^{-1} in pure water. The samples ($200 \text{ } \mu\text{g ml}^{-1}$) were tested for contaminating proteins and nucleic acids by scanning spectrophotometrically between 200 and 400 nm. The absence of absorption peaks at 260 nm and 280 nm indicated the samples were free from nucleic acid and protein contaminants, respectively.

2.6.3 Mini-phenol-water (MPW) extraction

A rapid MPW extraction procedure was employed to obtain the small amounts of pure cell-surface glycoconjugates required for thin-layer chromatography (TLC) and SDS-PAGE analysis while allowing the screening of numerous isolates due to the efficiency of the procedure. The procedure was developed by Mills *et al.* (1992) and was applied

to *C. jejuni* by Prendergast *et al.* (2001). Initially, biomass from two agar plates of confluent growth was harvested and washed three times using sterile PBS. The washed biomass was resuspended in 0.75 ml of dH₂O followed by the addition of an equal amount of 90% phenol (pre-heated to 65°C). The sample was vortexed for 1 min then incubated at 65°C for 10 min, with vortexing for 30 s at 2 min intervals. Subsequently, the sample was cooled on ice and centrifuged at 15,000 × g for 3 min. The upper aqueous phase was then pipetted to a pre-weighed Eppendorf centrifuge tube. Extraction of residual phenol was carried out by layering 0.5 ml diethyl ether over the aqueous phase a total of three times, with the diethyl ether discarded each time. Remaining diethyl ether was allowed to evaporate off in the fume-hood before the sample was lyophilised, weighed and dissolved at 2 mg ml⁻¹ in pure water.

2.6.4 Sodium Acetate Extraction of CPS extraction

Capsular polysaccharide extraction was performed as described previously (Liu *et al.*, 1971). Briefly, bacterial biomass was precipitated with calcium chloride and absolute ethanol at the following ratio, biomass:calcium chloride:ethanol (5:1:54), and left overnight at 4°C. The precipitate was collected by centrifugation (3,000 × g, 30 min), and washed twice with absolute ethanol, twice with acetone and twice with diethyl ether, with centrifugation steps (3,000 × g, 30 min), between each washing. Subsequently, the precipitate was extracted five times with 0.1 M sodium acetate (pH 7.0, 200 ml per 20 g precipitate), centrifuged (20,000 × g, 15 min) and supernatants combined. To this calcium chloride and absolute ethanol were added (supernatant:calcium chloride:ethanol, 9:1:2.5), refrigerated overnight, and the resulting precipitate collected by centrifugation (26,000 × g, 30 min). This precipitate was then lyophilised and constituted crude PS. For purification this PS was dissolved vigorously in 0.1 M Tris-HCL buffer (pH 7.5) to a concentration of 10 mg ml⁻¹, incubated with Rnase A and Dnase II (both at 1 mg per 10 mg of PS) at 37°C overnight, and then with proteinase K (0.1 mg per 10 mg of PS) for 4 h at 37°C, and subsequently for 30 min at 65°C. The resulting preparation was dialysed against pure water for 3-4 days, with 2 changes of water per day, and lyophilised to yield purified PS.

2.7 Electrophoretic analysis

Two systems of electrophoresis were used in this study: the discontinuous buffer system described by Laemmli (1970) (glycine-SDS-PAGE); and, the tricine-SDS-PAGE system described by Schägger (2006).

2.7.1 Preparation of samples for SDS-PAGE

Before electrophoresis WCE and PKWCEs, which were stored at -20°C were incubated at 60°C for 20 min to ensure solubilisation. Using a pipetter, samples were added to the wells of the stacking gel, which was first submerged in running buffer. The first and last wells were loaded with sample buffer, as samples in these wells tend to migrate at an angle, these empty wells also served as negative controls. As positive control and molecular-mass marker in gels containing WCEs a low- M_r protein standard (Bio-Rad) was used, gels were then stained with silver or Coomassie blue. The marker consisted of the following proteins: rabbit muscle phosphorylase b (97 kDa); BSA (66 kDa); hen egg white ovalbumin (45 kDa); bovine carbonic hydrase (31 kDa); soybean trypsin inhibitor (21 kDa); and, hen egg white lysozyme (14 kDa). For gels that were to be stained with Alcian blue, a ColorPlus pre-stained broad range protein marker (New England Biolabs, County Rd., Ipswich, MA, U.S.A.) was used as a positive control and consisted of the following proteins: Maltose binding protein (MBP)- β -galactosidase (175 kDa); MBP-truncated- β -galactosidase (80 kDa); MBP-chitin binding domain (CBD, 58 kDa); CBD-*Mxe* Intein-2CBD (46 kDa); CBD-*Mxe* Intein (30 kDa); CBD-*E. coli* parvulin-like protein (23 kDa); Lysozyme (17 kDa), and; Aprotinin (7 kDa).

2.7.2 Preparation of SDS-PAGE gels

SDS-PAGE gels were prepared by the polymerisation of acrylamide and N, N'-methylene bisacrylamide. The gel was composed of a lower resolving gel that was overlaid by an upper stacking gel. The constituents of the resolving gel were combined and pipetted between two glass plates, 0.75 mm apart. Approximately 100 μl of *n*-butanol was overlaid to prevent atmospheric oxygen coming into contact with the gel, as this inhibits polymerisation. After 1 h the *n*-butanol was wicked off with filter paper (Whatman paper Ltd.) and the combined stacking gel solution was pipetted over the resolving gel. A ten-lane gel comb (BioRad) was then immediately inserted into the stacking gel, and allowed to set for 1 h.

2.7.3 Glycine-SDS-PAGE

Separation of WCE, PK, HMW and MPW extracts was carried out using glycine-SDS-PAGE.

2.7.3.1 Solutions required for glycine-SDS-PAGE

All reagents were prepared using analytical grade water (dH₂O). Acrylamide and gel buffers were stored in the dark at 4°C for up to 1 month.

Ammonium persulfate (APS) solution (10%)

APS (Sigma-Aldrich)	1 g
dH ₂ O	10 ml

The solution was prepared freshly before use.

Acrylamide

Acrylamide (Sigma-Aldrich)	29.2 g
N, N'-methylene bisacrylamide	0.8 g
dH ₂ O	100 ml

Acrylamide solution was degassed under a vacuum for 30 min, before storage.

Resolving gel buffer

Tris base (Sigma-Aldrich)	18.15 g
dH ₂ O	100 ml

Resolving gel buffer of 1.5 M tris-HCl was prepared by dissolving tris in 50 ml of dH₂O and adjusting to pH 8.8 with 1 M HCl, in a graduated cylinder. Final volume was then prepared by making up to 100 ml with dH₂O.

Stacking gel buffer

Tris base	6 g
dH ₂ O	100 ml

Stacking gel buffer of 0.5 M tris-HCl was prepared by dissolving tris base in 50 ml of dH₂O and adjusting to pH 6.8 with 1 M HCl. Final solution was then prepared by making up to 100 ml with dH₂O.

Sodium dodecyl sulfate (SDS) solution (10%)

SDS	1 g
dH ₂ O	10 ml

Solution was stored at RT (~20°C).

Electrode running buffer (5×)

Tris base	15 g
Glycine (Sigma-Aldrich)	72 g
SDS	5 g
dH ₂ O	1 l

Running buffer was diluted 1:4 in dH₂O before use.

2.7.3.2 Glycine SDS-PAGE gels for separation of proteins

For the separation of WCEs, gels consisted of a 12% resolving gel overlaid with a 4% stacking gel. Gel composition for preparation of two 0.75 cm thick gels is given in Table 2.5.

Table 2.5 Constituents of glycine-SDS-PAGE gels for protein separation.

Reagent	Stacking gel (4%)	Resolving gel (12%)
Acrylamide-bis (30% T)	870 µl	5.34 ml
Stacking gel buffer	1.66 ml	-
Resolving gel buffer	-	3.34 ml
dH ₂ O	4.06 ml	4.46 ml
10% SDS	75.0 µl	150.0 µl
APS solution	33.0 µl	607.0 µl
TEMED ^a	3.33 µl	6.7 µl

^aTEMED, N, N, N', N'-Tetramethylethylenediamine.

2.7.3.3 Glycine-SDS-PAGE gels for separation of glycoconjugates

For the separation of PK, HPW, or MPW extracts a 15% resolving gel was overlaid with a 5% stacking gel. Gel composition for the preparation of two 0.75 cm thick gels is given in Table 2.6.

Table 2.6 Constituents of glycine-SDS-PAGE gels for glycoconjugate separation.

Reagent	Stacking gel (5%)	Resolving gel (15%)
Urea	-	2.9 g
Acrylamide-bis (30% T)	1.0 ml	7.2 ml
Stacking gel buffer	1.56 ml	-
Resolving gel buffer	-	3.1 ml
dH ₂ O	3.44 ml	1.6 ml
10% SDS	60.0 µl	120.0 µl
APS solution	60.0 µl	60.0 µl
TEMED	3.0 µl	4.2 µl

2.7.3.4 Electrophoresis conditions for glycine-SDS-PAGE

Glycine-SDS-PAGE was carried out in a Mini-PROTEAN II electrophoresis cell (Bio-Rad), under a constant current of 35 mA until dye front was at bottom of the gel.

2.7.4 Tricine-SDS-PAGE

Tricine SDS-PAGE was used for the separation of low-M_r mass glycoconjugates.

2.7.4.1 Solutions required for tricine-SDS-PAGEAcrylamide

Acrylamide (Sigma-Aldrich)	48 g
N, N'-methylene bisacrylamide	1.5 g
dH ₂ O	100 ml

Gel buffer

Tris base	36.3 g
SDS	300 mg
dH ₂ O	100 ml

Gel buffer for resolving and stacking gels was 3 M tris-HCl which was prepared by dissolving tris base in 50 ml of dH₂O and adjusting to pH 8.5 with 1 M HCl. Final volume was then prepared by making up to 100 ml with dH₂O.

Cathode running buffer (10×)

Tris base	121.12 g
Tricine (Sigma-Aldrich, T0377)	179.17 g
SDS	100 mg
dH ₂ O	1000 ml

To prepare working concentration, the cathode running buffer (10×) was diluted 1:10 in dH₂O. Solution was stored at RT.

Anode buffer (10×)

Tris base	121.12 g
dH ₂ O	1000 ml

Anode running buffer (10×) consisting of 1 M tris-HCl was prepared by dissolving tris in 700 ml of H₂O and adjusting to pH 8.9 with 1 M HCl. Final volume was then prepared by making up to 100 ml with dH₂O. Working buffer was prepared by diluting 1:10 in dH₂O.

2.7.4.2 Tricine-SDS-PAGE gels for separation of glycoconjugates

For the separation of PK, HPW and MPW extracts using the tricine-SDS-PAGE system a 12% resolving gel was overlaid with a 4% stacking gel. Gel composition, for preparation of two 0.75 cm thick gels, is given in Table 2.7.

Table 2.7 Constituents of Tricine-SDS-PAGE gels.

Reagent	Stacking gel (4%)	Resolving gel (12%)
Urea	-	2.7 g
Acrylamide-bis (49.5% T)	500 μ l	2.5 ml
Gel buffer	1.5 ml	2.5 ml
dH ₂ O	4.0 ml	800 μ l
APS solution (10%)	45 μ l	25 μ l
TEMED	5 μ l	5 μ l

2.7.4.3 Electrophoresis conditions for Tricine-SDS-PAGE

Tricine-SDS-PAGE was carried out in a Mini-PROTEAN II electrophoresis cell (Bio-Rad). Initially a current of 40 V was applied until the samples entered the stacking gel, after which the current was increased to 130 V until dye front reached the bottom of the gel.

2.7.5 Staining of SDS-PAGE gels

Immediately after SDS-PAGE, gels were extracted from plates, stacking gel was removed and gels were stained using one a number of different methods.

2.7.5.1 Coomassie blue staining

Coomassie blue staining was carried out on electrophoretically separated WCEs and to detect protein contamination in HPW and MPW extracts.

Coomassie blue stain

Glacial acetic acid (Merck, Darmstadt, Germany)	100 ml
Methanol (Merck)	450 ml
dH ₂ O	450 ml
Coomassie Brilliant Blue R-250 (Biorad)	2.5 g

Destaining solution

Glacial acetic acid	100 ml
Methanol	450 ml
dH ₂ O	450 ml

Immediately after SDS-PAGE, gels were placed in Coomassie blue stain for 30 min at RT, with gentle shaking. Stain was aspirated off, and destain solution was added to gels, and left at RT with shaking until background staining was sufficiently removed. Coomassie blue stains proteins non-specifically, and they appear as blue bands with clear background.

2.7.5.2 Silver staining

Silver staining was carried out according to the procedure of Tsai & Frasch (1982), for the detection of LOS and LPS separated by SDS-PAGE. Omission of the periodic acid oxidation step enabled silver staining of proteins to be achieved. The following solutions were required for silver staining.

Fixer solution

Glacial Acetic acid	50 ml
Ethanol (Merck)	450 ml
dH ₂ O	550 ml

0.7% (w/v) Oxidising solution

Fixer solution	150 ml
Periodic acid (Sigma-Aldrich)	1.05 g

Oxidising solution was prepared fresh before use.

20% (w/v) Silver nitrate solution

Silver nitrate (Sigma)	1 g
dH ₂ O	5 ml

Silver nitrate solution was prepared fresh before use.

0.1 M Sodium hydroxide solution

Sodium hydroxide pellets	4 g
dH ₂ O	1000 ml

Staining solution

20% silver nitrate	5 ml
25% ammonia solution	2 ml
0.1 M NaOH	28 ml

To prepare the stain the NaOH and ammonia solutions were combined and the silver nitrate solution was added dropwise with stirring, allowing the black precipitate that initially developed to disappear. The solution was brought up to a final volume of 150 ml with dH₂O. The staining solution was prepared fresh before use.

Developer solution

37% (v/v) formaldehyde	0.5 ml
Citric acid	50 mg

The solution was made up to 1 L with dH₂O.

Shrink solution

Glycerol	2 ml
dH ₂ O	448 ml
Methanol	550 ml

All steps in silver staining were carried out at RT with gentle shaking. Initially the gel was immersed in fixer solution for 1 h. The reduced fixation step was used to minimise elution of polysaccharide molecules that may occur with long fixation steps. The fixer was aspirated off and the oxidizing solution was added for 5 min, to open up carboxyl

and sulphhydryl groups on polysaccharides, for interaction with silver ions. The gel was then washed 3 times for 10 min in dH₂O, and then immersed in silver staining solution for 12 min. The silver stain solution was then aspirated off and the gel was washed 3 times for 10 min in dH₂O. The silver stained components were then visualized by adding developer solution until bands had developed sufficiently (2-5 min). A stop solution of 5% acetic acid was then added for 15 min to halt the reaction, and the gels were then rinsed in dH₂O.

2.7.5.3 Alcian blue staining

In order to visualise CPS in SDS-PAGE gels, Alcian blue staining was carried out.

Alcian blue stain

Glacial acetic acid	50 ml
Ethanol	400 ml
dH ₂ O	550 ml
Alcian blue 8 GX (Sigma-Aldrich)	1 g

Alcian blue destain

Glacial acetic acid	50 ml
Ethanol	400 ml
dH ₂ O	550 ml

After electrophoresis gels were washed 3 times for 10 min, with shaking in destain solution to remove residual SDS that would inhibit binding of the cationic dye to the CPS. The gel was then immersed in the staining solution for 30 min at 50°C with gentle shaking. The stain was then aspirated off and destain was added until background staining was removed sufficiently.

2.7.6 Drying and storage of SDS-PAGE gels

Gels were digitally scanned after staining with an Epson Perfection 1260 scanner and the accompanying software (Epson Inc., Long Beach, CA., U.S.A.). To allow long term storage of gels they were shrunk and dried.

Shrink solution

Methanol	550 ml
dH ₂ O	448 ml
Glycerol	2 ml

Gels were immersed in shrink solution overnight. Two pieces of BioDesign Gel Wrap (Biodesign Inc., Carmel, NY, U.S.A.) were cut 2 cm larger than the gel and soaked in shrink solution overnight also. The gel was then sandwiched and clamped between the two pieces of wrap and a mass was placed upon it to keep it the gel flat. The gel was then allowed to dry at RT for 3-4 days.

2.8 Thin-Layer Chromatography

Separation by TLC combined with chemical and immuno-staining was used to further characterise *C. jejuni* HPW and MPW extracts

2.8.1 Solutions required for TLC

Ganglioside developing solution

Chloroform	50 ml
Methanol	45 ml
dH ₂ O	10 ml
CaCl ₂	2.2 mg

LOS/ LPS developing solution

n-propanol	60 ml
dH ₂ O	30 ml

25% ammonia solution	10 ml
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Chemical staining reagents

Resorcinol-HCL

HCl	33 ml
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Resorcinol (2%)	10 ml
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CuSO ₄ .5H ₂ O (0.1M)	0.25 ml
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dH ₂ O	57 ml
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After development TLC plate was sprayed with a light mist of resorcinol-HCl using TLC atomiser (Fisons, San Carlos, CA, U.S.A.) in a fume-hood. The TLC plate was allowed dry, then was clamped between two clean glass plates, and placed at 100°C for 20 min.

Diphenylamine-aniline

Diphenylamine	500 mg
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Acetone	50 ml
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Aniline	0.5 ml
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Phosphoric acid (85%)	5 ml
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After development, the TLC plate was sprayed in a fume-hood with a fine-mist of diphenylamine-aniline spray using a TLC atomizer (Fisons). The plate was air-dried and placed at 130°C for 10 min.

Charring reagent

Ethanol	80 ml
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H ₂ SO ₄ (37.7 N)	20 ml
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The TLC plate was dipped in charring reagent, allowed to air-dry and was placed at 100°C for 15 min.

Gangliosides

Ganglioside controls (Table 2.8) were dissolved in chloroform/ methanol (2:1, v/v) at 1 mg ml⁻¹.

Table 2.8 Ganglioside controls used in TLC analysis.

Ganglioside	Source
GM ₁	Sigma-Aldrich (G9652)
GM ₂	Sigma-Aldrich (G9398)
Mix 1 (GD ₃ , GD _{1a} , GD _{1b})	Matreya (1509- Matreya LLC., Pleasant Gap, PA, U.S.A.)
Mix 2 (GM ₁ , asialo-GM ₁ , GD _{1a} , GD _{1b} , GT _{1b})	Matreya (1511)

Polyisobutylmethacrylate (PIBM) solution

PIBM	1.2 g
Chloroform	6 ml
<i>n</i> -hexane	594 ml

Sonication was used to aid dissolving the PIBM in chloroform, before mixing with *n*-hexane.

Gelatin-PBS

Gelatin (Biorad)	0.3 g
PBS	100 ml

The constituents were heated to 37°C to dissolve the gelatin. This solution was used as a blocker for non-specific binding and for diluting antibodies.

Color-development buffer

4-chloro-1-naphtol (4-CN, Biorad)	60 mg
Methanol	620 ml
H ₂ O ₂ (30%)	594 ml
TBS	100 ml

To visualise peroxidase-conjugated antibodies and ligands a color development system was used. The 4-CN was dissolved in the methanol, and kept away from the light. Immediately before use the H₂O₂ was added to the TBS, and the 4-CN solution was then also added. The solution was overlaid on the TLC plate until sufficient color development had occurred. The TLC plate was then washed 3 times for 5 min with shaking, to stop the reaction.

2.8.2 Separation of glycoconjugates

Gangliosides, HPW or MPW extracts were applied to precoated silica gel 60 glass plates (Merck), using a 5 µl syringe (Hamilton Bonaduz AG, Switzerland), 1 cm from the bottom of the plate. The TLC plate was carefully placed in a 20 cm × 20 cm glass TLC chamber (Fisher Scientific International Inc., Hampton, NH, U.S.A.), containing 100 ml of the appropriate development solvent. The solvent front was allowed to develop until 1 cm from the top of the TLC plate, then the plate was removed, the solvent front marked, and the plate dried in a fume-hood.

2.8.3 TLC with immunostaining

For the detection of ganglioside mimicry in *C. jejuni* LOS, TLC with immunostaining was carried out according to the method of Saito *et al.* (1983), as modified by Schwerer *et al.* (1995). Developed and air-dried TLC plates were dipped in the 0.2% PIBM solution for 1.5 min, and air-dried. The PIBM helps maintain the silica phase throughout the procedure, and reduces non-specific binding. Non-specific binding was further prevented by blocking in the gelatin-PBS solution for 1 h at RT, with shaking. TLC plates were then overlaid with primary antibody (Anti-GM₁ and anti-asialo-GM₁; diluted 1:100 in gelatin-PBS), and incubated at 4°C overnight, with shaking in a humid chamber. Subsequently, the primary antibody was aspirated off and the plates were

washed three times in cold PBS. The plates were then overlaid with a secondary antibody of peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich, A0545), diluted to a concentration of 1:1000, for 1h at RT, with shaking. Following this the plates were washed 3 times for 5 min in cold PBS and then color development buffer was added for reaction with Hrp-conjugated secondary antibody.

2.8.4 Binding experiments with ligands

TLC plates were overlaid with cholera toxin B-subunit Hrp-conjugate (Sigma-Aldrich, C3741), diluted 1:1000, as well as peanut agglutinin (PNA) Hrp-conjugate (Sigma-Aldrich, L7759), at a concentration of 1:50. The procedure was carried out as with anti-ganglioside antibodies, however, Hrp detection was carried out after one overlay step, due to direct Hrp-conjugation to primary ligand.

2.9 Glycan microarray analysis

Glycan microarray analysis of *C. jejuni* HS:41 GBS and enteritis-related strains was carried out on an array consisting of 60 amine-linked oligosaccharides (Table 2.9).

2.9.1 Preparation of SuperAmine® glass slides

Propylamino-glass slides (SuperAmine2 glass slides, Array It Microarray Technologies) were functionalised with a polyethylene glycol spacer moiety as previously described (Bradner *et al.*, 2006). Glass slides were incubated for at least 18 h in a solution of Fmoc-8-amino-3,6-dioxaoctanoic acid (10 mM), PyBOP (10 mM), diisopropylethylamine (20 mM) in dry DMF at room temperature in an anhydrous environment. After functionalisation the slides were rinsed in DMF, the Fmoc protecting group removed by incubating the slides in piperadine (10% v/v) in DMF for 30 min, and the slides were subsequently rinsed in DMF. The slides were then activated with isocyanate functionality by incubation in a DMF solution of 1,6-disocyanatohexane (10% v/v) for 30 min at room temperature, rinsed once in DMF, once in THF, centrifuged dry ($500 \times g$ for 2 min). The slides were stored under vacuum in desiccator for up to 1 week prior to use.

2.9.2 Preparation of glycoamines

Glycans obtained Dextra Laboratories (Reading, UK) and Glycoseperations (Moscow, Russia), and functionalised with an amine group using a modification of a previously described methodology by Vetter and Gallop (1995). One to five milligrams of each glycan and ammonium carbonate (1.3 equivalents) were dissolved in ~ 25% ammonium hydroxide (500 μ l) in a sealed glass vial and incubated at 50°C for 3 days. The vials were unsealed, allowed to remain at 50°C for 2 h to dissipate excess ammonia and carbon dioxide, frozen and concentrated by lyophilisation. The glycosylamines were subsequently dissolved in anhydrous DMSO (100 μ l), diluted further with an equal volume of anhydrous DMF and stored in sealed vials at 4°C until required.

2.9.3 Preparation of glycan microarrays

Microarrays were printed by a robotic non-contact dispensing (Piezoarray, Non-Contact Dispensing System, PerkinElmer) of 1500 pl of 10 mM glycosylamines in DMSO/DMF (1:1) onto isocyanate functionalised glass slides in replicates of four. Two identical sub-arrays were printed per slide with each sub-array comprising 432 spots (60 glycans and 8 controls in replicates of 4, Table 2.9). Once the microarrays had been printed they were placed in a vacuum desiccator fitted with a gas tap, which was evacuated under high vacuum. The vacuum was sealed off and a round-bottomed flask containing pyridine (~1 ml) attached via hosing and the desiccator was re-equilibrated with catalytic pyridine vapour for at least 8 h. The slides were then neutralised by incubating them in ethylene glycol (1 M) and pyridine (1% v/v) in DMF for 30 min at room temperature. The glycan array slides were rinsed in DMF, then ethanol and centrifuged dry (500 \times g for 2 min), at which stage they were ready for binding assays.

2.9.4 Preparation of *C. jejuni* for glycan microarray analysis

An overnight culture of *C. jejuni* biomass adjusted to $\sim 2 \times 10^6$ in PBS was centrifuged at 13000 rpm for 1 min. Pellet was washed three times in PBS. The pellet was resuspended in 1 ml PBS and 500 μ l of 2.5 μ M carboxyfluorescein diacetate, succimidyl ester (CFDA-SE; Molecular probes/ Invitrogen) was added, prepared to manufacturers instruction in PBS and ethanol. Bacterial/dye suspension was incubated in the dark at 37°C for 30 mins, for development of fluorescence. Excess dye was removed by washing cells in 1 ml of PBS eight times. Washed cell pellet was

resuspended in 1 ml of an PBS containing 2 mM of both CaCl_2 and MgCl_2 to facilitate the ligand-receptor binding interactions.

Table 2.9 Glycan structures present on the glycan microarray.

<u>ID</u>	<u>Glycan structure</u>	<u>ID</u>	<u>Glycan structure</u>
1A	Gal β (1,3)GlcNAc	7J	Gal β (1,3)[Fuc α (1,4)]GlcNAc
1B	Gal β (1,4)GlcNAc	7K	GalNAc α (1,3)[Fuc α (1,2)]Gal
1C	Gal β 1,4Gal	7L	Fuc α (1,2)Gal β (1,4)[Fuc α (1,3)]Glc
1D	Gal β 1,6GlcNAc	7M	Gal β (1,3)[Fuc α (1,2)]Gal
1E	Gal β 1,3GalNAc	7N	Fuc α (1,2)Gal β (1,4)[Fuc α (1,3)]GlcNAc
1F	Gal β 1,3GalNAc β 1,4Gal β 1,4Glc	7O	Fuc α (1,2)Gal β (1,3)GlcNAc
1G	Gal β 1,3GlcNAc β 1,3Gal β 1,4Glc	7P	Fuc α (1,2)Gal β (1,3)[Fuc α (1,4)]GlcNAc
1H	Gal β 1,4GlcNAc β 1,3Gal β 1,4Glc	8A	SO ₃ ,3Gal β (1,3)[Fuc α (1,4)]GlcNAc
1I	Gal β 1,4GlcNAc β 1,6(Gal β 1,4GlcNAc α 1,3)Gal β 1,4Glc	8B	SO ₃ ,3Gal β (1,4)[Fuc α (1,3)]GlcNAc
1J	Gal β 1,4GlcNAc β 1,6(Gal β 1,3GlcNAc α 1,3)Gal β 1,4Glc	8C	Gal β (1,3)GlcNAc β (1,3)Gal β (1,4)[Fuc α (1,3)]GlcNAc β (1,3)Gal β (1,4)Glc
1K	Gal α 1,4Gal β 1,4Glc	8D	Gal β (1,4)[Fuc α (1,3)]GlcNAc β (1,6)[Gal β (1,3)GlcNAc β (1,3)]Gal β (1,4)Glc
1L	GalNAc α 1,O,Ser	8E	Gal β (1,4)[Fuc α (1,3)]GlcNAc β (1,6)[Fuc α (1,2)Gal β (1,3)GlcNAc β (1,3)]Gal β (1,4)Glc
1M	Gal β (1,3)GalNAc α 1,O,Ser	8F	Gal β (1,4)[Fuc α (1,3)]GlcNAc β (1,6)[Fuc α (1,2)Gal β (1,3)[Fuc α (1,4)]GlcNAc β (1,3)]Gal β (1,4)Glc
1N	Gal α (1,3)Gal	10A	Neu5Ac α (2,3)Gal β (1,3)[Fuc α (1,4)]GlcNAc
1O	Gal α (1,3)Gal β (1,4)GlcNAc	10B	Neu5Ac α (2,3)Gal β (1,4)[Fuc α (1,3)]GlcNAc
1P	Gal α (1,3)Gal β (1,4)Glc	10C	Neu5Ac α (2,3)Gal β (1,3)GlcNAc β (1,3)Gal β (1,4)Glc
2A	Gal α (1,3)Gal β (1,4)Gal α (1,3)Gal	10D	Gal β (1,4)[Fuc α (1,3)]GlcNAc β (1,6)[Neu5Ac α (2,6)Gal β (

2B	Gal β (1,6)Gal		1,4)GlcNAc β (1,3)]Gal β (1,4)Glc
5C	Man α (1,2)Man	10K	Neu5Ac α (2,3)Gal β (1,4)GlcNAc
5D	Man α (1,3)Man	10L	Neu5Ac α (2,6)Gal β (1,4)GlcNAc
5E	Man α (1,4)Man	10M	Neu5Ac α (2,3)Gal β (1,3)GlcNAc β (1,3)Gal β (1,4)Glc
5F	Man α (1,6)Man	10N	Gal β (1,3)[Neu5Ac α (2,6)]GlcNAc β (1,3)Gal β (1,4)Glc
5G	Man α (1,6)[Man α (1,3)]Man	10O	Neu5Ac α (2,6)Gal β (1,4)GlcNAc β (1,3)Gal β (1,4)Glc
		10P	Neu5Ac α (2,3)Gal β (1,3)[Neu5Ac α (2,6)]GlcNAc β (1,3)Gal β (1,4)Glc
5H	Man α (1,6)[Man α (1,3)]Man α (1,6)[Man α (1,3)]Man	11A	Neu5Ac α (2,3)Gal β (1,4)Glc
7A	Fuc α (1,2)Gal β (1,3)GlcNAc β (1,3)Gal β (1,4)Glc	11B	Neu5Ac α (2,6)Gal β (1,4)Glc
7B	Gal β (1,3)[Fuc α (1,4)]GlcNAc β (1,3)Gal β (1,4)Glc	11C	(Neu5Ac α 2,8Neu5Ac) _n (n<50)
7C	Gal β (1,4)[Fuc α (1,3)]GlcNAc β (1,3)Gal β (1,4)Glc	11D	Biantennary 2,6,sialylated N-glycan-Asn
7D	Fuc α (1,2)Gal β (1,3)[Fuc α (1,4)]GlcNAc β (1,3)Gal β (1,4)Glc		
7E	Gal β (1,3)[Fuc α (1,4)]GlcNAc β (1,3)Gal β (1,4)[Fuc α (1,3)]Glc		
7F	Fuc α (1,2)Gal		
7G	Fuc α (1,2)Gal β (1,4)Glc		
7H	Gal β (1,4)[Fuc α (1,3)]Glc		
7I	Gal β (1,4)[Fuc α (1,3)]GlcNAc		

2.9.5 Glycan microarray analysis of *C. jejuni*

The two sub-arrays of the glycan microarray were isolated using a 1.7 × 2.8 cm Gene Frame (Abgene), and slides were labelled with diamond-tip pen. Added to each subarray was 130 µl of the labelled bacterial suspension for hybridisation reaction. Coverslide was attached causing solution to spread evenly across surface and array was incubated in a humidifier at 37°C in the dark, for 30 min. Coverslide was then removed and array was washed by inverting in 50 ml of PBS in a sterile centrifuge tub. Then, PBS was pipetted over the array a number of times to remove any non-specific binding. The microarray was then submerged in 3.7% formaldehyde for fixation fixative step. Before scanning slide was dried in a centrifuge at 500 × g for 4 min. For each strain analysed, duplicate arrays were performed using cultures prepared on separate days.

2.9.6 Data acquisition and analysis

Microarrays were scanned using the ProScanArray Microarray 4-laser Scanner (PerkinElmer), using the Blue Argon 488 excitation laser set to FITC (494 excitation, 518 emission). Microarray spot fluorescent intensities were measured and data was analysed using the ProScanArray imaging software ScanArray Express (PerkinElmer). Multilevelled statistical analysis was conducted in Stata 12 (StataCorp. 2011, Stata Statistical Software: Release 12. College Station, TX: StataCorp LP).

2.10 Neoglycoconjugate (NGC) and lectin microarrays

Neoglycoconjugate microarrays and lectin microarrays were prepared to analyse *C. jejuni* glycan recognition and *C. jejuni* cell-surface glycan expression, respectively.

2.10.1 Neoglycoconjugate arrays

Neoglycoconjugate microarrays were printed to include oligosaccharides relevant to human cell surface expression for the analysis of *C. jejuni* host-glycoconjugate interaction. Neoglycoconjugate microarrays differed from the previous glycan microarray since glycans were attached to bovine serum albumin (BSA) or human serum albumin (HSA) backbone via a non-native linker to form NGCs, before spotting on microarray slide.

2.10.1.1 Neoglycoconjugate synthesis

Several mono- and disaccharide neoglycoproteins were synthesised in-house from their commercially available phenylisothiocyanate (ITC), *p*-nitrophenyl (pNP) or 4-aminophenyl (4AP) derivatives conjugated to periodate-treated conjugation-grade BSA. The ratio of carbohydrate to protein of the neoglycoconjugates was determined using microBCA and Monsigny assays. Oligosaccharide neoglycoconjugates were also purchased from Dextra Laboratories (UK).

2.10.1.2 NGC array printing, incubation and scanning

Neoglycoconjugate (NGC) array slides were prepared as outlined in Kilcoyne *et al.* (2012). Poly-L-lysine slides were functionalised with sulfhydryl-reactive maleimide groups by incubation of the slide surface with 10 mM sulfo-SMCC prepared in PBS, pH 7.4 (137mM NaCl, 2.7 mM KCL, 2 mM, KH₂PO₄ and adjusted to correct pH with Na₂HPO₄ and NaH₂PO₄) for 1 h at RT, in a humidity chamber. Functionalised slide were then washed twice in dH₂O, centrifuged dry (500 × g, 5 min) and stored at 4°C with desiccant until required.

NGCs were prepared at a concentration of 1 mg ml⁻¹ in PBS, pH 7.4, based on BCA assay (NGCs) or mass (glycoproteins) and printed at approximately 1 nL per feature on functionalised poly-L-lysine slides, or Nexterion® Slide H microarray slides (Schott AG, Mainz, Germany), in humidity (62% ± 2%), using a SciFLEXARRAYER S3 (Scienion AG, Germany) equipped with a 90 µm uncoated glass nozzle. Each slide was printed with six subarrays, with each probe spotted in replicates of twelve per subarray. Slides were incubated in a humid atmosphere overnight after printing for complete conjugation. Functional groups on poly-L-lysine were deactivated or capped with 1.4 mM β-mercaptoethanol in PBS pH 7.4 for 1 h at RT and the Nexterion® Slide H with 100 mM ethanolamine in 50 mM sodium borate, pH 8 for 1 h at RT. The slides were washed with PBS, pH 7.4 with 0.05% Tween-20 (PBS-T) three times and once with PBS. Slides were then centrifuges dry (500 × g, 5 min) and stored dry at 4°C with desiccant until required.

Table 2.10 List of neoglycoconjugates on the array.

<u>Abbreviation</u>	<u>Neoglycoconjugate</u>
Fetuin	Fetuin
ASF	Asialofetuin
OV	Ovalbumin
RB	RNase B
Xferrin	Transferrin
α -C	α -Crystallin
GlcNAcBSA	GlcNAc-BSA
LacNAcBSA	LacNAc-BSA
4APBSA	4AP-BSA
LNFPIBSA	Lacto-N-fucopentaose I-BSA
LNFPIIBSA	Lacto-N-fucopentaose II-BSA
LNFPIIIBSA	Lacto-N-fucopentaose III-BSA
LNDHIBSA	Lacto-N-hexaose I-BSA
SLexBSA14	3'Sialyl Lewis x-BSA
6SuLexBSA	6-Sulfo Lewis x BSA
6SuLeaBSA	6-Sulfo Lewis a-BSA
3SuLeaBSA	3-Sulfo Lewis a-BSA
LeaBSA	Lewis a-BSA
BGABSA	Blood Group A-BSA
BGBBSA	Blood Group B-BSA
3SLNBSA	3'SialylLacNAc-BSA
GGGNHSA	Gal α 1,3Gal β 1,4GlcNAc-HSA
M3BSA	Man α 1,3(Man α 1,6)Man-BSA
3SLexBSA	3'Sialyl Lewis x-BSA
LexBSA	Lewis x-BSA
LebBSA	Lewis b-BSA
2FLBSA	2'Fucosyllactose-BSA
3SFLBSA	3'Sialyl-3-fucosyllatose-BSA
Gb4GBSA	Gal β 1,4Gal-BSA
H2BSA	H Type II-APE-BSA
3SLacHSA	3'-Sialyllactose-APD-HSA
LNnTHSA	Lacto-N-neotetraose-APD-HSA
GlobTHSA	Globotriose-APE-HSA
6SLacHSA	6'-Sialyllactose-APD-HSA
LeyHSA	Lewis y-tetrasaccharide-APE-HSA
aGM1HSA	Asialo-GM1-pentasaccharide-APD-HSA
LNTHSA	Lacto-N-tetraose-APD-HSA
GlobNTHSA	Globo-N-tetraose-APD-HSA
3LexHSA	Tri-Lex-APE-HSA
DiLexHSA	DiLex-APE-HSA

DFPLNHSA	Difuco-para-lacto-N-hexaose-
SLNFVHSA	APD-HSA
MMLNnHSA	Sialyl-LNF-V-APD-HSA
	Monofucosyl, monosialyllacto-
	<i>N</i> -neohexaose-APD-HSA
3FeyHSA	Tri-fucosyl-Ley-
	heptasaccharide-APE-HSA
DiLexBSA	Di-Lex-APE-BSA
SLNnTHSA	Sialyl-LNnT-penta-APD-HSA
GM1HSA	GM1-pentasaccharide-APD-
	HSA
3SuLexBSA	3-Sulfo Lewis x-BSA
Ga3GBSA	Gal α 1,3Gal-BSA
Ga2GBSA	Gal α 1,2Gal-BSA

2.10.2 Lectin microarray construction and bacterial incubation

A panel of lectin (Table 2.11) was printed on Nexterion® Slide H microarray slides in a 62% (+/-2%) humidity environment using a SciFLEXARRAYER S3 equipped with a 90 μ m uncoated glass nozzle. Lectins were diluted to their print concentration of 0.5 mg/mL in phosphate buffered saline (PBS; 10 mM sodium phosphate, 137 mM NaCl, 2 mM KCl, 2 mM KH₂PO₄, pH 7.4) supplemented with 1 mM of their respective haptenic simple sugars to protect their carbohydrate recognition domains during conjugation to the slide surface (Table 2.11). Each microarray slide was printed with eight replicate subarrays, with each lectin (probe) spotted in replicates of six. Slides were incubated in a humidity chamber overnight after printing to facilitate complete conjugation and were then blocked with 100 mM ethanolamine in 50 mM sodium borate, pH 8.0, washed four times in PBS-T for 2 min each, once with PBS and centrifuged dry (500 \times g, 5 min). Printing and performance of the conjugated lectins was verified by incubation with fluorescently labelled glycoproteins. Microarray slides were stored dry with desiccant at 4°C until use.

Fluorescently labelled glycoproteins or bacteria diluted in TBS-T were incubated on the lectin microarray using an 8 well gasket (Agilent Technologies, Cork, Ireland) at room temperature for 1 h with gentle rotation (4 rpm) in the dark. For bacterial incubations, 30 μ l of the bacteria at OD₆₀₀ of 2.0 was diluted to a final volume of 70 μ l per well with TBS-T. Three biological replicate experiments were performed, with each

experiment for uninhibited binding done in duplicate. To verify carbohydrate-mediated binding samples were co-incubated with appropriate haptenic carbohydrates in parallel to give a final concentration of approximately 57 mM monosaccharide. Inhibited intensity values were then compared to an uninhibited subarray incubated on the same slide. The slides were washed four times with TBS-T, once with TBS, centrifuged dry and scanned immediately with the 543 nm laser (90% laser power, 70% PMT, TRITC emission filter, 5 μ m resolution) of a ScanArray Express HT microarray scanner (Perkin-Elmer, Waltham, MA). The entire procedure from bacterial harvest after culture, to microarray scan took approximately 2.5 h.

Table 2.11 Lectins printed, their binding specificities and the simple print sugars (1 mM).

Abbreviation	Source	Species	Common name	Binding specificity*	Print sugar
AIA, Jacalin	Plant	<i>Artocarpus integrifolia</i>	Jack fruit lectin	Gal (sialylation independent)	Gal
RPbAI	Plant	<i>Robinia pseudoacacia</i>	Black locust lectin	Gal	Gal
PA-I	Bacteria	<i>Pseudomonas aeruginosa</i>	Pseudomonas lectin	Gal, Gal derivatives	Gal
SNA-II	Plant	<i>Sambucus nigra</i>	Sambucus lectin-II	Gal/GalNAc	Gal
SJA	Plant	<i>Sophora japonica</i>	Pagoda tree lectin	β -GalNAc	Gal
DBA	Plant	<i>Dolichos biflorus</i>	Horse gram lectin	GalNAc	Gal
APP	Plant	<i>Aegopodium podagraria</i>	Ground elder lectin	GalNAc	Gal
SBA	Plant	<i>Glycine max</i>	Soy bean lectin	GalNAc	Gal
VVA-B4	Plant	<i>Vicia villosa</i>	Hairy vetch lectin	GalNAc	Gal
BPA	Plant	<i>Bauhinia purpurea</i>	Camels foot tree lectin	GalNAc/Gal	Gal
WFA	Plant	<i>Wisteria floribunda</i>	Japanese wisteria lectin	GalNAc/sulfated GalNAc	Gal
ACA	Plant	<i>Amaranthus caudatus</i>	Amaranthin	Sialylated/Gal- β -(1,3)-GalNAc	Lac
PNA	Plant	<i>Arachis hypogaea</i>	Peanut lectin	Gal- β -(1,3)-GalNAc	Lac
GSL-II	Plant	<i>Griffonia simplicifolia</i> (<i>Bandeiraea simplicifolia</i>)	Griffonia/Bandeiraea lectin-II	GlcNAc	GlcNAc
sWGA	Plant	<i>Triticum vulgare</i>	Succinyl WGA	GlcNAc	GlcNAc
DSA	Plant	<i>Datura stramonium</i>	Jimson weed lectin	GlcNAc	GlcNAc
STA	Plant	<i>Solanum tuberosum</i>	Potato lectin	GlcNAc oligomers	GlcNAc
LEL	Plant	<i>Lycopersicon esculentum</i>	Tomato lectin	GlcNAc- β -(1,4)-GlcNAc	GlcNAc

NPA	Plant	<i>Narcissus pseudonarcissus</i>	Daffodil lectin	α -(1,6)-Man	Man
GNA	Plant	<i>Galanthus nivalis</i>	Snowdrop lectin	Man- α -(1,3)-	Man
HHA	Plant	<i>Hippeastrum hybrid</i>	Amaryllis agglutinin	Man- α -(1,3)-Man- α -(1,6)-	Man
ConA	Plant	<i>Canavalia ensiformis</i>	Jack bean lectin	Man, Glc, GlcNAc	Man
Lch-B	Plant	<i>Lens culinaris</i>	Lentil isolectin B	Man, core fucosylated, agalactosylated biantennary <i>N</i> - glycans	Man
PSA	Plant	<i>Pisum sativum</i>	Pea lectin	Man, core fucosylated trimannosyl <i>N</i> -glycans	Man
WGA	Plant	<i>Triticum vulgaris</i>	Wheat germ agglutinin	NeuAc/GlcNAc	GlcNAc
MAA	Plant	<i>Maackia amurensis</i>	Maackia agglutinin	Sialic acid- α -(2,3)-linked	Lac
SNA-I	Plant	<i>Sambucus nigra</i>	Sambucus lectin-I	Sialic acid- α -(2,6)-linked	Lac
PHA-L	Plant	<i>Phaseolus vulgaris</i>	Kidney bean leucoagglutinin	Tri- and tetraantennary β - Gal/Gal- β -(1,4)-GlcNAc	Lac
PCA	Plant	<i>Phaseolus coccineus</i>	Scarlet runner bean lectin	GlcNAc in complex oligosaccharides	Lac
PHA-E	Plant	<i>Phaseolus vulgaris</i>	Kidney bean erythroagglutinin	Biantennary with bisecting GlcNAc, β -Gal/Gal- β -(1,4)- GlcNAc	Lac
RCA-I/120	Plant	<i>Ricinus communis</i>	Castor bean lectin I	Gal- β -(1,4)-GlcNAc	Gal
CPA	Plant	<i>Cicer arietinum</i>	Chickpea lectin	Complex oligosaccharides	Lac
CAA	Plant	<i>Caragana arborescens</i>	Pea tree lectin	Gal- β -(1,4)-GlcNAc	Lac

ECA	Plant	<i>Erythrina cristagalli</i>	Cocks comb/coral tree lectin	Gal- β -(1,4)-GlcNAc oligomers	Lac
AAL	Fungi	<i>Aleuria aurantia</i>	Orange peel fungus lectin	Fuc- α -(1,6), - α -(1,3)	Fuc
LTA	Plant	<i>Lotus tetragonolobus</i>	Lotus lectin	Fuc- α -(1,3)	Fuc
UEA-I	Plant	<i>Ulex europaeus</i>	Gorse lectin-I	Fuc- α -(1,2)	Fuc
EEA	Plant	<i>Euonymous europaeus</i>	Spindle tree lectin	α -Gal	Gal
GSL-I-B4	Plant	<i>Griffonia simplicifolia</i> (aka <i>Bandeiraea simplicifolia</i>)	Griffonia/Bandeiraea lectin-I	α -Gal	Gal
MPA	Plant	<i>Maclura pomifera</i>	Osage orange lectin	α -Gal	Gal
VRA	Plant	<i>Vigna radiata</i>	Mung bean lectin	α -Gal	Gal

* Reported recognition based on literature consensus

2.10.3 Preparation of *C. jejuni* for array analysis

C. jejuni strains NCTC 81116 and 81-176 were grown on 5% Columbia blood agar at 37 and 42°C for 24 h in a microaerobic atmosphere. Bacteria were harvested, pelleted by centrifugation (5,000 x g, 5 min) and washed twice in Tris-buffered saline supplemented with Ca²⁺ and Mg²⁺ ions (TBS; 20 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.2). Bacteria were diluted to an OD₆₀₀ of 1.0 (~5 × 10¹⁰ cfu/ml) in TBS, and 1 ml of the bacterial suspension was pelleted by centrifugation and then resuspended in 0.5 ml of TBS. Bacteria were incubated with 20 μM SYTO 82 (Life Technologies, Carlsbad, CA) orange fluorescent cell-permeable nucleic acid dye (λ_{ex} 541 nm, λ_{em} 560 nm) at 37°C for 1 h with rotation. After incubation, the fluorescently-labelled cell suspension was washed seven times in TBS to remove excess dye, and finally resuspended in 0.5 ml of TBS with 0.05% Tween-20 (TBS-T) for immediate use on the lectin microarrays.

To determine the optimum SYTO 82 concentration for each strain, different concentrations of dye were added to the washed bacterial suspensions to give a final range of 5 - 100 μM. After incubation, 100 μl of the bacterial samples, both with and without post-staining wash steps, was loaded into 96 well black microtitre plate and fluorescence was measured on a SpectraMax M5e microplate reader (Molecular Devices, Inc., Berkshire, UK). The optimal concentration was determined based on maximum fluorescence. Similar fluorescence intensities were noted when bacteria were incubated in TBS or PBS.

2.10.4 Data acquisition and analysis

Raw intensity values were extracted from the image files using GenePix Pro v6.1.0.4 (Molecular Devices, Berkshire, U.K.) software. Circular features were extracted using a proprietary *.gal file (containing identification and location data for all printed features) applied to the image and an adaptive diameter (70-130%) feature fitting approach based on 230 μm feature diameter. Numerical intensity data was exported as text to Excel (version 2007, Microsoft) where all calculations were performed. Local background was subtracted and background-corrected median feature intensity (F543median-B543) was used for each feature intensity value. The median of six replicate spots per subarray was handled as a single data point for graphical and statistical analysis ($n = 3$). Data intensities were normalised to the per-subarray total intensity mean of six replicate microarray slides and binding data was presented in histogram form of mean intensity with average deviation of six replicates from three biological experimental replicates. The significance of inhibition data was evaluated using a standard Student's t-test (paired, two-tailed, Appendix ii).

Unsupervised clustering of lectin microarray data was performed using Hierarchical Clustering Explorer v3.5 (HCE 3.5, University of Maryland, <http://www.cs.umd.edu/hcil/hce/hce3.html>). Normalised data was imported into HCE 3.0 for hierarchical clustering by Euclidean distance with complete linkage.

Similar to the method of Tateno, *et al.*, principle component analysis (PrCA) was performed on normalized lectin microarray data with Minitab 16 software (Minitab, Inc., State College, PA, USA). Two-dimensional score plots for two principal components were generated from total lectin microarray data and lectin microarray data selected by significance as assessed by standard Students's t-test (paired, two-tailed, p values ≤ 0.05 selected from Appendix).

2.11 Lectin agglutination

In addition to lectin array format, a microwell-plate lectin agglutination assay was performed

2.11.1 Lectin preparation

The following plant lectins were used in the agglutination assay: AIA, SNA-II, SBA, BPA, WFA, PNA, sWGA, STA, LEL, NPA, ConA, LchA, WGA, SNA-I, ECA, GSL-1-B₄, MPA, VRA, MOA (Table 2.11). The carbohydrate specificity of each lectin shown in Table 2.11. Lectins were dissolved in TBS containing 0.02% calcium chloride and 0.02% magnesium chloride at 0.5 mg ml⁻¹ and 0.8 mg ml⁻¹.

2.11.2 Bacterial sample preparation

Bacteria for lectin typing were prepared as described previously (Hynes *et al.*, 2002). *C. jejuni* was harvested and biomass was washed three times in PBS. For proteolytic treatment, whole cell samples were incubated in PBS (adjusted to pH 4 with 1 M hydrochloric acid) for 30 min, at 20°C to allow autolysis. Cellular debris was then washed twice in PBS, resuspended in PBS containing proteinase K at 0.5 mg ml⁻¹, incubated at 60°C for 1 h, and then at 100°C for 10 min, followed by centrifugation. Both whole cell samples and proteolytically-treated pellets were then adjusted in TBS to an OD₅₅₀ of 0.9.

2.11.3 Performance of lectin agglutination assay

Lectin agglutination assay was performed as described previously (Hynes *et al.*, 2002). Whole cell or proteinase-k treated samples (40 µl) were added to 10 µl of lectin in round-bottomed wells of 96-well microtitre plates (Nunc, Thermo Fisher Scientific, Dublin, Ireland) and mixed by gentle rocking for 5 s, or alternatively, with 10 µl TBS as a negative control, then left undisturbed overnight at 20°C. As a positive control, 40 µl of a 0.75% solution of human erythrocytes, type O, was mixed with 10 µl of each lectin solution, and was inspected for agglutination after 2 h. Results were determined by visual inspection. Positive agglutination of bacterial samples with lectins was indicated by a carpet of debris on the well-bottom. A negative reaction was indicated by a dot of

debris located centrally on the well-bottom, and confirmed with movement of the debris by tilting the plate at 45°. Each reaction was carried out in duplicate.

2.12 Caco-2 cell culture

Caco-2 cell adherence assays with lectin and carbohydrate inhibition, were used to verify carbohydrate-mediated adherence by *C. jejuni*. Caco-2 cells were grown in at 37°C in a controlled atmosphere incubator, and examined microscopically.

2.12.1 Media and reagents used in tissue culture

Caco-2 cells were routinely cultured in the standard growth medium Dulbecco's Modified Eagle's Medium (DMEM). The following supplements were added to 500 ml of DMEM (Sigma-Aldrich, D6546), to make the complete DMEM for culture.

Complete DMEM

DMEM	500 ml
Fetal calf serum (FCS), heat inactivated (Sigma-Aldrich)	20% (v/v)
L-Glutamine	1% (v/v)
Penicillin-streptomycin (Sigma-Aldrich, P4333)	1% (v/v)
Non-essential amino acids (Sigma-Aldrich, M7145)	1% (v/v)

The solution was aseptically divided into 50 ml aliquots for storage at 4°C for up to 1 month.

Cryopreservative

Complete DMEM	10 ml
FCS	1 ml
DMSO, cell-culture grade (Sigma-Aldrich, D2438)	1 ml

Cryopreservation medium was freshly prepared before use.

2.12.2 Maintenance of Caco-2 cell cultures

Caco-2 cells were routinely grown in 25 cm² (T25) or 75 cm² (T75) tissue culture flasks with gas-exchange lids (Sarstedt AG & Co., Wexford, Ireland). Caco-2 cells were grown from frozen stocks by adding the contents of a frozen stock to 5 ml of complete DMEM prewarmed to 37°C, in a T25 flask. Caco-2 cells were grown to ~80% confluency before passaging, plate seeding or cryopreservation.

2.12.2.1 Passaging of Caco-2 cells

Flasks were checked microscopically for ~80% confluency and media was pipetted off. Cells were rinsed with sterile PBS to remove residual media. Trypsin-EDTA (Sigma-Aldrich, T3924) was added at 0.5 ml per 25 cm² of flask growth surface and incubated at 37°C with periodic monitoring microscopically, until cells were no longer adherent-up to 15 min. To inactivate the trypsin, 10 ml of complete DMEM was added and pipetted over the surface to ensure complete removal of adherent cells. Cell suspension was then transferred to a 15 ml centrifuge tube and centrifuged at 500 × g for 5 min. Supernatant was discarded and the pellet was resuspended in 5 ml of complete DMEM. The solution was passed through a 21-gauge hypodermic needle and 1 ml was added to 5 ml of pre-warmed complete DMEM, before incubation at 37°C. Alternatively, cells were counted and diluted to appropriate density, with 100,000 cells taking ~7 days to reach 80% confluency in a T25 flask, and 300,000 cells taking approximately 5 days in a T25 before the need to passage. Fresh growth media was replaced every 3 days.

2.12.2.2 Counting of Caco-2 cells

Caco-2 cells were counted using the Kova Glasstic Slide 10 (Hycor, Garden Grove, CA, U.S.A.). After passing through the hypodermic needle as above, 10 µl of the well-mixed cell suspension was added to Kova slide and cells were counted with the aid of a microscope, according to manufacturers instructions. Briefly, starting at top left of the grid, all cells within nine separate squares were counted, plus an additional one. Importantly, cell debris or cells more than halfway out of a grid line were not counted. Next, in bottom right corner of grid, cells were counted in nine squares, plus an

additional one. This was then repeated in the centre of the grid. The cell count was then averaged and multiplied by nine thousand, to give the average number of cells per ml.

2.12.2.3 Cryo-preservation of Caco-2 cells

Cells from a T25 flask at ~80% confluency were trypsinated, centrifuged, resuspended and passed through a needle as above. The percentage viability of the cell population was determined by adding 50 μ l of the cell suspension to 10 μ l of trypan blue (Sigma-Aldrich, T8154), in a 1.5 ml centrifuge tube. After 5 min, 10 μ l of the suspension was added to the Kova slide as above. The percentage of dead cells (blue) compared to viable cells (white) was determined and not less than 85% viability was deemed suitable for cryopreservation. For cryopreservation, cell suspensions were centrifuged at $500 \times g$ for 5 min and resuspended in 1 ml of cryopreservative, then added to cyrotube (Fisher Scientific). Cells were cooled to 4°C for 4 h, then, placed at -20°C overnight, before long-term storage in liquid nitrogen.

2.12.3 Caco-2 adherence assay with lectin inhibition

Initially, Caco-2 cells were prepared and counted as above and were diluted in complete DMEM to a concentration of 5×10^5 cells per ml. The cell suspension was well mixed, and 1 ml was added to each well of a 24-well tissue culture plate (Sarstedt), and incubated for 72 h. Before adherence assays each well was inspected microscopically for the presence of a confluent monolayer of Caco-2 cells, with tight junctions visible as black perimeters of each cell. For lectin inhibition, 50 μ g (5 μ l of 1 mg ml⁻¹) of lectins ECA, UEA-I, ConA, MAA, SNA (Table 2.9), LFA (*Limax flavus* agglutinin, Neu5Ac-specific), were added to the wells and rotated at 200 rpm for 5 min, before incubation at 37°C for 1 h, to allow lectin binding to Caco-2 cell-surface carbohydrates. Each lectin was tested in triplicate each day. The media was then pipetted off and the cell monolayers were washed twice with pre-warmed PBS, to remove unbound lectin and the antibiotic-containing media. An overnight culture of *C. jejuni* was harvested, washed once in PBS and was adjusted to an OD₆₀₀ of 0.3, taken to be $\sim 3 \times 10^8$ cfu ml⁻¹. The bacterial suspension was further diluted to 2.5×10^7 cfu ml⁻¹ in DMEM, which did not contain antibiotics, and 1 ml of the suspension was added to each test well of the 24-

well plate. The plate was then centrifuged at $200 \times g$ for 5 min to encourage bacterial interaction with the monolayer, and incubated at 37°C for 1 h, to allow adherence to proceed. Following this, each well was washed three times in PBS to remove non-adherent bacteria and the cell monolayer was then lysed by adding 0.1% Triton-X 100 (Sigma-Aldrich) with rotating at 250 rpm for 10 min. Adherent bacteria were enumerated by serial dilution and viable counts following plating on Columbia agar with 5% horse blood and growth at 42°C for 36 h. The cell concentration of the inoculum was also verified by serial dilution with viable counts.

2.13 Structural analysis

C. jejuni 81116 LOS was structurally characterised using mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

2.13.1 Preparation of *C. jejuni* LOS for NMR and MS analysis

Anhydrous hydrazine was used for *O*-deacylation of the LOS prior to MS analysis (Holst *et al.*, 1993). For NMR analysis acid-hydrolysed core oligosaccharide was obtained by incubating LOS in 1% acetic acid for 90 min at 100°C , and recovered in the aqueous phase following centrifugation ($8000 \times g$, 20 min). Purification of delipidated LOS for NMR was carried out using anion-exchange chromatography (HiTrap Q HP column) on an AKTA explorer system (Amersham Biosciences) with subsequent removal of contaminating NaCl with HiTrap desalting columns (Amersham Biosciences).

2.13.2 Mass Spectrometry of *C. jejuni* LOS

O-deacylated LOS samples were analysed by capillary electrophoresis coupled to electrospray ionisation mass spectrometry (CE-ESI-MS) as described before (Li *et al.*, 2005).

2.13.3 NMR analysis of *C. jejuni* core OS

^1H and ^{13}C NMR spectra were recorded by using a Varian Inova 500 MHz spectrometer in 100% D_2O solutions with acetone standard (2.225 ppm for ^1H and 31.5 ppm for ^{13}C)

by using standard pulse sequences correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) (mixing time 120ms), nuclear Overhauser effect spectroscopy (NOESY) (mixing time 200ms), heteronuclear single-quantum correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) (long-range transfer delay 100 ms), as described before (Houliston *et al.*, 2011).

Chapter 3.
Phenotypic cell-surface characterisation of *C.*
***jejuni* clinical and food isolates**

3.1 Introduction

The *C. jejuni* cell-surface glycoconjugate repertoire includes glycoproteins and glycolipids. *C. jejuni* produces two types of glycolipid, LOS and a polysaccharide capsule CPS. The structures of these poly- or oligosaccharides can vary widely, and this variability has an important role in *C. jejuni* virulence (Guerry *et al.*, 2002; Karlyshev *et al.*, 2005). Furthermore, molecular mimicry of peripheral nerve gangliosides in the LOS of certain *C. jejuni* strains is linked with the development of the autoimmune polyradiculoneuropathy GBS. Central to the development of GBS is the induction of autoreactive antibodies against gangliosides found in the axon of the nerve fibre (Willison & Yuki, 2002). *C. jejuni* cell surface lipooligosaccharide LOS structures have been shown to be not only central to the virulence of *C. jejuni* (Guerry *et al.*, 2000; 2002) but also to the induction of the autoantibodies implicated in GBS development (Prendergast & Moran, 2000; Moran & Prendergast, 2001; Moran *et al.*, 2005). Moran *et al.* (1991) was first to show that *C. jejuni* LOS is sialylated, and LOS of certain strains mimic the gangliosides GM_{1a} and GD_{1a} (Aspinall *et al.*, 1993) that are present in the axons of nerve tissue. Numerous studies have documented that *C. jejuni* LOS chain termini, particularly strains associated with GBS, mimic one or more of the human gangliosides: GM_{1a}, GM₂, GM_{1b}, GD₃, GD_{1a} or GT_{1a} (Aspinall *et al.*, 1994; Prendergast & Moran, 2000; Moran & Prendergast, 2001; Godschalk *et al.*, 2004; Takahashi *et al.*, 2005; Godschalk *et al.*, 2007). Thus, it has been postulated that an infection by a *C. jejuni* strain mimicking gangliosides, that induces an immune response can trigger GBS development in humans (Aspinall *et al.*, 1993; Yuki *et al.*, 1993; Moran, 1995; Moran *et al.*, 2002).

This study was carried out in order to characterise the level of glycoconjugate production by a collection of genotyped and non-genotyped *C. jejuni* isolates, from clinical and food sources on the island of Ireland. Furthermore, the isolates were screened for ganglioside-mimicry, an important predisposing factor for the development of GBS.

3.1.1 *C. jejuni* isolates

A group of 25 *Campylobacter jejuni* isolated from clinical (12 isolates) and food (13 isolates) sources throughout the island of Ireland was obtained for analysis. The twelve *C. jejuni* clinical isolates were from human cases of gastroenteritis from public health laboratories in Belfast City Hospital (BC: Belfast clinical), Cherry Orchard hospital, Dublin (DC: Dublin clinical), and University College Hospital, Galway (GC: Galway clinical). The thirteen *C. jejuni* strains were isolated from food samples obtained from large retail outlets and dedicated butchers shops in Belfast (BF: Belfast food), Dublin (DF: Dublin food) and Galway (GF: Galway food). *C. jejuni* was isolated from contaminated food such as poultry products (9 isolates), beef mince (1 isolate), duck leg (1 isolate), cow liver (1 isolate), mushroom (1 isolate). Furthermore, a single *C. coli* isolate (2134 GF) was used for comparison, and as a negative control throughout the study.

A group of 16 of the *C. jejuni* isolates examined (Table 3.1) was characterised by pulsed-field gel-electrophoresis (PFGE) in a genotyping study carried out previously (O'Leary *et al.*, 2011). In that study the isolates were analysed using the CAMPYNET *Sma*I PFGE protocol and compared using clustering dendograms, to determine the genetic relatedness of the isolates (O'Leary *et al.*, 2011). The typed *C. jejuni* isolates represented a highly diverse group, with overall cohort similarity of 20.5%. Two isolate patterns were said to be indistinguishable if their patterns were greater than 90% similar. Conversely, strains with less than 90% fragment pattern similarity were said to be distinguishable.

Combined cluster analysis of the entire isolate sample (both clinical and food isolates) from the island of Ireland had previously grouped 4/ 16 isolates used in the current study into 2 separate clusters that consisted of indistinguishable isolates. Isolates 260 BC and 1473 BF were clustered in group J3 (Table 3.1), consisting of 5 isolates all

from the Belfast sampling centre (4 Belfast clinical, 1 Belfast food). Isolates 592 BF and 1450 GF were grouped in cluster J7 (Table 3.1). Cluster J7 consisted of a group of 44 isolates obtained from all three sampling centres, and was the predominant *C. jejuni* pattern identified overall in the genotyping study, and also represents the predominant PFGE pattern in *C. jejuni* worldwide (O'Leary *et al.*, 2011).

Cluster analysis of PFGE data from individual food and clinical cohorts revealed the level of genetic diversity between the *C. jejuni* isolates within each cohort. The four genotyped Belfast clinical isolates analysed (32 BC, 300 BC, 301 BC and 260 BC) shared 54% similarity, with 32 BC and 300 BC having 88% similarity. The five genotyped Belfast food isolates (591 BF, 592 BF, 1474 BF, 2325 BF, 1473 BF) were 35% similar overall, with 591 BF and 592 BF sharing 84% similarity. The genotyped Dublin food isolates used in this study (1373 DF, 1644 DF and 2265 DF) were 54% similar, with 1373 DF and 1644 DF sharing 73% similarity. The genotyped Galway food isolates used in this study (2118 GF and 1450 GF) were 58% similar.

Table 3.1 *C. jejuni* clinical and food isolates genotyped by PFGE

<u>PFGE-typed <i>C. jejuni</i> isolates</u>	
<u>PFGE clusters</u>	<u>Isolate I.D.</u>
J3:	260 BC
	1473 BF
J7:	592 BF
	1450 GF
<u>Clinical isolates</u>	
Belfast:	32 BC
	300 BC
	301 BC
Dublin:	16 DC
Galway:	38 GC
<u>Food isolates</u>	
Belfast:	591 BF
	1474 BF

	2325 BF
Dublin:	1373 DF
	1644 DF
	2265 DF
Galway:	2118 GF

The remaining 9/ 25 isolates analysed in this study were clinical and food *C. jejuni* which were not previously genotyped (Table 3.2).

Table 3.2 Untyped *C. jejuni* clinical and food isolates

<u>Untyped <i>C. jejuni</i> isolates</u>	
<u>Clinical isolates</u>	<u>Isolate I.D</u>
Belfast:	250 BC
Dublin:	2 DC
	18 DC
	26 DC
	28 DC
	180 DC
<u>Food isolates</u>	
Belfast:	1607 BF
	462 BF
Dublin:	248 DF

3.2 Analysis of glycoconjugates from *C. jejuni* isolates

This study was carried out to assess the level of LOS and CPS expression and diversity, which is an important virulence factor, amongst the group of isolates, and to compare the levels of expression in food and clinical isolates. Furthermore, closely related genotyped strains and un-typed isolates were compared for their production of these glycoconjugate molecules. In addition, the level of mimicry of gangliosides important in

GBS pathogenesis was examined in the isolates, comparing clinical and food isolates as well as genotyped and untyped isolates.

Isolates were cultured at 37°C and the glycoconjugates were extracted using the PK and MPW extraction methods. The PK extracts were subjected to SDS-PAGE analysis, followed by staining with silver or Alcian blue. Mini-phenol-water extracts were further analysed using TLC with chemical staining. Furthermore, mimicry of gangliosides GM₁ and asialo-GM₁ in the isolates was determined, by performance of immuno-staining with specific anti-sera and ligands.

3.2.1 SDS-PAGE analysis of *C. jejuni* glycoconjugates

Initially, PK extracts were prepared and separated using SDS-PAGE, with the Laemmli discontinuous buffer system, using glycine as the trailing ion. Silver staining of the separated extracts revealed a pattern of bands that migrated near the bottom of the gel (Fig. 3.1). This low-M_r banding is characteristic of LOS, composed of lipid A and core oligosaccharide only. No high-M_r banding was detected, indicating an absence of O-chain like LPS structures in these *C. jejuni* isolates. In comparison, a ladder-like banding pattern is observed in smooth-type *Salmonella* LPS (Fig. 3.1, lane 1), which contains a complete O-chain structure, with each band representing LPS molecules with a different number of the repeating O-chain units.

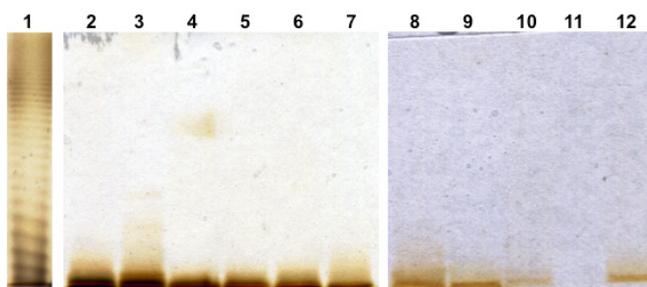


Figure. 3.1 Silver-stained glycine SDS-PAGE gels of representative proteinase-K extractions from *C. jejuni* isolates. Lanes: 1, *Salmonella* LPS as positive control and as glycolipid size comparison; 2, 592 BF; 3, 462 BF; 4, 1474 BF; 5, 1607 BF; 6, 2325 BF; 7, 1473 BF; 8, 2128 GF; 9, 2134 GF; 10, 1644 DF; 11; Sample buffer (negative control); 12, 2265 DF.

As there was an inability to discriminate between isolates using this method of SDS-PAGE, it was decided to use an SDS-PAGE system using tricine as the trailing ion. This system is designed to aid separation of protein molecules in the low- M_r region, but can equally be applied in the separation of low- M_r glycoconjugates. With tricine in the running buffer, and the addition of 6 M urea to the gel, separation of molecules in the 5 kDa range is possible. Furthermore, the tricine-SDS-PAGE system allows the use of protein markers to estimate the molecular-mass of the microbial glycolipids, unlike glycine-SDS-PAGE where protein markers cannot be accurately used to estimate molecular-masses of glycolipids. Tricine-SDS-PAGE had a greatly enhanced discriminatory power, enabling better comparison of the isolates. With tricine-SDS-PAGE clear differences were observed between the M_r of the LOS of many of the isolates, as well as heterogeneity within the samples, that was previously undetected (Fig. 3.2). Therefore, the decision was made to use the tricine-SDS-PAGE system, in all subsequent analyses of *C. jejuni* extracts.

Figures 3.2 – 3.4 with silver stained tricine-SDS-PAGE gels, demonstrate the heterogeneity that existed in the isolate LOS. Examination of the banding pattern revealed that only 32 BC (Fig. 3.4, lane 5) had a single band. A total of 20/ 25 (80%) of isolates had a doublet silver-stain banding pattern. This constituted 11/13 (84%) of food (Fig. 3.2: 2325 BF, 591 BF, 1474 BF and 1644 DF; Fig 3.3: 462 BF, 592 BF, 248 DF and 2118 GF; Fig. 3.4: 1450 GF; Fig 3.5: 1473 BF and 1373 BF) and 9/ 12 (75%) of

clinical (Fig 3.4: 16 DC, 18 DC, 180 DC and 301 BC; Fig 3.5: 250 BC, 300 BC, 260 BC, 26 DC and 28 DC) isolates. The doublet pattern consisted of a prominent upper band of apparent higher density, and a more faint band at a lower- M_r , potentially representing a truncated or altered structure. With most isolates the prominent band migrated at a similar distance, in comparison to the salmonella control LPS. However, certain isolates had a different staining pattern, for example the clinical isolate 16 DC (Fig 3.4, lane 2) and the food isolate 248 DF (Fig. 3.3, lane 6) had doublet-banding consisting of an upper band of an apparent lower M_r than all other isolates, with a very faint minor structure at a lower- M_r .

Even greater heterogeneity in the form of a triplet-banding pattern was seen in 16% of the isolates. This comprised 15.3% of food (1607 BF and 2265 DF: Figs. 3.2 and 3.3, lanes 6 and 3, respectively) and 15.3% of clinical (2 DC and 38 GC: Figs. 3.2 and 3.4, lanes 5 and 6, respectively) isolates. The genotyped isolate 38 GC (Fig. 3.4: lane 6) had a unique profile amongst the isolates, consisting of a triplet-banding pattern which consisted of two prominent bands, and a fainter band of lower- M_r . Unlike the other isolates which had one prominent band, the upper band of 38 GC was a similar apparent density to the middle, and had an apparently higher- M_r than any other band visualized amongst the isolates. The LOS banding patterns were distributed evenly amongst the sampling centres and type of food samples involved.

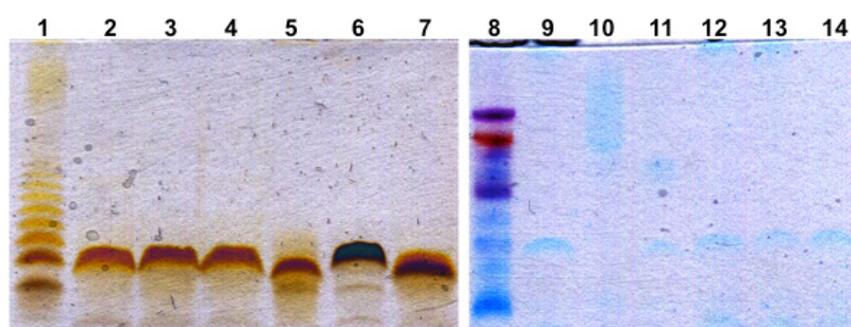


Figure 3.2 Tricine SDS-PAGE gels of representative proteinase-K extracts from *C. jejuni* isolates. Lanes 1-7, silver stained. Lanes 8-14, Alcian blue stained. Lanes: 1/ 8, *Salmonella*/ prestained protein molecular-mass marker (Biorad); 2 & 9, 2325 BF; 3 & 10, 591 BF; 4 & 11, 1474 BF; 5 & 12, 2 DC; 6 & 13, 1607 BF; 7 & 14, 1644 DF.

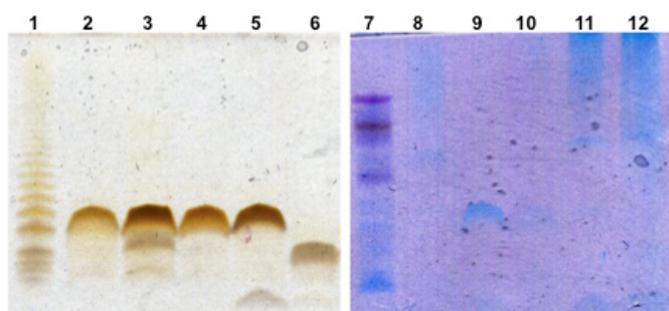


Figure 3.3 Tricine SDS-PAGE gels of representative proteinase-K extracts from *C. jejuni* isolates. Lanes 1-6, silver stained. Lanes 7-12, Alcian blue stained. Lanes: 1/ 7, *Salmonella*/ prestained protein molecular-mass marker (Biorad); 2 & 8, 462 BF; 3 & 9, 2265 DF; 4 & 10, 592 BF; 5 & 11, 2118 GF; 6 & 12, 248 DF.

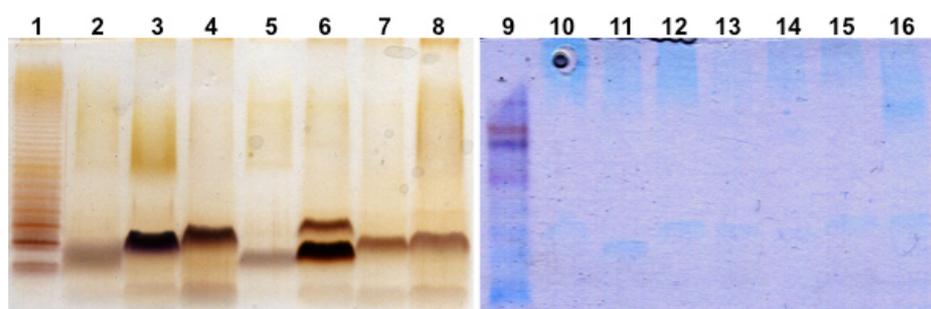


Figure 3.4 Tricine SDS-PAGE gels of representative proteinase-K extracts from *C. jejuni* isolates. A: silver stained. B: Alcian blue stained. Lanes A: 1/9, *Salmonella* LPS/ prestained protein molecular-mass marker (Biorad); 2 & 10, 16 DC; 3 & 11, 1450 GF; 4 & 12, 18 DC; 5 & 13, 32 DC; 6 & 14, 38 GC; 7 & 15, 301 BC; 8 & 16, 180 DC.

It was revealed that none of the typed isolates had a single band, while 85.7% had a doublet and 14.3% had a triplet. The LOS phenotype of genetically related strains was found to be identical. The genetically indistinguishable isolates 1473 BF and 260 BC (Fig 3.5: lanes 5 and 7, respectively), which were grouped in cluster J3 (Table 3.1), had an identical profile consisting of a doublet.

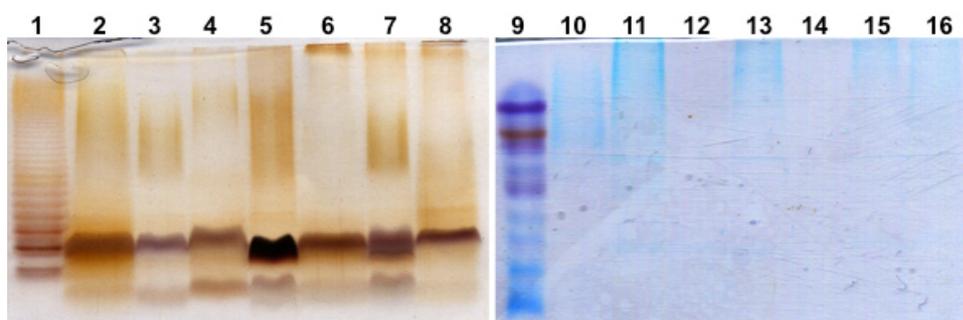


Figure 3.5 Tricine SDS-PAGE gels of representative proteinase-K extracts from *C. jejuni* isolates. Lanes 1-7, silver stained. Lanes 8-14, Alcian blue stained. Lanes: 1/ 9, *Salmonella*/ prestained protein molecular-mass marker (Biorad); 2 & 10, 250 BC; 3 & 11, 26 DC; 4 & 12, 28 DC; 5 & 13, 1473 BF; 6 & 14, 1373 DF; 7 & 15, 260 BC; 8 & 16, 300 BC.

Furthermore, the isolates 592 BF and 1450 GF, which were grouped in cluster J7, and had an identical SDS-PAGE profile, although isolated from different sampling centres, demonstrating that this LOS phenotype is stable, and well distributed geographically. The strains 591 BF and 592 BF (Fig. 3.2, lane 3 and Fig. 3.3, lane 4, respectively), which shared 84% similarity, had a similar profile consisting of a doublet with a prominent upper band and very faint lower- M_r band. Furthermore, isolates 1644 DF and 1373 DF (Fig 3.2, lane 7 and Fig 3.5, lane 6, respectively), which shared 72% similarity, had a doublet profile consisting of a prominent upper band and very faint lower- M_r band. Analysis of the untypeable strains reveals that they had a range of SDS-PAGE profile also, one had a single band (32 BC: Fig. 3.4, lane 5), 72.7% had a doublet and 18.9% had a triplet.

In order to detect capsular polysaccharide production in the *C. jejuni* isolates, SDS-PAGE and staining with the cationic dye Alcian blue was carried out. Capsular polysaccharide production by *C. jejuni* is a virulence factor involved in host colonisation and infection, as well as environmental survival. Alcian blue reacted with 20/ 25 (80%) isolates extracts examined, in the mid- to high- M_r region, which would indicate the production of capsular polysaccharide by these isolates. Some faint staining in the low- M_r region of many isolates would indicate cross-reaction of the stain with LOS. A total of 10/12 (83.3%) clinical isolates and 10/13 (76.9%) food isolates reacted

with Alcian blue. Of the food isolates 46.1% of the chicken isolates reacted with Alcian blue in the mid-high- M_r region, as did strain 462 BF (Fig. 3.3: lane 8) isolated from duck leg, strain 1474 BF from beef mince, and strain 2118 GF (Fig. 3.3 lane 11) isolated from cow liver.

Overall, 11/ 16 (68.7%) of the genotyped strains reacted with Alcian blue in the mid-high M_r region, while 6/ 9 (66.6%) of the untypeable strains had a reaction indicating the presence of CPS. The isolates 1473 BF and 260 BC (Fig. 3.5, lanes 13 and 15, respectively), which were grouped in cluster J3 (Table 3.1), had identical Alcian blue staining. This highlights that the ability to express CPS was not effected by environmental storage (1473 BF) or passage *in vivo* (260 BC). In comparison, the isolates 592 BF (Fig. 3.3, lane 10) and 1450 GF (Fig. 3.4, lane 11, which grouped in cluster J7 (Table 3.1), had different Alcian blue profiles. Despite being genetically indistinguishable only 1450 GF reacted with Alcian blue in the mid-high- M_r region. Furthermore, with the isolates 591 BF and 592 BF, which shared 84% similarity, only isolate 591 BF (Fig. 3.2, lane 10) had staining in the high- M_r region, suggesting the presence of CPS. Overall, 5/ 25 (20%) strains did not react with Alcian blue in the mid-high M_r region. This included 4 food strains, 1644 DF (Fig. 3.2, lane 14), 592 BF, 2265 DF (Fig. 3.3, lanes 9 and 10, respectively), and 1373 DF (Fig. 3.5, lanes 14), as well as a 1 clinical strain 28 DC (Fig. 3.5, lane 12).

3.2.1.1 Summary of silver and Alcian blue staining

A range of silver stain tricine-SDS-PAGE profiles was observed in the food and clinical isolates consisting of single, doublet or triplet low- M_r bands. The majority (80%) of isolates reacted with Alcian blue stain in the mid-high M_r , which would suggest the presence of CPS. Closely related strains 1473 BF and 260 BC (95% similarity) had identical silver and Alcian blue staining profiles, demonstrating genotypic and phenotypic homogeneity. A stable geno/phenotype was also seen in strains 1450 GF and 591 BF, which shared 92% similarity and, had a doublet silver stain profile, as well Alcian blue staining. However, the strain 592 BF that was closely related to both 1450 GF and 591 BF did not have any Alcian blue staining.

3.2.2 Thin-layer chromatography with chemical staining

Due to the presence of highly charged chemicals such as SDS in the PK samples, they are unsuitable for TLC analysis. To complement SDS-PAGE analysis of *C. jejuni* isolates a MPW extraction procedure was employed and combined with TLC, followed by chemical staining. Mini phenol-water extraction, although more time consuming to carry out than PK extraction, resulted in a sample of higher purity that could be analysed by TLC.

C. jejuni MPW extracts (8 µg LOS) were developed in a TLC solvent system consisting of *n*-propanol/ water/ 25% NH₄OH (60:30:10 [vol/vol/vol]). Ganglioside preparations (4 µg) were developed using a solvent system of chloroform/ methanol/ 0.22% CaCl₂.2H₂O (50:45:10 [vol/ vol/ vol]). TLC separated glycolipids were visualized with either of two carbohydrate-specific stains, diphenylamine-aniline and resorcinol-HCl stains. It was observed that TLC separated gangliosides when stained with resorcinol-HCl, stained purple, whereas non-sialylated gangliosides stained yellow/brown (Fig. 3.6).

A comparison of chemical staining of *C. jejuni* extracts with either diphenylamine-alanine or resorcinol-HCL found that both stains detected the presence of glycolipids, with identical retention factor (R_f) values (Fig. 3.7- Fig. 3.9). Although diphenylamine-alanine stained the *C. jejuni* glycolipids adequately, due to the ability of resorcinol-HCL to discriminate between sialylated and non-sialylated carbohydrates it may provide more information regarding the nature of individual bands on TLC chromatographs of *C. jejuni* glycolipids.

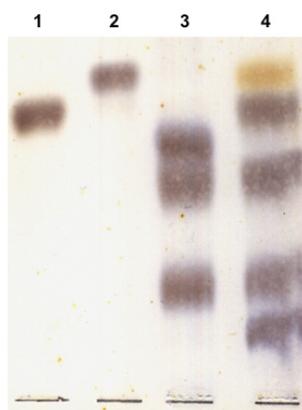


Figure 3.6 Resorcinol-HCL staining of gangliosides. Lanes: 1, GM₁ (sigma); 2, GM₂ (sigma); 3, disialo ganglioside mix containing, from bottom to top, GD_{1b}, GD_{1a}, GD₃; 4, ganglioside mix containing, from bottom to top, GT_{1a}, GD_{1b}, GD_{1a}, GM₁, asialo-GM₁.

A comparison of chemical staining of *C. jejuni* extracts with either diphenylamine-alanine or resorcinol-HCL found that both stains detected the presence of glycolipids, with identical retention factor (R_f) values (Fig. 3.7- Fig. 3.9). Although diphenylamine-alanine stained the *C. jejuni* glycolipids adequately, due to the ability of resorcinol-HCL to discriminate between sialylated and non-sialylated carbohydrates it may provide more information regarding the nature of individual bands on TLC chromatographs of *C. jejuni* glycolipids.

Chemical staining of *C. jejuni* extracts revealed differences between isolates, which represents differences in the size and composition of the glycolipids extracted. The presence of a band with an R_f value of ~ 0.3 , was common to all 100% isolates tested. A second band developing further up the TLC plate, with an R_f value of ~ 0.45 , was detected in 18/ 25 (72%) of the isolates. A third band with an R_f value of ~ 0.2 was detected in 6/ 25 (24%) of the isolates, while the isolate 1607 BF had two bands one with an R_f value of 0.52, and the other 0.55 (Fig 3.7: lane 10), and the isolate 18 DC has a diffuse band ranging from R_f of 0.5- 0.6 (Fig. 3.8: lane 11). An R_f value of 0.36 was previously determined to represent LOS (Prendergast *et al.*, 2004). However, in this study with many cases with resorcinol-HCL staining represented in figure 3.7 (A), and faintly in figures 3.8 (B) and 3.9 (B) the lower band stained yellow-brown, whereas the higher band stained purple, indicating the presence of sialic acid in

the higher band only. This may represent a sialylated LOS molecule that is truncated, which accounts for its altered mobility on the TLC chromatograph. The presence of multiple bands in the extracts indicates the existence of a range of molecular species or glycoconjugate forms produced by the isolates. The single *C. coli* strain included in the study had a band with an R_f value of 0.3 and a second band with an R_f value of 0.48.

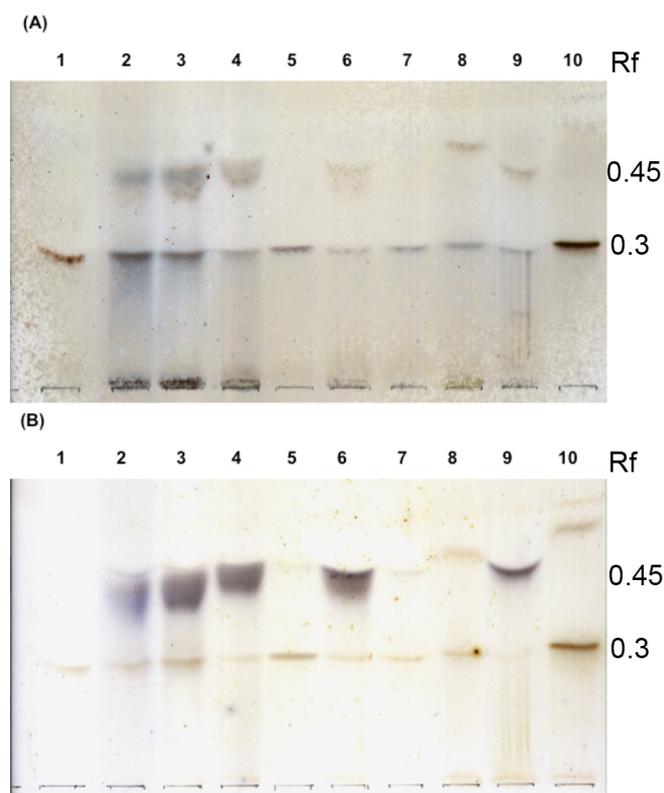


Figure 3.7 Chemical-stained TLC plates of glycolipids from *C. jejuni* isolates. (A) Diphenylamine-aniline stained. (B) Resorcinol-HCL stained. Lanes: 1, 26 DC; 2, 2 DC; 3, 28 DC; 4, 1473 BF; 5, 1474 BF; 6, 260 BC; 7, 1450 GF; 8, 2134 GF; 9, 300 BC; 10, 1607 BF.

In consideration of the food isolates, 3/ 12 (25%) had a band with an R_f value of ~ 0.25 and 13/ 13 (100%) had a band with an R_f value of ~ 0.30 . Similarly, 25% of the clinical isolates had a band with an R_f value of ~ 0.25 , whereas 12/ 12 (100%) had a band with an R_f value of ~ 0.3 .

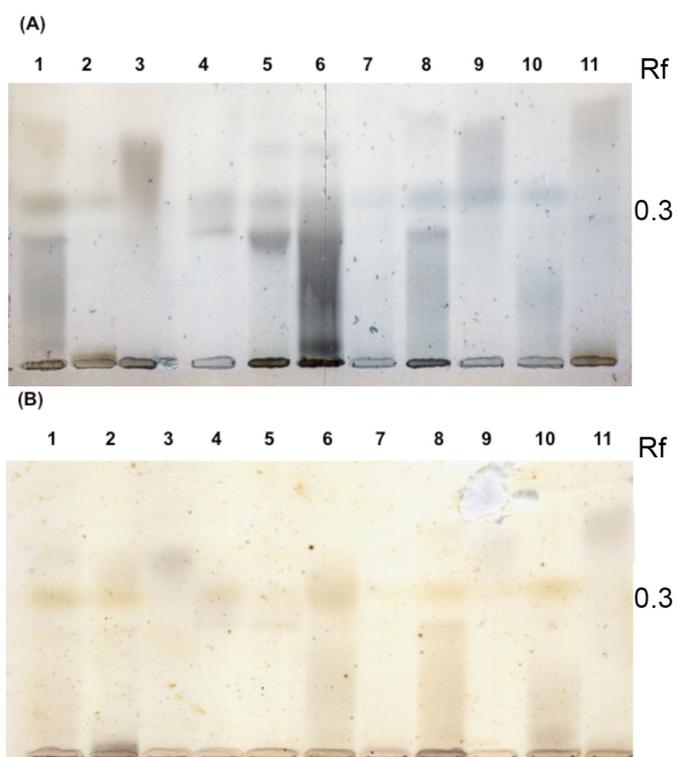


Figure 3.8 Chemical-stained TLC plates of glycolipids from *C. jejuni* isolates. (A) Diphenylamine-aniline stained. (B) Resorcinol-HCL stained. Lanes: 1, 250 BC; 2, 462 BF; 3, 32 DC; 4, 16 DC; 5, 2265 DF; 6, 248 DF; 7, 1373 DF; 8, 591 BF; 9, 2325 BF; 10, 1644 DF; 11, 18 DC.

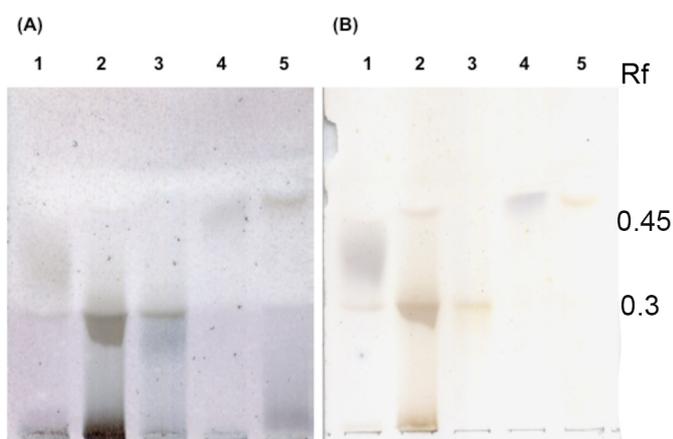


Figure 3.9 Chemical stained TLC plates with glycolipids from *C. jejuni* isolates. (A) diphenylamine-aniline stained. (B) resorcinol-HCL stained. Lanes: 1, 38 GC; 2, 592 BF; 3, 2118 GF; 4, 301 GC; 5, 180 DC.

Analysis of the genotyped isolates revealed that in addition to the ubiquitous band at R_f 0.3, 4/ 16 (25%) had a band with R_f of \sim 0.2, and 12/ 16 had a band with R_f of \sim 0.5. Examination of the closely related isolates 260 BC and 1473 BF, which were grouped in cluster J3 (Table 3.1), revealed they shared an identical TLC profile, with two bands (R_f values of 0.3 and 0.5). The isolates 592 BF and 1450 GF which grouped in cluster J7 also had identical TLC staining with a band each with R_f values of 0.3 and 0.45. However, the isolates 591 BF and 592 BF, which shared 84% similarity, had slightly different profiles. Both isolates had banding with R_f values of 0.3 and 0.5, while only isolate 591 BF had an additional band at R_f 0.25.

Analysis of the genotyped isolates revealed that in addition to the ubiquitous band at R_f 0.3, 4/ 16 (25%) had a band with R_f of \sim 0.2, and 12/ 16 had a band with R_f of \sim 0.5. Examination of the closely related isolates 260 BC and 1473 BF, which were grouped in cluster J3 (Table 3.1), revealed they shared an identical TLC profile, with two bands (R_f values of 0.3 and 0.5). The isolates 592 BF and 1450 GF which grouped in cluster J7 also had identical TLC staining with a band each with R_f values of 0.3 and 0.45. However, the isolates 591 BF and 592 BF, which shared 84% similarity, had slightly different profiles. Both isolates had banding with R_f values of 0.3 and 0.5, while only isolate 591 BF had an additional band with an R_f value of 0.25.

3.2.3 Comparison of TLC chemical staining and SDS-PAGE profiles

Prendergast *et al.* (2004) suggested that a TLC R_f value of 0.36 represented *C. jejuni* LOS. In this study all of the isolates examined has a band with an R_f value of \sim 0.3, which would correlate with the presence of LOS in these samples. Therefore, the additional TLC bands present in 84% of the isolates tested, potentially represented truncated LOS molecules, or other glycoconjugates such as CPS. In total, 80% of isolates had a doublet silver stain banding pattern, and 62.5% of the isolates had a second TLC band with an R_f of \sim 0.4. In many cases this second upper TLC band stained purple with resorcinol-HCL (Fig. 3.7, B), so sialylated structures are present at these R_f values, indicating the bands represent LOS molecules. Isolate 1607 BF had a unique TLC profile (Fig. 3.7, lane 10) consisting of a band with an R_f value of 0.3, and

unlike any of the other isolates two higher bands at an R_f value of 0.55 and 0.6. The silver stain profile of 1607 BF was also unique with a triplet-banding pattern (Fig 3.2, lane 6). The other isolate with a silver stain triplet-banding pattern, 2265 DF, also had a triplet of TLC bands at R_f values of 0.24, 0.32 and 0.44 (Fig. 3.8, lane 5).

3.2.4 TLC with immuno-overlay

To determine the level of ganglioside mimicry in the *C. jejuni* isolates, immuno-overlay of TLC plates with antisera and ligands specific for human gangliosides, was carried out. Extracts separated by TLC, were probed with anti-sera specific for the gangliosides GM₁ and asialo-GM₁. Extracts were also tested for reactivity with Hrp-conjugated cholera toxin B-subunit (CTB) a ligand specific for ganglioside GM₁, as well as PNA, a lectin from *Arachis hypogaea*, specific for the asialo-GM₁ structure, Gal β (1,3)GalNAc. A correlation between anti-GM₁ antibodies in GBS, and preceding *C. jejuni* infection has frequently been reported (Jacobs *et al.*, 1998; Moran & Prendergast 2001; Yuki 2007). Approximately half of patients with preceding *C. jejuni* infection have anti-GM₁ reactivity (Moran 2010). However, there are also reports of anti-ganglioside antibodies in *C. jejuni* infected GBS patients reactive against gangliosides other than GM₁ including asialo-GM₁ (Ogawa *et al.* 2009)

3.2.4.1 GM₁ mimicry

Of the 25 isolates examined, 14/ 25 (56%) reacted with anti-GM₁ antibody (Fig. 3.10). The reactive band had an R_f value of ~0.3 in 11/ 14 isolates that reacted with anti-GM₁ antibody. However, isolates 26 DC, 2 DC and 28 DC (Fig. 3.10, lanes 1, 2 and 3) had two reactive bands each for anti-GM₁ overlay, with R_f values of 0.3 and 0.45. Interestingly, the isolate 18 DC (Fig. 3.10, lane 21) had a reactive band with an R_f value of 0.25 and some faint reaction with an R_f value of 0.5. In total, 8/ 12 (66.6%) of the clinical isolates reacted with anti-GM₁ antibody, whereas only 6/ 13 (46%) food isolates reacted. Therefore, there was higher frequency of GM₁ mimicry in clinical isolates than food isolates, suggesting that the source of infection in certain clinical cases was not food.

It was observed that 8/ 16 (50%) genotyped isolates reacted with anti-GM₁ antibody, compared to 5/ 13 (66.6%) of untyped isolate. Both 260 BC and 1473 BF

(Fig. 3.10, lanes 6 and 9, respectively) which were grouped in cluster J3, reacted with anti-GM₁. However, the reactive band with 260 BC had an R_f of 0.3, compared to 0.4 for 1473 BF. Neither isolate 592 BF or 1450 GF (Fig. 3.10, lanes 7 and 18, respectively) grouped in from Cluster J7, reacted with anti-GM₁ antibody. The isolates 592 BF and 591 BF which shares 84% similarity had a different reaction with anti-GM₁, with only 591 BF reacting with the antibody (Fig. 2.10, lane 24). Neither isolate, 1373 DF or 1644 DF (Fig. 3.10, lanes 17 and 20, respectively) which shared 72% similarity reacted with anti-GM₁ antibody.

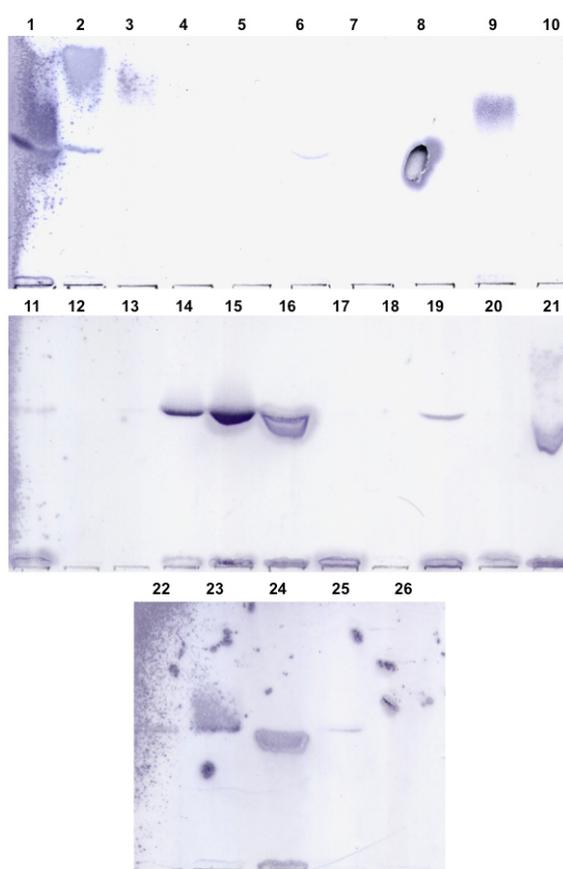


Figure 3.10 TLC plates with immunostaining using anti-GM₁ antibodies of glycolipid extracts from *C. jejuni* isolates. Lanes:1, 26 DC; 2, 2 DC; 3, 28 DC; 4, 180 DC; 5, 301 BC; 6, 260 BC; 7, 1450 GF; 8, 300 BC; 9, 1473 BF; 10, 1607 BF; 11, 250 BC; 12, 462 BF; 13, 32 BC; 14, 16 DC; 15, 2265 DF; 16, 248 BF; 17, 1373 DF; 18, 592 BF; 19, 2325 BF; 20, 1644 DF; 21, 18 DC; 22, 1474 BF; 23, 38 GC; 24, 591 BF; 25, 2118 GF; 26; 2134 GF.

Cholera toxin B-subunit, which reacts with GM₁, reacted with 28% (7/ 25) of the isolates (Fig. 3.11). Six of the isolates that reacted with CTB had reactive band with R_f values of 0.4, whereas 38 GC and 2118 GF (Fig 3.11, lanes 22 and 24) had diffuse bands with R_f values of 0.3-0.45. The isolates 2265 DF and 18 DC (Fig. 3.11, lanes 15 and 21) had reactive bands with R_f values of 0.3 and 0.5, respectively. In total, 5/ 25 (41.6%) of the clinical isolates reacted with CTB, whereas 2/ 13 (15.3%) of the food isolates reacted.

It was observed that 4/ 16 (25%) genotyped isolates reacted with CTB whereas 3/ 16 (18.7%) of the untyped isolates reacted. Both of the indistinguishable isolates 260 BC and 1473 BF (Fig 3.11, lanes 6 and 9, respectively), which were grouped in Cluster J3, reacted with CTB. Neither of the indistinguishable isolates 1450 GF or 592 BF (Fig. 3.11, lanes 7 and 18, respectively, which grouped in cluster J7 reacted with CTB. The isolates 592 BF and 591 BF which shares 84% similarity had a different reaction with CTB, with only 591 BF reacting with the antibody (Fig. 3.11, lane 23). Neither isolate, 1373 DF or 1644 DF (Fig. 3.11, lanes 17 and 20, respectively) which shared 72% similarity reacted with anti-GM₁ antibody.

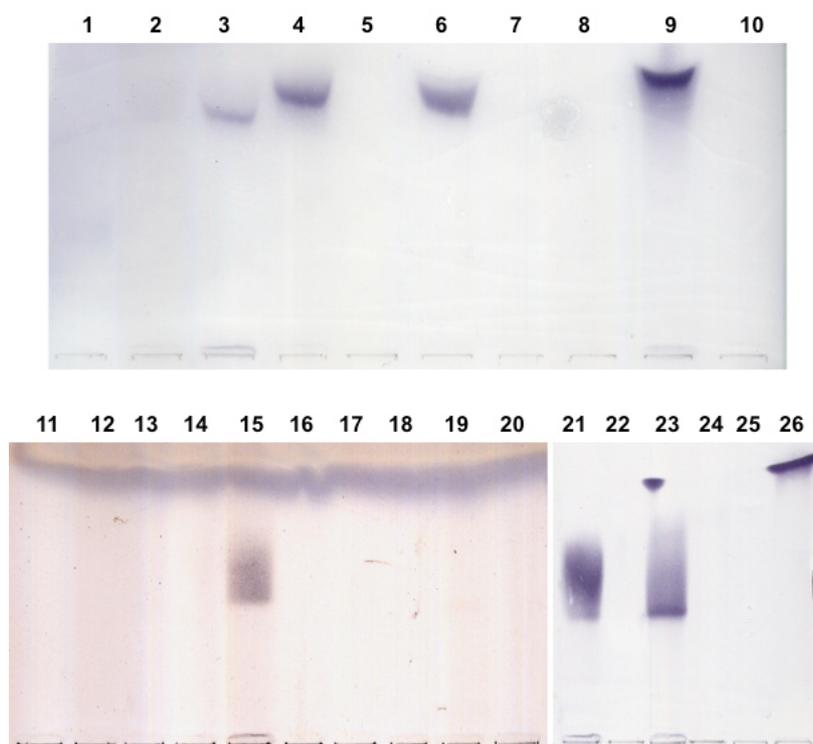


Figure 3.11 Hrp-conjugated cholera toxin-B subunit staining of TLC plates of glycolipid extracts from *C. jejuni* isolates. Lanes: 1, 26 DC; 2, 2 DC; 3, 28 DC; 4, 180 DC; 5, 301 BC; 6, 260 BC; 7, 1450 GF; 8, 300 BC; 9, 1473 BF; 10, 1607 BF; 11, 250 BC; 12, 462 BF; 13, 32 BC; 14, 16 DC; 15, 2265 DF; 16, 248 BF; 17, 1373 DF; 18, 592 BF; 19, 2325 BF; 20, 1644 DF; 21, 18 DC; 22, 38 GC; 23, 591 BF; 24, 2118 GF; 25, 1474 BF; 26, positive control (ganglioside GM₁).

Reaction with both GM₁ ligands was seen in 6/ 25 (24%) of the isolates (260 BC, 1473 BF, 591 BF, 2265 DF, 18 DC and 28 DC) which reacted with both anti-GM₁ antibody and CTB. Furthermore, 7/ 14 (50%) of isolates that reacted with anti-GM₁ did not react with CTB (16 DC, 38 GC, 250 BC, 248 DF, 2325 BF, 26 DC and 2 DC, Tables. 3.2 and 3.3). Only 1 of the isolates (180 DC, Table 3.3) which reacted with CTB, did not react with anti-GM₁. These differences in recognition by GM₁ ligands may be explained by differences in the conformation or presentation of the epitopes recognised by either ligand. Cholera toxin-B subunit is known to have high affinity for GM₁, but may bind other related ganglioside structures (Sack *et al.*, 1998), while the anti-

GM₁ antibody used is polyclonal and may react with a number of discreet epitopes on a GM₁ ganglioside.

3.2.4.2 Asialo-GM₁ mimicry

Anti-asialo-GM₁ antibody reacted with 44% (11/ 25) of the isolates (Fig 3.12). The reactions observed were all of low R_f value ranging from 0.05 (Fig. 3.12, lane 9) to 0.2 (Fig 3.12, lane 20) and often diffuse (Fig. 3.12, lane 24). This low diffuse band reactive with asialo-GM₁ antibody was visualized previously with chemical staining, for example 300 BC and 248 DF (Figs. 2.7 and 3.8, lanes 9 and 6, respectively), and may be a minor structure produced. Asialo-GM₁ is truncated form of GM₁ lacking the sialic acid residue, which can be formed due to phase variation in sialic acid transferase. Conversely, the reactivity of two (32 BC, 301 BC) of the isolates with anti-asialo-GM₁ separately from any of the anti-GM₁ ligands indicates asialo-GM₁ structures can be expressed as an independantly to GM₁, as well as in a heterogeneous sample with GM₁-mimicking LOS. It was observed that 7/ 11 (63.6%) of the isolates reactive with anti-asialo-GM₁ (16 DC, 248 BF, 591 BF, 2265 DF, 2118 GF and 2325 BF), were also reactive with either or both of of the GM₁ ligands, CTB and anti-GM₁. The isolates 2265 DF and 591 BFF reacted with all three ligands (anti-GM₁, anti-asialo-GM₁, and CTB), suggesting the presence in these isolates of asialo-GM₁ and GM_{1a} mimicry No isolate reacted with only anti-asialo-GM₁ and CTB.

In total, 6/13 (46.1%) of the food isolates and 5/ 12 (41.6%) of clinical isolates were reactive with anti-asialo-GM₁. A total of 6/11 (54.5%) of asialo-GM₁ reactive isolates were from food sources (2265 DF, 248 DF, 2325 BF, 591 BF, 1473 BF, 2118 GF) and 44.5% of these were clinical (32 BC, 300 BC, 301 BC, 16 DC, 38 GC), so this form of mimicry was equally distributed amongst food and clinical isolates..

It was observed that 10/ 16 (62.5%) of the genotyped isolates reacted with asialo-GM₁, whereas only 1/ 9 (11%) of the untyped isolates reacted. Regarding the indistinguishable isolates 260 BC and 1473 BF which were grouped in Cluster J3, and the isolates 592 BF and 1450 GF, grouped in Cluster J7, no reaction with asialo-GM₁ was observed. The isolate 591 BF reacted with asialo-GM₁, whereas the closely related 592 BF (84% similarity) didnt react. Neither isolate 1373 DF or 1644 DF (Fig. 3.11,

lanes 17 and 20, respectively), which shared 72% similarity, reacted with anti-GM₁ antibody.

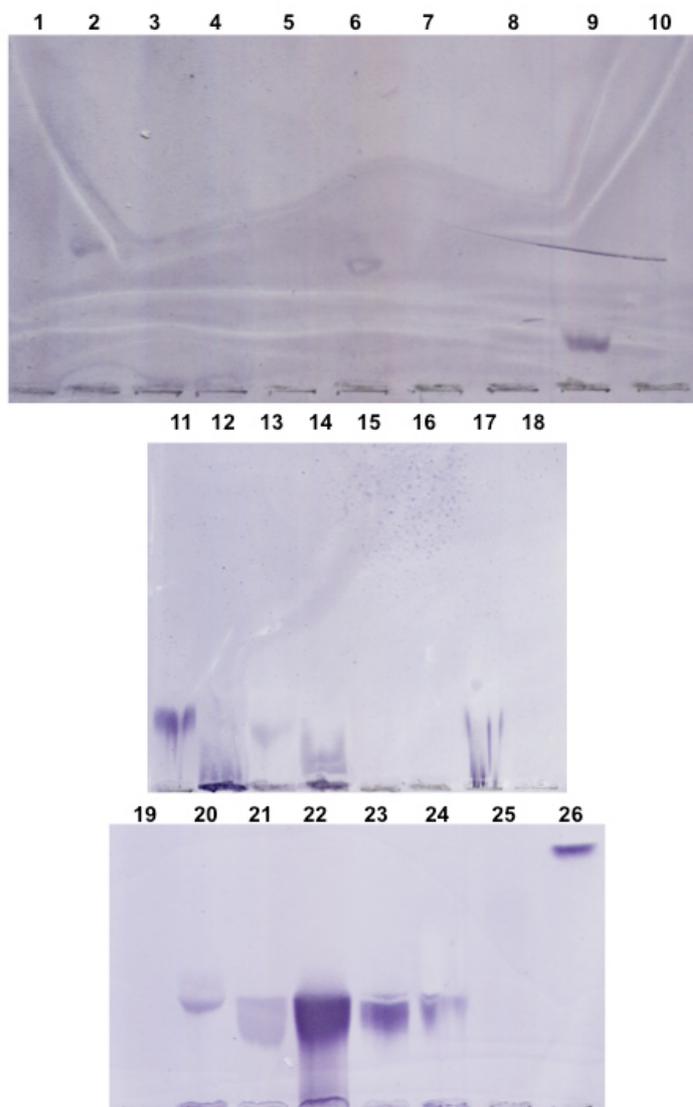


Figure 3.12 TLC plates with immunostaining using anti-asialo GM₁ antibodies of glycolipid extracts from *C. jejuni* isolates. Lanes: 1, 26 DC; 2, 2 DC; 3, 28 DC; 4, 180 DC; 5, 1644 DF; 6, 260 BC; 7, 1450 GF; 8, 1473 BF; 9, 300 BC; 10, 1607 BF; 11, 32 BC; 12, 16 DC; 13, 2265 DF; 14, 248 DF; 15, 462 BF; 16, 1373 DF; 17, 2325 BF; 18, 592 BF; 19, 250 BC; 20, 38 GC; 21, 591 BF; 22, 2118 GF; 23, 301 BC, 24, 1474 BF; 25, 18 DC; 26, positive control (ganglioside asialo-GM₁).

Due to sample depletion, only 17 of the isolates were probed with PNA, specific for the asialo-GM₁ structure, Gal β (1-3)GalNAc, and of these only 2 (11.8%) reacted (Fig. 3.13). This included the isolates 26 DC (Fig. 3.13, lane 1) which reacted with both anti-asialo-GM₁ and PNA, and 2118 GF (Fig. 3.13, lane 14), which reacted with PNA only. However, it should be noted that there was no reaction between PNA and the positive control, which consisted of ganglioside asialo-GM₁. Plant lectins may react differently with non-plant carbohydrates and can only be used as a guide to the exact sugar moieties present.

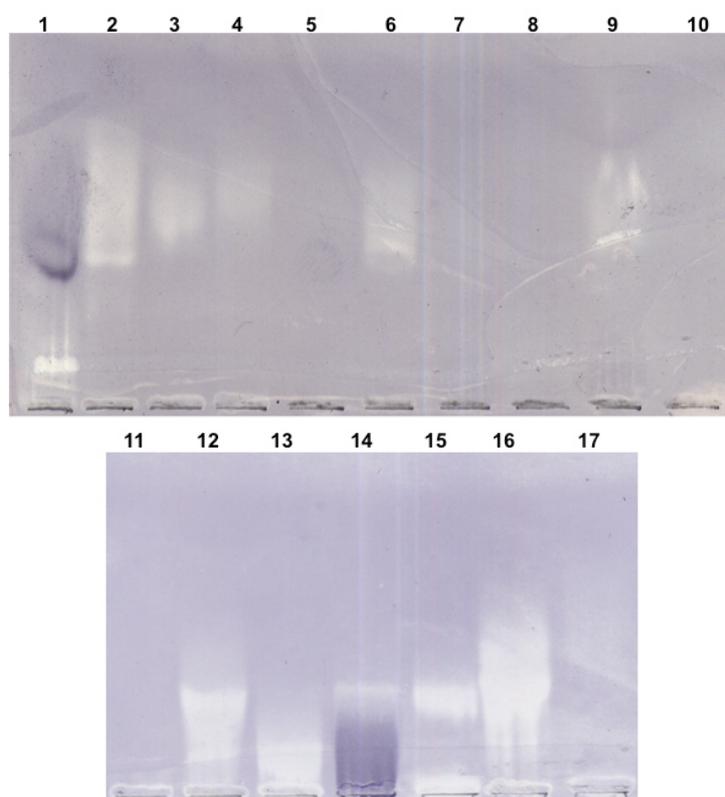


Figure 3.13 Hrp-conjugated lectin from *Arachis hypogaea* (peanut agglutinin, PNA) staining of TLC plates of glycolipid extracts from *C. jejuni* isolates. Lanes: 1, 26 DC; 2, 2 DC; 3, 28 DC; 4, 180 DC; 5, 31 BC; 6, 250 BC; 7, 1450 GF; 8, 2134 GF; 9, 300 BC; 10, 1607 BF; 11, 1474 BF; 12, 38 GC; 13, 591 BF; 14, 2118 GF; 15, 1473 BF; 16, 301; 17, 1373 DF.

3.2.4.3 Summary of TLC with immuno-overlay

The results of the TLC with immuno-overlay, summarized in tables 3.2 and 3.3 show that a total of 56% of the isolates reacted with anti-GM₁ antibody, and 28% reacted with other GM₁ ligand, CTB. A comparison of clinical and food isolates revealed a greater amount of GM₁ mimicry in clinical isolates, which reacted with both GM₁ ligands (anti-GM₁ antibody and CTB), at higher rates than food isolates. This may indicate that the infection source of some clinical cases was not food. Furthermore, 11 of the isolates reacted with anti-asialo-GM₁ at lower R_f values than the reactions with the GM₁ ligands. It was observed that asialo-GM₁ structures are produced as truncated version of GM₁ in heterogeneous LOS, or as independent LOS forms without GM₁.

It was observed that 7/11 of the isolates reactive with anti-GM₁ antibody were not reactive with CTB, and that only one isolates (180 DC) reacted with CTB only. This highlights the differences in fine specificity of the reaction the GM₁ ligands, and indicates the value of using multiple ligands for detection of ganglioside mimics. CTB may react with other gangliosides to GM₁ (Sack *et al.*, 1998). Furthermore, the fine specificity of the antibody used is unknown, and was potentially reactive with either or both of GM_{1a} and GM_{1b}, or may, due to its polyclonality, recognise various epitopes on the LOS molecule, accounting for the differences observed. Therefore, potentially CTB-reactive LOS could stimulate GM₁ reactive anti-ganglioside antibodies, and the isolate 180 DC that reacted with CTB only may still be neuropathic, despite non-reactivity with polyclonal anti-GM₁ antibody. Alternatively, isolate 180 DC expressed mimicry of different ganglioside. Furthermore, one strain (26 DC) reacted with the asialo-GM₁ ligand PNA, but not anti-asialo-GM₁ antibody.

The indistinguishable isolates which were clustered, 260 BC and 1473 BF (Cluster J3) and 592 BF and 1450 GF (Cluster J7), had identical reaction patterns with all of the anti-ganglioside ligands. Furthermore, the isolates 1373 DF and 1644 DF which had 72% similarity had identical reaction patterns. However, the isolated 591 BF and 592 BF, which had 84% similarity had different reaction patterns, with isolate 591 BF having reactivity with anti-GM₁ antibody, CTB and asialo-GM₁.

Overall 12/ 25 (48%) of the isolates reacted with one or more anti-ganglioside ligand, an indication of the heterogeneity which exists in the structure of *C. jejuni* LOS. It was observed that 6/ 25 (24%) of the isolates did not react with any of the ligands

tested, and all of these were food isolates (592 BF, 462 BF, 1450 GF, 1607 BF, 1374 DF and 1644 DF).

Table 3.3 Results of TLC with immuno-overlay carried out on *C. jejuni* isolates genotyped by PFGE.

PFGE-typed <i>C. jejuni</i>					
<u>Isolate Group</u>		<u>Anti-ganglioside antibody/ ligand</u>			
<u>PFGE Clusters</u>		<u>Anti GM₁</u>	<u>CTB</u>	<u>Anti-asialo GM₁</u>	<u>PNA</u>
J3:	260 BC	+	+++	-	-
	1473 BF	+++	++++	-	-
J7:	592 BF	-	-	-	-
	1450 GF	-	-	-	-
<u>Clinical</u>					
Belfast:	32 BC	-	-	+++	nd
	300 BC	-	-	+++	-
	301 BC	-	-	+++	-
Dublin:	16 DC	++++	-	++	nd
Galway:	38 GC	+++	-	+++	-
<u>Food isolates</u>					
Belfast:	591 BF	+++	++++	+++	-
	1474 BF	-	-	++	-
	2325 BF	++	-	++	nd
Dublin:	1374 DF	-	-	-	nd
	1644 DF	-	-	-	nd
	2265 DF	++++	+++	++	nd
Galway:	2118 GF	+	-	++++	+++

Table 3.4 Results of TLC with immuno-overlay carried out untyped *C. jejuni* isolates.

<u>Untyped <i>C. jejuni</i> isolates</u>					
<u>Anti-ganglioside antibody/ ligand</u>					
<u>Clinical isolates</u>	<u>Anti-GM₁</u>	<u>CTB</u>	<u>Anti-asialo GM₁</u>	<u>PNA</u>	
Belfast: 250 BC	+	-	-		nd
Dublin: 2 DC	+++	-			
18 DC	+++	++++	-		nd
26 DC	+++	-	-		+++
28 DC	+	++	-		-
180 DC	-	+++	-		-
<u>Food isolates</u>					
Belfast: 1607 BF	-	-	-		-
462 BF	-	-	-		nd
Dublin: 248 DF	++++	-	++		nd

3.2.5 Summary of isolate characterisation

This study was carried out in to characterise the level of glycoconjugate production (an important virulence factor) by a collection of 25 genotyped and non-genotyped *C. jejuni* isolates, from clinical and retail sources, from the island of Ireland. The isolates were also screened for ganglioside-mimicry, a predisposing factor in the development of the neurological disorder GBS.

Nine isolates had two or more SDS-PAGE silver stain bands, and combined reactivity with anti-GM₁ antibody/ CTB as well as anti-asialo-GM₁ / PNA including 2265 DF, 2325 BF, 591 BF, 2118 GF, 1473 BF, 16 DC, 26 DC, 38 GC and 301 BC. Since asialo-GM₁ is a truncated, lower-M_r form of GM₁ they would be expected to migrate at different R_f in the SDS-PAGE gel. In all cases the upper band in SDS-PAGE was apparently the most dense, therefore, these isolates may produce predominantly

GM₁ mimicking LOS as well as a lower-M_r asialo-GM₁ structure. Three silver stain bands as well as GM₁ and asialo-GM₁ mimicry were observed in the isolates 2265 DF and 38 GC. The third LOS band may represent a further truncated structure which does not mimic either of the ganglioside structures examined for in this study.

Collectively, the isolates represented a heterogenous group with multiple SDS-PAGE and TLC profiles and a wide ranging interactions with anti-ganglioside ligand. In the genotyped isolates, this phenotypic diversity reflected the genetic heterogeneity observed with PFGE. The isolates which were grouped in clusters were genetically and phenotypically indistinguishable. This included isolates 260 BC and 1473 BF which grouped in cluster J3, and isolates 592 BF and 1450 GF which grouped in cluster J7. However, isolates 591 BF and 592 BF which shared 84% similarity had different SDS-PAGE and TLC profiles, and different reaction patterns with anti-ganglioside ligands, despite a high degree of genetic relatedness. It would be rewarding to examine in future a greater cohort of genotypically similar strains, in order to investigate this phenomenon.

3.3 Discussion

Phenotypic diversity between *C. jejuni* isolates has been observed for almost every characteristic identified as having a role in pathogenicity, including LOS and CPS (Wassenaar & Blaser, 1999). This study was carried out in order to assess the level of phenotypic diversity in LOS and CPS in a group of *C. jejuni* clinical and food isolates, in addition to determining the level of LOS ganglioside mimicry. SDS-PAGE analysis of *C. jejuni* isolate extracts revealed a variety of silver stain profiles with differences in molecular-masses of silver stained components apparent, representing the diversity of LOS structures that can be produced by *C. jejuni*. The gene content of LOS biosynthesis loci varies between strains, and is responsible for variations in LOS structure (Gilbert & Wakarchuk, 2001). A majority of the isolates in this study (24/ 25) had multiple silver stained bands of different apparent molecular-masses. Studies have shown that these represent distinct LOS glycoforms, which are variably expressed due to phase variation of LOS biosynthesis genes (Guerry *et al.*, 2002). Phase-variation of ganglioside-mimicking LOS has been observed *in vitro* (Guerry *et al.*, 2002) and *in vivo*

(Prendergast *et al.*, 2004), related to the *cstIII*, *cgtA* and *cgtB* genes, coding for sialyl- and two galactosyltransferases, respectively (Gilbert & Wakarchuk, 2001). In the current study, the genotyped isolates that clustered together (indicating genetic similarity of greater than 90%) had an identical silver stain profile likely reflecting shared LOS biosynthesis gene loci. Furthermore, similar silver stain profiles were apparent for the isolates that shared 84% and 72% similarity. This analysis reveals that production of multiple LOS glycoforms is a common feature of *C. jejuni* isolates from numerous sources, representing its importance as a pathogenicity factor. Additionally, the high rates of Alcian blue reactive polysaccharide expression (indicative of CPS) in food and clinical *C. jejuni* isolates, demonstrates the importance of this trait in the *C. jejuni* lifestyle (Karlyshev *et al.*, 2005). *C. jejuni* cannot respire aerobically and human-to-human transfer is rare, so there exist mechanisms by which *C. jejuni* can survive in the environment until it comes into contact with a suitable host. Production of CPS may protect the cell from desiccation during environmental exposure, contributing to survival, which in turn contributes to the risk of infection from the contaminated foodstuff (Taylor & Roberts, 2005). In the host, CPS production is thought to be involved in protection from bacteriophages and involved in aspects of pathogenesis, such as in host cell adhesion, immune evasion, and protection from complement (Bacon *et al.*, 2001; Guerry *et al.*, 2012). Although both isolates 1450 GF and 591 BF reacted with Alcian blue, the isolate 592 BF, which shared >90% and 84% similarity respectively, did not. Similar to LOS, CPS biosynthesis genes are highly variable due to genetic mechanisms including phase variation that can switch production on/ off at any one time, which may account for the differences in similar isolates (Karlyshev *et al.*, 2004). Furthermore, since the cationic dye Alcian blue interacts with negatively charged macromolecules on the CPS (Karlyshev *et al.*, 2001), modification of the CPS with charged moieties that are under the control of phase variation could effect staining. Alternatively, variations in the structure of the CPS lipid anchor in different isolates may influence the lability of the CPS of the particular isolates, resulting in cleavage from the cell and loss of CPS during the extraction procedure (Corcoran *et al.*, 2006).

Initially, TLC with chemical staining was carried out for carbohydrate visualization that revealed a range of distinct components in the extracts. A band with an R_f of ~0.3 was detected in all the isolates, which has previously been reported to

represent LOS (Prendergast *et al.*, 2001). However, purple staining by resorcinol-HCL highlighted the presence of sialylation on structures that had resolved further up the TLC plate, indicating sialylated LOS at R_f of ~0.45. These may represent truncated LOS structures that retained sialylation. Presence of additional TLC bands in a number of isolates may represent capsular polysaccharide, and other *C. jejuni* cell-surface glycoconjugates.

Previous studies have highlighted that GBS-related strains are more likely to have GM₁-mimicry than non-GBS isolates (Prendergast *et al.*, 1998; Nachamkin *et al.*, 1999; Yuki, 2010). Presence of anti-GM₁ antibodies following *C. jejuni* infection is strongly linked with the purely motor form of the disease, AMAN (Yuki, 2010). An *ex vivo* model in which *C. jejuni*-induced anti-GM_{1a} antibodies bound with the neuronal axon at the nodes of Ranvier of human nerve tissue, identical to the pathology seen in GBS patients (Moran *et al.*, 2005). Consistent with this model, another study has shown that accumulation of anti-GM_{1a} antibodies at the nodes of Ranvier can cause of disruption of Na⁺ and K⁺ channels, and thus, interfere with nerve conduction (Moran *et al.*, 2005). In the current study, TLC with immuno-overlay revealed that 56% of the total isolates had anti-GM₁ antibody reactivity, comprising 66.6% of clinical and 46.4% of food isolates. Furthermore, 28% of the isolates reacted with CTB, and 71.4% of these were clinical isolates. Therefore, a higher incidence of GM₁ reactivity was observed in clinical isolates in this study. This may indicate that the source of infection in certain clinical cases was not food. Approximately 20% of cases of *C. jejuni* infection are due to vehicles of infection other than food, including water (Meade *et al.*, 1999).

A previous study, sampling enteritis isolates in the USA, and using a similar methodology as in the current study, reported 26.2% of 275 enteritis isolates had GM₁-like epitope (Nachamkin *et al.*, 1999). None of the clinical isolates in the current study were associated with GBS, therefore, a higher rate of GM₁ mimicry in Irish clinical (enteritis) isolates was found than in randomly chosen enteritis isolates from the USA (Nachamkin *et al.*, 1999). This may be due to differences in the prevalence of specific *C. jejuni* strains, which can be geographically related (Prendergast *et al.*, 1998). It was suggested by Moran *et al.* (2002) that mimicry of relatively undecorated gangliosides such as GM₁ might cause this more severe form of GBS due to the structural simplicity of the saccharide mimic. Potentially, the presence of bulky sugar moieties in other, more

elaborate *C. jejuni* LOS mimics, prevents the exposure of the sialosyl-disaccharide structures, which are the crucial ganglioside mimicking components of LOS (Moran *et al.*, 2002).

Use of cholera toxin to determine the presence of GM₁ does have some limitations since although GM₁ is the natural ligand for CTB, it may not be the only ganglioside bound by CTB (Sack *et al.*, 1998). *C. jejuni* expressing GM₁ was expected to be bound by cholera toxin, but reaction with *C. jejuni* presenting other LOS ganglioside mimics could potentially occur (Sack *et al.*, 1998). Only one strain (180 DC) reacted with CTB without also reacting with anti-GM₁ antibody, suggesting that this strain may not express an alternative mimic to GM₁.

Anti-asialo-GM₁ reactivity was observed in 44% of the isolates. In total, 63.6% of anti-asialo-GM₁ reactivity isolates were also reactive with either of the GM₁ isolates. Since the reactive component resolved lower on the TLC plate than the GM₁ ligands, this indicates these isolates produce both sialylated and asialylated-GM₁ LOS mimics. This is likely due to the phase variation in LOS sialyltransferases gene such as *neu1B* or *cstII* (Guerry *et al.*, 2002). Mice immunized with non-sialylated LOS mutants develop high-titres of anti-asialo-GM₁ antibodies, compared to the wild type LOS, which induces anti-GD_{1a} antibodies (Godschalk *et al.*, 2004). Therefore, both sialylated and non-sialylated LOS phase variants may induce an antibody response, although the role of asialo-GM₁ antibodies in GBS is not well understood.

Both the identical genotyped isolates 260 BC and 1473 BF, which clustered in J3, reacted with CTB, anti-GM₁ and anti-asialo-GM₁ antibodies. Comparatively, the isolates 591 BF and 592 BF, which had 84% similarity, had different anti-ganglioside ligand reaction patterns, and slightly different chemical stained TLC profiles, which was not detected by SDS-PAGE with silver staining. Thus, it appears that the 16% divergence in genetic identity was responsible for the phenotypic difference between these closely related isolates. Other genetic mechanisms besides phase variation that could be responsible for this divergence include horizontal gene transfer, gene rearrangement and inactivation (Karlyshev *et al.*, 2005). Furthermore, the substrate specificity and affinity of the glycosyl transferases are frequently altered with single point mutations (Gilbert *et al.*, 2002). Comparison of a greater quantity of closely related genotypes would be valuable in future. Interestingly, structural analysis of the

purified LOS of *C. jejuni* strains does not always agree with the structure predicted on the basis of the gene content (Godschalk *et al.*, 2007; Semchenko *et al.*, 2012).

Semchenko *et al.* (2012) described a *C. jejuni* clinical strain, 520, with the ability to produce a heterogenous LOS mix of GM₁-like, GM₂-like, asialo-GM₁-like, and asialo-GM₂-like, and despite this strain having identical LOS biosynthesis cluster type as the genome strain 11168, they have different LOS phenotypic profiles (Oldfield *et al.*, 2002; St Michael *et al.*, 2002; Semchenko *et al.*, 2012).

As expected, no reaction was seen with the *C. coli* isolate 2134 GF that was used as a negative control, as it does not express ganglioside mimicry. In the case of the unreactive *C. jejuni* isolates, they either expressed ganglioside mimics distinct from those tested for, or similar to *C. coli* they do not express ganglioside-mimicry. Certain *C. jejuni* strains are unable to express ganglioside mimics as they lack the genetic machinery for the expression of sialic acid, for example the HS:3 serostrain (Aspinall *et al.*, 1995). Importantly, the study showed that 18/ 25 isolates reacted with at least one anti-ganglioside ligand tested. The presence of carbohydrate structures, which are cross-reactive with anti-ganglioside ligands, in food isolates may represent a concern for the consumer. However, since anti-ganglioside ligand reactivity was also found in isolates from clinical cases of uncomplicated enteritis, it indicates that other factors in addition to ganglioside mimicry are required for the development of GBS.

Chapter 4.
Glycan recognition by Guillain-Barré syndrome
and enteritis-related *Campylobacter jejuni*
isolates

4.1 Introduction

Campylobacter jejuni, a Gram-negative microaerophile, is the leading bacterial cause of diarrhoeal disease globally (Prendergast & Moran, 2000). Clinically, disease primarily manifests as self-limiting gastroenteritis (Blaser, 1997). However, *C. jejuni* is also recognised as the most frequent antecedent infection in the development of the neurological disorder GBS, the most common cause of acute muscular paralysis worldwide (Allos, 2001). Collectively, studies demonstrating the reactivity of anti-ganglioside antibodies with *C. jejuni* LOS have confirmed the presence of ganglioside related epitopes in *C. jejuni* LOS (Yuki *et al.*, 1995; Neisser *et al.*, 1997; Yuki, 2005). The molecular mimicry hypothesis was proposed as a pathogenic mechanism for *C. jejuni*-related GBS, whereby an immune response is induced by infection resulting in the generation of cross-reactive anti-ganglioside antibodies that target neural gangliosides, leading to tissue damage and nerve dysfunction (Bowes *et al.*, 2002).

Although the presence of ganglioside-like structures in the LOS of *C. jejuni* is thought to be a pre-requisite for the development of *C. jejuni*-related GBS, it does not seem to be the only factor involved. Certain *C. jejuni* Penner heat-stable (HS) serotypes show a strong association with GBS, particularly *C. jejuni* HS:19 which was found to be a predominant serotype isolated from GBS patients in Japan and the United States (Mishu & Blaser, 1993; Yuki, 1997) as well as serotype HS:41 which was over-represented in cases of GBS in South Africa (Lastovica *et al.*, 1997). Genetic characterisation of South African *C. jejuni* HS:41 isolated over a period of 17 years, found that they represented a genetically homogeneous, clonal population (Wassenaar *et al.*, 2000). Interestingly, these isolates were recovered from patients who had developed GBS, but also from patients who suffered uncomplicated enteritis only (Lastovica *et al.*, 1997). Significantly, ganglioside mimicking structures, were found in the LOS of both neuropathic and enteritis isolates (Prendergast *et al.*, 1998). Therefore, the evidence suggests the involvement of largely unidentified bacterial and host-related factors in GBS pathogenesis. Undoubtedly, valuable insight can be gained from comparing closely-related isolates with different clinical consequences (Gilbert *et al.*, 2004).

Central to the pathogenesis of this bacterium is colonisation of the intestine, not only the mammalian intestine in which disease occurs, but also as a commensal in

poultry which act as the major source for transmission to humans (Young *et al.*, 2007). Colonisation is mediated by the presence of adhesins which attach to components of the extracellular matrix and glycan structures expressed on the surface of intestinal epithelial cells (Young *et al.*, 2007). Comparison of the glycan binding profile of neuropathic and non-neuropathic strains of *C. jejuni* may contribute to the understanding of the bacterial factors involved in the development of GBS. Therefore, this study was carried out in order to compare the glycan-binding behavior of a group of closely related *C. jejuni* strains with different clinical outcomes.

4.2 Results

Initially, *C. jejuni* HS:41 isolates were compared using a cutting-edge microarray consisting of 60 host-related glycan structures, followed by an *in vitro* Caco-2 cell adherence assay.

4.2.1 Glycan microarray analysis of *C. jejuni* HS:41 strains

Fluorescence intensity was measured eight times for each of 60 glycans (2 biological replicates) (Table 2.7) across six bacterial strains. The 60 glycans can be grouped into five glycan classes, based on related structural features: glycans 1A (lacto-N-biose I) - 2B (Gal β 1-6 Gal) are terminal-Gal-containing structures; 5A-5H are mannosylated glycans; 7A-8F are fucosylated glycans, and; 10A-11D are sialylated glycans. Six *C. jejuni* HS:41 strains were from two clinical groups, enteritis-related (238 and 176.83), and GBS-related (2813.94, 299.95, 233.95 and 260.94). The six strains and the two clinical groups were compared in their adherence to the glycan families on the array, with fluorescent intensity taken as an indication of the level of binding.

C. jejuni bound to a range of terminal-Gal-, Man-, Fuc-, and Neu5Ac-containing glycans on the array. To enable statistical comparison of *C. jejuni* strains and clinical groups multilevel modeling was employed. Since measurements of fluorescence intensity were measured repeatedly within glycan structures these data were most suitably modeled using multilevel models. This model allowed variation in intensity within glycans to be modeled separately from variation of intensity between glycans. Initially, overall fluorescent intensity of all strains for all glycans was compared to

determine the distribution of fluorescent intensities. The intensity data were positively skewed (Fig. 4.1) but contained non-positive values, where no adherence to glycans occurred, which made a log transformation impossible. Therefore, for stringent comparative analysis, a $\log(x+1)$ transformation was used to incorporate the intensities of 0. This led to a distribution made up of two parts, a group of 689 zeros (where no binding occurred) and 4687 approximately normally distributed log intensities (Fig. 4.2).

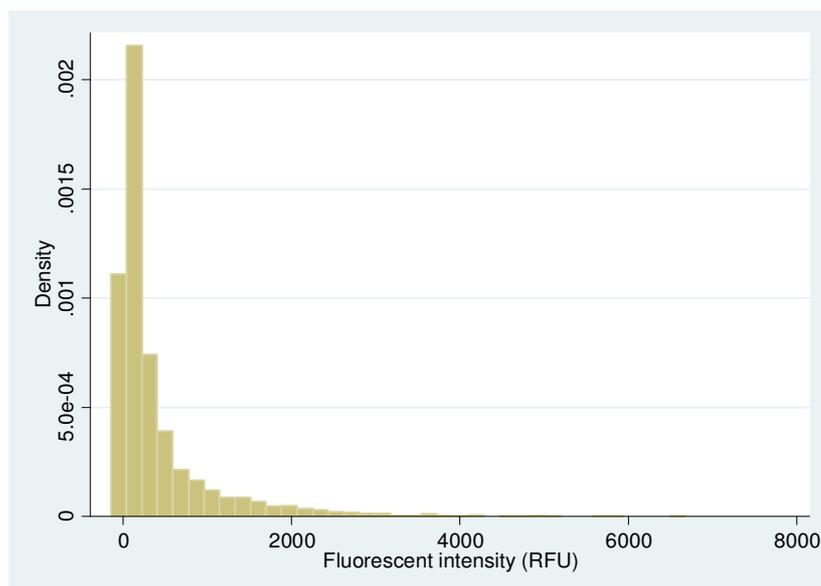


Figure 4.1 Fluorescent intensity for all HS:41 strains and glycan structures

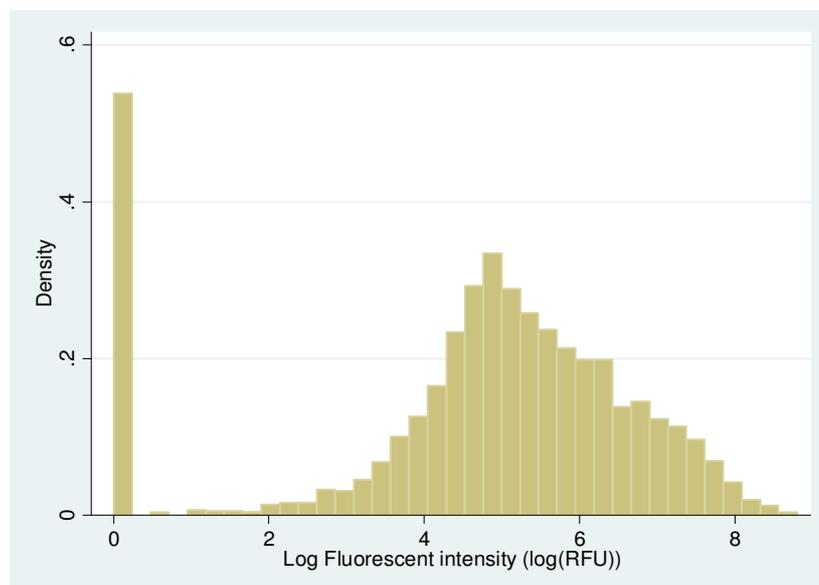


Figure 4.2 Log (fluorescent intensity + 1) for all HS:41 strains and glycan structures

Reported are results from the multilevel models for each of the four glycan families. The log-normal intensities (bell-shaped distribution in Fig 4.2) were modeled to estimate differences between (1) clinical groups and (2) bacterial strains separately since bacterial strains were nested within clinical group. Using multilevel models differences in $\log(\text{intensity})$ between clinical groups and between bacterial strains were observed, while controlling for the multilevel structure of the data.

4.2.1.1 Binding to terminal-Gal glycans

From the multileveled modelling in the terminal-Gal family, the average $\log(\text{intensity})$ for the enteritis-related group was about 0.25 higher than in the GBS-related group (Fig. 4.3). This was the estimated difference in average $\log(\text{intensity})$ in the sample. However, it was more relevant to see if the observed difference was evidence for a true difference in the population, not just the average. Therefore, confidence intervals were calculated to give a range of credible values for the true difference between comparators. Importantly, if the interval contained a zero then it cannot be ruled out that zero was the true difference, and thus it cannot be said there was evidence for a difference. However, if zero is not in the interval then there is evidence that there is a

difference between groups or strains. Using the 95% confidence interval, it is likely that the GBS-related group had a log(intensity) which was between 0.08 and 0.42 lower on average than the enteritis-related group (Fig. 4.3 A; $p = 0.004$). Thus there was some evidence to suggest a true difference between clinical groups in binding to the terminal-Gal glycans

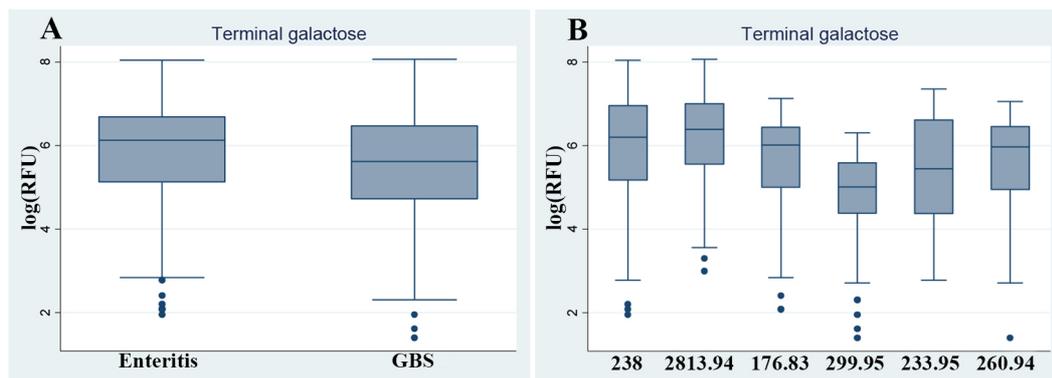


Figure 4.3 Box plots comparing enteritis-related and GBS-related *C. jejuni* HS:41 A) clinical groups, and B) strains in their binding to the terminal Gal family of glycans on the array.

Comparing the strains, 2813.94 had the highest fluorescent intensity that was statistically indifferent to enteritis strain 238. Strains 176.83 and 260.94 bound at similar rates to the terminal-Gal glycans, and there was evidence ($p < 0.05$) to suggest that average log(intensity) of GBS-related strain 260.94 and enteritis-related strain 176.83 had a lower average log(intensity) than 238, indicating that 238 was driving the difference between clinical groups. There was strong evidence that strains 299.95 and 233.95 had lower average log intensities than strains 238 for the terminal-Gal family. For example, the model estimated an average log(intensity) 1.02 lower in 299.95 than in 238. However, using the 95% confidence interval, it was likely that the true average log(intensity) was between 0.77 and 1.27 lower in strain 299.95 than in strain 238 (Fig 4.3 B, $p < 0.0005$).

4.2.1.2 Binding to mannose-containing glycans

From the multileveled modeling of the mannose family of glycans, there was weak evidence ($p = 0.47$) that the enteritis-related group bound at a higher rate GBS-related

group. The average log(intensity) for the enteritis-related group was about 0.27 higher than in the GBS-related group. It is likely that the GBS-related group had a log(intensity) which was between 0.54 and 0.003 lower on average than the enteritis-related group (Fig. 4.4 A; $p = 0.047$).

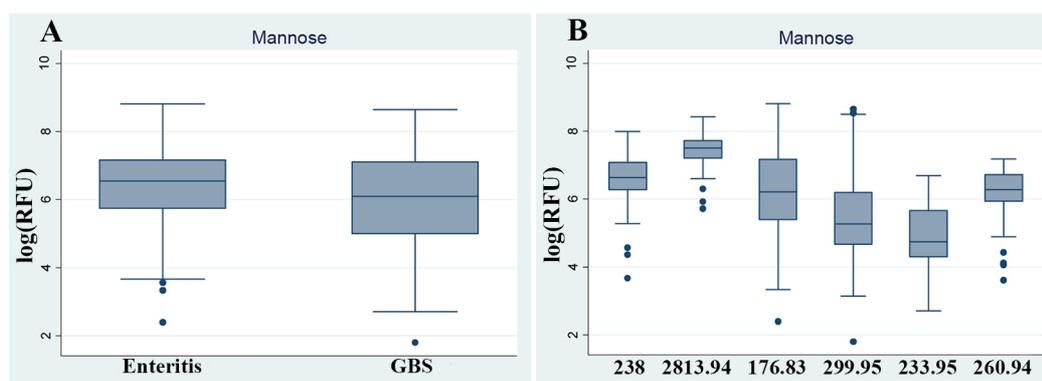


Figure 4.4 Box plots comparing enteritis-related and GBS-related *C. jejuni* HS:41 A) clinical groups, and B) strains, in their binding to the mannose family of glycans on the array

In comparison of the strains (Fig. 4.4 B), the GBS-related strain 2813.94 had the highest rate of binding to the mannose family of glycans. There was strong evidence ($p < 0.0005$) that strain 2813.94 bound at a higher intensity than 238. Strains 233.95, 299.95 and 260.94 bound at statistically lower rates than 238 ($p < 0.0005$). Comparing the enteritis isolates, there was some evidence to suggest that 176.83 bound at a lower rate than 238 ($p = 0.014$), indicating that strain 238 was driving the difference between clinical groups.

4.2.1.3 Binding to fucosylated glycans

With the fucosylated glycans, there was strong evidence (Fig. 4.5 A, $p < 0.0005$) that the enteritis-related group bound at a higher rate GBS-related group. The average log(intensity) for the GBS-related group was about 0.35 lower than the enteritis-related group. It is likely that the GBS-related group had a log(intensity) which was between 0.51 and 0.20 lower on average than the enteritis-related group.

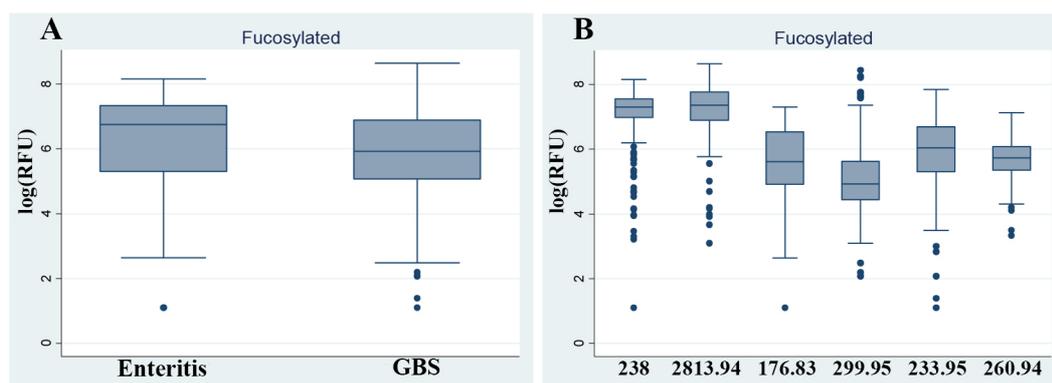


Figure 4.5 Box plots comparing enteritis-related and GBS-related *C. jejuni* HS:41 A) clinical groups, and B) strains in their binding to the fucosylated family of glycans.

Again, strain 2813.94 had the highest average intensity for the fucosylated glycans, which was statistically indifferent to enteritis strain 238. There was strong evidence that strain 238 and 2813.94 bound at higher rates than GBS strains 233.95, 299.95 and 260.94 (Fig. 4.5 B: $p < 0.0005$). Furthermore, binding by enteritis strain 176.83 was significantly lower (Fig. 4.5 B: $p < 0.0005$) than strain 238, so strain 238 was driving the difference between clinical groups.

4.2.1.4 Binding to sialylated glycans

Considering the sialylated glycans, again there was strong evidence that the enteritis-related group bound at a higher rate GBS-related group (Fig. 4.6 A). The average log(intensity) for the enteritis-related group was about 0.32 higher than the GBS-related group. It is likely that the GBS-related group had a log(intensity) which was between 0.47 and 0.16 lower on average than the enteritis-related group (Fig. 4.6 A; $p < 0.0005$).

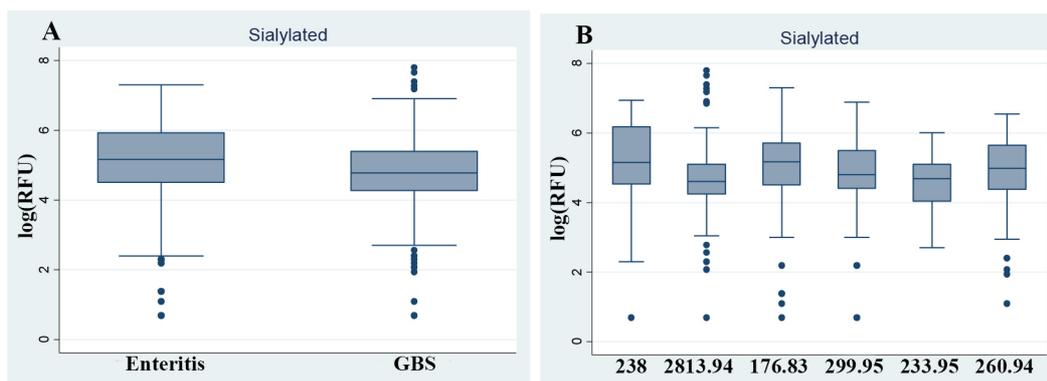


Figure 4.6 Box plots comparing enteritis-related and GBS-related *C. jejuni* HS:41 A) clinical groups, and B) strains in their binding to the sialylated family of glycans.

There was no difference between enteritis-related strains in binding to sialylated glycans. There was no difference between strain 260.94 and 238. However, strains 2813.94, 233.95 and 299.95 bound at lower rates (Fig. 4.6 B, $p < 0.05$) than strain 238 sialylated glycans.

4.2.2 Caco-2 adherence assay

Cultured eukaryotic cell assays are a standard technique in the study of *Campylobacter* adhesion (Hanel *et al.*, 2004). In addition to observations of *C. jejuni* glycan-mediated adherence on the glycan array, an *in vitro* cell adherence assay was carried out, using Caco-2 cells pre-treated with a lectin to determine specificity of glycan-mediated binding of *C. jejuni* to Caco-2 cell surface. Bacterial adherence to Caco-2 cells is known to be glycan-mediated (Angeloni *et al.*, 2005). Specific lectins were selected to represent the range of glycan structural families present on the glycan array. These included *Erythrina cristigalli* agglutinin (ECA; Gal β 1,4GlcNAc/ GlcNAc > Gal), *Ulex europaeus* agglutinin 1 (UEA-1; terminal α 1,2 Fuc), concanavalin A (Con A; Man), *Limax flavus* agglutinin (LFA; terminal sialic acid), *Maackia amurensis* agglutinin (MAA; Neu5Ac α 2,3 > Gal) and *Sambucus nigra* agglutinin (SNA; Neu5Ac α 2,6 > Gal). The influence of lectin pre-treatment on *C. jejuni* binding was determined relative to the level of adherence to non-pretreated Caco-2 cells, which was set at 100%.

All the strains adhered to the Caco-2 cells at low rates, but at rates, which are consistent with the literature for the methodology employed (Friis *et al.*, 2005). Adherence rates of the strains are compared in Figure 4.7. The enteritis-related strain

238 adhered at the highest rate of all the strains (2.35%), while the other enteritis-related strain 176.83 adhered at a rate of 1.39%. GBS-related strain 2813.94 had the second highest adherence rate of 1.43%, while the remaining other GBS-related strains 260.94, 299.95 and 233.95 adhered at lower rates of 0.39%, 0.68% and 0.97%, respectively.

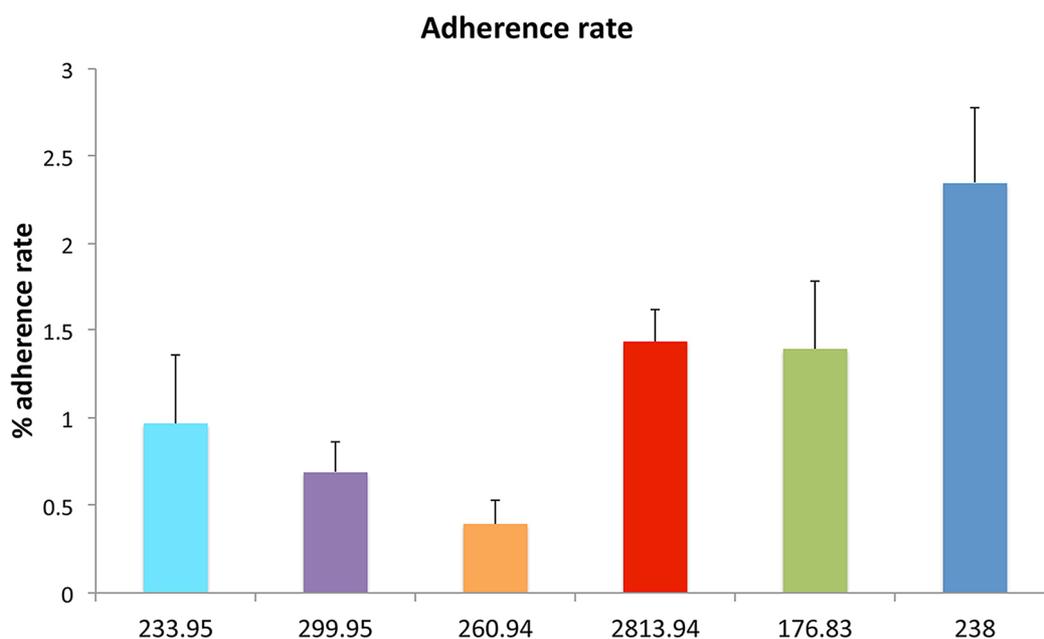


Figure 4.7 Rate of adherence of *C. jejuni* HS:41 strains to Caco-2 cells. Rate determined as a percentage of total cells added. Histograms show the mean of three biological replicates, carried out in triplicate.

The influence of lectin pre-treatment on *C. jejuni* adherence was investigated (Fig 4.8) Pre-treatment with the lectin ECA, specific for terminal lactose/ Gal structures on the Caco-2 cell surface was observed to reduce *C. jejuni* adherence. Caco-2 pre-treatment with ECA resulted in statistically significant reduced adherence in all six strains. Both enteritis-related strains 176.83 and 238, as well as GBS-related strain 233.95 had very significant ($p < 0.01$) reductions in adherence following ECA-pretreatment, by 82.7%, 78.5% and 59.8%, respectively. Adherence of GBS strains 260.94, 2813.94, and 299.95 to Caco-2 was significantly reduced with ECA pretreatment by 47.5%, 46.7% and 45% ($P < 0.05$), respectively.

Caco-2 pretreatment with the lectin UEA-I was carried out to assess the involvement of $\alpha(1,2)$ -linked Fuc on *in vitro* adherence of the *C. jejuni* strains. Statistically significant reductions in adherence were observed with all *C. jejuni* strains. Observed reductions in adherence of enteritis-related strains 176.83 and 238, were 85.2% ($p = 0.004$) and 79.1% ($p = 0.001$), respectively. In addition, reductions in adherence of GBS-related strains of 299.95, 233.95, 2813.94, and 260.94, were greater than 75% ($p < 0.01$)

The involvement of cell-surface mannosylated glycans on Caco-2 adherence by *C. jejuni* was investigated using ConA. Caco-2 pretreatment resulted in a statistically significant reduction in adherence of all six strains. Significant reductions in adherence of 84.4% ($p = 0.003$) and 79.1% ($p = 0.005$) were observed for enteritis-related strains 176.83 and 238, respectively. Pretreatment with ConA reduced adherence of GBS-related strains by 37.6-85.5% ($p < 0.05$).

Initial cell binding assays using LFA to block all terminal Neu5Ac residues present on Caco-2 cells were performed to evaluate the involvement of Neu5Ac on *in vitro* adherence of *C. jejuni* HS:41 strains. Statistically significant reductions in adherence were observed in only two strains, 176.83 and 2813.94, with observed reductions in adherence of 20.1% ($p = 0.003$) and 33.3% ($p < 0.03$), respectively. While reductions in adherence of enteritis-related strain 176.83, and GBS-related strains 233.95, 299.95, 260.94, following LFA pretreatment were not statistically significant reductions in adherence of up to 50% were observed ($p > 0.05$).

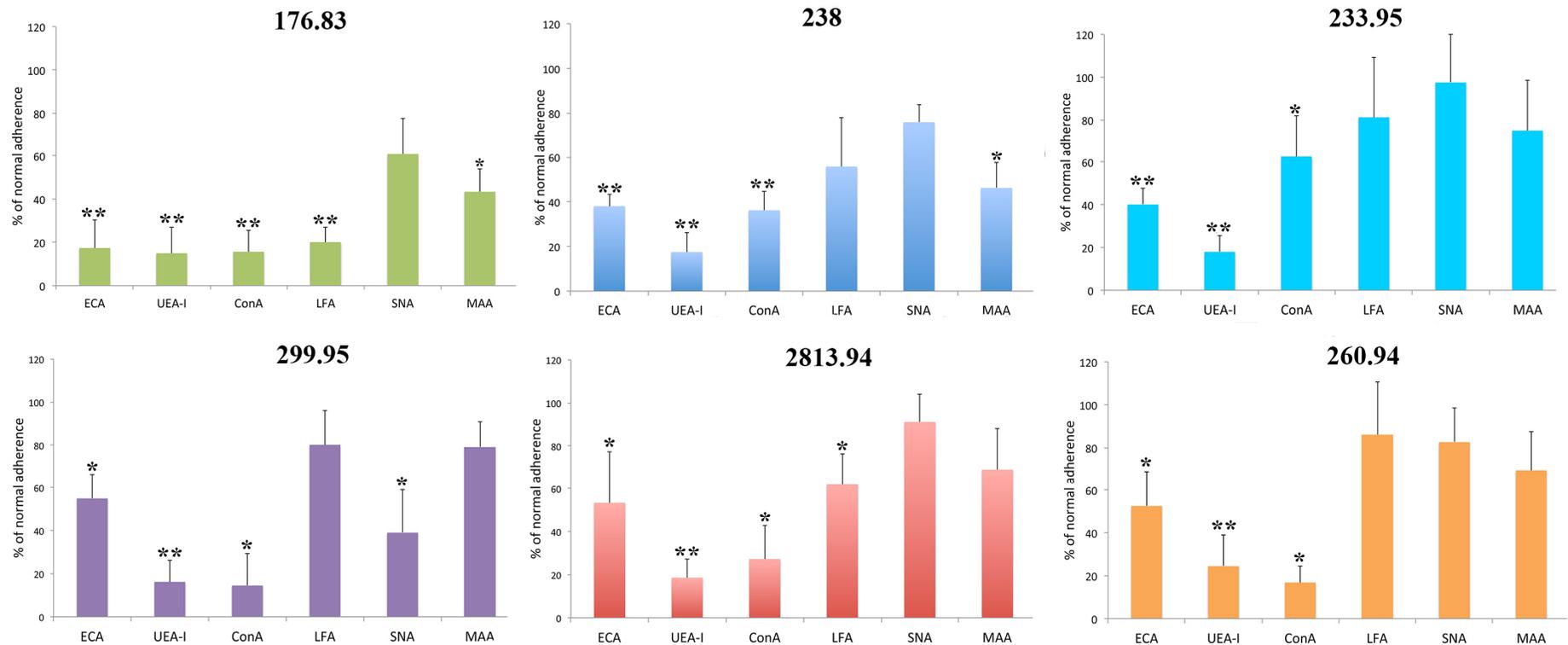


Figure 4.8 Lectin inhibition of *C. jejuni* adherence to Caco-2 cells. Percentage reduction of *C. jejuni* HS:41 176.83 (green), 238 (blue), 233.95 (turquoise), 299.95 (purple), 2813.94 (red), 260.94 (orange), adherence to Caco-2 cells pretreated with ECA, UEA-I, ConA, LFA, SNA and MAA. (* $p < 0.05$, ** $p < 0.01$; significant difference to non-lectin treated controls). Each experiment was carried out at least three times.

To obtain further information on the specific nature of Neu5Ac binding, if present, of *C. jejuni* adherence to Caco-2 cells, lectins MAA (Neu5Ac α 2,3Gal, specific) and SNA (Neu5Ac α 2,6Gal-specific) were chosen. With SNA a statistically significant reduction in adherence was observed for only one strain, 299.95, which was reduced by 60.9% ($p = 0.01$). Reduction of adherence of 299.95 following pretreatment with MAA was not significant ($p = 0.10$), indicating a binding preference of this strain for α (2,6)-linked Neu5Ac.

Only enteritis strains 176.83 and 238 had statistically significant reductions in adherence following pretreatment with MAA of 66.6% and 46.2% ($p < 0.05$), respectively. This would indicate a binding specificity of enteritis-related strains for α (2,3)-linked Neu5Ac, although reductions in adherence of up to 68.4% for 176.83 ($p = 0.06$) and 32.8% for 238 ($p = 0.10$), were observed after SNA pretreatment. No statistically significant difference ($p > 0.05$) was observed for the remaining strains following MAA. However, reduction in adherence of strain 2813.94, 233.95 and 260.94 of 21.2-39.1% ($p > 0.05$), were observed following MAA pretreatment. Furthermore, non-significant reductions of 46.4-61% ($p > 0.05$) were observed for these strains following SNA pretreatment.

4.2.3 Summary of glycan microarray and Caco-2 inhibition assay

Glycan array analysis of six clonally-related, genetically and serologically indistinguishable *C. jejuni* isolates, which were associated with different clinical outcomes, GBS (4) or enteritis-only (2), was carried out. Binding of glycans by clinical groups and strains was compared using multilevel modelling. The first model compared average log(intensity) of clinical groups, which provided evidence that enteritis-related strains had higher rates of binding than GBS-related strains to terminal-Gal, Man-, Fuc-, and Neu5Ac-containing glycans.

The second model compared the average log(intensity) of strains in binding to the glycans. Strains 2813.94 and 238 had similar high rates of binding to terminal-Gal, and Fuc-containing glycans, although, strain 2813.94 bound at the highest rate to mannosylated glycans. Statistical analysis revealed that strain 238 was driving the difference in binding of clinical groups to terminal-Gal, Man- and Fuc- containing

glycans. However, there was no difference in binding of sialylated glycan by enteritis-related isolates, and enteritis-related isolates bound to sialylated glycans at higher rates than 3/4 GBS-related isolates. Evidence of strain specificity in glycan binding was observed. Binding data indicated the following binding preferences of strains: 238, Fuc > Man \geq Gal > Neu5Ac; 176.83, Man > Fuc \geq Gal > Neu5Ac; 2813.94, Man > Fuc > Gal > Neu5Ac; 299.95, Man > Fuc > Neu5Ac \geq Gal; 233.95, Fuc > Gal > Man \geq Neu5Ac; 260.94, Man > Gal \geq Fuc > Neu5Ac.

The adherence of all six strains to Caco-2 cells was reduced following pretreatment with the lectins UEA-I, ECA and ConA, demonstrating the role of $\alpha(1,2)$ -linked Fuc, terminal-Gal and Man, respectively, in adherence of *C. jejuni* to Caco-2 cells. However, strain differences were observed on the level and significance of inhibition. Pretreatment with the Neu5Ac-specific lectin LFA significantly reduced adherence in only two strains 176.83 and 2813.94 indicating Neu5Ac-mediated adherence. However, pretreatment with lectin SNA or MAA did not significantly reduce adherence of 2813.94. Both enteritis-related strains 238 and 176.83 had statistically significant reduction in adherence following MAA pretreatment, indicating preference of enteritis-related strains for $\alpha(2,3)$ -linked Neu5Ac on Caco-2 cells. No GBS strain was significantly reduced following MAA pretreatment, and only strain 299.95 was reduced significantly following SNA pretreatment, indicating $\alpha(2,6)$ -linked Neu5Ac mediated adherence. Lectin pretreatment of Caco-2 cells inhibited adherence of HS:41 strains in the following order: 238, ConA \geq ECA > UEA-I \geq MAA > LFA > SNA; 176.83, UEA-I \geq ConA \geq ECA \geq LFA > MAA > SNA; 2813.94, UEA-I > ConA > ECA > LFA > MAA > SNA; 299.95, ConA \geq UEA-I \geq SNA > ECA > MAA > LFA; 233.95, UEA-I > ECA > ConA > MAA > LFA > SNA; 260.94, ConA > UEA-I > ECA > MAA > SNA > LFA. This correlates well with binding preferences observed on the glycan array.

4.3 Discussion

It is clear that apart from antecedent infection with *C. jejuni* other unknown bacterial and/or host factors are involved in the GBS development. Potentially, strain-specific variations in bacterial virulence may influence the outcome of *C. jejuni* infection, which ultimately, can result in GBS or uncomplicated enteritis alone. A number of studies

have highlighted that GBS and enteritis strains do not appear to be part of distinct *C. jejuni* lineages, and in many cases neuropathic strains are genetically related to enteritis strains (Endtz *et al.*, 2000; Dingle *et al.*, 2001; Leonard *et al.*, 2004; Taboada *et al.*, 2007). *C. jejuni* HS:41 strains were isolated in South Africa from cases of GBS and uncomplicated enteritis (Lastovica *et al.*, 1997). Although a rarely isolated serotype, overrepresentation of *C. jejuni* HS:41 in GBS cases in South Africa, suggests that strain-specific factors are associated with a neuropathic potential for this serotype. *C. jejuni* HS:41 have a class A1 LOS biosynthesis locus, which is the most frequently associated LOS locus class associated with GBS, but is also found in enteritis-only patients (Godschalk *et al.*, 2004; Taboada *et al.*, 2007; Parker *et al.*, 2008). Taken together, these studies indicate that additional factors to HS:41 ganglioside mimicry are involved in the neuropathogenesis. The present study may indicate that glycan-mediated host cell adherence is linked with clinical outcome in this group of strains. *C. jejuni* interaction with glycans expressed in the host GI tract is a critical virulence step in the establishment of infection, and potentially the glycan-binding phenotype of particular *C. jejuni* strains may determine the severity or course of disease, and therefore influence the clinical outcome of infection, GBS or uncomplicated-enteritis alone.

Glycan structures were grouped into classes of related structures consisting of terminal-Gal containing, mannosylated, fucosylated and sialylated glycans to enable comparison statistical of clinical groups and strains, by analysis through multilevel modelling. From the multilevel models there was strong statistical evidence ($p < 0.0005$) that enteritis-related strains bound at a higher rate than GBS-related strains to Fuc- and Neu5Ac-containing glycans. Additionally, there was weaker evidence ($p < 0.05$) that enteritis-related strains bound to terminal-Gal and Man-containing glycans than GBS-related. Therefore the results tentatively suggested that *C. jejuni* HS:41 strains adhere to these host-related glycans at a higher rate than GBS-relates isolates. Furthermore, enteritis strain 238 had the highest rate of adherence to Caco-2 cells, and the other enteritis-related strain 176.83 had a higher Caco-2 cell adherence rate than 3/4 GBS isolates. It has been postulated that more invasive strains are associated with GBS (Louwen *et al.*, 2008), and other post-infectious sequelae such as reactive arthritis (Mortensen *et al.*, 2009), since *C. jejuni* host cell invasion would generate a humoral immune response leading to neuropathic auto-reactivity (Perera *et al.*, 2007). GBS

patients infected with *C. jejuni* HS:41 have a particularly severe form of the disease, necessitating much longer hospitalization and ventilation than patients infected with non-HS:41 *C. jejuni*, and *Campylobacter*-negative GBS patients (Lastovica *et al.*, 1997), which may suggest increased strain virulence. Importantly, even highly-related strains such as HS:41 can have significantly different virulence-associated phenotypes (Carrillo *et al.*, 2004). While adherence is thought to be a prerequisite for epithelial cell invasion by *C. jejuni*, adherence may not always be followed by invasion, and adherence and invasion by *C. jejuni* are distinct phenomena (Hänel *et al.*, 2004). So, although less adherent than enteritis strains, GBS-related strains may still express levels of invasion, which would contribute to the development of the neuropathy.

It is important to note that the number of strains used in the current study was quite low, and despite their unique relatedness, a larger sample size may be needed to see if HS:41 enteritis strains are more adherent in general. It should also be noted that a single enteritis strain 238, was driving the difference between clinical groups in binding to terminal-Gal, Man- and Fuc- containing glycans. Strain 238 which had a highest Caco-2 adherence rate of all strains of 2.35%, although this is not hyper-adherent since *C. jejuni* adherence rates to Caco-2 cells can vary from 0.4-12.7% (Hänel *et al.*, 2004). However, combined with the high rates of binding on the glycan array, this suggests a comparatively greater adherent phenotype for this strain. Also, 2813.94 had the second highest Caco-2 adherence rate of compared of 1.45%, and was consistently a high binder to glycans on the array, further demonstrating correlation of Caco-2 cell adherence rate with glycan array binding.

While strain 238 was driving the difference between clinical groups for some classes of glycans, for Neu5Ac-containing glycans both enteritis-related strains bound at a similar rate that was higher than all four GBS-related strains. Furthermore, both enteritis-related strains were significantly reduced in their adherence to Caco-2 cells after MAA pretreatment, suggesting a greater affinity of enteritis strains for sialylated glycans, particularly $\alpha(2,3)$ -linked Neu5Ac. Pretreatment with LFA also reduced adherence of enteritis strains, although with 238 the inhibition was not statistically significant. Sialic acid is highly expressed on less than 1-week old Caco-2 cell monolayers (Angeloni *et al.*, 2005), thus, higher rates of enteritis strain adherence to Caco-2 cells observed in the present study may be linked to increased affinity for

$\alpha(2,3)$ -linked Neu5Ac. Sialic acid is also a common constituent of glycoconjugates in the GI tract (Severi *et al.*, 2007), and it could be hypothesised that higher rates of Neu5Ac binding by enteritis-related strains may determine bacterial localisation and host cell tropism, potentially influencing subsequent interaction with the immune system, and therefore the course and clinical outcome of infection.

There may be some disadvantages with using lectins as inhibitors of adherence as a means of extrapolating individual glycan specificities. Lectin specificities are determined using inhibitory sugars, but in addition to specific lectin-glycan interactions, non-specific or non-carbohydrate-mediated interactions of lectins with the Caco-2 cells may occur (Gerlach *et al.*, 2011). Furthermore, lectins bound to Caco-2 cells, may not only inhibit binding by *C. jejuni* to the specific glycan bound by the lectin, but potentially the lectin molecule could block additional binding sites, non-specifically, due to steric or electrostatic effects (Dr. J. Gerlach, personal communication). However, a previous study has shown high rates of lectin binding to Caco-2 cell glycoproteins, indicating the presence of $\alpha(2,3)$ -linked and $\alpha(2,6)$ -linked Neu5Ac, Fuc and terminal-Gal moieties (Angeloni *et al.*, 2005). Furthermore, in the present study, binding data generated from the glycan array correlated well with the *in vitro* model of adhesion.

Overall, the evidence suggests that *C. jejuni* has broad glycan binding specificity. *C. jejuni* showed strong affinity for terminal-Gal, Fuc-, Man- containing glycans, and some recognition of Neu5Ac-containing glycans. *C. jejuni* lacks any orthologs to glycan-binding adhesins found in other bacteria, including the related organism *H. pylori* which expresses a number of proteins with well defined carbohydrate-binding activities (Walz *et al.*, 2005). Therefore, the molecular basis of the diversity in glycan recognition exhibited by *C. jejuni* is unexplained.

High rates of *C. jejuni*-infection in the population, combined with a low *C. jejuni*-related GBS incidence rate (Poropatich *et al.*, 2010), suggests host factors may predispose the individual to the development of GBS. A particularly strong indication of the influence of host-related factors is that outbreaks of *C. jejuni* infection are relatively common, but clusters of GBS-following *C. jejuni* infection are rare (Khoury, 1978; Sliman, 1978; Yuki *et al.*, 1995, Blaser *et al.*, 1997, Ang *et al.*, 2000). However, the work presented suggests that there may be a link between *C. jejuni* glycan-mediated

adherence and the subsequent development of GBS following enteritis, representing another potential bacterial factor in GBS pathogenesis.

Chapter 5.

**Development and application of a
neoglycoconjugate microarray for the
investigation of glycan recognition by
*Campylobacter jejuni***

5.1 Introduction

Campylobacter jejuni has the ability to survive in diverse environments, such as those experienced in the susceptible human host and during commensal relationships with birds, as well as the conditions encountered in the natural environment outside the host and within the food supply chain. *C. jejuni* adaptation to the chicken or human host is a multifactorial process which is triggered by environmental stimuli that may be specific to the particular host environment. One factor which differs between the chicken and human host, and which may act as signal for specific host adaptation is host physiological temperature (37°C in humans, 42°C in chickens). Differential transcription of numerous *C. jejuni* genes, including several genes involved in heat shock response such as *groESL*, *grpE*, *dnaK*, *dnaJ*, *clpA*, *lon*, and *hslU*, have been observed due to temperature shift from 37 to 42°C, using a whole genome-microarray (Stintzi, 2003). *C. jejuni* chemotaxis and binding to host-related glycoconjugates was shown to be influenced by growth at host physiological temperatures (Khanna *et al.*, 2006; Day *et al.*, 2009). Therefore, temperature may provide an important trigger for specific host and environmental adaptation in *C. jejuni*, with commensalism or pathogenesis as the potential outcome (Young *et al.*, 2007).

It is believed that *C. jejuni* binding to mucin and epithelial cell-surface glycans may be an important part the host-cell adherence process (Ruiz-Palacios *et al.* 2003). The principal constituent of mucus is mucin, a high molecular-mass glycoprotein secreted from specialized mucus producing goblet cells in the gut epithelium (Tu *et al.*, 2008). Oligosaccharide chains consisting of GlcNAc, GalNAc or Gal, with terminal-Fuc and/ or Neu5Ac extend from the underlying protein backbone of the mucin, and constitute up to 50% of the entire mass of the glycoprotein. Evidence suggests that *C. jejuni* is attracted to mucus and L-Fuc (Hugdahl *et al.*, 1988), and exposure to Fuc and mucin has been shown to upregulate flagellar promoter activity (Allen & Griffiths, 2001; Jones *et al.*, 2004). Furthermore, mucus and Fuc-mediated *C. jejuni* binding to epithelial cells has been reported (McSweegan & Walker, 1986). Fucosylated glycoconjugates are also expressed on the surface of epithelial cells (Ruiz-Palacios *et al.*, 2003; Day *et al.*, 2009). *C. jejuni* binding to multiple glycans, including cell-surface

associated histo-blood group antigens containing fucose, has been observed on a glycan microarray (Day *et al.*, 2009).

Microarray technology overcomes many of the challenges of traditional techniques for the analysis of carbohydrates and their interactions with other biological materials. The benefits of using glycan microarray technology include small sample quantities, and the ability to display a large panel of carbohydrates in a limited chip space (Feizi *et al.*, 2003). Compared to conventional molecular/immunological assays such as 96-well plates, each carbohydrate is spotted in a much-reduced amount. Furthermore, glycan arrays have reliable, higher detection sensitivities than most conventional molecular/immunological assays (Oyelaran & Gildersleeve, 2009).

A range of glycan microarrays have been developed with different surface chemistries and glycan-containing molecules, including neoglycolipids (Liu *et al.*, 2007), natural- and neo-glycoproteins (Tateno *et al.*, 2008; Gao *et al.*, 2010), polysaccharides (Kosik *et al.*, 2010), glycosaminoglycans (Rogers *et al.*, 2011) and mucins (Kilcoyne *et al.*, 2012). Described here is the development of a microarray consisting of NGCs, with the aim of elucidating potential mechanisms of glycosylation-mediated adherence of *C. jejuni* to the host cell surface in humans and birds, by comparison of the glycan interaction of *C. jejuni* grown at 37 and 42°C, respectively.

5.2 NGC microarray development

The methods/chemistry of glycan array construction vary in technical features and suitability for specific purposes or practical application. NGCs were used as opposed to simply printing glycans directly to an array, since glycans are always presented as glyconconjugates in a biological system (Varki, 1993).

5.2.1 Poly-L-lysine slide chemistry

Neoglycoconjugate array v.1 consisted of poly-L-lysine slides which were functionalized with sulfo-SMCC (sulfosuccinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate), a bifunctional linker with an amine-reactive *N*-hydroxysuccinimide (NHS) ester which bound to the slide (Fig 5.1), and a sulfhydryl-reactive maleimide group which reacted with the denatured BSA of the NGC



Figure 5.1 Slide chemistry of poly-L-lysine slide (Piercenet.com). Figure has been removed due to copyright restrictions

Theoretically, this gave good 3D presentation of the carbohydrate on the array surface. The array consisted of 20 probes, with 6 replicates of each probe on the array, and each slide consisting of 6 sub-arrays (Fig. 5.2). The probes consisted of NGCs with different anomeric linkages (α or β) and linkers, as well as controls. Linkers consisted of diazonium coupling of 4AP glycosides to BSA as well as conjugation of ITC glycosides to the protein.

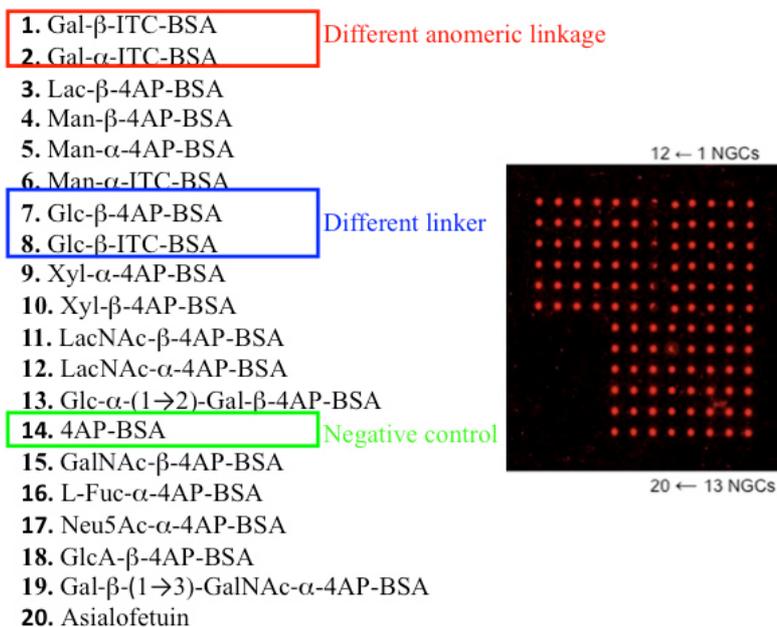


Figure 5.2. List of neoglycoconjugates presented on the poly-L-lysine slide array, with red-reflect image of a sub-array. Each slide consisted of 6 sub-arrays.

This array was validated with binding experiments using TRITC-conjugated lectins and inhibitions with 100 mM of various carbohydrates. The specificity of lectin interactions confirmed the satisfactory presentation of the carbohydrates, and low background binding was detected for most lectins apart from VVA, which interacted non-specifically with the array background.

5.2.2 Bacterial staining optimisation

Dye uptake titration curves were constructed for both strains of *C. jejuni*, using maximum fluorescence intensity achieved to determine the optimal dye concentration. Aside from the possibility of the highly charged dye molecule interfering in or altering binding events, the excess unbound dye lead to fluorescence quenching when not removed from the solution and resulted in a lower optimum dye concentration compared to that determined after washing (Fig. 5.3). Thus, the stained bacteria were thoroughly washed after staining and resuspended in TBS-T for incubation on the lectin microarray. Before washing the optimal dye concentration was perceived to be 10 μM , however after thorough washing the concentration at which fluorescence was saturated was 20 μM . Therefore, *C. jejuni* was fluorescently stained with a dye concentration of 20 μM for subsequent microarray hybridisation experiments.

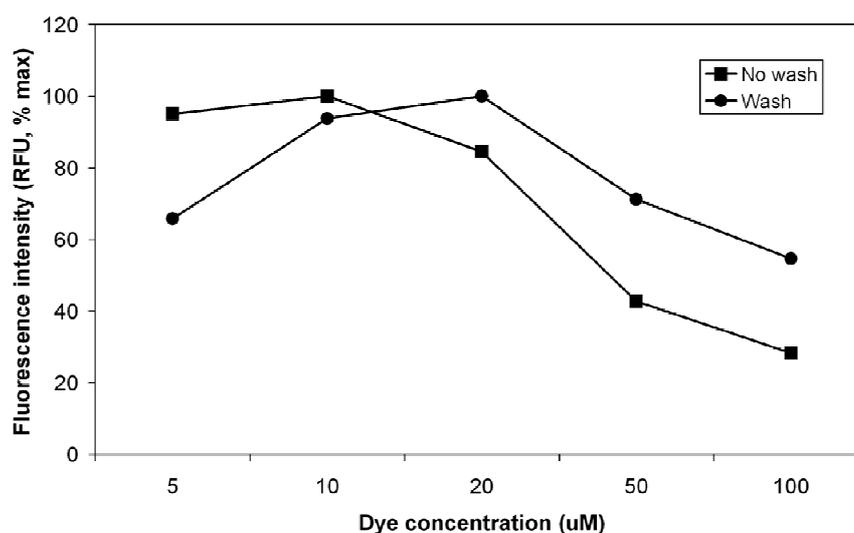


Figure 5.3 Dye uptake concentration curve normalised to 100% of each condition (maximum signal intensity), comparing the effect of post-staining washing steps on signal intensity in the strain 81116 at 37°C.

Similar results were observed with *C. jejuni* strain 81116 grown at 42°C and strain 81-176 at 37 and 42°C (not shown) and hence, the same optimal concentration was used for all strains at all temperatures.

5.2.3 Bacterial binding to NGC microarray

Bacterial adhesion experiments were carried out with labeled bacteria. After incubation with bacteria, binding to microarray spots was detected although background binding was unacceptably high. To minimise background, blocking assessment was carried out using a range of solutions containing thiols and periodate-treated BSA, to neutralise any remaining active sites on the slide and reduce non-specific binding. Blockers used included 3% BSA + 0.2mM mercaptohexanol, 3% BSA + 1mM mercaptohexanol, 3% BSA + 1mM mercaptoethanol + 0.1% BSA in wash buffer, 3% BSA-4-aminophenol (BSA-4AP) conjugate + 1mM mercaptoethanol, 1mM mercaptohexanol, 5mM mercaptohexanol, 25mM mercaptohexanol, 25mM mercaptoethanol (Fig. 5.4). The highest background intensities were observed after blocking with thiols in conjunction with an absence of BSA. Addition of BSA in the blocking solution served to reduce the average background intensity. The most effective reduction in background intensity occurred with a blocking solution of 1mM mercaptoethanol and 3% BSA conjugated to

4AP. Despite markedly reducing non-specific background binding by *C. jejuni*, there was still an unacceptable level present, and this did not allow differentiation between signal and background noise. High background noise reduces the sensitivity of the binding by reducing the signal to noise ratio, and by decreasing dynamic range (Seuryneck-Servoss *et al.*, 2007) and therefore can mask any actual binding events which occur on the array surface.

Thus, it was concluded that the poly-L-lysine slide was not suitable for bacterial adhesion experiments. While, the poly-L-lysine platform would be useful for screening labeled glycoproteins and certain lectins, and for some suitable applications it may be cost preferential, it was not useful as a universal platform. Therefore, an alternative slide, the SCHOTT Nexterion® Slide H, was then tested for its suitability to bacterial adhesion experiments.

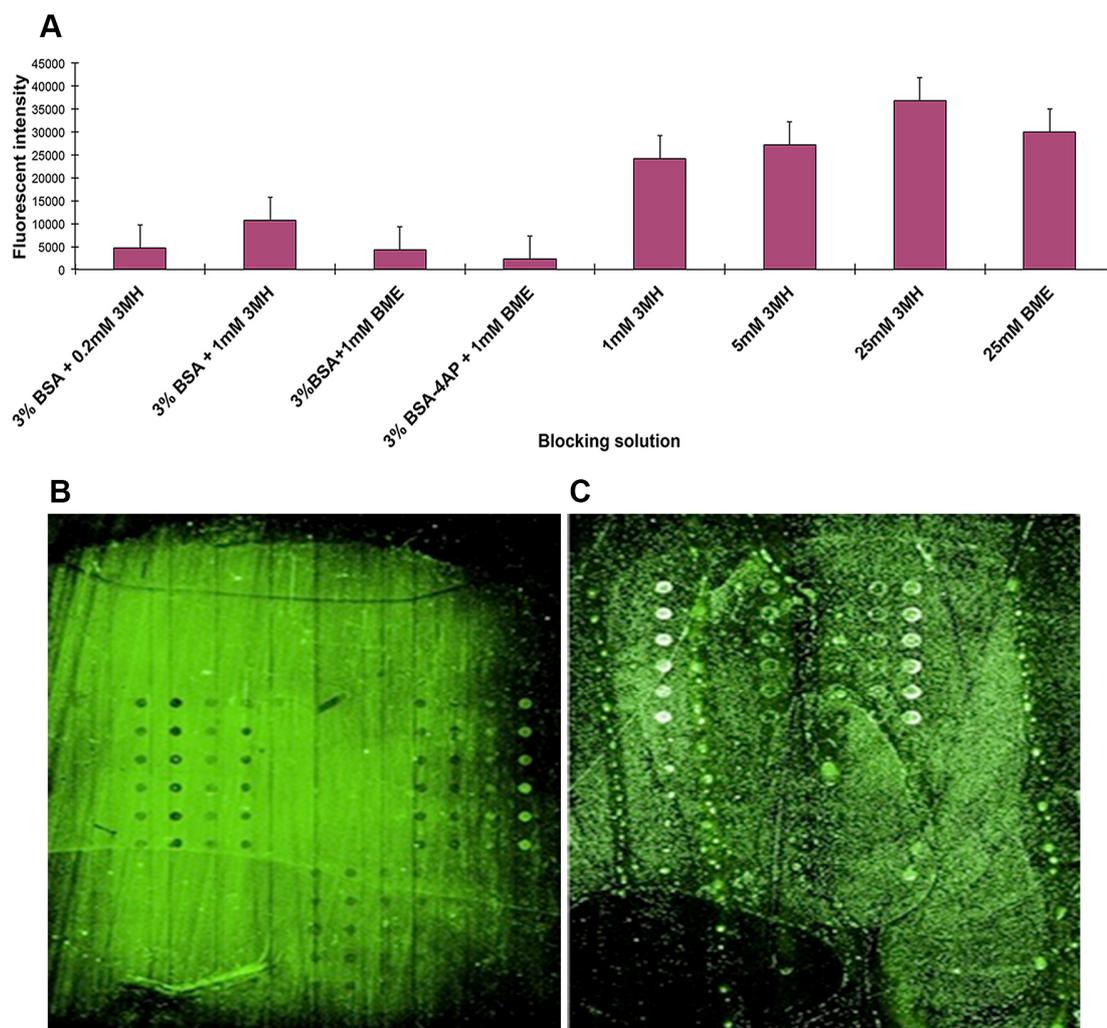


Figure 5.4 Testing of blockers to reduce non-specific binding to poly-L-lysine slide. A) Histogram depicts the mean background intensity of three subarrays after blocking with a range of chemicals (3MH-mercaptohexanol, BME-beta mercaptoethanol). NCG array images showing high level of binding by labeled *C. jejuni* to slide background after blocking with B) 25 mM mercaptohexanol, and C) 3% BSA + 1 mM mercaptohexanol.

5.2.4 Change of slide chemistry: SCHOTT Nexterion® Slide H

The coating on the Nexterion® Slide H consisted of a cross-linked, multi-component polymer layer activated with *N*-hydroxysuccinimide (NHS) esters to provide covalent immobilization of amine groups. The Nexterion® Slide H coating has been engineered

to exhibit a very low intrinsic non-specific background without the need for blocking with BSA (Fig. 5.5).

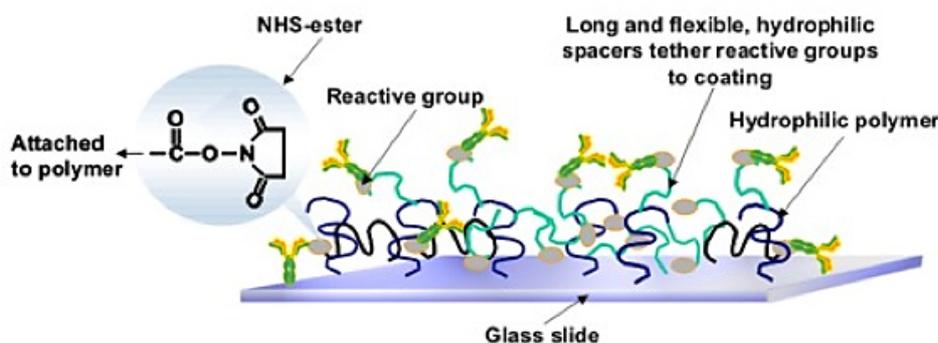


Figure 5.5 SCHOTT Nexterion® Slide H surface chemistry (schott.com)

Due to the change of slide chemistry, immobilisation of the NGCs was then through amide linkage, as opposed to the involvement of sulfhydryl groups in binding to the poly-L-lysine slide.

Neoglycoconjugate microarray v.2 was printed to include oligosaccharides relevant to human cell surface expression, including histo-blood group antigens and milk oligosaccharides. The carbohydrates were covalently attached to human- and bovine-serum albumin backbones to facilitate attachment to the array surface and also to allow a more natural three-dimensional presentation of the sugars (Table 2.7). Furthermore, 4AP-BSA, consisting of unmodified linker attached to a protein backbone, and PBS were spotted as negative controls. The NGC was validated with binding experiments using TRITC-conjugated lectins. Figure 5.6 represents binding of lectins MAA and AIA, at 20 and 15 $\mu\text{g ml}^{-1}$ in TBS-T, respectively. The NGC array data agreed with the known binding specificity of MAA for terminal $\alpha(2,3)$ -linked Neu5Ac, in particular to that substituted on $\text{Gal}\beta(1,3/4)\text{GlcNAc}$, with high rates of binding to 3'Sialyl-LacNAc-BSA [3SLNBSA: Neu5Ac α 2,3Gal β 1-4Glc] and sialyl-lacto-N-neotetraose-penta-APD-HSA [SLNnTHSA: Neu5Ac α 2-3Gal β 1,4GlcNAc β 1,3Gal β 1,4Glc], but also to lacto-N-neotetraose [LNnTHSA: Gal β 1-4GlcNAc β 1,3Gal β 1,4Glc], which does not contain Neu5Ac, but does have the Gal β 1,4GlcNAc disaccharide. A striking example of the specificity of MAA was the

strong interaction with sialylated fetuin, compared to low intensity interaction with asialofetuin (ASF).

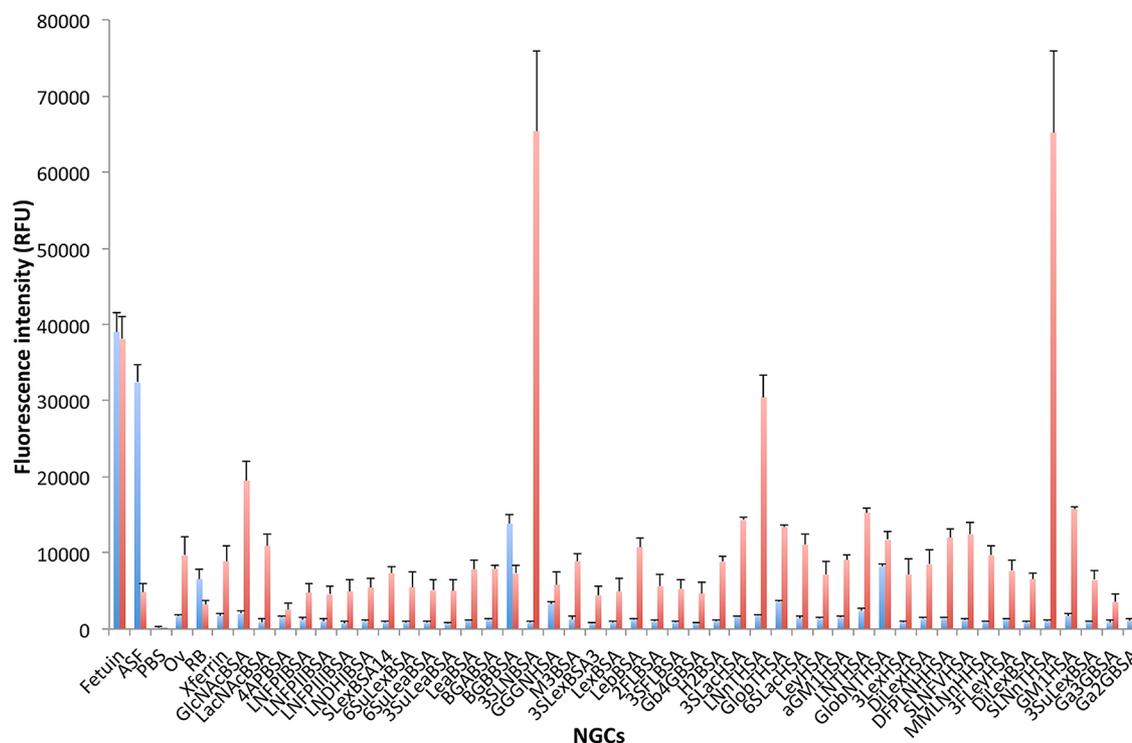


Figure 5.6 Histogram representing the mean fluorescence intensities resulting from incubation of two replicate NGC microarray slides with lectins AIA (Blue: *Artocarpus integrifolia* agglutinin, specific for galactose) and MAA (Red: *Maackia amurensis* agglutinin, specific for $\alpha(2,3)$ -linked sialic acids). Error bars depict standard deviation of the mean of four replicate microarray slides. NGCs are listed in table 2.7.

The lectin AIA, which is specific for terminal β -linked Gal and has highest affinity for Gal $\beta(1,3)$ GalNAc, bound at high rates to fetuin and ASF, which have *N*-glycans containing terminal Gal residues, and also bound at lower rates to Blood Group B-BSA [BGBBSA: Gal β 1-3(Fuc α 1,2)Gal], which also contains terminal Gal. However AIA did not bind to other Gal containing glycans such as Ga2GBSA [Gal α 1,2Gal-BSA] or Ga3GBSA [Gal α 1,2Gal-BSA], indicating the lectins preference for β -linked Gal as well as potential slide or linker influences, or involvement of other glycan residues in binding of AIA.

Overall, the pattern of lectin binding was highly reproducible, with an average % coefficient of variation (CV) of 19% for both lectins, which is on the lower end for protein-based microarrays (Guilleaume *et al.*, 2005). As technical controls for each microarray in all subsequent bacterial incubation experiments, two subarrays were incubated with each of these lectins to confirm that all NGCs were printed correctly and were available for binding interactions with bacteria.

5.2.5 Analysis of *C. jejuni* 81-176 and 81116 at 37 and 42°C

Following incubation of bacteria with the array, consistent low background intensity was detected (Fig. 5.7: average 362.7 RFU), facilitating observation and extraction of the binding intensity signals. After applying the threshold of four times average local background (Fig. 5.8: 1451.5 RFU), the highest level of binding for both strains was seen with α -crystallin, a major glycoprotein in the bovine lens. However, binding to this component was probably due to electrostatic effects due to the high levels of phosphorylation on the α -crystallin molecule (Spector *et al.*, 1985), rather than a specific molecular recognition event.

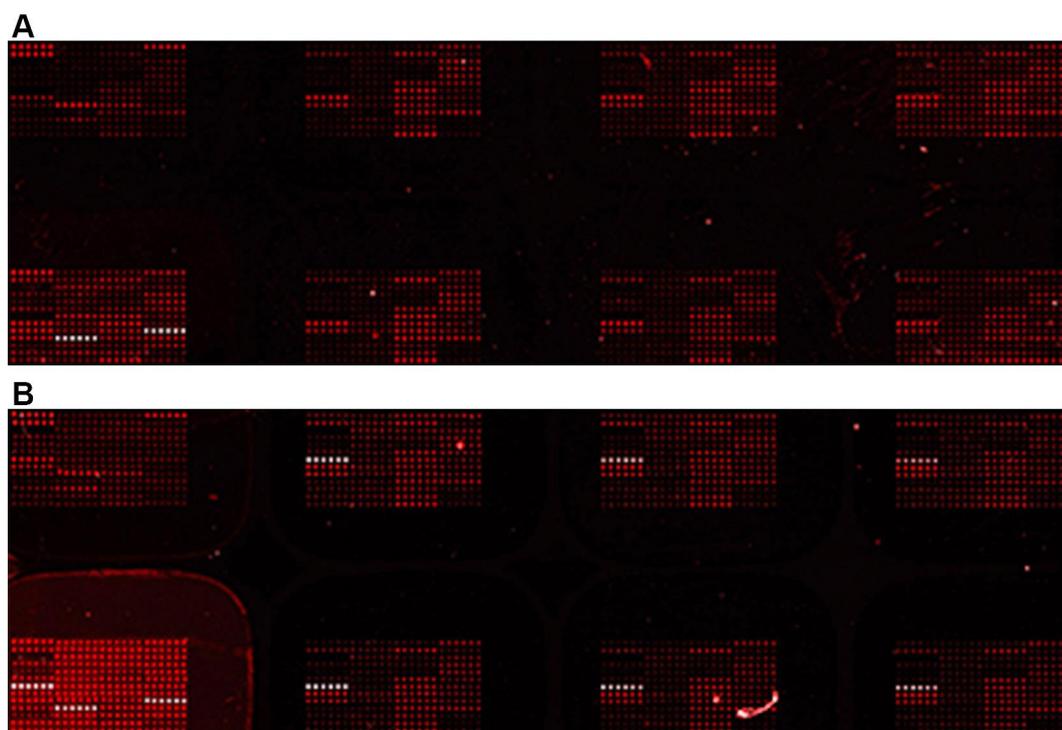


Figure 5.7 False-colored, fluorescence images of representative NGC arrays after incubation with *C. jejuni* A) 81116, and B) 81-176. 8 sub arrays incubated with clockwise bottom left Tritc-conjugated lectins AIA and MAA, 37°C controls and inhibitory sugars.

The NGC bound at the highest rate by both strains at 37°C was lacto-*N*-tetraose-APD-HSA [LNTHSA: Gal β 1,3GlcNAc β 1-3Gal β 1-4Glc], with binding intensities of 5837.6 and 6073.5 RFUs, for 81116 and 81-176, respectively. However, for both strains at 42°C, 3'-sialyllactose-APD-HSA [3SLacHSA: Neu5Ac α 2,3Gal β 1,4Glc] was bound at the highest rate, with binding intensities of 5920.1 and 5595.9 RFUs, for 81116 and 81-176, respectively.

While there were some subtle differences between 81-176 and 81116 when grown at both 37 and 42°C, the overall binding profile of both strains at both temperatures was similar. Unsupervised clustering analysis using the NGC binding data for the two strains at the two temperatures was unable to distinguish strains from each other (Fig. 5.9 A).

However, comparison of the strains revealed that a number of NGCs were bound at higher rates by 81-176 than 81116, however there were no structures bound by 81116 at a higher rate than 81-176. Two structures (H2BSA and LeyBSA) were bound at a significantly higher rate by 81-176, at both 37 and 42°C. The structures GlcNAcBSA, LebBSA, 2FLBSA, GlobNTHSA, SLNnTHSA DiLexBSA were bound at a significantly (Student's t-test; unpaired, two-tailed) higher rate by 81-176, compared to 81116, at 37°C only. There were no structures bound at significantly different rate between the strains at 42°C.

Comparing the influence of growth at 37 and 42°C on glycan binding by individual *C. jejuni* strains, strain 81116 demonstrated no statistically significant difference between binding at 37 and 42°C. However, 81-176 bound four NGCs (GlcNAcBSA, BGABSA, 2FLBSA, aGM1BSA) at a significantly higher ($p < 0.05$) rate at 37°C compared to 42°C, which may indicate a host-related change in glycan binding. Clustering analysis carried out on individual strains at 37 and 42°C did not reveal any clear difference between the strains at both temperatures (Fig 5.9, B & C).

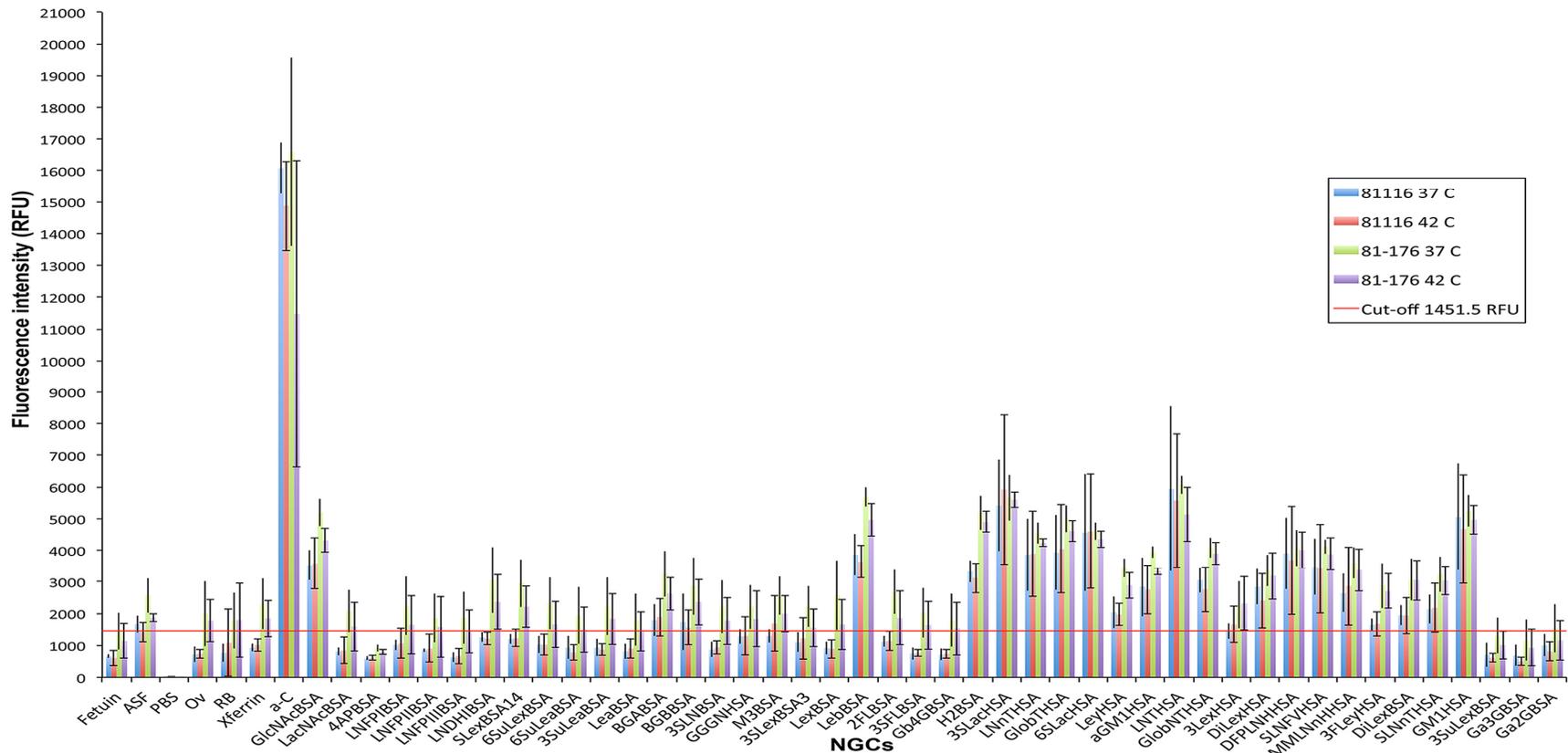


Figure 5.8 Histogram representing differences in binding of *C. jejuni* strains 81116 (blue and red) and 81-176 (green and purple), grown at 37 and 42°C, respectively, to printed NGCs. Histograms represent the mean of two biological replicate experiments, each experiment done in duplicate. Error bars depict one average deviation of the mean of the two experiments. Data from each microarray is the median of six data points. Cut-off for binding was calculated as four times the average local background intensity.

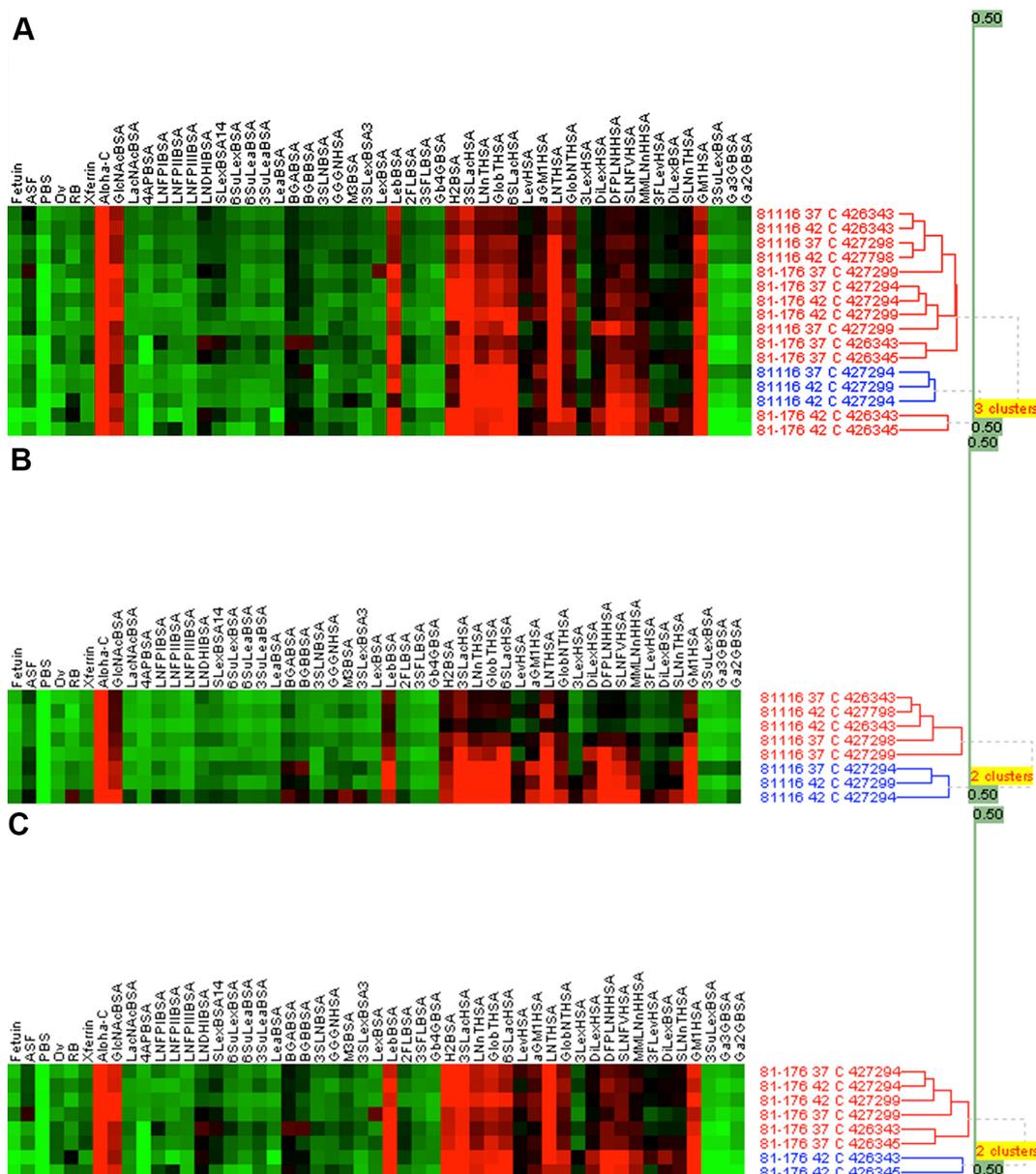


Figure 5.9 Comparison of NGC array data for A) 81116 and 81-176 at 37 and 42°C, B) 81116 at 37 and 42°C, and C) 81-176 at 37 and 42°C. Clustering was done with total intensity mean normalisation, complete linkage, Euclidean distance and all 52 probes printed (including controls) were included.

To verify carbohydrate-mediated attachment of *C. jejuni* to NGCs, co-incubation with 100 mM Gal, Man, Fuc and GlcNAc was carried out. With 81-176 there were a number of cases where the presence of monosaccharide resulted in modest reductions in adherence to NGCs, when grown at 42°C only. Co-incubation with Fuc resulted in

inhibition of binding of 81-176 DiLexHSA (24.5% reduction, $p = 0.039$), SLNFVHSA (34.6% reduction, $p = 0.029$) and MMLNnHHSA (27.3% reduction, $p = 0.02$), which were NGCs containing Fuc as part of their structure. Furthermore, co-incubation with Gal at 42°C reduced adherence of 81-176 to 3SLacHSA (21.3% reduction, $p = 0.04$), MMLNnHHSA (17.5% reduction, $p = 0.039$) and SLNnTHSA (12.9% reduction, $p = 0.037$), which contain Gal in the oligosaccharide backbone, thus potentially indicating the involvement of the particular sugar in adherence to these NGCs at these growth temperatures. However, the presence of monosaccharides at 100 mM concentration did not reduce binding by *C. jejuni* to the NGCs in the majority of cases. No reduction in adherence was observed for 81116 when co-incubated with monosaccharides.

The similarity of the NGC data indicates a common glycan binding affinity for these *C. jejuni* strains, and provided some insights into the nature of glycan binding by this pathogen. *C. jejuni* bound to histo-blood groups and related antigens presented on the array. Both strains bound at the highest intensity to the type-I structure Le^b-BSA [LebBSA: Fuc α 1,2Gal β 1-3(Fuc α 1-4)GlcNAc] at both temperatures, and also bound to Le^y-BSA [LeyBSA: Fuc α 1,2Gal β 1,4(Fuc α 1,3)GlcNAc], a type-II structure. *C. jejuni* did not bind with Le^a-BSA [LeaBSA: Gal β 1,3(Fuc α 1,4)GlcNAc], monomeric Le^x-BSA [LexBSA: Gal β 1,4(Fuc α 1,3)GlcNAc], or either sulfated or sialylated Le^b structures. However, binding of both strains to divalent Le^x-BSA and DiLexHSA did occur. Strain 81-176 bound at both temperatures to BGABSA and BGBBSA, while 81116 did not. Both strains bound to the H-antigen [H2BSA: (Fuc α 1,2)Gal β 1,4GlcNAc], but not 2'Fucosyllactose-BSA [2FL-BSA: Fuc α 1,2Gal β 1,4Glc], which are related structures containing a terminal α (1,2)-linked Fuc, and differ only in that H2-BSA has a GlcNAc at the reducing end of the backbone disaccharide, whereas 2FL-BSA contains a Glc. This demonstrates the influence of subterminal sugar residues on binding, and the difference in binding may be due to the presence of the GlcNAc in H2-BSA. Consistent with an involvement of GlcNAc in *C. jejuni* binding, high rates of binding to GlcNAc-BSA on the array were observed for all bacterial samples (Fig. 5.8).

Sialylation of NGCs was generally associated with an increase in binding intensity by all *C. jejuni* strains, in particular strain 81116 where three of the four NGCs bound at highest intensity at both 37 and 42°C, were sialylated structures. Both strains

bound at a higher intensity to 3SLacHSA, and the $\alpha(2-3)$ -linked Neu5Ac containing ganglioside GM₁-APD-HSA [GM1HSA:

Gal β 1,3GalNAc β 1,4(Neu5Ac α 2,3)Gal β 1,4Glc], compared to 6'-sialyllactose-APD-HSA [6SLacHSA: Neu5Ac α 2,6Gal β 1.4Glc]. Binding by both strains to GM1HSA (containing a $\alpha(2,3)$ -linked Neu5Ac), was significantly higher ($p < 0.05$) than to the non-sialylated variant, asialo-GM₁ [Gal β 1,3GalNAc β 1,4Gal β 1,4Glc].

5.3 Discussion

The glycan microarray has become an important tool in recent years, for the robust high-throughput screening of carbohydrate-protein interactions (Oyelaran & Gildersleeve, 2009). The format enables a parallel analysis of multiple binding interactions unrivalled for efficiency in use of reagents and analytes. A range of glycan-containing macromolecules, and array surface chemistries, has been used in the display of carbohydrates on microarray slides. This study demonstrates the development of an array for use in *C. jejuni* glycan-binding analysis.

Two slide chemistries were compared in their suitability for analysis of bacterial binding interactions. Initially, a poly-L-lysine slide was assessed, which would have provided a very cost-effective platform. However unacceptable levels of background noise, and non-specific binding suggested that this slide was unsuitable. In comparison, the 3D hydrogel coated slide had consistently low levels of background, and showed reproducible levels of binding of control lectins. Therefore, the hydrogel slide was selected as the most suitable substrate on which a panel of 52 NGC molecules and controls, representing part of the human glycome, was printed. Contrastingly, in another study it was found that the 2D slide surface exhibited lower background than 3D slides in binding of antigen to an antibody array (Seuryneck-Servoss *et al.*, 2008), indicating that slide performance varies depending on the nature of the ligand/analyte.

The NGC array generated a binding profile for *C. jejuni* 81-176 and 81116 that was similar for both strains indicating common glycan binding affinities for the strains. Unsupervised clustering analysis was unable to clearly differentiate between the strains at both temperatures. However, eight NGCs were bound at significantly higher rate by 81-176 than 81116. These higher rates of glycan binding may reflect the significantly

higher rates of virulence associated with strain 81-176 compared to 81116 (Hofreuter *et al.*, 2006; Fearnley *et al.*, 2008). Strain 81116 is known to have relatively low adhesion and invasiveness of epithelial cells *in vitro* (Fearnley *et al.*, 2008), whereas strain 81-176 is considered a hyper-invasive strain with corresponding high levels of epithelial cell adherence (Hofreuter *et al.*, 2006). Comparative genetics of hyper-invasive and low invasive *C. jejuni* strains revealed only two candidate genes, which may be associated with the invasive phenotype (Fearnley *et al.*, 2008). Potentially, characteristics such as higher rates of host glycan binding may contribute to a more virulent phenotype.

The effect of growth at 37 and 42°C on glycan binding was compared in order to shed light on possible host-related adaptations. Although some clustering of replicate arrays was observed, clustering analysis could not clearly differentiate between the strains when grown at these temperatures. With strain 81116 there were no NGCs, which were bound at a significantly different rate between growth temperatures. Four NGCs were bound by 81-176, at a significantly higher rate at 37 than at 42°C, potentially indicating a strain-specific host-related change in glycan binding. It is believed that host-pathogen interactions have exerted an evolutionary pressure responsible for the species-specific differences in the glycan repertoire of different animals (Varki, 1993). The preference of this strain at 37°C for these NGCs presenting human glycosylation motifs may suggest that if the array included glycans that reflected structures originating from the chicken gut, greater temperature-related influences would have been observed.

Competition assays involving co-incubation of *C. jejuni* were performed with a selection of monosaccharides representing some of the building blocks of the glycans presented on the printed NGCs. Through competitive monosaccharide inhibition bacteria would adhere to the monosaccharide using specific receptors, thereby reducing the number of free receptors able to bind to corresponding sugar residues on the NGCs, thus providing confirmation of the glycan-binding specificity. Furthermore, any influence of co-incubation on bacterial binding, was not expected to be due to metabolic reasons, since these particular *C. jejuni* strains are asaccharolytic (Muraoka & Zhang, 2011). However, co-incubation resulted in only modest reductions in adherence to a small number of NGCs, but in the majority of cases monosaccharides had little or no influence on binding, despite the presence of 100 mM of monosaccharide, which is a

considerable concentration (Gerlach *et al.*, 2011). The affinity of glycan binding proteins including bacterial adhesins is typically in the millimolar to micromolar range, but avidity is increased through multivalent interactions (Krishnamoorthy & Mahal, 2009). One advantageous technical aspect of the glycan array is the ability for the multivalent display of glycans replicating the presentation of carbohydrates on a cell-surface. Therefore, the general inability of monosaccharides to inhibit *C. jejuni* adherence may reflect that bacterial affinity for the multivalent NGC oligosaccharide was greater than for the competitive monosaccharide.

Both *C. jejuni* strains bound to fucosylated NGCs on the array, however binding occurred primarily with NGCs containing a terminal $\alpha(1,2)$ -linked Fuc residue, in particular Leb-BSA, H2-HSA, Ley-HSA, and DFPLNH-HSA (Fig. 5.8). One exception was strain 81-176 which bound at low but detectable rates to BGA-BSA and BGB-BSA which contain subterminal $\alpha(1,2)$ -linked Fuc. This correlates with a study carried out by Cervantes *et al.* (1995) suggested *C. jejuni* has particular affinity for the $\alpha(1,2)$ fucosyl motif. *C. jejuni* did not bind well with other fucosylated NGCs on the array containing subterminal Fuc and Fuc with $\alpha(1,3/4)$ -linkage.

The H-antigen is widely distributed in humans, and its presence is dependent on the expression of either of two fucosyltransferases (*FUT*), *FUT1* (H) on red blood cells, or *FUT2* (Se) on exocrine secretions and intestinal mucosa (Reguine-Arnould 1995; Bry 1996). *C. jejuni* bound avidly to Chinese hamster ovary cells (CHO) which were transfected with human *FUT2* and expressed the H-2 antigen, whereas binding to untransfected cells was not observed (Ruiz-Palacios *et al.*, 2003). Adherence was inhibited using $\alpha(1,2)$ -specific monoclonal antibodies, fucose-specific lectins (UEA-I and LTA), as well as H-2 mimetics, such as a H-2 neoglycoprotein, 2'-fucosyllactose and fucosylated human milk oligosaccharides (Ruiz-Palacios *et al.*, 2003). This elaborate study strongly presented the H-2 antigens of epithelial cells as the primary host cell receptors for *C. jejuni* adherence. The NGC array data in present study, also highlights preference of *C. jejuni* for $\alpha(1,2)$ -linked Fuc.

Human milk is a rich source of the neutral and acidic glycans including gangliosides which were bound by *C. jejuni* on the array. Also in Ruiz-Palacios *et al.* (2003), transgenic mouse dams transfected with human *FUT1* expressed

oligosaccharides consisting of the human H-2 antigen in their milk, provided mouse pups with significant protection against experimental *C. jejuni* colonisation. Furthermore, the fucosylated oligosaccharides in human milk inhibited *C. jejuni* adhesion to the H-2 antigen in a solid phase assay, and prevented adherence to Hep-2 cells and colonisation of mice *in vivo* (Ruiz-Palacios *et al.*, 2003). Recently, Lane *et al.* (2012) have demonstrated inhibition of *C. jejuni* adherence through the use of bovine colostrum oligosaccharides.

In addition to fucosylated NGCs on the array, *C. jejuni* also bound to sialylated NGCs including ganglioside GM₁-HSA. Human milk contains large amounts of gangliosides, which are essential for development of intestinal microbiota in newborns (Salcedo *et al.*, 2013). Previously, co-incubation and pre-treatment with gangliosides such as GM₁ were found to prevent *C. jejuni* attachment to Caco-2 cells (Salcedo *et al.*, 2013). Overall, these studies highlight the significant role of milk oligosaccharides in infant protection against microbial pathogens such as *C. jejuni*. The current study demonstrates the successful development of an NGC microarray that has potential for identifying strain-specific anti-adhesive prophylactics, such as milk oligosaccharides.

Chapter 6.
***Campylobacter jejuni* strain discrimination and
temperature-dependent glycome expression
profiling by lectin microarray**

Michelle Kilcoyne,¹ Marcus E. Twomey,² Jared Q. Gerlach,¹ Anthony P. Moran,^{2,†}
Lokesh Joshi¹

¹ Glycoscience Group, National Centre for Biomedical Engineering Science, National
University of Ireland, Galway, Ireland

² Microbiology, School of Natural Sciences, National University of Ireland, Galway,
Ireland

† Prof. Anthony P. Moran, RIP 1960 – 2010.

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6.1 Introduction

Gram-negative *C. jejuni* is the leading cause of bacterial gastroenteritis in humans worldwide and is the most frequently identified antecedent infection associated with GBS, the debilitating autoimmune neuropathy characterised by progressive paralysis (Allos, 2001; Hughes & Cornblath, 2005). Consequently, *C. jejuni* infection represents a significant health and economic burden worldwide (Buzby & Roberts, 1997). While *C. jejuni* is pathogenic in humans, it is a commensal in many avian hosts and colonises in high numbers asymptotically (Young *et al.*, 2007).

Consumption of contaminated food products, particularly poultry, is the main risk factor for infection (Young *et al.*, 2007). The mechanisms which lead to infection or persistent colonisation of *C. jejuni* in humans or chickens, respectively, are poorly understood. *C. jejuni* displays an extensive array of cell-surface glycoconjugates, having distinct biosynthetic loci for the expression of LOS/ LPS, CPS, as well as protein *O*- and *N*-linked glycosylation (Fry *et al.*, 2000; Karlyshev *et al.*, 2005a; Guerry & Szymanski, 2008). As virulence factors, *C. jejuni* LOS and CPS play a role in adhesion and invasion of host cells and protection from host immune defences (Bacon *et al.*, 2001; Guerry *et al.*, 2002; Logan *et al.*, 2002). Flagellar *O*-glycosylation (Logan *et al.*, 2002; Guerry, 2007) and *N*-linked surface protein glycosylation (Karlyshev *et al.*, 2005) (Fig. 6.1), have also been identified as important virulence factors (Young *et al.*, 2007). Antigenic variability of the cell surface glycoconjugates (Guerry *et al.*, 2002; Bacon *et al.*, 2001; Logan *et al.*, 2002), as well as structural mimicry of host glycans in the LOS of certain *C. jejuni* strains (Prendergast *et al.*, 2004), constitute immune evasion strategies. In particular, molecular mimicry of gangliosides in *C. jejuni* LOS is thought to elicit the production of cross-reactive anti-ganglioside antibodies which are involved in GBS pathogenesis (Hughes & Cornblath, 2005).

Considerable intra-species variation can exist within *C. jejuni* (Young *et al.*, 2007). The extensively studied, genome-sequenced strains *C. jejuni* 81-176 and 81116, originally isolated from human hosts, are genetically and phenotypically distinct (Hofreuter *et al.*, 2006; Pearson *et al.*, 2007). The highly virulent 81-176 strain, which belongs to serogroup HS:23/36 and was originally isolated in a raw milk-borne case of colitis, is hyper-invasive to intestinal cells *in vitro* (Hofreuter *et al.*, 2006). It produces

two independent CPSs, one of which is phase variable (Bacon *et al.*, 2001; Papp-Szabo *et al.*, 2005) (Fig. 6.1), and phase variable gene expression enables the potential production of LOS structures which mimic the gangliosides GM₂, GM₃, GD_{1b} and GD₂ (Guerry *et al.*, 2002). *C. jejuni* 81116 types as both HS:6 and HS:7, was first isolated in a waterborne case of gastroenteritis and has comparatively low invasiveness (Palmer *et al.*, 1983; Fearnley *et al.*, 2008). This strain was found to express two independent polysaccharides, one LPS-related and the other CPS-derived (Muldoon *et al.*, 2002; Kilcoyne *et al.*, 2006) (Fig. 6.1), and may also express an LOS-like molecule (Holden *et al.*, 2012).

Among the many physiological differences between the human and chicken host environment, core body temperature (37 and 42°C in humans and chickens respectively) is known to affect *C. jejuni* genetically and phenotypically (Young *et al.*, 2007). Differential transcription of numerous *C. jejuni* genes, including genes involved in modulation of the cell surface glycosylation, have been observed due to temperature shift from 37 to 42°C (Stintzi, 2003). Temperature-related variation of *C. jejuni* LOS and polysaccharide expression has been described previously (Corcoran & Moran, 2007; Semchenko *et al.*, 2010), and *C. jejuni* chemotaxis and binding to host-related structures was shown to be influenced by growth at host physiological temperatures (Khanna *et al.*, 2006; Day *et al.*, 2009). Therefore, temperature may provide an important trigger for specific host and environmental adaptation in *C. jejuni*, with commensalism or pathogenesis as potential outcomes (Young *et al.*, 2007).

Lectins, non-enzymatic proteins of non-immune origin which bind specifically to carbohydrates, are frequently used as an analytical tool to detect and identify specific carbohydrate moieties associated with certain cell types or stages of differentiation (Gerlach *et al.*, 2011). While not fully characterised for bacterial-type glycosylation, plant lectins have been used as a profiling tool for microbes for identification or differentiation between strains, usually in the context of simple and cheap agglutination assays (Yakovleva *et al.*, 2011). The stability of plant lectins during storage and their ready commercial availability makes them a good reagent choice (Aabenhus *et al.*, 2002). When adapted to a microarray format (Hsu & Mahal, 2006), lectins can provide a useful platform for profiling differences between bacteria in a higher throughput manner than traditional plate assays, and quantitative readouts can facilitate more

straightforward comparison between strains and help identify the classes of glycoconjugates responsible for any alterations. Lectin microarrays have been recently employed to discriminate populations of glycoprotein glycoforms (Gerlach *et al.* 2014), distinguish the differentiation stages of human embryonic and induced pluripotent stem cells (Toyoda *et al.* 2011), and profile different bacterial species (Gao *et al.*, 2010).

In this work, the two pathogenic *C. jejuni* strains 81116 and 81-176 were cultured at the temperatures of 37 and 42°C and profiled on a lectin microarray to investigate temperature-related glycosylation alterations at the cell surface microenvironment. The strains were differentiated from one another on the basis of the microarray profiles and culture temperatures were discriminated for both strains using careful selection of subsets of lectin data demonstrating significant and stable differences between strains and conditions. Different classes of glycoconjugates were implicated as the major accessible carbohydrate determinants in the two strains. The lectin interaction profiles of the strains were compared between the microarray and traditional agglutination assay platforms and contrary behaviour of the lectins was noted in several cases. The lectin microarray platform was shown to be a useful tool for in profiling global glycomic changes in response to host and environmental changes.

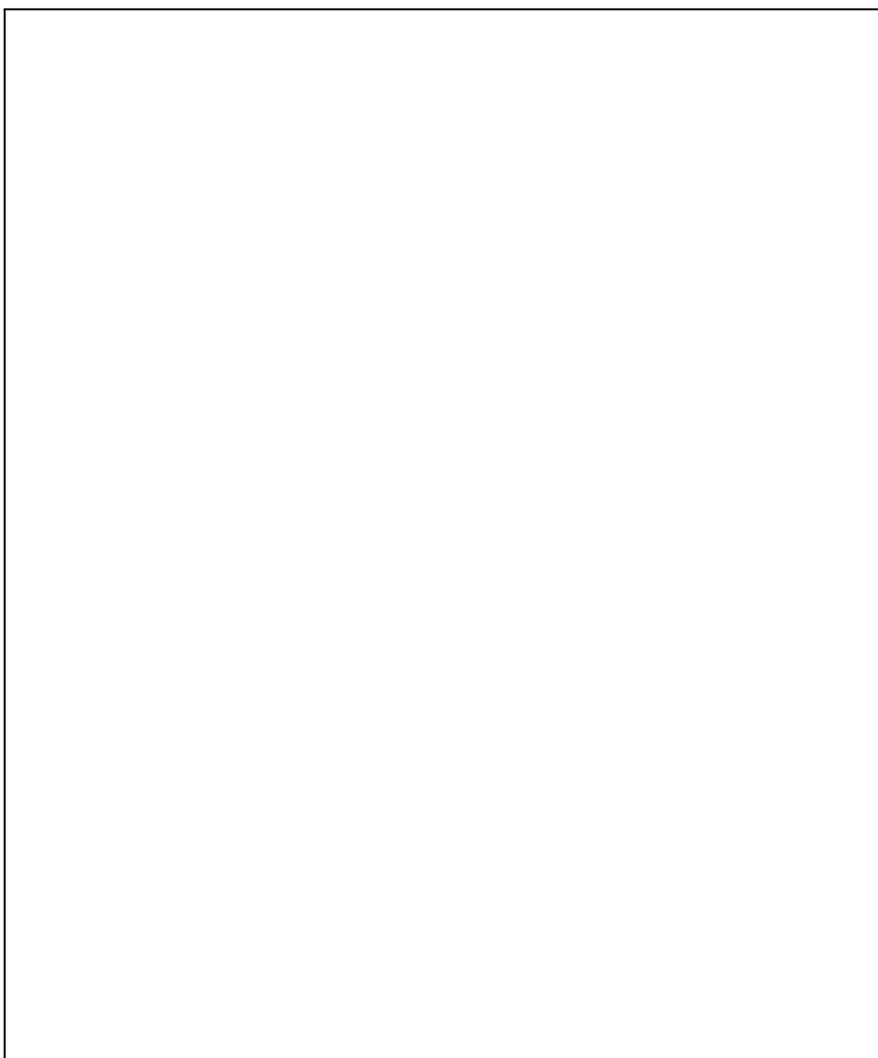


Figure 6.1 Polysaccharide structures from capsular (CPS) and lipopolysaccharide (LPS) from *C. jejuni* 81116 (Muldoon *et al.*, 2002; Kilcoyne *et al.* 2006), CPS^{13, 60} and predominant 3.6 and 3.8 kDa lipooligosaccharide (LOS) core structures from *C. jejuni* 81-176 (Aspinall *et al.*, 1992; Papp-Szabo *et al.*, 2005). The Gal residue of *C. jejuni* 81-176 can be substituted by non-stoichiometric amounts of *O*-methyl phosphoramidate (MeOPN) at C-2 (Kanipes *et al.*, 2006). **A.** GM₂-like predominant component of 3.8 kDa LOS core and minor component of 3.6 kDa LOS core. **B.** GM₃-like predominant component of the 3.6 kDa LOS core. The conserved *N*-linked glycan structure (Karlyshev *et al.*, 2005) of *C. jejuni* is depicted at the bottom of the figure, where Bac is bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucopyranose) and is linked to asparagine. Figure has been removed due to copyright restrictions

6.2 Results

6.2.1 Lectin microarray analysis of two strains at human and avian temperatures

The lectin microarrays were constructed to include lectins previously reported to differentiate between strains of *Campylobacter* spp., namely WGA, Con A, MAA, Lch-B, BPA, ECA, PNA, STA and WFA, (Wong *et al.*, 1985; O'Sullivan *et al.*, 1990; Aabenhus *et al.*, 2002; Yakovleva *et al.*, 2011) in addition to thirty-two others for a total of forty-one lectins (Table 2.11). Incubation of *C. jejuni* strains 81116 and 81-176 grown at 37 and 42°C showed good feature coverage and different lectin microarray profiles for the strains at each temperature (Fig. 6.2 & 6.3 A), which implied that different carbohydrate structures were interacting with the lectins. The profiles of strain 81116 varied greatly between temperatures but the differences between temperatures of strain 81-176 were more subtle. In addition, there was considerable variability in the responses of the lectins PA-I, BPA, WFA and CAA between biological replicates of strain 81116 at 37°C in contrast to the greater consistency of all other lectin responses and the generally very high consistency between all other biological replicates (Fig. 4A).

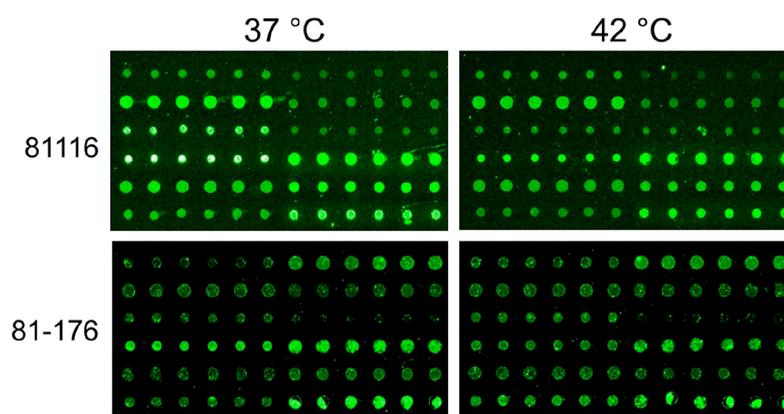


Figure 6.2. Representative scan of the same segment of lectin microarray subarrays incubated with fluorescently labelled *C. jejuni* strains 81116 and 81-176 cultured at 37 and 42°C.

Lectins bind specifically to distinct carbohydrate moieties but may also contain one or more non-carbohydrate ligand binding sites (Borondes, 1988) and thus, carbohydrate-mediated binding must be confirmed by inhibition of binding in the presence of haptenic sugars (Gerlach *et al.*, 2011; Kilcoyne *et al.*, 2012a; b). Co-incubation with Gal inhibited binding to SNA-II, LEL and RCA-I, and GlcNAc inhibited binding to LEL for strain 81116 at both temperatures and to SNA-II and RCA-I at 42°C (Fig. 6.4 A and B). In addition, binding to CAA at 42°C was inhibited by both Gal and GlcNAc (Fig. 6.4 B). The binding of strain 81-176 at both growth temperatures to WGA and LEL was inhibited with GlcNAc, AAL and RCA-I with Fuc and RCA-I with Gal (Fig. 6.6 A and B). Galactose also reduced bacterial binding to SNA-I and AAL at 37°C for strain 81-176 (Fig. 6.6 A). The ‘cross-inhibitability’ of typically Fuc-binding AAL with Gal and Gal-binding RCA-I with Fuc is perhaps not surprising considering that Fuc is in the *galacto* configuration (Klein *et al.*, 2000). Except for the cases mentioned, lectin microarray profiles of the *C. jejuni* strains remained similar to uninhibited profiles (Figs. 6.4 & 6.6). Co-incubation with Man did not inhibit binding of either strain at either temperature (data not shown). In particular, it was interesting to note that the interaction of both bacterial strains with the lectin ACA was non-carbohydrate-mediated at both temperatures (Figs. 6.4 & 6.6).

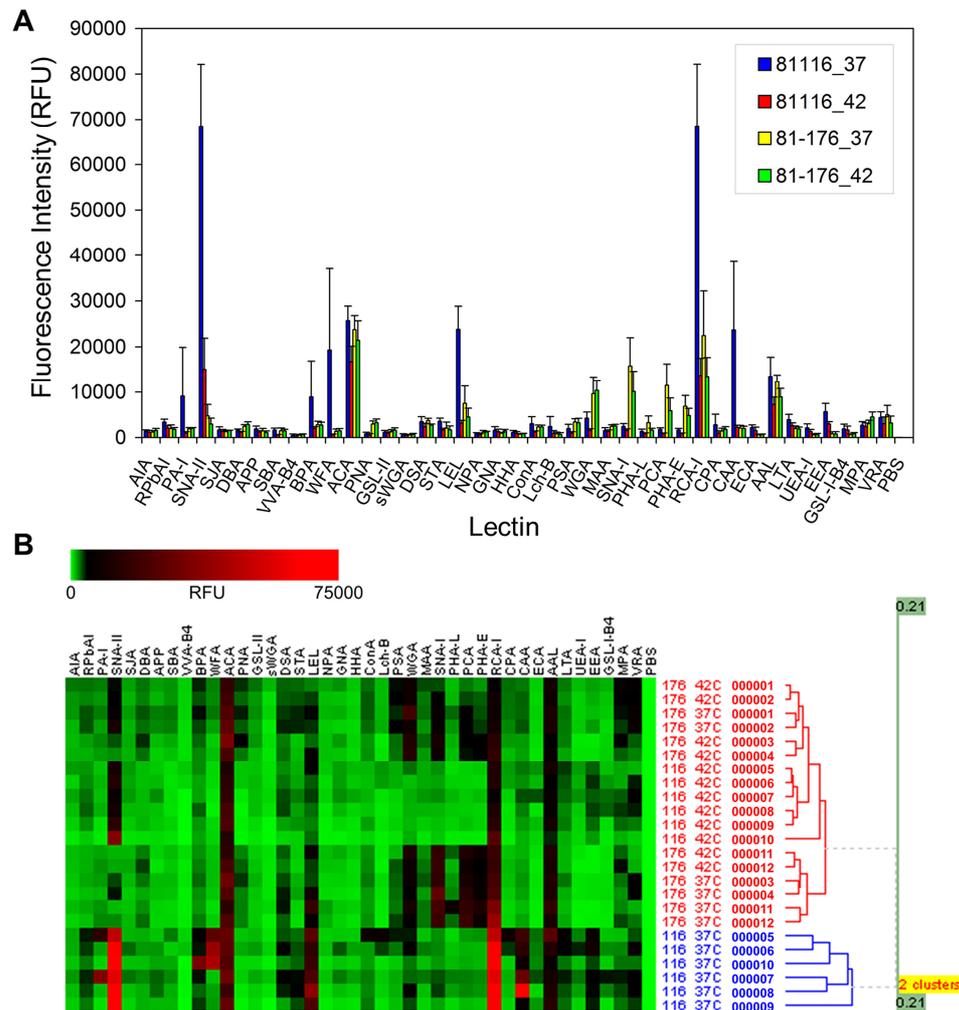


Figure 6.3 (A) Histograms representing the differences in recognition of printed lectins by *C. jejuni* strains 81116 (blue and red) and 81-176 (yellow and green) grown at 37 and 42°C (respectively). Histograms represent the mean of three biological replicate experiments, each experiment done in duplicate, and error bars are one average deviation of the mean of the three experiments. Data from each microarray is the median of six data points. **(B) Comparison of lectin microarray data for strains 81116 (designated 116) and 81-176 (designated 176) grown at 37 and 42°C.** Clustering was done with total intensity mean normalisation, complete linkage, Euclidean distance and all 42 probes printed (41 lectins and PBS) were included. Individual replicates (data points) were labelled in the format 'strain_temperature_experiment number'.

In terms of carbohydrate-mediated binding, *C. jejuni* strain 81116 at 37°C bound to lectins characterised to have affinity for mammalian glycosylation motifs containing Gal and/or GalNAc (PA-I, EEA, SNA-II, BPA, WFA), GlcNAc (LEL) and lactose (RCA-I and CAA) (Table 2.11 and Fig. 6.4) which are residues present in the LPS-like polysaccharide structure (Fig. 6.1). The structure previously elucidated for the CPS of strain 81116 at 37°C contains Man and Glc residues (Muldoon *et al.*, 2002; Kilcoyne *et al.*, 2006), but typical Man or Glc recognising lectins such as GNA bound with low intensity, which suggested that the CPS of this strain did not take part in binding to the microarray (Fig. 6.1).

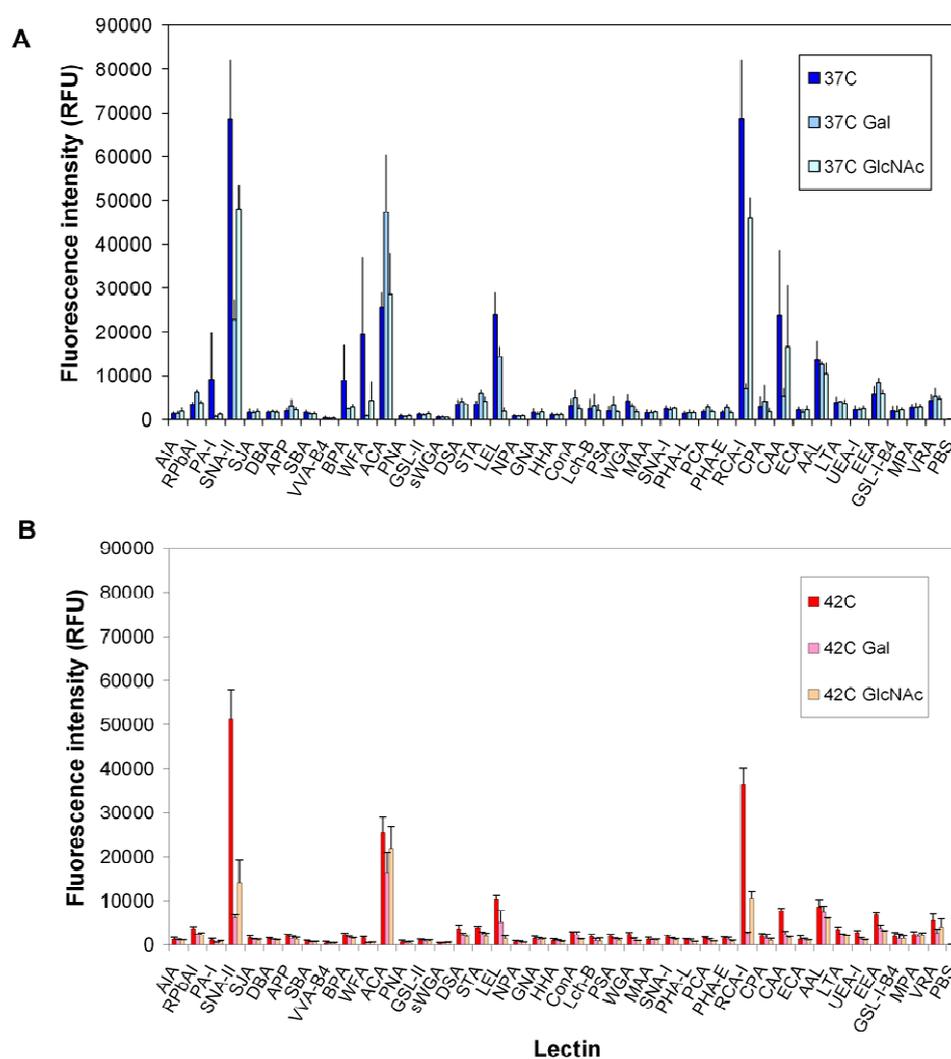


Figure 6.4 Histograms representing the inhibition of binding to printed lectins by *C. jejuni* strain 81116 grown at 37 and 42°C in the presence of haptenic monosaccharides. **(A)** Strain 81116 grown at 37°C co-incubated with Gal or GlcNAc. **(B)** Strain 81116 grown at 42°C co-incubated with Gal or GlcNAc. Histograms represent the mean of three biological replicate experiments and error bars are one average deviation of the mean of the three experiments. Each experiment is the median of six data points.

However, the presence of CPS in MPW extracts of both strains at both temperatures, even after nine wash steps, was confirmed by Alcian Blue staining of SDS-PAGE electrophoresed samples (Fig. 6.5). Hence, the CPS of both strains should have available for interaction during bacterial profiling.

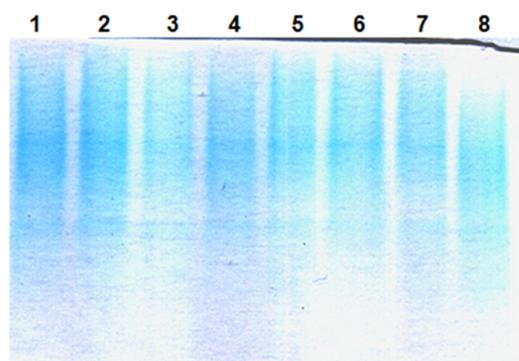


Figure 6.5 Alcian blue stained SDS-PAGE gel demonstrating the presence of capsular polysaccharide (CPS) in the mid-high M_r region after multiple wash steps from mini phenol water extracts of *C. jejuni* strains 81116 and 81-176, at both 37 and 42°C. Lanes: 1, Strain 81116, 37 °C, 1 wash; 2, 37 °C, 9 wash; 3, 42 °C, 1 wash; 4, 42 °C, 9 washes; 5, Strain 81-176, 37 °C, 1 wash; 6, 37 °C, 9 washes; 7, 42 °C, 1 wash, and 8, 42 °C, 9 washes.

In addition to interacting with lectins with affinity for GlcNAc and Gal/GalNAc-containing motifs (LEL, WGA, RCA-I, SNA-II and PCA), strain 81-176 also bound to lectins which typically indicate the presence of sialic acid (WGA and SNA-I) (Table 2.11 and Fig. 6.3A). This agrees with known CPS structures for strain 81-176 at 37°C, which contain GlcNAc residues, and both CPS and LOS, which contain Gal residues. However, sialic acid residues have only been described as components of the LOS of strain 81-176. Although SNA-I typically binds to α -(2,6)-linked sialic acid in mammalian glycosylation, the known Neu5Ac linkage in *C. jejuni* 81-176 LOS is α -(2,3). It should be noted that the characterised mammalian glycan specificity of a particular lectin may not be the same for bacterial glycans as the relevant residues are present in different linkages and orientations e.g. as polysaccharide components compared to mammalian *N*-linked oligosaccharides, and for some bacterial-specific residues, e.g. talosamine and shewanellose (Nazarenko *et al.*, 2003), no lectins with corresponding affinity are known. In addition, AAL typically interacts with α -(1,6)- and α -(1,3)-linked Fuc for mammalian-type glycosylation (Matsumura *et al.*, 2009) (Table 2.11), but Fuc has not been previously described as a component of either LOS or CPS

of strain 81-176 (Fig. 6.1). However, it is possible that this lectin interacted with a residue from the CPS in the α -galacto configuration (Klein *et al.*, 2000).

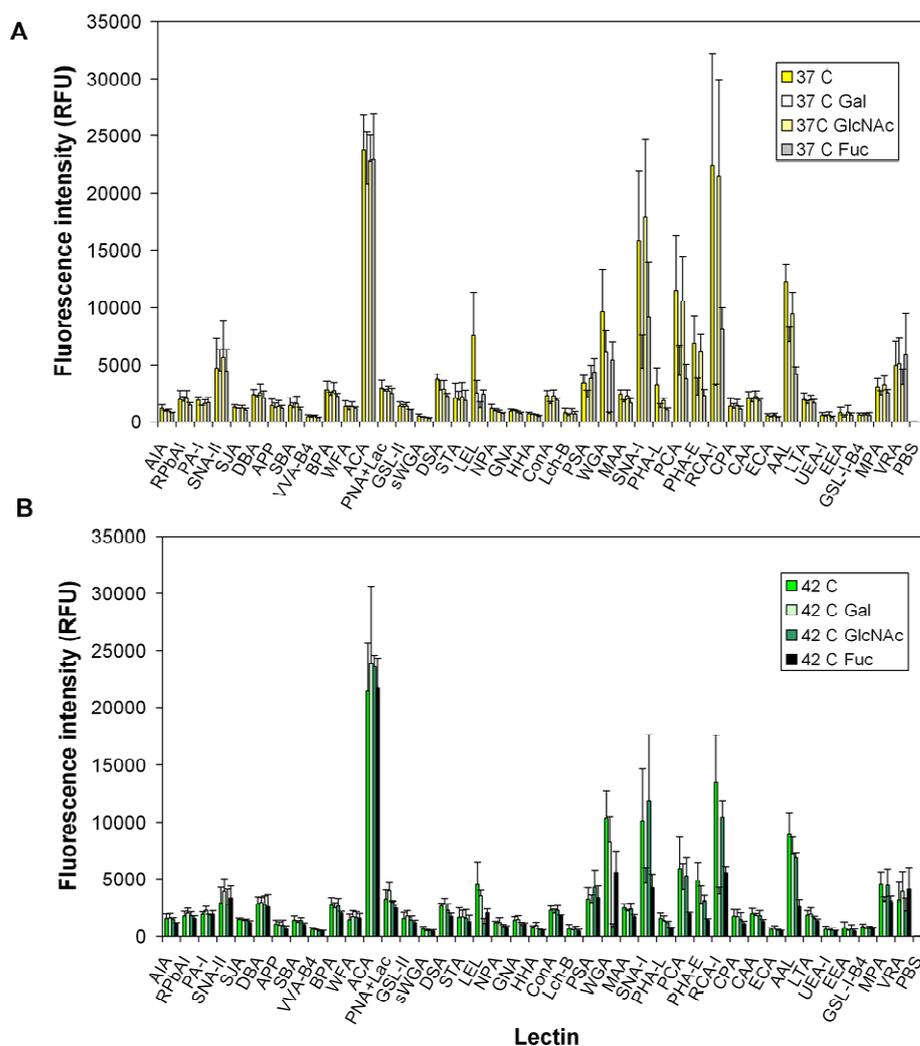


Figure 6.6 Histograms representing the inhibition of binding to printed lectins by *C. jejuni* strain 81-176 grown at 37 and 42°C in the presence of haptenic monosaccharides. **(A)** Strain 81116 grown at 37°C co-incubated with Gal, GlcNAc or Fuc. **(B)** Strain 81-176 grown at 42°C co-incubated with Gal, GlcNAc or Fuc. Histograms represent the mean of three biological replicate experiments and error bars are one average deviation of the mean of the three experiments. Each experiment is the median of six data points.

Clustering analysis of the two strains at the two temperatures showed that the lectin microarray platform distinguished the strains from each other at 37°C but not at 42°C (Fig. 6.3 B), as was readily visible from difference in profiles (Fig. 6.3 A). When the profiles for only strain 81116 were clustered, there was a clear distinction between the profiles at the two culture temperatures with 21% minimum similarity (not shown). However, the 81-176 strain cultured at either temperature could not be distinguished between temperatures nor from 81116 grown at 42°C by unsupervised clustering analysis despite slight differences in profiles observed.

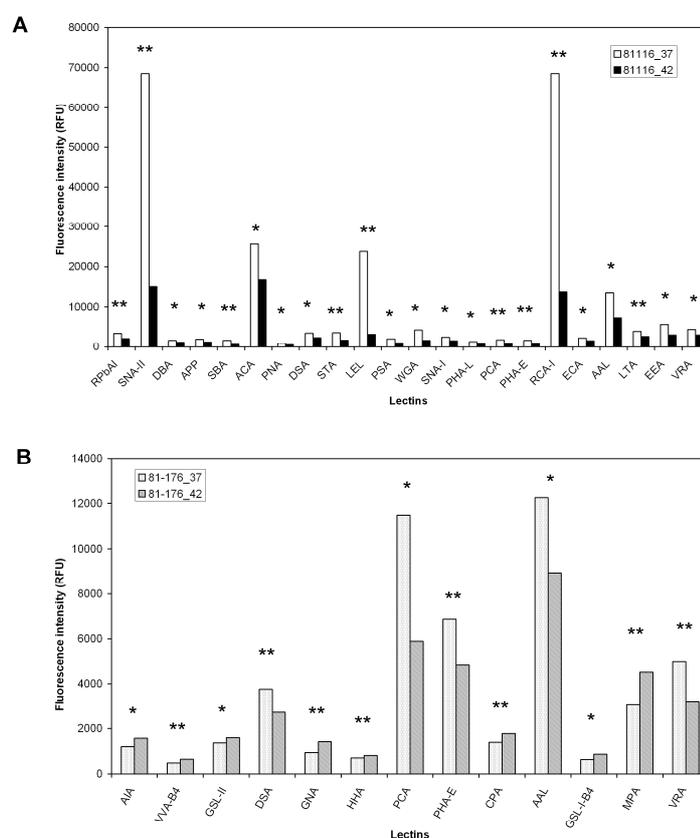


Figure 6.7 Histograms representing the binding of *C. jejuni* strains 81116 and 81-176 grown at 37 and 42°C to only printed lectins selected for significantly different binding intensities at the two culture temperatures (*, $p < 0.05$; **, $p < 0.01$). **(A)** Strain 81116 grown at 37 and 42°C. **(B)** Strain 81-176 grown at 37 and 42 °C. Histograms represent the mean of three biological replicate experiments and each experiment is the median of six data points.

6.2.2 Multivariate analysis of lectin microarray data

The ability of PrCA of the lectin microarray data to distinguish strains 81116 and 81-176 at the two temperatures was also evaluated. For strain 81-176, inclusion of all lectin data (41 lectins in total) produced considerable overlap in the two-dimensional score plots of replicates grown at 37 and 42°C (Fig. 6.8 A). However, for strain 81116, very well-defined groups emerged when all lectin data was included in the principal component calculations (Fig. 6.8 C). PrCA performed for 37 and 42°C cultures of 81-176 using only a subset of lectins which showed significantly different binding intensities ($p < 0.05$) between the two temperatures (13 lectins, Table 6.1 and Fig. 6.7) decreased the overlap of replicate groups considerably (Fig. 6.8 B) and also decreased the overall plot area of the groups, which suggested that these lectin responses contributed substantially to the uniqueness of each glycosylation profile. Similarly, a p value-based pre-selection of lectin data for the 37 and 42°C cultures of strain 81116 (22 lectins, Appendix and Fig. 6.7) also reduced the plot area for the replicate groups at each temperature and enhanced the spatial separation of the two groups (Fig. 6.8 D). It is important to note that some of the lectin responses which differed with high significance levels had only low intensity binding, e.g. SBA binding intensity for strain 81116 was only 1502.72 and 568.06 RFU at 37 and 42°C, respectively, although the p value was 0.0068 (Appendix i and Fig. 6.7). All lectins demonstrating significantly different binding for strain 81116 showed lower intensity at 42°C compared to 37°C. For strain 81-176 changes in intensities of significant lectins were more subtle.

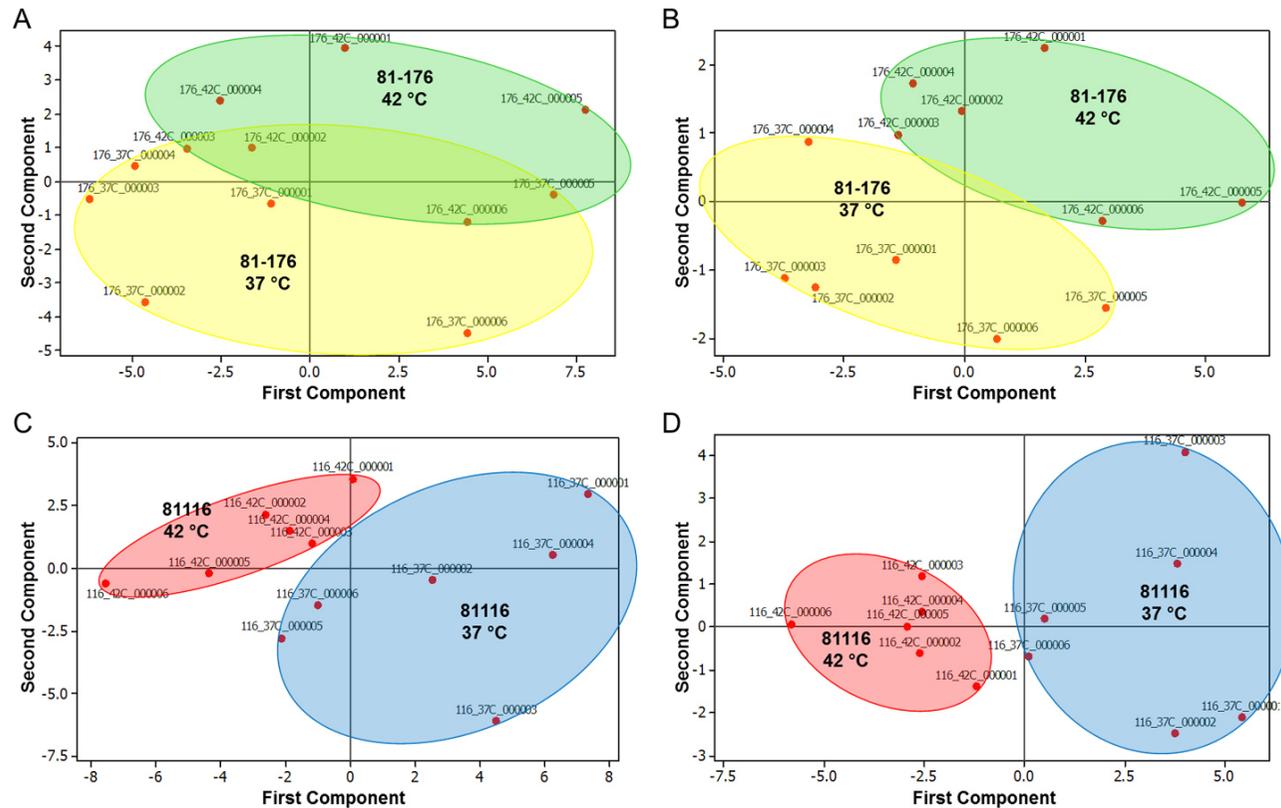


Figure 6.8 PrCA score plots representing the lectin profile pattern differences for *C. jejuni* strains 81116 and 81-176 grown at 37 and 42°C. **(A)** Strain 81-176 grown at 42°C (yellow) versus 37°C (red) as resolved by all lectins. **(B)** Strain 81-176 at 42°C and 37°C resolved only by lectins with significant differences between conditions ($p < 0.05$, AIA, VVA, GSL-II, DSA, GNA, HHA, PCA, PHA-E, CPA, AAL, GSL-I-B4, MPA, VRA). **(C)** Strain 81116 grown at 42°C (blue) versus 37°C (green) as resolved by all lectins. **(D)** Strain 81116 at 42°C and 37°C resolved only by lectins with significant differences between conditions ($p < 0.05$, RPbAI, SNA-II, DBA, APP, SBA, ACA, PNA, DSA, STA, LEL, PSA, WGA, SNA-I, PHA-L, PCA, PHA-E, RCA-I, ECA, AAL, LTA, EEA, VRA). Individual replicates (data points) are labelled in the format 'strain_temperature_experiment number'.

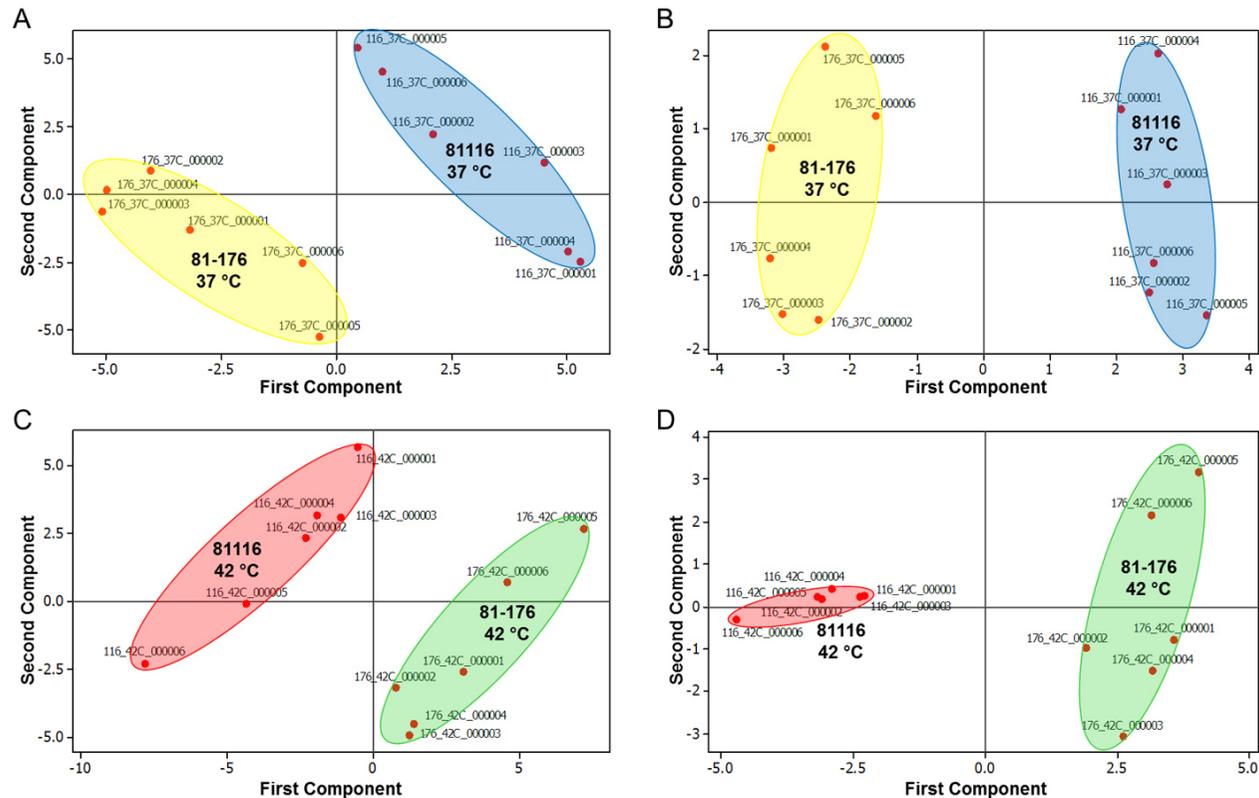


Figure 6.9 PrCA score plots representing the lectin microarray profile pattern differences between *C. jejuni* strains 81116 and 81-176. (A) Strain 81116 (green) versus 81-176 (red) grown at 37°C as resolved by all 41 lectins. (B) Strain 81116 (green) versus 81-176 (red) grown at 37°C as resolved by only lectins with significant differences between strains ($p < 0.05$, SNA-II, DBA, PNA, LEL, MAA, SNA-I, PCA, PHA-E, RCA-I, ECA, LTA, UEA-I, EEA). (C) Strain 81116 (blue) versus 81-176 (yellow) grown at 42°C as resolved by all 41 lectins. (D) Strain 81116 (blue) versus 81-176 (yellow) grown at 42°C as resolved by only lectins with significant differences between strains ($p < 0.05$, PA-I, SNA-II, DBA, SBA, WFA, PNA, NPA, Con A, PSA, WGA, MAA, SNA-I, PCA, PHA-E, EEA, MPA). Individual replicates (data points) are labelled in the format 'strain_temperature_experiment number'.

In contrast to distinguishing growth temperature within a strain, distinguishing strains from each other was more straightforward at a single temperature when all lectin data was used to generate two-dimensional score plots (Fig. 6.9 A and C). Comparison of strains 81116 and 81-176 grown at 37°C using PrCA generated from only lectins with significant differences between strains ($p < 0.05$, 13 lectins, Appendix i) minimised the replicate group spread and further enhanced the separation of the two strains' glycosylation profiles (Fig. 6.9 B). Similarly, comparison of strains 81116 and 81-176 grown at 42°C using PrCA generated from most significant lectin responses ($p < 0.05$, 16 lectins, Appendix) greatly reduced the spread of the 81-176 replicate group (Fig. 6.9 D).

6.2.3 Lectin agglutination assay

A microtitre plate lectin agglutination assay was employed to test the microarray lectin binding results in a different format. A representative group of lectins, both binding and non-binding to *C. jejuni* on the lectin microarray, was selected. Lectin agglutination of human RBCs (type O) was used as a positive control. RBCs agglutinated with expected lectins and did not autoagglutinate (Shibuya, 2001; Tan *et al.*, 2010), as demonstrated by no agglutination in TBS (Table 2.11, Fig. 6.10).

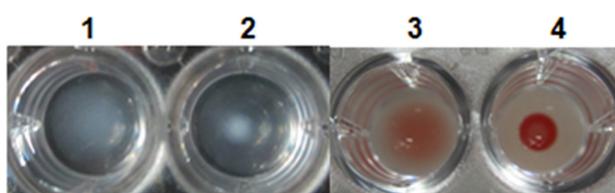


Figure 6.10 Representative results of microwell plate lectin agglutination.

(1) *C. jejuni* positive (+++) and (2) negative (-), and (3) RBC control positive (+++) and (4) negative (-).

The lectin agglutination patterns of WC and PK preparations of *C. jejuni* strains 81116 and 81-176, cultured at both 37 and 42°C, were compared. WC samples of strain 81116 at both 37 and 42°C could not be typed due to auto-agglutination in the control well and are not shown. However, PK preparations of strain 81116 did not

autoagglutinate along with WC and PK preparations of strain 81-176 at both temperatures.

PK preparations of strain 81116 grown at 42°C agglutinated with 5 out of 18 lectins tested, WFA, PNA, STA, LEL and WGA, and at 37°C, agglutination with the same lectins was observed but with an additional two lectins, SBA and BPA (Table 6.9). Thus, in common with the lectin microarray incubation, culture temperature differentiation was possible with the agglutination assay but information on only cell surface glycosylation was not possible to discern from this due to the ruptured cell. WC samples of 81-176 grown at 37 °C agglutinated with 6 out of 18 lectins, BPA, WFA, STA, LEL, WGA and ECA, which were the same lectins as the PK samples of strain 81-176. However, WC preparations of strain 81-176 at 42°C agglutinated with only 4 of these, BPA, WFA, LEL and ECA, and PK samples agglutinated with an additional two lectins, STA and WGA, to result in an identical agglutination profile to strain 81-176 WC and PK at 37°C (Table 6.1). The extra lectins bound after disruption of the cell with proteinase treatment indicated that additional epitopes were available, most likely intracellular carbohydrates. Although it may be possible to distinguish growth temperature of strain 81-176 by agglutination assay of WC preparations, it was apparent from these results that proteolytic pre-treatment was required for accurate lectin typing of all strains in agglutination assays, in agreement with previous studies (Hynes *et al.* 2002).

While differences in the degree of agglutination of the bacterial samples at both temperatures were observed (Table 6.1), the benefit of significantly increase dynamic range and quantitation of the lectin microarray were apparent where similar lectins interacted across platforms for WC samples e.g. LEL. It was possible to distinguish between strains on the basis of agglutination or no agglutination with two lectins, PNA and ECA. PNA strongly agglutinated PK preparations of strain 81116 at both temperatures but not with WC or PK preparations of 81-176 at either temperature. On the other hand, ECA agglutinated both WC and PK preparations of strain 81-176 at both 37 and 42°C but not 81116 at either temperature (Table 6.1). These lectins also exemplified the contrasting behaviour of several lectins across the two microarray and assay platforms. Interestingly, PNA showed highest lectin microarray intensity values with the 81-176 strain grown at either temperature (Fig. 6.6) and this inverse

relationship compared to the agglutination assay platform was also seen with ECA.

ECA bound most intensely with strain 81116 in the lectin array format (Fig. 6.4) which was opposite to the agglutination assay results.

Table 6.1. Lectin typing of *C. jejuni* 81116 and 81-176 agglutination. Results presented were carried out in duplicate and were identical for lectins concentrations at both 0.5 and 8 mg/mL. WC, whole cell suspensions, PK, proteinase K treated, RBC, red blood cells (type O). WC of strain 81116 at both 37 and 42°C were untypeable due to auto-agglutination in the control well and results are not presented.

Lectin or TBS	<i>C. jejuni</i> strain, culture temperature (°C)						RBCs		
	81116, 37		81116, 42		81-176, 37			81-176, 42	
	PK	PK	WC	PK	WC	PK			
AIA	- ^a	-	-	-	-	-	+++		
SNA-II	-	-	-	-	-	-	+++		
SBA	++	-	-	-	-	-	+++		
BPA	+++	-	+++	+++	+++	++	+++		
WFA	+++	+++	+++	+++	+++	+++	+++		
PNA	+++	+	-	-	-	-	-		
sWGA	-	-	-	-	-	-	+++		
STA	+++	+	++	+	-	+++	+++		
LEL	++	+++	++	++	+	+	+++		
NPA	-	-	-	-	-	-	-		
ConA	-	-	-	-	-	-	+++		
Lch-B	-	-	-	-	-	-	+++		
WGA	+++	+++	++	+	-	+	+++		
SNA-I	-	-	-	-	-	-	+++		
ECA	-	-	+++	+	+++	+	+++		
GS-I-	-	-	-	-	-	-	-		
B ₄									
MPA	-	-	-	-	-	-	-		
VRA	-	-	-	-	-	-	-		
TBS	-	-	-	-	-	-	-		

^a +++, strong agglutination; ++, moderate agglutination; +, weak agglutination; -, no agglutination

6.2.4 SDS-PAGE analysis of cell surface glycolipids

Cell surface glycolipids were extracted from strains 81116 and 81-176 cultured at both 37 and 42°C and were analysed by SDS-PAGE. Silver staining of the MPW extracts revealed a pattern of low- M_r bands in both strains at both temperatures (which were not co-incident with Alcian blue-stained CPS, Fig. 6.5), which indicated the presence of LOS. A doublet of low- M_r species was detected for *C. jejuni* 81116, which corresponded to the approximately 3.8 and 3.6 kDa bands previously reported (Guerry *et al.*, 2002). A greater quantity of the '3.8 kDa' M_r band was present at both temperatures and the distribution of LOS species did not change between temperatures (Table 6.2, Fig. 6.11, lanes 2 and 3). This heterogeneity was also present in MPW extracts of strain 81-176, which also migrated as a doublet of low- M_r bands at both temperatures. However, only 18% of the total LOS was present as the lower M_r band at 37°C compared to 42% in the 42°C extract of this strain (Table 6.2, Fig. 6.11, lanes 4 and 5).

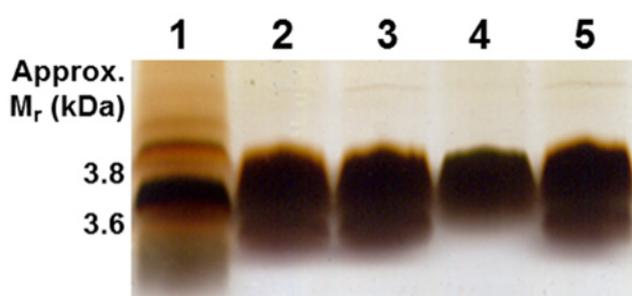


Figure 6.11 Silver stained SDS-PAGE gel of *C. jejuni* glycolipids isolated using the MPW procedure. Lanes: 1, *E. coli* AM1 clinical strain LPS; 2, *C. jejuni* 81116 37°C; 3, *C. jejuni* 81116 42 °C; 4, *C. jejuni* 81-176 37°C; 5, *C. jejuni* 81-176 42°C.

Table 6.2 Densitometry analysis of LOS species apparent molecular mass (in kDa) distribution for *C. jejuni* strain 81116 and 81-176 at 37 and 42 °C.

<i>C. jejuni</i> strain	LOS species (kDa)	37°C	42°C
		% distribution	
81116	3.8	55	55
	3.6	45	45
81-176	3.8	82	58
	3.6	18	42

6.3 Discussion

A diverse range of cell surface glycans are expressed by *C. jejuni*, including LOS, CPS, glycoproteins and other PS-related molecules, and these glycans are important in bacterial-host interaction and may be important factors in both virulence and commensalism (Bacon *et al.*, 2001; Karlyshev *et al.*, 2004; Bachtiar *et al.*, 2007). The inhibitable lectin responses for *C. jejuni* 81116 suggested interaction with Gal, lactose and GlcNAc containing structures which corresponded to the LPS-like structure previously characterised for this strain at 37°C (Muldoon *et al.*, 2002; Kilcoyne *et al.*, 2006). The lectins BPA and WFA typically bind to GalNAc-containing structures and binding in strain 81116 indicated interaction with bacterial *N*-linked glycans, which have a terminal α -linked GalNAc residue (Guerry & Szymanski, 2008).

The lectin SBA is often used to indicate the presence of bacterial *N*-linked glycans (Jervis *et al.*, 2012), but very little binding of either *C. jejuni* strain was observed with this lectin in the microarray format. The observed AAL binding could be the result of interaction with the α -linked Gal in the LPS-like structure and/or the terminal α -linked GalNAc of bacterial *N*-linked glycans. These motifs may also be recognised by the lectins EEA, VRA and MPA, which typically bind to terminal α -linked Gal. The binding of strain 81116 to the GalNAc-specific macrophage galactose-like C-type lectin (MGL) was previously reported to be *N*-glycan-mediated and mutation in the critical *N*-

glycan biosynthesis *pgl* gene decreased Caco-2 cell adherence and chick colonisation (Karlyshev *et al.*, 2005; van Sorge *et al.*, 2009).

In this work, no lectin interaction indicative of the presence of Glc or Man residues was observed for strain 81116, which implied that CPS was not recognised by the lectins despite its availability for interaction as shown by SDS-PAGE analysis after extensive washing. Thus, the LPS-like polysaccharide and *N*-linked glycans were most likely the dominant cell surface structures of this strain. Interestingly, the CPS of this strain was reported to be involved in human epithelial cell binding but not to have a significant role in colonisation of the chicken gut (Bachtiar *et al.*, 2007).

C. jejuni is capable of adaptation and survival in a range of hosts and environments and can express specific factors to survive in diverse ecological niches. Environmental and host physiological temperature is an important regulatory signal prompting *C. jejuni* to modify expression of certain virulence or colonisation factors, which include cell-surface carbohydrates (Corcoran & Moran, 2007; Semchenko *et al.*, 2010). Importantly, it was possible to clearly distinguish between the two strains when cultured at 37°C with the lectin microarray. The greater variability of response of many lectins with *C. jejuni* 81116 cultured at 37°C compared to 42°C may indicate more variability of chain length of the outer polysaccharide at the lower temperature, and thus greater diversity of conformation of presented epitopes for interaction combined with a greater accessibility of the bacterial *N*-linked glycans. This is reflected in the relative increase in area for this group in the PrCA score plots (Fig. 6.8 C and D). Differential expression of 336 genes in *C. jejuni* NCTC 11168 has been reported in response to temperature change from 37 to 42°C including genes involved in cell surface and flagellar glycosylation (Stintzi, 2003) and LOS structural modifications were detected with change in temperature (Semchenko *et al.*, 2010). In this work, strain 81116 cultured at 37°C was significantly differentiated from the same strain cultured at 42°C by 22 lectins. Many of these lectins indicated a change in the LPS-like structure which may imply that this structure became less dominant and that the *N*-linked glycans were more accessible to the microenvironment at the higher temperature. Interestingly, a gene involved in LPS biosynthesis in strain 81116, *galE* (Fry *et al.*, 2000), was reported to be upregulated with increased temperature (Stintzi, 2003) supporting the hypothesis of temperature-related alteration to the LPS-like structure in *C. jejuni* 81116. Furthermore,

the temperature-dependent expression of a high- M_r polysaccharide with a ladder like electrophoretic banding pattern characteristic of LPS has been described for *C. jejuni* NCTC 11168 in liquid culture which was expressed at 37 but not at 42°C (Corcoran & Moran, 2007) and a similar phenomenon may have occurred here.

In contrast to strain 81116, *C. jejuni* 81-176 interacted with typically Gal/GalNAc and GlcNAc recognition lectins, which implied binding to the known CPS structure. Binding to AAL probably indicated interaction with the α -linked Gal residues in the CPS and/or bacterial *N*-linked glycans although other typically GalNAc recognising lectins such as BPA and WFA had low intensity binding. However, the interaction with VRA and MPA may have indicated terminal α -GalNAc recognition. Binding of PHA-E, which is known to bind to complex biantennary *N*-linked mammalian glycans typically with Gal residue termini, may reflect interaction with terminal Gal residues in LOS that are spaced correspondingly. The binding of WGA and SNA-I lectins indicated that sialic acid was an accessible residue for interaction and Neu5Ac has been characterised on the LOS of strain 81-176 (Guerry *et al.*, 2002; Kanipes *et al.*, 2004). Expression of CPS and LOS by *C. jejuni* 81-176 has numerous roles in virulence including adhesion and invasion of epithelial cells as well as evasion of the immune system (Bacon *et al.*, 2001; Kanipes *et al.*, 2004). Further, the LOS is structurally similar to human nerve gangliosides and this ganglioside mimicry is involved in the development of GBS (Hughes & Cornblath, 2005). Here, the binding of typically Gal and sialic acid recognising lectins and the low to negligible binding of typical GalNAc-specific lectins suggested that mimics of GM₃ and GD_{1b} gangliosides which lack a terminal GalNAc were mainly recognised. Bacterial expression of certain host ganglioside mimics may have particular biological roles since GM₃-mimicking strain 81-176 has been reported to be more invasive to epithelial cells *in vitro* (Guerry *et al.*, 2002), and anti-ganglioside antibodies were induced *in vivo* (Prendergast *et al.*, 2004). Sialic acid and linkage-dependent *C. jejuni* binding to siglecs was reported as leading to dendritic cell activation and the production of the cross-reactive anti-sera that are central to GBS pathogenesis (Bax *et al.*, 2011). In summary, the main cell surface structures recognised for strain 81-176 were CPS, LOS and possibly *N*-linked glycosylation.

Distinguishing between culture temperatures using lectin microarray profiling was less clear with strain 81-176 compared to 81116, which implied an overall relative consistency of accessible surface structures at both temperatures. The 13 lectins which showed relatively small though significant intensity changes between temperatures did not include sialic acid specific lectins which indicated that the overall expression of LOS was not altered (Fig. 6.7). The slight increases in intensity of the two lectins which have specificity for α -linked Glc or Man, HHA and GNA, indicated that the α -glucan (Papp-Szabo *et al.*, 2005) was more accessible or present in greater relative proportion among the CPSs produced by strain 81-176. This change in distribution of CPS structures at 42°C was supported by changes in binding intensities to lectins which indicated the presence of the other known CPS structures, i.e. lectins with affinities for GlcNAc and α -linked Fuc and Gal residues (Fig. 6.1). Changes in the relative expression and distribution of known LOS structures were also demonstrated by slight changes in lectins which indicated the presence of β -linked Gal and GalNAc residues and complex structures.

It was previously reported that LOS from strain 81-176 produced two bands of approximately M_r 3.8 and 3.6 kDa (Guerry *et al.*, 2002), and this double band was also observed in this work. Guerry, *et al.* had also shown that the 3.8 kDa band consisted of primarily GM₂ and minor amounts of GD₂ and GD_{1a}-mimicking LOS. The 3.6 kDa LOS band consisted of a GM₃-mimicking structure, which is a truncated form of the major GM₂-mimicking structure formed by phase variable expression of *cgtA*, an *N*-acetylgalactosaminyltransferase that when turned off, prevents terminal GalNAc transfer to the LOS core. Instances of an increase in the relative quantity of the lower- M_r LOS species produced at 42 compared to 37°C have been reported in certain *C. jejuni* strains human and chicken-derived strains (Semchenko *et al.*, 2010). The change in relative expression and distribution of LOS structures in *C. jejuni* 81-176 between temperatures indicated by the lectin microarray profiles indicated a similar increase in relative expression of the lower M_r LOS and this was subsequently confirmed by SDS-PAGE analysis (Table 6.2). In contrast, strain 81116 produced both higher and lower- M_r LOS at both human and avian host temperatures and the relative proportion of both LOS bands were the same at both temperatures, in agreement with a previous study (Semchenko *et al.*, 2010). In certain strains of *C. jejuni*, it appears that the higher- M_r

LOS species, or a subset of this molecular population, interacts with lectins of the host immune system (Van Sorge *et al.*, 2009). The greater expression of the lower- M_r LOS species at the avian host temperature of 42°C may have a major role in the differences in the pathogenesis of *C. jejuni* between human and chicken hosts. Expression of the '3.6 kDa' M_r GM₃-mimicking LOS was reported to result in greater invasiveness in human intestinal epithelial cells *in vitro* (Guerry *et al.*, 2002), but invasion of chicken cells by *C. jejuni* occurs at a lower rate compared to human cells *in vivo* (Van Deun *et al.*, 2008). Why more of this lower- M_r LOS is produced at 42°C is unclear but it may have alternative functions in *C. jejuni* adaptation to the avian host. Antibody recognition of oligosaccharides was previously shown to be modulated by structurally heterogeneous neighbouring oligosaccharides and varying densities on a microarray platform. Interestingly, when the target oligosaccharide was presented in a mixed population with shorter oligosaccharides at the same density, antibody recognition was decreased (Liang *et al.*, 2011). Thus, examination of the neighbouring effects of the lower- M_r LOS species on the modulation of biological response from the higher- M_r LOS may yield valuable insights for immune system interaction and commensalism studies.

Although the characterised mammalian glycan specificity of a particular lectin may not be the same for bacterial glycans, structurally related residues appear to interact with lectins previously characterised to have a similar binding specificity e.g. the interaction of Pse with LFA, which has been characterised as a sialic acid-specific lectin (Doig *et al.*, 1996). However, the effect of non-carbohydrate substitutions on lectin binding, such as PEtn on the LOS or the MeOPN side chain on CPS of strain 81-176, has not been investigated (Guerry *et al.*, 2002; Kanipes *et al.*, 2004). The employment of recent pioneering developments such as the construction of a microarray of bacterial oligosaccharide antigens (Blixt *et al.*, 2008) and the present work can help to build a picture of defined lectin interactions with known bacterial glycosylation. In this work, the known cell surface glycosylation of strains 81-176 and 81116 cultured at 37°C had been previously structurally elucidated by our groups and others (Aspinall *et al.*, 1993; Aspinall *et al.*, 1993; Guerry *et al.*, 2002; Muldoon *et al.*, 2002; Kanipes *et al.*, 2004; Papp-Szabo *et al.*, 2005; Kilcoyne *et al.*, 2006) and could be well correlated with the lectin profiles for the same temperature.

Previously, lectin typing of *Campylobacter* spp. by agglutination assay has been carried out on untreated bacterial cells as well as cells subjected to prior enzymatic or heat treatment (Wong *et al.*, 1985; O'Sullivan *et al.*, 1990; Aabenhus *et al.*, 2002) while bacteria have been used directly after washing for a quartz crystal microbalance technique (Yakovleva *et al.*, 2011). Cell pre-treatment with proteases is often required to inhibit auto-agglutination of bacteria for agglutination assays but it also enables intracellular glycans as well as those from the cell-surface to interact with the lectins. In contrast, on the lectin microarray only surface-accessible carbohydrates are available for lectin binding as intact cells are analysed. This method provides more biologically relevant information since the cell-surface glycans of the living bacterial cell are those involved in bacteria-host cell interactions. Furthermore, profiling of bacterial cell surface glycosylation using the lectin microarray method in this study took 2.5 h, from harvesting bacteria after culture to data acquisition, compared to typical microtitre plate agglutination assays requiring proteolytic and/or heat treatment followed by overnight incubation. Bacterial auto-agglutination also did not occur during the microarray method, which was possibly due to the significantly shorter time for the experiment.

The contrasting performance of certain lectins in the two assay formats, e.g. PNA strongly agglutinated strain 81116 but not 81-176 in the plate assay while it bound more intensely to 81-176 compared to 81116 in the lectin microarray platform, may be explained by the effect of attachment of the lectin to the microarray surface. Conjugation of the lectin to a surface, or indeed to an enzyme or fluorescent label, can potentially alter the presentation and conformation of the carbohydrate recognition domain (CRD) of the lectin and hence affect its binding behaviour. While printing the microarray, the CRDs of the lectins were protected by including a suitable carbohydrate in the print buffer to maintain the CRD conformation during conjugation and the performance of the surface-immobilised lectins were subsequently verified by incubation with fluorescently-labelled glycoproteins with well characterised glycosylation (data not shown). However, lectin behaviour in response to multivalent presentation of carbohydrate epitopes, i.e. avidity, may not be the same when the lectin is immobilised on a surface compared to in solution, as arrangement of the lectin subunits or accessibility of the CRDs may differ. Platform-dependent performance for antibody-carbohydrate interactions has been recently demonstrated by Pochechueva, *et*

al., across three array platforms; microarray, suspension array and enzyme-linked immunosorbent assay (ELISA) (Pochechueva *et al.*, 2011). Thus, when lectins are under consideration for various applications, such as strain discrimination in this case, their behaviour must be assessed in the relevant application platform, as lectin performance and behaviour may not directly translate from one platform or orientation to another.

In addition to specific lectin-glycan interactions, non-specific or non-carbohydrate-mediated interactions with lectins may occur (Gerlach *et al.*, 2011). Genuine carbohydrate-lectin interactions were confirmed in this study by haptenic sugar inhibitions. However, non-carbohydrate-mediated binding to ACA for both strains at both culture temperatures was noted. This binding may have been mediated by a protein or hydrophobic constituent, of the bacterial cell surface. The possibility of adhesins on the surface of *C. jejuni* interacting with glycosylation on the lectins themselves must also be considered when inferring motif expression changes from lectin data. Haptenic inhibition controls can usually confirm that expected binding has occurred, i.e. that the expected motif of the lectin CRD was recognised as opposed to the glycosylation of the lectin itself. Use of recombinant, non-glycosylated lectins has also been proposed as a solution for this potential problem (Rakus *et al.*, 2011). However, low yields, formation of insoluble aggregates and reduced carbohydrate affinity mean that this is not an option for many lectins (Streicher *et al.*, 2003; Sahasrabudde *et al.*, 2004). For increased confidence in the interpretation of lectin binding data, there is value in having multiple lectins that have similar specificities including any available recombinant lectins. However, this is only practical using the efficient lectin microarray format. Profiling bacterial strains against an expanded feature diversity not only provides greater opportunities for motif identification, but also increases the probability for discrimination between strains and conditions and can monitor even subtle changes in glycome expression rather than isolated constituents which reflects a dynamic bacterial adaptation.

Chapter 7.

Interaction of avian cells with *Campylobacter jejuni* CPS and LOS, and molecular characterisation of temperature-related lipid A variation

7.1 Introduction

Profiling of caecal innate immune gene expression in *C. jejuni*-colonised chickens has highlighted an absence of a significant pro-inflammatory response (Smith *et al.*, 2008; Shaughnessy *et al.*, 2009). Additionally, global gene expression analysis has failed to demonstrate upregulation of important inflammatory mediators following caecal colonisation by *C. jejuni* (Li *et al.*, 2010; Hermans *et al.*, 2011)).

Therefore, there is a possibility that commensalism may be a result of *C. jejuni* evasion or subversion of the chicken immune response. Conflictingly, however, activation of PRRs by live *C. jejuni* has been observed *in vitro* in chicken-specific epithelial cells (Borrmann *et al.*, 2007; Larson *et al.*, 2008; Li *et al.*, 2010), kidney cells (Smith *et al.*, 2005) and macrophages (Smith *et al.*, 2005; de Zoete *et al.*, 2010). This apparent discrepancy between the *in vivo* and *in vitro* situation may reflect adaptation of *C. jejuni* to a commensal relationship with its avian host. This hypothesis is strengthened by the fact that the microbe produces different transcriptional and phenotypic profiles when grown between 37 and 42°C, the respective core body temperatures of humans and birds (Stintzi, 2003; Corcoran & Moran, 2007; Semchenko *et al.*, 2010).

Functionally, these adaptations may involve downregulation or modification of certain PAMPs, resulting in avoidance of chicken immune detection. Considering this, better characterisation of *C. jejuni* PAMP interaction with chicken PRRs would potentially provide insight into the underlying basis of the commensalism.

C. jejuni is composed of numerous components which could be recognised as PAMPs, including glycosylated flagella, lipopeptides, nucleic acids and glycoconjugates such as LOS and CPS. Research into the interaction of chicken PRRs such as TLRs is in its infancy. However it is known that the *C. jejuni* flagella is not recognised by either chicken TLR5, or its mammalian counterpart (de Zoete *et al.*, 2010a; b). In comparison, chicken TLR2 activation can occur, probably as a result of exposure to *C. jejuni* lipopeptides from lysed cells (de Zoete *et al.*, 2010a). Chicken TLR21 sensing of *C. jejuni*-specific DNA is also described, and certain *C. jejuni* LOS has been shown to activate chicken TLR4 (van Mourik *et al.*, 2010).

Endotoxins are among the most potent inciters of inflammatory responses (Opal, 2010), and considering that TLR4 activation is critical to the establishment of *C.*

jejuni-mediated intestinal immunopathology in the murine model (Bereswill *et al.*, 2011), detailed characterisation of chicken-specific immune effectors induced in response to *C. jejuni* LOS is needed. Additionally, since differential transcription of numerous *C. jejuni* genes, including LOS biosynthesis genes, have been observed due to temperature shift from 37 to 42°C (Stintzi, 2003), LOS structural modification may be involved in *C. jejuni* host adaptation. Moreover, the immunogenic potential of *C. jejuni* CPS in chickens is yet to be determined, and since it is the outermost component of the microbe it may be the first to come into contact with avian epithelial PRRs, with potential to act as a shield to other underlying PAMPs (Young *et al.*, 2007).

Initially, the aim of this study was to characterise chicken macrophage and fibroblast responses to the LOS of *C. jejuni* grown at 37 and 42°C, as well as *C. jejuni* CPS. Following the observation of temperature-related differences in response to *C. jejuni* LOS, the molecular basis of this was determined, using mass spectrometry. Moreover, complete structural elucidation of the *C. jejuni* 81116 core OS was attempted using NMR.

7.2 Results

7.2.1 Investigation of the pro-inflammatory effects of *C. jejuni* LOS and CPS in chicken cells

Little is known about how chicken cells respond to *C. jejuni* cell surface components, such as LOS and CPS. LOS and CPS the major outer membrane structures and could interact with chicken epithelial and immune cells and play undefined roles in the commensal nature of the *C. jejuni*-chicken relationship. Therefore, LOS and CPS was extracted and purified for investigation of their immunostimulatory influence on a variety of chicken and human macrophages, as well as chicken embryonic fibroblast (CEF) cells, using *E. coli* LPS as a control. Furthermore, the immunological impact of potential growth temperature-related LOS variation, between LOS grown at 37 and 42°C, was investigated.

7.2.1.1 Isolation and purification of *C. jejuni* 81116 LOS and CPS

Biomass was grown on 7% blood agar, at 37°C (3.2 g dry mass) and 42°C (1.6g dry mass) and subjected to hot-phenol water extraction, followed by enzymatic purification with RNase A, DNase II, and proteinase K. To isolate CPS, sodium acetate extraction was carried out with 1g of dried biomass, followed by enzymatic purification as above. The purified LOS and CPS samples were dissolved at 2mg ml⁻¹ in endotoxin free water, for use in immunostimulation of human THP-1, and chicken HD11 macrophage cell lines, as well as CEF cells, in collaboration with Prof. Cliona O'Farrelly and Dr. Ronan Shaughnessy of Trinity College Dublin.

7.2.1.2 Macrophages responses to *C. jejuni* 81116 LOS and CPS

Macrophages express a wide range of PRRs, and respond by producing high levels of pro-inflammatory mediators, such as IL6, IL8 (pro-inflammatory chemokines) and IL1 β (pro-inflammatory cytokine), making them appropriate candidates for investigating the immunostimulatory properties of *C. jejuni* cell-surface glycoconjugates. *C. jejuni* CPS (1 μ g/ml) appeared to induced low *IL1 β* (42-fold) and *IL6* (46-fold) gene expression increases in avian HD11 cells (a macrophage-monocyte-derived cell line) at 4 h post-challenge (Fig. 7.1). However, treatment of the CPS preparation with PMB, an antibiotic that neutralises lipid A toxicity (Cardoso *et al.*, 2007), abrogated the pro-inflammatory effect (Fig. 7.1), indicating that low levels of LOS contamination were present in the CPS after sodium acetate extraction. The presence of PMB (10 μ g/ml) eliminated the low level of cytokine gene expression observed in the absence of the antibiotic, presumably as a result of PMB binding the contaminating LOS and preventing its pro-inflammatory influence on the macrophages (Fig. 7.1). Therefore, this work indicates that *C. jejuni* CPS is not detected by avian macrophages, since no pro-inflammatory influence on these cells was detected here.

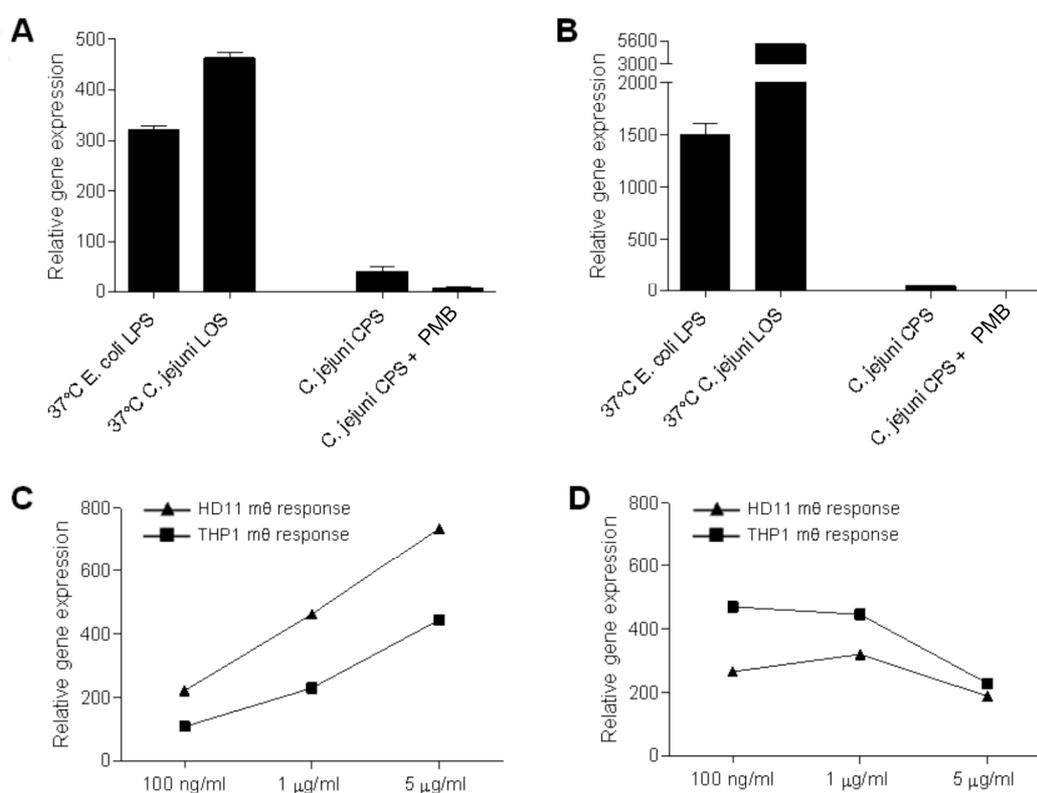


Figure 7.1 Chicken and human macrophage responses to *C. jejuni* surface components. (A) *IL1β* and (B) *IL6* gene expression is increased in chicken HD11 macrophages in response to *E. coli* LPS (1 μg/ml) and *C. jejuni* LOS (1 μg/ml) at 4 h post-challenge. Minimal increases are evident in response to 1 μg/ml purified *C. jejuni* CPS while CPS in the presence of polymyxin B (PMB) fails to induce cytokine expression. (C) Increased *IL1β* gene expression in both HD11 and THP1 macrophages in response to increasing *C. jejuni* LOS doses. (D) Decrease in *IL1β* gene expression in both HD11 and THP1 macrophages in response to increasing *E. coli* LPS doses.

Treatment of HD11 macrophages with *E. coli* LPS (1 μg/ml) induced high *IL1β* (~300-fold) and *IL6* (~1500-fold) gene expression. In comparison, LOS (1 μg/ml) from *C. jejuni* 81116 grown at 37°C, induced higher *IL1β* (~400-fold) and *IL6* (~5200-fold) gene expression. The opposite was true for human THP-1 macrophages, where *E. coli* LPS (1 μg/ml) induced ~400-fold *IL1β* and ~300-fold *IL8* gene expression increases in THP1 macrophages while *C. jejuni* LOS (1 μg/ml) induced *IL1β* and *IL8* gene expression increases by >200-fold (Fig. 7.1).

Sequentially increasing the challenge dose of *C. jejuni* LOS and *E. coli* LPS revealed differences in the cell response. Gradual increases in cytokine gene expression in both human THP1 and chicken HD11 macrophages were observed in response to incremental 100 ng/ml, 1 µg/ml and 5 µg/ml LOS challenges (Fig. 7.1 C). In response to LPS, a plateau in cytokine gene expression was reached at 1 µg/ml, and a reduced response to 5 µg/ml LPS was observed, with comparable gene expression increases from 100 ng/ml LPS (Fig. 7.1 D).

Challenge studies with endotoxin in the presence of PMB were carried out to confirm that the chicken cells were responding to the lipid A component of the *C. jejuni* LOS molecule. Presence of this cationic antibiotic, which binds lipid A, completely prevented the induction of *IL1β* gene expression in HD11 cells by *E. coli* LPS. However, only partial reduction (~60%) of LOS-mediated induction occurred due to the presence of PMB (Fig. 7.2).

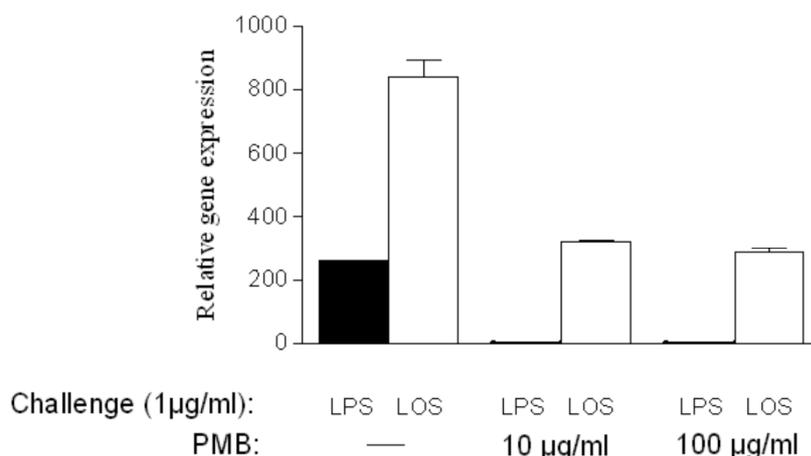


Figure 7.2 Inhibition of TLR4 signalling. Polymyxin B (PMB) completely inhibits *E. coli* LPS-mediated induction of *IL1β* gene expression in chicken HD11 macrophages while *C. jejuni* LOS-mediated induction is only partially reduced. All data represents 4 h post-challenge.

To further investigate avian responses to *C. jejuni* LOS and *E. coli* LPS, *TLR* gene expression was profiled in challenged cells. Responses were similar to both endotoxins, in which *TLR15* upregulation (15-fold) was evident while *TLR2*, *TLR4*, *TLR5* and *TLR21* were not differentially expressed (Fig. 7.3 A). Profiling *TLR15* gene

expression in HD11 cells in response to LOS across a 4 h timecourse revealed that the receptor was upregulated as early as 30 min post-challenge (Fig. 7.3 B).

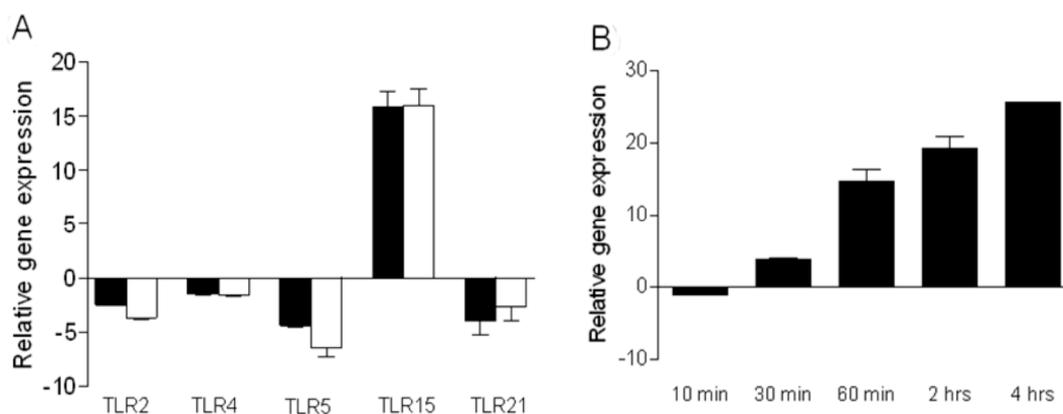


Figure 7.3 Differential pattern recognition receptor (PRR) and signalling pathway gene expression in response to 1 µg/ml *E. coli* LPS and *C. jejuni* LOS in HD11 macrophages. (A) LPS (black) and LOS (white) both upregulate *TLR15* gene expression 4 h post-challenge. (B) *TLR15* mRNA upregulation is evident as early as 30 minutes post-*C. jejuni* LOS challenge.

7.2.1.3 Chicken macrophages response to *C. jejuni* 81116 42°C LOS

Subsequent to the demonstration of the pro-inflammatory influence of 37°C 81116 LOS in chicken and human macrophages, LOS from *C. jejuni* 81116 grown at 42°C was then compared in its ability to stimulate chicken HD11 macrophages. At 4 h post-challenge 42°C LOS had a comparable effect on chicken HD11 macrophages as 37°C LOS, with induction of *IL1β* (800-fold), *IL6* (3500-fold), and *IL8* (600-fold) gene expression (Fig. 7.4).

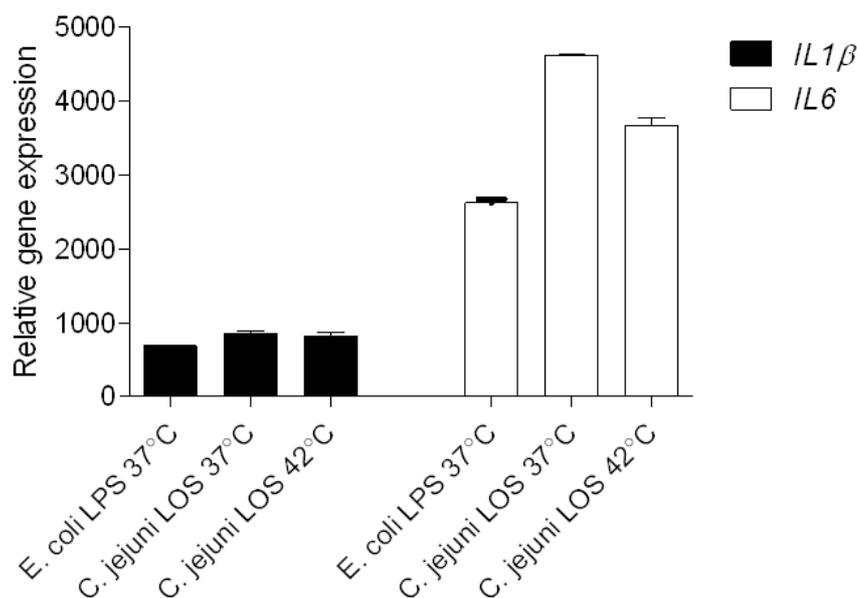


Figure 7.4 Comparison of pro-inflammatory effects of 37 and 42°C LOS on HD11 macrophages. LOS (1 $\mu\text{g/ml}$) from *C. jejuni* grown at both 37 and 42°C induce comparable *IL1 β* and *IL6* gene expression increases at 4 h post-challenge in chicken HD11 macrophages.

7.2.1.4 Response of chicken embryonic fibroblast cells to *C. jejuni* 81116 LOS and CPS

Fibroblast-like cells are major constituents of the intestinal lamina propria that may or may not have a pro-inflammatory response to *C. jejuni* components. Therefore, CEF cells were challenged with *C. jejuni* LOS and CPS (Fig. 7.5). CPS induced *IL8* gene expression, which was abrogated with the addition of PMB, again implicating LOS contamination of the CPS as the mediator of the pro-inflammatory effects. Furthermore, *E. coli* LPS failed to induce pro-inflammatory responses in CEF cells. In contrast, upregulation of *IL8* gene expression by 12-fold and 27-fold was observed in response to 1 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ 37°C LOS concentrations, respectively. It was revealed that at the mRNA level CEF cells express *TLR2*, *TLR5*, *TLR15* and *TLR21*, but lack *TLR4*, which is associated with LOS detection (van Mourik *et al.*, 2010). This reveals that the 37°C LOS was detected by an avian PRR other than TLR4 in these cells (Fig. 7.5). TLR gene expression profiling revealed *TLR15* gene expression increases, but not *TLR2*, *TLR5* and

TLR21 gene expression increases, in response to 37°C LOS. Significantly, compared to 37°C LOS, challenge of CEF cells with 42°C LOS did not induce pro-inflammatory gene expression increases in (Fig. 7.5), indicating 42°C LOS evasion of the undefined PRR that was activated by 37°C LOS.

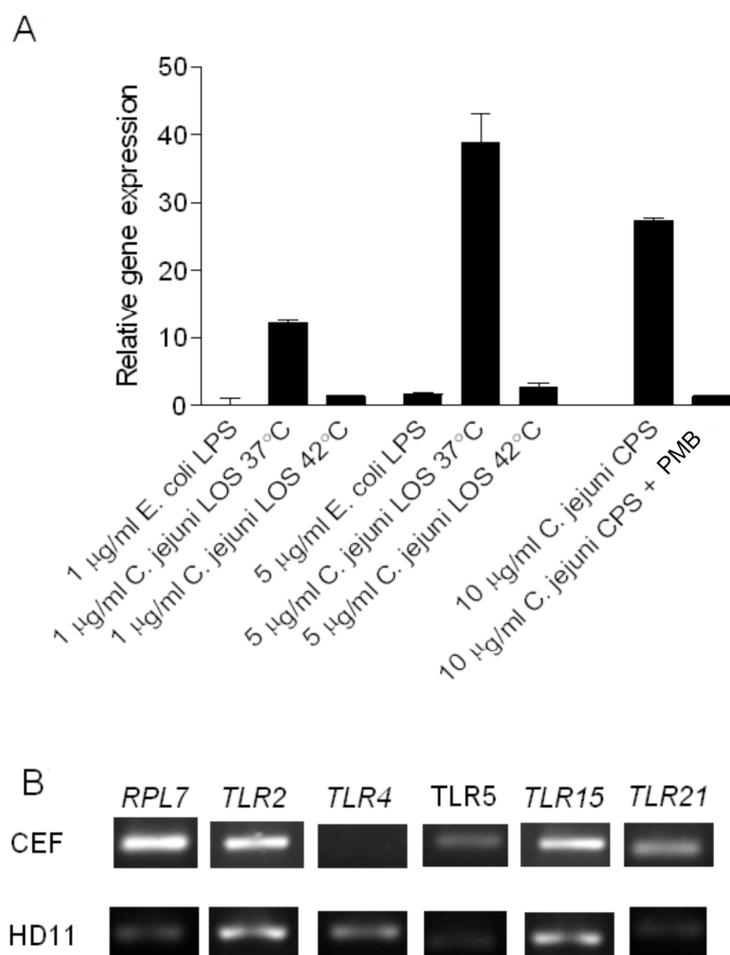


Figure 7.5 Differential PRR recognition of LOS between *C. jejuni* grown at 37°C and 42°C. **(A)** Chicken embryonic fibroblasts (CEF) induce *IL8* gene expression in response to LOS purified from *C. jejuni* grown at 37°C but not to LOS purified from *C. jejuni* grown at 42°C. *C. jejuni* CPS and *E. coli* LPS are also not inductive of *IL8* gene expression in CEFs. **(B)** In comparison to chicken HD11 macrophages, CEFs do not express basal *TLR4* mRNA. *RPL7* served as a housekeeper gene.

7.2.1.5 Summary of immunostimulation studies

Chicken HD11 macrophages and CEF cells were challenged with *C. jejuni* CPS and LOS. LOS was obtained from *C. jejuni* grown at both 37°C and 42°C. LOS induced cytokine (>700-fold) gene expression, and also upregulated *TLR15* gene expression (15-fold) in chicken HD11 cells. Chicken embryonic fibroblast cells responded differently to the LOS types, as 37°C LOS, but not 42°C LOS, induced pro-inflammatory gene expression. Thus, *C. jejuni* LOS produced in a 42°C environment, such as the chicken intestine, may fail to induce pro-inflammatory gene expression in certain cell types. Furthermore, CPS failed to induce cytokine and chemokine gene expression in either HD11 or CEF cells, which suggest that encapsulated *C. jejuni* evades PRR detection.

7.2.2 Molecular basis of temperature-related differences observed in immunostimulation studies

Immunological challenges with LOS in the presence of PMB reduced the pro-inflammatory effects of the LOS, suggesting that the lipid A is the immunostimulatory component of the molecule. In an attempt to elucidate the molecular basis for the differential *in vitro* cell responses observed after challenge with 37 and 42°C LOS, isolation of LOS was performed for comparative analysis. In contrast to the classical enterobacterial lipid A, exemplified by *E. coli*, where the lipid A backbone consists of GlcN β (1,6)GlcN disaccharide, the *C. jejuni* lipid A can consist of three different backbone disaccharides: a major species with a backbone consisting of GlcN₃N β (1,6)GlcN (Fig. 7.6 1), with three *N*-linked fatty acids, and one *O*-linked fatty acid; and, two minor species with backbones disaccharides consisting of GlcN₃N β (1,6)GlcN₃N (Fig. 7.6 2), with four *N*-linked fatty acids; and, GlcN β (1,6)GlcN (Fig. 7.6 3), with two *N*-linked fatty acids, and two *O*-linked fatty acid. These lipid A species can be substituted with variable phosphorylation.

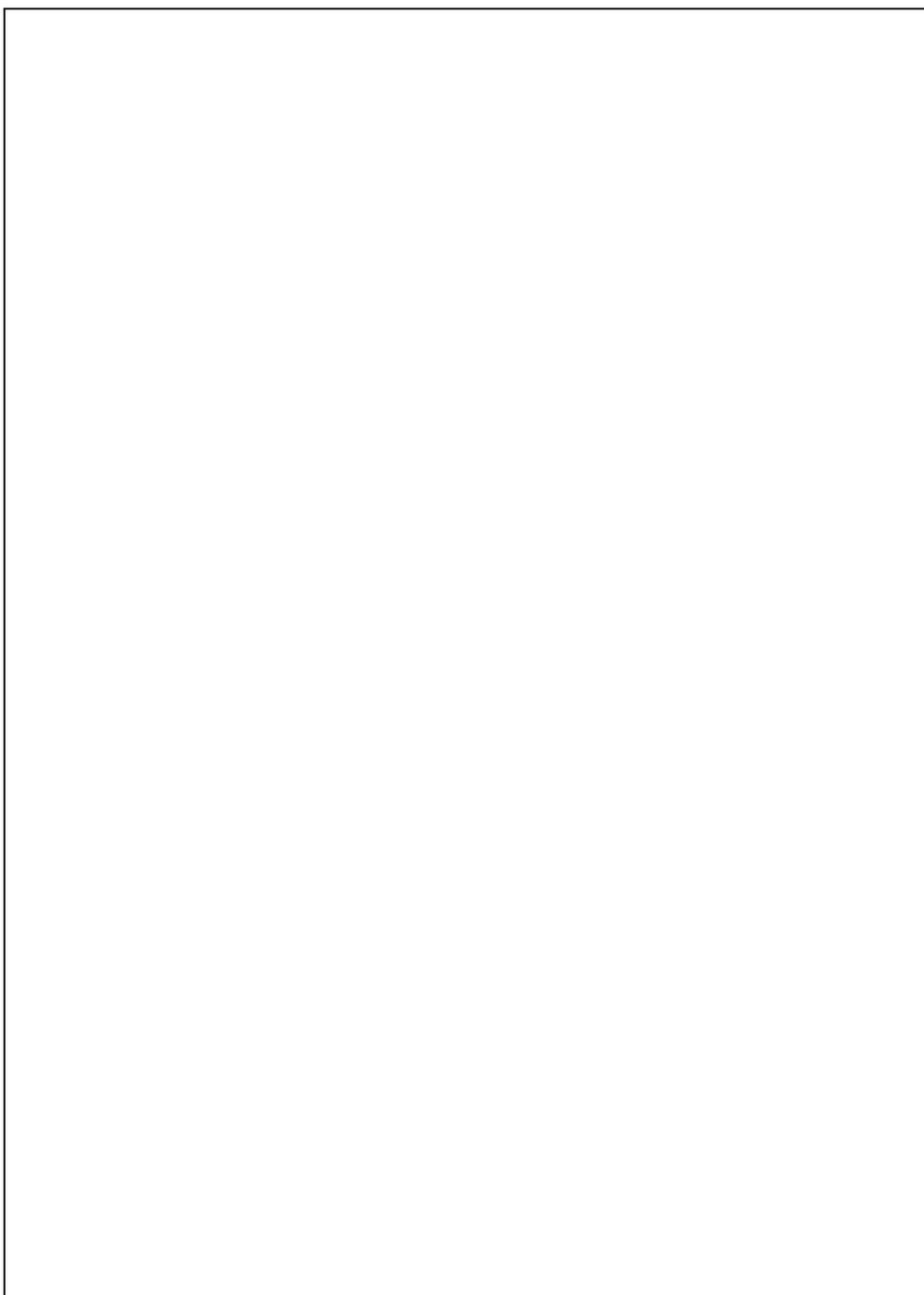


Figure 7.6 Chemical structure of *C. jejuni* lipid A species. (1) Major lipid A species composed of a GlcN3N-GlcN disaccharide backbone, three *N*-linked fatty acids, and one *O*-linked fatty acid. (2) Minor lipid A species composed of GlcN3N-GlcN3N, with four *N*-linked fatty acids (3) Minor lipid A species composed of GlcN-GlcN, with two *N*-linked fatty acids, and two *O*-linked fatty acids. Variable phosphorylation indicated by brackets (Adapted from Szymanski *et al.*, 2003). Figure has been removed due to copyright restrictions.

7.2.2.1 Comparative structural analysis of *C. jejuni* 81116 LOS grown at 37 and 42°C

Five biological replicate mini-phenol-water (MPW) preparations were prepared from *C. jejuni* 81116 grown at each of 37 and 42°C, and each preparation yielded approximately 1mg of material per replicate. Tricine-SDS-PAGE with silver staining demonstrated similar electrophoretic mobility and M_r between the samples, which indicated no large structural differences between 37 and 42°C extracts (Fig. 7.7). A doublet of low- M_r species was detected the MPW extracts for *C. jejuni* 81116. Densitometry analysis revealed strain 81116 produced approximately 55% of the higher M_r form and 45% of the lower- M_r form, at both 37 and 42 °C.

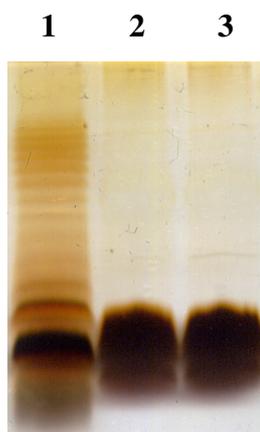


Figure 7.7 Tricine SDS-PAGE of one replicate mini-phenol water extract from *C. jejuni* 81116 grown at 37 and 42°C. Lanes: 1, *E. coli* LPS used for molecular-mass comparison and as a positive control; 2, MPW from *C. jejuni* 81116 grown at 37°C; 3, MPW from *C. jejuni* 81116 grown at 42°C. Although, not clear from image lanes 2 & 3 have two bands each. No differences are observed between 37 and 42°C samples.

For more detailed structural analysis MPW samples were *O*-deacylated by base treatment, and subjected to analysis by CE-ESI-MS, as described in Li *et al.* (2005). *O*-Deacylation removed the *O*-linked fatty acids from the lipid A backbone, leaving only *N*-linked acyl chains attached to each GlcN. Unlike the traditional approach to LOS

analysis which involves acid hydrolysis, the mild conditions employed in *O*-deacylation (anhydrous hydrazine, 37°C, 1 h) prevents the destruction of important acid labile constituents of the LOS molecule, such as *PEtn*, which can be lost with hydrolysis, and allows analysis of the deacylated molecule, without inadvertent modifications. Furthermore, improved solubility and reduced formation of LOS aggregates, allows capillary electrophoresis to separate even closely related LOS glycoforms due to differences in molecular conformations and ionic charge distributions. This separation facilitates acquisition of molecular-masses by CE-ESI-MS, which combined with knowledge of published *C. jejuni* LOS structures, enables proposal of chemical structures for the LOS glycoforms present in the samples. Since *O*-deacylation only removes *O*-linked fatty acids, CE-ESI-MS observation of *O*-deacylated LOS enables the determination of the number of *N*-linked fatty acids present, and thus the form of lipid A backbone present in the species (whether GlcN₃N-GlcN, GlcN-GlcN, or GlcN₃N-GlcN₃N), as well as the degree of phosphorylation with phosphate residues (P), *PEtn* and pyrophosphoethanolamine (*PPEtn*).

CE-ESI-MS analysis of *O*-deacylated LOS from *C. jejuni* 81116 yielded multiple mass species at both 37 and 42°C, which represented the presence of distinct LOS glycoforms. Table 7.1 shows representative CE-ESI-MS data for one replicate of each of 37 and 42°C, and the proposed compositions of LOS glycoforms detected based on molecular-masses of known structures. Three LOS core oligosaccharide glycoforms were detected, which varied due to the type of lipid A disaccharide backbone present, and the degree of lipid A phosphorylation (number of phosphate residues, and presence/absence of *PEtn*/*PPEtn*). The MS data suggested that LOS glycoforms contained dideoxyhexosamine (ddHexN), which was either modified with an acetyl group (Ac) to form *N*-acetyl dideoxyhexosamine, or with a formyl group (Fo) to form *N*-formyl dideoxyhexosamine.

Table 7.1 Representative negative ion ESI-MS data and proposed compositions for O-deacylated LOS of *Campylobacter jejuni* 81116 grown at 37 and 42°C. Highlighted in grey are the main series of ions used to quantify relative ratios of lipid A N-acylation. Main series have same composition other than N-linked fatty acids (main series peaks measured for 2 N-,3 N- and 4 N-linked species, both in the 3- and 4- ion series).

Strain	Observed ions (m/z)			Proposed compositions			
	<u>[M-4H]⁴⁻</u>	<u>[M-3H]³⁻</u>	<u>[M-2H]²⁻</u>	<u>Calculatedⁱ</u>	<u>Core oligosaccharide</u>	<u>Phosphorylation in lipid A</u>	<u>Acylation in lipid A</u>
81116 37°C		1063.8		3194.15	ddHexN ₁ •HexNAc ₂ •Hex ₂ •Hep ₃ •Kdo ₂	PEtn, P	3 N-(C14:0 3-OH)
	818.7	1092.0		3277.98	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn, P	2 N-(C14:0 3-OH)
	837.3	1116.9		3352.26	ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	P,P	3 N-(C14:0 3-OH)
	844.5	1126.2		3380.30	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	P,P	3 N-(C14:0 3-OH)
	847.8	1131.0		3395.33	ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn, P	3 N-(C14:0 3-OH)
		1140.6		3423.37	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn, P	3 N-(C14:0 3-OH)
	868.2	1157.7		3475.31	ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn, P	3 N-(C14:0 3-OH)
	875.1	1167.0		3503.35	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn, P	3 N-(C14:0 3-OH)
	879.0	1172.1		3518.38	Na ⁺ •NH ₄ ⁺ •ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn ₂ , P	3 N-(C14:0 3-OH)
	900.6	1201.5		3605.67	Na ⁺ •NH ₄ ⁺ •ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	P,P	4 N-(C14:0 3-OH)
		1232.7		3626.40	Na ⁺ •NH ₄ ⁺ •ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn ₂	4 N-(C14:0 3-OH)
	924.5	1232.7		3700.68	ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn, P	4 N-(C14:0 3-OH)
	931.5	1242.3		3728.72	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn, P	4 N-(C14:0 3-OH)
	935.1	1246.8		3743.78	Na ⁺ •NH ₄ ⁺ •ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	PEtn ₂ , P	4 N-(C14:0 3-OH)

81116 42°C					
	1063.5	3194.15	ddHexN ₁ •HexNAc ₂ •Hex ₂ •Hep ₃ •Kdo ₂	<i>PP</i> Etn, P	3 N-(C14:0 3-OH)
818.7	1091.7	3277.98	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	<i>PP</i> Etn, P	2 N-(C14:0 3-OH)
	1096.8	3293.01	ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	<i>PE</i> tn ₂ , P	2 N-(C14:0 3-OH)
	1116.9	3352.26	ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	P,P	3 N-(C14:0 3-OH)
844.2	1125.9	3380.80	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	P,P	3 N-(C14:0 3-OH)
848.1	1131.3	3395.33	ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	<i>PP</i> Etn	3 N-(C14:0 3-OH)
	1140.6	3423.37	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	<i>PP</i> Etn	3 N-(C14:0 3-OH)
867.9	1157.7	3475.31	ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	<i>PP</i> Etn, P	3 N-(C14:0 3-OH)
875.1	1167.0	3503.35	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	<i>PP</i> Etn, P	3 N-(C14:0 3-OH)
878.7	1171.8	3518.38	Na ⁺ ₁ •NH ₄ ⁺ ₁ •ddHexNFo ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	<i>PE</i> tn ₂ , P	3 N-(C14:0 3-OH)
905.7	1208.1	3626.40	Na ⁺ ₁ •NH ₄ ⁺ ₁ •ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	<i>PP</i> Etn ₂	3 N-(C14:0 3-OH)
931.5	1242.0	3728.72	ddHexNAc ₂ •HexNAc ₂ •Hex ₂ •Hep ₃ •P ₁ •Kdo ₂	<i>PP</i> Etn, P	4 N-(C14:0 3-OH)

[†] Isotope-average mass units were used for calculation of molecular-mass values based on proposed compositions as follows: HexN, 161.16; HexN₃N, 160.17; C14:0 3-OH, 226.36; *PE*tn, 123.05; Kdo, 220.18; *P*, 79.98; Hep, 192.17; Hex, 162.14; HexNAc, 203.20; ddHexNAc, 187.20; ddHexNFo, 173.17; ddHexN, 145.16; NH₄⁺, 17.03; Na⁺, 21.98; H₂O, 18.0.

The CE-ESI-MS yielded multiple mass species, which is typical for *O*-deacylated LOS from *C. jejuni* (Li *et al.*, 2005), which varied in *N*-linked fatty acids, the degree of phosphorylation of the lipid A, and composition of the core OS. Three core OS glycoforms were detected at both 37 and 42°C. LOS grown at both 37 and 42°C had a major triply deprotonated ion peak at m/z 1167, which corresponded to an LOS species with a mass of 3503.3 Da (GlcN3N-GlcN backbone with *PPEtn* and P, and a core OS with presence of an acetylated ddHexN). The core oligosaccharide of this species, representing the predominant LOS glycoform produced, was proposed to be composed of three heptoses (Hep; 192.17 Da), two hexoses (Hex; 162.14 Da), two *N*-acetyl hexosamines (HexNAc; 203.2 Da), two ddHexNAc (ddHexNAc; 187.20) residues, two Kdo (Kdo; 220.18) residues, and a single phosphate (P; 79.98) residue (ddHexNAc2•HexNAc2•Hex2•Hep3•P1•Kdo2). Lipid A variants of this glycoform common to 37 and 42°C (having identical core oligosaccharide structures) included the 2 *N*-acylated lipid A variant that had a triply deprotonated ion peak at m/z 1092 which corresponds to an LOS species with a mass of 3277.9 Da (GlcN-GlcN backbone similar to classical enterobacterial lipid A, but with *PPEtn* and P), as well as the 4 *N*-acylated lipid A variant that had a triply deprotonated ion peak at m/z 1242 which corresponds to an LOS species with a mass of 3728.7 Da (GlcN3N-GlcN3N backbone with *PPEtn* and P) (Table 7.1).

The second glycoform that was detected in both 37 and 42°C LOS had a major triply deprotonated ion peak at m/z 1157.7, which corresponds to an LOS species with a mass of 3475.3 Da (GlcN3N-GlcN backbone with *PPEtn* and P, and contained a core OS with apparent presence of a formylated ddHexN). The core oligosaccharide of this species was proposed to consist of three heptoses, two hexoses, two *N*-acetyl hexosamines, two ddHexNFo (ddHexNFo; 173.17) residues, two Kdo residues, and a single phosphate residue (ddHexNFo2•HexNAc2•Hex2•Hep3•P1•Kdo2). A lipid A variant of this glycoform with 4 *N*-linked fatty acids was also detected at both 37 and 42°C LOS had a triply deprotonated ion peak at m/z 1242.3 which corresponds to an LOS species with a mass of 3728.72 Da (GlcN3N-GlcN3N backbone with *PPEtn* and P). However, the 2 *N*-acylated lipid A variant of this LOS glycoform was only detected in 42°C LOS having a triply deprotonated ion peak at m/z 1096.8, which corresponds to an LOS species with a mass of 3293.0 Da; GlcN-GlcN backbone similar to classical enterobacterial lipid A, but with *PPEtn* and P) (Table 7.1).

A third LOS glycoform was present in both 37 and 42°C LOS had a major triply deprotonated ion peak at m/z 1603, which corresponds to an LOS species with a mass of 3194.1 Da (GlcN3N-GlcN backbone with *PPEtn* and *P*). The lower M_r of this glycoform compared to the other two, was due to the loss from the core OS of a single ddHexN residue, lack of acetylation/ formylation of the remaining ddHexN residue, as well as lack of core OS phosphorylation, and represents a truncated structure. Therefore, the core OS of this species (3194.1 Da) had three heptoses, two hexoses, two *N*-acetyl hexosamines, one ddHexN residue, and two Kdo residues (ddHexN1•HexNAc2•Hex2•Hep3•Kdo2; Table 7.1). This truncated LOS glycoform may constitute the lower- M_r band detected in SDS-PAGE analysis as presented in Figure 7.7.

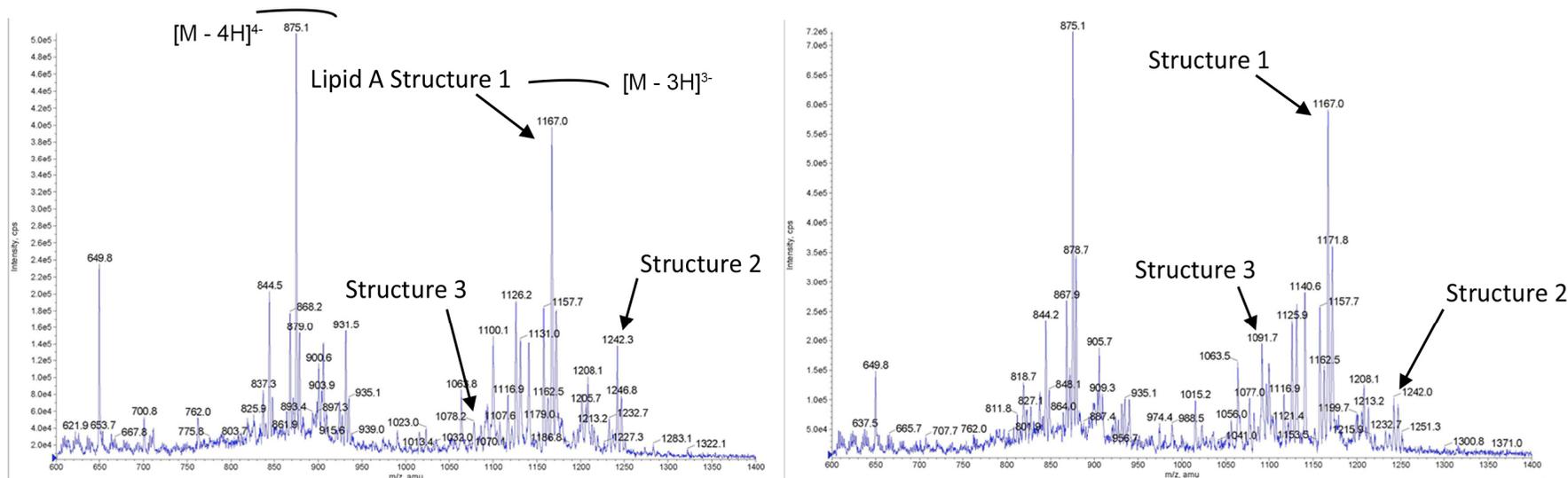


Figure 7.8 Representative ESI-MS spectra from single replicate of *O*-deacylated LOS from *C. jejuni* 81116. (A) 37°C, and (B) 42°C. The arrows indicate the peaks of the main series of ions in the 3⁻ ion series, used to quantify relative ratios of lipid A *N*-acylation. Main series have same composition other than number of *N*-linked fatty acids (main series measured for 2 *N*-, 3 *N*- and 4 *N*-acylated lipid A species, in both the [M-3H]³⁻ ion and [M-4H]⁴⁻ ion series). Refer to Figure 7.6 for structure of three lipid A species with either 2 *N*-, 3 *N*- or 4 *N*-linked acyl chains. Lipid A structures 1, 2 and 3 composed of GlcN3N-GlcN (3 *N*- and 1 *O*-linked fatty acids), GlcN3N-GlcN3N (4 *N*-linked fatty acids), and GlcN-GlcN (2 *N*- and 2 *O*-linked fatty acids), respectively.

Qualitatively, there were reproducible differences in the ratios of lipid A structure 3 (with 2 *N*-linked fatty acids) and lipid A structure 2 (with 4 *N*- fatty acids), as is represented in Figure 7.8. At 37°C, a greater amount of lipid A structure 2, consisting of a GlcN3N-GlcN3N lipid A backbone was present compared to lipid A structure 3. The opposite was apparent with the 42°C samples where a greater amount of lipid A structure 3 consisting of a GlcN-GlcN lipid A backbone, was present. For a semi-quantitative approximation of the ratios of 2 *N*-, 3 *N*- and 4 *N*-acylation the CE-ESI-MS peak intensities of the main series of ions were used, which was limited to six ions, highlighted in grey in Table 7.1, which was the major series at both 37 and 42°C. The analysis was limited to this series since it was the major glycoform series were peaks for 2 *N*-, 3 *N*- and 4 *N*- were measured in both the $[M-3H]^{3-}$ and $[M-4H]^{4-}$ ion, with many of the triply and quadruply charged ions having too low intensity and were lost in background. The mean percentage of lipid A species present in this series of all five replicates, at both 37 and 42°C are presented in Table 7.2. These figures were determined for each of the replicates by taking the sum of the $[M-3H]^{3-}$ and $[M-4H]^{4-}$ ion peak intensities of each of the 2 *N*-, 3 *N*- and 4 *N*-linked fatty acid mass species, and calculated as a percentage of total peak intensity of the 2 *N*-, 3 *N*-, and 4 *N*-linked fatty acid species. From these figures, the mean percentage ratios of lipid A with 2 *N*-linked fatty acids, 3 *N*-linked fatty acids and 4 *N*-linked fatty acids, were calculated, as in Table 7.2 and presented as a histogram in Figure 7.9.

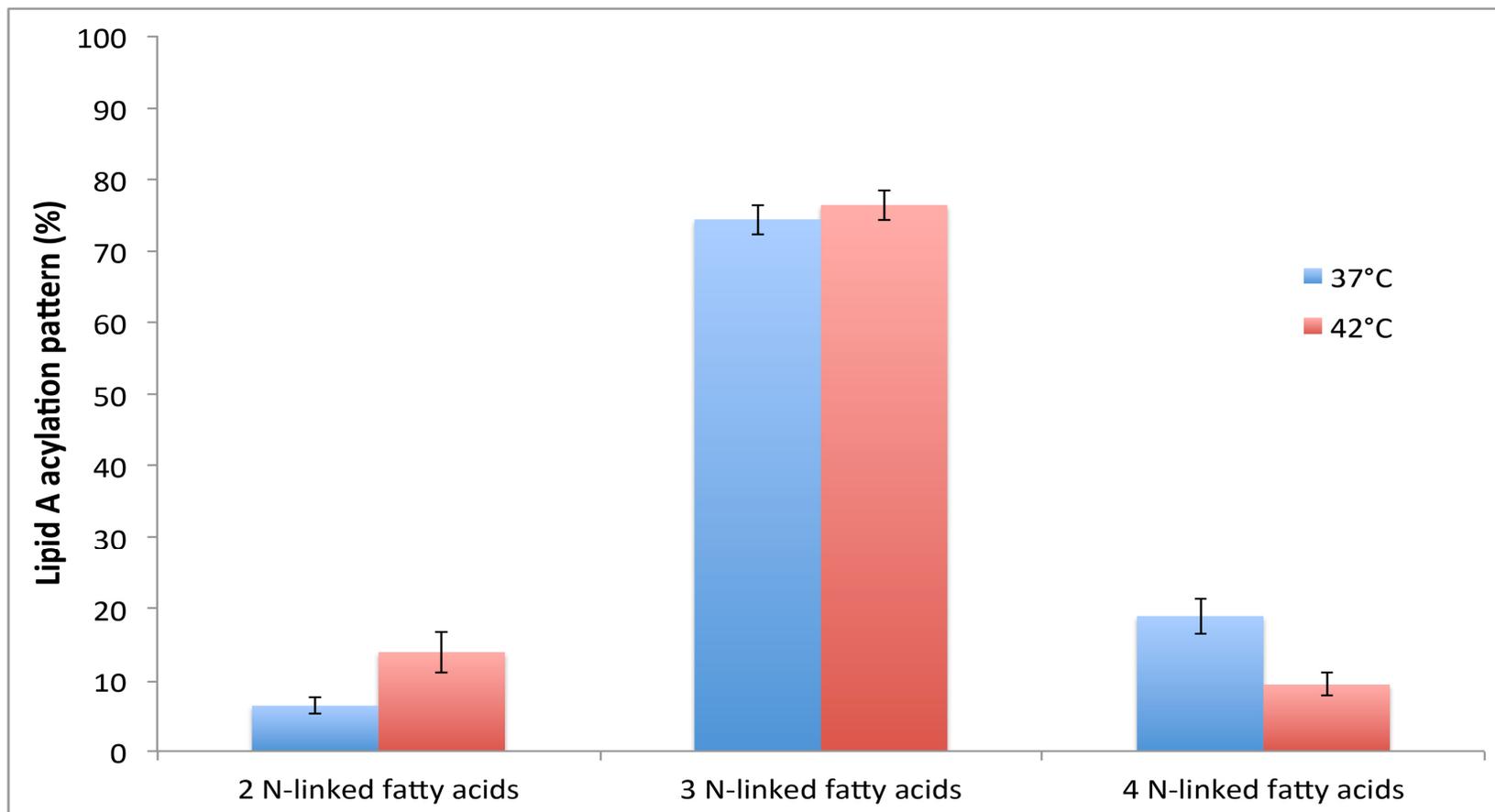


Figure 7.9. Histogram representing the relative abundance of lipid A species with 2 *N*-, 3 *N*- and 4 *N*-linked fatty acids in *C. jejuni* 81116 grown at 37 (blue) and 42°C (red). Temperature-related variation in ratio of 2 *N*- and 4 *N*-linked fatty acids can be observed between 37 and 42°C sample. Histograms represent the mean of five biological replicate CE-ESI-MS experiments, and error bars are one average deviation of the mean of the five independent extractions.

Both the 37 and 42°C samples have approximately 75% (Fig. 7.9 & Table 7.2) of the 3 *N*-acylated lipid A variant. At 37°C, there is greater amount of the 4 *N*-acylated (19.0% ± 2.6%) lipid A compared to the 2 *N*-acylation (6.5% ± 1.2%) variant. In contrast, at 42°C there is a greater amount of the 2 *N*-acylated (14.0% ± 3.1%), than 4 *N*-acylated (9.6% ± 1.6%) variant. Furthermore, this data shows that the ratio of 2 *N* to 4 *N*-acylation and therefore GlcN-GlcN to GlcN3N-GlcN3N is approximately reversed at 37°C compared to 42°C. Although the quantitation of lipid A acylation was only carried out for the main series, the trend appears to be the same for all three LOS glycoforms (Dr. J. Lee; personal communication). This is the first report of temperature-related lipid A variation in *C. jejuni*.

Table 7.2 Ratio of 2N:3N:4N- acylation of lipid A in *C. jejuni* 81116 grown at 37 and 42°C

<i>N</i> -acylation	Percentage ratio of lipid A <i>N</i> -acylation	
	<i>C. jejuni</i> 81116 37°C	<i>C. jejuni</i> 81116 42°C
2- <i>N</i>	6.5 ± 1.2%	14.0 ± 3.1%
3- <i>N</i>	74.6 ± 2.0%	76.4 ± 2.3%
4- <i>N</i>	19.0 ± 2.6%	9.6 ± 1.6%

7.2.2.2 Summary of comparative structural analysis of *C. jejuni* 81116 37 and 42°C LOS

O-deacylated LOS from *C. jejuni* 81116 grown at both 37 and 42°C was analysed by CE-ESI-MS revealing three major LOS glycoforms, which varied due to the composition of the core OS. Furthermore, variation in lipid A structure, and degree of lipid A phosphorylation, was detected. Three lipid A variants were detected, which varied due to the composition of the backbone disaccharide, and consequently the number of *N*- and *O*-linked acyl chains present. Lipid A with a hybrid backbone disaccharide (GlcN3N-GlcN, structure 1, Fig. 7.6) with 3 *N*-linked fatty acids was most common at both 37 and 42°C. However, at 37°C there was a greater amount of lipid A with a 4 *N*-linked fatty acids (GlcN3N-GlcN3N, structure 2, Fig. 7.6) than at 42°C. The

presents difficulty in the full characterisation by NMR, and is likely the reason why the structure for 81116 LOS has not been fully determined, to date.

7.2.3.1 Structural analysis of *C. jejuni* 81116 core OS by NMR

For the best opportunity to fully characterise the structure of the 81116 LOS core, large quantities of biomass were prepared from 800 blood agar plates (dry mass biomass: 11.3 g). Hot-phenol water extraction was carried out, followed by enzymatic treatment with RNase A, DNase II and proteinase K, yielding 390 mg of purified extract (3.4% yield, based on dry mass of bacterial mass). The material was ultracentrifuged at $100,000 \times g$ for 18 h, at 4°C, which obtained 131 mg in the pellet, and 227 mg in the supernatant. The pellet containing lipidated material (LOS) was kept for analysis. Acid hydrolysis of the LOS pellet contained was carried out by incubation in 1% acetic acid for 90 min, at 100°C, and the liberated core oligosaccharides were retrieved from the aqueous phase after centrifugation ($8000 \times g$, 20 min). Core oligosaccharide was fractionated by anion-exchange chromatography (HiTrap Q HP column) on an AKTA explorer system (Amersham Biosciences), followed by removal of contaminating NaCl with HiTrap desalting columns (Amersham Biosciences). Two main acidic fractions were isolated and NMR analysis was performed. The spectra were significantly heterogeneous with many overlapping signals, and the degree of heterogeneity in the sample made full assignment of the structure impossible. However, a partial structure for the main species was deduced. Although other minor components were present, it was not possible to characterise all species without further separation.

1D ^1H NMR spectra revealed four carbohydrate residues and their linkages based on coupling constants of the anomeric proton signals, two α - and two β -linked. The residues and their linkages were identified and proton and carbon signals were assigned based on 2D NMR experiments including COSY, TOCSY, HSQC and HMBC. A non-reducing β -Gal was twice substituted at the 2- and 3- positions by α -GlcNAc and α -GalNAc, respectively. Terminal α -GalNAc was substituted at the 3-position with β -Qui3NFo. The residue sequence was confirmed using the HMBC experiment. The observed partial structure was deduced to consist of β -Qui3NFo-(1,3)- α -GalNAc-(1,3)-[α -GlcNAc-(1,2)]- β -Gal (Fig.7.11). This partial structure deviated from the preliminary structure proposed in Holden *et al.* (Fig. 7.10). It was shown here that the two HexNAc

residues were substituted on the same Gal rather than present as a chain as was previously reported.

NMR data for more acidic product (Table 7.3), enabled the following characterisations. Residue Z (Qui3NFo), was deduced to be β -linked because of upfield proton chemical shift (lower energy) (^1H at 4.80 ppm) with larger coupling constant and lower field anomeric carbon shift (^{13}C δ 103.4 ppm). Residue Z had ring carbons C-2, 4 and 5 (^{13}C δ 76.7, 74.0 and 73.5 ppm, respectively), a C-N bond at C-3 (^{13}C δ 56.1 ppm), and a methyl group at C-6 (δ 17.9 ppm). Combined with the presence of an *N*-formyl signal (^1H δ 8.4 ppm), this indicated that residue Z was β -linked 3-*N*-formyl-quinovosamine (β -Qui3NFo/ 3-formamido-3,6-dideoxyglucose).

Residue E,D was deduced to be α -linked due to downfield proton shift (^1H at 5.31 ppm) and smaller coupling constant. Residue E,D had an amine group at C-2 (^{13}C δ 48.9), ring carbons C-3, 4 and 5 (^{13}C δ 77.9, 68.9 and 71.7 ppm, respectively), and a methylene group at C-6 (^{13}C δ 62.1), indicating that it was α -GalNAc. Residue K,L was deduced to be β -Gal with high proton shift (^1H at 4.88/ 4.95 ppm), and ring carbons at C-2, 3 and 5 (^{13}C δ 73.5, 75.2 and 75.6 ppm, respectively). The unusually high field carbon shift of ED-1 and C-4 of β -Gal could not be explained but may be due to some unidentified structural component.

Residue A was deduced to be α -GlcNAc with low field shift (^1H δ 5.63 ppm), an amine group at C-2 (^{13}C δ 54.3 ppm), ring carbons C-3, 4, 5 (71.9, 70.7 and 73.4 ppm, respectively) and a methylene group at C-6 (^{13}C δ 62.2 ppm).

Residue E,D gave a very strong nuclear Overhauser effect (NOE) to KL4, but since KL C-4 was not substituted (^{13}C at 63.9 ppm), it should have been linked to KL-3. However, a weak NOE of residue ED H1 to residue KL H3 was present. No NOE of residue A H1 to residue KL H1 was present, which was not in agreement with the presented structure (Fig. 7.11), but there seems to be no other explanation of the observed NOE residue A H1 to residue KL H2 or H3. There were no other suitable signals to identify and determine, additional components and linkages.

Table 7.3. ^1H and ^{13}C NMR chemical shift data for the core oligosaccharide of *C. jejuni* 81116 LOS.

	1	2	3	4	5	6
β -Qui3NFo Z	4.80	3.78	4.07	3.31	3.64	1.40
	103.4	76.7	56.1	74.0	73.5	17.9
α -GalNAc E,D	5.31	4.42	4.14	4.39	4.23	3.94
	92.5	48.9	77.9	68.9	71.7	62.1
β -Gal K,L	4.88	4.05	4.07	4.33	3.78	
	99.9	73.5	75.2	63.9	75.6	
α -GlcNAc A	5.63	4.11	3.87	3.62	4.07	4.04
	97.3	54.3	71.9	70.7	73.4	62.2

N-formyl proton shift 8.4 ppm,

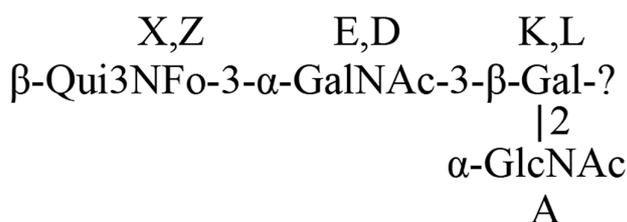


Figure 7.11 Partial structure for *C. jejuni* 81116 LOS glycoform determined by NMR. Qui3NFo (3-*N*-formylquinovosamine), GalNAc (*N*-acetylgalactosamine), GlcNAc (*N*-acetylglucosamine), Gal (galactose).

The structure proposed based on NMR analysis showed that the two HexNAc residues were substituted on the same Gal rather than being present as a chain as suggested in Holden *et al.* (2012). The partial structure showed that the ddHexN was modified with a formyl group, which is consistent with the CE-ESI-MS data presented in this study as well as that in Holden *et al.* (2012), where the presence of either formylation or acetylation of ddHexN was suggested in the form of *N*-formylquinovosamine or *N*-acetylquinovosamine, respectively.

7.2.3.2 Proposed structures for three *C. jejuni* 81116 core OS glycoforms

Based on the CE-ESI-MS and NMR data gathered in this study putative structures for the three *C. jejuni* 81116 core OS glycoform molecules, detected at 37 and 42°C, are proposed in Figure 7.12. With Glycoform A, the partial structure determined by NMR, confirmed the identity of certain sugar residues and linkages, and was combined with the compositional evidence provided by CE-ESI-MS. However, it can be assumed that the sugar residues and linkages for the core as determined by NMR, are common to all glycoforms. NMR confirmed a β -Gal residue in the core structure, which likely links to the inner core via substitution on a heptose residue. A β -Gal residue is typically found linking the outer core to the inner core in *C. jejuni* (Aspinall *et al.*, 1993). This β -Gal is twice substituted at the 2 and 3 positions by α -GlcNAc and α -GalNAc, respectively. The terminal GalNAc residue is twice substituted with ddHexN residues; NMR confirms one Qui3NFo residue is attached to position 3 of the GalNAc residue, as described above, CE-ESI-MS analysis would suggest that the other ddHexN is also Qui3NFo but linkage was not confirmed. The proposed structure for Glycoform B resembles Glycoform A, although both ddHexNFo residues are replaced with ddHexNAc, which are taken to be Qui3NAc. Glycoforms A and B represent major structures and are likely the constituents of upper silver stained bands observed with SDS-PAGE (Fig. 7.7). Proposed Glycoform C is a truncated structure similar to both Glycoforms A and B, but with only a single QuiN residue that lacked acetylation/formylation substituted on the GalNAc, and absence of core OS phosphorylation. Glycoform C was likely the constituent of the lower M_r silver stain band observed with SDS-PAGE (Fig. 7.7). Lack of core OS phosphorylation in Glycoform C would suggest that phosphorylation in Glycoforms A and B was located on the terminal Qui3NAc/ Qui3NFo.

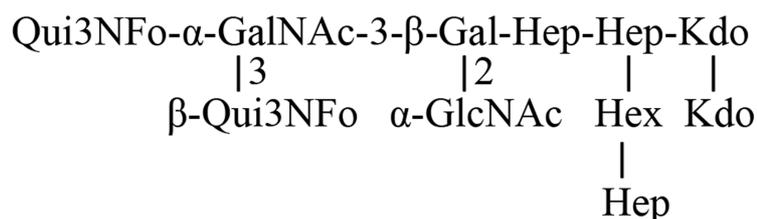
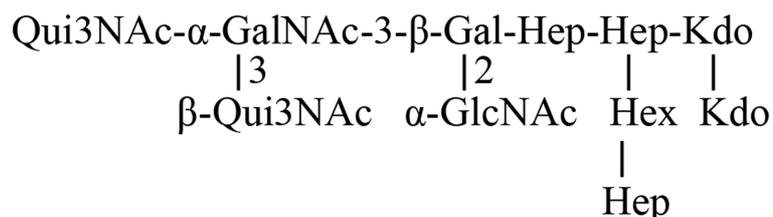
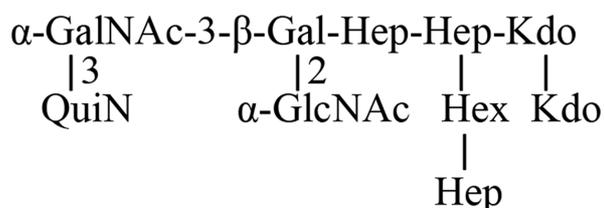
Proposed Glycoform A**Proposed Glycoform B****Proposed Glycoform C**

Figure 7.12 Proposed structures for *C. jejuni* 81116 LOS glycoforms based on combination of CE-ESI-MS and NMR data. NMR analysis could confirm not all carbohydrate linkages, although the inner core region is conserved in *C. jejuni*. Substitution of the core OS with a phosphate residue is also likely present, based on MS data. Qui3NFo (3-*N*-formylquinovosamine), Qui3NAc (3-*N*-acetylquinovosamine), QuiN (quinovosamine), GalNAc (*N*-acetylgalactosamine), GlcNAc (*N*-acetylglucosamine), Gal (galactose), Hep (heptose), Hex (hexose), Kdo (3-deoxy-*D*-manno-oct-2-ulosonic acid).

7.3 Discussion

Campylobacter jejuni induces acute pro-inflammatory responses in humans but not chickens. Certain *C. jejuni* components may contribute to immune evasion or subversion, and consequently play a role in commensalism that exists between *C. jejuni* and chickens. In the related *H. pylori*, existence of chronic infection may be linked to low bioactivity of *H. pylori* LPS, due to difference in lipid A structure compared to

endotoxically potent *E. coli* LPS (Moran *et al.*, 1996). Potentially, *C. jejuni* chronic colonisation of avian species may be influenced by lipid A modification.

To explore this hypothesis, HD11 chicken macrophages and CEF cells were challenged with *C. jejuni* CPS and ultrapure LOS extracted from *C. jejuni* grown at both 37 and 42°C. Human THP1 monocytes that were differentiated into macrophages, and chicken HD11 macrophages were compared in their response to challenge with *C. jejuni* 37°C LOS and *E. coli* LPS. The cells responded to both endotoxins with a similar pro-inflammatory response, suggesting minimal difference in recognition of these PAMPs by human and chicken macrophages. Recently, de Zoete *et al.* (2010a) reported increased pro-inflammatory cytokine expression in both chicken HD11 macrophages and human MM6 monocytes in response to *C. jejuni* LOS. However, with variation of endotoxin dosage, there was indication that *C. jejuni* LOS and *E. coli* LPS were differentially recognised. A low *E. coli* LPS dose induced the highest cytokine gene expression in both macrophage types, while reduction in expression was observed at higher doses, potentially indicating macrophage-mediated tolerance to LPS (Nomura *et al.*, 2000). In the case of LOS, a gradual increase in cytokine gene induction occurred, indicating differences in recognition of these PAMPs. The saccharide side chain of rough and smooth LPS is known to alter the interaction with the TLR4/MD2 complex (Jiang *et al.*, 2005). Therefore, in this case *C. jejuni* LOS, which lacks an O-chain may act like rough LPS.

Using PMB, it was possible to show that HD11-mediated pro-inflammatory responses to *E. coli* and *C. jejuni* endotoxins were a result of lipid A recognition. In the presence of PMB, increases in cytokine gene expression in response to *E. coli* LPS were eliminated, and reduced in response to *C. jejuni* LOS. Treatment with PMB binds lipid A molecules, neutralizing the immunostimulatory properties of LPS (Cardoso *et al.*, 2007). Such incomplete neutralisation of LOS is likely an indication that the endotoxin is somewhat resistant to PMB binding, on the basis of previous studies. PMB specifically binds to phosphate groups of lipid A but with *C. jejuni* lipid A, these groups can be substituted by *PP*Et_n, thus, reducing binding efficiency (Moran *et al.*, 1991). Further evidence of resistance comes from observation that live *C. jejuni* are resistant to PMB killing (Cullen & Trent, 2010; van Mourik *et al.*, 2010).

Chicken HD11 macrophages were also challenged with LOS from *C. jejuni* grown at 42°C. Both 37 and 42°C LOS induced cytokine gene expression increases in HD11

cells that were similar in magnitude, which were slightly higher than the cytokine increases observed in response to *E. coli* LPS challenge. This suggested that both LOS types equally stimulated pro-inflammatory responses in chicken cells.

In addition to macrophages, fibroblast-like cells are also found in the chicken intestinal lamina propria (Illemann *et al.*, 2004), and thus, CEF cells were compared in their response to 37 and 42°C LOS. In response to 37°C LOS, increased *TLR15* and *IL8* gene expression by CEF cells was observed. In contrast, neither *C. jejuni* 42°C LOS or *E. coli* LPS induced pro-inflammatory mediator gene expression in these cells. Gene expression analysis in this study revealed that CEF cells express at the mRNA level *TLR2*, *TLR4*, *TLR5*, *TLR15* and *TLR21*, but lack *TLR4* gene expression. Therefore, it appears that 37 and 42°C LOS can be recognised by chicken *TLR4* (expressed on HD11 cells), and that 37°C LOS can also be recognised by CEF cells in a *TLR4*-independent manner. Although it is not clear which other PRR was involved, a potential candidate is *TLR2*, since it is expressed at a high level in CEF cells. Lipopolysaccharide from the related microbe *H. pylori* is known to activate both human *TLR2* (Yokota *et al.*, 2007) and *TLR4* (Ishihara *et al.*, 2004). However, *TLR15* may be involved since it upregulated in CEF cells challenged with 37°C LOS, and is most similar to chicken *TLR2* with 30% amino acid sequence homology (Higgs *et al.*, 2006). Future investigations are required to confirm the identity of the PRR involved in detection of 37°C LOS. Importantly, cell-surface PRRs do not function in isolation, and often require the co-operation of co-receptors (Triantafilou *et al.*, 2002), as well as adaptor and signaling proteins (Jiang *et al.*, 2005). Alternative receptors on mammalian cells including sialoadhesin (Sn) have been observed to bind *C. jejuni* LOS in a sialic acid-specific manner, and have accessory roles in *TLR* activation in macrophages and dendritic cells (Klaas *et al.*, 2012). Although strain 81116 does not express sialylated LOS, it is decorated with the related sugar quinovosamine, that may influence the interaction with avian PRRs (Holden *et al.*, 2012).

To determine the molecular basis of differential response of CEF cells to 37 and 42°C LOS, multiple replicates of *O*-deacylated *C. jejuni* 81116 LOS were analysed by CE-ESI-MS. Focusing here on the lipid A, since it was demonstrated to be involved in PRR activation, a major lipid A species that was composed of a hybrid backbone GlcN3N-GlcN disaccharide was detected. This constituted ~75% of lipid A in *C. jejuni* 81116 grown at both 37 and 42°C. In addition, at 37°C, strain 81116 was found to

produce ~6.5% GlcN-GlcN, and ~19% GlcN3N-GlcN3N lipid A. However, when strain 81116 was grown at 42°C the level of expression of these lipid A forms was approximately reversed compared to 37°C, with GlcN-GlcN present at ~14%, and GlcN3N-GlcN3N present at ~9.5%. This suggests a temperature-related change in the lipid A backbone disaccharide and membrane fatty acid composition in a subset of *C. jejuni*, was responsible for differences observed in CEF cell response to 37 and 42°C LOS. The biological role of *N*-linked fatty acyl chains in pathogenesis is undetermined, although, it has been speculated that increased amide-linked acyl chains provides greater stability to the outer membrane under harsh conditions such as elevated temperature, due to greater bond strength (Trent *et al.*, 2006). This is somewhat contradicted by the present study, where a lesser quantity of *N*-linked fatty acids was observed at the higher temperature of 42°C. This may suggest a fundamental role of the variation in *C. jejuni* host adaptation.

In other pathogens, lipid A modification is an important component of host adaptation, and is influenced by host environmental conditions. In *Yersinia pestis*, modification of lipid A is dependent on temperatures mimicking the conditions found throughout the host cycle. At 20-25°C, which is the temperature experienced in the pikelethermic flea, *Y. pestis* produces a mixture of tetracyl, pentacyl and hexacylated lipid A. An increase in temperature to 37°C results in a decrease in acylation, and the production of primarily tetracylated lipid A. This occurs due to temperature-regulated differential expression of *Y. pestis* lipid A biosynthesis genes. Adaptation mechanisms such as lipid A modification occur due to regulation of specific genes, often under the control of two-component regulatory systems. Whole genome microarray analysis of *C. jejuni* 11168 gene transcription in temperature shift from 37 to 42°C, showed differential regulation of genes involved in biosynthesis of surface structures. Numerous genes involved in core OS and lipid A synthesis were differentially expressed indicating that surface structure modification plays a role in *C. jejuni* adaptation to growth at 42°C. The *C. jejuni htrB* gene, homologous to the *E. coli lpxL* gene, which is a lipid A biosynthesis laurosyl acyl transferase in *E. coli*, is upregulated during heat shock at 44°C (Phongsisay *et al.*, 2007), further suggesting that lipid A modification may be important in *C. jejuni* heat adaptation. Therefore, differential expression of lipid A biosynthesis genes, may account for the lipid A variation detected in the current study, and constitute a host-related adaptation.

The biological impact of lipid A modification includes evasion and modulation of the immune system. The cytokine-inducing activity of LPS is mediated by lipid A structure (Park *et al.*, 2007), and the reduced acylation in *Y. pestis* lipid A at 37°C, results in significantly reduced biological activity for these molecules (Knirel & Anisimov, 2012). In contrast to the hexacylated *Y. pestis* lipid A, the lower-acylated form is not recognised by TLR4, and does not activate innate immunity, resulting in decreased TNF α production in human and mouse macrophages, and lower IL12 secretion by DCs (Robinson *et al.*, 2008; Telepnev *et al.*, 2009). Furthermore, the underacylated lipid A produced by *S. typhimurium* has a significantly reduced ability to stimulate TLR4 (Kawasaki *et al.*, 2004). The nature of the linkage of acyl chains has previously been shown to influence the biological activity of *C. jejuni* LOS. Van Mourik *et al.* (2010) investigated the biological significance of the increased *N*-acylation found in the mixed lipid A produced by *C. jejuni*. The genes *gnnA* and *gnnB*, encoding a putative oxidoreductase and a transaminase, respectively (Trent *et al.*, 2006), were found to enable the synthesis of a GlcN3N precursor in the *C. jejuni* lipid A backbone (van Mourik *et al.*, 2010). Orthologues of these genes present in all Gram-negative organisms known to produce GlcN3N-substituted lipid A (Trent *et al.*, 2006). Mass spectrometry confirmed that knockout mutagenesis of either of these genes resulted in substitution of an *N*-linked acyl chain with an hydroxyl-linked acyl chain on the backbone disaccharide (van Mourik *et al.*, 2010). This increased *O*-acylation resulted in an LOS that was more biologically active towards human TLR4/MD2. The authors postulated that the increased *N*-acylation in *C. jejuni* lipid A may, in part, contribute to the lower overall biological activity of *C. jejuni* lipid A in humans, compared to *E. coli* lipid A (Moran, 1995; van Mourik *et al.*, 2010). Potentially temperature-related variation in expression of the genes *gnnA* and *gnnB* involved in the addition of *N*-linked acyl chains to the lipid A backbone may account for the differences observed in *C. jejuni* 81116 acylation in the current study. However, in the present study, in contrast to van Mourik *et al.*, increased *N*-linked fatty acids in 37°C lipid A were associated with pro-inflammatory effects on CEF cells, indicating that *C. jejuni* LOS produced at 37°C evades human TLR4 (van Mourik *et al.*, 2010), but it appears with certain chicken PRRs the reverse is true and a reduction of *N*-linked fatty acids is associated with PRR evasion.

The biological activity of lipid A is directly correlated with its molecular shape

(Seydel *et al.*, 2003). A conical molecular shape is required for endotoxicity (agonism) and a cylindrical one for antagonism (Schromm *et al.*, 2000; Seydel *et al.*, 2003). Hexacylated lipid A from *E. coli* has a conical/concave shape is associated with its high rates of bioactivity, whereas pentaacylated lipid A is antagonistic to TLR4 (Schromm *et al.*, 2000). It has been suggested that the presence of less ester-linked acyl chains, and a greater amount of amide-linked acyl chains in lipid A makes the molecule less biologically active toward TLR4 (Moran, 1997; Schromm *et al.*, 2000; van Mourik *et al.*, 2010). A previous study determined that *C. jejuni* lipid A is not antagonistic to TLR4 owing to its slight tendency toward a conical/concave shape (Schromm *et al.*, 2000). However, *C. jejuni* expresses three distinct lipid A variants and the supramolecular shape and bioactivity of each of these individual variants has not been described. Furthermore, their comparative interaction with alternative PRRs to TLR4 is unknown and the ligand specificity of human, murine and chicken TLRs and co-receptors varies (Hajjar *et al.*, 2002; Keestra & van Putten, 2008)

Potentially, at 37°C a high enough concentration of bioactive lipid A was produced, that was stimulatory to the unidentified PRR on the CEF cells, while at 42°C the concentration of bioactive lipid A was reduced below the threshold of bioactivity for this receptor. This argument is strengthened from the observation that *E. coli* LPS did not stimulate pro-inflammatory induction on the CEF cells either, and that 42°C lipid A has greater resemblance to *E. coli* lipid A with the presence of a greater amount of the GlcN-GlcN backbone disaccharide (Structure 3, Fig. 7.6).

Alternatively, it could be proposed that the increased level of *O*-acylated LOS produced at 42°C may have an antagonistic effect, and inhibit the induction of a pro-inflammatory response through the unidentified PRR. This effect is observed with LPS produced by *Y. pestis* at 37°C, which is antagonistic to TLR4, and inhibits a TLR4-dependent response to pro-inflammatory LPS in human macrophages and DCs (Matsuura *et al.*, 2009; Telepnev *et al.*, 2009). Additionally, in *H. pylori*, LPS can act antagonistically, preventing TLR4 activation by *E. coli* LPS (Lepper *et al.*, 2005). The periodontal pathogen *Porphyromonas gingivalis* can produce two LPS types, one with a pentaacylated lipid A which is a TLR4 agonist, and the other with a tetraacylated lipid A which inhibits TLR4 activation (Reife *et al.*, 2006). Although, no change the number of LOS acyl chains was detected in this study, the alteration of the lipid A backbone that was observed could potentially result in similar phenomena, due to associated changes

in the supramolecular structure of the LOS molecule which, in turn, would have an influence on LOS-PRR interaction.

Hypothetically, the PRR that recognised 37 but not 42°C LOS on CEF cells, would not be activated by exposure to *C. jejuni* in the chicken intestine. Under these circumstances TLR4 expressed on other epithelial cells would potentially recognise the LOS, although in unchallenged birds, TLR4 is lowly expressed in the caecum at the mRNA level, compared to other PRRs including TLR2 and TLR15 (Higgs *et al.*, 2006). Therefore, in these conditions, TLR4 activation by 42°C *C. jejuni* LOS would be negligible, and the alternative LOS PRR would be inhibited or evaded, resulting in minimal pro-inflammatory effects. Furthermore, the contribution of *C. jejuni* CPS to chicken immune evasion was also demonstrated in this study, by challenging chicken HD11 macrophages and CEF cells with *C. jejuni* CPS.

Human epithelial cells have been shown to produce IL6 in response to crude *C. jejuni* CPS (Friis *et al.*, 2009). Pro-inflammatory gene expression increases were detected in response to the purified *C. jejuni* CPS preparation in both chicken HD11 macrophages and CEF cells. However, the pro-inflammatory response observed was minimal and since there have been few published reports of cytokine response to bacterial CPS, it was clear that *C. jejuni* LOS contamination may have induced the response. Treatment of the cells with CPS in the presence of PMB completely abrogated the pro-inflammatory influence of the material in HD11 and CEF cells, indicating that LOS contamination was responsible for the cytokine response. Therefore, these results suggest that chicken CPS is not immunogenic in chickens. Since CPS is the outermost component of the microbial cell, it may thus form an immunologically inert shield, protecting antigenic *C. jejuni* outer membrane components from chicken immune system detection. Thus, CPS expression, and temperature-related lipid A modification by *C. jejuni* may be a significant factor in immune evasion and modulation contributing to commensalism in the chicken host. However, further investigation is required, in particular in determining the significance of these findings in the chicken host *in vivo*.

Holden *et al.* (2012) proposed a putative structure based on CE-ESI-MS analysis of *O*-deacylated LOS, and reported the presence of a number of LOS glycoforms (Holden *et al.*, 2012). Using a similar approach in the current study, CE-ESI-MS analysis suggested the presence of three core OS glycoforms, two major structures with either formylation or acetylation, and a lower- M_r structure. This correlated with the

SDS-PAGE profile for this strain, which consisted of two silver stain bands, with a greater amount of the higher M_r band likely representing the major structures, and a lower- M_r band likely consisting of the truncated structure. This resembles strain 81-176, where phase variation has been determined to result in the production of multiple LOS glycoforms which express molecular mimicry of either GM_{1a} , GM_2 or GM_3 gangliosides (Bacon *et al.*, 2001; Guerry *et al.*, 2002; Karlyshev *et al.*, 2004; Bachtiar *et al.*, 2007). Importantly, the lower- M_r GM_3 phase variant was shown to be more invasive *in vitro*, demonstrating a specific role for LOS phase variation, in addition to presentation of variable cell surface antigens in *C. jejuni* pathogenesis (Guerry *et al.*, 2002; Muldoon *et al.*, 2002; Kilcoyne *et al.*, 2006).

In this study, full structural characterisation of strain 81116 LOS was also attempted. As a genome sequenced strain, and the subject of much research into *C. jejuni* pathogenesis, knowledge of this structure would be valuable. However, high-levels of heterogeneity, and difficulties in separating the various cell-surface components have meant that complete structural elucidation of the *C. jejuni* 81116 LOS core has eluded investigators for a decade. In order fully characterise the structure for 81116 LOS NMR analysis of purified phenol-water extract was carried out. Due to the heterogeneous nature of the material, spectral interpretation was complicated, and some structural components were not identified. However, the determination of a partial structure for the core OS of 81116 was achieved that differed from the structure proposed by Holden *et al.* (2012). Although not identical, this partial structure shares many features of the core OS of the *C. jejuni* HS:3 (ATCC 43431) serostrain characterised by Aspinall *et al.* (1993). This is not surprising since these strains have similar but not identical LOS biosynthesis loci (Parker *et al.*, 2005; Holden *et al.*, 2010). *C. jejuni* 81116 has a class E LOS biosynthesis locus, containing 19 genes, which included homologues of glycosyltransferases, a lipid A acyltransferase, and 7 genes for ddHexN biosynthesis (Parker *et al.*, 2005; Guerry & Szymanski, 2008). In comparison, the *C. jejuni* ATCC 43431 has a class H LOS biosynthesis locus, consisting of 18 genes, 17 of which share sequence similarity to the 81116 LOS biosynthesis locus (Parker *et al.*, 2005; Jervis *et al.*, 2012). Furthermore, the OS of strain ATCC 43431 is modified with the acetylated form of ddHexN, Qui3NAc, which was indicated to be present in 81116 based on CE-ESI-MS analysis (Aspinall *et al.*, 1995; Karlyshev *et al.*, 2005; van Sorge *et al.*, 2009). The Qui3NAc residues characteristic of *C. jejuni* ATCC 43431 have also been detected in *C. jejuni* clinical isolates (Bachtiar *et al.*, 2007; Poly *et al.*,

2008), as well as in *C. coli* (Penner & Aspinall, 1997), and is a characteristic of the LPS of a number of other species including *E. coli* and *Salmonella* (Clark *et al.*, 2009). However, the NMR data presented here confirms for the first time the presence of formyl modification of *C. jejuni* LOS, in the form of Qui3NFo. *N*-formyl modified amino sugars have been detected in the LPSs of other Gram-negative bacteria (Katzenellenbogen *et al.*, 1995; Ovchinnikova *et al.*, 2007). The exact role of this modification is unknown, but it is likely another of the multiple elements used to add variability to Gram-negative surface glycans. Bacteria must constantly vary their surface glycans in order to avoid not only the host immune system, but also phage binding (Sorensen *et al.*, 2011). In *Providencia alcalifaciens*, which contains *N*-formylquinovosamine as part of its LPS O-chain, a novel formyl transferase gene (*vioF*) was recently described, which is responsible for the conversion of dTDP-Qui4N to dTDP-Qui4NFo (Liu *et al.*, 2012). Few genes in the *C. jejuni* 81116 LOS biosynthesis locus have been functionally characterised, primarily due to lack of complete knowledge of LOS structure, and a *C. jejuni* formyltransferase homologue has not been described to date.

Previous chemical analysis of *C. jejuni* 81116 reported a “polysaccharide B” (Fig. 6.1) with a repeating unit that contained two residues of GlcNAc and one each of Glc and Gal (Muldoon *et al.*, 2002). A subsequent investigation confirmed that “polysaccharide B” was LPS-like and not associated with the capsule (Kilcoyne *et al.*, 2006). However, this polysaccharide was not identified in any fraction analysed in the present study, and it is unclear where this “O-chain” would be connected to the core structure described in the current study.

Combination of MS and NMR data enabled the proposal of putative structures for three LOS glycoforms expressed by *C. jejuni* 81116. In another study, structural analysis of the LOS of individual *C. jejuni* colonies indicated that production of distinct *C. jejuni* LOS glycoforms is due to phase variation (Li *et al.*, 2005). Two of the glycoforms proposed in this study differ only in that that core OS is modified with either ddHexNAc or ddHexNFo. The remaining glycoform is a truncated structure with no acylation or formylation of ddHexN present, and lacking core phosphorylation. The ability of strain 81116 to variably express unmodified ddHexN, ddHexNAc and ddHexNFo, probably due to phase variation, would provide an efficient evasion mechanism from phage and host immune receptors. Phage resistance is also a rational reason for a genus, such as *Campylobacter*, to have hundreds of serotypes i.e. avoiding

having common receptors for phage (Dr. M. Gilbert 2012, personal communication). However, this theory remains to be proven. Overall, the data gathered in this study indicate that in *C. jejuni* both the core oligosaccharide, and the lipid A backbone are subject to structural variation, which may be an important factor in environmental and host adaptation.

Chapter 8.
Discussion

Cell-surface glycoconjugates play a critical role in the lifestyle of *C. jejuni*, those expressed by both the bacterium and the host cell. Previous studies have highlighted the wide variety of glycan molecules produced by *C. jejuni* strains, which have diverse roles as virulence factors (Moran *et al.*, 1991; Aspinall *et al.*, 1992; Aspinall *et al.*, 1993; Aspinall *et al.*, 1994; Aspinall *et al.*, 1995; Hanniffy *et al.*, 1999; Bacon *et al.*, 2001; Muldoon *et al.*, 2002; Oldfield *et al.*, 2002; Karlyshev *et al.*, 2005; McNally *et al.*, 2005; Papp-Szabo *et al.*, 2005). High rates of antigenic variation in *C. jejuni* cell-surface glycoconjugates, as well as structural mimicry of host glycans, are strategies employed by the bacterium for evasion of host defenses, enabling infection of humans to proceed, and chicken colonisation to persist. Significant, also is the pathogenic link between *C. jejuni* cell-surface glycoconjugates, (specifically LOS) and the peripheral nerve neuropathy, GBS. Furthermore, interaction with host-cell surface glycoconjugates is important step in infection and host colonisation (Ruiz-Palacios *et al.*, 2003). Consequently, the aim of this study was to investigate glycoconjugate production and binding by *C. jejuni*, and their involvement in the pathogenesis of infection and the development of GBS.

In Chapter 3.1 experimentation focused a large selection of clinical and retail *C. jejuni* isolates from the island of Ireland, using electrophoretic and serological techniques to characterise the variety of glycan production, and the extent of ganglioside mimicry in these strains. The isolates expressed a range of silver stain profiles, suggestive of heterogeneous LOS expression. Isolates that were genetically related had similar silver stain profiles, indicating shared LOS biosynthesis loci. However, potential variation in production of CPS by certain genetically related strains was observed. This may be related to the phase-variation in CPS-biosynthesis/ modification genes that potentially turned off expression of CPS (Guerry, 2012), or alternatively, had altered CPS structure so that the cationic dye Alcian blue was no longer able to bind to the molecule (Karlyshev & Wren, 2001). The highly variable presence/ absence of methyl, ethanolamine, phosphoramidate and aminoglycerol modifications of CPS has been reported (St Michael *et al.*, 2002; Szymanski *et al.*, 2003; McNally *et al.*, 2007), a factor which is associated with resistance to phage (Holst Sørensen *et al.*, 2012).

It has been postulated that an infection by a ganglioside-mimicking *C. jejuni* strain can induce an immune response that can trigger GBS development, in humans (Yuki *et al.*, 1993; Aspinall *et al.*, 1993; Schwerer *et al.*, 1995; Moran and O'Malley,

1995; Moran *et al.*, 2002), and that cross-reactive antibodies generated against ganglioside mimicking *C. jejuni* LOS are central to this (Yu *et al.*, 2006). Serological profiling with anti-GM₁ ligands revealed that 56% of the total isolates had anti-GM₁ antibody reactivity, comprising 66.6% of clinical and 46.4% of food isolates. Therefore there was a higher incidence of GM₁ reactivity in clinical isolates, than food isolates in this study. Furthermore, this indicated a higher rate of GM₁ mimicry in *C. jejuni* isolates in Ireland than previously reported figures for the USA.

Anti-asialo-GM₁ reactivity was observed in 44% of the isolates, with 63.6% of these isolate also reactive with the GM₁ ligands. Production of sialylated and non-sialylated LOS in *C. jejuni* is a virulence factor likely due to phase variation. The ability of pathogenic bacteria, including *C. jejuni*, to modulate the expression of surface antigens, particularly LOS, is required for avoiding the host immune response (Guerry *et al.*, 2000), and adaptation to varying intestinal environments. This is particularly pertinent to *C. jejuni* that is able to infect animals, with very different intestinal cell surface receptors, such as birds and mammals (Kilcoyne *et al.*, 2012b). The role of LOS sialylation in *C. jejuni* pathogenesis is well described. Louwen *et al.* (2008), observed significantly greater invasiveness of sialylated *C. jejuni* strains in Caco-2 and T84 cells, compared to non-sialylated strains and mutants lacking sialic acid. Habib *et al.* (2009) also correlated *C. jejuni* LOS sialylation with increased invasion of Caco-2 cells. *C. jejuni* strains expressing sialylated and ganglioside mimicking LOS are associated with more severe clinical symptoms (Mortensen 2009), which correlates with the higher rates of invasion and translocation of epithelial cells associated with these strains *in vitro* (Habib *et al.*, 2009; Louwen *et al.*, 2008; Louwen *et al.*, 2012). Furthermore, mutants lacking in sialylation are more serum sensitive (Guerry *et al.*, 2002), thus, the production of non-sialylated LOS by *C. jejuni in vivo* may seem counterintuitive for an infecting pathogen. However, recent studies have demonstrated macrophages mediated phagocytosis of *C. jejuni*, and pro-inflammatory responses in macrophages and DCs dependant on *C. jejuni* LOS sialylation (Klass *et al.*, 2012; Huizinga *et al.*, 2012). Thus, in certain cases non-sialylated *C. jejuni* would avoid detection and destruction by the immune system, highlighting the advantage to an infecting *C. jejuni* of producing both sialylated and non-sialylated LOS in a dynamic fashion, as bestowed by phase variation.

Given the high rate of ganglioside mimicry observed in *C. jejuni* retail and clinical isolates in this study, and its well-reported role in GBS pathogenesis, combined with knowledge of the almost ubiquitous contamination of poultry products in Ireland

(Eurosurveillance editorial team, 2013), this must serve to sufficiently warn of the risk in Ireland of contracting *C. jejuni* that may stimulate the production of GBS. However, the high rates of ganglioside mimicry observed in clinical isolates would indicate that in addition to ganglioside mimicry, other factors are involved in development of GBS that preceded infection by *C. jejuni*. Potentially, the low incidence of immune-mediated disease following infection may be due to rare, uncharacterised features of neuropathogenic *C. jejuni*. In Japan, South Africa and Bangladesh, GBS is associated with *C. jejuni* strains of the HS:19, HS:41 and HS:23 serotypes, respectively (Fujimoto *et al.*, 1992; Lastovica *et al.*, 1997; Islam *et al.*, 2009), indicating the existence of pre-disposing, serotype-specific features.

In Chapter 4.1 comparative glycan microarray analysis of a clonal group of *C. jejuni* that had been isolated from cases of GBS and uncomplicated enteritis-only, revealed a strain-specific glycan binding profile. However, enteritis-related isolates bound to glycans at a higher rate than GBS-related isolates, correlating with higher rates of Caco-2 cell adherence for these isolates. It is proposed that the rate of *C. jejuni* adherence rate may influence the course and clinical outcome of infection, which could be enteritis or ultimately GBS. Results of a Caco-2 cell lectin inhibition assay confirmed glycan-binding profiles observed on the array, demonstrating the utility of the glycan microarray as a high-throughput tool. Since this technology is still in its infancy, continued efforts at technological development are key to full realisation of the potential of glycan microarrays in glycobiological research.

In Chapter 5.1, a microarray format was envisaged that could display glycans covalently bound to a protein linker, in the form of NGCs, which would more accurately represent the natural expression of glycans as glycoconjugates. Successful development of an NGC microarray enabled its use in examining the influence of growth temperature on *C. jejuni* glycan recognition. *C. jejuni* strains 81116 and 81-176 were cultured at 37 and 42°C to simulate human and avian host conditions, respectively. The disease that follows *C. jejuni* interaction with humans, contrasts to the benign commensal relationship it has with avian species. Therefore, it was postulated that temperature-related changes in *C. jejuni* would provide clues to the basis of the different outcomes of *C. jejuni*-host interaction. *C. jejuni* interaction with fucosylated NGCs on the array correlated with previous studies (Ruiz-Palacios *et al.*, 2003; Newburg *et al.*, 2005). However, only minor temperature-related differences in *C. jejuni* glycan-recognition were observed. Thus, attention was focused to the influence of growth

temperature on the *C. jejuni* cell-surface glycome.

In Chapter 6.1 a lectin array consisting with a wide range of binding specificities was able to differentiate between *C. jejuni* 81116 and 81-176. *C. jejuni* 81116 profiles indicated that the previously characterised LPS-like molecule and *N*-linked glycans were the dominant cell surface structures with the interaction of Gal, GlcNAc, and GalNAc-binding lectins. With strain 81-176 CPS, LOS and *N*- and *O*-linked glycosylation were best recognised on the array. It was possible to distinguish the strains grown at either 37 or 42°C, although the profiles were closer across the two temperatures for strain 81-176. For *C. jejuni* 81-176, a change in the relative distribution of CPS and LOS structures was indicated at the higher temperature. Initially, differences observed with strain 81116 grown at 42°C were associated with reduced dominance of the LPS-like structure and greater accessibility of surface exposed *N*-linked glycan. However, subsequent chemical characterisation revealed a partial structure for 81116 LOS composed of, among other components, GlcNAc, Gal and GalNAc residues, suggesting that 81116 LOS may have been involved in interactions with lectins on the array. Furthermore, the LPS-like molecule described in Kilcoyne *et al.* (2006) was not detected in any of the fractions analysed in this study, by NMR or CE-ESI-MS. Moreover, in the LOS subjected to CE-ESI-MS analysis there was no temperature-related change observed in the core OS. Combined, this indicates that the temperature-related change on the lectin array was due to variation in cell-surface *N*-glycosylation alone. Therefore, it appears with 81116 grown at 37°C the lectins on the array interacted primarily with LOS, whereas when grown at 42°C cell-surface *N*-glycosylation became more dominant, with greater binding of GalNAc-specific lectins. Concordantly, a previous DNA microarray study reported up-regulation in response to temperature up-shift from 37 to 42°C, of a *galE* gene, located in a 16-kb region named the *pgl/wla* locus (Fry *et al.*, 2000; Stintzi, 2003). Although, initially described as being involved in 81116 LPS expression (Fry *et al.*, 2000), genes in this locus are also part of the *N*-glycosylation system in *C. jejuni* (Szymanski *et al.*, 1999; Stintzi, 2003). Therefore, the present study suggests a novel temperature-related increase in *C. jejuni* *N*-glycosylation, which may have a role in adaptation to the avian environment. Previously, it was reported that disruption of *N*-glycosylation reduces colonisation of chickens and mice *in vivo* (Szymanski *et al.*, 2002; Hendrixson & DiRita, 2004; Alemka *et al.*, 2013).

Difficulty in understanding the function of *C. jejuni* *N*-glycosylation is due in part to the pleiotropic impact of *pgl* locus gene mutation, since diverse proteins are affected (Alemka *et al.*, 2013). Given that greater than 65 *C. jejuni* proteins, with a variety of functions are known to be *N*-glycosylated (Nothaft *et al.*, 2010), attributing a direct role for *N*-glycosylation in colonisation is tenuous. It was recently shown that *C. jejuni* *N*-glycosylation of surface proteins protects them from cleavage by chicken gut proteases, thereby increasing bacterial fitness and facilitating persistent colonisation (Alemka *et al.*, 2013), indicating a more general, but nonetheless essential role for *C. jejuni* *N*-glycosylation. Therefore, an increased production of cell-surface *N*-glycan expression by *C. jejuni* at 42°C may be associated with survival and persistence in the harsh ecological niche provided by the avian GI tract.

The lectin array offers an unrivalled capacity to profile dynamic changes in the bacterial cell surface glycome. Nonetheless, there is value in taking a more reductionist approach and focusing on individual, highly bioactive components such as LOS. In Chapter 7.1 *C. jejuni* 81116 LOS induced cytokine (>700-fold) gene expression in chicken HD11 macrophages, and also upregulated *TLR15* gene expression (15-fold) in HD11 cells. Chicken embryonic fibroblast cells responded differently to the LOS grown at 37 and 42°C, in which exposure to 37°C LOS resulted in a TLR4-independent pro-inflammatory reaction, possibly mediated by TLR2 or TLR15, whereas 42°C LOS or *E. coli* LPS, did not induce a response. Subsequent structural analysis of 81116 LOS identified a temperature-related lipid A variation correlating with the differential response of CEF cells *in vitro*. Thus, it was proposed that in a 42°C environment, such as the chicken intestine, *C. jejuni* may fail to induce pro-inflammatory gene expression in certain cell types. The difference in innate immune recognition was determined to be due to a temperature-related change in the ratio of lipid A backbone disaccharide variants, with varying numbers of lipid A *N*-hydroxyacyl chains present. This subtle change in lipid A expression in 81116 LOS may have altered the interaction with a PRR and potential co-receptors on the CEF cell, resulting in an attenuation of the pro-inflammatory response. Potentially, at 37°C a high enough concentration of bioactive lipid A was produced, which enabled it stimulate the unidentified receptor on the CEF cells, while at 42°C the concentration of bioactive lipid A was reduced below the threshold of bioactivity for this receptor. Alternatively, innate receptor antagonism may have been responsible for the lack response to 42°C LOS requiring (in order to engage

with a PRR *in vivo*) to be shed from the cell-surface. Outer membrane vesicles offer an explanation how periplasmic and membrane bound components such as lipid A could interact with such host PRRs, allowing them to modify host responses, without requiring prior cell lysis. Outer membrane vesicles have been proposed as an alternate means of *C. jejuni* virulence factor delivery (Lindmark *et al.*, 2009), but could also be involved in release of immune modulating factors, such as modified lipid A, by commensal *C. jejuni* in chickens. Outer membrane vesicles have roles in virulence, host colonisation and immune modulation in a range of Gram-negative bacteria (Kulp *et al.*, 2010). In recent study by Elmi *et al.* (2012), *C. jejuni* OMVs were found to consist of LOS, and at least 151 outer membrane and periplasmic proteins, including adhesins and sixteen *N*-glycoproteins. Overall, the present study highlights the complexity and specificity of PRR-PAMP interaction, and further suggests an important role for cell surface modification in promoting of adaptation of *C. jejuni* to its various hosts.

Adaptation mechanisms such as lipid A and glycoprotein modification occur due to regulation of specific genes, often under the control of two-component regulatory systems (TCRS). Two-component regulatory systems consist of sensor (S) kinases and response (R) regulators which cooperate to coordinate gene transcription, generating an adaptive response to environmental stimuli. In *Salmonella typhimurium*, modification of lipid A is controlled by the PhoP/PhoQ two-component regulatory system, which regulates the gene products PagL and PagP, a 3-*O*-deacylase and a palmitoyltransferase, respectively whose expression results in production of a lipid A with decreased acylation (Kawasaki *et al.*, 2004; Apel *et al.*, 2012). The *C. jejuni* genome predicts a total of 11 response regulators, 6 sensor kinases and one hybrid sensor regulator protein (Parkhill *et al.*, 2000). The RacR/ RacS signal transduction system, one of the first TCRSs to be reported in *C. jejuni*, is found in 98% of *C. jejuni* isolates (Apel *et al.*, 2012). A study by Bràs *et al.* (1999) reported that in *C. jejuni* 81116 the RacR/ RacS TCRS is required for chick colonisation and for optimal growth *in vitro* at 42°C (Bras *et al.*, 1999). Conversely, a recent study determined only a modest involvement of RacR/ RacS in growth of *C. jejuni* 81-176 at 42°C (Apel *et al.*, 2012). However, strain differences may account for discrepancy between the two studies, which correlate with the differences observed between strains 81116 and 81-176 observed in the present study. Therefore, the RacR/ RacS TCRS may be important for adaptation of certain *C. jejuni* strains such as 81116 to survival at 42°C, and the

genes which are responsible for the lipid A and surface *N*-glycan modification observed in this study may be under the control of this TCRS.

It may be helpful to consider the *C. jejuni*-chicken interaction in terms the human relationship with its gut microbiome, which is has been the subject of far more research. The modification or downregulation of certain PAMPs is a strategy employed by members of the human gut flora, to limit the pro-inflammatory effects of their presence. For instance commensal *Bacteroides* spp., lack flagella and express penta-acylated lipid A, which reduces their activation of TLR5 and TLR4, respectively (Erridge *et al.*, 2004; Wexler, 2007; Comstock, 2009). *C. jejuni* cell surface adaptations including the production of CPS may be important in adaptation to commensal colonisation of chickens. Expression of CPS by human commensals is correlated with avoidance of macrophages-mediated phagocytosis (Yasuda *et al.*, 2008), and immune evasion (Wexler, 2007). However, *C. jejuni* CPS is immunogenic in humans, evident from the fact that strain-specific differences in CPS structure form the basis of *C. jejuni* serotyping (Maue *et al.*, 2012). Interestingly, the observations in this study suggest that *C. jejuni* CPS is not immunogenic in chickens, which reflects the status of CPS in human commensals. In the immunostimulation experiments, purified 81116 CPS did not induce the production of pro-inflammatory cytokines in chicken HD11 macrophages or CEF cells. Furthermore, lectin array data indicated the non-recognition of 81116 CPS, based on its known structure (Kilcoyne *et al.*, 2006). Therefore, this may suggest that *C. jejuni* CPS avoids interaction with chicken immune lectins, thus limiting a pro-inflammatory response, potentially due to modification with non-carbohydrate substitutions (Holst-Sørensen *et al.*, 2012). In addition, chickens are known to have an incomplete response to T-cell independent Type-2 antigens (usually polysaccharides), which can activate B-cells without T-cell involvement (Young *et al.*, 2007). Taken together, the chicken immune system may be more tolerant of *C. jejuni* which is encapsulated in polysaccharide, since it has a reduced capacity to detect and respond to these carbohydrate antigens.

Numerous unsuccessful attempts at the complete structural characterisation of *C. jejuni* 81116 LOS highlight the difficulties posed to investigators due to the high levels of cell-surface variation expressed by *C. jejuni* (Muldoon *et al.*, 2002; Kilcoyne *et al.*, 2006; Holden *et al.*, 2012). In the present study, NMR analysis enabled the elucidation of a partial structure for strain 81116 LOS, which was observed to resemble *C. jejuni* HS:3 serostrain LOS, with which it shares similar LOS biosynthetic loci (Aspinall *et al.*,

1995; Parker *et al.*, 2008). Unique to strain 81116 was the identification of Qui3NFo, which is an unprecedented confirmation of an *N*-formyl modified hexose in *C. jejuni*. The ability of *C. jejuni* to synthesise unusual, strain-specific sugar residues such as Neu5Ac, Qui3NAc and Qui3NFo, probably enables *C. jejuni* interaction with diverse host cell receptors to facilitate immune modulation (Avril *et al.*, 2006; Heikema *et al.*, 2010; Bax *et al.*, 2011). Furthermore, the proposal of three structures in this study for 81116 LOS from combined NMR and MS analysis, demonstrates the level of heterogeneity expressed in *C. jejuni* LOS alone. This combined with other cell surface modifications, such as those observed in CPS and *N*-glycosylation undoubtedly represent strategies for evasion of phage and host immune receptors. *C. jejuni* strain-specific variation, many forms of which were highlighted in this study, illustrate the difficulty in forming general models of *C. jejuni* pathogenesis, and requires that each strain must be dealt with on an individual basis (O’Croinin & Backert, 2012). This is exemplified in this study by the unique cell-surface glycome expressed by *C. jejuni* 81116. Furthermore, cell-surface heterogeneity hampers identification of universal carbohydrate vaccine for *C. jejuni*. However, the advent of cutting-edge technologies such as microarrays is helping unravel the lifestyle of this enigmatic pathogen. Combined with continual development and improvement of more traditional tools in carbohydrate analysis, such as MS and NMR, this will help in identification of novel vaccine candidates, and prophylactic and therapeutic agents. The primary aim of research on *C. jejuni* is for improved control, prevention and treatment of the disease. However, it is also shedding light on the very nature of host-microbe interaction, which can be probiotic or, as this study has shown, circumstantially commensal or pathogenic. Consequently, this will broaden understanding of our relationship with our own microbiota, which is the focus of intense research, and is of major medical importance.

8.1 Conclusions

- (i) *C. jejuni* clinical and retail isolates from Ireland have highly variable glycan phenotypes, and high rates of ganglioside mimicry. Ganglioside mimicry in enteritis-related strains suggests that other bacterial and/or host factors are involved in GBS pathogenesis.
- (ii) Higher glycan-mediated adherence of *C. jejuni* HS:41 enteritis strains compared to closely-related GBS strains, indicates that glycan binding by *C. jejuni* influences clinical outcome of infection. Greater adherence of Neu5Ac by enteritis-related strains may be involved in this phenomenon. Recognition of glycans on a microarray correlates with glycan-mediated adherence in an *in vitro* cell model.
- (iii) Plant lectins printed on a microarray can differentiate between *C. jejuni* strains, and it was possible to distinguish the strains grown at either 37 or 42°C from one another on the lectin microarray. Growth at the higher temperature was associated with a change in the relative distribution of CPS and LOS structures in *C. jejuni* 81-176. With *C. jejuni* 81116 there was reduced dominance of LPS/LOS and greater recognition of *N*-linked glycans at 42°C, indicating a host-related adaptation.
- (iv) Superior differentiation is possible with lectin microarrays compared to agglutination assays, along with significantly faster experimentation time and the availability of quantitative data. Opposite behaviour of certain lectins occurs between the plate agglutination assay compared to the microarray platform, demonstrating platform-dependent lectin performance.
- (v) Immunostimulation of avian cells revealed differences in lipid A-mediated recognition of LOS prepared from *C. jejuni* 81116 grown at 37 and 42°C. *C. jejuni* 81116 LOS grown at 37°C is detected by TLR4 on chicken macrophages, as well as by an unidentified non-TLR4 PRR on CEF cells. However, both 42°C LOS and *E. coli* LPS evade detection of the unidentified PRR on CEF cells.

- (vi) Structural characterisation of *C. jejuni* 81116 LOS identified temperature-related changes in the ratio of lipid A structural variants, which account for the different immunostimulatory properties observed, and represent a host-related adaptation.
- (vii) Capsular polysaccharide is not pro-inflammatory to chicken cells *in vivo* suggesting a role in immune evasion that may contribute to chicken colonisation. Combined expression of modified lipid A, increased *N*-glycosylation and CPS by *C. jejuni* may facilitate persistent chicken colonisation.
- (viii) *C. jejuni* 81116 expresses a unique LOS structure that consists of an *N*-formyl modified hexose previously unidentified in the species.

Chapter 9.

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Appendix

Table (i) Comparison of growth at 37 and 42°C on lectin binding of *C. jejuni* 81-176

Lectin	81-176 37°C	81-176 42°C	p value	Sig
AIA	1131.51	1330.47	0.01226	*
RPbAI	1570.24	1606.68	0.49137	
PA-I	1893.08	1951.62	0.56575	
SNA-II	3905.25	2619.28	0.09816	
SJA	1289.93	1469.25	0.07990	
DBA	2586.29	2574.44	0.22597	
APP	1193.18	1055.58	0.06604	
SBA	1175.46	1356.96	0.97860	
VVA-B4	420.72	589.57	0.00746	**
BPA	2559.80	2564.55	0.50137	
WFA	1106.36	1175.59	0.32662	
ACA	23569.77	20483.28	0.20649	
PNA	2697.28	2888.07	0.35982	
GSL-II	1229.83	1548.67	0.04293	*
sWGA	480.82	572.04	0.20415	
DSA	3885.45	2767.84	0.00065	**
STA	1531.21	1180.92	0.12630	
LEL	5755.12	3553.91	0.06823	
NPA	1167.21	1150.64	0.84377	
GNA	862.38	1299.55	0.00531	**
HHA	699.78	789.68	0.00047	**
ConA	2226.93	2095.91	0.96787	
Lch-B	619.16	442.08	0.05062	
PSA	3233.71	2936.20	0.46407	
WGA	8398.56	9980.23	0.80972	
MAA	2538.29	2541.42	0.67492	
SNA-I	16997.64	9207.53	0.09791	
PHA-L	2514.12	1378.76	0.05200	
PCA	12214.67	4611.08	0.01292	*
PHA-E	6882.77	5130.25	0.00989	**
RCA-I	21490.16	15426.21	0.05192	
CPA	1026.35	1552.78	0.00255	**
CAA	1805.11	1765.33	0.42510	
ECA	481.71	611.85	0.09455	
AAL	11253.43	9236.02	0.02916	*
LTA	1994.42	1831.49	0.26979	
UEA-I	513.38	512.86	0.28897	
EEA	615.74	465.94	0.72130	
GSL-I-B4	640.92	822.77	0.01249	*
MPA	3029.90	4623.48	0.00521	**
VRA	5518.23	2664.61	0.00953	**

All t-tests paired, two-tailed based on 6 replicate data sets. Values shown for each strain and temperature are median of 6 replicate data sets (* $p < 0.05$; ** $p < 0.01$)

Table (ii) Comparison of growth at 37 and 42°C on lectin binding of *C. jejuni* 81116

Lectin	81116 37°C	81116 42°C	p value	Sig
AIA	1370.61	1052.25	0.17871	
RPbAI	3537.47	2103.58	0.00782	**
PA-I	1449.35	1113.49	0.20667	
SNA-II	63369.73	11586.30	0.00080	**
SJA	1255.52	1453.97	0.31583	
DBA	1466.57	1230.89	0.01727	*
APP	1771.43	1051.82	0.01994	*
SBA	1502.72	568.06	0.00681	**
VVA-B4	227.54	122.44	0.32187	
BPA	4399.22	1791.24	0.21329	
WFA	14131.13	687.38	0.07229	
ACA	27200.59	15738.73	0.02475	*
PNA	797.68	336.23	0.03769	*
GSL-II	1058.55	1128.44	0.21724	
sWGA	492.06	550.33	0.22598	
DSA	3654.04	2321.36	0.01347	*
STA	3296.34	1674.82	0.00073	**
LEL	22954.48	2877.49	0.00109	**
NPA	772.42	543.89	0.06355	
GNA	1117.63	1490.75	0.39407	
HHA	936.55	881.87	0.26285	
ConA	2266.80	1230.75	0.10171	
Lch-B	911.84	503.87	0.17022	
PSA	1473.14	826.03	0.04215	*
WGA	3780.59	1430.46	0.01678	*
MAA	1176.84	1441.39	0.31509	
SNA-I	2113.33	1467.44	0.04004	*
PHA-L	1176.24	837.66	0.04955	*
PCA	1673.60	880.20	0.00475	**
PHA-E	1635.86	813.28	0.00148	**
RCA-I	63331.21	12514.52	0.00067	**
CPA	1283.44	679.48	0.13922	
CAA	16956.70	2204.68	0.05238	
ECA	2293.41	1256.22	0.02000	*
AAL	12021.31	7252.34	0.01921	*
LTA	3719.80	2629.75	0.00559	**
UEA-I	2042.39	1229.92	0.07760	
EEA	4825.62	2966.70	0.01551	*
GSL-I-B4	1609.29	1524.83	0.41963	
MPA	2581.40	2201.27	0.30136	
VRA	4233.98	3302.63	0.02027	*

All t-tests paired, two-tailed based on 6 replicate data sets. Values shown for each strain and temperature are median of 6 replicate data sets (* $p < 0.05$; ** $p < 0.01$)

Table (iii) Comparison of lectin binding of *C. jejuni* 81-176 and *C. jejuni* 81116 grown at 37°C

Lectin	81-176 37°C	81116 37°C	p value	Sig
AIA	1131.51	1370.61	0.56230	
RPbAI	1570.24	3537.47	0.08984	
PA-I	1893.08	1449.35	0.25946	
SNA-II	3905.25	63369.73	0.00028	**
SJA	1289.93	1255.52	0.43831	
DBA	2586.29	1466.57	0.02312	*
APP	1193.18	1771.43	0.33837	
SBA	1175.46	1502.72	0.71100	
VVA-B4	420.72	227.54	0.83987	
BPA	2559.80	4399.22	0.24944	
WFA	1106.36	14131.13	0.08429	
ACA	23569.77	27200.59	0.48912	
PNA	2697.28	797.68	0.00580	**
GSL-II	1229.83	1058.55	0.57189	
sWGA	480.82	492.06	0.91463	
DSA	3885.45	3654.04	0.68024	
STA	1531.21	3296.34	0.11802	
LEL	5755.12	22954.48	0.00276	**
NPA	1167.21	772.42	0.12295	
GNA	862.38	1117.63	0.22776	
HHA	699.78	936.55	0.15933	
ConA	2226.93	2266.80	0.51095	
Lch-B	619.16	911.84	0.28183	
PSA	3233.71	1473.14	0.12534	
WGA	8398.56	3780.59	0.09617	
MAA	2538.29	1176.84	0.01930	*
SNA-I	16997.64	2113.33	0.00734	**
PHA-L	2514.12	1176.24	0.13893	
PCA	12214.67	1673.60	0.00851	**
PHA-E	6882.77	1635.86	0.00771	**
RCA-I	21490.16	63331.21	0.01231	*
CPA	1026.35	1283.44	0.38434	
CAA	1805.11	16956.70	0.05596	
ECA	481.71	2293.41	0.00631	**
AAL	11253.43	12021.31	0.50075	
LTA	1994.42	3719.80	0.04545	*
UEA-I	513.38	2042.39	0.04162	*
EEA	615.74	4825.62	0.00717	**
GSL-I-B4	640.92	1609.29	0.08656	
MPA	3029.90	2581.40	0.60641	
VRA	5518.23	4233.98	0.69399	

All t-tests paired, two-tailed based on 6 replicate data sets. Values shown for each strain and temperature are median of 6 replicate data sets (* $p < 0.05$; ** $p < 0.01$)

Table (iv) Comparison of lectin binding of *C. jejuni* 81-176 and *C. jejuni* 81116 grown at 42°C

Lectin	81-176 42°C	81116 42°C	p value	Sig
AIA	1052.25	1330.47	0.30766	
RPbAI	2103.58	1606.68	0.51766	
PA-I	1113.49	1951.62	0.00701	**
SNA-II	11586.30	2619.28	0.02310	*
SJA	1453.97	1469.25	0.53926	
DBA	1230.89	2574.44	0.00102	**
APP	1051.82	1055.58	0.77990	
SBA	568.06	1356.96	0.00263	**
VVA-B4	122.44	589.57	0.23270	
BPA	1791.24	2564.55	0.14461	
WFA	687.38	1175.59	0.00875	**
ACA	15738.73	20483.28	0.18504	
PNA	336.23	2888.07	0.00287	**
GSL-II	1128.44	1548.67	0.14700	
sWGA	550.33	572.04	0.35674	
DSA	2321.36	2767.84	0.46472	
STA	1674.82	1180.92	0.98563	
LEL	2877.49	3553.91	0.15991	
NPA	543.89	1150.64	0.01421	*
GNA	1490.75	1299.55	0.68625	
HHA	881.87	789.68	0.58823	
ConA	1230.75	2095.91	0.00755	**
Lch-B	503.87	442.08	0.63392	
PSA	826.03	2936.20	0.01912	*
WGA	1430.46	9980.23	0.00036	**
MAA	1441.39	2541.42	0.01133	*
SNA-I	1467.44	9207.53	0.01145	*
PHA-L	837.66	1378.76	0.07147	
PCA	880.20	4611.08	0.01823	*
PHA-E	813.28	5130.25	0.00540	**
RCA-I	12514.52	15426.21	0.95184	
CPA	679.48	1552.78	0.16537	
CAA	2204.68	1765.33	0.63244	
ECA	1256.22	611.85	0.08226	
AAL	7252.34	9236.02	0.23787	
LTA	2629.75	1831.49	0.14805	
UEA-I	1229.92	512.86	0.10357	
EEA	2966.70	465.94	0.00718	**
GSL-I-B4	1524.83	822.77	0.19886	
MPA	2201.27	4623.48	0.03381	*
VRA	3302.63	2664.61	0.92108	

All t-tests paired, two-tailed based on 6 replicate data sets. Values shown for each strain and temperature are median of 6 replicate data sets (* $p < 0.05$; ** $p < 0.01$)
