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Identification of Host-pathogen Interacting Molecules of *Campylobacter jejuni* using Phage Display Technology and in silico Sequence Analysis

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To all, I say, thank you and God bless!

Dr Harry Hoffman ABRUQUAH, BSc, MB ChB, MPhil.
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DEDICATION

This Thesis is dedicated to my son,
Harold Avery Ato Abruquah, who has been my inspiration since his birth,
his mum and my sweet wife, Akua, who has been my backbone
&
his grandma and my mum, Auntie Becky, who gave me her all.

DECLARATION

With the exception of references to the work of other scientists, I, Harry Hoffman ABRUQUAH, do hereby declare that all the work described in this thesis and the thesis thereof is my own, and that this thesis has not been submitted, neither in whole or in part, elsewhere for another degree.

(25/03/2009)
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ABREVIATIONS

ABC	ATP-binding cassette	**kDa**	Kilo Dalton
AFLP	Amplified fragment length polymorphism	**LB**	Luria Bertani/Luria Broth
AIDP	Acute inflamm. demyelinating polyneuropathy	**LEP**	Laboratory of Enteropathogens
AMAN	Acute motor axonal neuropathy	**LimC**	Lactoferrin-interact molecule, *Campylobacter*
AMSAN	Acute motor-sensory axonal neuropathy	**LOS**	Lipooligosaccharide
AP	Antarctic Phosphatase	**LPS**	Lipopolysaccharide
AS	Ankylosing spondylitis	**MAP**	Mitogen-activated protein
AT	Adenine-Thymine	**MCP**	Methyl-accepting chemotaxis protein
ATP	Adenosine Triphosphate	**MFD**	Mutation Frequency Decline, *mfd* gene
BLAST	Basic	**MFS**	Miller Fisher syndrome
bp/Mbp	Base pairs/mega base pairs	**MH**	Mueller-Hinton
CadF	Campylobacter adhesion to fibronectin	**MOMP**	Major Outer membrane Protein
CapA	Campylobacter Adhesion Protein A	**MUC2**	Mucin 2
CBF1	Cell-binding factor 1, same as PEB1	**NF-kB**	Nuclear factor-kappa B
CCDA	Charcoal-cefoperazone-desoxycholate agar	**NOD**	Nucleotide-binding oligomerization domain
CCL20	Chemokine (C-C motif) ligand 20	**NPL**	Natural peptide library, see RPL
CDT	Cytolethal Distending Toxin	**Nramp**	Natural resistance-assoc macrophage protein
CO_2	Carbon Dioxide	**O_2**	Oxygen
CpG	— Cytosine —phosphate— Guanine —	**Omp**	Outer membrane protein
CPS	Capsular Polysaccharide	**QRDR**	Quinolone Resistance Determining Region
dNTPs	Deoxynucleotide triphosphates	**PAMP**	Pathogen-associated molecular pattern
dATP	Deoxy Adenosine Triphosphate	**PAS**	Per, ARNT and Sim proteins
dTTP	Deoxy Thymidine Triphosphate	**PBS**	Phosphate Buffered Saline
DNA	Deoxyribonucleic acid	**PCR**	Polymerase Chain Reaction
cDNA	Chromosomal DNA	**PEB1**	protein Pei, Ellison and Blaser 1, same as CDF1
ssDNA	Single-stranded DNA	**PFGE**	Pulsed-field gel electrophoresis
dsDNA	Double-stranded DNA	**PMN**	Polymorphonuclear leukocyte
rDNA	Ribosomal DNA	**PRR**	Pattern-recognition receptor
EDTA	Ethylenediaminetetraacetic acid	**RAPD**	Random amplified polymorphic DNA
ELISA	Enzyme-linked ImmunoSorbent Assay	**RFLP**	Restriction fragment length polymorphism
ERK	Extracellular regulated kinases	**RNA**	Ribonucleic acid
ETEC	Enterotoxigenic *E. coli*	**rRNA**	Ribosomal RNA
GBS	Guillain-Barré Syndrome	**tRNA**	Transfer RNA
GC	Guanine-cytosine	**ReA**	Reactive Arthritis
GIT	Gastro-intestinal tract	**RPL**	Random peptide library, see NPL
H_2	Hydrogen	**SOB**	Super optimal broth
HAMP	Histidine, adenylyl, methyl-accept phosphatase	**SOC**	Super optimal broth with catabolite repress
HL	Heat labile	**SVR**	Short variable region
HLA	Human leukocyte antigen	**SpA**	Spondyloarthritis / spondyloarthritide
HMW	High molecular weight	**SPR**	Surface Plasmon Resonance
HS	Heat stable	**TAE**	Tris-acetate EDTA
IAM	Invasion-associated marker	**TLR**	Toll-like receptor
IFN	Interferon	**TNF**	Tumour necrosis factor
IL	Interleukin	**TTSS**	Type III secretion system, also T3SS
IV	Intravenous	**Th1**	Helper T cell 1
Ig	Immunoglobulin	**g1-g8**	Gene 1 – gene 8
		pI-pXI	Protein I – protein XI

Abstract

The Gram-negative, spiral, microaerophilic bacterium, *Campylobacter jejuni,* has established itself as the leading cause of food- and water-borne human gastroenteritis world-wide, and this gastroenteritis is sometimes followed by unprecedented complications, ranging from localized peritonitis, pericarditis and encephalopathy to generalised neuropathy and bacteraemia. Although much progress has been made in the past three decades in regards to its virulence determinants, our understanding of the molecular basis of *C. jejuni* pathogenesis still lags behind that of other enteric pathogens.

Bacterial pathogenesis is often dependent on the panoply of proteinaceous virulence factors which are either transported to the bacterial cell surface or released into the external environment. Understanding the molecular basis of interactions between these proteins and host cells is necessary in understanding and controlling infections.

Background: Previously identified adhesins of *C. jejuni* NCTC11168 have still not provided us with a total understanding of the pathogenesis of campylobacteriosis. It is hypothesized that as yet unidentified *C. jejuni* surface proteins interact with host proteins in a number of ways which contribute to colonisation and pathogenesis, through processes such as adhesion, toxigenicity, and iron acquisition. This study, thus, sought to screen the whole genome of *C. jejuni* NCTC11168 to identify additional genes that may code for other adhesins to some key components of host extracellular molecules and other host-secreted proteins such as lactoferrin, using phage display technology and in silico sequence analysis.

Phage display has been widely used in peptide-ligand interaction studies, including whole microbial genome libraries in identification of microbial adhesins that are vital for colonization. By searching the genome of *C. jejuni* for homologues of virulence factors previously described in other pathogens, it is possible to predict the role of such genes in *C. jejuni*.

Auto-transported proteins or autotransporters have become a rapidly growing family of proteins among Gram-negative bacteria due to their diverse passenger domains and different functions. These proteins have been known to function as adhesins, proteases, mucinases, and toxins or to mediate serum resistance.

Methods: A phage display library was constructed by the insertion of randomly fragmented chromosomal DNA of *C. jejuni* NCTC11168 into the phagemid vector pG8SAET. Following three rounds of affinity panning of the library against holo- and apo-lactoferrin, enriched clones were further screened with ELISA to identify affinity-binding clones. Several phage clones were randomly selected and their *C. jejuni* DNA inserts sequenced and analysed with bio-informatic tools.

In order to identify novel autotransporter proteins of *C. jejuni*, the amino acid sequences of previously described adhesion-associated and other well-characterized autotransporters were employed in BLAST searches of the predicted coding sequences of *C. jejuni* NCTC11168 genome database.

Results: The screening of the phage display library resulted in the identification of a unique *C. jejuni* NCTC11168 gene, Cj0609c, which encodes an uncharacterized *ca.* 45-kDa putative periplasmic protein, predicted to be a periplasmic member of the SGNH-family of hydrolases, a diverse family of lipases and esterases.

Using the amino acid sequences of known autotransporters to search the genome of *C. jejuni* NCTC11168, Cj0628 was identified as coding for a *ca.*116-kDa protein that bears all of the typical characteristics of autotransporters and was designated CapA. CapA was demonstrated to be surface-exposed, mutants of which had a significantly lowered ability to associate with or invade Caco-2 cells and failed to colonize chicken guts, indicating that CapA plays a role in host association and colonization by *Campylobacter*.

Conclusion: Cj0609c possesses signal peptide sequences and although periplasmic, may be involved in binding of *C. jejuni* to lactoferrin for iron acquisition *in vivo,* and/or play further role in adhesion, colonization and internalisation of *C. jejuni* into host cells. We therefore designate Cj0609c as lactoferrin-interacting molecule of *Campylobacter jejuni* (LimC).

CapA also possesses signal peptide sequences and is surface exposed, playing a role in adhesion. In view of its demonstrated role, CapA is proposed to stand for *Campylobacter* adhesion protein A. Further characterization of these identified proteins, CapA and LimC, should therefore contribute to our understanding of the pathogenesis of *C. jejuni*.

Chapter One

General Introduction

Identification of Host-pathogen Interacting Molecules of *Campylobacter jejuni* using Phage Display Technology and in silico Sequence Analysis

CHAPTER ONE

1.0 General Introduction

1.1 *Campylobacter jejuni*

Campylobacter jejuni subspecies *jejuni*, hereby referred to as *Campylobacter jejuni* in this thesis, is a member of the delta-epsilon group of Proteobacteria, a phylum which contains 1534 species or 32.3% of all known bacteria (Ludwig & Klenk, 2001). It is a slim (1.5-6.0 µm by 0.2-0.5 µm), microaerophilic, caprophilic (requiring 3-15% O_2 and 3-5% CO_2 concentrations), Gram-negative, spiral, moderately thermophilic bacterium (growing best at 42°C) with a polar flagellum at one or both ends (Bolton & Coates, 1983; Ketley, 1997; Smibert, 1978). Despite emerging as the leading cause of acute diarrhoea in humans with several debilitating sequelae such as Guillain-Barré syndrome and its milder variant, Miller-Fisher syndrome and several other unprecedented complications, the pathogenesis of its diseases continues to elude the scientific community (Samuel *et al.*, 2004; Young *et al.*, 2007; Zilbauer *et al.*, 2008). Poultry and livestock remain a major risk in the transmission of *Campylobacter* in most developed countries (Yamazaki *et al.*, 2009).

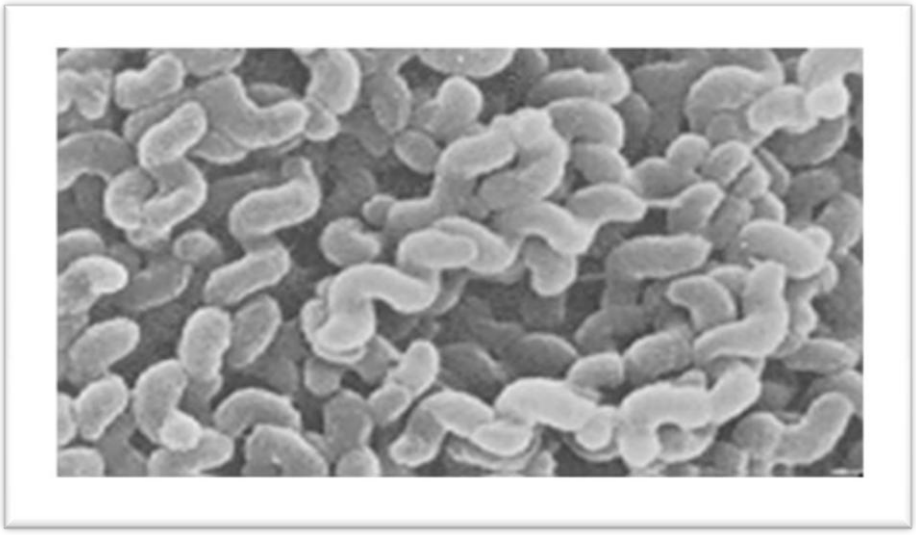

Figure 1. 1 | Electron micrograph of *Campylobacter jejuni* cells. Photograph shows the typical spiral morphology of healthy *C. jejuni* bacteria. Older cells progressively degenerate to coccoid (spherical) bodies via unusual curled, and even donut-shaped forms, with some becoming aflagellate and thus non-motile, straight and even fusiform in some cases [Courtesy: Professor D E Taylor, University of Alberta, Canada].

1.2 The Genus *Campylobacter*

The Genus *Campylobacter* comprises of a large group of closely related Gram-negative, non-sporing, mostly microaerophilic bacteria that are known to colonize the gastrointestinal tracts of humans and a wide range of animal hosts. The majority of *Campylobacter* species may be commensals, but *Campylobacter jejuni* and *Campylobacter coli* are known human enteropathogens responsible for acute enteritis worldwide (Acke *et al.*, 2009; French *et al.*, 2009; Ketley, 1997; Young *et al.*, 2009).

First cultured from samples from aborting ewes as far back as 1906 by McFadyean in the United Kingdom (Figure 1.2), the first species of this genus was described in detail in 1913 and thought to belong to the genus *Vibrio* (Butzler, 2004; McFadyean & Stockman, 1913; Skirrow, 2006). It was formally assigned to the genus *Vibrio* in 1919, with the species name *fetus* following similar work by Smith and Taylor (Smith, 1919; Smith & Taylor, 1919) and belonged here until 1963 when the name *Campylobacter* was proposed due to fundamental differences between these and the well-known species of *Vibrio*, *Vibrio cholerae,* such as GC content and growth requirements (Sebald & Veron, 1963; Veron, 1966).

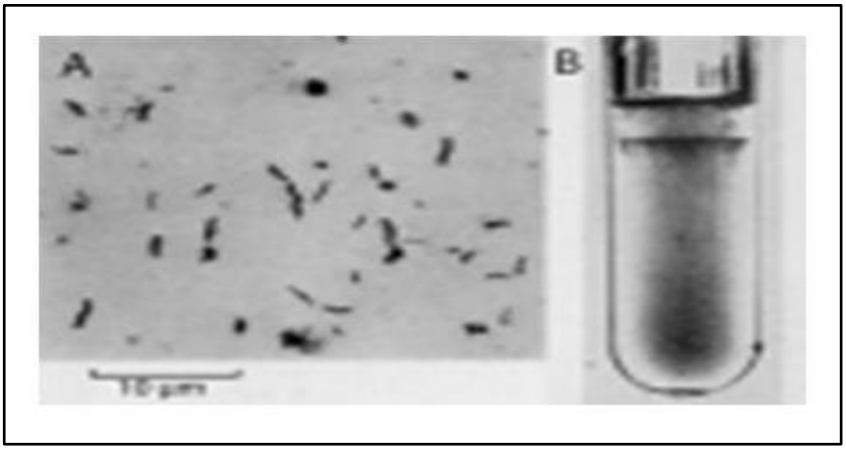

Figure 1. 2 | McFadyean's photographs of the "*Vibrio*" species. John McFadyean and Stewart Stockman observed these organisms in smears of uterine exudate from an infected ewe (A), and a culture of the "*Vibrio*" species in agar (B), which had been inoculated and mixed while liquid and then solidified. The characteristic subsurface band of growth, where there is reduced oxygen tension, resembles that of *Brucella abortus* [Courtesy: Professor M B Skirrow, Microbiologist Public Health Laboratory, Gloucestershire Royal Hospital, Gloucester, UK].

It is purported that Theodor Escherich's observation of spiral bacteria in the faeces of infants with diarrhoea and the colons of children who died of "cholera infantum" in 1886 could have been *Campylobacter*, but he regarded them as non-culturable and strangely did not consider them to be of great clinical significance (Butzler, 2004; Kist, 1985a; Siegel *et al.*, 1990). Kist argued that the organisms observed by Theodor Escherich was *Campylobacter* because of the typical morphology of the organism and its association with enteritis in neonates, infants and kittens, the failure to grow on solid medium despite microscopic detection and the fact that to date no other bacteria with comparable morphology had been associated with human enteric infections (Kist, 1986). However, Escherich's drawing of these spiral bacteria (Figure 1.3) leaves little doubt that they were campylobacters, just as he did not consider these to have any relation to the aetiology of intestinal diseases (Kist, 1985b).

Figure 1. 3 | Escherich's drawing of *Vibrio*-like organisms. Theodor Escherich observed that the colons of children who died of cholera infantum had *Vibrio*-like organisms which were unculturable. Strangely, he did not consider these organisms to be of great clinical significance [Courtesy: Professor M B Skirrow, MicrobiologistPublic Health Laboratory, Gloucestershire Royal Hospital, Gloucester, UK].

By 1973, several Gram-negative bacteria, from microaerophilic to aerophilic, and fermentative to oxidase-positive, had been added to the genus including the species originally described under the names *Vibrio jejuni* (Jones *et al.*, 1931), *V. coli* (Doyle, 1944), *V. fetus venerealis* (Stegenga & Terpstra, 1949), *V. fetus intestinalis* (Florent, 1959), *V. sputorum* (Prevot, 1940; Tunnicliff, 1914), and *V. bubulus* (Florent, 1953) whilst some scientists also proposed that the genus be

redefined to include and exclude some bacteria (Loesche *et al.*, 1965; Veron & Chatelain, 1973). The description of isolation methods by Butzler and co-workers in 1972 and its simplification by Skirrow in 1977 sparked enormous interest in *Campylobacter* and related organisms (Dekeyser *et al.*, 1972; Skirrow, 1977).

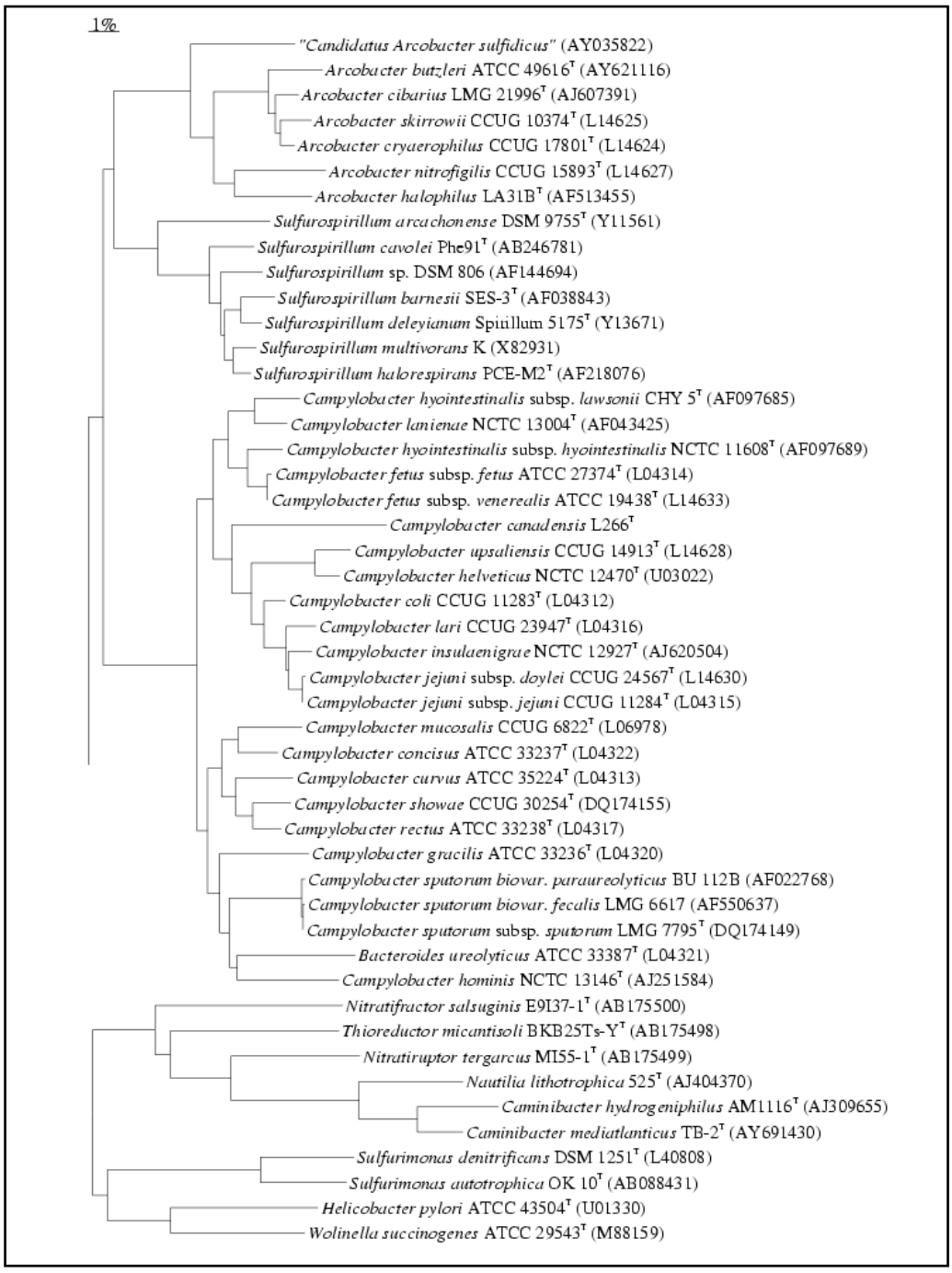

Figure 1. 4 | Phylogenetic tree of *Campylobacteraceae* family. This is given alongside its closest genetic neighbours based on the percentage difference between the compared 16s RNA gene sequences [Courtesy: Prof. Dr. Peter A. R. Vandamme, Universiteit Gent, Belgium].

The following decade saw several phylogenetic studies that sought to clarify this ill-defined genus, and by 1992 16S/23S rRNA sequence analyses and DNA-DNA hybridization experiments had revealed the presence of four independent branches within this group of organisms (Lau *et al.*, 1987; Paster & Dewhirst, 1988; Schumacher *et al.*, 1992; Vandamme *et al.*, 1991). The name *Campylobacter* was maintained for the *C. fetus* lineage, whereas *Helicobacter* was proposed to accommodate *C. pylori*, *C. mustelae*, *C. cinaedae*, and *C. fennelliae*. *Arcobacter* was suggested for *C. nitrofigilis*, *C. cryaerophilus*, and *C. butzleri* whilst the fourth branch was referred to as *Sulfuospirillum*.

The genus *Campylobacter* now contains 16 species and 6 subspecies namely; *C. coli*, *C. concisus*, *C. curvus*, *C. fetus* (*C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*), *C. gracilis*, *C. helveticus*, *C. hyointestinalis* (*C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii*), *C. jejuni* (*C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*), *C. lari*, *C. lanienae*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum*, *C. upsaliensis*, and an unusual species which is not yet properly characterized, Candidatus *C. hominis* [Campynet: http://campynet.vetinst.dk/].

1.3 Genetics and Bio-Informatics of *Campylobacter jejuni*

Belonging to the 16S rRNA superfamily VI of the delta-epsilon (**δ-ε**) Proteobacteria, *Campylobacter* species are known to have small, AT-rich genomes of between 1.6 and 1.7 Mbp, with a low GC content of around 30% (Newnham *et al.*, 1996; Taylor *et al.*, 1992). The genome of the *Campylobacter jejuni* type strain NCTC11168, which was the first *C. jejuni* strain to be sequenced, is a circular chromosome of 1,641,481 base pairs (bp) encoding 1,643 proteins (previously thought to be 1,654) and is known to have 29 homopolymeric G tracts i.e. tracts of ≥ 7 consecutive G residues, in genes encoding products required for the biosynthesis or modification of surface structures such as the capsule, lipooligosaccharides (LOS) and flagellum, or in closely linked genes of unknown function (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000).

Likewise, the genomes of the other sequenced *C. jejuni* strains, RM1221, 81-176, and NCTC11828 also present with small sizes of 1,777,831; 1,594,651 and 1,628,115 respectively, and contain 25, 19, and 17 homopolymeric tracts respectively (Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Parker *et al.*, 2006; Pearson *et al.*, 2007). These homopolymeric tracts have the potential to cause multiple phase variation events, gene duplications and deletions, frameshift and point mutations, which are subsequently translated into the surface structures and may be responsible for the adaptation and persistence of *C. jejuni* in its hosts as well as manifestation of disease (Gilbert *et al.*, 2002; Guerry *et al.*, 2002; Karlyshev *et al.*, 2002; Karlyshev *et al.*, 2005; Linton *et al.*, 2000b). Phase variation, which is a heritable, though reversible, "on and off" biological switch that regulates the expression of a gene or an operon, results in a heterogenic phenotype of a clonal bacterial population, in which some cells do express the phase-variable protein(s) whilst others do not (van der Woude & Baumler, 2004; Young *et al.*, 2007). In other instances a clonal population may express one of multiple antigenic forms of the protein causing antigenic variation. This phenomenon is well noted in *Campylobacter* (Gilbert *et al.*, 2002; Guerry *et al.*, 2002; Karlyshev *et al.*, 2002; Karlyshev *et al.*, 2005; Linton *et al.*, 2000b).

Of the 1,643 predicted coding sequences of *C. jejuni*, at least 20 appear to be pseudogenes, with 94.3% of the genome coding for proteins. Despite its small genome size functional information is known for only 77.8% of *C. jejuni* sequences, whereas 13.5% have similarity with genes of unknown function, with 8.7% having no database match or any functional information (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000). The small genome size has been cited as the likely explanation for the requirement of complex growth media, and the inability of *C. jejuni* to ferment carbohydrate or degrade complex substances (Griffiths & Park, 1990).

Due to its natural competence, *C. jejuni* is able to take up DNA in the environment, allowing genetic exchange and recombination between strains to generate the extensive genetic diversity associated with the organism (de Boer *et al.*, 2002; Wilson *et al.*, 2003). This horizontal transfer of genetic material has

been shown to occur both *in vivo* and *in vitro*, indicating the importance of natural transformation in the spread of adaptive factors such as antibiotic resistance among strains (Avrain *et al.*, 2004). Although it is believed that DNA transfer is more likely to occur between different strains of *C. jejuni* than between different species, Sheppard *et al.* has recently reported patterns of genetic exchange that show that *C. jejuni* and *C. coli* are converging as a consequence of recent changes in gene flow (Sheppard *et al.*, 2008; Wilson *et al.*, 2003). Although the mechanisms are yet to be fully elucidated, it is believed that natural transformation in *C. jejuni* requires some components of type II secretion system, some components of a plasmid encoded type IV secretion system, a putative DNA-processing enzyme, a homologue of *H. pylori* DprA, as well as genes involved in *N*-linked glycosylation and LOS biosynthesis (Bacon *et al.*, 2000; Fry *et al.*, 2000; Larsen *et al.*, 2004; Takata *et al.*, 2005). Recently Jeon *et al.* reported the likely involvement of yet two additional genes in *C. jejuni* natural transformation, Cj1211 and Cj0011c, defined as probable integral membrane protein and possible non-specific DNA binding protein respectively (Jeon & Zhang, 2007; Jeon *et al.*, 2008).

It has often been reported that genes from *Campylobacter* species are difficult to clone and subsequently analyze, an observation which is believed to be due to several possible factors, including the high AT content, which results in promoter-like sequences, the lack of required accessory factors in *Escherichia coli* or both, as well as different patterns of methylation or codon usage and the frequent changes in the hypervariable G tracts present in the genomes (Pearson *et al.*, 2007; Taylor *et al.*, 1992). Strain NCTC11828 is, however, thought to be fairly stable due to the presence of fewer hypervariable G tracts (Pearson *et al.*, 2007).

1.4 Epidemiology of *Campylobacter jejuni*

The Gram-negative bacterium, *Campylobacter jejuni*, is a major cause of zoonotic bacterial gastroenteritis in the world, the leading cause of bacterial gastroenteritis in most developed countries and has therefore become an

important public health problem worldwide (Samuel *et al.*, 2004; Young *et al.*, 2007; Zilbauer *et al.*, 2008).

A population based cohort study undertaken in England in 1999 to identify the causative microorganisms in infectious diarrhoea reported *Campylobacter* species as the commonly isolated organism among 12.2% of 2893 cases (Tompkins *et al.*, 1999). Likewise, *Campylobacter* species was the most frequently reported pathogen in a similar study in Scotland (Noone *et al.*, 2000).

In the United Kingdom as a whole, epidemiologic and microbiologic data indicate that *C. jejuni* accounts for 93% of the cases of campylobacteriosis, with *C. coli* constituting 7%, *C. lari* <1%, and *C. fetus* <1%. Epidemiologic data from the Caribbean also indicate that *C. jejuni* constitutes 63.6% of isolates from stools whereas *C. coli* constituted 31.8% (Gillespie *et al.*, 2002; Workman *et al.*, 2006).

The risk factors for *Campylobacter* enteritis, as identified by case-control studies and outbreak reports, are numerous and varied but contaminated food is a common source of human infection, with consumption or handling of poultry considered as the single most important route of infection, at least in developed countries (Gormley *et al.*, 2008; Nichols, 2005; Stern *et al.*, 2001). Generally, it is believed that most veterinary sources are reservoirs of pathogenic campylobacters since populations of veterinary and human isolates overlap (Danis *et al.*, 2009; Manning *et al.*, 2003). Based on circumstantial evidence such as the low infective dose and the ubiquitous nature of the organism, the isolation of *Campylobacter* from flies has led to the hypothesis that the latter could be major carriers of small quantities of materials infected with the organism onto foods (Ekdahl *et al.*, 2005; Szalanski *et al.*, 2004; Wright, 1983). However, the consumption of inadequately cooked poultry, both at home and in restaurants, barbecued meats, untreated water, unpasteurized milk, contact with pets, particularly puppies, and occupational and recreational exposure to farm animals remain the major known risk factors (Danis *et al.*, 2009; Nichols, 2005; Rushton *et al.*, 2009; Stern *et al.*, 2001; Verhoeff-Bakkenes *et al.*, 2008). Travel abroad and swimming have been associated with some incidences of campylobacteriosis (Allos, 2001; Studahl & Andersson, 2000). Nevertheless, a

large proportion of sporadic cases remain unexplained by these commonly recognized risk factors (de Mattos *et al.*, 2002).

Cases of campylobacteriosis are higher in the warmer months in most developed countries, and between April and July in developing tropical countries indicating the propensity for infection in warmer months (Asrat *et al.*, 1999; Banmali *et al.*, 2006). It is more common in young children and young adults and affects more men than women (Banmali *et al.*, 2006; Unicomb *et al.*, 2008).

Within England and Wales over 45,000 laboratory-confirmed campylobacteriosis cases are reported annually, whereas an estimated 500,000 community cases are reported in England alone per annum (Tam *et al.*, 2006a). An estimated 2 million people were infected with *Campylobacter* species in the United States each year during the period 1996-1999 (Samuel *et al.*, 2004).

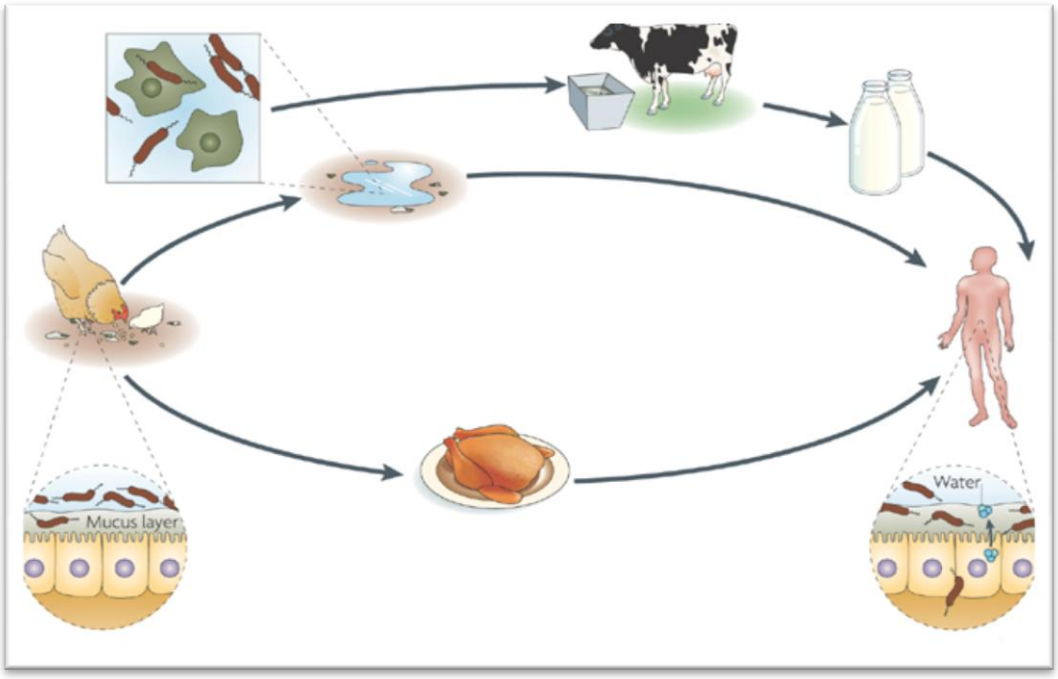

Figure 1. 5 | The Ecological cycle of *Campylobacter jejuni*. Wild birds, livestock and chicken constitute the major reservoirs of *C. jejuni*. It colonizes the gastrointestinal mucosa of chicken in high numbers, from where it is passed between chicks within a flock through the faecal–oral route or enters the water supply. *C. jejuni* infect humans directly through drinking of contaminated water or the consumption of contaminated animal products, such as unpasteurized milk or meat, particularly poultry. In humans, *C. jejuni* invades the intestinal epithelial layer, resulting in inflammatory diarrhoea [Adopted with permission from Young *et al.*, 2007; ***Nat Rev Microbiol*** 5, 665-679).

Although the incidence of campylobacteriosis has declined in both the UK and the USA in recent years, it still remains a major cause of bacterial diarrhoea affecting around 1% of their populations which underscores the need to better understand the pathogenesis of the disease (Konkel *et al.*, 2005; Samuel *et al.*, 2004). Likewise, the incidence of campylobacteriosis in the European Union has declined in the last five years, an observation which although encouraging still warrants that attention is given to this organism and its pathogenesis (Janssen *et al.*, 2008).

In 2007 New Zealand reportedly had one of the highest incidences of human campylobacteriosis of 396 cases per 100,000 persons, whereas the United States reported one of the lowest cases of 12.7 cases per 100,000 persons (Ailes *et al.*, 2008; Baker *et al.*, 2007). This clearly shows that the global incidences of *Campylobacter* cases differ from country to country. But with little or no data from several countries especially developing ones, this might also be a reflection of differences in reporting in different countries (Janssen *et al.*, 2008; WHO, 2001).

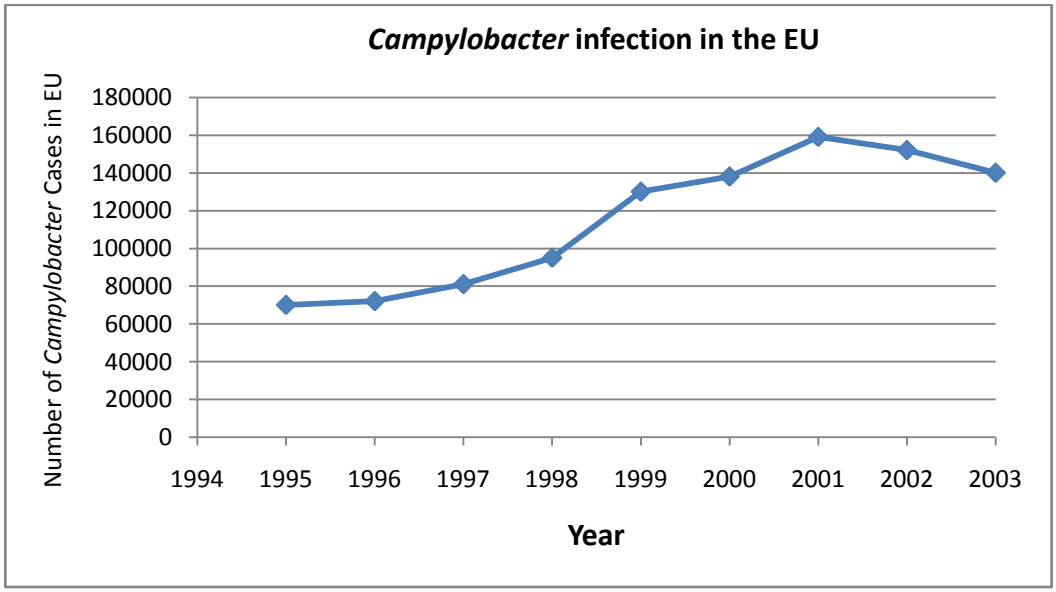

Figure 1. 6 | Number of *Campylobacter* cases in the European Union until 2003. Data after 2003 are not shown because several new European Union (EU) member states with a high reported incidence of campylobacteriosis joined the European Union (Table 1) [Adopted from Janssen *et al.*, 2008; *Clin Microbiol Rev* **21**, 505-518].

This same epidemiological picture is depicted by the number of human campylobacteriosis cases reported per 100,000 persons within the European Union which also differs widely among the countries averaging 51.6 as shown in Table 1 (EFSA, 2006).

Country	No. of confirmed cases	No. of cases/100,000
Austria	5,065	61.7
Belgium	6,879	65.8
Czech Republic	30,268	302.7
Denmark	3,677	68
Estonia	124	9.2
Finland	4,002	76.4
France	2,049	3.3
Germany	62,114	75.3
Hungary	8,288	82.1
Ireland	1,794	43.7
Lithuania	694	20.3
Luxembourg	194	42.6
Malta	91	22.6
The Netherlands	3,761	46.2
Poland	47	0.1
Slovakia	2,204	40.9
Spain	5,513	2.8
Sweden	5,969	66.2
United Kingdom	52,686	88.5
Total	**195,419**	**51.6**

Table 1. 1 | Country-specific campylobacteriosis cases in the European Union in 2005 [Modified from Janssen, *et al*. 2008; *Clin Microbiol Rev* 21, 505-518].

1.5 Isolation, culture and storage of *Campylobacter*

There are two main methods for the isolation of *Campylobacter* species. The first involves selective filtration of specimen through membrane filters with pore sizes of 0.45 µm, 0.65 µm and 0.8 µm as initially described by Dekeyser and Butzler (Butzler *et al.*, 1973; Dekeyser *et al.*, 1972). This is based on the principle that *Campylobacter* cells are very small and highly motile, and thus are capable of penetrating these membranes. The filtrate is then inoculated onto selective media and incubated in microaerophilic atmosphere containing both elevated CO_2 and H_2 concentrations and at a temperature of around 42-43°C.

The second method involves plating of specimen directly unto a selective medium as initially described by Skirrow (Skirrow, 1977). Skirrow's medium

contained 10 µg/ml of vancomycin, 2.5 IU/ml of polymyxin B and 5 µg/ml of trimethoprim. The inoculated plates are then incubated at 43°C in an atmosphere of 5% oxygen, 10% carbon dioxide, and 85% hydrogen (Skirrow, 1977). Following this breakthrough by Skirrow, several selective media have been described containing different supplements at different concentrations, but none of these supports the growth of all the *Campylobacter* species (Aspinall *et al.*, 1993; Butzler *et al.*, 1983; Goossens *et al.*, 1986; Goossens *et al.*, 1989; Griffiths & Park, 1990; Griffiths, 1993).

It must, however, be stated here that there is no gold standard for the isolation of campylobacters, but the predominant species of human infections, *C. coli* and *C. jejuni*, can be readily cultured on selective media under microaerophilic conditions without the addition of H_2 (Butzler *et al.*, 1983). It has been suggested that all 18 serogroups of *C. coli* and *C. jejuni*, could even be cultured on solid media in an atmosphere of 10% CO_2 in moist air at a relative humidity of 99%, thus minimizing the cost of isolation of the organism (Fraser *et al.*, 1992). Similarly, it has been shown that buffered peptone water (BPW) supplemented with blood and antibiotics could be used in the isolation of both *C. jejuni* and *C. coli* (Oyarzabal *et al.*, 2006). *Campylobacter* can best be grown and propagated in/on Mueller-Hinton broth/agar and for laboratory storage purposes high cell concentrations could be mixed with 10% glycerol or dimethyl sulfoxide (DMSO) and stored at -80°C (Davis & DiRita, 2008).

Whereas *Campylobacter* grows best at 42-43°C, *Helicobacter* thrives best at 37°C both under microaerophilic conditions, but *Arcobacter* is highly aero-tolerant and thermo-intolerant differentiating the three related genera from each other (Davis & DiRita, 2008; Hamill *et al.*, 2008; Jalava *et al.*, 1998; Sainsus *et al.*, 2008; Skirrow, 1977). Although of limited value in routine diagnostics, analysis of respiratory quinones has been used in differentiation of *Campylobacter* strains from *Helicobacter* and *Arcobacter* until the recent development of new methods such as polymerase chain reaction (PCR), sequencing and PCR-restriction fragment length polymorphisms (Gonzalez *et al.*, 2006; Hill *et al.*, 2006; Neubauer & Hess, 2006; Quinones *et al.*, 2007; Samie *et al.*, 2007).

1.6 Identification of *Campylobacter* species

The importance of identifying a strain up to species and subspecies levels cannot be underestimated due to the enormous diversity within campylobacters. This importance ranges from estimation of the prevalence and significance of different species to source tracing and clinical management (Babiker *et al.*, 2001).

Two main forms of identification are available; phenotypic tests such as biochemical tests, fatty acid or protein profiling and genotypic tests such as DNA fingerprints, sequencing, pulsed-field gel electrophoresis (PFGE), polymerase chain reaction (PCR) and microarray-based methods (Keramas *et al.*, 2004; Wassenaar & Newell, 2000).

Following isolation and culture under the conditions described above, *Campylobacter* species produce non-haemolyitc spreading, droplet-like colonies on blood agar whilst producing grey, moist, flat-spreading colonies on improved blood-free agar, with some strains having a green hue or a dry appearance with or without a metallic sheen (Cheesbrough, 2006). Under light microscopy, a wet mount of *Campylobacter* species would exhibit the typical darting and corkscrew motility whilst a Gram-stain would show the typical spiral morphology (Butzler *et al.*, 1973; Butzler & Skirrow, 1979). *Campylobacter* is oxidase and catalase positive and hydrolyzes hippurate to benzoate (Harvey, 1980; Krausse & Ullmann, 1985).

1.6.1 Typing Methods

Since the advent of the development of novel culture techniques for their isolation, several phenotypic and genotypic methods have been developed to identify *Campylobacter* to the species level.

i. Serotyping

The widely used phenotypic method is serotyping. Several agglutination techniques have been described, involving haemagglutination, slide agglutination, and latex agglutination using antisera generated against various *Campylobacter* strains and species and which could be used to test unknown

strains (Hodinka & Gilligan, 1988; Lior *et al.*, 1981; Miller *et al.*, 2008; Nachamkin & Barbagallo, 1990; Penner & Hennessy, 1980). These are all based on two main serological schemes developed in the 1980s in Canada; the Penner scheme, developed by John Penner of the University of Toronto, is based on a passive haemagglutination serological technique using soluble antigen extracts of *Campylobacter* isolates to raise specific antisera that detect the heat-stable (HS) or somatic O antigens of *C. jejuni* and *C. coli*; and the Lior scheme, developed by Hermy Lior at Canada's national Laboratory for Enteric Pathogens in Ontario, is based on a slide agglutination serology, which uses live bacteria together with absorbed and unabsorbed antisera to detect heat-labile (HL) or flagellar antigens of *Campylobacter* strains (Lior *et al.*, 1981; Penner & Hennessy, 1980).

The HS or O antigen was originally thought to be the *C. jejuni* lipooligosaccharide or lipopolysaccharide, but was subsequently shown to be the previously unrecognised capsular polysaccharide antigen by Karlyshev and co-workers (Karlyshev *et al.*, 2000; Preston & Penner, 1987). Although flagella was suggested to be the major antigenic determinant of some Lior HL serotypes including LIO5, LIO6, LIO7 and LIO17, it has also been shown that non-flagella antigen may be the serodeterminant for this scheme (Alm *et al.*, 1991; Wenman *et al.*, 1985; Yoshida *et al.*, 1987).

The Penner scheme, which comprises 65 serotypes, including 48 serotypes of *C. jejuni* and 17 serotypes of *C. coli*, was modified in the UK to comprise of 118 serotypes for use in the Laboratory of Enteropathogens (LEP) of the Public Health Laboratory Service (Frost *et al.*, 1998). In this modification, specific antisera are used to detect antigens by direct bacterial agglutination of heated suspensions in microtiter plates.

A comparative analysis of the serotyping results of 9,024 sporadic human isolates of *C. jejuni* using the Penner and Lior schemes revealed conserved associations between specific HS (O) and HL antigens (O/HL serovars), making the case for the combined use of O and HL serogrouping as a practical and phylogenetically valid method for investigating the epidemiology of sporadic *C. jejuni* infection (Jackson *et al.*, 1998).

Using *C. jejuni* polyclonal antibodies, Hochel *et al.* developed an indirect competitive ELISA for detection of somatic antigen O:23 of *C. jejuni* in foods with high sensitivity (Hochel *et al.*, 2004).

ii. Cellular Fatty Acid Profiling

The identification of bacteria could also be achieved through the profiling of their cellular fatty acid (CFA) and whole-cell protein using mass spectrometry (MS) (On, 2005). Using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS or Pyrolysis MS (Py-MS), specific moieties from intact bacteria or pyrolysed *Campylobacter* cells could be separated by their mass to charge ratio and the results presented in a dendrogramatic form alongside that of known taxa (On, 2005). Identification is made on the basis of cluster analysis.

iii. Phage Typing

The lytic activity of some *Campylobacter* phage has long been shown to differ in different species of the organism (Bryner *et al.*, 1970; Bryner *et al.*, 1973). This selective lysis by bacteriophage derived from *C. jejuni*, *C. coli* and other *Campylobacter* species has been employed in the identification of unknown strains in what is referred to as phage typing (Grajewski *et al.*, 1985). Several *Campylobacter* phage types have been identified that can be used in further characterization of various strains, notably *C. jejuni* and *C. coli* (Frost *et al.*, 1999; Khakhria & Lior, 1992; Salama *et al.*, 1990). Phage typing of *Campylobacter* strains, alongside serotyping, lends a further level of discrimination that enhances the epidemiological typing of *Campylobacter* species (Frost *et al.*, 1999).

iv. Pulsed Field Gel Electrophoresis

With the advancement of molecular biology techniques, pulsed field gel electrophoresis (PFGE) became a gold standard in epidemiological studies of pathogenic organisms, proving superior to the conventional agarose gel electrophoresis in separating larger DNA fragments, and providing invaluable information for molecular genotyping of *Campylobacter* (Fitzgerald *et al.*,

2001; Karenlampi *et al.*, 2007; Klein *et al.*, 2007; Ribot *et al.*, 2001). This involves the cleavage of chromosomal DNA using selected restriction enzymes, such as *Sma*I, *Sal*I, *Apa*I, *Kpn*I, *Xho*I and *Bss*HII, that cut DNA infrequently, and the resultant fragments which are generally very large, separated by agarose gel electrophoresis under special conditions (Wassenaar & Newell, 2000). This produces genotypic profiles that could be interpreted to identify the species of an unknown strain.

Scientists have also made use of the high variability of the flanking regions of rRNA gene loci coding for 5S, 16S and 23S rRNA, as well as the strong conservation of portions of some non-rRNA genes in the identification of *Campylobacter* species (Wassenaar & Newell, 2000). Following restriction digestion of genomic DNA with one or two of the enzymes *Pst*I, *Hae*III, *Hin*dIII or *Pvu*II and agarose gel electrophoresis, southern blot hybridization is done with probes specific for rRNA genes to produce ribotype profiles akin to PFGE in high level species identification (Denes *et al.*, 1997).

v. Polymerase Chain Reaction

Of equally significant value in the species identification of *Campylobacter* is the use of polymerase chain reaction (PCR) in amplification of conserved portions of specific genes which are unique to a particular species (Burnett *et al.*, 2002). In this regard, several genes, including *flaA*, *mapA*, *ceuE*, *pflA*, *gyrA*, *flgE* and *hipO* as well as some variable regions of the 16S rRNA/rDNA have been identified as being unique to particular species and have been utilised in that manner (Harmon *et al.*, 1997; Klena *et al.*, 2004; Oyarzabal *et al.*, 1997; Waegel & Nachamkin, 1996). Using randomly designed 10-mer primers, several polymorphic genes could be amplified in a single assay known as the multiplex PCR in the species identification of strains (Burnett *et al.*, 2002; Klena *et al.*, 2004; Wesley *et al.*, 1997).

Furthermore, fluorescently labelled PCR oligonucleotide probes designed to match the highly variable DNA segment within the *flaA* short variable region (SVR) of *Campylobacter jejuni* or *C. coli* could be used to identify a

Campylobacter strain to the species level in a LightCycler-based assay in what is referred to as real-time PCR (Jensen *et al.*, 2005; Ridley *et al.*, 2008).

The presence of several unique genes of *Campylobacter* species could be detected in an ordered arrangement on a microarray glass slide. This involves the PCR amplification of specific regions in the target genes, such as *fur*, *glyA*, *cdtABC*, *ceuB-C*, and *fliY*, followed by microarray-based analysis of these amplified DNAs (Volokhov *et al.*, 2003).

vi. Restriction Fragment Length Polymorphism

An extension to the PCR methods of typing is the use of rare-cutting restriction enzymes in the digestion of resultant amplicons, called restriction fragment length polymorphism (RFLP) or PCR-RFLP (Takahashi *et al.*, 2006). Nachamkin *et al.* described the observation of 18 RFLP patterns following digestion of the amplified *flaA* gene with the restriction enzyme *Dde*I, whilst Zorman *et al.* observed 12 RFLP patterns when same gene was digested with *Cfo*I (Nachamkin *et al.*, 1993; Zorman *et al.*, 2006). Other restriction endonucleases that have been successfully used to type *Campylobacter* strains are *Mbo*I, *Mse*I, and *Alu*I (Fujimoto *et al.*, 1997).

vii. Amplified Fragment Length Polymorphism

A variation of the RFLP which has become important in speciation of campylobacters is the amplified fragment length polymorphism (AFLP) which involves restriction digestion of the genomic DNA of a strain, followed by ligation of adaptors to the sticky ends of the restriction fragments (Wassenaar & Newell, 2000). A subset of the restriction fragments are then amplified using radioactively or fluorescently labelled primers complementary to the adaptor and part of the restriction site fragments (Fang *et al.*, 2006; Koene *et al.*, 2008). Using the SVR of *flaA* and 16S rRNA it has now become possible to detect and enumerate specific *Campylobacter* strains in mixed populations using a novel molecule-based method, that uses strain- and genus-specific oligonucleotide probes (Elvers *et al.*, 2008).

viii. Multi-Locus Sequence Typing

Despite the extensive variability of the SVR of the *flaA* gene and the invaluable information it provides in species identification following PCR and restriction digestion, direct nucleotide sequencing of PCR amplicons of specific portions of this and some other genes of *Campylobacter* strains have proven to be superior to RFLP or AFLP giving birth to the current state-of-the-art multi-locus sequence typing (MLST) technique (Kinana *et al.*, 2007; Levesque *et al.*, 2008; Price *et al.*, 2006). This involves profiling a *Campylobacter* strain based on the DNA sequences of short sequences at a number of loci within selected housekeeping genes (Al Amri *et al.*, 2007; Dingle *et al.*, 2001).

ix. Biosensor

Moving even further in the typing of *Campylobacter*, Ivnitski *et al.* described an ion-channel biosensor based on supported lipid bilayer membrane for direct and fast detection of *Campylobacter* species (Ivnitski *et al.*, 2000). This system incorporated *C. jejuni* antibodies in an artificial lipid bilayer membrane covering the sensing element of the biosensor, which served as channel forming proteins in detecting *Campylobacter* species. Wei and co-workers have also recently described the development of a surface plasmon biosensor based on antigen-antibody binding using *C. jejuni* polyclonal antibodies for the rapid identification of *C. jejuni* (Wei *et al.*, 2007).

1.7 Pathogenesis of *Campylobacter jejuni* Infection

Despite being recognized as the leading cause of bacterial gastroenteritis in developed countries, the pathogenic mechanisms of *C. jejuni* are still far from being well-understood (Samuel *et al.*, 2004; Young *et al.*, 2007; Zilbauer *et al.*, 2008). To establish an infection sufficient to cause disease, the organism would have to survive the physiological stresses associated with external and internal environments such as temperature fluctuations, variations of pH, different hosts, host immunity, oxidative stress and limited nutrient supply (Snelling *et al.*, 2005).

Like all other enteropathogenic bacteria, *Campylobacter* species have evolved attributes, many of which are virulence determinants that have helped them to circumvent the innate host defences of humans and other mammalian and avian hosts (Finlay & Falkow, 1997; Guerry, 2007).

This section looks at the general features and virulence factors that determine the course of a *Campylobacter* disease.

1.7.1 General Features of *Campylobacter* Pathogenesis

i. Adhesion and Colonization

The role of adhesins, flagellae, cellular and extracellular matrix proteins, and a host of other factors as observed in other bacteria such as *Salmonella*, *Yersinia* species, *Shigella* and *Escherichia coli* cannot be overlooked in *C. jejuni* pathogenesis (Falkow *et al.*, 1992; Finlay & Falkow, 1997). Adhesion to host cells, colonization and invasion of host cells are needed in order for several bacterial pathogens to successfully establish infection and induce diseases, whereas others may employ toxins to induce disease (Finlay & Falkow, 1997). In the case of *C. jejuni* adhesion and invasion of mammalian cells have been demonstrated *in vitro* and are considered to be important factors in its pathogenicity *in vivo* (Coutte *et al.*, 2003; Grant *et al.*, 1993; Ketley, 1997). Furthermore, motility, chemotaxis, iron acquisition, quorum sensing as well as host immune evasion also play various roles in *C. jejuni* pathogenesis (Elvers & Park, 2002; Ketley, 1997).

ii. Invasion

Following colonization of the human intestinal mucus layer *C. jejuni* invades the intestinal epithelial cells where it may modify the signal transduction pathways of its host, thus, aiding entry and easy passage through the epithelial monolayer (Konkel *et al.*, 2005). This invasion is thought to be initiated by signaling events on the surface of the host cell, coupled with a unique caveolae-dependent entry pathway and downstream signals that trigger microfilament and microtubule depolymerization and subsequent membrane

ruffling and internalization of the bacterium (Hu *et al.*, 2006b; Watson & Galan, 2008; Wooldridge & Ketley, 1997).

This theory is buttressed by recent studies in which depolymerization or stabilization of microfilaments and microtubules [see Figure 1.7], as well as inhibition of host cell tyrosine kinases, receptor-mediated endocytosis, endosome acidification, and phosphatidylinositol-3-kinases, resulted in reduction in or inhibition of, invasion by *C. jejuni* (Biswas *et al.*, 2000; Biswas *et al.*, 2003; Oelschlaeger *et al.*, 1993; Wooldridge *et al.*, 1996).

It has also been observed that epithelial cell membrane pseudopods, which are known to contain microtubules, do extend towards and envelope *C. jejuni* (Biswas *et al.*, 2000; Hu & Kopecko, 1999; Kopecko *et al.*, 2001).

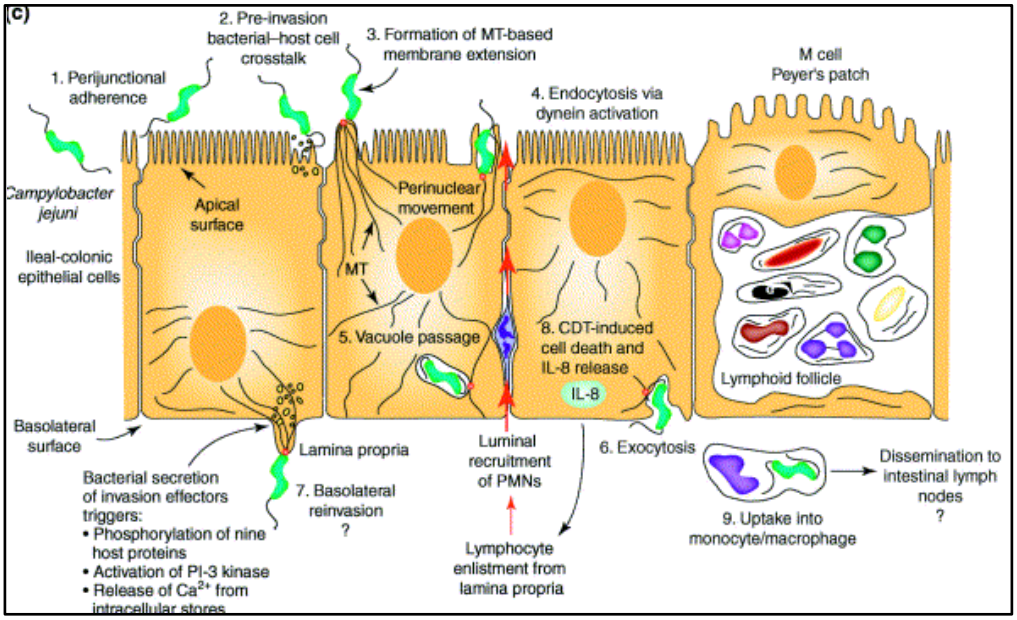

Figure 1. 7 | Illustration of the current working model of *Campylobacter jejuni* pathogenesis. (1) Initial adhesion of *C. jejuni* is believed to start at the apical cell surface at the peri-junctional region. (2) Where it secretes putative 'invasion' effectors into the host cell, which in turn trigger phosphorylation of host proteins and the release of Ca^{2+} from intracellular stores. (3) Host signalling cascades trigger a localized disruption of cortical actin filaments and an extension of microtubules (MTs) to form a membrane protrusion to meet the adjacent bacterium. (4) Host-pathogen interaction at caveolae leads to an endocytosis of the bacterium via membrane invagination. (5) The engulfed *C. jejuni* within a membrane-bound vacuole moves via dynein along MTs to the basolateral surface for exocytosis (6), and then re-enter the epithelium basolaterally (7). (8) Infected epithelial cells secrete interleukin (IL)-8 basolaterally, which leads to proliferation of lymphocytes from the lamina propria. (9) *C. jejuni* apparently survive in macrophages/monocytes for several days, which aids their local dissemination [Adopted with permission from Kopecko, *et al.* 2001; *TRENDS in Microbiology* **9,** 389-396].

Also, there have been reports of the involvement of caveolae, which are non-clathryn-coated small invaginations, 50-100 nm in diameter, found in the plasma membrane of many vertebrate cell types, in the internalization of *C. jejuni* (Schnitzer *et al.*, 1994; Wooldridge *et al.*, 1996). Transport across caveolae is known to be inhibited by filipin III, a sterol-binding agent, and this phenomenon has been demonstrated in Caco-2 cells co-cultured with *C. jejuni* (Schnitzer *et al.*, 1994; Wooldridge *et al.*, 1996).

Although, this model of caveolae-mediated, microtubule-dependent epithelial cell invasion has become well accepted, there have been other reports which seek to suggest that *C. jejuni* gut invasion via other mechanisms could also result in dysentery (Kopecko *et al.*, 2001).

For instance, there have been previous reports in which inhibition of endosome acidification, phosphatidylinositol-3-kinases or protein kinase-C did not inhibit *C. jejuni* invasion of epithelial cell lines, although the same studies reported some of the observations above (Biswas *et al.*, 2000; Oelschlaeger *et al.*, 1993). The question that arises is, should we ignore these contradictory reports? Another study that used the same inhibitors as above to inhibit the various structures and processes found no involvement of any of microfilaments, microtubules or clathryn-coated pits in *C. jejuni* invasion of epithelial cell lines (Russell & Blake, 1994). Recently, van Alphen and co-workers reported a cell entry mechanism involving active and rapid migration of the pathogen into the epithelial sub-cellular spaces (termed "subvasion"), followed by bacterial invasion at the cell bases (van Alphen *et al.*, 2008). Although, these contradictory reports may be possibly due to the differences between different strains in terms of how they elicit cellular invasion they still leave us with no clear explanation of the mechanisms involved in the pathogenesis of *Campylobacter* infection.

iii. Intracellular Survival

Immediately following the unique caveolae-dependent entry or invasion, *C. jejuni*-containing vacuoles appear to roll along microtubules towards the perinuclear region of the epithelial cell through interactions with dynein, thus,

deviating from the canonical endocytic pathway, and hence avoiding delivery into lysosomes (Hu & Kopecko, 1999; Watson & Galan, 2008). The bacterium remains largely confined to membrane bound vacuoles, although some have been observed freely in the cytoplasm (Konkel *et al.*, 1992a; Russell *et al.*, 1993). Infected epithelial cells exhibit cytoplasmic swelling, loss of microvilli and premature apoptosis due to the cytotoxic effect of surviving *C. jejuni*, and are finally exfoliated into intestinal lumen, disrupting the intestinal epithelium (Konkel *et al.*, 1992a; Russell *et al.*, 1993).

iv. Trans-Epithelial Translocation

Using polarized Caco-2 cell monolayers grown on micro-porous membrane filters, Konkel and co-workers showed that *C. jejuni* is actively translocated across the epithelial monolayer, a process that can be inhibited by chloramphenicol (Konkel *et al.*, 1992b). Whereas some studies have shown that trans-epithelial translocation is aided by an overall reduction in the trans-epithelial electrical resistance and disruption of the cellular tight junctions, others have shown that none of these changes do occur (Bras & Ketley, 1999; Chen *et al.*, 2006; MacCallum *et al.*, 2005b). This translocation of *C. jejuni* across the intestinal epithelial cell barrier to the lamina propria is considered important in *C. jejuni* pathogenesis as it leads to tissue damage and inflammation (Wine *et al.*, 2008; Wooldridge & Ketley, 1997). The organism continues to proliferate in the lamina propria, eliciting an inflammatory response that disrupts the normal function of the intestinal cells causing diarrhoea (MacCallum *et al.*, 2005b).

1.7.2 Virulence Factors

It is generally accepted that most bacterial virulence factors are located on the cell surface or are secreted (Finlay & Falkow, 1997; Wilson *et al.*, 2002). Several adhesins of *C. jejuni* have been identified, and although adhesion is known to be the first step in the invasion process, the involvement of these adhesins in pathogenesis has been a subject of much research (Finlay & Falkow, 1997; Min *et al.*, 2009). Adhesins that have been firmly characterized are the 28-kDa protein Pei, Ellison and Blaser one (PEB1), encoded by the

peb1 gene (Leon-Kempis *et al.*, 2006; Pei & Blaser, 1993), the 46-kDa *Campylobacter* adhesion to fibronectin (CadF) protein encoded by the *cadF* gene (Konkel *et al.*, 1997; Konkel *et al.*, 2005; Mamelli *et al.*, 2006), the 46-kDa pore-forming major outer membrane protein (MOMP), encoded by the *porA* gene (Schroder & Moser, 1997), the 42.3-kDa protein JlpA, encoded by the *jlpA* gene (Jin *et al.*, 2001; Jin *et al.*, 2003), and a lipoprotein autotransporter adhesin, CapA (described later), encoded by the *capA* gene (Ashgar *et al.*, 2007). Two putative fibronectin-binding proteins as well as three other PEB adhesins have also been described (Asakura *et al.*, 2007; Fouts *et al.*, 2005; Min *et al.*, 2009; Rangarajan *et al.*, 2007).

i. The Flagella

Like all other bacterial flagella, the basic structure of the *C. jejuni* flagellum consists of at least three parts, namely a basal body, which is embedded within the matrix of the cell wall and which constitutes the motor of the flagellum, a hook, which functions as a universal joint and an outer filament which functions as a propeller [see Figure 1.8] (Minamino *et al.*, 2008). The motor, which is capable of both clockwise and counterclockwise rotation is driven by proton or sodium ion motive force across the cell membrane (Minamino *et al.*, 2008).

The flagellar export apparatus consists of six integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ and FliR or its homologues), three soluble components (FliH, Flil and FliJ), and cytoplasmic chaperone proteins which together facilitate the export of substrates in an injectisome manner akin to the type III secretion system (TTSS/T3SS) involved in translocation of virulence factors into host cells (Minamino *et al.*, 2008).

Equipped with a single unsheathed flagellum at one or both ends, *Campylobacter* is rendered highly motile, which aids the organism in overcoming intestinal peristalsis and facilitate entry into the mucous layer (Guerry, 2007). The *C. jejuni* flagellum is composed of two 59-kDa, highly homologous flagellins, FlaA and FlaB, and regulated by σ^{28} (encoded by *fliA*) through the sensor kinase FlgS and σ^{54} (encoded by *rpoN*) through the

response regulator FlgR respectively, as well as a two-component regulatory system (Guerry *et al.*, 1991; Hendrixson & DiRita, 2003; Nuijten *et al.*, 1990; Poly *et al.*, 2007b).

Phase variation, which is a common feature of both *C. jejuni* and *C. coli*, is also observed in the expression of the flagellins through slip-strand mismatch repair (Caldwell *et al.*, 1985; Hendrixson, 2006; Park *et al.*, 2000).

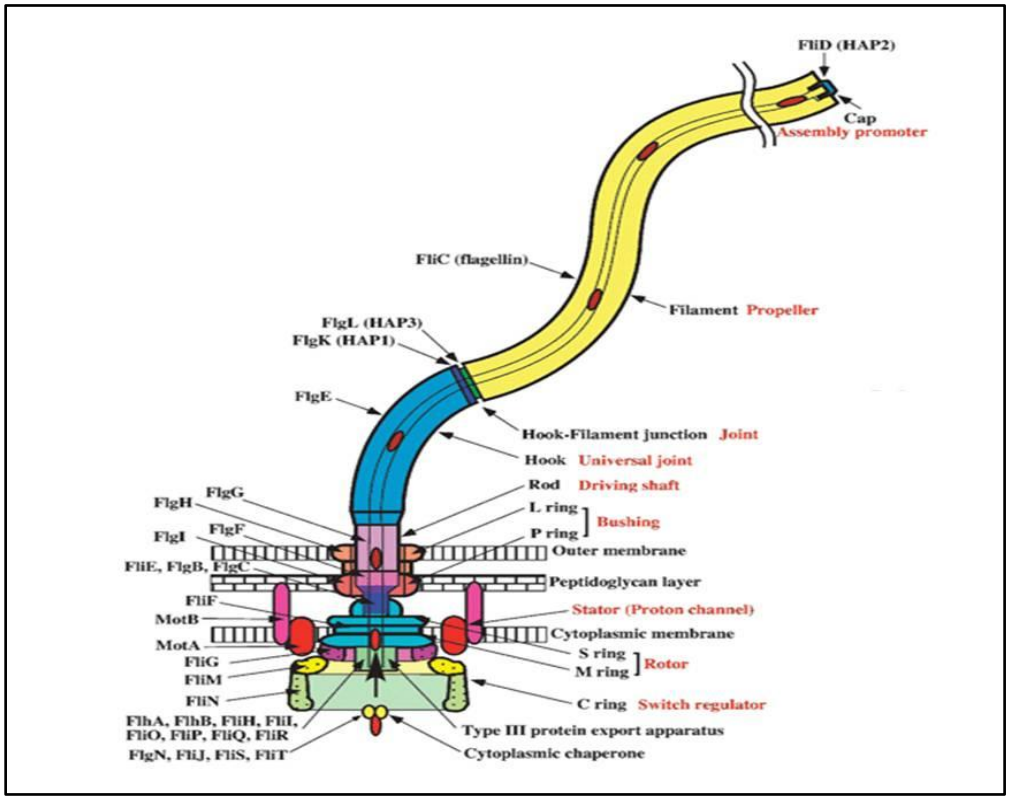

Figure 1. 8 | Schematic representation of bacterial flagellum. Flagellar components are synthesized in the cytoplasm and then transported to the periplasmic space, the outer membrane, or the extracellular space where their assembly occurs. Apart from FlgA, which is proposed to be a periplasmic chaperone for the P-ring assembly, and two other proteins, the external component proteins are translocated into the central channel and to the distal end of the growing flagellar structure through type III export apparatus (see later). The *C. jejuni* flagella are not only important in motility but are also employed in the secretion of virulence proteins [Adopted from Minamino *et al.*, 2008; Mol. BioSyst., 4, 1105-1115].

It has long been documented that motility is important in *C. jejuni* colonization and invasion of intestinal epithelial cells (Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1991). However, *C. jejuni* flagella are not only important in motility but are also employed in the secretion of virulence proteins, notably,

CiaB, FlaC and FspA, as well as in chemotaxis, autoagglutination, microcolony formation and evasion of host immune responses (Guerry, 2007).

Evasion of host immune responses is aided by the absence of Toll-like receptor 5 (TLR 5) recognition sites in the *C. jejuni* flagella, whereas the glycosylation of the flagellin at 19 serine or threonine residues mediate autoagglutination and microcolony formation (Andersen-Nissen *et al.*, 2005; Guerry *et al.*, 2006; Jeon *et al.*, 2003; Misawa & Blaser, 2000).

Furthermore, several non-flagellar genes such as Cj0040, Cj0062c, Cj0428 and Cj0977c have been identified to be up-regulated in very virulent and actively motile strains of *C. jejuni* and have been confirmed to be regulated by the same flagellin sigma factors, σ^{28} and σ^{54}, leading to the hypothesis that they may be involved in virulence (Carrillo *et al.*, 2004; Guerry, 2007). In fact, the flagellin sigma factors, σ^{28} and σ^{54}, play an important role in *C. jejuni* pathogenesis since they are also involved in secretion of Cia proteins (Fernando *et al.*, 2007).

ii. Lipooligosaccharide and Capsular Polysaccharide

Like all other mucosal pathogens, *C. jejuni* expresses two classes of surface-located glycolipid: lipo-oligosaccharide (LOS) and capsular polysaccharide (CPS) (Linton *et al.*, 2001). *C. jejuni* LOSs are unique in the sense that they are the only known bacterial LOSs capable of mimicking human gangliosides structurally and capable of eliciting immune reactions, the antibodies of which can cross-react with host gangliosides causing Guillain-Barré syndrome and Miller Fisher syndrome (Ang *et al.*, 2002; Yuki *et al.*, 2000).

Phase variations within one of the LOS locus genes, *cgtB* (also known as *wlaN*), encoding a galactosyltransferase involved in LOS biosynthesis, is known to cause changes in LOS ganglioside mimicry, whilst mutants of its homologue in *C. jejuni* 81-176, *cgtA* encoding an N-acetylgalactosaminyltransferase, has also been implicated in the adhesion and invasion of host epithelial cells and increased protection from complement-mediated killing (Guerry *et al.*, 2002; Kordinas *et al.*, 2005; Linton *et al.*,

2000). A recent study, however, found no association between these genes and adhesion or invasion (Muller *et al.*, 2007).

Again, the capsule is a common feature on bacterial surfaces and is important in bacterial survival and persistence in the environment, whilst its polysaccharides often contributed to pathogenesis (Roberts, 1996). CPSs are known to exhibit structural variation, host antigen mimicry as well as providing resistance to phagocytosis and complement-mediated killing. In *C. jejuni*, CPSs were previously described as high molecular weight "lipopolysaccharides" (HMW LPSs), until the initiation of the first genome-sequencing project, that led to the identification of *kps* genes involved in capsule biosynthesis and subsequent identification in other *C. jejuni* strains (Karlyshev *et al.*, 1999; Karlyshev *et al.*, 2001; Karlyshev & Wren, 2001). CPSs were again confirmed as the major antigens in the Penner serotyping scheme (Karlyshev *et al.*, 2000). However, our understanding of the role of *C. jejuni* CPS in protecting the organism against host immune mechanisms remains poor. Whereas some studies of capsule-deficient mutants of 81-176 and 81116 strains exhibited reduced adherence, invasion or virulence, both *in vitro* and *in vivo*, other studies found no significant differences between the wild type and the mutants (Bachtiar *et al.*, 2007; Bacon *et al.*, 2001).

Again, in a study of the bactericidal effect of epithelial β-defensins on *C. jejuni*, it was demonstrated that the presence or the absence of the bacterial polysaccharide capsule did not have any effect on the innate immune responses induced by *C. jejuni* or the survivability of the organism in recombinant β-defensins, thus casting more doubt on the role of CPS in protecting *C. jejuni* (Zilbauer *et al.*, 2005a). However, it must be stated that *C. jejuni* CPS may not be involved in its survival inside the host since the main role of the capsule is to prevent bacterial desiccation (Zilbauer *et al.*, 2008).

iii. Adhesins

PEB1, also known as cell-binding factor 1 (CBF1), is a homologue of the amino acid ATP-binding cassette (ABC) transporters, and has been confirmed to bind specifically to aspartate and glutamate (Pei & Blaser, 1993; Pei *et al.*,

1991). Although it is a periplasmic protein, PEB1 is believed to be surface exposed due to the presence of signal peptidase II recognition site, a motif akin to that seen in surface-localized lipoproteins, coupled with its identification in some culture supernatant (Leon-Kempis *et al.*, 2006). Other PEB variants are PEB3 and PEB4 (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000).

CadF, which is thought to be similar to outer membrane protein A (OmpA) of *E. coli*, is believed to be required by *C. jejuni* for maximal adherence and invasion at the basolateral surface of intestinal epithelial cells (Monteville & Konkel, 2002; Monteville *et al.*, 2003). JlpA is a surface-exposed lipoprotein and has been demonstrated to bind to heat shock protein 90 (Hsp90) α leading to the activation of p38 mitogen-activated protein (MAP) kinase and nuclear factor-kappa B (NF-κB) (Jin *et al.*, 2001; Jin *et al.*, 2003).

In contrast to other autotransporters, CapA is believed to be retained on the cell surface, the mutant of which was reported to have led to reduced ability by *C. jejuni* to associate with and invade Caco-2 cells *in vitro* (Ashgar *et al.*, 2007). Lastly, a periplasmic glycoprotein, encoded by Cj1496c, has been shown to mediate *C. jejuni* invasion of human epithelial cells (Kakuda & DiRita, 2006). Other putative adhesins include a collagen-binding surface protein, SpaP, an outer membrane adhesin that is believed to be involved in iron acquisition and a putative periplasmic adhesin which is also believed to function as initial receptors in ABC transport of metal ions (Fouts *et al.*, 2005; Gundogdu *et al.*, 2007).

iv. Toxins

The most well-characterized *C. jejuni* toxin is the cytolethal distending toxin (CDT), named after the morphological changes of cytoplasmic distension associated with its action (Johnson & Lior, 1988). CDT, which has three subunits, CdtA, -B and -C, is encoded by three adjacent genes, *cdtABC*, with the B subunit being the active component of the toxin (Young *et al.*, 2007). CDT is involved in eukaryotic cell maturation arrest in the G2/M phase of the

cell cycle leading to epithelial cell death (Abuoun *et al.*, 2005; Asakura *et al.*, 2008; Carrillo *et al.*, 2004; Kalmokoff *et al.*, 2006; Muller *et al.*, 2006).

The effect of CdtB is mainly in the nuclear compartment of the host cell and it has been demonstrated that it is delivered into the host cell through the binding of CdtA and CdtC to the host cell (Lee *et al.*, 2003). Furthermore, being membrane bound protein, CDT has been demonstrated to induce interleukin-8 (IL-8) secretion in polarized human intestinal epithelial cells (Zheng *et al.*, 2008). Several studies have confirmed the involvement of CDT in *C. jejuni* disease pathogenesis (Jain *et al.*, 2008; Kalischuk *et al.*, 2007). Recently, it has been demonstrated that CDT may have carcinogenic potential *in vivo* (Ge *et al.*, 2008; Kalischuk *et al.*, 2007).

Historically, physiological and immunological relatedness has been demonstrated between a *C. jejuni* enterotoxin, the *Vibrio cholerae* enterotoxin, and the heat-labile cholera-like *Escherichia coli* enterotoxin. However, nucleotide sequence similarity between *C. jejuni* DNA and either the *toxA*, *toxB*, *eltA* or *eltB* genes remained to be shown (Calva *et al.*, 1989; Collins *et al.*, 1992; Klipstein & Engert, 1985). Recently, Albert and co-workers have demonstrated that this cross-reactivity with cholera toxin is embedded in the *C. jejuni* major outer membrane protein, which could be explored as a vaccine candidate (Albert *et al.*, 2008).

v. Chemotaxis

Chemotaxis is a necessary phenomenon in all bacteria and other unicellular organisms in directing their movements according to certain chemicals in their ecology and associated processes (Frymier *et al.*, 1993; Parkinson *et al.*, 2005). The acidic environment of the gastrointestinal tract (GIT), and the numerous chemicals secreted therein means that *C. jejuni* uses chemotaxis to move towards or away from chemicals (Takata *et al.*, 1992). Thus, chemotaxis is important in the pathogenesis of *C. jejuni* and this has been demonstrated via the investigation of phenotypic changes in non-chemotactic mutants *in vivo*, one of which in a mouse model showed that chemotactic mutants failed

to colonise the intestines (de Melo & Pechere, 1990; Hendrixson & DiRita, 2004; Takata *et al.*, 1992).

Most features of *C. jejuni* chemotaxis have been likened to chemotaxis systems in *E. coli* and *Bacillus subtilis* based on genome wide analysis (Marchant *et al.*, 2002). Firstly, based on chemotaxis systems in *E. coli* and *Bacillus subtilis* twelve methyl-accepting chemotaxis proteins (MCPs) or chemoreceptors can be identified from the *C. jejuni* genome, namely, Cj0019c, Cj0144, Cj0246c, Cj0262c, Cj0448c, Cj0923c, Cj0924c, Cj0951c, Cj1110c, Cj1190c, Cj1506c and Cj1564, previously referred to as transducer-like proteins (Tlp) (Gundogdu *et al.*, 2007; Marchant *et al.*, 2002). However, only two of these, Cj0019c (DocB) and Cj0262c, have so far been shown to have an effect on chicken colonization (Hendrixson & DiRita, 2004).

Furthermore, *C. jejuni* motility has been shown to be dependent on two energy taxis or aerotaxis proteins, CetA (Tlp9/Cj1190c) and CetB akin to *E. coli* HAMP domain (named for the presence of histidine kinases, adenylyl cyclases, 46 methyl-accepting chemotaxis proteins and phosphatases) and PAS domain (named after three proteins Per, ARNT and Sim) (Gundogdu *et al.*, 2007; Hendrixson *et al.*, 2001). The PAS domain is believed to interact directly with the HAMP domain to transmit an energy taxis signal parallel to the inner membrane through detection of redox changes in the electron transport system or cytoplasm when the bacteria enter or leave a hypoxic microniche (Taylor, 2007; Watts *et al.*, 2008). Recently, it has been demonstrated that whilst CetA and CetB interact to provide energy for motility, only CetA contributes to *C. jejuni* invasion of human epithelial cells (Elliott & Dirita, 2008; Elliott *et al.*, 2008).

Additionally, three genes, *cheA, cheV* and *cheY*, have also been shown to encode polypeptides of response regulator domain characteristics, the CheY of which has long been predicted to interact with the flagellar motor to influence the direction of rotation (Baker *et al.*, 2006; Marchant *et al.*, 2002; Yao *et al.*, 1997). Although the roles of CheA and CheV have not been fully elucidated in *C. jejuni*, they are believed to play a role in adaptation, where CheA may phosphorylate CheY or the presence of multiple response regulator domains

may serve as a "phosphate sink" to remove phosphate groups from CheY (Marchant *et al.*, 2002; Young *et al.*, 2007).

It has long been known that *C. jejuni* migrate towards some components of mucus as well as towards amino acids in the chicken GIT (Hugdahl *et al.*, 1988). Recently, it has been shown that *C. jejuni* utilizes MUC2, the most abundant of the secreted mucins in the human intestine, as an environmental cue for the modulation of expression of genes with various functions including colonization and pathogenicity (Tu *et al.*, 2008). Again, the regulation of the CmeABC multidrug efflux pump is believed to be mediated by the presence of bile salts through CmeR (Lin *et al.*, 2005a; Lin *et al.*, 2005c).

vi. Iron Acquisition

The importance of iron in *C. jejuni* pathogenesis cannot be overemphasised given that it is an essential nutrient for bacterial growth and survival playing a major role in the catalysis of many biochemical reactions (Andrews *et al.*, 2003). Bacteria, including *C. jejuni*, are known to access iron that is present in the body fluids of its mammalian host that are complexed to high affinity iron-binding proteins (van Vliet *et al.*, 1998). The serum iron is present in complexes with haemoglobin (in small amounts from lysis of erythrocytes) or transferrin, while iron exists as a complex with lactoferrin in secretions on mucosal surfaces (Payne & Finkelstein, 1978). It was previously thought that *C. jejuni* did not utilise iron from these sources, but recent developments have shown that *C. jejuni* is capable of utilizing lactoferrin-bound and transferrin-bound iron (Miller *et al.*, 2008; Tom-Yew *et al.*, 2005).

Three iron-uptake systems have been described in *C. jejuni*: (i) a haemin/haemoglobulin uptake system called *chuABCD*; (ii) a periplasmic binding-protein-dependent transport (PBT) system for the uptake of a ferric enterochelin (*cfrA* and *ceuBCDE*); and (iii) a ferric siderophore uptake system, *cfhuABD* (Galindo *et al.*, 2001; Palyada *et al.*, 2004; Richardson & Park, 1995; Ridley *et al.*, 2006). Two uncharacterized iron uptake systems, Cj1658-Cj1663 and Cj0173c-Cj0178 have also been predicted (Parkhill *et al.*, 2000). Though not extensively characterised, the *C. jejuni* genome also encodes an

iron transporter operon akin to the *E. coli feoAB* operon. Recently, Naikare *et al.* demonstrated that FeoB is actually involved in ferrous iron acquisition in *C. jejuni*, contributing significantly to colonization of the gastrointestinal tract during both commensal and infectious relationships, contradicting the previous assertion by Raphael and Jones that this operon played no role in iron acquisition (Naikare *et al.*, 2006; Raphael & Joens, 2003).

The response of the organism to environmental changes in iron concentrations is regulated by another gene, the ferric uptake regulator (*fur*) gene (Holmes *et al.*, 2005; van Vliet *et al.*, 1999; Wooldridge *et al.*, 1994). Furthermore, the lipoprotein component of the *ceuBCDE* operon, encoded by *ceuE*, is thought to confer a haemolytic phenotype thus complicating our understanding of iron acquisition in *C. jejuni* (Park & Richardson, 1995).

vii. Other virulence Genes/Proteins

Several other *Campylobacter* virulence genes have been identified such as thermotolerance gene, *dnaJ*, and the structural gene for a phospholipase A, *pldA* involved in hemolysis (Grant *et al.*, 1997; Konkel *et al.*, 1998). Carvalho and co-workers reported the identification of an invasion-associated marker (IAM) using random amplified polymorphic DNA (RAPD) techniques, which was later found to be encoded by the *iam* gene (Carvalho *et al.*, 2001; Konkel *et al.*, 1999b). Another gene, *virB11*, carried by the virulence plasmid pVir, was reported to be involved in *Campylobacter* disease pathogenesis (Bacon *et al.*, 2000).

However, whilst we may think of invasion as being mediated by *ciaB*, *pldA*, *iamA* and *virB11*, the presence of all these putative virulence genes in a *Campylobacter* species does not guarantee successful colonization and invasion *in vivo* since some of these genes, notably *virB11* gene, have been found to be absent in some invasive *Campylobacter* isolates (Talukder *et al.*, 2008; Zheng *et al.*, 2006). The *virB11* gene is strongly associated with antibiotic resistance rather than with invasion (Bang *et al.*, 2003; Bang *et al.*, 2004).

DksA, a well known bacterial regulatory protein involved in the transcription of rRNA and control of expression of virulence genes in pathogenic bacteria, was recently reported to also play an important regulatory role in numerous metabolic events and the virulence of *C. jejuni*, with a *C. jejuni* DksA-like protein mutant exhibiting a decreased ability to invade intestinal cells or induce IL-8 secretion from intestinal cells (Yun *et al.*, 2008). Again, CmeR, which is known to be a transcriptional repressor regulating the expression of the CmeABC multidrug efflux pump, is believed to also regulate the expression of multiple genes with diverse functions and is required for *Campylobacter* adaptation in the chicken host (Guo *et al.*, 2008; Lin *et al.*, 2005a; Lin *et al.*, 2005c).

Furthermore, *C. jejuni* is known to secrete a set of eight proteins called *Campylobacter* invasive antigens (Cia) A-H, the CiaB of which has been well-characterized as a 73.1-kDa protein and believed to be required for the invasion of cultured epithelial cells as well as colonization of the chicken gut (Konkel *et al.*, 1999b; Ziprin *et al.*, 2001).

The mechanism of secretion of these Cia proteins and their role in cellular invasion have been likened to type III secretion systems (TTSS/T3SS) where the effector proteins are injected directly into host cells (Guerry, 2007; Konkel *et al.*, 1999c). It is believed that the secretion of CiaB, as well as the other Cia proteins, is mediated by the flagellar export apparatus, emphasizing the importance of the flagella in *C. jejuni* pathogenesis (Konkel *et al.*, 1999c; Konkel *et al.*, 2004b; Song *et al.*, 2004).

Recently, it has been shown that two additional virulence proteins, a 26.6-kDa FlaC protein, and a 15.5-kDa FspA protein, are also secreted through the flagellar filament (Poly *et al.*, 2007a; Song *et al.*, 2004).

1.7.3 Protein Glycosylation in *Campylobacter*

Post-translational glyco-conjugation of protein has long been recognised as essential in eukaryotic cellular biology, modulating protein structure and function, including sorting, targeting, localization, stability, and antigenicity (Kelly *et al.*, 2006). There are two forms of glycosylation: *O*-linked and *N*-

linked, the former involving attachment of glycans to the hydroxyl groups of serines or threonines and the latter involving the attachment of glycans to amino groups of asparagines in the sequon Asn-Xaa-Ser/Thr, where Xaa is any amino acid except for proline (Benz & Schmidt, 2002; Messner & Schaffer, 2003; Szymanski *et al.*, 2005).

This post-translational protein modification was first identified in bacteria in *C. jejuni* flagellin by Logan and co-workers (Logan *et al.*, 1989), with Szymanski demonstrating that an *N*-linked glycosylation is responsible for the synthesis of a conserved bacillosamine-containing heptasaccharide that modifies over thirty secreted *Campylobacter* proteins (Szymanski *et al.*, 1999; Szymanski *et al.*, 2003).

Despite the predominance of *N*-linked glycosylation in bacteria, current data indicate that *C. jejuni* contains both *N*- and *O*-linked glycosylation systems [see Figure 1.9], with the latter responsible for the addition of pseudaminic acid and related sugars to the immunodominant *C. jejuni* flagellin protein, FlaA (Szymanski *et al.*, 2003).

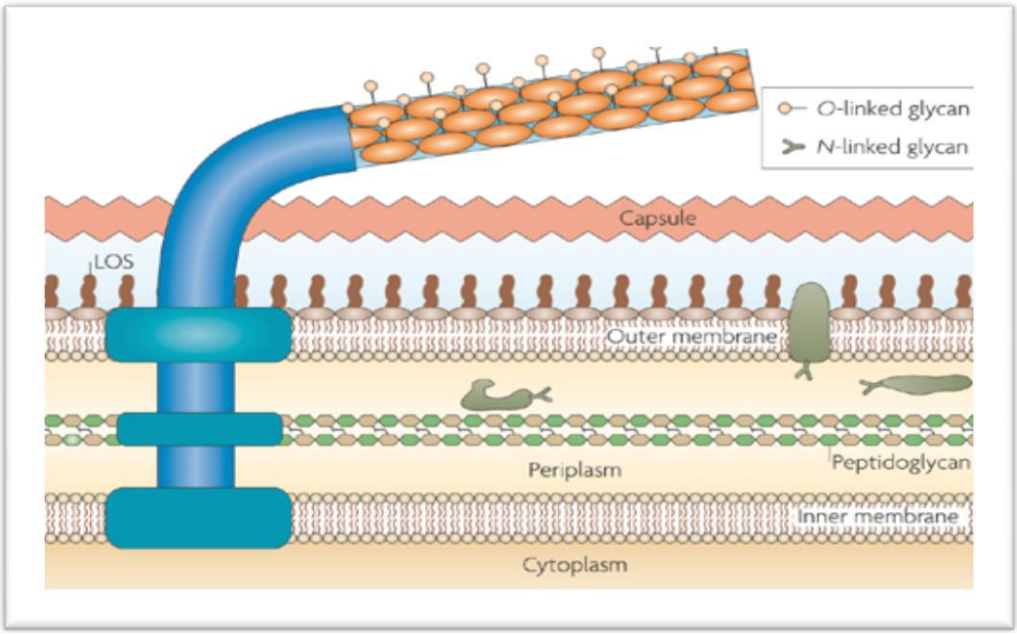

Figure 1. 9 | A cross-sectional view of *C. jejuni* cell wall. The photo depicts the flagellum, capsule, outer membrane protein and its associated lipooligosaccharides, and the *O*- and *N*-linked glycans [Adopted with permission from Young *et al.*, 2007; *Nat Rev Microbiol* 5, 665-679].

The precise functions of these glycosylations are yet to be elucidated but there is evidence that loss of the conserved heptasaccharide of the *N*-linked glycosylation results in changes in antigenicity, reduction in *in vitro* adherence and invasion, loss of *in vivo* colonization, and loss of type IV protein complex assembly and DNA uptake, whereas the high variability of the *O*-linked glycosylation suggests a role in immune evasion (Kelly *et al.*, 2006; Szymanski *et al.*, 2003).

Furthermore, Guerry and co-workers have demonstrated that alterations in the glycosylation of *Campylobacter* flagellin proteins result in reduced adherence and invasion (Guerry *et al.*, 2006).

1.7.4 Host Factors in *C. jejuni* infection

Infection with *C. jejuni* is usually self-limiting but has varied manifestations depending on the host and the host's geographical location, thus, prompting the importance of host immune responses in determining disease outcome (Blaser *et al.*, 1980; Zilbauer *et al.*, 2008). Fucosylated sugars in breast milk have been demonstrated not only to inhibit *C. jejuni* binding and infection but also prevent diarrhoea in infants, whilst the bactericidal effect of human serum has long been demonstrated, underlining the importance of host innate responses in containment of infection with *C. jejuni* (Blaser *et al.*, 1985b; Morrow *et al.*, 2004; Ruiz-Palacios *et al.*, 2003).

Following ingestion, *C. jejuni* does not only have to survive the acidic environment of the stomach, but also has to bypass the mucus lining of the intestinal epithelium which are important barriers, the depletion of which leads to increased susceptibility to *Campylobacter* infection (Doorduyn *et al.*, 2008; Neal *et al.*, 1996; Tu *et al.*, 2008). The epithelial lining plays a crucial role in microbial sensing to initiate appropriate innate immune responses such as the production of chemokines, cytokines, and antimicrobial peptides (Eckmann, 2005; Zilbauer *et al.*, 2005b). Epithelial β-defensins, a family of host-defence peptides known to be modulated during *C. jejuni* infection and which have been demonstrated to be also present in the avian intestinal tract, play a very

crucial role in mucosal innate defence through their chemotactic and bactericidal activity (Byrne *et al.*, 2007; Ganz, 2003).

Initiation of innate immunity is known to be mediated by the interaction of host pattern-recognition receptors (PRRs) with conserved microbial signature motifs called pathogen-associated molecular patterns (PAMPs) (Akira *et al.*, 2006; Sanderson & Walker, 2007). The phylogenetically conserved Toll-like receptors (TLRs), which are expressed at the basolateral surface of the intestinal epithelium, constitute one of the commonest PRRs involved in flagella recognition and activation of host NF-κB, a family of mammalian proteins that regulate transcription of pro-inflammatory genes involved in early host immunity against infection (Gewirtz *et al.*, 2001; Ghosh *et al.*, 1998; Hayashi *et al.*, 2001).

One of the early immunological challenges encountered by infecting *C. jejuni* is presumably the presence of these TLRs, which are present in most vertebrates including humans and chickens (Takeda *et al.*, 2003; Young *et al.*, 2007). The stimulation of innate immune responses through TLR5 and TLR9, which are responsible for the recognition of primary flagellin structure and unmethylated CpG dinucleotides respectively, is known to be deficient in *C. jejuni* (Andersen-Nissen *et al.*, 2005; Dalpke *et al.*, 2006; Johanesen & Dwinell, 2006; Watson & Galan, 2005). However, a recent demonstration that mice deficient in MyD88, an important molecule downstream of TLRs, are more susceptible to *C. jejuni* infection has rekindled the belief that TLRs are important in the pathogenic mechanisms of campylobacters, providing host-immune defences (Watson *et al.*, 2007). This assertion is supported by previous but recent reports that NF-κB is readily activated in *in vitro* models and that NF-κB-gene deficient mice are also highly susceptible to *C. jejuni* infection (Chen *et al.*, 2006; Fox *et al.*, 2004; Jin *et al.*, 2003; Johanesen & Dwinell, 2006; Jones *et al.*, 2003; Zilbauer *et al.*, 2005b). Also, innate immunity has been shown to be partly mediated by other PRRs such as the nucleotide-binding oligomerization domain one (NOD1), and the natural resistance-associated macrophage protein one (Nramp1) (Watson *et al.*, 2007; Zilbauer *et al.*, 2007).

The interaction of *C. jejuni* with intestinal epithelial cells also results in the activation of MAP kinases, which in turn induce host responses such as the induction of interleukin 8 (IL-8) production by p38MAP kinases and extracellular regulated kinases (ERK) (MacCallum *et al.*, 2005a; Watson & Galan, 2005). The interaction of *C. jejuni* surface protein, JlpA, with the heat shock protein 90 (Hsp90) of epithelial cells leads to the activation of both p38MAP and NF-κB (Jin *et al.*, 2003). In addition to TLR-mediated activation of NF-κB, bacterial adhesion and invasion and the presence of CDT can stimulate IL-8 production (Hickey *et al.*, 1999; Hickey *et al.*, 2000; Zheng *et al.*, 2008).

The release of IL-8, and other pro-inflammatory cytokines such as interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα), IL-2 and IL-β, in response to bacterial infection is not only considered to be crucial in bacterial clearance but also in the development of diarrhoea, through recruitment of polymorphonuclear leukocytes (PMNs), especially neutrophils to the site of local inflammation, recruitment of activated macrophages, inhibition of the absorptive functions of the intestinal epithelium and disruption of its cellular tight junctions (Al-Banna *et al.*, 2008; MacCallum *et al.*, 2005b; Zilbauer *et al.*, 2008). However, the degree of IL-8 induction has been shown to vary in *in vitro* models of infection and correlates well with pathological changes (Al-Banna *et al.*, 2008; MacCallum *et al.*, 2006).

The interaction of neutrophils (PMN) with infecting *C. jejuni* leads to phagocytosis of the bacterium resulting in the production of reactive oxygen species which, may lead to bacterial killing depending on the level of induction (Wooldridge & Ketley, 1997). Monocytes and macrophages have long been shown to internalize and kill infecting *C. jejuni*, with the killing mediated by reactive nitrogen species (Al-Banna *et al.*, 2008; Iovine *et al.*, 2008; Wassenaar *et al.*, 1997; Wooldridge & Ketley, 1997). However, some studies have also demonstrated that a significant proportion of *C. jejuni*-infected monocytes do undergo apoptosis (Hickey *et al.*, 2005; Siegesmund *et al.*, 2004). Furthermore, *C. jejuni* has been shown to survive in porcine and

murine peritoneal macrophages for days, and this survival is attributed to the production of catalase by the bacterium (Day *et al.*, 2000).

Recently, *C. jejuni* has been shown to also induce specific cell-mediated immune responses *in vitro* leading to the maturation of dendritic cells and secretion of associated pro-inflammatory cytokines (Hu *et al.*, 2006a; Johanesen & Dwinell, 2006; Rathinam *et al.*, 2008). Johanesen *et al.* demonstrated an increased expression of CCL20, a chemo-attractant cytokine of dendritic cells, following exposure of T84 epithelial cell line to *C. jejuni*, which goes to buttress the involvement of cell-mediated immunity in *C. jejuni* pathogenesis (Johanesen & Dwinell, 2006). In another study, Jones *et al.* have shown that maturation of dendritic cells is bacterial LOS-dependent and that it leads to internalization of *C. jejuni* (Jones *et al.*, 2003). Also, in an *in vivo* study by Fox and co-workers, it was demonstrated that IgG2a is produced in response to both the wild-type strain and a *cdtB C. jejuni* mutant, through Th1, and was responsible for clearance of the bacterium from infected mice (Fox *et al.*, 2004). Although the significance of the protective effect of γδ T-cells against *Campylobacter* infection is not known, it has been reported that non-protein extracts of *Campylobacter* can induce *in vitro* expansion of these (Van Rhijn *et al.*, 2003).

Several studies have described humoral immune responses following human infections with *C. jejuni* (Blaser & Duncan, 1984; Blaser *et al.*, 1985a; Blaser *et al.*, 1986; Lane *et al.*, 1987; Martin *et al.*, 1989; Mizuno *et al.*, 1985). Likewise, similar responses have been reported in several animal models including monkeys, rabbits, hamsters and mice (Humphrey *et al.*, 1985; Russell *et al.*, 1989). This immune response has been attributed to some components of the bacterium, such as the flagella, MOMP, LOS, CDT and outer membrane proteins (Omps) (Abuoun *et al.*, 2005; Guerry *et al.*, 2000; Huang *et al.*, 2007; Mills & Bradbury, 1984; Nachamkin & Yang, 1989).

Infection with *C. jejuni* has also been reported to generate a secretory immunoglobulin A (sIgA) in both humans and experimental rabbits (Burr *et al.*, 1988). In infants, *C. jejuni* infections result in the generation of low levels of IgA, IgG and IgM, which is thought to be due to the presence of maternal

antibodies (Ruiz-Palacios *et al.*, 1990; Young *et al.*, 2007). Antibodies produced as a result of *C. jejuni* infection have long been shown to be protective (Black *et al.*, 1988; Dolby & Newell, 1986; McSweegan *et al.*, 1987; Ruiz-Palacios *et al.*, 1990). A pentaglobulin preparation containing *C. jejuni* specific IgM is reported to have been used to successfully treat hypoglobulinemic patients who were suffering from chronic campylobacteriosis (Borleffs *et al.*, 1993). Children in developing countries and persons who consume high amounts of raw milk tend to have high levels of IgG which is thought to protect them against bloody diarrhoea (Blaser *et al.*, 1985a; Blaser *et al.*, 1986; Blaser *et al.*, 1987). Following infection, the level of IgG is thought to persist longer than IgA or IgM levels, which affirms the superior protective effect of the former (Cawthraw *et al.*, 2002; Russell *et al.*, 1989).

1.7.5 Mechanisms of *Campylobacter* Diarrhoea

Whereas gastroenterologists define diarrhoea as the passage of more than 200 g of stool per day, many patients and doctors describe diarrhoea in terms of increased stool frequency, with lose or watery consistency (Boon *et al.*, 2006). Following passage through the human gastrointestinal tract (GIT) by *Campylobacter*, the illness that may ensue could and has been described as diarrhoea, which could be either watery or bloody (Janssen *et al.*, 2008).

Following a thorough review of available literature four mechanisms can be said to be responsible for the induction of diarrhoea based on clinical syndromes and intestinal biopsies:

i. adherence and colonization of *Campylobacter* to the intestine (Byrne *et al.*, 2007; Smith *et al.*, 2008; Tu *et al.*, 2008; Van Deun *et al.*, 2008; Wine *et al.*, 2008)

ii. production of toxins (Ge *et al.*, 2008; Jain *et al.*, 2008; Kalischuk *et al.*, 2007; Wassenaar, 1997; Zheng *et al.*, 2008)

iii. invasion of intestinal epithelial cells (Kakuda & DiRita, 2006; Louwen *et al.*, 2008; van Alphen *et al.*, 2008; Watson & Galan, 2008) and

iv. initiation of an inflammatory response (Al-Banna *et al.*, 2008; Borrmann *et al.*, 2007; Hu *et al.*, 2006a; Li *et al.*, 2008; MacCallum *et al.*, 2006; Zheng *et al.*, 2008; Zilbauer *et al.*, 2007).

For infection to ensue, *Campylobacter* must first adhere to the intestinal epithelium, which is believed to be achieved through a number of adhesins including the CadF (Konkel *et al.*, 1997; Konkel *et al.*, 2005; Mamelli *et al.*, 2006), JlpA (Jin *et al.*, 2001; Jin *et al.*, 2003), PEB1 (Leon-Kempis *et al.*, 2006; Pei & Blaser, 1993), several outer membrane proteins including MOMP (Schroder & Moser, 1997), CPS and a host of other secreted proteins such as Cia proteins (Konkel *et al.*, 1999b; Ziprin *et al.*, 2001), CapA (Ashgar *et al.*, 2007), FlaC and FspA (Poly *et al.*, 2007a; Song *et al.*, 2004) as discussed previously.

This initial adherence results in the immediate disturbance of the intestinal micro flora, a time-dependent decrease in trans-epithelial electrical resistance, a redistribution of the protein occludin, which is the major component of the epithelial tight junctions, resulting in malabsorption and hence diarrhoea (Chen *et al.*, 2006; MacCallum *et al.*, 2005b). Colonization is aided by these proteins which also help the *Campylobacter* cells to adhere to each other through biofilm formation further disturbing the intestinal micro flora and initiating activation of NF-κB, MAP kinases and IL-8, leading to malabsorption and hence diarrhoea (Chen *et al.*, 2006; Fields & Thompson, 2008; Hanning *et al.*, 2008; Joshua *et al.*, 2006).

Cytolethal distending toxin (CDT) has been extensively proven to be a major factor in the pathogenesis of *Campylobacter* enteritis, although some other toxins have been mentioned in the past (Jain *et al.*, 2008; Poly & Guerry, 2008). The production of this toxin results in apoptosis-like cell death, due to developmental arrest in the G2/M phase of the intestinal cell cycle (Lara-Tejero & Galan, 2001).

Furthermore, it has long been known that *Campylobacter* cells invade intestinal epithelial cells (van Spreeuwel *et al.*, 1985). This internalization of *C. jejuni* into cells is aided by *Campylobacter* secreted proteins on one hand and host-cell depolymerization of microtubule and microfilaments on the other (Biswas *et al.*, 2003). Also, it is believed that the interaction between *Campylobacter* cells and intestinal caveolae results in membrane ruffling and subsequent phagocytosis (Wooldridge & Ketley, 1997). Although *Campylobacter* cells are thought to be confined to membrane-bound cell vesicles once inside the cell, some have been observed in the cytoplasmic space (Russell *et al.*, 1993).

The three processes described, namely, adherence and colonization, toxin production, and invasion would inadvertently elicit an immune response that may result in inflammatory diarrhoea (Hu & Hickey, 2005; Mamelli *et al.*, 2006; Smith *et al.*, 2008; Zheng *et al.*, 2008). The end result is mucoid diarrhoea that could be bloody, with leucopenia.

1.8 Clinical Significance of *Campylobacter jejuni*

Campylobacter jejuni has been known to be a causative agent of gastroenteritis in both humans and animals for several decades (Blaser *et al.*, 1979; Butzler & Skirrow, 1979; Karmali & Fleming, 1979; King, 1957; Skirrow, 1977; White, 1967) and is still considered a major cause of the disease in the 21st century (Butzler, 2004; Danis *et al.*, 2009; French *et al.*, 2009; Hariharan *et al.*, 2009; Huang *et al.*, 2009; Taylor *et al.*, 2009; Wesley *et al.*, 2009; Yoda & Uchimura, 2006; Young *et al.*, 2007; Zilbauer *et al.*, 2008).

The first well-documented case of human infection with *C. jejuni* is reported to have taken place in Illinois in 1938 which involved a milk-borne outbreak of diarrhoea in 355 inmates of two adjacent institutions (Levy, 1946). In 1947 Vinzent and co-workers isolated the organism from the blood of three pregnant women admitted with pyrexia of unknown origin, two of whom later aborted (Vinzent *et al.*, 1947). Then in 1957 Elizabeth King described a vibrio of human origin with similar features as the one described by Vinzent and proposed the name "related Vibrios" (King, 1957). By 1972, only 12 cases of "related *Vibrio*"

infections had been reported in literature comprising of seven infants, two children and three adults (Butzler, 2004).

The story is different now, and there is no doubt that *C. jejuni* is the commonest cause of bacterial enteritis in the world, with the rate of infection in some developed countries exceeding 400 per 100,000 persons (Baker *et al.*, 2007). The World Health Organisation's last report on the health impact of *Campylobacter* infections recognizes the tremendous increase in the incidence of human campylobacteriosis in the last two decades and estimates *Campylobacter* mortality at 30 days post-infection to be as high as 4 deaths per 1000 infections (WHO, 2001). Guillain-Barré Syndrome (GBS), a debilitating autoimmune disorder of the peripheral nervous system characterized by symmetrical leg weakness has become a clearly recognised sequelae of *C. jejuni* enteritis, and *C. jejuni* is the inducing antecedent infection in approximately 15% and 30% of cases of GBS in the UK and the US respectively (Nachamkin, 2001; Smith, 2002; Tam *et al.*, 2003).

C. jejuni has also been implicated in post-infectious musculoskeletal disorders such as reactive arthritis and Miller Fisher syndrome, which is a milder form of GBS (Hannu *et al.*, 2002; Hannu *et al.*, 2004a; Koga *et al.*, 2005). Furthermore, the involvement of *C. jejuni* in irritable bowel syndrome as well as immuno-proliferative small intestinal diseases has been well-documented (Lecuit *et al.*, 2004; Malinen *et al.*, 2005).

As all foodborne infections could be potentially hazardous to both the mother and the fetus in pregnancy, *C. jejuni* bacteraemia during pregnancy may also result in intrauterine infection of the fetus, with a resultant abortion, stillbirth, or early neonatal death and even death of the mother (Meyer *et al.*, 1997; Smith, 2002). Neonatal enteritis, bacteraemia, and/or meningitis have resulted from *Campylobacter* infection of an infant during parturition or shortly after (Fujihara *et al.*, 2006; Simor *et al.*, 1986; Smith, 2002).

Although gastroenteritis remains the main presentation of *C. jejuni* infection, there have been reports of other presentations such as osteitis of the foot (Pedler & Bint, 1984), severe bacteraemia in immunocompromised persons (Manfredi *et al.*, 1999), osteomyelitis (Vandenberg *et al.*, 2003), myelitis (Aberle *et al.*,

2004), uveitis (Hannu *et al.*, 2004b), cellulitis (Monselise *et al.*, 2004), and myocarditis (Braun *et al.*, 2008; Reda & Mansell, 2005). With reports of aortoiliac aneurysms infected with *C. fetus* (Cochennec *et al.*, 2008; Yano *et al.*, 2008), it makes sense to suggest that *C. jejuni* could pose a similar risk.

In England and Wales, the estimated cost of treatment of a case of *Campylobacter* enteritis is £315 which is higher than the average cost of treatment of other infectious intestinal disease estimated at £253 (Edwards *et al.*, 2000). It is therefore obvious that the clinical significance of *Campylobacter jejuni* and hence the economic impact of campylobacteriosis cannot be overlooked.

1.8.1 *Campylobacter* Enteritis

The main route of infection in humans is the oral route. Gastroenteritis remains the most common end-presentation of *Campylobacter* infection (Blaser, 1997). Although birds could invariably remain asymptomatic even in the face of heavy infestations of up to 10^9 *Campylobacter* organisms, human disease could ensue with as few as 10^2 (Black *et al.*, 1988; Robinson, 1981).

The mode of infection of this organism is binding and colonization of the distal ileum and jejunum of the gastrointestinal tract, inducing diarrhoea which may be indistinguishable from diarrhoea caused by other enteropathogens such as *Salmonella* and *Shigella* (Nachamkin, 2002). Generally, a large proportion of the ingested organisms may be killed, but the few that may survive the transit through the GIT would then adhere to the intestinal epithelia causing the diarrhoea observed or lead to an asymptomatic carrier state (Cabrita *et al.*, 1992).

There are two main manifestations of the disease, based on epidemiological studies; bloody, mucoid diarrhoea which is usually self-limiting is the common presentation among young adults in the developed world, whereas in developing countries it manifests as non-inflammatory watery diarrhoea mostly in children (Blaser *et al.*, 1980).

1.8.1.1 Clinical Features of *Campylobacter* Enteritis

The clinical features of *Campylobacter* enteritis caused by *C. jejuni* may not be sufficiently different from that caused by other campylobacters or other bacterial pathogens such as *Salmonellae* to allow for easy and reliable diagnosis without laboratory tests (Gascon, 2006; Nachamkin, 2002). However, it is believed that within 24-72 hours of exposure to *C. jejuni*, an acute diarrhoeal illness could ensue, followed by a non-specific syndrome of fever, chills, myalgia and headache (Blaser, 1997). Individuals may have 8-10 diarrhoeal bouts in a day and the stool may be loose, watery and/or bloody, with associated faecal leucocytes and erythrocytes in the majority of cases (Young & Mansfield, 2005).

The disease is usually associated with nausea, but only a small proportion of patients actually vomit (Janssen *et al.*, 2008; Pacanowski *et al.*, 2008). The diarrhoea may subside within 3-5 days but carriage of the organism may persist even during convalescence for well over 30 days (Kapperud *et al.*, 1992).

Although diarrhoeal diseases constitute a major risk among infants and older children in developing countries, *C. jejuni* infection is yet to be considered a major causative organism, since asymptomatic shedding of the organism has been reported in children two years and older (Megraud *et al.*, 1990; Pazzaglia *et al.*, 1991). However, where inflammatory enteritis ensues, there is often significant morbidity and mortality associated with campylobacteriosis among infants than among adults (Young & Mansfield, 2005).

Whereas *C. jejuni* may pass unnoticed in the stools of most adults in the developing world, in advanced countries young adults are the most common group with disease (Cabrita *et al.*, 1992; Robinson *et al.*, 2004). Nevertheless, asymptomatic carriage has been reported in 0.75% of adults studied in the United Kingdom (Wheeler *et al.*, 1999).

This differential expression of the disease between developed and developing countries could be attributed to the early acquisition of immunity

in childhood following hyperendemic and/or polymicrobial exposure, which persists into adulthood (Calva *et al.*, 1988; Mansfield *et al.*, 2003).

1.8.1.2 *Campylobacter* Infection in Animals

The course of infection of *Campylobacter* in most domesticated animals cannot be likened to that in humans, since in most animals colonisation can occur without significant pathology. Several experiments have described the course of infection as the same following inoculation of *C. jejuni* by various routes in pigs (Babakhani *et al.*, 1993; Mansfield *et al.*, 2003), rabbits (McSweegan *et al.*, 1987), dogs (Prescott *et al.*, 1981) and chickens (Meinersmann *et al.*, 1991). However, natural infections with *C. jejuni* in which enteritis has ensued have also been reported in a wide range of domesticated animals ranging from weaning ferrets and juvenile macaques (Bell & Manning, 1990; Fox *et al.*, 1986b; Russell *et al.*, 1993) to cats and dogs (Fox *et al.*, 1986a; Fox *et al.*, 1988). However, infection is more common and more pronounced in puppies and kittens, as it is in infants (Fox *et al.*, 1986a; Young & Mansfield, 2005).

The clinical signs and symptoms following infection with *C. jejuni* are obvious, and are usually watery to bloody mucoid, bile-stained diarrhoea lasting 5-15 days, with associated fever and leucopenia (Young & Mansfield, 2005).

Asymptomatic carriage of the organism in healthy domesticated animals has also been documented (Cave *et al.*, 2002; Hackett & Lappin, 2003), with several studies reporting that over 50% of commercially raised pigs excrete *C. jejuni* suggesting early exposure (Moore *et al.*, 2002a; Moore *et al.*, 2002b). In pigs therefore, it is believed that this early exposure confers some level of acquired immunity to *C. jejuni*, with diarrhoea resulting only in co-infection with other pathogens such as *Trichuris suis* or bacteria (Babakhani *et al.*, 1993).

Most domesticated animals could therefore be considered as significant sources of *C. jejuni*. However, it is believed that inoculating gnotobiotic or colostrum-deprived piglets with *C. jejuni* would result in clinical signs and

symptoms akin to that observed in acute infections in humans (Babakhani *et al.*, 1993).

Although *C. jejuni* is known to also cause diarrhoea in sheep and cattle late-pregnancy abortion has been the commonest presentation of infection (Delong *et al.*, 1996; Varga *et al.*, 1990a; Varga *et al.*, 1990b). This is not a unique occurrence in sheep or cattle as *C. jejuni* is reported to have been isolated in the vaginal discharge of three German Shepherd bitches after late-pregnancy abortions (Odendaal *et al.*, 1994).

Although birds are known to have a higher carriage and infection rate than other animals, *Campylobacter jejuni* rarely causes any disease in them (Pearson *et al.*, 1993; Young & Mansfield, 2005). This is true of free-living birds such as gulls, waterfowls and crows (Maruyama & Katsube, 1990), as well as domesticated poultry, especially chickens, which constitute a major reservoir of *C. jejuni* causing 50-70% of human infections (Adak *et al.*, 1995; Allos, 2001). That said, *C. jejuni* infestation of pet birds such as parrots and finches has a high morbidity and mortality rate resulting from anorexia, weight loss, lethargy, hepatitis and associated yellow diarrhoea (Young & Mansfield, 2005).

1.8.2 *Campylobacter* Reactive Arthritis or Reiter's syndrome

Reactive arthritis (ReA), oftentimes referred to as Reiter's syndrome, refers to the development within 2-4 weeks of sterile inflammatory arthritis as a sequel to a distant infection, most commonly gastrointestinal or genitourinary infections involving *Campylobacter*, *Salmonella*, *Yersinia*, *Shigella* and *Chlamydia* (Hannu *et al.*, 2006; Hill Gaston & Lillicrap, 2003). In the strictest sense, Reiter's syndrome is characterized by a triad of arthritis, conjunctivitis, and urethritis, but in reality not all three symptoms occur in all affected individuals due to the post-infectious nature of the onset of the arthritis and the two terms are used interchangeably (Dworkin *et al.*, 2001; Hill Gaston & Lillicrap, 2003).

ReA has been classified as part of the group of spondyloarthritide (SpA) diseases that share common features such as an association with HLA-B27,

the absence of rheumatoid factor, a tendency towards family aggregation and the typical non-articular manifestations of urethritis, iritis, conjunctivitis, enthesopathy (McColl *et al.*, 2000). This group of SpA includes ankylosing spondylitis (AS), sacroiliitis, oligoarthritis, psoriasis arthropathy, and inflammatory bowel disease-associated arthritis (Rudwaleit *et al.*, 2001).

1.8.2.1 Clinical Features of *Campylobacter* Reactive Arthritis

Reactive arthritis typically involves inflammation of one joint (monoarthritis) or four or fewer joints (oligoarthritis), preferentially affecting those of the lower extremities; the pattern of joint involvement is usually asymmetric (Rudwaleit *et al.*, 2001). Inflammation is common at enthuses (places where ligaments and tendons attach to bone), especially the knee and the ankle (Rudwaleit *et al.*, 2001).

1.8.2.2 Pathogenesis of *Campylobacter* Reactive Arthritis

As indicated earlier, there exists an association between reactive arthritis and the human leukocyte antigen (HLA) B27 genotype, the major histocompatibility complex in humans (Hill Gaston & Lillicrap, 2003). These proteins displayed on the surfaces of nucleated somatic cells with high concentrations in white blood cells, are thought to affect the elimination of the infecting bacteria or an individual's immune response, thus, becoming a predisposing factor in reactive arthritis (Barth & Segal, 1999). Although HLA-B27 is not responsible for the initial predisposition to the infection itself, it is believed to increase the risk of developing arthritis that is more likely to be severe and prolonged, a risk that is slightly greater for *Salmonella* and *Yersinia*-associated arthritis than with *Campylobacter* (Hill Gaston & Lillicrap, 2003).

1.8.3 Guillain Barré Syndrome

Guillain-Barré Syndrome (GBS) is a debilitating autoimmune disorder of the peripheral nervous system that is characterized by a symmetrical ascending paralysis (Boon *et al.*, 2006). Evolving over a period of several days to weeks it has become a clearly recognised sequela of *C. jejuni* enteritis, and is the

inducing antecedent infection in approximately 15% and 30% of cases of GBS in the UK and the US respectively (Nachamkin, 2001; Smith, 2002; Tam *et al.*, 2003).

1.8.3.1 Clinical Features of Guillain Barré Syndrome

The illness initially presents as weakness in the lower limbs that may spread to the upper limbs and the face in an ascending order, along with complete loss of deep tendon reflexes (Boon *et al.*, 2006).

Frequently, the lower cranial nerves may be affected which results in bulbar weakness and its accompanying dysphagia and respiratory difficulties that may warrant hospitalization, with nearly 20-30% requiring ventilatory assistance (Nachamkin *et al.*, 1998b). Sensory loss, which usually takes the form of loss of proprioception (position sense) is a common finding, alongside deep aching pain in the weakened muscles akin to overexercising (Boon *et al.*, 2006). There may be bladder dysfunction in severe cases but this is usually transient.

Although GBS is largely self-limited and the progressive weakness may be followed by partial or complete recovery, about 15-20% of patients may be left with permanent neurologic deficits (Hiraga *et al.*, 2005; Hughes & Cornblath, 2005). Two forms of treatment exist, namely, the administration of high-dose intravenous immunoglobulins (IV-Ig) or plasmapheresis, which involves the removal of patient's blood for separation of cellular and plasma components and re-administration of the cellular components back into the patient in artificial plasminate (Nachamkin *et al.*, 1998a).

Based on the target of the immune response, GBS could be classified into three groups, namely, an acute inflammatory demyelinating polyneuropathy (AIDP) which is the common type, a more severe variant involving both motor and/or sensory fibres called acute motor axonal neuropathy/acute motor-sensory axonal neuropathy (AMAN/AMSAN) or a milder form that involves only sensory fibres called the Miller-Fisher syndrome (MFS) (Nachamkin *et al.*, 1998a). The MFS manifests as a descending paralysis in

the reverse order of AIDP and is characterized by an acute onset of ataxia, areflexia and ophthalmoplagia (Davidson & Abbott, 1987).

1.8.3.2 *C. jejuni* Infection and Guillain Barré Syndrome

Although GBS had long been known to be an important sequel of many infections, especially viral upper respiratory infections, it was not until 1982 that the first case of its association with *Campylobacter* was reported in a 45-year old man who developed severe GBS following *Campylobacter* enteritis (Rhodes & Tattersfield, 1982). Several reports later described cases in which *C. jejuni* infection has been the preceding infection in GBS (Hagensee *et al.*, 1994; Isaacson, 1998; Mishu *et al.*, 1993; Rees *et al.*, 1995; Speed & Kaldor, 1985). New cases of GBS that could be directly linked to an infection with *C. jejuni* continue to be reported (Kopyta & Wardak, 2008).

Several studies that have analysed antecedent infections to cases of GBS have concluded that *C. jejuni* plays a major role in the onset of disease and that *C. jejuni*-associated GBS can even be more severe and more likely to be of the AMSAN variant (Sinha *et al.*, 2007; Tam *et al.*, 2006b; Yuki & Koga, 2006). Again, considering the time course of *C. jejuni* infection in relation to the time of onset of the neurological symptoms of GBS, it is obvious that immunopathogenic mechanisms were in play (Nachamkin *et al.*, 1998a).

Other studies have also demonstrated that patients with the Guillain-Barré syndrome are more likely than controls to have serologic evidence of *C. jejuni* infection in the weeks before the onset of neurologic symptoms (Ho *et al.*, 1995; Mishu *et al.*, 1993). In a case-control study by Sinha and co-workers in which they sought to confirm the association between campylobacteriosis and GBS using both culture and PCR diagnostics, it was observed that 19% of the GBS subjects were positive for *C. jejuni* whereas none of the control subjects was (Sinha *et al.*, 2004). In another study in Japan that spanned a period of 13 years involving 378 hospitals and 1,049 sufferers of GBS, culture results of 11% were positive for *C. jejuni* whereas serotyping showed further that there is a high prevalence of HS:19 strain in

AIDP as opposed to the prevalence of HS:02 in MFS (Takahashi *et al.*, 2005). Again, in Kuwabara's study using ELISA to measure *C. jejuni* antibodies he observed that 14% of 159 GBS sufferers were positive for *C. jejuni* (Kuwabara *et al.*, 2004). Tam *et al.*, estimated that 15% of all 157 GBS cases reported between 1 April 2000 and 31 March 2001 in England were attributable to symptomatic *C. jejuni* infection (Tam *et al.*, 2003). Again, using the General Practice Research Database, he estimated the incidence of GBS in a cohort of patients presenting with *Campylobacter* enteritis to be 1.17/1000 person-years, a rate 77 times greater than that in the general population (Tam *et al.*, 2006b).

1.8.3.3 Anti-ganglioside Antibodies and Guillain Barré Syndrome

Representing nearly 10% of the total lipid content of the peripheral and the central nervous systems, gangliosides are a heterogeneous family of glycosphingolipids with one or more sialic acid(s) linked to an oligoglycosyl backbone attached to a ceramide base (Mocchetti, 2005). Synthesised in neuronal somata, they are transported to specific sites of neuronal function such as synapses and nodes of Ranvier (Nachamkin *et al.*, 1998a).

The number of sialic acid residues in a ganglioside is used to classify the different species, with one sialic acid designated by M (monosialo), two by D (disialo), giving rise to names such as GM1 (monosialotetrahexosylganglioside), GM2 and GM3 (Mocchetti, 2005). Ganglioside localization studies have shown that GM1-like epitopes are concentrated at the nodal regions and the paranodal myelin loops whereas GM1 are abundant in the motor nerve myelin of the human sensory and motor nerves (Ogawa-Goto *et al.*, 1992; Sheikh *et al.*, 1998).

Again, myelin-associated glycoprotein occurring on the adaxonal surface of Schwann cells are known to bind to the axolemma through GT1b and GD1a (Yang *et al.*, 1996).

The identification of antibodies to these gangliosides in both AMAN and AIDP patients has been unequivocal suggesting the involvement of anti-ganglioside antibodies in the pathogenesis of GBS (Ilyas *et al.*, 1992;

Kornberg *et al.*, 1994; Pestronk *et al.*, 1994a). Although less than 50% of Chinese AMAN sufferers have been found to be positive for IgG anti-GM1, it is believed that its identification is more specific for AMAN cases than it is in AIDP cases and is even rarely found in other anomalies lending further support to this theory (Ho *et al.*, 1995; Kornberg *et al.*, 1994).

Furthermore, the identification of IgG and complement in the nodal region as well as the paranodal myelin loops of autopsied AMAN patients is more than enough proof of the role of anti-ganglioside antibodies in the pathogenesis of this debilitating disease (Griffin *et al.*, 1996; Hafer-Macko *et al.*, 1996).

1.8.3.4 Is GBS the Result of Molecular Mimicry?

Following the strong epidemiological data that linked GBS to previous *C. jejuni* infection, scientists were eager to elucidate the association and in 1993 Yuki and co-workers reported that their gas-liquid chromatography-mass spectrometric analysis of acute-phase sera from patients with GBS after *C. jejuni* infection has shown that the purified LOS contained Gal, GalNAc, and NeuAc [see Table 1.2], which are sugar components of GM1 ganglioside (Yuki *et al.*, 1993).

The following year they further isolated *C. jejuni* from 2 patients with Miller-Fisher syndrome subsequent to enteritis and then extracted and separated crude lipo-oligosaccharide fractions from the bacteria by thin-layer chromatography. Using monoclonal antibodies to GQ1b ganglioside (GMR13 and 7F5) they showed that both lipo-oligosaccharide fractions reacted to the anti-GQ1b antiganglioside antibodies, indicating that the lipo-oligosaccharides bear the GQ1b epitope (Yuki *et al.*, 1994b).

These were the first reports and probably the first evidence of molecular mimicry between neural tissue components and the often associated antecedent infectious agents of AIDP and MFS. A further work illustrated that anti-GM1 and anti-GD1a antibodies reacted with the LOSs of serotypes HS:01, HS:04, and HS:19 (Yuki *et al.*, 1994a).

Ganglioside Mimic	Structure
GM1	Gal (β1-4)GalNAc(β1-4)Gal (β1-4)Glc(β1-4)-Ceramide (α2-3) NeuNAc
GD1a	Gal (β1-4)GalNAc(β1-4)Gal (β1-4)Glc(β1-4)-Ceramide (α2-3) (α2-3) NeuNAc NeuNAc
GD1b	Gal (β1-4)GalNAc(β1-4)Gal (β1-4)Glc(β1-4)-Ceramide (α2-3) NeuNAc (α2-3) NeuNAc
GT1a	Gal (β1-4)GalNAc(β1-4)Gal (β1-4)Glc(β1-4)-Ceramide (α2-3) (α2-3) NeuNAc NeuNAc (α2-8) NeuNAc
GT1b	Gal (β1-4)GalNAc(β1-4)Gal (β1-4)Glc(β1-4)-Ceramide (α2-3) (α2-3) NeuNAc NeuNAc (α2-8) NeuNAc
GQ1b	Gal (β1-4)GalNAc(β1-4)Gal (β1-4)Glc(β1-4)-Ceramide (α2-3) (α2-3) NeuNAc NeuNAc (α2-8) (α2-8) NeuNAc NeuNAc
GM2	GalNAc(β1-4)Gal (β1-4)Glc(β1-4)-Ceramide (α2-3) NeuNAc
GD3	Gal (β1-4)Glc(β1-4)-Ceramide (α2-3) NeuNAc (α2-8) NeuNAc
GM4	Gal (β1-4)-Ceramide (α2-3) NeuNAc

Table 1. 2 | Structures of different gangliosides with terminal saccharide structures. These are the reported variants for isolates of *C. jejuni* (Courtesy: Nachamkin *et al.* 1998).

In 1994, a further collaborative work between Aspinall and Penner elucidated the structure of the core oligosaccharide regions of *C. jejuni* strains HS:04, and HS:19 and concluded that they mimic human gangliosides in structure (Aspinall *et al.*, 1994a; Aspinall *et al.*, 1994b). In that same year, other reports of the immunological mimicry of *C. jejuni*

were published sparking intense research into *C. jejuni*-associated GBS (Kornberg *et al.*, 1994; Pestronk *et al.*, 1994a; Pestronk *et al.*, 1994b).

Sensitization of female guinea pigs with lipo-oligosaccharides from serotype HS19 of *C. jejuni* has been shown to result in increased titres of anti-GD3 antibodies and subsequently cause decreases of nerve conduction velocity and conduction block (Shu *et al.*, 2007). These anti-GD3 antibodies can also block transmission in neuromuscular junctions of spinal cord-muscle cell cocultures and thus are thought to be involved in the development of Guillain-Barré syndrome (Seigo Usuki, 2006). Again, axonal degeneration has also been observed with the expression of ganglioside-mimicking structures of *C. jejuni* lipooligosaccharides and is considered essential for the induction of antiganglioside antibodies that lead to Guillain-Barré syndrome (Xiao Mei Shu, 2006).

Now it is known that *C. jejuni* sialyltransferase gene *cst-II*, which functions in the biosynthesis of ganglioside-like LOSs, determines the transferase activity and that a mutation of asparagine to threonine at amino acid position 51 (Asn-51-Thr) leads to the expression of GM1 and GD1a epitopes, whereas GBS patients infected with *cst-II* (Thr51) strains have anti-GM1 or anti-GD1a IgG antibodies (Yuki, 2007; Yuki & Kuwabara, 2007).

1.9 Management and Control of *C. jejuni* Infection

C. jejuni infections are usually self-limiting and treatment may not be necessary except in severe diarrhoeal and/or vomiting cases where replacement of fluid and electrolytes may be warranted (Boon *et al.*, 2006; Poly & Guerry, 2008; Zilbauer *et al.*, 2008).

However, the long-term consequences of infections caused by this organism can be potentially dangerous for some individuals. Bacteraemia, toxic megacolon, haemolytic uraemic syndrome, Reiter's syndrome, Guillain-Barré syndrome, Miller-Fisher syndrome and reactive arthritis (ReA) are the most serious known long-term consequences of *C. jejuni* infections (Nachamkin, 2002).

1.9.1 Chemotherapy

On this backdrop, it is imperative that antimicrobial treatment is instituted to hasten the clearance rate of the organism and reduce the period of asymptomatic shedding, although this does not lead to immediate cessation of the disease process itself (Janssen *et al.*, 2008). Again, antibiotics are recommended in severe cases or in cases where the disease becomes protracted with prolonged enteritis and/or septicaemia (Zilbauer *et al.*, 2008).

The first choice of antibiotic for confirmed cases of *C. jejuni* infection is the macrolide antibiotic, erythromycin, whereas ciprofloxacin, a fluoroquinolone, has been used in suspected travellers' diarrhoea (Boon *et al.*, 2006). Nalidixic acid, doxycycline as well as tetracycline are also effective in the treatment of *Campylobacter* enteritis (Boon *et al.*, 2006). It has recently been shown that the newer macrolides such as azithromycin and clarithromycin are equally effective and that the efficacy of azithromycin compares with fluoroquinolones (Allos, 2001; Sanders *et al.*, 2007).

In serious bacteraemia and other systemic infections due to *Campylobacter*, intravenous aminoglycosides are recommended (Aarestrup & Engberg, 2001). Meropenem has also been successfully used in the treatment of a severe case of *Campylobacter* meningitis (Burch *et al.*, 1999).

1.9.2 Education

The main goal of the public health physician is in reduction of transmission through public education of the dangers of unpasteurised milk, the necessity of proper food handling practices, an awareness of the potential for cross-contamination in the kitchen, the importance of thoroughly cooking meat and the vigilant attention to personal hygiene with particular regard to hand-washing before eating, after contact with pets and after using the toilet (Allos, 2001; Crushell *et al.*, 2004; Humphrey *et al.*, 2001).

1.9.3 Farm-To-Fork Strategies

Despite the complex nature of the transmission dynamics, coupled with the relative contribution of different sources in *C. jejuni* infections it is still

reasonably clear that food-producing animals such as poultry, cattle and sheep remain the important routes through which organisms could enter the food chain (Allen *et al.*, 2007; Corry & Atabay, 2001; Gormley *et al.*, 2008; Nichols, 2005). This means that the incidence of campylobacteriosis could be dramatically reduced by enhanced on-farm biosecurity or preventing food-borne transmission.

The most promising control strategy suggested by leading scientists is "scheduled processing" which involves keeping colonised and non-colonised flocks apart during slaughter (Wagenaar *et al.*, 2006). The *Campylobacter*-free meat can be supplied as fresh meat to the meat market, whilst the *Campylobacter*-infected meat is treated to reduce the *Campylobacter* concentration before supplying to the market. In a study in Belgium that sought to obtain insight into the effect of food practices on *Campylobacter* transmission, it was reported that unskinned chicken meat preparations had an almost 2.2-fold probability of being positive for *Campylobacter*, while chicken meat preparations made from frozen meat, or partly containing pre-frozen meat, had a significantly lower probability of being positive for *Campylobacter* (Sampers *et al*., 2008).

Chlorate- and nitro-containing salts employed in the feed and water of various farm animals have proven efficacious in reducing concentrations of *Campylobacter* as well as *E. coli* and *Salmonella* in the gut of cattle, sheep, swine, and poultry (Anderson *et al.*, 2005). This technology is based on the ability of Enterobacteriaceae to catalyze the reduction of chlorate to the lethal chlorite. Again, the use of caprylic acid supplement in feeds has also been shown to be effective in reducing *C. jejuni* colonisation of chickens (Solis de Los Santos *et al.*, 2008).

C. jejuni colonisation of chicken can be inhibited by feeding freshly hatched chicken with some strains of *Campylobacter* in what is referred to as competitive exclusion (Barrow & Page, 2000; Zhang *et al.*, 2007a). The first commercially available competitive exclusion product, broilact, has proven to be efficacious in preventing colonisation of several enteric pathogens including *Campylobacter* (Hakkinen & Schneitz, 1999). Whereas some

scientists have argued that this effectiveness of competitive exclusion is dependent on the bird strain, others also argue about the safety of this method (Laisney *et al.*, 2004; Wagner, 2006).

Recently, the experimental treatment of *Campylobacter* colonized broiler flocks with bacteriophage has proven effective in the control of transmission at the poultry farm level (Bigwood *et al.*, 2008; Havelaar *et al.*, 2007).

1.9.4 Vaccines

Although the use of vaccine in the prevention of transmission has been an option in the minds of many scientists, the setback has been the theoretical risk of triggering GBS in recipients (Crushell *et al.*, 2004). Several attempts have been made using previously known approaches for vaccine development, such as the demonstration by Rollwagen *et al.* of the immune response and protective effect following the oral administration of killed *Campylobacter* in mice (Rollwagen *et al.*, 1993). Similarly, Baqar and co-workers demonstrated the immunogenicity and protective efficacy of a prototype *Campylobacter* whole-cell killed vaccine in mice and non-human primates (Baqar *et al.*, 1995a; Baqar *et al.*, 1995b). Guerry and co-workers described the development of a *recA* mutant *C. jejuni* for inclusion in live-attenuated vaccines (Guerry *et al.*, 1994). Recently, it was demonstrated that feeding ferrets with inactivated whole cell *C. jejuni* led to prevention of disease (Burr *et al.*, 2005). Subunit vaccine, containing truncated FlaA flagellin of *Campylobacter coli* VC167 was shown to induce a protective sIgA when administered intranasally (Lee *et al.*, 1999).

In a rather twisted development, a novel approach in which a live-attenuated vaccine of *Salmonella typhimurium* harbouring PEB1 of *C. jejuni* was tested, significant levels of anti-PEB serum IgG were induced, but no protection was observed when recipients were challenged with oral administration of *C. jejuni* (Sizemore *et al.*, 2006).

Through proteomics, Prokhorova and co-workers have recently identified eight *C. jejuni* surface proteins, which showed high protective efficacies following expression in *E. coli* and immunization of mice (Prokhorova *et al.*,

2006). With all these promising developments, the question that still remains is who should be vaccinated? Vaccination of poultry is probably a more likely prospect.

1.10 *Campylobacter* and Antibiotic Resistance

Antibiotic resistance in pathogenic bacteria has always been a threat to both the clinician and the patient, and threatens to curtail the euphoria that came with the discovery of these drugs in the early 1900s. Bacterial antibiotic resistance evolves over time through the acquisition of chromosomal mutations that are passed on to daughter cells (Cirz *et al.*, 2005). *Campylobacter* species are known to possess the genetic machinery for natural transformation and conjugation; hence, acquired antibiotic resistance genes are easily transferred between strains (Jeon & Zhang, 2007; Jeon *et al.*, 2008; Kim *et al.*, 2008).

The re-annotation of the *C. jejuni* NCTC11168 genome has revealed the presence of 12 antibiotic resistance genes; three of them have been described as multidrug efflux pump CmeABC, another three predicted to encode for an efflux pump CmeDEF, two additional putative efflux pumps Cj0035c/Cj1687, a putative periplasmic β-lactamase Cj0299, a putative acetyltransferase Cj1715, and two hypothetical proteins Cj1296/ Cj1297 (Gundogdu *et al.*, 2007).

The result of acquisition of these antibiotic resistance genes is the development of one or more of several mechanisms that lead to reduced uptake of the antibiotic into the cell, active efflux of the antibiotic from the cell, modification of the antibiotic target to eliminate or reduce binding, overproduction of the antibiotic target (titration), sequestration of the antibiotic by protein binding, binding of specific immunity proteins to the antibiotic, and inactivation of the antibiotic by enzymatic modification (Davies, 1994). Three of these mechanisms appear to be involved in antibiotic resistance in *C. jejuni*, namely, efflux of the antibiotic, mutation of the target site, and protection of the target site (Alfredson & Korolik, 2007; Taylor & Tracz, 2005).

Three main groups of antibiotics are important in *Campylobacter* treatment and control, namely, the fluoroquinolones (ciprofloxacin, nalidixic acid, etc), the macrolides (erythromycin, azithromycin, etc), and the tetracyclines (Allos,

2001; Coker *et al.*, 2002). Resistance to these antibiotics as well as other antibiotics that could be used in the treatment of *Campylobacter* infections has become a major public health concern globally for some time now (Nachamkin *et al.*, 2002). *C. jejuni* resistance to various antibiotics including fluoroquinolones, macrolides and lincosamides, tetracyclines, aminoglycosides, chloramphenicol, cotrimoxazole, tylosine, ampicillin and other β-lactams has been reported (Moore *et al.*, 2006). Of major importance in the emergence of resistance to these antibiotics by *Campylobacter* is their usage in food-producing animals and the subsequent transmission of these antibiotic resistant *Campylobacter* strains to humans (Alfredson & Korolik, 2007).

1.10.1 Fluoroquinolone Resistance

DNA gyrases and/or topoisomerase IV are the sole targets of fluoroquinolone action where binding results in altered enzyme conformation, causing fluoroquinolone-mediated complexes with the two enzymes to impede DNA re-ligation after cleavage during DNA replication, transcription, recombination and maintenance of genomic stability (Drlica, 1999; Tse-Dinh, 2007). Resistance to fluoroquinolone is mediated by single nucleotide polymorphisms of Ala-70, Thr-86, or Asp-90 within the quinolone resistance-determining region (QRDR) of the *gyrA* sequence encoding the enzyme DNA gyrase (Drlica, 1999; Hooper, 2001; Luo *et al.*, 2003; Payot *et al.*, 2006). Thr-86-Ile substitution in the DNA Gyrase A protein is the commonest mutation and is associated with a higher level of resistance to ciprofloxacin and nalidixic acid in both *C. jejuni* and *C. coli* (Jesse *et al.*, 2006; Kinana *et al.*, 2006).

The involvement of the multidrug efflux pump, CmeABC, encoded by the *cmeABC* operon (Figure 1.10), in *C. jejuni* resistance to fluoroquinolone as well as other antibiotics has also been well documented, paving way for its inhibition as a promising approach in curbing antibiotic resistance of *Campylobacter* in both humans and animals (Berggard *et al.*, 2002; Martinez & Lin, 2006; Yan *et al.*, 2006). Despite the existence of several efflux pumps among bacteria, the CmeABC is the only well-documented efflux pump in

Campylobacter that is known to mediate antibiotic drug resistance (Ge *et al.*, 2005; Luo *et al.*, 2003). A mention has been made of a second efflux pump, CmeDEF, which is described as being uniformly distributed among multiple strains of fluoroquinolone resistant *C. jejuni* but its involvement in antimicrobial resistance is yet to be proven (Lin *et al.*, 2005b; Payot *et al.*, 2006). Recently, a mutation frequency decline (*mfd*) gene, which encodes a transcription-repair coupling factor involved in DNA repair, was found to promote the emergence of spontaneous fluoroquinolone resistant mutants and the development of fluoroquinolone resistance in *C. jejuni* in contrast to its previously known function in the decline of mutation frequency in other bacterial organisms (Han *et al.*, 2008; Park *et al.*, 2002b; Selby & Sancar, 1993).

Fluoroquiniolone resistance in campylobacters has been noted since the early 1990s (Acar *et al.*, 1993; Adler-Mosca & Altwegg, 1991; Endtz *et al.*, 1990; Nachamkin *et al.*, 2002; Rautelin *et al.*, 1991; Reina & Alomar, 1990). In his review, Engberg noted that due to the then increased incidences of fluoroquinolone resistance among *Campylobacter* isolates erythromycin and other macrolides should remain the drugs of choice across the globe (Engberg *et al.*, 2001).

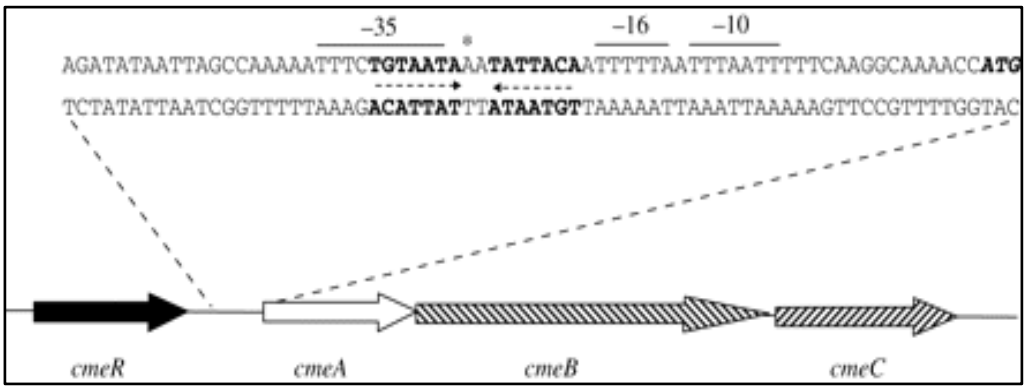

Figure 1. 10 | Genomic organization of the operon coding for CmeABC efflux system. The figure also shows features of the intergenic region between *cmeR-cmeABC*. ORFs are indicated by boxed arrows. The start codon (ATG) of the *cmeA* gene is in bold italics, and the sequences that form the inverted repeats are highlighted in bold and indicated by dashed arrows. The predicted –10, –16, and –35 regions of P_{cmeABC} are overlined. The nucleotide deleted in a laboratory-selected efflux mutant, showing an over-expression of CmeABC efflux system, is indicated by an asterisk [Courtesy: Gibreel & Taylor, 2006].

In a survey in the United Kingdom, foreign travel was strongly associated with the acquisition of ciprofloxacin resistant *C. jejuni* (Collaborators, 2002).

Likewise, in 2006 Norstrom reported increased ciprofloxacin resistance among *Campylobacter* isolates from persons returning from foreign travel than among isolates from local broilers (Norstrom *et al.*, 2006). Since the recognition of antibiotic resistant *Campylobacter* among humans, the efficacy of ciprofloxacin is far discouraging than that of erythromycin (Wardak *et al.*, 2007). Nevertheless, erythromycin resistance has also been reported in isolates from humans and animals, as well as food (Saenz *et al.*, 2000).

The situation has not been much different in isolates from cattle, pigs or poultry, with reports of quinolone resistance as high as 89.3% among cattle in a Washington state farm (Bae *et al.*, 2005; Humphrey *et al.*, 2005; Taylor *et al.*, 2008).

1.10.2 Macrolide Resistance

The mechanism of action of the macrolides is based on inhibition of bacterial protein synthesis whereby the antibiotic binds reversibly to the 50S subunit of the bacterial ribosome, to inhibit translocation of peptidyl tRNA (Nakajima, 1999). In high antibiotic concentrations this action, which is mainly bacteriostatic, can also be bactericidal. Macrolides tend to accumulate within leukocytes, and are therefore transported to the site of infection (Nakajima, 1999). Three main mechanisms of macrolide resistance in bacteria have been described, namely, (i) target modification mediated by rRNA methylases, specific point mutations in the 23S rDNA and/or alteration of the 50S ribosomal proteins, (ii) enhanced efflux of the antibiotic from the organism, and (iii) macrolide hydrolysis by esterase or phosphorylation mediated by the genes, *ere* and *mph*, respectively leading to inactivation/degradation of the antibiotic (Gibreel & Taylor, 2006; Nakajima, 1999; Payot *et al.*, 2006).

Although a previous study by Yan *et al.*, ruled out the involvement of any of the above three mechanisms in macrolide resistance in *Campylobacter*, a recent study by Caldwell and co-workers has come to confirm the role of the above in erythromycin resistance (Caldwell *et al.*, 2008; Yan & Taylor, 1991).

Caldwell *et al.* reported that point mutations in domain V of the 23S rRNA target gene of A2074C or A2075G akin to A2058G or A2058C in *E. coli* (Figure 1.11) correlated well with high erythromycin resistance and inactivation of CmeABC efflux pump led to increased sensitivity to erythromycin (Caldwell *et al.*, 2008). This serves as further evidence in support of previous studies that demonstrated the involvement of CmeABC efflux pump in both intrinsic and acquired resistance to erythromycin among *C. jejuni* as well as *C. coli* strains (Corcoran *et al.*, 2006; Mamelli *et al.*, 2005; Payot *et al.*, 2004a). It is believed that *cmeB* over-expression may be associated not just with erythromycin resistance, but rather a multidrug resistance phenotype of *C. jejuni* (Payot *et al.*, 2006).

Again, Gibreel *et al.* demonstrated that there was no significant association between macrolide resistance and alterations in the 50S ribosomal subunit proteins L4 and L22, which are portions of the exit tunnel within the 70S ribosome, but a recent study by Caldwell *et al.* asserts the reverse as true (Caldwell *et al.*, 2008; Gibreel *et al.*, 2005). Again, Corcoran *et al.* has described the presence of the characteristic A2075G polymorphism alongside one or more amino acid substitutions in both L4 and L22 in 13 *Campylobacter* isolates (Corcoran *et al.*, 2006). These findings suggest that the mechanism of macrolide resistance is multifactorial and that the involvement of each of the above factors may be independently selected by macrolide levels, with one factor modifying or increasing the effect of the other.

Macrolide, specifically erythromycin, resistance in *Campylobacter* has been reported since the 1970s (Brunton *et al.*, 1978; Karmali *et al.*, 1980; Walder & Forsgren, 1978). However, the increasing incidence of macrolide resistance in *Campylobacter* in both developed and developing countries, with the situation worsening in developing countries, makes it a public health concern (Engberg *et al.*, 2001; Moore *et al.*, 2006; Payot *et al.*, 2004b).

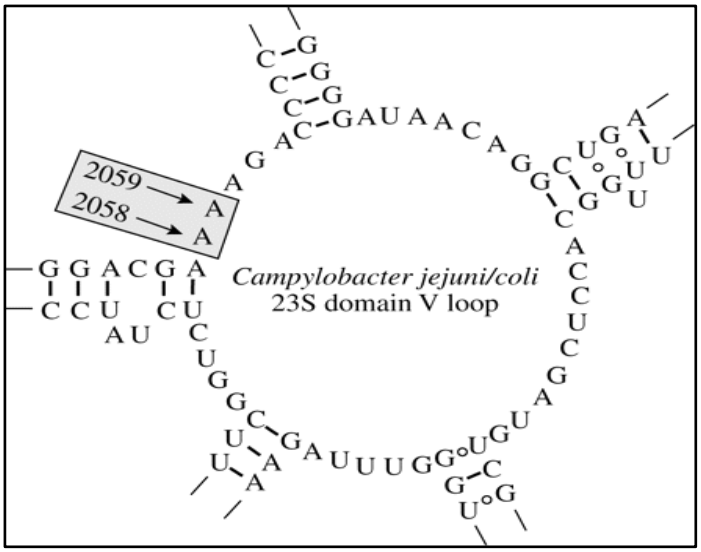

Figure 1. 11 | The peptidyl-transferase loop in domain V of the *C. jejuni/coli* 23S rRNA. This is the secondary structure showing positions 2058 and 2059 (based on *E. coli* numbering) as the location of the mutations associated with erythromycin resistance [Adopted with permission from Gibreel & Taylor, 2006; *J Antimicrob Chemother*, 58(2):243-55].

1.10.3 Tetracycline Resistance

There are two main ways through which tetracyclines produce their antibiotic effect on bacteria; the atypical tetracyclines such as anhydrotetracycline produce their effect by disrupting bacterial cell membranes, whereas the typical tetracyclines such as tetracycline and minocycline produce their effect by binding to bacterial ribosome to inhibit binding of the aminoacyl transfer RNA (aa-tRNA) to the ribosomal A site, thereby preventing the elongation phase of protein synthesis (Chopra & Roberts, 2001; Oliva *et al.*, 1992; Rasmussen *et al.*, 1991).

Bacterial resistance to tetracycline is achieved through the disruption of the ribosomal binding site of tetracycline by ribosomal protection proteins (RPPs) and through efflux pumps (Chopra & Roberts, 2001; Connell *et al.*, 2002). There are, however, other mechanisms through which resistance could also be achieved such as enzymatic degradation of tetracycline as described in *Bacteroides* and mutations in the 16S rRNA gene as described in *H. pylori* and *Propionibacterium acnes* (Gerrits *et al.*, 2002; Speer *et al.*, 1992; Trieber & Taylor, 2002).

In both *C. jejuni* and *C. coli*, resistance to tetracycline is mediated by an RPP named Tet(O), which is encoded by a self-transmissible plasmid *tet(O)* gene (Aarestrup & Engberg, 2001). This gene, which is mainly located on a plasmid, may also be carried on the chromosome of *Campylobacter* strains lacking plasmids (Gibreel *et al.*, 2004b). An insertion element, IS*607**, akin to that found in *H. pylori*, has been reported in *tet(O)*-carrying plasmids in *C. jejuni*, prompting the belief that mobile genetic elements other than transmissible plasmids may be involved in the acquisition and transfer of *tet(O)* (Gibreel *et al.*, 2004a).

Tetracycline resistance in *C. jejuni* was first reported in the 1970s by Butzler *et al.*, and it still continues to be a major setback in the poultry industry as resistance among strains could reach as high as 100% in one production cycle (Butzler *et al.*, 1974; Luangtongkum *et al.*, 2008).

1.11 Aims and Objectives of Study

It is now generally accepted that bacterial pathogenesis is often dependent on the panoply of proteinaceous virulence factors which are either transported to the bacterial cell surface or released into the external environment (Finlay & Falkow, 1997; Wilson *et al.*, 2002). The interactions of these bacterial proteins with host cellular molecules, including the extracellular matrix proteins, are important in the pathogenesis of many bacterial infections (Casadevall & Pirofski, 2001; Patti *et al.*, 1994). Understanding the molecular basis of interactions between these proteins and host cells is necessary in understanding and controlling infections. Although previously identified adhesins and other virulence determinants of *C. jejuni* have added to our understanding of the pathogenesis of campylobacteriosis, our understanding of the molecular basis still lags behind that of other enteric pathogens (Poly & Guerry, 2008; Zilbauer *et al.*, 2008). Again, with the increasing incidence of campylobacteriosis around the globe and increasing antibiotic resistance, there is a need to fully understand the molecular basis of *Campylobacter* disease pathogenesis to enable us control these infections. One important approach to this lies in the inhibition of the initial adhesion and colonization, which requires an extensive knowledge of the adhesins involved.

It is highly likely that as yet unidentified *C. jejuni* surface proteins interact with host proteins in a number of ways which contribute to colonisation and pathogenesis. Indeed, over 20% of the 1,643 coding sequences of the *C. jejuni* NCTC11168 genome are still uncharacterized including 150 putative membrane proteins, 105 putative periplasmic proteins, over 50 putative surface structures and several uncharacterized secreted proteins, coupled with the fact that new information on previously characterized genes or proteins continues to emerge (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000).

This study seeks to screen the genome of *C. jejuni* NCTC 11168 to identify additional genes that may code for other adhesins to key components of host extracellular matrix molecules such as, collagen, fibronectin, fibrinogen, vitronectin, laminin and heparin sulphate, as well as host-secreted proteins such as lactoferrin/transferrin given that they are abundant and directly involved in

many bacterial processes in the ecological niche of *C. jejuni* in its human host (Patti *et al.*, 1994). One approach to finding such genes is to utilise phage display technology.

Molecular and proteomics approaches in the study of host-pathogen interactions, combined with host and pathogen driven epidemiological findings are important in elucidating the pathogenesis of *Campylobacter* infection for effective control strategies to be developed.

1.11.1 Main Objective

The main aim of this work was to identify *Campylobacter jejuni* surface proteins that may contribute to the pathogenesis of campylobacteriosis in its human host.

1.11.2 Specific Objectives

The specific objectives of this work were:

- To construct a phage display DNA library of *Campylobacter jejuni* NCTC11168. Such a library will provide an important resource for future research.
- To pan the resultant library against host cell protein ligand(s) known to be involved in the processes of *Campylobacter jejuni* pathogenesis, specifically lactoferrin and/or transferrin.
- To identify the *Campylobacter jejuni* DNA sequences responsible for the peptides that may bind to the host protein ligand(s) as stated above.
- To analyse the identified sequences based on available data for their possible roles in the pathogenesis of *Campylobacter* infection.
- To identify putative autotransporter proteins from the genome sequence of *Campylobacter jejuni* NCTC11168 using in silico analyses.
- To attempt expression of one of the identified proteins for further characterization in the future.

Chapter Two

Construction of Phage Display Library of *Campylobacter jejuni* NCTC11168

Identification of Host-pathogen Interacting Molecules of *Campylobacter jejuni* using Phage Display Technology and in silico Sequence Analysis

CHAPTER TWO

2.0 Construction of a Phage Display Library of *Campylobacter jejuni* NCTC11168

2.1 Introduction

The involvement of proteinaceous virulence factors, that are either transported to the bacterial cell surface or released into the external milieu, has long been generally accepted in the early mechanisms of bacterial pathogenesis (Finlay & Falkow, 1997). Understanding the molecular basis of the interactions between these proteins and host cells is not just necessary in understanding the disease but also in controlling infections. Phage display offers a molecular link between surface proteins and their genetic coding sequences, thereby allowing the experimental phenotype to be directly linked to an encapsulated genotype through sequencing (Wang *et al.*, 2008).

The superiority of phage display over traditional expression libraries in identifying virulence factors lies in the fact that the laborious screening procedure of the latter is replaced with affinity selection or panning, followed by amplification and repanning which results in enrichment of clones with the highest binding affinity to a specific ligand (Jacobsson & Frykberg, 2001b; Wang *et al.*, 2008).

This chapter describes the construction of a phage display library of *Campylobacter jejuni* NCTC11168, based on the filamentous phage M13. This library will be utilized to identify *C. jejuni* proteins that may be involved in binding to lactoferrin and fibronectin present in the host intestinal mucosa.

2.2 Phage Display Technology

First described in 1985 by GP Smith (Smith, 1985), phage display involves the incorporation of foreign DNA sequences into the genome of a filamentous phage resulting in the expression of the foreign peptide or protein in fusion with one of the coat proteins of the phage particle (Kay *et al.*, 2001). A viable wild-type phage particle expresses two kinds of coat proteins: the major and minor

coat proteins. The M13 coat is composed of 2,700 copies of the major coat protein pVIII encoded by its gene 8 (g8) and 5 copies each of four minor coat proteins (pIII, pVI, pVII, pIX) (Webster, 1996). Thus, a foreign peptide/protein in fusion with one of the coat proteins can be selected or "panned" for by allowing the phage particles to interact with selected ligands immobilized on an appropriate surface (Mullen *et al.*, 2006; Siegel, 2008).

Two types of phage display library exist, random peptide libraries (RPLs) and natural peptide libraries (NPLs). Whereas the peptides displayed in RPLs are encoded by synthetic degenerate oligonucleotide inserts (Cwirla *et al.*, 1990; Lundin *et al.*, 1996), NPLs display natural peptides encoded by random fragments of genomic DNA from selected organisms especially pathogenic ones (Azzazy & Highsmith, 2002; Kehoe & Kay, 2005). They are therefore preferred since they are more successful in eliciting specific antibody response to the native intact pathogen than those of RPLs and hence are useful in the identification of vaccine candidates and bacterial adhesins (Jacobsson *et al.*, 2003; Matthews *et al.*, 2002; Siegel, 2008).

2.3 The Filamentous Phage

Bacteriophages are viruses that infect a variety of Gram-negative bacteria using mainly pili as receptors, but can also use other surface molecules such as lipopolysaccharides, teichoic acid, outer membrane proteins or even flagella (Azzazy & Highsmith, 2002; Häusler, 2006). The Ff filamentous phage constitutes a type of bacteriophage which is rod-like or filament-shaped and infect *E. coli* strains that carry the F episome or sex pilus (Azzazy & Highsmith, 2002; Häusler, 2006). The best characterized filamentous phage are the M13, f1 and fd strains whose genome sequences have approximately 98% identity (Webster, 1996).

2.3.1 Structure of the M13 Virion

The wild-type M13 filamentous phage is a long cylindrical virion, about 930 nm in length and 6.5 nm in diameter, containing a covalently closed 6,407-base single-stranded DNA (ssDNA) genome encased in a protein coat as

depicted in Figure 2.1 (Kehoe & Kay, 2005; Webster, 1996). The genome consists of eleven genes encoding five structural proteins, which form the viral coat, and six non-structural proteins which are involved in DNA replication and assembly (Kehoe & Kay, 2005).

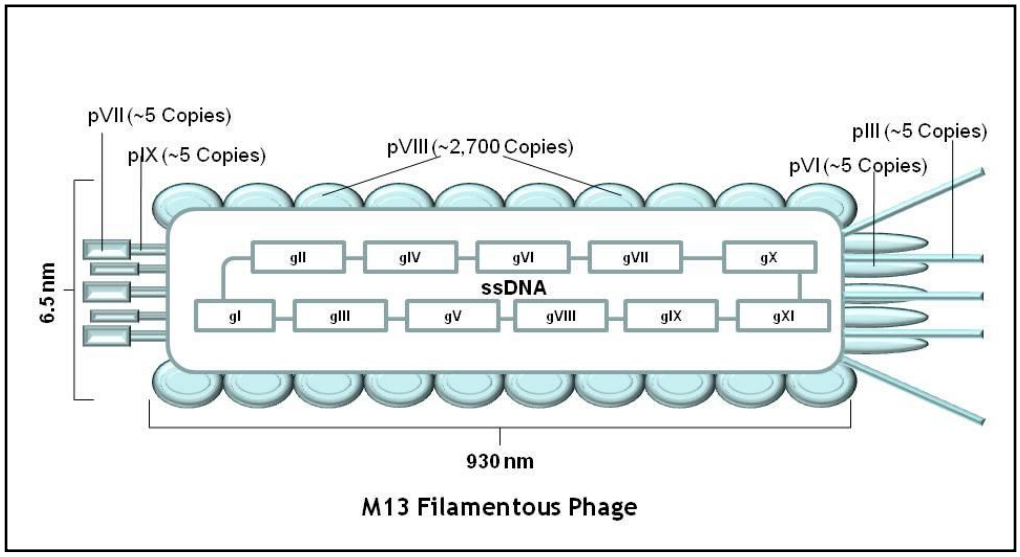

Figure 2. 1 | A Schematic Representation of the M13 Filamentous Phage. The dimensions, architecture, and copy numbers of each of the structural proteins are shown [Redrawn/Modified from Mullen *et al*, 2006 and Kehoe & Kay, 2005].

2.3.2 The Life Cycle of the M13 Virion

Unlike the lysis induced in bacteria following infection with lytic phage such as T4, infection of *E. coli* with the M13 filamentous phage induces a state in which the infected bacterium produces and secretes phage particles indefinitely without cell lysis albeit in reduced numbers over time (Azzazy & Highsmith, 2002). As depicted in Figure 2.2, the infection process is initiated by the attachment of pIII to the F pilus of a male *E. coli* strain such as *E. coli* TG1 (Azzazy & Highsmith, 2002; Kehoe & Kay, 2005). The pilus then retracts to bring the phage particle to the surface of the bacterium, with pIII binding through the *E. coli* membrane protein, TolA (Riechmann & Holliger, 1997). The phage particle then transfers its ssDNA genome into the host cell, with its outer coat fusing with the bacterial membrane (Nakamura *et al.*, 2003). The DNA replication enzymes of the host bacterium initiate conversion

of the phage's ssDNA genome into the double-stranded (ds) replicative form (RF), which then serves as a template for the replication of ssDNA and synthesis of M13's 11 proteins (Azzazy & Highsmith, 2002; Kehoe & Kay, 2005).

Assembly of phage progeny begins as phage proteins and ssDNA accumulate within the bacterial cell. The structural proteins pIII, pVI, pVII, pVIII, and pIX have been reported to insert into the bacterial cell membrane whilst pV forms complexes with ssDNA for packaging and extrusion through a pIV-gated pore in the bacterial cell membrane (Kehoe & Kay, 2005). The ssDNA is initially coated with nearly 2,700 copies of pVIII and 5 copies each of pVII and pIX at one end of the phage, whilst 5 copies each of pIII and pVI are incorporated at the opposite end as the phage is extruded (Feng *et al.*, 1997; Rakonjac & Model, 1998).

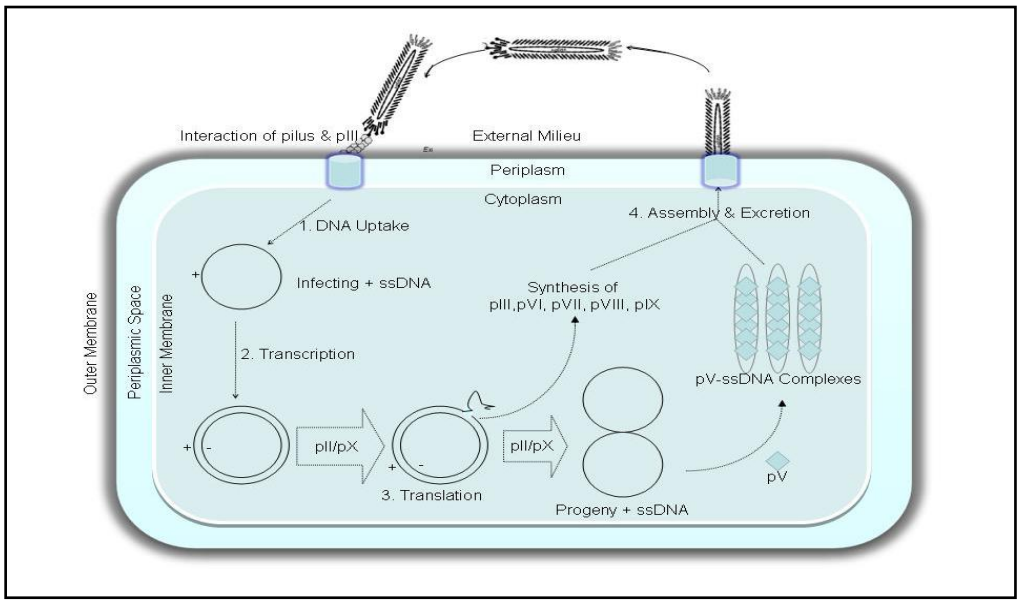

Figure 2. 2 | A Schematic Diagram of the Life Cycle of an M13 Filamentous Phage. Through the pIII coat protein, phage particle binds to the *E. coli* through the F pilus which retracts to bring the phage to the bacterial cell surface to interact with TolA. The ssDNA (+strand) viral genome is then injected into the bacterium (1), which is then converted to the dsDNA (RF) (2). This serves as a template for the synthesis of viral proteins and replication of ssDNA (3). Progeny components of the phage are assembled by pV and then extruded out of the bacterial cell without cell lysis [Redrawn/Modified from Mullen *et al*, 2006 and Kehoe & Kay, 2005].

Whilst all five coat proteins of the phage particle have been utilized in phage display technology, the most common practice has been to fuse foreign sequences to the amino terminus of pIII and pVIII (Kehoe & Kay, 2005).

Nevertheless, the possibility of peptide fusions to the carboxy termini of pVI, as well as an artificial pVIII and pIII have also been reported (Fuh & Sidhu, 2000; Hufton *et al.*, 1999; Weiss & Sidhu, 2000). Peptides or proteins in fusion with phage proteins are known to be completely exposed on the phage surface and could be present in each copy of its partner, thus, allowing interaction with other proteins (Kehoe & Kay, 2005).

2.4 Phage Cloning Vectors

It is clear from the life cycle of the M13 bacteriophage that viral DNA from the phage particle is single-stranded whereas that from an infected bacterium is double-stranded. Thus, dsDNA isolated from infected bacteria is usually used for cloning of a target DNA fragment, although cloning into ssDNA is also possible (Jacobsson & Frykberg, 2001b).

Most vectors available for exogenous expression of a foreign peptide or protein have utilized both g3 and g8 (Kehoe & Kay, 2005). Phage vectors that incorporate foreign DNA sequences into either g3 or g8 are termed type 3 or 8 respectively, as detailed in Figure 2.3 (Smith, 1993). Whereas pIII is known to tolerate a variety of proteins and short peptides, pVIII on the contrary, seems to be able to harbour only short inserts of five to six amino acids due probably to the closely packed nature of the viral surface (Greenwood *et al.*, 1991; Kishchenko *et al.*, 1994). Again, sequences may not be well-displayed on the surface of the phage due to defects in assembly which may ultimately affect viral stability and infectivity (Armstrong *et al.*, 1996). This latter problem is compensated for in two other groups of vectors.

In one group of vectors, an additional wild-type copy of the gene of interest is added to the recombinant genome. These are referred to as type 33 or 88 (Smith, 1993). Infection of bacterial cells with these phage vectors results in phage particles displaying both the wild-type and the fusion copies of pIII or pVIII (Figure 2.3).

Termed 3+3 or 8+8, the third group of vectors contains the so-called phagemid-based vectors. These are designed such that they carry only one copy of the gene of interest, and do not possess the viral machinery needed for the synthesis and packaging of new bacteriophages (Smith, 1993). Bacterial cells carrying these phagemids therefore require the assistance of an M13 helper phage, which carries the full machinery for the synthesis of the phage coat but is defective in replication, in order to secrete phage particles that will display both wild-type and the pIII or pVIII fusion protein of interest (Figure 2.3 f & g). This process is termed "phage rescue".

In contrast to the multivalency of the 8+8 vector system, the displayed peptide in the 3+3 vector system is mainly monovalent which is important in eliminating the avidity in protein-protein interactions. The peptide concentration in both systems is diluted by the presence of the wild-type copies contributed by the helper phage in spite of the fact that it is advantageous when the resultant phage particles are not stable or infectious (Armstrong *et al.*, 1996).

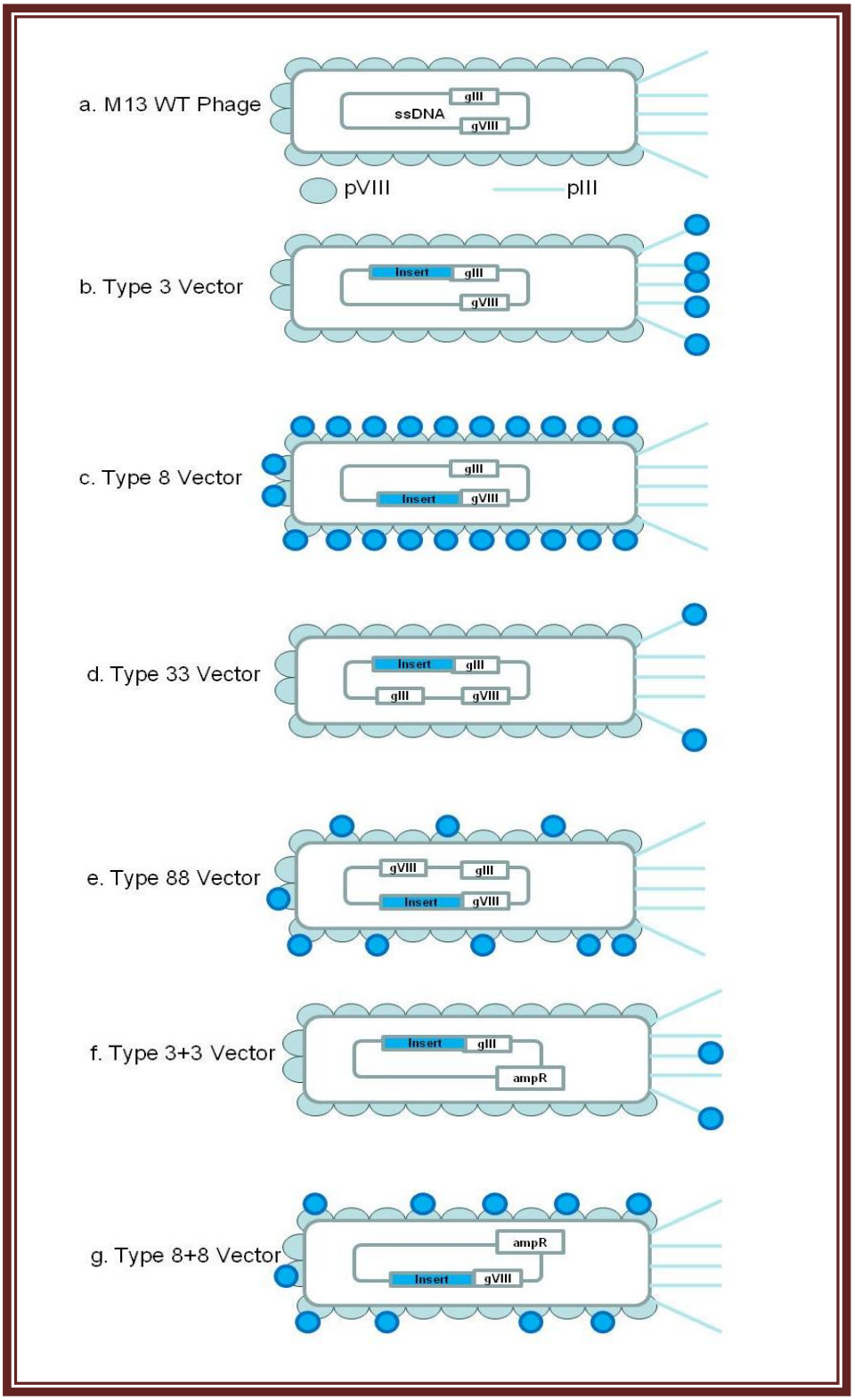

Figure 2. 3 | Representative M13 phage display vectors and viral particles. The dark blue spheres depict the translation of the dark blue foreign DNA insert as a fusion protein with either pIII or pVIII respectively. Each vector is named based on the host viral gene. Type 3 or 8 vectors have single copies of the viral genes (b & c), whereas type 33 or 88 vectors have two copies (d & e). Types 3+3 and 8+8 are phagemid-based (f & g), producing viral particles only when bacterial cells carrying the phagemid are infected with helper phage [Courtesy, Brian Kay; Redrawn].

2.4.1 Phagemid Vector

Phagemid cloning vectors, or simply phagemids, are hybrids of phage and plasmid vectors [see Figure 2.4]. They are designed to contain the origin of replication of both M13 and a plasmid origin of replication from *E. coli*. In addition to these, they include the bacteriophage gene to be fused to foreign DNA sequences, appropriate cloning sites to enable the insertion of foreign DNA sequences, and an antibiotic resistance cassette, but lack all the structural and non-structural genes required for the synthesis of a complete phage (Azzazy & Highsmith, 2002; Jacobsson & Frykberg, 1995; Jacobsson & Frykberg, 2001b).

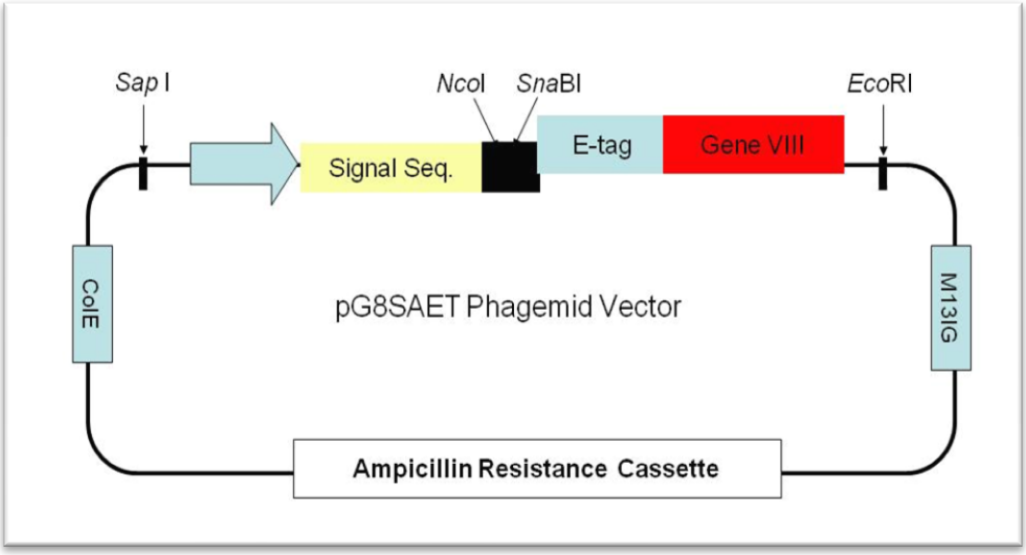

Figure 2. 4 | A schematic Diagram of a g8-based Phagemid vector, pG8SAET. The vector contains origin of replication of M13 and of *E. coli*, in addition to gene 8 and *Nco*I/*Sna*BI cloning sites for insertion of foreign DNA sequences. An ampicillin resistance cassette is also present in the vector sequence. This vector was used in the construction of the phage display library of *C. jejuni* NCTC11168 described in this study [Redrawn with permission from Jacobsson *et al*., 2003].

As with all phage vectors, the phagemid vector also allows the incorporation of foreign DNA sequences into its genome, the display of the said sequences as peptides or proteins on the surface of the phage or in the periplasmic space of the *E. coli* host cell, the recovery of the packaged gene encoding the said peptide and, more importantly, allows selective culture of phage-infected bacterial cells over uninfected ones (Azzazy & Highsmith, 2002).

2.4.2 Manipulation of Phagemids

An important feature in phagemids is the presence of multiple restriction sites which enable foreign DNA sequences to be inserted into g3 or g8 (Azzazy & Highsmith, 2002).

Phagemids have been designed such that the displayed peptide is separated from pIII or pVIII by short peptide linkers, the sequence of which is often a variation of GGGGS (Armstrong *et al.*, 1996). Insertion of a proteolytic cleavage site between the displayed peptide and pIII or pVIII, such as Factor Xa cleavage site in M13mp18Xa, has also been utilized in some phagemids. This has been used to recover the displayed peptide (Nagai & Thogersen, 1984). In addition, disruption of the g3 or g8 reading frames has been used to reduce the percentage of non-recombinant phage particles in phage libraries (Jacobsson & Frykberg, 2001b). This is achieved through introduction of frame-shifts in the stuffer sequences or restriction sites of the vector, the reading frame of which is restored when exogenous DNA sequences are inserted.

Another important factor in the choice of phage display vector is the presence of a marker such as an antibiotic resistance cassette, antibody recognition tags, *lac*Z expression sequence or a stop codon, which are important in selecting recombinant phage (Jacobsson & Frykberg, 1998). Table 2.1 shows the names of published phage display vectors, with the gene of expression, restriction sites and markers.

It is known that for infection to ensue, there must be an interaction between N1 terminal domain and TolA of *E. coli* and this is only achieved through a disruption of the two terminal domains, N1 and N2, of pIII (Lubkowski *et al.*, 1999). Thus, replacement of N1 with an exogenous sequence results in phage progeny which are not infectious. It is therefore possible to manipulate the phagemid vector by replacing the N-terminal domains of pIII with peptide- or protein-encoding DNA sequences such that the resultant phage particles are selectively infective (Crissman & Smith, 1984; Nelson *et al.*, 1981). These vectors are engineered such that the missing N-terminal domains necessary for

infection are harboured in adaptor molecules made up of the ligand covalently coupled to the replacement DNA sequences. Following recovery, therefore, non-infective phage mixed with adaptor molecules restores infectivity to the phage particles that display peptides or proteins that can bind to the ligand which provides the missing N-terminal domains of pIII (Krebber *et al.*, 1997). This selectively infective phage (SIP) has an advantage in selecting for high affinity binders.

Vector	Gene	Rest Site	Marker	Reference
fUSE1	III	*Pvu*II	tet^R	Parmley and Smith, 1988
fUSE2	III	*Bgl*II	tet^R	Parmley and Smith, 1988
fUSE5	III	*Sfi*I	tet^R	Scott and Smith, 1990
pHEN1	III	*Sfi*I/*Not*I	amp^R	Hoogenboom *et al.*, 1991
λSurfZap	III	*Not*I/*Spe*I	amp^R	Hogrefe *et al.*, 1993
p8V5	VIII	*Bst*XI	amp^R	Affymax
f88	VIII	*Pst*I/*Bam*HI	tet^R	Scott and Smith, 1990
pG8SAET	VIII	*Sna*BI/*Nco*I	amp^R	Jacobsson *et al.*, 1998

Table 2. 1 | Names of published phage display vectors, with gene of expression, restriction sites and markers. All of these vectors have been used successfully in the construction of phage display libraries as referenced in the table.

Peptides and proteins attached to the domain 2 of pIII are more accessible, and hence this domain has become the preferred site of DNA insertion in phage display, such as the fusion of human growth hormone to a truncated form of pIII within amino acid position 198 to 406 (Armstrong *et al.*, 1996; Lubkowski *et al.*, 1999).

Manipulating the phagemid vector to contain a signal sequence, Frykberg and co-workers developed a system that displays only exported bacterial proteins, thus enabling isolation of adhesin-encoded genes and genes of other exported proteins (Karlstrom *et al.*, 2004; Rosander *et al.*, 2002). The phage vector can therefore be manipulated in several ways to suit one's aims and objectives in any phage display library.

2.5 Construction of Phage-Display Libraries

As depicted in Figure 2.5, the basic principle of the construction of a phage display library is the same irrespective of the type of library needed, although minor modifications of the procedures may be necessary. The foreign DNA fragments to be displayed as fusion peptides or proteins are introduced into a linearized vector DNA by ligation. The ligated fusion vector-DNA fragment is then introduced by electroporation into an appropriate *E. coli* strain and then amplified to produce a library (Jacobsson *et al.*, 2003).

In the construction of RPLs, double-stranded DNA fragments are usually obtained through the assembly of degenerate oligonucleotides (Adey *et al.*, 1996). Whereas some researchers anneal only three oligos and then proceed to ligate into a vector (Cwirla *et al.*, 1990), others may amplify these fragments by PCR, digest with restriction enzymes to obtain the desired ends, before ligating into a vector (Scott & Smith, 1990; Sparks *et al.*, 1998).

Phage display technology has been applied in many areas of research including the study of infectious diseases (Mullen *et al.*, 2006). The principle of the construction of a phage library of a bacterial genome is very much the same as that described for random peptide libraries and antibody libraries. DNA fragments can be obtained through sonication of genomic DNA in what is referred to as the shot-gun phage display libraries (Jacobsson *et al.*, 2003; Mullen *et al.*, 2007b). The resulting fragmented DNA is separated by agarose gel electrophoresis and DNA in the required size range excised from the gel and purified for downstream applications (Jacobsson & Frykberg, 2001b). The disadvantages with this method lie in the fact that the blunt-ended ligation results in low insert yields, and that the inserted fragments are usually small. The percentage of insert following ligation can be improved by restriction digestion of the genomic DNA with an infrequent cutter such as *Sau*3AI (or its isoschizomer, *Bfu*CI), which also gives DNA fragments with cohesive end-termini. However, using restriction digested DNA in this kind of library also has a big disadvantage since they can only cut where there are enzyme sites which means that some genes may never be represented as expressed proteins in the library.

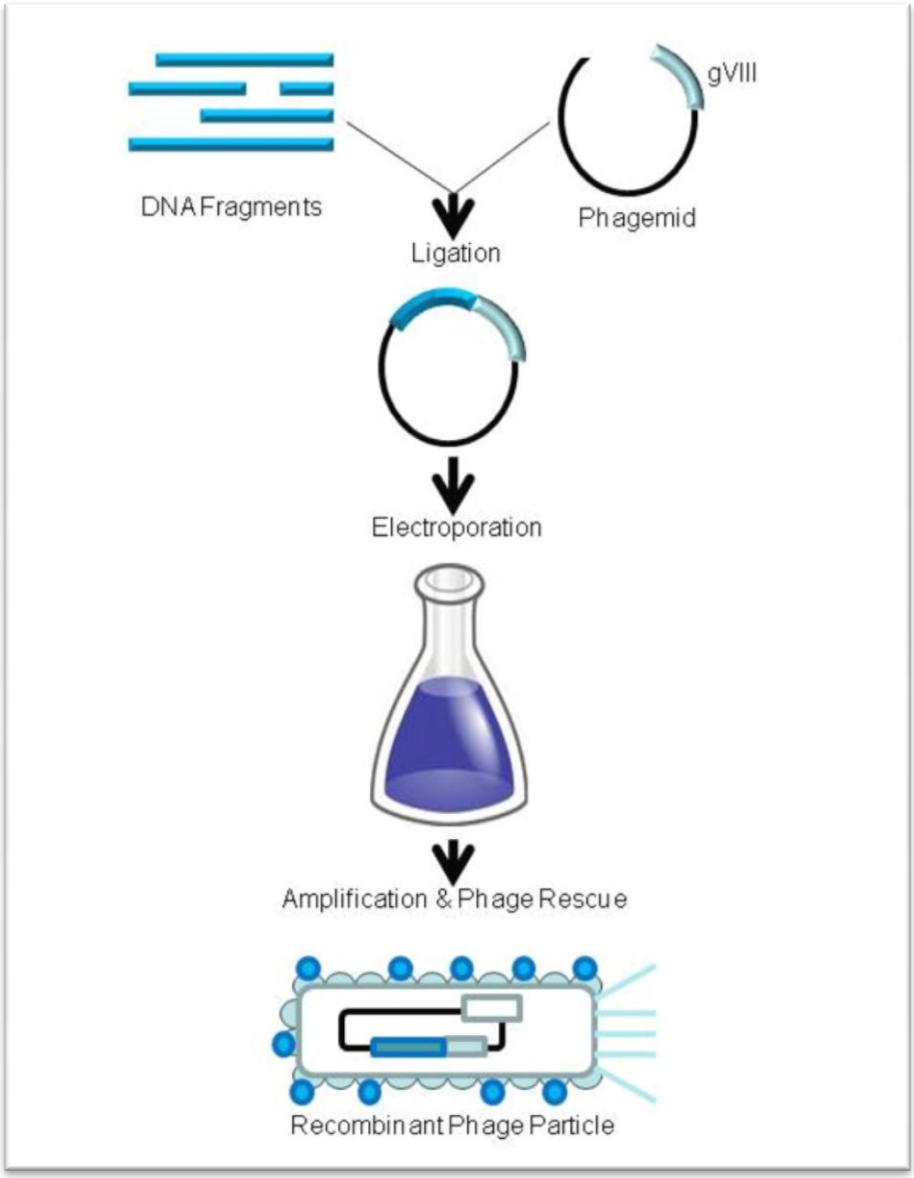

Figure 2. 5 | Schematic representation of phage display library construction. Foreign DNA fragments, obtained from sonication or restriction digestion of chromosomal DNA or through the assembly of degenerate oligonucleotides, are ligated into linearized phagemid vector and then used to transform an appropriate *E. coli* host. Cells containing cloned foreign DNA fragments are then cultured in an amplification step. In order to secrete phage particles that will display both the wild-type and pIII or pVIII fusion proteins, the *E. coli* host cells are super-infected with helper phage in a phage rescue step [Drawing based on principles as described in literature].

2.5.1 Applications of Phage Display Libraries

Following Smith's demonstration of the display of foreign peptides on the surface of bacteriophage, the technology has been used in the generation of several fusion proteins with phage capsid, the applications of which have been

numerous. Random peptide libraries have been shown to have the potential to mimic linear, conformational and non-proteinaceous epitopes (Smith, 1991).

RPLs have been used to identify peptides that specifically bind to a wide range of targets such as antibodies (Scott & Smith, 1990), the lectin protein concanavalin A (Scott *et al.*, 1992), streptavidin (Devlin *et al.*, 1990), and ribonucleases (Smith *et al.*, 1993). Phage display has been used to identify peptides that could be incorporated into conductive apatite layers in bone engineering to promote cell adhesion, proliferation and ultimately differentiation into bone tissue, leading to increased osteogenesis (Segvich *et al.*, 2009).

Through their binding activities, peptides identified through phage display have been shown to act as agonists or antagonists, whereas others that neutralize immunoglobulins have been employed as therapeutic agents in some autoimmune diseases (Blank *et al.*, 1999; Doorbar & Winter, 1994; Goodson *et al.*, 1994; van Groen *et al.*, 2008). Panning a 12-mer phage display library against human hepatocellular carcinoma cell line HepG2, a novel peptide was identified by Zhu *et al.* which can be employed as a delivery carrier in target diagnosis or therapy in hepatocellular carcinoma (Zhu *et al.*, 2008).

Cheng *et al.* showed that using phage display techniques amino acid sequences that are capable of affecting biologically significant protein-DNA interactions can be identified from random peptide libraries (Cheng *et al.*, 1996). Using a plaque dilution assay for affinity screening of their 23-mer random peptide library, they identified four groups of oligo-binding phage that showed preference for: (1) single-stranded oligos irrespective of sequence; (2) double-stranded oligos irrespective of sequence; (3) sequence-specific binding to single-stranded oligos; and (4) weak non-specific binding to all types of oligos tested. The beauty of the analysis of this was the identification of the consensus motifs responsible for the binding of the specific oligos.

As knowledge in phage display technology increased, so did the length of DNA sequences cloned and several scientists attempted cloning longer peptides including functional peptides (Cwirla *et al.*, 1990).

Antibodies became the first functional proteins to be displayed in this manner following several innovations using RPLs (McCafferty *et al.*, 1990; Parmley & Smith, 1988). The technology was used to express fragments of various antibodies for epitope mapping and protein interaction studies (Chang *et al.*, 1991; Clackson *et al.*, 1991). The technique can now be used to isolate high-affinity antibodies, which are known to have several advantages over monoclonal antibodies generated by hybridoma technology (Azzazy & Highsmith, 2002). Cheng *et al.* have recently identified single chain antibodies specific for three of the four human Toll-like receptor-2 homologues using a phage display antibody library (Chen *et al.*, 2009).

In 1993, Kay and colleagues successfully constructed an M13 RPL displaying random 38-amino-acid peptides, the length of which led to intense interest in the display of functional peptides other than antibodies (Kay *et al.*, 1993). The enzymes, alkaline phosphatase, trypsin, and β-lactamase have since been expressed through this technology without any of them losing their catalytic activities (Corey *et al.*, 1993; McCafferty *et al.*, 1991; Siemers *et al.*, 1996).

2.5.2 Phage Display Technology in the study of Infectious Diseases

As is clear now, interaction between bacterial adhesins and host cellular proteins are important in the events leading to the establishment of many bacterial infections (Finlay & Falkow, 1997; Patti *et al.*, 1994). Phage display has long been thought of as a good system for displaying specific or random peptides of important infectious agents as antigens, which could lead to the development of cheap and effective vaccines, as well as in the identification of peptides or adhesins in pathogenic bacteria that are not easily grown in the laboratory, or for which genetic manipulations are not straightforward (Antonara *et al.*, 2007; Greenwood *et al.*, 1991; Willis *et al.*, 1993). *Campylobacter* falls in the last two categories.

By constructing phage display libraries of fragments of genomic DNA of pathogenic bacteria and panning against various ligands, Frykberg and Jacobsson identified several genes encoding bacterial proteins that were found to interact with host proteins (Jacobsson & Frykberg, 1995; Jacobsson &

Frykberg, 1996). Using phage display, Jacobsson and co-workers identified 52 novel extracellular and transmembrane proteins from *Lactobacillus reuteri*, an organism routinely used in probiotics (Wall *et al.*, 2003). Phage display technology was used in the identification and characterization of a plasminogen-binding protein B (PgbB) of *Helicobacter pylori* (Jonsson *et al.*, 2004), whilst the proteins FnBPA of *Staphylococcus aureus* (Ingham *et al.*, 2004), Embp, FnBPA and FnBPB of *Staphylococcus epidermidis* (Ingham *et al.*, 2004; Williams *et al.*, 2002), ScpB of *Streptococcus agalactiae* (Beckmann *et al.*, 2002), DemA of *Streptococcus dysgalactiae* (Vasi *et al.*, 2000), and FnBP of *Streptococcus equi* (Lindmark & Guss, 1999), were all found to be adhesins that bind to fibronectin through phage display technology.

In the same way, the proteins Fbe of *Staphylococcus epidermidis* (Nilsson *et al.*, 1998), FgagV1, FgagV2 and FgagV3 of *Streptococcus agalactiae* (Jacobsson, 2003), and Fb1 of *Staphylococcus lugdunensis* (Nilsson *et al.*, 2004a), were also found to be adhesins that bind to fibrinogen through phage display technology. The F17a-G adhesin of bovine enterotoxigenic *E. coli* (ETEC) which is involved in the attachment of the bacterium to the intestinal epithelium was displayed as an amino-terminal fusion with the phage protein pIII and was found to elicit an IgG response against F17a-G after intra-peritoneal immunisation of mice (Van Gerven *et al.*, 2008).

Phage display technology has also been employed in the identification of vaccine candidates against infectious diseases. In one study using a phage display cDNA library of *Brugia malayi*, the causative agent of lymphatic filariasis, five antigens were identified that bound to antibodies from infected persons, one of which was later proved to provide protection against infection in animal models of the disease (Gnanasekar *et al.*, 2004). Again, phage libraries of single chain variable fragments have been used to identify potential vaccine candidates for infectious agents such as *Clostridium difficile*, *Bacillus subtilis* and the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) (Deng *et al.*, 2003; Knurr *et al.*, 2003; Liu *et al.*, 2005).

2.5.3 Innovations in Phage Display Technology

Following identification of proteins or peptides that bind to a particular ligand, the specific binding domains could be identified through an innovative approach to phage display where a single-gene phage display library is constructed from the fragments of the gene of interest. This innovation has been used to identify the IgG and albumin-binding domains of two novel receptors of group C streptococci as MAG and ZAG, whilst mapping the fibronectin-binding activity of *Streptococcus equi* to two C-terminal domains of FnBP (Jacobsson *et al.*, 1997; Lindmark *et al.*, 1996; Lindmark & Guss, 1999). Zhang and co-workers also identified the binding domains of the Sbi protein of *Staphylococcus aureus* to IgG and β_2-glycoprotein through this method (Zhang *et al.*, 1999). Using a RPL, Benedek and co-workers identified laminin-binding motifs of the *Yersinia pestis* plasminogen activator (Benedek *et al.*, 2005).

Another innovation in the use of phage display is the identification of bacterial proteins that bind to host proteins deposited on biomaterials of routinely used medical devices in clinical practice, such as intravenous catheters and prosthetic heart valves. By panning a genomic DNA library of *Staphylococcus aureus* against a freshly removed central intravenous catheter, several bacterial proteins were identified as binding to fibrinogen or β_2-glycoprotein I (Bjerketorp *et al.*, 2004).

2.5.4 Advantages and Disadvantages of Phage Display Technology

Put simply, the benefits of phage display technology are therefore three-fold: (i) the ability to very effectively produce a large number of differing peptides and proteins (up to 10^{10} particles per ml of library) (ii) the ability to screen libraries expressing billions of unique peptides and proteins by way of binding to a ligand(s) and (iii) the capacity to maintain a molecular link between the displayed protein and the DNA sequence encoding it (Azzazy & Highsmith, 2002; Mullen *et al.*, 2006).

However, it must be stated that phage display technology has its attendant drawbacks. For instance, very high phage titres containing large numbers of

clones are needed in NPLs since the majority of clones from such libraries are non functional. Furthermore, only one in eighteen clones would be in frame with the vector sequences as only one clone in three will start in frame with the coat protein, only one in three will end in frame with the coat protein, and one in two will be in the right orientation (Jacobsson & Frykberg, 2001b; Mullen *et al.*, 2006).

Clonal variation in the display levels of even closely related sequences is a known shortcoming of phage display technology, where differences in translation, transport, folding or stability of the fusion can negatively affect the expression levels of the recombinant protein (McCafferty, 1996).

Furthermore, affinity selection is also affected by the number of recombinant phage particles present in any particular panning, where incubation of low numbers of phage particles results in about 34% of binding, with higher inputs yielding less (McCafferty, 1996). Therefore, careful optimization and strict adherence to the optimized protocol is required for panning of phage display libraries if consistent results are to be obtained.

Post-translational protein modification is another important factor in phage display of proteins and peptides. In *C. jejuni*, glycosylation is known to play an important role in the functionality of many of its surface proteins (Young *et al.*, 2007; Zilbauer *et al.*, 2008) and its absence could be expected to affect interaction of phage expressed *C. jejuni* proteins with the appropriate ligands. Post-translational modification may therefore have a negative or a positive effect depending on the protein in question.

Although *E. coli* remains one of the most widely used hosts for the production of heterologous eukaryotic proteins, the expression of toxic proteins could also be toxic to the host cells, thus, affecting propagation of phage clones containing such proteins.

2.5.5 Aims of The Work Described in This Chapter

As stated earlier, this chapter describes the construction and validation of a phage display library of *Campylobacter jejuni* NCTC11168, based on the filamentous phage M13. This library will be utilized to identify *C. jejuni* proteins that may be involved in binding to lactoferrin and fibronectin present in the host intestinal mucosa. The phagemid vector, pG8SAET (Jacobsson & Frykberg, 2001a), will be utilized for this purpose.

2.6 Materials and Methods

2.6.1 Bacterial Strains and Culture conditions

The library was constructed using chromosomal DNA from wild type *Campylobacter jejuni* NCTC11168 obtained from a laboratory stock maintained in a 50:50 mixture of glycerol and Mueller-Hinton (MH) broth and stored at -80°C. *C. jejuni* NCTC11168 was grown at 42°C on charcoal-cefoperazone-desoxycholate agar (CCDA) or in MH broth (Oxoid) with shaking, in a MACS VA500 Microaerophilic workstation (Don Whitley Scientific) in atmospheric conditions of 6% O_2, 5% CO_2, 3%H_2 and 86% N_2.

E. coli TG1 electroporation-competent cells [*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5* (r_K^- m_K^-) [F´ *traD36 proAB lacI*^{q}*ZΔM15*] (Stratagene) were used for the propagation of the phagemids, the construction of phage display libraries and the production of phage stocks. *E. coli* TG1 was grown at 37°C in Luria-Bertani (LB) broth (Fischer Scientific) with shaking or on LB agar (Fisher Scientific), that had been supplemented with 200 µg/ml of ampicillin or 50 µg/ml of kanamycin and 2% (w/v) filter-sterilized glucose when appropriate.

2.6.2 Phagemids, Helper Phage and Plasmids

The phagemid vector, pG8SAET (Figure 2.4), was used in the construction of the *C. jejuni* NCTC11168 phage display library (Jacobsson & Frykberg, 2001a). This was a kind gift from Lisa Mullen, Division of Microbial Diseases, Eastman Dental Institute, University College London, London, UK. The vector was used with permission from its originators, Lars Frykberg and Karen Jacobsson, Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

R408 helper phage (Promega) was employed for the packaging and secretion of the pG8SAET-chromosomal DNA hybrids as single-stranded DNA (ssDNA) viral particles.

The plasmid, pJMK30 (Figure 2.6), containing a kanamycin resistance gene, *aphA-3*,as well as an ampicillin resistance cassette (van Vliet *et al.*, 1998), was used to prepare "insert models" to mimic the chromosomal DNA inserts.

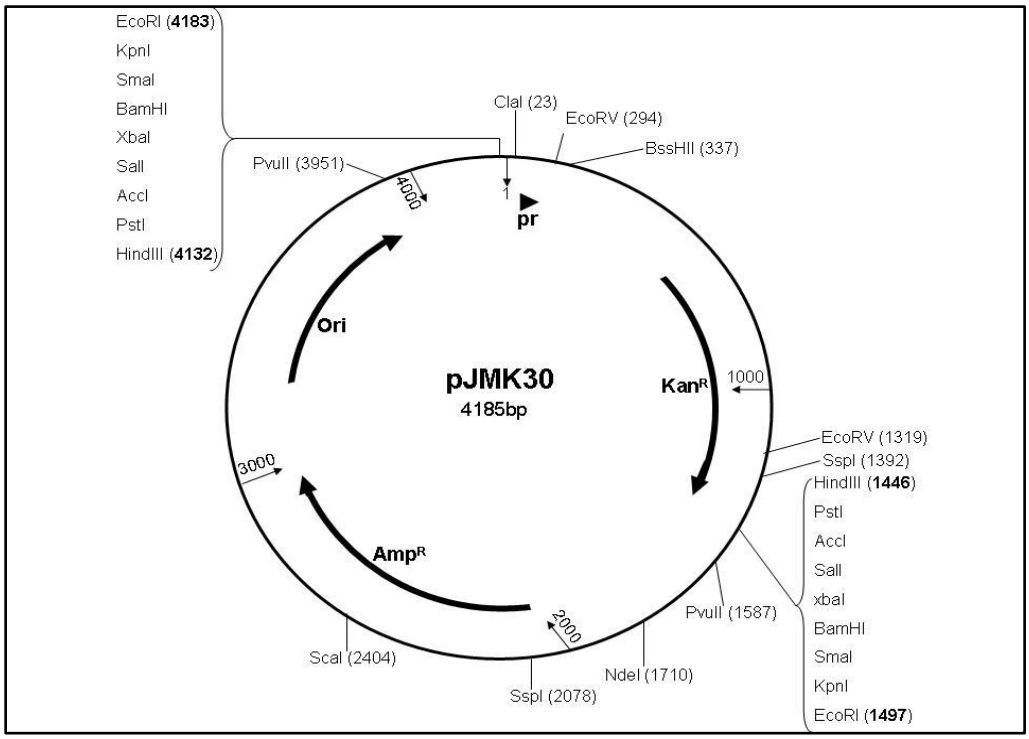

Figure 2. 6 | Schematic representation of the pJMK30 plasmid. The figure shows all restriction enzyme sites. The kanamycin resistance cassette was employed in the creation of insert mimics [Redrawn from van Vliet *et al*, 1998].

2.6.3 Antibiotic Supplements

Ampicillin and kanamycin were obtained from Sigma-Aldrich. Stock solutions were prepared with distilled water to final concentrations of 100 mg/ml and 50 mg/ml respectively, and then filter-sterilized with a 0.22-µl pore sterile Millex® filter unit (Millipore) before use. Antibiotic stocks were stored at 4°C until used.

2.6.4 Culture Media

i. CCDA *Campylobacter* Selective Agar (Skirrow)

CCDA otherwise known as Skirrow's *Campylobacter* selective agar, obtained from Oxoid, was used in the culture of *C. jejuni* NCTC11168. This medium, which is designed to be used at 42°C for optimum selective effect, is based on

Skirrow's early recipe that contained 23 g/l of special peptone, 1 g/l of starch, 5 g/l of NaCl and 10 g/l of agar and supplemented with 10 µg/ml of vancomycin, 2.5 IU/ml of polymyxin B, and 5 µg/ml of trimethoprim (Skirrow, 1977).

ii. Mueller-Hinton Broth

Mueller-Hinton (MH) Broth powder (Oxoid) suspended in distilled water according to manufacturer's instructions and autoclaved was used for liquid culture of *C. jejuni* NCTC11168. This microbiological media typically contains 30% beef infusion, 1.75% of casein hydrolysate and 0.15% of starch. An addition of 1.7% of agar results in solidification of the medium to give Mueller-Hinton agar (Atlas, 2004).

iii. SOC Media

Super optimal broth with catabolite repression (SOC) is made from super optimal broth (SOB medium) by the addition of 0.3% glucose (Hanahan, 1983). Premade SOC medium was obtained from Invitrogen, but was otherwise made up from 2% w/v bacto-tryptone (20g), 0.5% w/v bacto-yeast extract (5g), and 0.01 M NaCI (0.584g), 0.005 M KCI, and 0.02 M $MgSO_4$ (2.408g) or 10mM $MgCl_2$ (0.952g), made up to a final volume of 1 litre in distilled water. The broth was sterilized by autoclaving, and stored at 4°C until required. Immediately prior to use, 0.02 M glucose (3.603 g/l), filter sterilized using 0.22 µm pore sterile Millex® filter unit (Millipore Corporation) was added.

iv. LB Media

Pre-made Luria-Bertani (LB) was obtained from Fischer Scientific of which a specified amount of its powder was suspended in distilled water according to manufacturer's instructions and autoclaved as appropriate. It was otherwise prepared according to the Miller recipe by dissolving 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in 1 litre of distilled water (Miller, 1972). The broth was then sterilized by autoclaving at 121°C, 15 lb. $inch^{-2}$ for 15 min.

LB agar was made in an identical fashion except for the addition of 1% (w/v) agar to LB broth prior to autoclave sterilization. Following cooling to

approximately 40°C, any necessary antibiotics were added and the agar transferred to Petri dishes.

v. Soft Agar (Soft LB Agar)

Soft agar was made by adding 0.05 % (w/v) of agar to LB broth prior to autoclave sterilization. Following cooling, bottles containing soft agar were kept at room temperature until required. This was used in the amplification of phage infected *E. coli* TG1 cells.

vi. Luria-Bertani with Glucose

Both LB broth and LB agar, fortified with glucose, were used in the propagation of phage-infected *E. coli* TG1 cells prior to amplification of these clones. LB-glucose was obtained by adding 2% (w/v) glucose, filter sterilized using 0.22 µm pore sterile Millex® filter unit, to either of LB broth or LB agar prior to use.

2.6.5 Miscellaneous Buffers and Solutions

i. 0.5 M EDTA

0.5 M Ethylenediaminetetraacetic acid was prepared by adding 186.1 g to 900 ml of distilled water and adjusting the pH to 8.0 using 10 M NaOH. The volume was made up to 1 litre and the solution sterilized by autoclaving at 121°C, 15 lb. $inch^{-2}$ for 15 min.

ii. TAE Buffer

Tris-acetate EDTA (TAE) buffer was routinely used in preparation of agarose gels. A 50 × TAE buffer was prepared by dissolving 242 g of Tris base [2-amino-2-hydroxymethyl-propane-1,3-diol, ≡ 2 M], 57.1 ml glacial acetic acid [≡ 1 M of 100% acetic acid], and 100 ml 0.5 M Na_2 EDTA (pH 8.0) in 1 litre of distilled water.

iii. Phosphate Buffered Saline

Phosphate buffered saline (PBS) was prepared by dissolving 1 tablet of pre-made PBS, obtained from Oxoid, in 100 ml distilled water [≡ 1× PBS] and sterilized by autoclaving at 121°C, 15 lb. $inch^{-2}$ for 15 min. It was otherwise prepared from 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 dissolved in 1 litre of distilled water and the pH adjusted to 7.3. This was then sterilized by autoclaving.

PBS-Tween 20 (PBS-T) was made by adding 200 µl of Tween 20 to 400 ml of 1× PBS.

iv. 3 M Sodium Acetate

This was prepared by dissolving 40.8 g of sodium acetate salt in 90 ml of distilled water and adjusting the pH to 5.2 using glacial acetic acid and the volume made up to 100 ml. The resulting solution was then sterilized by autoclaving at 121°C, 15 lb. $inch^{-2}$ for 15 min.

v. Phenol-Chloroform-Isoamyl Alcohol

Phenol: chloroform: isoamyl alcohol 25:24:1 was obtained from Sigma-Aldrich and used routinely in the purification of DNA following ligation of *C. jejuni* NCTC11168 chromosomal DNA fragments into pG8SAET vector.

2.6.6 Restriction Enzymes

The restriction endonucleases/enzymes [*Sna*BI, *Bgl*II, *Bam*HI, *Sma*I, *Eco*RV and *Bfu*CI] were obtained from New England Biolabs, and were used according to the manufacturer's instructions with minor modifications.

2.6.7 DNA Modification Enzymes

i. Antarctic Phosphatase

Antarctic phosphatase (AP) was obtained from NEB and was used to catalyze the removal of the 5´ phosphate groups from linear DNA fragments, thus preventing self-ligation through the 5´ phosphoryl termini required by ligases

for ligation (Sambrook & Rusell, 2001). AP was used according to the manufacturer's guidelines. Typical reactions contained 5 µl [≡ 50 U] of AP, approximately 10 µg of vector DNA, 12 µl of 10× AP buffer and nuclease-free deionised water to a final volume of 120 µl. This was incubated at 37°C for 15 min.

ii. Klenow Fragment

Klenow Fragment (NEB) was employed to create blunt-ended chromosomal DNA fragments of *C. jejuni* NCTC11168 by "filling-in" 5´overhangs after sonication. A typical Klenow reaction contained 20 µg of fragmented chromosomal DNA, 20 U of Klenow [≡ 1 U of Klenow/µg of DNA], 5 µl of 2 mM dNTPs, 22 µl of 10× NEB buffer 2 and an appropriate amount of nuclease-free deionised water to a final volume of 220 µl. This was incubated at room temperature for 30 min, and then the reaction inactivated at 75°C for 10 min.

iii. T4 DNA Polymerase

T4 DNA polymerase (NEB) was employed to complement the Klenow blunting of sonicated-chromosomal DNA of *C. jejuni* NCTC11168. Without cleaning the initial Klenow reaction mixture, 7 µl of T4 DNA polymerase was added [≡ 1.05 U of T4 DNA polymerase/µg of DNA], alongside 10 µl of 2 mM dNTPs, 3 µl of 10× NEB buffer 2, 2.5 µl of 10× BSA, and an appropriate amount of nuclease-free deionised water to a final volume of 250 µl. This was then incubated at 12°C for 15 min, before the reaction was stopped by adding EDTA to a final concentration of 10 mM and inactivation at 75°C for 10 min.

iv. T4 DNA Ligase

T4 DNA ligase was employed in the ligation of chromosomal DNA fragments of *C. jejuni* NCTC11168 or insert models into linearized pG8SAET phagemid vector, by catalyzing the formation of a phosphodiester bond between juxtaposed 5´ phosphate and 3´ hydroxyl termini in DNA.

2.6.8 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used for a variety of purposes during this work and was carried out using a Techne 96-well thermocycler, using either *Taq* DNA Polymerase or Expand™ High Fidelity PCR System. All primers used in this study are listed in Table 2.2 and were synthesized by Eurofins MWG GmbH (Germany).

Primer	Sequence	Amplification of
pG8SAET_F1	5´ - GTACCCGGTGCGCCGGTGCCGTATCCG -3´	Linear vector with *Sna*BI site
pG8SAET_F1	5´ - GTACTGCCATGGTCATCGTGTTGCGCAGC - 3´	
pG8SAET_F2	5' - GGA**AGATCT**CCCGGTGCGCCGGTGCCGTATCCG - 3'	Linear vector with *Bgl*II Site
pG8SAET_R2	5' - GGA**AGATCT**CTGCCATGGTCATCGTGTTGCGCAGC - 3'	
pG8SAET_F	5´ - TATCTGGTGGCGTAACACCTGCT - 3´	cDNA insert across *Sna*BI/ *Bgl*II bridge
pG8SAET_R_Short	5´ - GATCGTCACCCTCGGATCCCTAGG - 3´	
pG8SAET_R_Long	5´ - GTGAAATACCGCACAGATGCGTAA - 3´	

Table 2. 2 | Primers used in PCR amplification of vector and inserts. The restriction sites are highlighted in maroon.

i. *Taq* DNA Polymerase

Taq DNA polymerase (5 U/µl, Roche) was used for the amplification of the linear vector as well as the chromosomal DNA inserts.

ii. Expand™ High Fidelity PCR System

The Expand™ High Fidelity (HiFi) PCR System (Roche) was used for high fidelity amplification of the pG8SAET vector as it gives a 3-fold yield, combined with higher fidelity and specificity compared with a conventional *Taq* DNA polymerase alone (Barnes, 1992; Barnes, 1994).

2.6.9 DNA Extraction from Bacterial Cells

Chromosomal DNA of *C. jejuni* NCTC11168 was isolated using the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. Following three-day growth of *C. jejuni* NCTC11168 on one CCDA plate, cells were harvested using 1 ml of PBS and DNA extracted from this. DNA was eluted from the membrane using nuclease-free deionised water.

Plasmid DNA was extracted using QIAprep spin miniprep kit (Qiagen) according to manufacturer's instructions. Single colonies of cells of interest were cultured overnight in 1.5 ml of LB broth containing appropriate antibiotics. Cells were harvested by centrifugation at 16,000 × *g* for 5 min and the pellet resuspended in the appropriate buffer for plasmid extraction. Plasmid DNA was eluted from the membrane using nuclease-free deionised water.

2.6.10 Confirmation and Quantification of DNA

i. Quantification of DNA by Spectrophotometer

All DNA preparations were re-suspended in an appropriate volume of nuclease-free deionised water and quantified using the Nanodrop ND-1000 Spectrophotometer (Thermo Scientific).

ii. Agarose Gel Electrophoresis

DNA fragments were resolved following DNA extraction, PCR, or restriction digestion using agarose gel electrophoresis. The agarose gel was prepared by dissolving 0.75% (w/v) agarose powder (Sigma) in an appropriate volume of 1× TAE buffer. The mixture was then boiled, cooled and ethidium bromide added to a final concentration of 0.5 µg/ml. The gel was then cast with an appropriate comb and after solidification, placed in a running tank containing 1× TAE buffer.

DNA samples were prepared for electrophoresis by adding 1 µl of 10× loading buffer to 9 µl of sample. DNA samples were then loaded alongside an appropriate molecular marker, usually GeneRuler^{TM} DNA Ladder (Fermentas). The gel was run at a constant voltage of 120 V/cm until the dye front had travelled through approximately 75% of the gel. DNA fragments were then visualized using ultra-violet light (290 nm) and photographed.

2.6.11 Purification of DNA

i. Agarose Gel Extraction and Purification of DNA

Purification of DNA fragments excised from agarose gels was done using Qiagen's gel extraction kit (Qiagen) according to manufacturer's instructions. DNA was eluted in 50 µl of nuclease-free deionised water by centrifugation at 14,000 × *g* for 1 min.

ii. Purification of DNA from PCR/Restriction Digests

DNA was purified using Qiagen's PCR purification kit (Qiagen) according to manufacturer's instructions, following PCR reactions, restriction digests or other enzymatic reactions. DNA was eluted in 50 µl of nuclease-free deionised water by centrifugation at 14,000 × *g* for 1 min.

iii. Phenol-Chloroform-Isoamyl alcohol Extraction of DNA

Ligation reaction mixtures were purified using phenol-chloroform-isoamyl alcohol. Nuclease-free water was added to the reaction mixture to a final volume of 500 µl, if the initial volume was less than this. An equal volume of phenol-chloroform-isoamyl alcohol was then added and thoroughly mixed until solution became cloudy. This was then centrifuged at 14,000 × *g* for 30 min, and 450 µl of the DNA-containing aqueous phase decanted into a fresh tube. To this, 0.7 volumes of 100% iso-propanol and 0.1 volumes of 3 M sodium acetate was added. 1 µl [≡10 µg/µl] of yeast tRNA (Sigma) was also added as a co-precipitant and samples incubated at -20°C for 30 min. The precipitated DNA was then pelletted by centrifugation at 14,000 × *g* for 30 min at 4°C, and then washed with 1 ml of 70% ethanol and re-pelletted by centrifugation at 14,000 × *g* for 30 min. The purified DNA was then air-dried for 15 min and re-suspended in an appropriate volume of nuclease-free deionised water.

2.6.12 Linearization of Vector DNA

2.6.12.1 Creation of Blunt-ended Linear Vector

Two methods were used in creating blunt-ended linear vector in this study, namely, restriction digestion with dephosphorylation and PCR.

i. *Sna*BI Restriction Digest of Circular Vector

The circular phagemid vector was linearized by restriction digestion using *Sna*BI. A typical digestion reaction contained *ca*. 20 µg of vector DNA, 100 U of *Sna*BI [≡ 5 U of *Sna*BI/µg of vector DNA], in a final volume of 100 µl in the recommended enzyme buffer and the mixture incubated at 37°C overnight. A 1-µl aliquot of this digestion reaction mixture, containing *ca*. 200 ng of vector DNA, was analysed by agarose gel electrophoresis to ascertain complete cleavage. To the remaining 99 µl of digestion reaction mixture, 10 µl of 10 × loading buffer was added and subjected to electrophoresis on a 0.75% agarose gel to size-separate the completely digested vector. The band corresponding to the completely digested vector was excised and purified using Qiagen Gel purification kit as described above, and dephosphorylated using AP.

ii. PCR of Digested Vector to Produce Blunt ends

The *Sna*BI-digested vector was amplified using the Expand^{TM} HiFi PCR System and primers pG8SAET_F1 and pG8SAET_R1 (Table 2.2). The PCR reaction mixture contained 10 ng of *Sna*BI-digested vector DNA, 60 pmol each of pG8SAET_F1 and pG8SAET_R1, 30 µl of 2 mM dNTPs, 30 µl of 10 × PCR buffer containing 1.5 mM $MgCl_2$, 15 U of Expand^{TM} HiFi PCR polymerase and nuclease-free deionised water to a final volume of 300 µl. This master mix was divided into 6 aliquots of 50 µl each and placed in a thermocycler with the following settings: an initial denaturation at 95°C for 3 min, and 30 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 74°C for 4 min, and a single final extension at 74°C for 5 min. After PCR, the samples were pooled,

cleaned up using Qiagen's PCR purification kit and eluted in 30 µl of nuclease-free deionised water, followed by dephosphorylation with AP.

2.6.12.2 Creation of A-Tailed Linear Vector

i. *Sna*BI-Digested/PCR Amplified Dephosphorylated Vector

Blunt-ended and dephosphorylated linear vector was A-tailed to improve ligation efficiency. A typical A-tailing reaction contained 10 µg of blunt-ended, dephosphorylated linear vector, 50 U of *Taq* polymerase [≡ 5 U of *Taq* polymerase /µg of DNA], 10 µl of 10 × PCR buffer with 1.5 mM $MgCl_2$, 10 µl of 2 mM dATP and nuclease-free deionised water to a final volume of 100 µl. The reaction mixture was incubated at 70°C for 1 h, cleaned up using Qiagen's PCR purification kit and quantified.

ii. PCR with *Taq* Polymerase

Using a *Taq* Polymerase (Roche), and primers pG8SAET_F1 and pG8SAET_R1 (Table 2.2), the linear vector was amplified using an aliquot of the *Sna*BI-digested vector as a template. This PCR took advantage of the low fidelity of the normal *Taq* polymerase, which results in the addition of an end A-tail to each single strand amplified.

The PCR reaction mixture contained 10 ng of *Sna*BI-digested vector DNA, 60 pmol each of pG8SAET_F1 and pG8SAET_R1, 30 µl of 2 mM dNTPs, 30 µl of 10 × PCR buffer containing 1.5 mM $MgCl_2$, 15 U of *Taq* polymerase and nuclease-free deionised water to a final volume of 300 µl. This master mix was divided into 6 aliquots of 50 µl each and placed in a thermocycler with the following settings: an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 74°C for 4 min, and a single final extension at 74°C for 5 min. After amplification, the samples were pooled, cleaned up using Qiagen's PCR purification kit and eluted in 30µl of nuclease-free deionised water.

2.6.12.3 Creation of Linear Vector with Cohesive End-termini

Primers were designed to amplify the vector yielding a *Bfu*CI/*Bgl*II recognition site. The PCR reaction mixture contained 10 ng of *Sna*BI-digested vector DNA, 90 pmol each of pG8SAET_F2 and pG8SAET_R2 (Table 2.2), 12 µl of 5 mM dNTPs, 30 µl of 10 × PCR buffer containing 1.5 mM $MgCl_2$, 42 U of Expand^{TM} HiFi PCR polymerase and an appropriate amount of nuclease-free deionised water in a final volume of 300 µl. This master mix was divided into 6 aliquots of 50 µl each and placed in a thermocycler with the following settings: an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 10 s, annealing at 54°C for 30 s, extension at 68°C for 5 min, and a single final extension at 68°C for 5 min. After amplification, the samples were pooled together and cleaned up using Qiagen's PCR purification kit and eluted in 30 µl of nuclease-free deionised water. This was followed by a *Bgl*II restriction digest and then dephosphorylation. The *Bgl*II restrictions sites are highlighted in maroon in Table 2.2.

A typical *Bgl*II digestion reaction contained 80 µl of PCR product (which typically yielded *ca.* 10 µg of phagemid DNA), 10 µl of *Bgl*II buffer, and 10 µl or 100 U of *Bgl*II enzyme [≡10 U of enzyme/µg of vector DNA]. This was incubated at 37°C overnight.

2.6.13 Preparation of Chromosomal DNA Inserts

2.6.13.1 Creation of Blunt-ended Chromosomal DNA Fragments

i. Sonication of Chromosomal DNA

100 µg of *C. jejuni* NCTC11168 chromosomal DNA was adjusted to 400 µl in nuclease-free deionised water. This was subjected to sonication in 2-4 s pulses. Aliquots of 2 µl [≡ *ca.* 500 ng] were analysed by agarose gel electrophoresis after each sonication to determine the optimal conditions needed to yield DNA fragments in the size range of 50 and 4,000 bp.

A portion of the sonicated-chromosomal DNA was used immediately in downstream applications, whilst the remainder was separated in one large

well of 0.75% agarose gel. The DNA smear corresponding to DNA fragment sizes of 50 to 4000 bp was carefully excised and replaced into a hole cut in an adjacent part of the gel. The orientation of current flow was reversed and the gel ran for an additional 15 min to re-focus the fragments prior to excision from the gel slice and purification carried out using Qiagen's Gel purification kit.

ii. Polishing of Fragmented Chromosomal DNA

Fragmented chromosomal DNA was subjected to polishing by the Klenow fragment, followed by further polishing with T4 DNA polymerase as described above.

2.6.13.2 Creation of Chromosomal DNA Fragments with Cohesive End-termini

i. Creation of T-tailed Chromosomal DNA Fragments

Blunt-ended or polished fragments of *C. jejuni* NCTC11168 chromosomal DNA were T-tailed to improve ligation efficiency. A typical T-tailing reaction contained 10 µg of polished fragments of *C. jejuni* NCTC11168 chromosomal DNA, 50 U of *Taq* polymerase [≡ 5 U of *Taq* polymerase /µg of DNA], 10 µl of 10 × PCR buffer with 1.5 mM $MgCl_2$, 10 µl of 2 mM dTTP and nuclease-free deionised water to a final volume of 100 µl. Reaction mixtures were incubated at 70°C for 1 h, cleaned up using Qiagen's PCR purification kit and quantified.

ii. *Bfu*CI Digestion of Chromosomal DNA

*Bfu*CI, an isoschizomer of *Sau*3AI, was used to digest chromosomal DNA of *C. jejuni* NCTC 11168. A typical *Bfu*CI restriction digest yielded DNA fragment sizes of between 50 and 4000 bp and contained *ca.* 10 µg of *C. jejuni* NCTC11168 chromosomal DNA, 10 µl of *Bfu*CI [≡ 0.1 U of *Bfu*CI i.e. 0.1U/µg of DNA], 5 µl of 10 × enzyme buffer, and nuclease-free deionised water to a final volume of 50 µl. This was incubated at 37°C for 20 min. The products of several reactions were pooled to obtain sufficient quantities of DNA for gel purification. A portion was directly purified

using a PCR purification kit, and the remainder was size-purified by agarose gel electrophoresis. The smear corresponding to DNA fragments in the size range 300-4000 bp was carefully excised and replaced into a hole cut in an adjacent part of the gel. The orientation of current flow was reversed and the gel ran for an additional 15 min to re-focus the fragments prior to excision from the gel slice and purification using Qiagen's Gel purification kit.

2.6.14 Preparation of Chromosomal DNA Insert Models

The plasmid, pJMK30 (Figure 2.5) was digested with *Eco*RV, *Sma*I and *Bam*HI to create insert models. These insert models were used to mimic chromosomal DNA fragments in optimization of experimental conditions.

2.6.14.1 Creation of Blunt-ended Insert Models

A typical *Eco*RV and *Sma*I digestion reaction contained 10 µg of pJMK30 plasmid DNA, 5 µl 10 × enzyme buffer, 5 µl enzyme [≡50 U of *Eco*RV or *Sma*I i.e. 5U of enzyme/µg of plasmid DNA], and nuclease-free deionised water to a final volume of 50 µl. The reaction mixture was incubated at 37°C overnight and aliquots analysed by agarose gel electrophoresis to ascertain whether there was complete digestion of the plasmid.

The remaining digests were separated by agarose gel electrophoresis and the *ca.* 3.1 kb fragment from the *Eco*RV digestion [pJMK30_*Eco*RV_Fr], and the *ca.* 1.4 kb fragment from the *Sma*I digestion [pJMK30_*Sma*I_Fr] containing the ampicillin and the kanamycin resistance cassettes respectively were carefully excised and purified using Qiagen's Gel purification kit.

To ensure that both chromosomal DNA fragments and pJMK30 fragments were subjected to the same conditions, the resulting DNA of the *Eco*RV or *Sma*I digestion of pJMK30 was polished by the Klenow fragment, followed by further polishing with T4 DNA polymerase as described above.

2.6.14.2 Creation of Insert Model with Cohesive End-termini

i. Creation of T-tailed Insert Model

The blunt-ended or polished DNA fragments from the *Eco*RV digestion [pJMK30_*Eco*RV_Fr] and the *Sma*I digestion [pJMK30_*Sma*I_Fr] were T-tailed to improve ligation efficiency. A typical T-tailing reaction contained 10 µg of polished fragments of pJMK30_*Eco*RV_Fr or pJMK30_*Sma*I_Fr, 50 U of *Taq* polymerase [≡ 5 U of *Taq* polymerase/µg of DNA], 10 µl of 10× PCR buffer with $MgCl_2$, 10 µl of 2 mM dTTP and nuclease-free deionised water to a final volume of 100 µl. This reaction mixture was incubated at 70°C for 1 h and then cleaned up using Qiagen's PCR purification kit, and quantified.

ii. *Bam*HI Digestion of pJMK30 Plasmid

The pJMK30 plasmid DNA was digested with *Bam*HI to yield DNA fragments with *Bfu*CI-compatible end-termini. A typical digestion reaction contained 10 µg of pJMK30 plasmid DNA, 5 µl 10 × enzyme buffer, 5 µl enzyme [≡50 U of *Bam*HI i.e. 5U of enzyme/µg of plasmid DNA], and nuclease-free deionised water to a final volume of 50 µl. The reaction mixture was incubated at 37°C overnight and an aliquot analysed by agarose gel electrophoresis to ascertain that there was complete digestion of the plasmid.

The remaining digest was separated by agarose gel electrophoresis and the *ca.* 1.4-kb fragment [pJMK30_*Bam*HI_Fr] containing the kanamycin resistance cassette was excised and purified using Qiagen's Gel purification kit.

2.6.15 Ligation and Cloning

Several test-ligations were carried out using various combinations of vector and insert prepared as described above, namely,

i. Blunt-ended vector + Blunt-ended inserts
ii. PCR generated Blunt-ended vector + Blunt-ended inserts

iii. A-tailed vector + T-tailed inserts

iv. PCR generated A-tailed vector + T-tailed inserts

v. PCR generated *Bfu*CI ended vector + *Bfu*CI-ended inserts

Ligation reactions were set up with amounts of vector and insert DNA based on the calculation given in box 1. Following initial test ligations, a vector-insert ratio of 1:3 was found to give the best results and was subsequently used in setting up ligation reactions.

Box 1:
Calculation of amounts of DNA needed in a ligation

$$\frac{\text{Amount of Insert DNA, } \chi \text{ (ng)}}{\text{Size of Insert DNA (kb)}} = \frac{\text{Amount of Vector DNA (ng)}}{\text{Size of Vector DNA (kb)}}$$

$$\frac{\chi \text{ (ng) of Insert DNA [cDNA/Kan}^r\text{_Fr]}}{1.5 \text{ kb}} = \frac{200\text{ng of pG8SAET}}{3.4 \text{ kb}}$$

➔ χ = **88ng**

Where, cDNA ≡ the average size of chromosomal DNA insert; ~1.5kb
Kan^r_Fr ≡ pJMK30_*Bam*HI_Fr or pJMK30_*Sma*I_Fr; ~1.5kb

➔ 88ng of insert DNA needed in a ligation in a 1:1 vector-insert ratio.
➔ 264ng of insert DNA needed in a ligation in a 1:3 vector-insert ratio.
➔ 440ng of insert DNA needed in a ligation in a 1:5 vector-insert ratio.

Source: Adopted from Technical Manual of Promega, Part# TM042, Revised 6/99, page 9; Optimizing Insert:Vector Molar Ratios.

A typical ligation reaction, therefore, contained *ca.* 200 ng of linear pG8SAET phagemid vector, *ca.* 264 ng of cDNA/ Kan^r_Fr, 1 µl T4 DNA ligase [≡ 0.5 U of ligase/µg of vector DNA], 2 µl of 10× ligase buffer, 2 µl of 2 mM ATP, and nuclease-free deionised water to a final volume of 20 µl. The reaction mixture was mixed and incubated at room temperature for 2 hours, followed by an overnight incubation at 4°C. It was then purified using the phenol-chloroform-isoamyl alcohol extraction method and then re-suspended in 10 µl of nuclease-free deionised water, for transformation into *E. coli* TG1 cells.

2.6.16 Electrotransformation of *E. coli* TG1 Cells

5 µl of each ligation was used to transform 50 µl of *E. coli* TG1 cells using a BioRad Gene Pulser at 1.7 kV and, resistance and capacitance of 100Ω and 25 µFD respectively. Immediately following electroporation, 950 µl of SOC medium (Invitrogen), pre-warmed to 37°C, was added to the *E. coli* TG1 cells, which were allowed to recover at 37°C for 1 h. Portions of the recovered cells were plated onto LB agar containing 200 µg/ml of ampicillin (LB-Amp agar) or 200 µg/ml of ampicillin and 50 µg/ml of kanamycin (LB-Amp/Kan agar) as appropriate, and incubated at 37°C overnight.

Viable colonies were counted manually, and the number of *E. coli* transformants and the percentage of recombinant colonies or ligation efficiency estimated. With regards to the insert model, Kan^r_Fr, this was estimated by dividing the number of colonies counted on an LB-Amp/Kan agar plate by the number of colonies counted on LB-Amp agar plate and multiplied by 100%.

2.6.17 Construction of *C. jejuni* NCTC11168 Phage Display Library

The library was constructed to cover the whole genome of *C. jejuni* NCTC11168. The number of clones, and hence the adequate number of ligations needed in such an experiment was arrived at based on the calculations in box 2.

Six ligation reactions were set up as described earlier. Following phenol-chloroform-isoamyl alcohol purification and ethanol precipitation, each reaction mix was re-suspended in a final volume of 5 µl and used to electro-transform 50 µl *E. coli* TG1 cells which were pooled and recovered in a total volume of approximately 6 ml SOC medium. 100 µl was plated for determination of the number of transformants and the percentage of transformants containing an insert. A number of colonies were selected for PCR analysis. The remaining 5.9 ml of culture was added to 60 ml of LB broth containing 200 µg/ml of ampicillin (LB-amp broth) in a 250 ml-flask and incubated at 37°C overnight.

Box 2:
Calculation of number of Clones needed for a good Chromosomal DNA Library of *C. jejuni* NCTC11168

A: Using simple reasoning based on

1. Estimated DNA fragment size of *ca.* 300 bp
2. Genome size of *Cj*11168 of 1,641,481 bp

➔ 1,641,481/ 300 = **5472** clones needed to cover genome. **BUT** in theory, only 1 in 18 clones will be in-frame.

➔ **5472** × 18 = **98489** (to make for 1 in 18 being in-frame)
➔ 10 × **98,489** = **984,890** (to make for good coverage of genome)
➔ ~ **1,000,000 Clones**

B: adopting the formula for calculation of above from Jacobsson *et al* (2003),

$$N = \frac{\ln (1 - P)}{\ln (1 - a/b)}$$

Where, P = probability that clone contains insert (0.8)
a = Average size of the DNA fragment (~300bp)
b = Size of genome (**1,641,481 bp** in *Cj*11168)

This implies that the number of clones needed to cover whole genome is:

$$N = \frac{\ln (1-0.8)}{\ln (1-300/1{,}641{,}481)} = \frac{-1.6094379}{-0.0001827}$$

N = **8810**
➔ 8810 × 18 (to make for 1 in 18 being in-frame) = 158,580
➔ 158,580 × 10 (to make for good coverage of genome) = 1,585,800
➔ **~ 1,600,000 Clones**

C: A *C. jejuni* DNA library aimed at anything between 1,000,000 and 1,600,000 clones would be of good genome coverage for the purpose of its construction.

The following day, this culture was divided into two portions and centrifuged briefly to pellet the cells. The supernatant from each portion was discarded and the pellets re-suspend in 5 ml of fresh LB-Amp broth each.

Phage Rescue: The resulting culture was divided into 10 × 1 ml portions and each portion infected with R408 helper phage at a multiplicity of infection of 100 (i.e. 100 µl of 1.3×10^{13} pfu/ml stock). Infection was allowed to proceed at room temperature for 1 h.

To each 1 ml aliquot of R408-infected culture, 25 ml of molten soft agar, pre-cooled to 45°C and containing 200 µg/ml of ampicillin, was added in 50 ml falcon tubes, mixed thoroughly and poured unto LB-Amp agar plates with approximate volume of 5 ml soft agar per plate. This was incubated at 37°C overnight.

Following overnight incubation, the soft agar from the 50 plates was transferred into a 1 L flask containing 250 ml of LB-Amp broth (i.e. 5 ml broth per plate giving a total volume of 500 ml). This was incubated at 37°C for 4 hours with shaking at 200 rpm. This culture was then divided into two portions of 250ml each in centrifuge flasks and centrifuged at 20,000 × *g* for 45 min, and the supernatant poured into fresh sterile tubes.

The supernatant (*ca.* 400 ml) was filtered using 0.45 µl pore sterile Millex® filter unit (Millipore Corp) to remove any remaining bacterial cells. This rescued phage constituted the library and was stored as 1.5-ml aliquots at -80°C.

2.6.18 Determination of Library Indices

i. Estimation of Number of *E. coli* Transformants/Transformation Efficiency

Following an hour's recovery of the transformed *E. coli* TG1 cells, containing g8-chromosomal DNA inserts in SOC medium, a 10-fold serial dilution of a 100-µl aliquot of this culture was made to a final dilution of 10^{-5} and 90 µl of each dilution was plated on LB-Amp agar plates and incubated at 37°C overnight.

The following day, colonies were counted and the number of *E. coli* transformants calculated.

ii. Colony Analysis/Complexity of Library

Several random colonies were selected from the LB-Amp agar plates for PCR analysis to confirm the presence or otherwise and the size of an insert for each clone [see Table 2.2].

Colony analysis PCR reaction mixtures contained 1 µl of 10× PCR buffer with 1.5 mM $MgCl_2$, 1 µl of 2 mM dNTPs, 0.1 µl of µl *Taq* polymerase, 0.2 µl each of primers pG8SAET_F1 and pG8SAET_R1_long, a colony [≡ ~ 0.5 µl] and 6.5 µl nuclease-free deionised water to a final volume of 10 µl.

The PCR machine was programmed for denaturation at 95°C for 2 min, then 30 cycles of 95°C denaturation for 10 s, annealing at 55°C for 30 s, elongation at 72°C for 3 min, and further elongation at 72°C for 8 min.

To increase sensitivity of this colony analysis, selected colonies were grown in 1.5 ml of fresh LB-Amp broth at 37°C overnight. DNA was then extracted from these cultures using Qiagen's MiniPrep kit and eluted in a final volume of 50 µl nuclease-free deionised water. PCR was done as described above using 0.5 µl of a 1 in 100 dilution of each sample as a template. A positive control template of a vector without an insert and a negative control of water were used alongside samples.

The resulting PCR product was resolved by agarose gel electrophoresis as described, and analysed for inserts and insert sizes. The percentage of colonies with inserts was calculated and multiplied by the number of *E. coli* transformants to reveal the complexity of the library.

iii. Titration of Library

A laboratory stock of *E. coli* TG1 was grown in fresh LB broth at 37°C until the optical density at 600 nm was between 0.5 and 0.6. A 90-µl aliquot of this culture was infected with 10 µl of rescued phage in an eppendorf tube. Infection was allowed to proceed at room temperature for 30 min. A 10-fold serial dilution of this was prepared and 90 µl of each dilution plated on LB-Amp agar plates and incubated at 37°C overnight.

The following day, colonies were counted and the number of *E. coli* transformants calculated.

2.7 Results

2.7.1 Purification of DNA

Two methods for DNA purification, phenol-chloroform-isoamyl alcohol with yeast tRNA and QIAquick PCR purification kit, were arrived at after evaluation of the percentage DNA recovered following various other purification procedures (Figure 2.7). QIAquick gel extraction kit was employed in size-selection of chromosomal DNA fragments.

Purification of DNA using QIAquick PCR purification kit could lead to the recovery of up to 91% of the starting amount of DNA, whilst phenol-chloroform-isoamyl alcohol with yeast tRNA led to the recovery of *ca.* 69% of the starting DNA. However, the use of phenol-chloroform-isoamyl alcohol was reserved for the purification of ligation reactions, prior to electrotransformation. This was based on the fact that the latter method eliminated silica which is usually present following column purification, as well as salt, following ligations thus preventing arcing during electroporation.

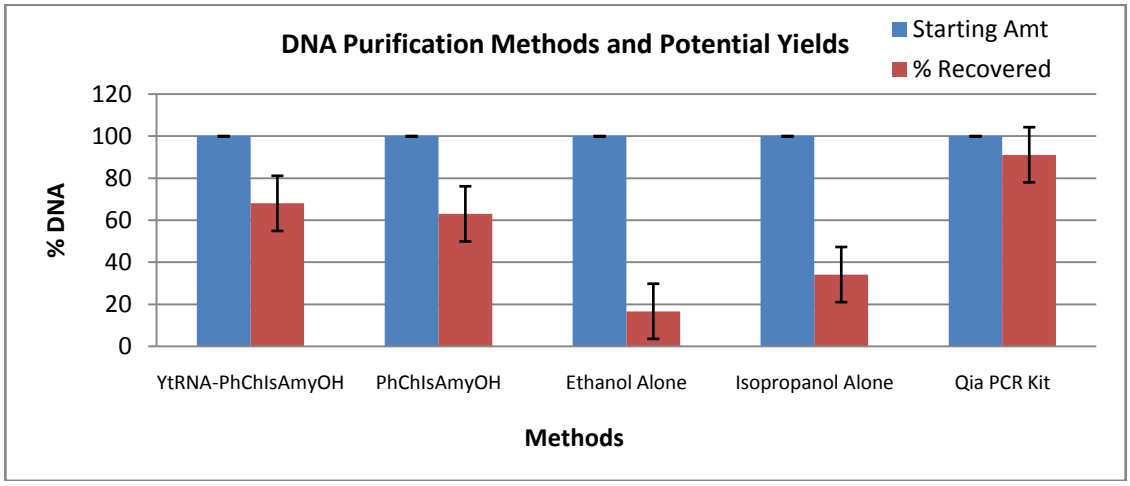

Figure 2. 7 | Efficiency of DNA Purification Methods. DNA preparations were purified using ethanol or iso-propanol alone, phenol-chloroform-isoamyl alcohol without yeast tRNA (PhChIsAmyOH) or with yeast tRNA (YtRNA-PhChIsAmyOH), as well as with the commercial kit QIAquick PCR purification kit (Qiagen). The resulting DNA was quantified and compared with that of the starting DNA.

2.7.2 Linearization of Vector DNA

Different types of the linear form of the vector, pG8SAET, were prepared and used in the preliminary stages of the study. The linear vector, **pG8SAET_V5**, was arrived at for the construction of the library following evaluation of the various forms.

2.7.2.1 Creation of Blunt-ended Linear Vector [pG8SAET_V2]

Two methods were used in creating blunt-ended linear vector in this study, namely, restriction digestion with dephosphorylation and then PCR.

i. *Sna*BI Restriction Digest of Circular Vector

The sequence of plasmid pG8SAET between nucleotides 283 and 336 is shown in Figure 2.8.

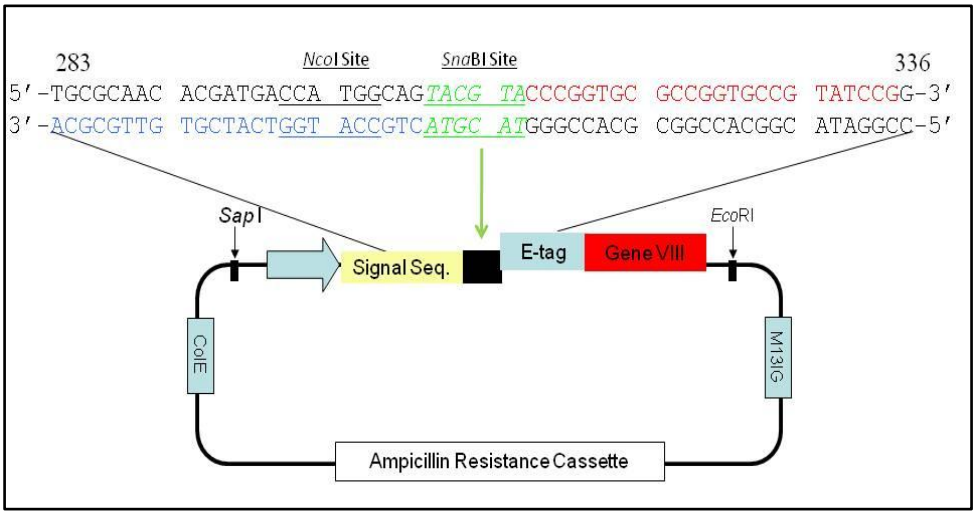

Figure 2. 8 | *Sna*BI Recognition Sequence site in pG8SAET Vector. The DNA sequences used in the design of PCR primers are depicted in blue and red. Nucleotides between positions 1-295 constitute the staphylococcal protein A promoter sequence followed by the signal peptide of the E-tag sequence from position 283 to 336. Between the staphylococcal protein A promoter sequence and the E-tag sequence are two enzyme recognition sites, a *Sna*BI site and an *Nco*I site. Digestion of the circular vector with *Sna*BI, therefore, yielded a blunt-ended linear vector and provided a site for insertion of a foreign DNA sequence [Modified from Jacobsson *et al*., 2003].

Following *Sna*BI digestion of pG8SAET, the linearized vector DNA was dephosphorylated and/or gel purified as described above. The efficiency of digestion was assessed by self-ligating various preparations of the vector

and using these to electro-transform *E. coli* TG1 cells. After plating aliquots of the electrotransformed *E. coli* cells on LB-Amp agar plates and incubating overnight, plates were described as either having colonies (+ve) or not (-ve) the results of which are given in Figure 2.9.

Although, the use of 5 U of *Sna*BI enzyme/µg of vector DNA was considered as yielding the best digest based on the appearance of the product on agarose gel, this yielded a large number of background colonies in all transformations.

To reduce the level of background colonies gel purification of digested vector was employed. This procedure reduced the number of background colonies but it appeared to reduce the ligation efficiency of the vector (Table 2.3).

Furthermore, analyses of a number of individual clones revealed that only 10-15% of them had inserts [see Table 2.5 further in text]. The highest percentage of colonies with insert obtained following further optimization was 44%.

Sample	Description	No. of Colonies (Column Purified)	No. of Colonies (Gel Purified)
Controls			
1	Undigested pG8SAET [+ve Control]	Confluent	Confluent
2	No DNA [-ve Control]	0	0
3	pJMK30 *Eco*RV Fragment	0	0
Ligations			
4	Digested, non-Dephosph Phagemid + Ligase	1260	161
5	Digested, non-Dephosph Phagemid - Ligase	720	70
6	Digested, Dephosph Phagemid + Ligase	312	62
7	Digested, Dephosph Phagemid - Ligase	316	61
8	1:3 pG8SAET:pJMK30_*Eco*RV_Fr + Ligase	301	31
9	1:3 pG8SAET:pJMK30_*Eco*RV_Fr - Ligase	580	42

Table 2. 3 | Colony counts following electrotransformation of different vector preparations and re-ligation and test-ligation with pJMK30_*Eco*RV_Fr. The highest percentage of insert obtained in this experiment was 15%.

Due to the low efficiency and high background colonies, vector preparation using *Sna*BI restriction digest, followed by dephosphorylation and/or gel purification was abandoned, and a new method that employed PCR adopted, as described earlier.

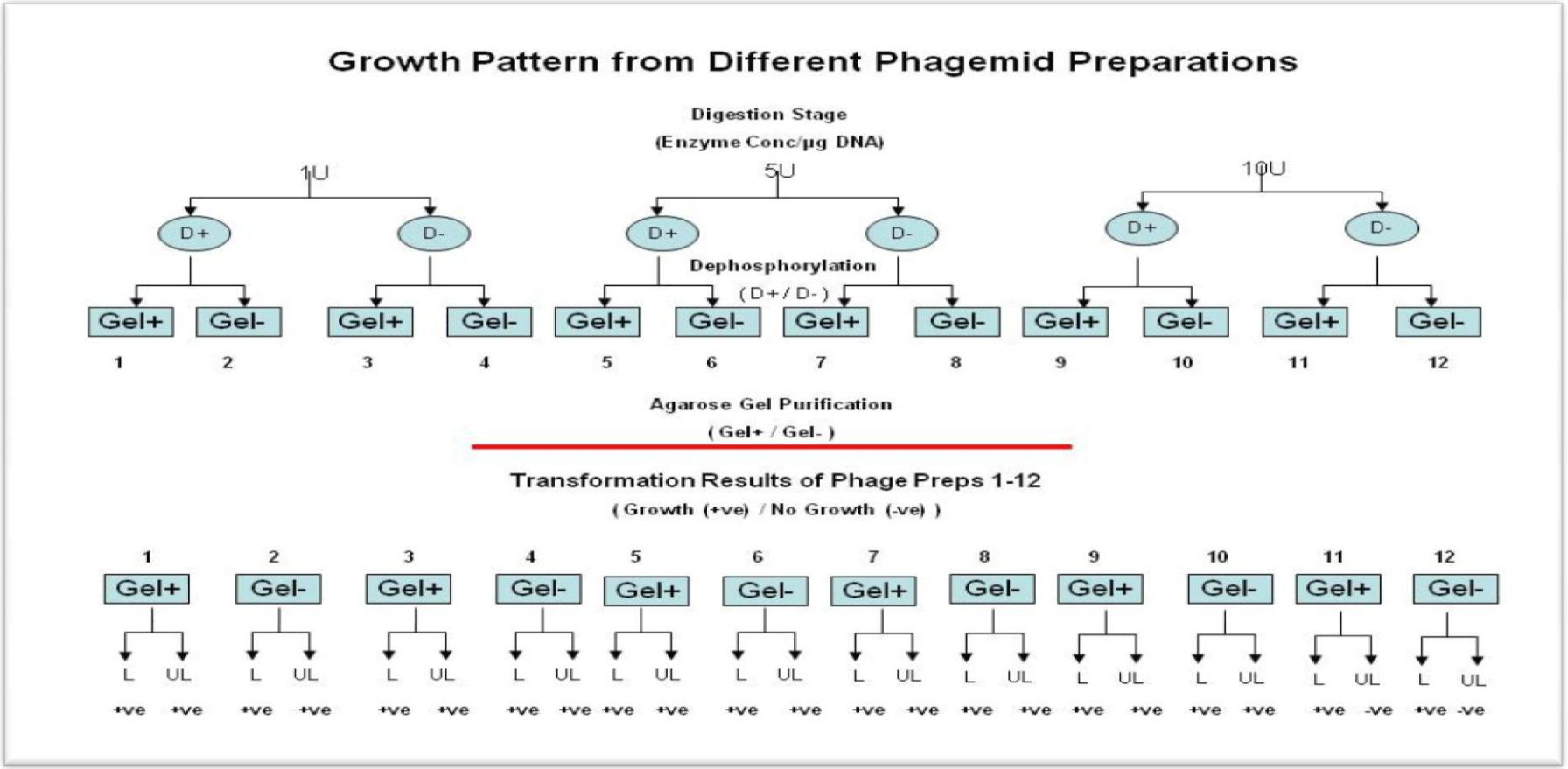

Figure 2. 9 | Linearization and purification of pG8SAET. The circular vector was digested with 1U, 5U and 10U of *Sna*BI enzyme/µg of vector DNA and half of the preparation dephosphorylated. The resulting DNA was either gel purified (Gel+) or used directly in a re-ligation experiment (L). These ligations were purified and transformed into *E. coli* TG1 cells, and assessed for background colonies (+ve/-ve). Complementary un-ligated DNA preparations (UL) were also transformed for comparison.

ii. PCR of Digested Vector to Produce Blunt ends [pG8SAET_V3]

A blunt-ended linear vector was obtained (Figure 2.10) by PCR amplification using an aliquot of a *Sna*BI-digested pG8SAET as a template, alongside the Expand™ HiFi PCR System and the primers pG8SAET_F1 and pG8SAET_R1, as described. The forward and reverse primers are highlighted as red and blue respectively in the vector sequence below (Figure 2.10).

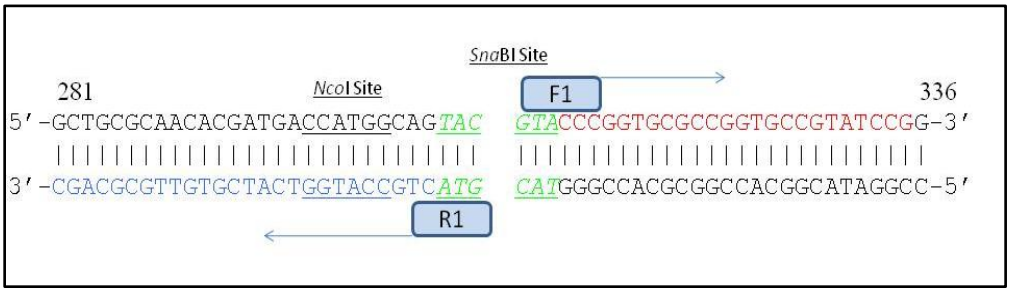

Figure 2. 10 | PCR Amplification of Vector using Primers pG8SAET_F1/R1. Primer sequences are highlighted in red and blue.

The resulting PCR product was test-ligated and used to electro-transform *E. coli* TG1 cells. These were assessed for background colonies of undigested vector by plating a 100-µl of serially diluted cells on LB-Amp agar plates and incubating overnight. As shown in Table 2.4, it was observed that amplification of the vector by PCR greatly reduced the number of background colonies resulting from uncut vector. The results further proved that the smallest amounts of uncut vector DNA have the potential to be propagated in electrotransformation, given that PCR that used an uncut vector as a template simply produced enormous number of colonies as shown below.

Sample	Description	No. of Colonies
Controls		
1	Undigested pG8SAET [+ve Control]	Confluent
2	No DNA [-ve Control]	0
3	pG8SAET [PCR with uncut template]	428
4	pG8SAET [PCR with cut template]	6
5	pJMK30_*Eco*RV_Fr	0
Ligations		
6	pG8SAET [PCR with uncut template] + Ligase	212
7	pG8SAET [PCR with uncut template] - Ligase	201
8	pG8SAET [PCR with cut template] + Ligase	7
9	pG8SAET [PCR with cut template] - Ligase	4
10	pG8SAET [PCR with uncut template]+pJMK30_*Sma*I_Fr +Ligase	221
11	pG8SAET [PCR with uncut template]+pJMK30_*Eco*RV_Fr +Ligase	198
12	pG8SAET [PCR with cut template] + pJMK30_*Sma*I_Fr + Ligase	9
13	pG8SAET [PCR with cut template] + pJMK30_*Eco*RV_Fr + Ligase	9

Table 2. 4 | Colony counts following Electrotransformation of different Vector Preparations using PCR and Re-ligation and Test-ligation with pJMK30_*Eco*RV_Fr.

Despite the reduction in the number of background colonies following vector preparation by PCR, colony analysis revealed that only 20-30% of colonies had inserts (data not shown). The highest percentage of colonies with insert obtained after further optimization was 44% [see Table 2.5 below].

2.7.2.2 Creation of A-Tailed Linear Vector [pG8SAET_V4]

*Sna*BI-digested linear vector and PCR-amplified, dephosphorylated vector DNA preparations were A-tailed [**pG8SAET_V4a**] by incubating with dATP and *Taq* polymerase as described above. Alternatively, using a *Taq* Polymerase (Roche) and primers pG8SAET_F1 and pG8SAET_R1, the *Sna*BI-digested vector was A-tailed through PCR [**pG8SAET_V4b**]. This PCR took advantage of the property of *Taq* polymerase to add an additional non-template-paired nucleotide to the 3' end of PCR products, and this nucleotide is normally A.

Test ligations with complementary T-tailed inserts of pJMK30_*Eco*RV_Fr gave similar results to those obtained with the blunt-ended vector. Colony analysis indicated that only 20-30% of clones contained an insert (data not shown).

2.7.2.3 Linear Vector with Cohesive End-termini [pG8SAET_V5]

i. Primers were designed to amplify a linear form of the vector incorporating a *Bfu*CI/*Bgl*II recognition site from an aliquot of the *Sna*BI-digested vector as described (Figure 2.11).

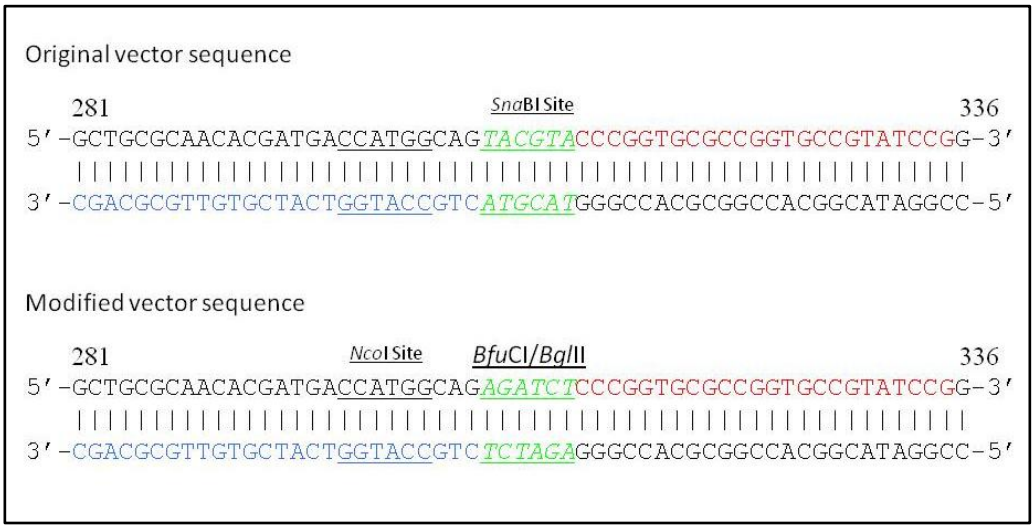

Figure 2. 11 | Modification of *Sna*BI Recognition Site of pG8SAET to *Bfu*CI/*Bgl*II Site. A *Sna*BI-digested vector was PCR amplified with primers containing *Bgl*II restriction sites, which after digestion with *Bgl*II and dephosphorylation yielded a linear vector with *Bfu*CI site.

A linear vector with *Bfu*CI/*Bgl*II recognition site was obtained (Figure 2.13) by PCR amplification using an aliquot of a *Sna*BI-digested pG8SAET as a template, alongside the Expand^{TM} HiFi PCR System and the primers pG8SAET_F2 and pG8SAET_R2, as described. The forward and reverse primers are highlighted as red and blue respectively in the vector sequences (Figures 2.11 and 2.12).

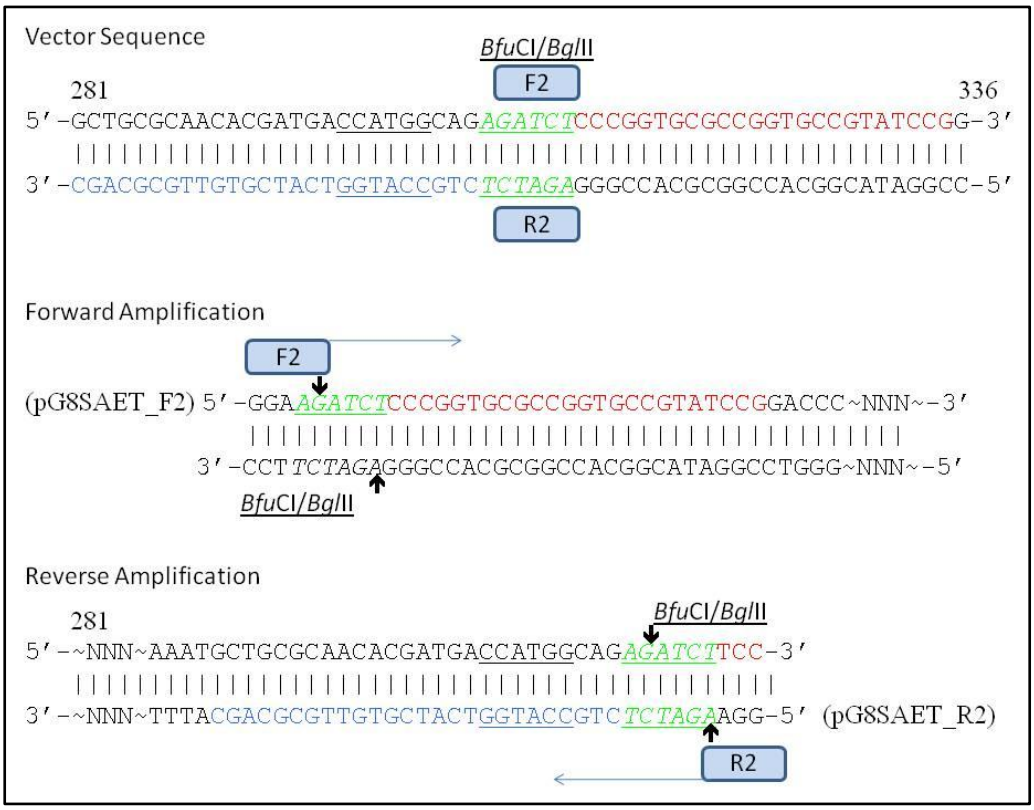

Figure 2. 12 | PCR Amplification of Vector using Primers pG8SAET_F2/R2. Primer sequences are highlighted in red and blue. A *Sna*BI-digested vector was PCR amplified with these primers containing *Bgl*II restriction sites, which after digestion with *Bgl*II and dephosphorylation yielded a linear vector with *Bfu*CI site.

ii. Following PCR amplification of the vector the primer ends were digested using *Bgl*II restriction enzyme to yield *Bfu*CI-compatible ends, to enable insertion of *Bfu*CI-digested chromosomal DNA fragments.

The different vector preparations were analysed by agarose gel electrophoresis and are shown in Figure 2.14.

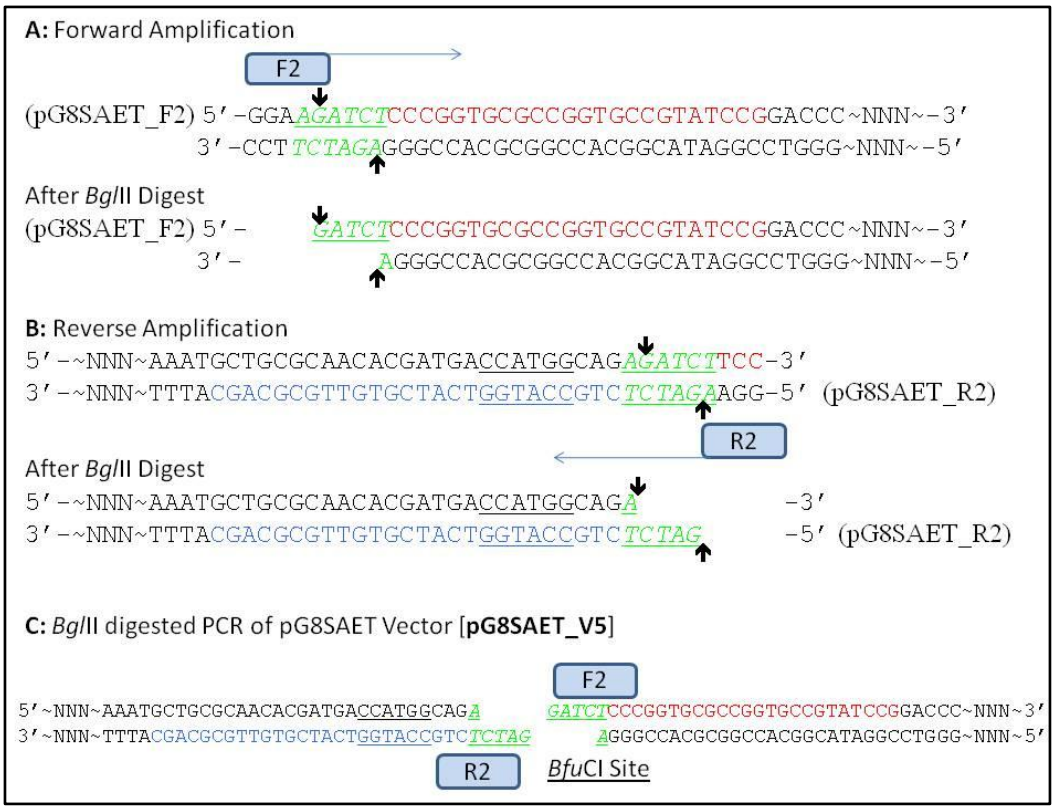

Figure 2. 13 | Linear vector with *Bfu*CI-end termini. Following PCR amplification of vector using primers pG8SAET_F2 and pG8SAET_R2, the PCR product was subjected to *Bgl*II digestion yielding a linear vector with *Bfu*CI end-termini. Primer sequences are highlighted in red and blue.

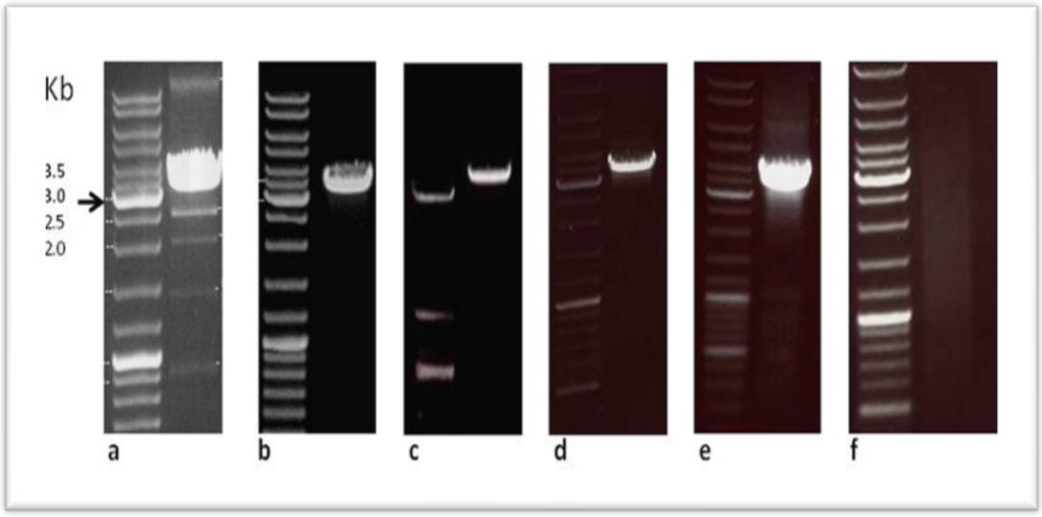

Figure 2. 14 | Agarose gel electrophoresis photo of DNA preparations of pG8SAET vector; following (a) Extraction from *E. coli* cells (*ca.* 3.5 kb), (b) after *Sna*BI digestion (c) after blunt-ended PCR using *Sna*BI-digested phagemid as template, (d) after PCR using an undigested phagemid as template, (e) after *Bfu*CI/*Bgl*II-ended PCR using *Sna*BI-digested phagemid as template and (f) nested PCR using a blunt-ended PCR product as template. The same marker was used in the different experiments.

2.7.3 Preparation of Chromosomal DNA Inserts

2.7.3.1 Creation of Blunt-ended Chromosomal DNA Fragments

i. Sonication of Chromosomal DNA and Polishing

C. jejuni NCTC11168 chromosomal DNA (cDNA) was fragmented by sonication as described above to obtain average insert sizes of 50 to 4,000 bp and the fragments resolved using agarose gel electrophoresis to determine the DNA fragment size range (Figure 2.15). Samples with fragments considered too large were sonicated again, while those containing fragments that were too small were discarded.

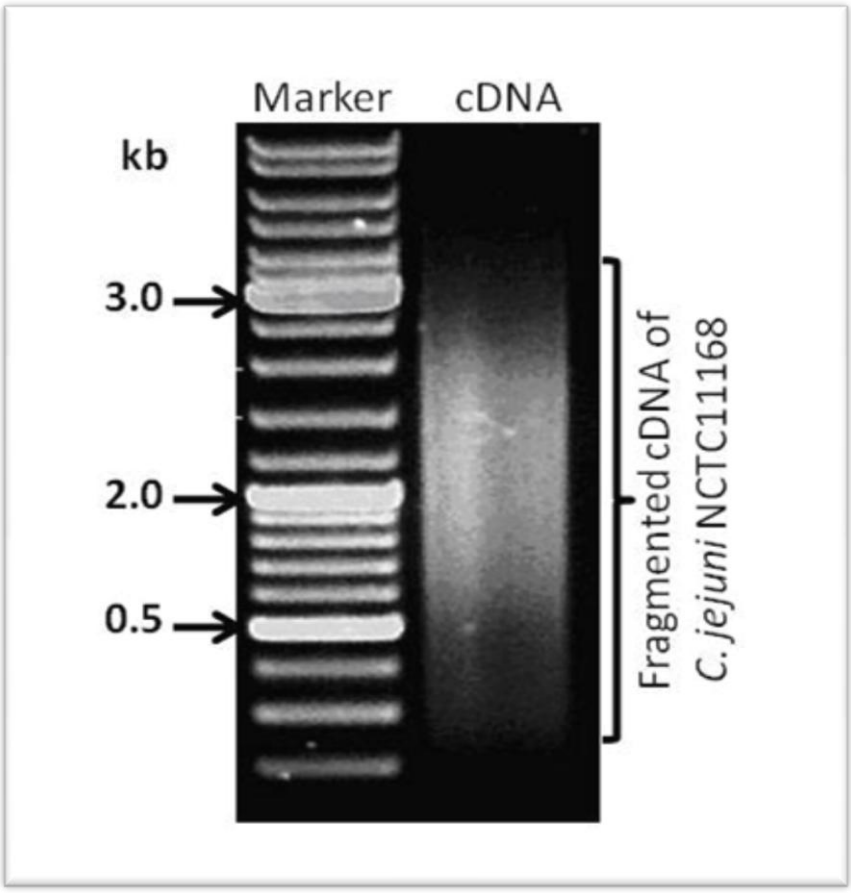

Figure 2. 15 | Agarose gel electrophoresis photo of sonicated cDNA of *C. jejuni* NCTC11168. Though the initial fragment sizes obtained ranged from 50 to 3,000 bp, much of it was between 500 and 3000 bp.

The sonicated chromosomal DNA was divided into two and a portion blunted immediately whilst the other portion was purified using QIAquick gel extraction kit as described above before blunting. The chromosomal DNA fragments were blunted by the Klenow fragment, followed by further polishing with T4 DNA polymerase as described above.

2.7.3.2 Creation of Chromosomal DNA Fragments with Cohesive End-termini

i. Creation of T-tailed Chromosomal DNA Fragments

The blunt-ended or polished fragments of *C. jejuni* NCTC11168 chromosomal DNA were T-tailed to improve ligation efficiency.

ii. *Bfu*CI Digestion of Chromosomal DNA

As described above, *Bfu*CI, an isoschizomer of *Sau*3AI, was used to digest chromosomal DNA of *C. jejuni* NCTC11168 to give DNA fragments with cohesive *Bfu*CI end-termini, as depicted in Figure 2.16. An enzyme-DNA concentration of 0.1 U/µg yielded the best usable fragments with sizes ranging between 50 and 6,000 bp (Figure 2.17). The digested chromosomal DNA was divided into two and a portion purified immediately using QIAquick PCR purification kit whilst the other portion was purified using QIAquick gel extraction kit as described above.

Figure 2. 16 | *Bfu*CI digestion of cDNA to yield fragments with *Bfu*CI compatible end-termini.

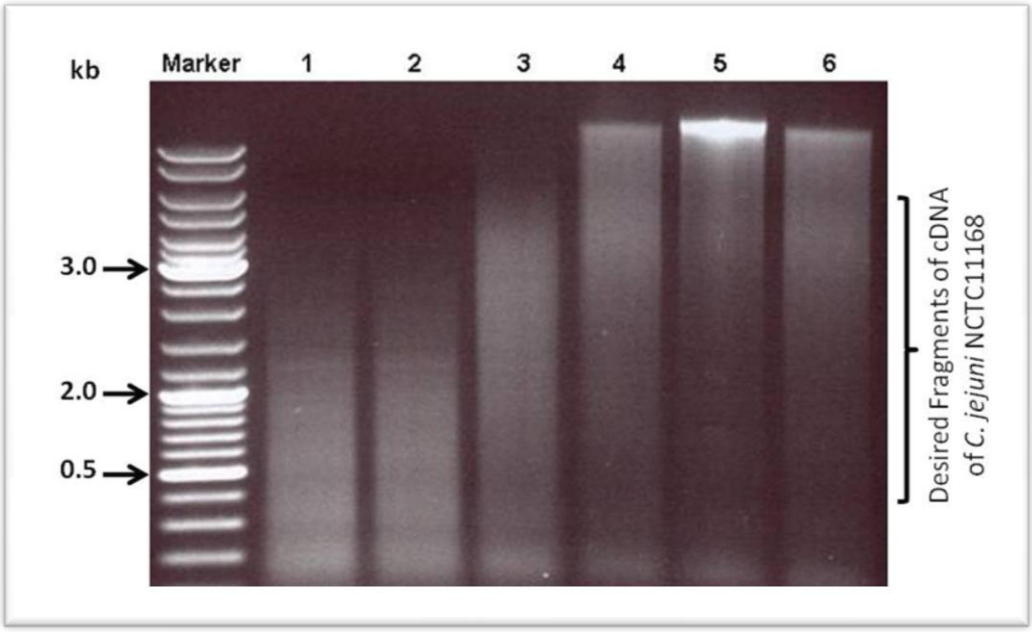

Figure 2. 17 | Agarose gel electrophoresis photo of cDNA digestion using serial dilutions of *Bfu*CI. Each 20-µl reaction volume contained *ca*. 1 µg of cDNA. 1: 0.4U, 2: 0.2U, 3: 0.1U, 4: 0.05U, 5: 0.025U, 6: 0.25U. An enzyme-DNA concentration of 0.1 U/µg was used in all subsequent reactions. Though the fragment sizes obtained ranged from 50 to 6,000 bp, gel purification was employed to select for sizes between 500 and 3000 bp.

2.7.4 Preparation of Chromosomal DNA Insert Models

The plasmid, pJMK30, containing a kanamycin resistance cassette, *aphA-3*, was selected for use to create a model to mimic chromosomal DNA fragments in the experimental stages of the study.

2.7.4.1 Creation of Blunt-ended Insert Models

i. Blunt-ended insert models were obtained through restriction digestion of pJMK30 with *Eco*RV and *Sma*I. *Eco*RV digestion yielded two blunt-ended fragments with approximate sizes of 3160 and 1025 bp, whilst *Sma*I digestion yielded two blunt-ended fragments with approximate sizes 2834 and 1359 bp [see Figure 2.6 and Figure 2.18]. The fragments of interest were the 1025-bp and the 1359-bp fragments from *Eco*RV and *Sma*I digestions [pJMK30_*Eco*RV_Fr and pJMK30_*Sma*I_Fr], the latter containing the kanamycin resistance cassette. Although the pJMK30_*Eco*RV_Fr did not contain a kanamycin resistance cassette, it was used as in sert model due to its smaller size.

ii. Polishing of *Eco*RV or *Sma*I Fragment of pJMK30

In order to prevent self-ligation of the *Eco*RV and *Sma*I digested fragments [pJMK30_*Eco*RV_Fr and pJMK30_*Sma*I_Fr], they were polished as previously described to produce blunt-ended fragments.

2.7.4.2 Creation of Insert Model with Cohesive End-termini

i. Creation of T-tailed Insert Model

The blunt-ended or polished pJMK30 fragments from the *Eco*RV and the *Sma*I digestion [pJMK30_*Eco*RV_Fr and pJMK30_*Sma*I_Fr] were T-tailed to improve ligation efficiency.

ii. *Bam*HI Digestion of pJMK30

Using *Bam*HI, pJMK30 was digested to yield two fragments with approximate sizes of 2834 and 1359 bp [see Figure 2.6 and Figure 2.18] with cohesive *Bfu*CI compatible end-termini as depicted in Figure 2.16. The smaller 1359-bp fragment containing the Kan^r cassette was used (Figure 2.18, 1).

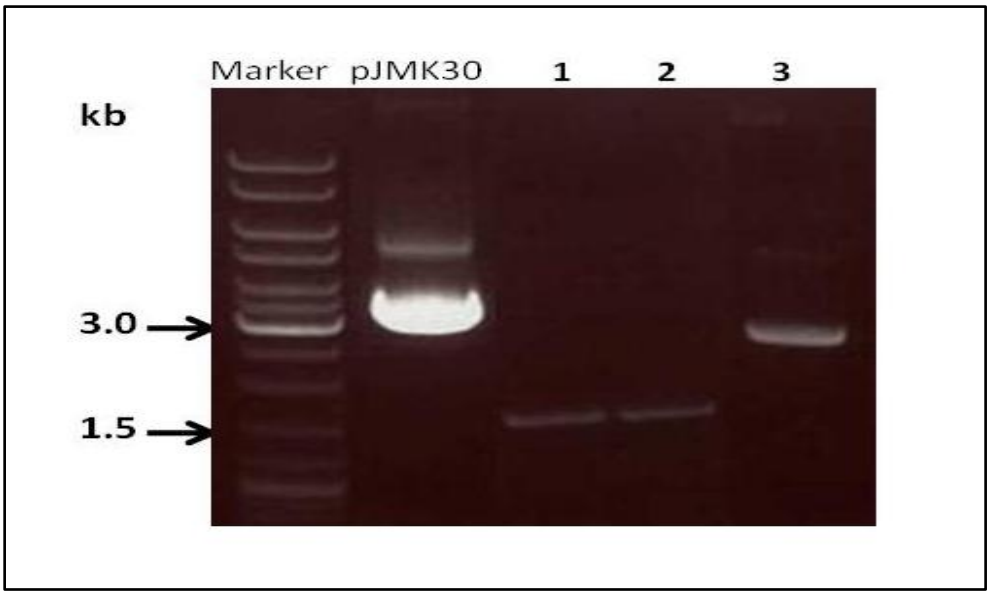

Figure 2. 18 | Agarose gel photo of pJMK30 and fragments. Purified fragments were (1) pJMK_*Bam*HI_Fr, (2) pJMK_*Sma*I_Fr and (3) pJMK_*Eco*RV_Fr, following restriction digest with *Bam*HI, *Sma*I and *Eco*RV respectively.

2.7.5 Test Ligations

Various combinations of vector and insert preparations as previously described, are given below.

i. **pG8SAET_V2** + Blunt-ended inserts
ii. **pG8SAET_V3** + Blunt-ended inserts
iii. **pG8SAET_V4a** + T-tailed inserts
iv. **pG8SAET_V4b** + T-tailed inserts
v. **pG8SAET_V5** + *Bfu*CI-ended inserts

Ligation reactions were set up with amounts of vector and insert DNA based on the calculation given in box 1. Following several test ligations, a vector-insert ratio of 1:3 was found to give best results and was subsequently used in setting up ligation reactions.

As described earlier, each single ligation reaction was re-suspended in 10 µl of nuclease-free deionised water after purification and 5 µl of the resulting DNA transformed into 50 µl of *E. coli* TG1 cells. After an hour's recovery in SOC medium, a 100-µl aliquot of the serially diluted transformed *E. coli* TG1 cells was plated on LB agar containing 200 µg/ml of ampicillin (LB-Amp agar) or 200 µg/ml of ampicillin and 50 µg/ml of kanamycin (LB-Amp/Kan agar) as appropriate, and incubated at 37°C overnight. Viable colonies were counted manually to estimate the number of *E. coli* transformants and the percentage of recombinant clones.

Following several ligation experiments, the highest obtainable percentage insert rate, using blunt-ended vector and blunt-ended inserts, was 16% (Table 2.5). Ligations using blunt-ended vector obtained from *Sna*BI digest [pG8SAET_V2] produced as low as 10% insert rate. T-tailing of inserts and A-tailing of the vector led to an improvement in ligation efficiency; the highest percentage insert rate obtained was 44%.

Using *Bfu*CI-ended vector and *Bfu*CI-ended inserts led to a significant increase in the ligation efficiency, yielding as high as 100% insert rate in several instances, with the least being 90% [see Table 2.6]. The ligation efficiency following this methodology of DNA preparation was also readily

visible on agarose gels when the reaction mixtures were resolved by electrophoresis (Figure 2.19). This method was therefore chosen for the construction of the *C. jejuni* NCTC11168 phage display libraries.

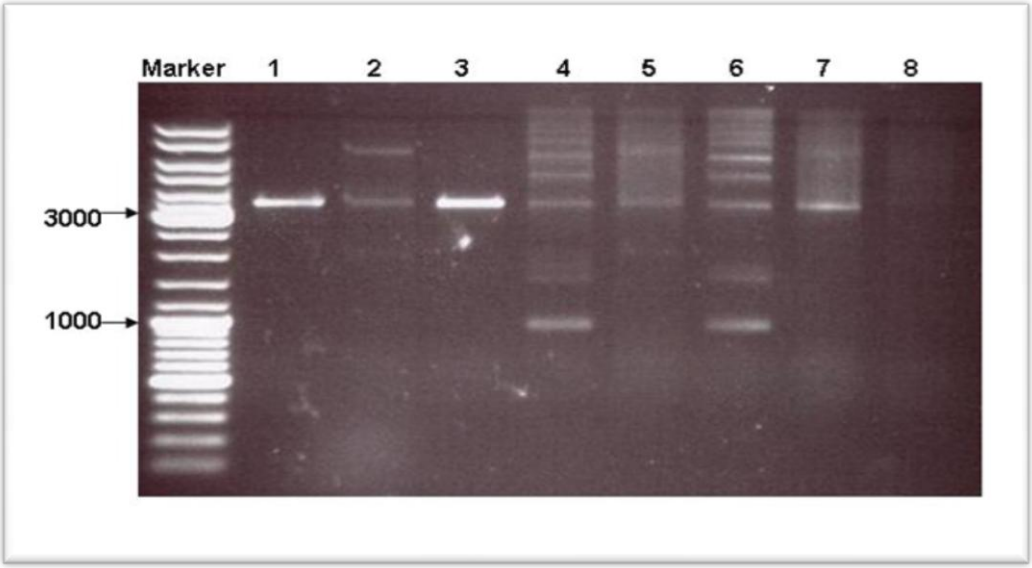

Figure 2. 19 | Agarose gel electrophoresis photo of test ligations using *Bfu*CI-compatible vector and inserts. 1: pG8SAET PCR product, before treatment with *Bgl*II does not self-ligate. 2: But self-ligates following treatment with *Bgl*II. 3: Dephosphorylation after treatment with *Bgl*II prevents the PCR of the vector from self-ligating. 4&5: Although some ligation is observed following *Bgl*II treatment of PCR-generated vector in the presence of pJMK30_*Bam*HI fragments or *Bfu*CI-digested cDNA fragments, the ligation is even better following dephosphorylation (6&7). 8: *Bfu*CI-digested cDNA.

Sample	Description	No. of Colonies Amp Plates	No. of Col Amp/Kan Plates	Percentage (%) Insert
Controls				
1	No DNA [-ve Control]	0	0	
2	pG8SAET_V1 [+ve Control of undigested pG8SAET]	Confluent	0	
3	pG8SAET_V2 [*Sna*BI-digested, Blunt-ended vector]	122	0	
4	pG8SAET_V3 [PCR-generated, Blunt-ended vector]	8	0	
5	pG8SAET_V4a [A-tailed pG8SAET_V3]	4	0	
6	pG8SAET_V4b [PCR-generated, A-tailed vector]	2	0	
7	pG8SAET_V5 [PCR-generated, *Bgl*II-digested vector]	4	0	
8	pJMK30_*Sma*I_Fr	2	2	
9	Blunt-ended cDNA Fragments	0	0	
10	pJMK30_*Bam*HI_Fr	2	1	
11	*Bfu*CI-ended cDNA Fragments	0	0	
Ligations				
12	pG8SAET_V2 + pJMK30_*Sma*I_Fr	132	13	10
13	pG8SAET_V2 + Blunt-ended cDNA Fragments	137	0	14
14	pG8SAET_V3 + pJMK30_*Sma*I_Fr	111	15	15
15	pG8SAET_V3 + Blunt-ended cDNA Fragments	109	0	16
16	pG8SAET_V4a + T-tailed pJMK30_*Sma*I_Fr	122	29	24
17	pG8SAET_V4a + T-tailed pJMK30_*Eco*RV_Fr	108	2	18
18	pG8SAET_V4a + T-tailed cDNA Fragments	126	0	20
19	pG8SAET_V4b + T-tailed pJMK30_*Sma*I_Fr	138	61	44
21	pG8SAET_V4b + T-tailed pJMK30_*Eco*RV_Fr	117	1	22
22	pG8SAET_V4b + T-tailed cDNA Fragments	134	0	32
23	pG8SAET_V5 + pJMK30_*Bam*HI_Fr	194	195	100
24	pG8SAET_V5 + *Bfu*CI-ended cDNA Fragments	188	0	95

Table 2. 5 | Colony counts following electrotransformation *E. coli* TG1 cells with test-ligations using different vector and insert preparations. Apart from the original *Sna*BI-digested vector, pG8SAET_V2, all vector preparations used in the above test-ligations were PCR-generated to reduce background colonies resulting from uncut vector as previously discussed. The presence of an insert was determined using PCR or the number of colonies on an Amp/Kan agar plate in the case of *Sma*I_Fr.

2.7.6 Parallel Libraries

To ascertain that the use of *Bfu*CI-ended vector and *Bfu*CI-ended inserts would yield libraries of sufficient complexities, parallel libraries were constructed using chromosomal DNA of *Neisseria meningitidis* MC58, *Helicobacter pylori* J99, as well as chromosomal DNA of *C. jejuni* NCTC11168, and their indices determined. As can be seen in Table 2.6, the transformation rates were almost the same for size-selected DNA preparations and column purified DNA preparations that included very small fragments of DNA as well as larger ones. However, the percentage insert rates or ligation efficiencies were higher for the former DNA preparations than for the latter. The resulting libraries from both forms of DNA preparations had very high titres of 10^{11} comparable to published data (Mullen *et al.*, 2007a; Zhu *et al.*, 2008).

Libraries	No. of *E. coli* Transformants (cfu/ml)	Percentage Insert %	Complexity (cfu/ml x %Ins)	Titre (pfu/ml)
1 : *N. Meningitidis* MC58				
(Column Purified DNA)	5.0×10^6	90	4.5×10^6	--
(Gel Purified DNA)	2.0×10^6	98	2.0×10^6	--
2: *H. pylori* J99				
(Column Purified DNA)	5.4×10^7	90	4.9×10^7	--
(Gel Purified DNA)	5.5×10^6	100	5.5×10^6	--
3: *C. jejuni* NCTC11168				
(Column Purified DNA)	2.7×10^7	96	2.6×10^7	2.0×10^{12}
(Gel Purified DNA)	4.4×10^6	100	4.4×10^6	2.6×10^{11}
4: *C. jejuni* NCTC11168				
(Column Purified DNA)	3.22×10^7	90	2.9×10^7	2.6×10^{11}
(Gel Purified DNA)	2.30×10^7	100	2.3×10^7	2.1×10^{11}

Table 2. 6 | Indices of Parallel Libraries using *Bfu*CI-ended vector and *Bfu*CI-digested chromosomal DNAs of *N. meningitidis* MC58, *H. pylori* J99, and *C. jejuni* NCTC1168.

2.7.7 Construction of *C. jejuni* NCTC11168 Phage Display Libraries

Two libraries, *Cj*11168 Library I and *Cj*11168 Library II, were constructed from *Bfu*CI-digested chromosomal DNA of *C. jejuni* NCTC11168. Whereas the DNA used in *Cj*11168 Library I was simply column-purified with QIAquick PCR purification kit without size-selection, the DNA used in *Cj*11168 Library II was gel-purified selecting for fragment sizes of 300-3000 bp. Each covered the whole genome of *C. jejuni* NCTC11168 several times

over. To determine the adequate number of ligations needed in such an experiment the following calculations were used:

i. Estimated number of Clones needed in this library

From the preliminary experiments, each 20 µl of ligation reaction contained 200 ng of phage vector and 264 ng of chromosomal DNA. Following clean up and re-suspension in a final volume of 10 µl, 5µl of this was used to transform 50 µl of *E. coli* TG1 cells and 950 µl of SOC medium was added to enable cells to recover. It was determined that 100 µl of this culture yielded approximately 18,000 colonies, which implied that 1,000 µl of culture would give 10 × 18,000 colonies i.e. 180,000. It could therefore be inferred that one ligation mixture of 10 µl would give twice as many colonies i.e. 2 × 180,000 [360,000] colonies or clones. To obtain 1,000,000 or 1,600,000 clones based on the calculations in box 2 above, **3 - 4 ligations** were needed to give 3 × 360, 000 **[1,080,000]** and 4 × 360, 000 **[1,800,000]** colonies.

Six ligation reactions were however set up for each library to ensure that each covered the entire genome of *C. jejuni* NCTC11168.

2.7.8 Determination of Library Indices

i. Estimation of Number of *E. coli* Transformants/Transformation Efficiency

A 10-fold serial dilution of the electrotransformed *E. coli* TG1 cells was plated on LB-Amp agar plates and incubated at 37°C overnight. Colonies were counted manually and the number of *E. coli* transformants determined.

The transformation efficiency for *Cj*11168 Library I was 3.42×10^6, whilst that of *Cj*11168 Library II was 2.13×10^6. These conformed to published data.

ii. Colony Analysis/Complexity of Libraries

The DNAs of 24 and 23 randomly selected clones from each of libraries I and II respectively were analysed for the presence or otherwise of an insert, the size of the insert, and whether or not the inserts were of *C. jejuni* NCTC11168.

Whilst the percentage insert rate for *Cj*11168 Library I was 95.8%, with an average insert size of *ca.* 600 (Figure 2.20), that of *Cj*11168 Library II was 100% with average insert size of *ca.* 1250 (Figure 2.21). The complexities of the libraries were calculated as the products of the percentage insert and the number of *E. coli* transformants i.e. the ratio of *E. coli* transformants carrying inserts. These are given in Table 2.7 below.

By sequencing 10 randomly selected clones from each of *Cj*11168 Library I and *Cj*11168 Library II, all the inserts were confirmed to have 100% identity with *C. jejuni* NCTC11168 by BLAST searching the bacterial genome database. All 20 clones matched different genes, indicating the insert diversity of the libraries.

iii. Titration of Libraries

Following infection of 90-µl aliquot of *E. coli* TG1 with 10 µl of rescued phage, a 10-fold serial dilution of this was carried out and 90 µl of each dilution plated on LB-Amp agar plates incubated at 37°C overnight. Colonies were counted for each dilution and the titres of each library determined.

The titre for *Cj*11168 Library I was 3.0×10^{11}, whilst that of *Cj*11168 Library II was 3.3×10^{11}. These conformed to published data (Mullen *et al.*, 2007a; Zhu *et al.*, 2008).

Phage Display Libraries of *C. jejuni* NCTC11168	No. of *E. coli* Transformants (cfu/ml)	Percentage Insert (%)	Complexity (cfu/ml × %Ins)	Titre of Libraries (pfu/ml)	Average Insert Size (bp)
Library I (Col Purified DNA)	3.42×10^7	95.8	3.28×10^7	3.0×10^{11}	611
Library II (Gel Purified DNA)	**2.13×10^7**	**100**	**2.13×10^7**	**3.3×10^{11}**	1247

Table 2. 7 | Summary of Library Indices of *Cj*11168 Library I and *Cj*11168 Library II. Library I was constructed from chromosomal DNA fragments that were not size-selected and hence contained small as well as large fragments, whilst library II was constructed from chromosomal DNA fragments that were size-selected using agarose gel extraction.

Figure 2. 20 | Agarose gel photo of PCR of 24 randomly selected clones of ***Cj*11168 Library I**. Chromosomal DNA used in the construction of this library was not size-purified and therefore contained some very small inserts. The analysis also shows that only one clone had no insert i.e. 95.8% insert rate. There were two clones with inserts as large as 2,500 bp.

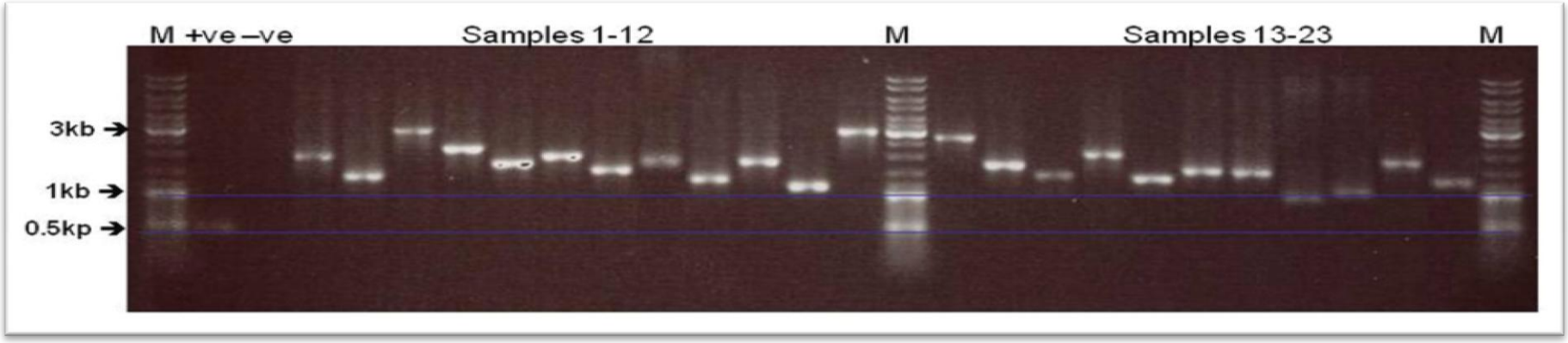

Figure 2. 21 | Agarose gel photo of PCR of 23 randomly selected clones of ***Cj*11168 Library II**. Chromosomal DNA used in the construction of this library was size-purified and therefore contained very large inserts of 500 to 2,500 bp. The analysis also shows that all the clones had inserts i.e. 100% insert rate.

2.8 Discussion

The phage display libraries, *Cj*11168 Library I and *Cj*11168 Library II, were constructed in the phagemid pG8SAET vector (Jacobsson *et al.*, 2003). The construction of phage display libraries involves meticulous manipulation of DNA, cloning and transformation of *E. coli* cells, the latter also involving the use of appropriately purified DNA. It was therefore imperative that DNA extraction and purification methods were employed to maximize DNA yield, eliminate salt and silica contaminants following enzymatic reactions to ensure efficient cloning and transformations.

The columns of the QIAquick PCR purification kit (Qiagen) can hold up to 10 µg of DNA according to the manufacturer's manual. Thus, using a starting DNA amount of *ca.* 10 µg, 91% of this was recoverable. The same amounts of DNA were employed in the other purification methods, facilitating comparison of the several methods employed in the construction of the libraries. The amount of recoverable DNA following the use of ethanol alone or iso-propanol alone was minimal necessitating the use of phenol-chloroform-isoamyl alcohol, which provided an aqueous inter-phase into which DNA aggregated for onward precipitation. The use of phenol-chloroform-isoamyl alcohol with yeast tRNA therefore proved superior to the other precipitation methods.

In order to insert a foreign DNA the circular vector, pG8SAET, needed to be linearized and processed such that self-ligation was minimised. This proved to be more difficult than anticipated. Several methods were assessed to arrive at a methodology that eliminated background colonies resulting from uncut phagemid to an acceptable level. Based on the appearance of the digested product on agarose gel, 5 U of *Sna*BI enzyme/µg of vector DNA incubated at 37°C overnight resulted in an optimal digest, whilst longer incubation periods or increased amount of enzyme resulted in star activity confirming that any restriction endonuclease can be made to cleave non-canonical sites under certain extreme conditions (Nasri & Thomas, 1986).

The resulting product was not devoid of background colonies from uncut phagemid, making this method unfit for the required purposes. Although

purification of the digested vector using QIAquick gel extraction kit led to a reduction in the level of background colonies, it also reduced the ligation efficiency of the vector [see Table 2.3 and Figure 2.9]. This led us to resort to the use of PCR in amplifying the linear vector. As depicted in Table 2.4, PCR amplification of the vector using *Sna*BI-digested DNA led to the reduction of background colonies, whilst using uncut vector DNA still gave a large number of background colonies.

Following elimination of the background colonies from uncut phagemid, blunt-ended ligation with chromosomal DNA fragments resulted in a very low ligation efficiency. This therefore warranted the manipulation of the linear vector and the chromosomal DNA inserts to maximize ligation efficiency. Whereas A-tailing of the linearized vector improved the ligation efficiency to 20-44% over the blunt-ended vector (Table 2.5), this was still significantly lower than ligation efficiencies reported by others (Jacobsson *et al.*, 2003; Mullen *et al.*, 2007b).

Forthwith, the *Sna*BI restriction site within the original vector sequence was manipulated by PCR to produce a *Bfu*CI restriction site. Ligations using such a *Bfu*CI-ended vector with chromosomal DNA fragments with compatible end-termini resulted in efficiencies of between 90 and 100% [see Figure 2.19 and Table 2.5]. This high ligation efficiency using *Bfu*CI compatible vector and insert was further proven in the construction of other libraries that used chromosomal DNA from *N. meningitidis* MC58, *H. pylori* J99 and *C. jejuni* NCTC11168 (see Table 2.6). However, given that restriction endonucleases can only cut where enzyme sites exist, employing restriction digested DNA fragments in this kind of library would mean that some genes within the genome may never be represented as expressed proteins in the library.

In panning experiments, true affinity selection is manifested by the isolation of clones with different inserts but emanating from the same gene i.e. overlapping sequences (Jacobsson *et al.*, 2003; Mullen *et al.*, 2007b). Isolation of overlapping sequences could be analysed to localise the binding domain of a gene to a specific ligand (Bjerketorp *et al.*, 2004; Zhang *et al.*, 2007b). However, with the use of restriction digested DNA fragments in this kind of

library, it may be theoretically impossible to obtain overlapping sequences in panning experiments.

To overcome these disadvantages, two libraries were constructed; *Cj*11168 Library I, which used column-purified DNA fragments made up of a wide range of different sizes from 50 to 4000 bp and *Cj*11168 Library II, which used gel-purified DNA fragments made up of sizes ranging between 300 and 3000 bp, with each covering the genome of *C. jejuni* NCTC11168 several times over.

The high insert rates in these phage display libraries of *C. jejuni* NCTC11168, together with the high complexities and high titres, are indicative of good genomic libraries (Jacobsson *et al.*, 2003; Mullen *et al.*, 2007b). Given that no such library has been described for *C. jejuni*, these will serve as rich resources for further host-pathogen protein interaction studies of *C. jejuni* for years to come.

Chapter Three

Panning of Phage Library of *C. jejuni* NCTC11168

Identification of Host-pathogen Interacting Molecules of *Campylobacter jejuni* using Phage Display Technology and in silico Sequence Analysis

CHAPTER THREE

3.0 Panning of Phage Library of *C. jejuni* NCTC11168

3.1 Introduction

Screening of phage display libraries is done through a technique called "panning" in which phage displaying different peptides or proteins are incubated with a ligand of interest and then bound phage eluted, amplified and analysed (Azzazy & Highsmith, 2002; Mullen *et al.*, 2007a; Newton & Deutscher, 2008). As depicted in Figure 3.1, panning is conceptually simple but can be fraught with technical difficulties that can render it cumbersome (Kehoe & Kay, 2005).

In theory, only one cycle of panning is ideal to select for phage particles that bind to the ligand of interest, but in practice the binding of non-specific phage particles places limitations on the use of single cycle enrichment (Azzazy & Highsmith, 2002; Kehoe & Kay, 2005). Thus, two or three rounds of panning may be necessary in selecting for true ligand-specific phage particles.

This chapter describes the panning of the phage display library of *C. jejuni* NCTC11168. Various modifications that have been applied to the basic principle of panning for screening phage display-libraries have also been discussed.

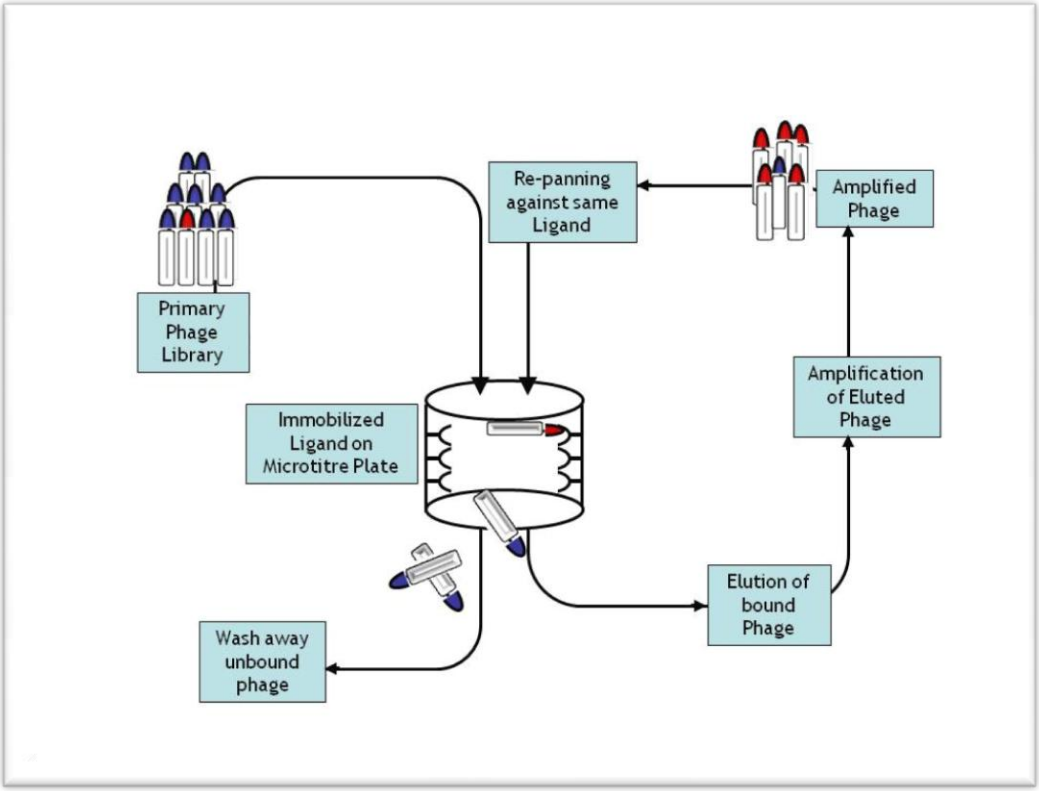

Figure 3. 1 | A Schematic representation of panning of phage-display library. Phage displaying different peptides or proteins are incubated with a ligand of interest, and then washed to remove any unbound phage particles. The bound phage particles are then eluted and amplified in an appropriate *E. coli* host. The process is repeated as needed to enrich clones that specifically bind to the ligand of interest [Drawing based on principle from Azzazy & Highsmith, 2002; Mullen *et al*, 2007].

3.1.1 Panning against Immobilized Ligands

Many researchers have performed panning of phage display libraries by immobilizing the ligands of interest, dissolved in appropriate buffer, on a plastic surface such as immunotubes (Maxisorb tubes, Nunc) or ELISA plates (Jacobsson & Frykberg, 2001a; Mullen *et al.*, 2007a). Phage particles are then added and allowed to interact with the immobilized ligand over time. The immunotubes or ELISA plates are then washed to discard non-binding clones, whilst specific binders are eluted by lowering the starting pH and then amplified in *E. coli* cells. Using affinity columns charged with an appropriate ligand, phage display libraries can be screened by passing the phage particles through these columns (Azzazy & Highsmith, 2002). The columns are then washed to discard non-binding clones, whilst specific binders are eluted and amplified in *E. coli* cells.

Despite its widespread use, immobilization of ligands on solid surfaces may affect the conformational integrity of the ligand. Thus, phage-displayed peptides or proteins that may bind to the ligand in this form may subsequently not bind the protein in its native form (Azzazy & Highsmith, 2002).

3.1.2 Panning against Ligands in Solution

To overcome the conformational changes associated with immobilization of ligands on solid surfaces, panning of phage display libraries can also be carried out in solution (Azzazy & Highsmith, 2002). The ligand can be biotinylated and incubated with the phage particles. The phage particles bound to the biotinylated-ligand are then recovered using streptavidin-coated paramagnetic beads and subsequently dissociated from the ligand.

Although this method eliminates the need for several rounds of panning, it can be limited by the presence or isolation of other peptides that may specifically bind to streptavidin.

3.1.3 Panning against Cells

Otherwise known as bio-panning, ligands present on cells can be employed in panning a library for peptides or proteins that bind to such molecules. This can be useful in selecting for antibodies or adhesion molecules to specific markers on cell surfaces from phage display libraries (Azzazy & Highsmith, 2002).

Typically, the phage library is incubated on monolayers of cells adherent to tissue culture flasks or in suspension with cells. The unbound phage particles are washed away by rinsing the tissue culture flask or by centrifugation of cell suspension, and then specifically binding phage isolated for subsequent infection of the appropriate *E. coli* strains.

To increase the sensitivity of this methodology, a competitive binding technique may be used, in which a small number of ligand-positive cells is diluted with an excess of ligand-negative "absorber" cells before the phage particles are added. The absorber cells serve as a sink for non-specific binders, thereby increasing the specificity of the panning for highly specific binders of the ligand-positive cells.

3.1.4 *In vivo* Panning

Injecting phage-displayed particles directly into live animals and examining tissues of the slaughtered animals for bound phage is another methodology used in panning libraries (Trepel *et al.*, 2008). This is best applied in natural peptide libraries, especially antibody libraries (Pasqualini & Ruoslahti, 1996a; Pasqualini & Ruoslahti, 1996b; Yao *et al.*, 2005). One advantage of this approach is that there is an inherent blocking step where phage-displayed peptides that recognise ubiquitous plasma and cell surface proteins are eliminated before reaching the targets of interest, thus, these targets are not missed (Trepel *et al.*, 2002). Furthermore, the binding-peptides may provide useful information on host-pathogen interactions and may lead to the identification of new drugs and drug receptors (Christianson *et al.*, 2007).

In summary, the principles of panning of phage display libraries may seem simple, but they cannot be said to have no practical difficulties since different laboratories have experienced and reported numerous problems (Azzazy & Highsmith, 2002). In many instances, pannings have failed to enrich for highly specific binders, resulting in recovery of only low-affinity peptides (Mullen *et al.*, 2007a). In many cases, comparison of insert sizes between different cycles of panning has shown that the fraction of phage with full-size inserts decreased following amplification since phage with smaller inserts tend to outgrow the former in mixed cultures (de Bruin *et al.*, 1999).

The panning of phage display library of *C. jejuni* NCTC11168 described here was done against immobilized ligands.

3.2 Selection of Ligands

3.2.1 Iron acquisition in *C. jejuni*

Iron is essential in several metabolic pathways of bacteria, including *C. jejuni*, but much of it is available in the human host as Fe^{3+} complex with host iron-binding and transport proteins such as haemoglobin (Hb), transferrin (Tf), and lactoferrin (Lf) (Payne & Finkelstein, 1978; Perez-Perez & Israel, 2000). In fact, excess free iron results in cellular damage through the formation of

harmful free radicals through the Haber-Weiss and Fenton reactions (Ratledge & Dover, 2000; Wally & Buchanan, 2007).

Bacterial pathogens can sequester iron through: (i) release of low-molecular-weight siderophores; (ii) active haem uptake; and (iii) host iron-binding and transport proteins (Parkkinen *et al.*, 2002). Thus, with iron mostly in complex with host iron-binding and transport proteins it is mostly accessed by bacteria through specialised iron uptake systems (Andrews *et al.*, 2003).

The importance of iron to *Campylobacter* has long been well-emphasised. *Campylobacter* cells grown in low-iron medium have been documented to exhibit slower growth rates and altered cellular morphology, as well as reduced toxin production (Field *et al.*, 1986; McCardell *et al.*, 1986). This importance is further emphasised by the presence of genes encoding multiple iron acquisition systems as well as detoxification systems indicating the central role that iron plays in *Campylobacter* gene regulation and virulence (Holmes *et al.*, 2005; van Vliet *et al.*, 2002).

As stated earlier, the three well-known uptake systems in the *C. jejuni* genome are: (i) a haem/haemoglobulin uptake system called *chuABCD*; (ii) a periplasmic binding-protein-dependent transport (PBT) system for the uptake of a ferric enterochelin (*cfrA* and *ceuBCDE*); and (iii) a ferric siderophore uptake system, *fhuABD* (Galindo *et al.*, 2001; Palyada *et al.*, 2004; Richardson & Park, 1995; Ridley *et al.*, 2006). The two predicted but yet to be characterized iron uptake systems are Cj1658-Cj1663 and Cj0173c-Cj0178 (Parkhill *et al.*, 2000). There has been some interest in the *C. jejuni* iron transporter operon akin to the *E. coli feoAB* operon for some time. The recent demonstration by Naikare *et al.* that FeoB is actually involved in ferrous iron acquisition in *C. jejuni*, contributing significantly to colonization of the gastrointestinal tract during both commensal and infectious relationships has rekindle our interest in this operon although these findings contradicts a previous assertion by Raphael and Jones that this operon played no role in iron acquisition (Naikare *et al.*, 2006; Raphael & Joens, 2003).

The response of the organism to environmental changes in iron concentrations is believed to be regulated by two paralogous genes, the ferric uptake regulator

(*fur*) gene and the peroxide repressor (*perR*) gene (Holmes *et al.*, 2005; van Vliet *et al.*, 1999; Wooldridge *et al.*, 1994).

Furthermore, the lipoprotein component of the *ceuBCDE* operon, encoded by *ceuE,* is thought to confer a haemolytic phenotype thus complicating our understanding of iron acquisition in *C. jejuni* (Park & Richardson, 1995).

It is therefore imperative that *Campylobacter* researchers sought further clarification on the mechanism of iron acquisition in the organism.

Although several pathogenic organisms including *H. pylori* and *N. meningitidis* have been reported to utilise iron bound to lactoferrin it has previously been thought that this iron source cannot be used by *C. jejuni* (Husson *et al.*, 1993; Pickett *et al.*, 1992). However, Miller and co-workers have recently shown that there is bacterial growth following supplementation of iron-deficient medium MEM with Lf-bound and Tf-bound iron, which is comparable to supplementation with $FeSO_4$ (Miller *et al.*, 2008). Mutation of an 84.1-kDa outer membrane protein gene, Cj0178, previously thought to be a probable siderophore, led to a significant reduction in growth in the presence of Lf-bound and Tf-bound iron, suggesting that the product of this gene may be involved in Lf or Tf binding for iron uptake, and possibly contributing to *C. jejuni* pathogenesis (Miller *et al.*, 2008; Palyada *et al.*, 2004).

Since the growth promoting effect of Lf is not completely abolished in Cj0178 mutants, it is likely that other, as yet unidentified, proteins may also be involved in the acquisition of iron from Lf in *C. jejuni* (Miller *et al.*, 2008). Cogan *et al.*, showed that exposure of *Campylobacter* to the mammalian neuroendocrine hormone norepinephrine causes an increase in the virulence-associated properties of the organism (Cogan *et al.*, 2007). Since norepinephrine is known to be capable of supplying iron for bacterial growth in the presence of Tf or Lf, it can be hypothesised that *Campylobacter* made use of Lf in Cogan's experiments (Anderson & Armstrong, 2008; Cogan *et al.*, 2007; Freestone *et al.*, 2000). Indeed, as *C. jejuni* is a mucosal pathogen, it is likely to rely on Lf for iron acquisition.

For these reasons, therefore, Lf was chosen as a ligand to pan the *C. jejuni* NCTC11168 phage display library. Lf represented an ideal ligand because the

C. jejuni gene, Cj0178, is a likely candidate protein that would provide a target for potential validation of the panning against Lf, in an attempt to identify other uncharacterised *Campylobacter* proteins that may be involved in binding to Lf, and hence in *C. jejuni* iron acquisition.

Tf, Fn and BSA were also used as control ligands in validation of the library and optimisation of the panning protocol.

3.2.2 Lactoferrin

Lactoferrin (Lf), belonging to the transferrin family of proteins, is a non-haem iron-binding protein that shares the same properties with serum Tf, ovotransferrin and melanotransferrin (Gonzalez-Chavez *et al.*, 2008; Gray-Owen & Schryvers, 1996; Singh *et al.*, 2002). It is an 80-kDa glycosylated protein of approximately 700 amino acids, with a simple folding structure of two symmetrical lobes, N and C, connected by a hinge (Legrand *et al.*, 2008). Each lobe is made up of α-helix and β-pleated sheets, with each capable of binding to an Fe^{2+} or Fe^{3+}, but also to Cu^{2+}, Zn^{2+} and Mn^{2+} (Gonzalez-Chavez *et al.*, 2008). It has a homology of 33-41% among different species, with the asparagine residues in both the N- and C-terminal lobes providing sites for *N*-glycosylation of the protein (Baker & Baker, 2009; Tomita *et al.*, 2009).

Due to its ability to bind reversibly to Fe^{3+}, Lf can exist free of the metal (apo-Lf) or in complex with it (holo-Lf) changing the three-dimensional conformation in so doing (Pierce & Legrand, 2009). Whereas apo-Lf has an open conformation, holo-Lf is closed and hence resistant to proteolysis (Legrand *et al.*, 2008).

Ubiquitous in most mammalian secretions, Lf is a glycoprotein produced by mucosal epithelial cells and is abundant in milk and gastrointestinal fluids including bile and saliva (Pierce & Legrand, 2009). Lf is also found in significant amounts in secondary neutrophils, tear, semen, vaginal fluid, urine, amniotic fluid, plasma and is most abundant in milk and colostrum, playing various physiological roles such as regulation of iron absorption in the bowel, immune response and anti-microbial activity (Gonzalez-Chavez *et al.*, 2008; Ochoa & Cleary, 2009; Puddu *et al.*, 2009; Wong *et al.*, 2009).

Aside its main function of transporting iron, Lf is also known for its anti-microbial, anti-carcinogenic, and enzymatic activities both *in vitro* and *in vivo*, as well as for its immunomodulatory and anti-inflammatory roles (Iigo *et al.*, 2009; Jenssen & Hancock, 2009; Leon-Sicairos *et al.*, 2009; Li *et al.*, 2009; Mohan & Abrams, 2009; Ochoa & Cleary, 2009; Paesano *et al.*, 2009; Puddu *et al.*, 2009; Wong *et al.*, 2009; Yen *et al.*, 2009). It is believed that the anti-microbial effect of Lf is achieved through two main mechanisms, namely, sequestration of iron at sites of infection, depriving micro-organisms of this nutrient which subsequently leads to a "bacteriostatic-like" effect, and interaction of the positive amino acids in Lf with anionic molecules on some bacterial, fungal, and parasite surfaces causing cell lysis (Gonzalez-Chavez *et al.*, 2008; Jenssen & Hancock, 2009; Leon-Sicairos *et al.*, 2009; Mohan & Abrams, 2009; Ochoa & Cleary, 2009; Onishi *et al.*, 2008). However, the interaction of the highly cationic lactoferricin moiety of Lf with the anionic surface structures of some organisms are known to inhibit the anti-microbial effect of Lf, enabling them to utilise Lf-bound iron (Senkovich *et al.*, 2007).

It is therefore not surprising that despite its wide range of anti-microbial activity, several micro-organisms including *Helicobacter pylori*, *Prevotella nigrescens*, *Clostridium* species have long been known to utilise Lf as an iron source (de Lillo & Fierro, 1997; Dhaenens *et al.*, 1999; Husson *et al.*, 1993; Jarosik & Land, 2000; Tomita *et al.*, 1998). It is also purported that Lf has a positive influence on the growth of *L. acidophilus* and *Bifidobacterium* species (Kim *et al.*, 2004). Other pathogens known to utilise Lf iron include *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus* species, *Bordetella* species, *Treponema* species, *Gardnerella vaginalis*, and *Tritrichomonas foetus* (Fang & Oliver, 1999; Grab *et al.*, 2001; Hammerschmidt *et al.*, 1999; Jarosik & Land, 2000; Menozzi *et al.*, 1991; Naidu *et al.*, 1992; Park *et al.*, 2002a; Schryvers & Morris, 1988; Schryvers, 1989; Staggs *et al.*, 1994). Other organisms also known to utilise Lf iron include *Enterobacter sakazakii* and *Entamoeba histolytica* (Kim *et al.*, 2002; Leon-Sicairos *et al.*, 2005; Wakabayashi *et al.*, 2008). Recently, two membrane proteins of the parasite, *Toxoplasma gondii*, have been described as being involved in Lf-binding and acquisition of iron (Dziadek *et al.*, 2007).

3.2.3 Transferrin

Transferrin (Tf), like Lf, is also a non-haem iron-binding protein of the transferrin protein family with a molecular weight of *ca.* 79 kDa (Gomme *et al.*, 2005). In the mammalian body, Tf functions as an iron transporter from sites of iron absorption and sites of iron recycling to all tissues involved in erythropoiesis or active cell division (Macedo & de Sousa, 2008). Again, Tf can exist free of Fe^{3+} (apo-Tf) or in complex with it (holo-Tf) changing the three-dimensional conformation in so doing (Hirayama *et al.*, 1990).

By binding to holo-Tf via bacterial Tf-like receptors, microbial pathogens can sequester iron through Tf (Parkkinen *et al.*, 2002). Although the utilisation of Lf- and Tf-bound iron has been described in several pathogenic organisms, this was not the case for *C. jejuni* until Miller *et al.* recently reported their observation in support of this (Miller *et al.*, 2008).

By virtue of the close similarity of Tf to Lf in its structure and function, it was also chosen as a target ligand for screening the library.

3.2.4 Fibronectin

Fibronectin (Fn) is a larger glycoprotein with a molecular weight of *ca.* 440 kDa that serves as a general cell adhesion molecule to anchor cells to collagen or proteoglycan substrates (Pankov & Yamada, 2002). A component of the extracellular matrix (ECM), Fn can mediate cellular interaction with the ECM through its binding to membrane-bound Fn receptors on the surfaces of numerous pathogens including *Campylobacter* (Graham *et al.*, 2008).

C. jejuni was shown to bind to Fn through a 37-kDa outer-membrane protein, CadF (*Campylobacter* adhesion to Fn) in the late 1990s (Konkel *et al.*, 1997). CadF has since been shown to mediate the binding of *Campylobacter* species to Fn, and has also been utilised in the identification of both *C. jejuni* and *C. coli* (Konkel *et al.*, 1999a; Ziprin *et al.*, 1999).

Prior to the identification of CadF, several studies had shown that *C. jejuni* was capable of binding to ECM and Fn (Kuusela *et al.*, 1989; Moser & Schroder, 1995; Moser *et al.*, 1997). Following its identification, CadF has

been shown to be not only necessary for adherence of *Campylobacter* to enterocytes, but also required for internalization of the organism (Graham *et al.*, 2008; Krause-Gruszczynska *et al.*, 2007; Monteville *et al.*, 2003). In fact, the native form, as well as truncated form, of this Fn-binding protein of *Campylobacter* has been expressed and the Fn-binding domain identified (Konkel *et al.*, 2005; Mamelli *et al.*, 2006).

Based on the above evidence, Fn was selected as one of the ligands for the panning experiments described in this chapter, purely to serve as a positive control, since it was expected to pick up all or parts of the *cadF* gene.

3.2.5 Bovine Serum Albumin

Bovine serum albumin (BSA), which is used in numerous biochemical and molecular biology applications including ELISAs and which has a molecular weight of *ca.* 66.4 kDa (Walcher *et al.*, 2003), was used as a blocking agent to block uncoated portions of the immuno-wells after coating with ligands. It was therefore also added to the phage library before incubation with the ligand to prevent non-specific binding. Initial panning against this protein also provided invaluable information in the optimisation of the panning procedure.

3.3 Materials and Methods

3.3.1 General Materials

3.3.1.1 Phage Library

Construction of the *C. jejuni* NCTC11168 phage display libraries used for the panning experiments described in this chapter is described in chapter two.

3.3.1.2 Bacterial Strains and Culture conditions

[See Section 2.6.1 above]

3.3.1.3 R408 Helper Phage

[See Section 2.6.2 above]

3.3.1.4 Antibiotic Supplements

[See Section 2.6.3 above]

3.3.1.5 Culture Media

[See Section 2.6.4 above]

3.3.1.6 Miscellaneous Buffers and Solutions

[See Section 2.6.5 above]

3.3.1.7 Polymerase Chain Reaction

[See Section 2.6.8 above]

3.3.2 Ligands

For the purposes of this study, all ligands used were of human source or origin. Lactoferrin (apo- and holo-Lf), holo transferrin (holo-Tf) and fibronectin (Fn) were obtained from Sigma, and were dissolved in sodium carbonate buffer.

3.3.3 ELISA Wells and Tubes

MaxiSorp ImmunoTubes® and MaxiSorp ImmunoWells® (Nunc) were both used as solid surfaces for immobilsing the ligands.

3.3.4 Growth stage of *E. coli* TG1 cells and Infectivity of Phage particles

To ensure reproducibility of amplification and titration of eluted and amplified phage, the growth stage of *E. coli* TG1 cells at which maximal phage infection can be obtained was determined. *E. coli* TG1 cells were grown to an optical density at 600 nm (OD_{600}) of 1.0. This was serially diluted with LB broth and grown for a further 4 h at 37°C with shaking until OD_{600} of between 0.3 and 1.0 were obtained. A 90-µl aliquot of each was infected with 10 µl of the phage library. Infection was allowed to proceed at room temperature for 30 min. A 10-fold serial dilution of this was prepared and 90 µl of each dilution plated on LB-Amp agar plates and incubated at 37°C overnight. The following day, the numbers of colonies of *E. coli* transformants were counted and the phage titres calculated.

3.3.5 Effect of Lf on *in vitro* infectivity of Phage particles

As discussed above, Lf is also known for its antiviral activity and to ensure that this did not affect the results of the panning of the phage library against Lf its effect on *in vitro* infectivity of phage particles was tested (Jenssen & Hancock, 2009; Leon-Sicairos *et al.*, 2009). A 180-µl aliquot of the phage library was incubated at room temperature for 2 h with 100 µl of apo- or holo-Lf dissolved in 50 mM sodium carbonate, pH 9.7, to a final concentration of 1, 10, and 100 µg/ml. This was further incubated for 1 h with 100 µl of 20 × blocking solution [i.e. 1% BSA in PBS-0.05% Tween 20 (PBS-T)] to yield a mixture containing 1% BSA.

A 10-µl aliquot of this mixture was used to infect 90 µl of freshly grown *E. coli* TG1 cells. As control, 9 µl of the phage library was used to infect 90 µl of freshly grown *E. coli* TG1 cells with optical density at 600 nm (OD_{600}) of 0.5-0.6. Infection was allowed to proceed at room temperature for 30 min. A 10-fold serial dilution of each infection was carried out and 90 µl of each dilution plated on LB-Amp agar plates and then incubated at 37°C overnight. The next day, colonies were counted for each dilution and the titres estimated.

3.3.6 Effect of Lf on *in vitro* growth of *E. coli* TG1 cells

As discussed above, Lf is also known for its bactericidal activity and to ensure that this did not affect the results of the panning of the phage library against Lf its effect on *in vitro* growth of *E. coli* TG1 cells was tested (Leon-Sicairos *et al.*, 2009). A 90-µl aliquot of freshly grown *E. coli* TG1 cells (OD_{600} of 0.5-0.6) was incubated at room temperature for 2 h with apo- or holo-Lf dissolved in 50 mM sodium carbonate, pH 9.7, to a final concentration of 1, 10, and 100 µg/ml. This culture was mixed with 20 × blocking solution to yield a mixture containing 1% BSA and further incubated for 1 h.

The resulting mixture containing *E. coli* TG1 cells was infected with 9 µl of the phage library. As control, 9 µl of the phage library was used to infect 90 µl of freshly grown *E. coli* TG1 cells. Infection was allowed to proceed at room temperature for 30 min. A 10-fold serial dilution of each infection was carried out and 90 µl of each dilution plated on LB-Amp agar plates and then incubated at 37°C overnight. The next day, colonies were counted for each dilution and the number of transformed *E. coli* TG1 cells estimated.

3.3.7 Panning

3.3.7.1 Preparation of Target Ligands and Phage

A solution of the protein ligand [apo-Lf, holo-Lf, apo-Tf, Fn or BSA] was made up in 50 mM sodium carbonate, pH 9.7 to a final concentration of 10-100 µg/ml and stored at 4°C until used.

200 µl of 10 × blocking solution was added to 1,800 µl of the phage library to give a mixture containing 1% BSA. This was incubated at room temperature for 1 h before being added to the ligand-coated wells.

3.3.7.2 The Panning Procedure

100-µl aliquots of the appropriate ligand (100 µg/ml of ligand dissolved in 50 mM sodium carbonate, pH 9.7) were immobilised in wells of ELISA-ready 96-well plates (Nunc™, Denmark) as described earlier and incubated at 4°C overnight.

The following day, wells were washed once with PBS and then blocked using 200 µl of blocking solution. Wells were incubated at room temperature for 1 h.

A 100-µl aliquot of phage library in blocking solution was then added to the wells and incubated at 4°C overnight. Unbound phage particles were removed by washing the wells three times with PBS–T, followed by two additional washes with PBS.

Bound phage particles were eluted by adding 100 µl of freshly grown *E. coli* TG1 cells [OD_{600} of 0.5-0.6], and incubating at 37°C for 1 h. *E. coli* TG1 cells were serially diluted and 10-µl aliquots plated on LB agar containing 200 µg/ml ampicillin (LB-Amp plates) for determining the titre of the eluted phage. The remaining phage-infected *E. coli* TG1 cells was also plated on LB agar containing 200 µg/ml ampicillin and 2% glucose as a catabolite suppressor (LB-Glu-Amp plate) for harvesting. Plates were incubated at 30°C overnight to minimize growth.

In the preliminary stages of the study, bound phage were eluted by adding 100 µl of 50 mM $Na_3C_3H_5O(COO)_3$/140 mM NaCl (pH 2.0) to each well and incubating at room temperature for 15 min. The solution was then neutralised by transferring eluted phage to a new well containing 50 µL neutralization solution (1M Tris-HCl, pH 8.0). The resulting solution was then used to infect freshly grown *E. coli* TG1 cells.

3.3.7.3 Amplification of Recovered Binding Phage

Cells were harvested from the LB-Glu-Amp plate into 1.5 ml LB-Glu-Amp broth and 16% glycerol added for storage. Aliquots of 100 µl of harvested cells were added to 5 × 5 ml of LB-Glu-Amp broth [start OD_{600} of 0.05-0.1]. These were incubated at 37°C until an OD_{600} of 0.5-0.6 was reached.

These cultures were infected with R408 helper phage at a multiplicity of infection of 20-50 (i.e. 100 µl of 1.3×10^{13} pfu/ml stock) and infection allowed to proceed at 37°C for a further 1 h with shaking (150 RPM). The R408-infected cultures were pooled together, and the cells pelletted by centrifugation at 4,500 × *g* for 5 min. Pelletted cells were re-suspended in 5

ml of fresh LB-Amp broth and 20 ml of molten soft agar, pre-cooled to 45°C and containing 200 µg/ml of ampicillin, added. The mixture was poured unto five LB-Amp agar plates, *i.e.* 5 ml of soft agar per plate and incubated at 30°C overnight.

The following day, the soft agar overlays were transferred into a 250-ml flask containing 25 ml of LB-Amp broth, *i.e.* 5 ml of broth per plate, to a final volume of approximately 50 ml. This was incubated at 37°C for 4 h with vigorous shaking. The resulting culture was centrifuged at 9,000 × *g* for 45 min, and the supernatant transferred into a fresh tube. This was then filter-sterilised (0.45 µm pore sterile Millex®) to remove any remaining bacterial cells. This filtered fraction constituted the final "ENRICHED PHAGE LIBRARY" fraction. This was stored in 1-ml aliquots at -80°C until required.

3.3.7.4 Preparation of Enriched Library for Repeat Panning

The enriched phage library following the first round panning was titrated. An aliquot of this was then mixed with 10 × blocking solution as described before. The same procedures, [sections 3.3.6.2 and 3.3.6.3], were carried out two more times to produce the second and third round enriched phage fractions.

3.3.8 Determination of Phage Titres after Amplification

At the end of each round, the number of phage particles was determined indirectly as the number of plaque- (colony-) forming units per milliliter (pfu/ml) of *E. coli* following infection with the phage.

A laboratory stock of *E. coli* TG1 was grown in fresh LB broth at 37°C until the optical density at 600 nm (OD_{600}) was between 0.5 and 0.6. A 90-µl aliquot of this culture was infected with 10 µl of rescued phage in a 1.5-ml Eppendorf tube. Infection was allowed to proceed at room temperature for 30 min. A 10-fold serial dilution of this was prepared and 90 µl of each dilution plated on LB-Amp agar plates and incubated at 37°C overnight. The following

day, the numbers of colonies of *E. coli* transformants were counted and the phage titres calculated.

3.3.9 Isolation of Plasmid for PCR and Sequencing

After determination of phage titres, twenty four colonies were randomly selected for PCR and sequencing. Clones were cultured in 1.5 ml of LB broth containing appropriate antibiotics at 37°C overnight. Cells were harvested by centrifugation at 16,000 × *g* for 5 min and the pellet resuspended in the appropriate buffer for plasmid extraction using QIAprep Spin Miniprep kit (Qiagen). The chromosomal DNA inserts were amplified by PCR with the primers pG8SAET_F and pG8SAET_R_Long to confirm the presence or otherwise and the size of the insert for each clone [see sections 2.6.8 and 2.6.18ii].

3.3.10 Nucleotide Sequencing of Chromosomal DNA Inserts

Sequencing of the chromosomal DNA inserts was performed with *Taq* polymerase-catalyzed cycle sequencing using fluorescent-labelled BigDye terminator kit, according to manufacturer's instructions, in an ABI 310 Genetic Analyser (ABI Perkin-Elmer) at the Biopolymer Synthesis and Analysis Unit, School of Biomedical Sciences, University of Nottingham. Primers pG8SAET_F and/or pG8SAET_R_Short were used to sequence the inserts in both the forward and reverse directions [section 2.6.8].

3.3.11 Bio-informatic Analysis of Chromosomal DNA Inserts

The Basic Local Alignment Search Tool (BLAST) of the Wellcome Trust Sanger Institute *C. jejuni* gene database (http://www.genedb.org/genedb/cjejuni/blast.jsp) was used to analyse the sequences obtained for similarity. The results were compared with similar BLAST searches at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Translation initiation sequences within each insert were searched for using the DNAMAN software (Lynnon Corporation), and then analysed for their

orientation with respect to the *Campylobacter* gene itself and with respect to gene 8 or protein VIII of the pG8SAET vector.

Clones were considered candidates if their inserts were in the right orientation and in frame with gene 8 of the vector and had a unique predicted function of possible interest. Where an insert was not in frame with gene 8 but was in the right orientation in the *Campylobacter* genome and of a unique predicted function, it was also considered as candidate gene. This is because it is believed that ribosomal slippage corrected the frameshifts during translation (Chen *et al.*, 2009; Jacobsson *et al.*, 2003).

Selected genes were analysed for the presence of signal sequences using the SignalP 3.0 Server for Gram-negative bacteria (http://www.cbs.dtu.dk/services/SignalP/).

Using the TMHMM 2.0 programme (http://www.cbs.dtu.dk/services/TMHMM-2.0/) an attempt was made to also identify transmembrane domains in the selected genes.

3.4 Results

3.4.1 Growth stage of *E. coli* TG1 cells and Infectivity of Phage particles

In order to ensure reproducibility of results generated from the use of *E. coli* TG1 cells, the optical density at 600 nm at which best infectivity with phage would be obtained was determined with a simple experiment as described [see section 3.3.4]. Following infection of *E. coli* TG1 cells at various stages of growth with an aliquot of phage library, the resulting ampicillin-resistant colonies were counted and the titres determined. It was observed the growth stage at which maximal infectivity of phage was obtained was OD_{600} of 0.4-0.6 (Figure 3.2). All subsequent titrations were therefore made using freshly grown *E. coli* TG1 cells with OD_{600} of 0.5-0.6.

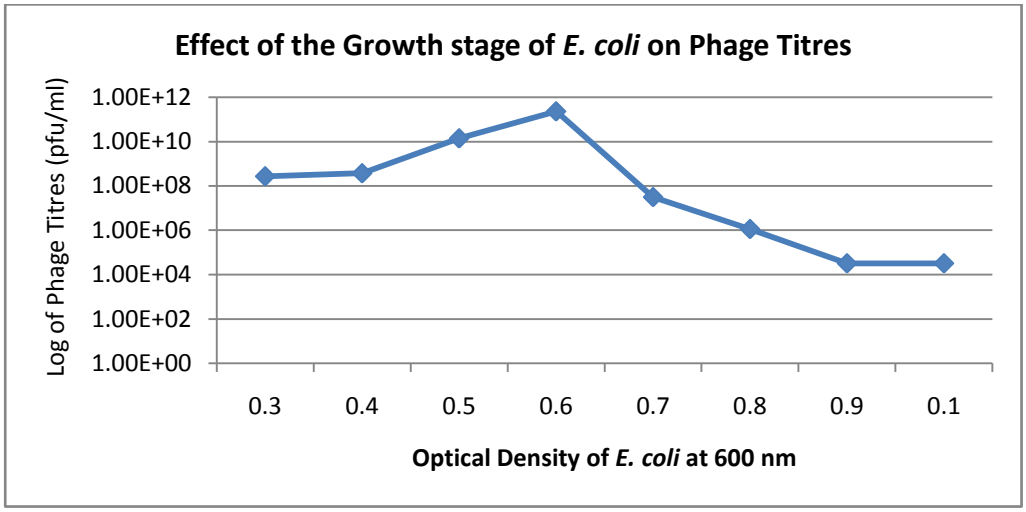

Figure 3. 2 | Effect of the growth stage of *E. coli* on phage titres. *E. coli* TG1 cells were grown to an OD_{600} of 1.0 and then serially diluted and grown for a further 4 h at 37°C to obtain OD_{600} of between 0.3 and 1.0. A 90-µl aliquot of each was infected with 10 µl of the phage library. The *E. coli* cells were then plated on LB-Amp agar and incubated at 37°C overnight and the number of ampicillin-resistant colonies counted to determine the titres.

3.4.2 Effect of Lactoferrin on *in vitro* infectivity of Phage particles

To ensure that Lf had no inhibitory effect on phage and phage infectivity in this study, phage particles were incubated with Lf and used to infect *E. coli* TG1 cells [see section 3.3.5]. Following incubation of the phage library with increasing amounts of apo- and holo-Lf, and the solvent Na_2CO_3 as a control, freshly grown *E. coli* TG1 cells were infected as described above. After spreading the cells on LB-Amp agar plates and incubating overnight at 37°C, the numbers of transformed cells were counted on each plate and the phage titres estimated.

As depicted in Figure 3.3 below, there were no significant differences between the titres of the apo-Lf treated phage and the holo-Lf treated phage or between the Lf-treated phage and the Na_2CO_3-treated phage. Compared with the titres of the starting phage no inhibitory effect on the abilities of the phage particles to infect the *E. coli* cells was observed following treatment with apo- or holo-Lf and Na_2CO_3.

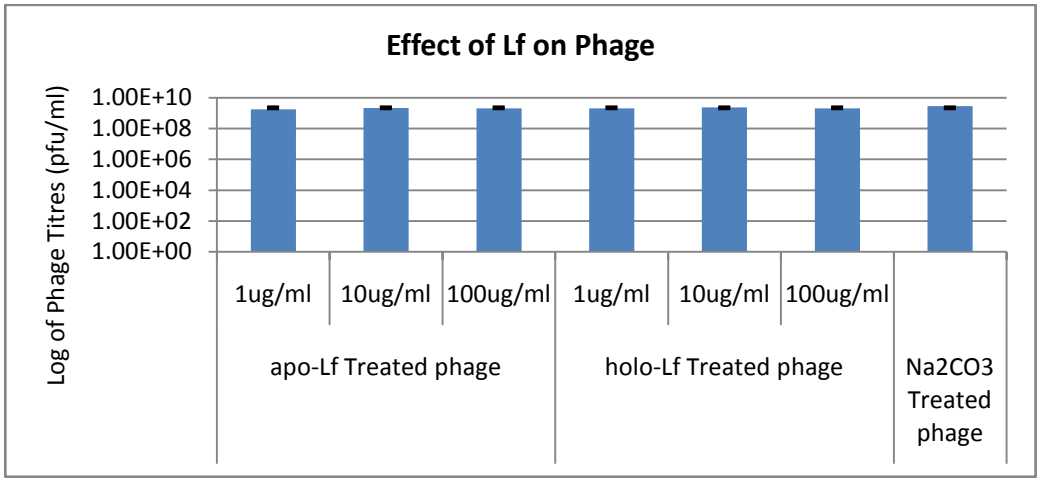

Figure 3. 3 | Effect of Lf on *in vitro* infectivity of phage particles. 180-µl aliquots of phage library were incubated with varying amounts of apo-Lf, holo-Lf or Na_2CO_3 as a control. Aliquots if these were used to infect 90 µl of freshly grown *E. coli* TG1 cells at room temperature over 30 min. A 10-fold serial dilution of each infection was carried out and 90 µl of each dilution plated on LB-Amp agar plates and then incubated at 37°C overnight. Ampicillin-resistant colonies were counted and titres estimated.

3.4.3 Effect of Lf on *in vitro* growth of *E. coli* TG1 cells

To ensure that Lf had no inhibitory effect on *E. coli* TG1 cells and infectivity with phage in this study, *E. coli* TG1 cells were incubated with Lf and then infected with phage [see section 3.3.6]. Following incubation of the *E. coli* TG1 cells with increasing amounts of apo- and holo-Lf, and the solvent Na_2CO_3 as a control, cells were infected with fresh phage particles as described above. After spreading the cells on LB-Amp agar plates and incubating overnight at 37°C, the numbers of transformed cells were counted on each plate and the phage titres estimated.

As depicted in Figure 3.4 below, there was no significant difference between the titres of the apo-Lf treated *E. coli* TG1 cells and the holo-Lf treated *E. coli* TG1 cells or between the Lf-treated *E. coli* TG1 cells and the Na_2CO_3-treated *E. coli* TG1 cells. Again, compared with the titres of untreated phage versus untreated *E. coli* no inhibitory effect on the *E. coli* cells was observed following treatment with apo- or holo-Lf and Na_2CO_3.

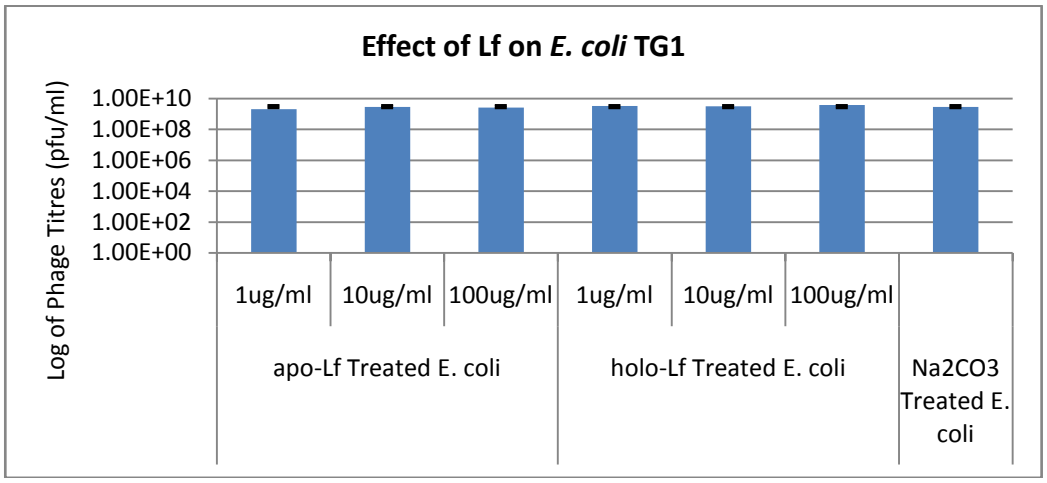

Figure 3. 4 | Effect of Lf on *in vitro* growth of *E. coli* TG1 cells. 90-µl aliquots of freshly grown *E. coli* TG1 cells were incubated with varying amounts of apo-Lf, holo-Lf or Na_2CO_3 as a control. Aliquots if these were then infected with 9 µl of phage library at room temperature over 30 min. A 10-fold serial dilution of each infection was carried out and 90 µl of each dilution plated on LB-Amp agar plates and then incubated at 37°C overnight. Ampicillin-resistant colonies were counted and titres estimated.

However, it must be stated that the titres obtained following treatment of *E. coli* with Lf were 5-10-fold lower compared with the titres following treatment of the phage particles with Lf. This can be attributed to the disruption of the optimal growth conditions for the *E. coli* cells during incubation with Lf at room temperature, rather than the effect of Lf.

3.4.4 Panning

Since *Bfu*CI was used in the fragmentation of the chromosomal DNA of *C. jejuni* NCTC11168, it would be expected that panning of both libraries I and II against any ligand would result in the enrichment of same peptides, plus additional smaller peptides in the case of library I.

Initial panning of library I yielded smaller single inserts, whilst initial panning of library II yielded larger inserts, including some multiple inserts. Library I was therefore used in all subsequent panning experiments to ensure the enrichment of single smaller inserts. Besides, gene 8 is supposed to display smaller inserts better than larger ones.

3.4.4.1 Panning against BSA and Fibronectin

To determine the superiority of one over the other for use in panning, MaxiSorp ImmunoWells® (wells) and MaxiSorp ImmunoTubes® (tubes) were coated with Fn and BSA as described in section 3.3.7. The phage library was panned against these ligands and the phage titres at the end of each round of panning determined as described. BSA was used in this instance as a control without any blocking step.

Firstly, phage eluted from both the wells and the tubes had similar titres in the range of 10^4 plaque-forming units (pfu) for BSA and 10^6 plaque-forming units (pfu) for Fn (Figure 3.5) indicating that neither the wells nor the tubes had demonstrable superiority in this respect. MaxiSorp ImmunoWells® (wells) were therefore used in all subsequent panning experiments for its convenience.

Secondly, panning against Fn generated titres of 10^5 compared with the titres of 10^4 generated after panning against the BSA control, indicating the ability of Fn to bind more phage than does BSA.

Thirdly, the titres of the eluted phage showed no consistent increases in the numbers of bound phage with successive pannings, indicating that the panning process did not result in significant enrichment of specific clones at the end of the third round of panning.

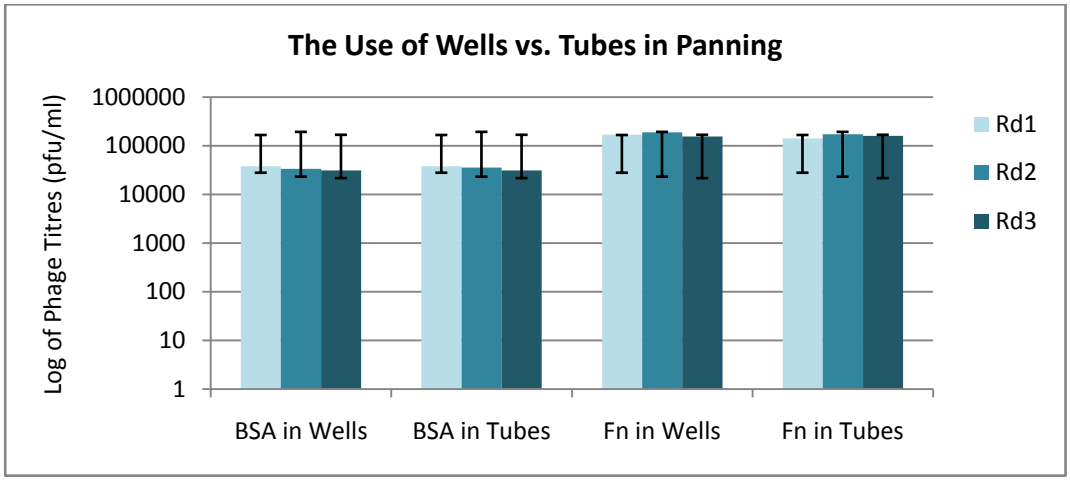

Figure 3. 5 | Titres of eluted-phage after panning of phage library in wells and tubes. The titres of bound phage after three successive rounds of panning (Rd1, Rd2 and Rd3) were determined by infecting *E. coli* TG1 cells with the eluted phage solution as described. The *E. coli* cells were then plated on LB-Amp agar and incubated at 37°C overnight and the number of ampicillin-resistant colonies counted. Phage titres obtained for the wells compared favourably with that obtained for the tubes, in both Fn and BSA although more phage were eluted from the Fn-coated wells/tubes than from BSA-coated wells/tubes.

The eluted-phage from the rounds 1 and 3 pannings against Fn and BSA in both wells and tubes were amplified in 0.2% soft agar as described. The eluted-phage particles from the round 2 pannings were amplified directly in LB-Amp broth overnight following infection with helper phage. The resulting phage for each panning was then titrated and compared (Figure 3.6).

Amplification in 0.2% soft agar produced phage titres 100-fold higher (Rd1 and Rd3) than direct amplification in LB-Amp broth (Rd2) as shown below. Again, the titres obtained for each round of panning for each experiment were consistent indicating the consistency of the amplification procedures, although amplification in 0.2% soft agar was preferred.

Amplification of a fourth round panning (data not shown) produced phage titres of 10^8 that were consistent with that obtained for Rd2. These data informed our decision on the use of soft agar in all subsequent amplification experiments.

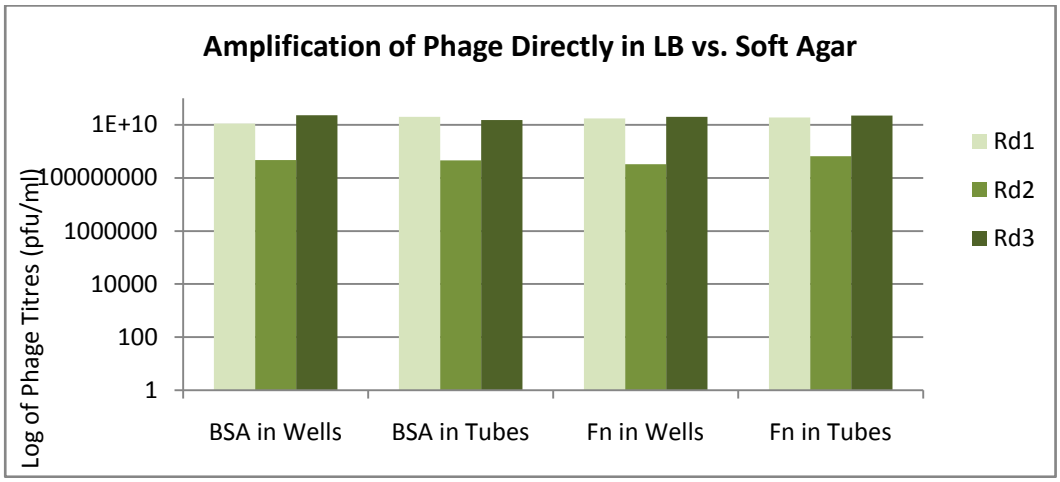

Figure 3. 6 | Titres of phage after amplification of eluted phage in LB broth or soft agar. After three successive rounds of panning (Rd1, Rd2 and Rd3), Rd1 and Rd3 phage were amplified in soft agar, whilst Rd2 was amplified directly in LB broth. TG1 *E. coli* cells were infected with the resulting amplified phage and plated on LB-Amp agar and incubated at 37°C overnight. The number of ampicillin-resistant colonies was counted and the titres calculated.

3.4.4.2 Panning against Several Ligands Concurrently

A repeat panning of the phage display library of *C. jejuni* NCTC11168 against Fn and BSA was done, alongside panning against apo-Lf and apo-Tf concurrently [see Figure 3.7]. Titration of the eluted phage at the end of each successive round consistently gave titres of between 10^4 and 10^5, with Fn producing the highest numbers of bound phage, reflecting the "stickiness" of Fn and hence the property to bind more phage than would the other ligands.

The titres of the eluted phage for each ligand showed no consistent increases in the numbers of bound phage with successive pannings, indicating that the pannings did not produce enrichment of specific clones at the end of the third round of panning.

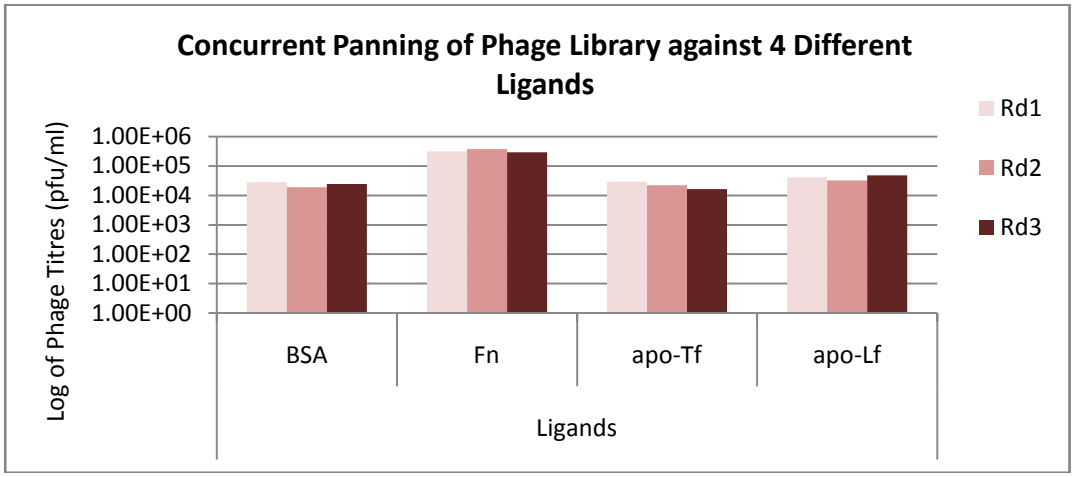

Figure 3. 7 | Titres of eluted phage after concurrent panning against BSA, Fn, apo-Tf and apo-Lf. After three successive rounds of panning, *E. coli* TG1 cells were infected with the eluted phage solution as described and plated on LB-Amp agar. These were incubated at 37°C overnight and the number of ampicillin-resistant colonies counted to determine the titres.

3.4.4.3 Elution of Bound Phage using *E. coli* TG1 cells or Acid Solution

As part of the initial panning experiments, bound phage were eluted with 50 mM Na-Citrate/140 mM NaCl, pH 2.0 and neutralised with 1M Tris-HCl,

pH 8.0 as described. The resulting solution was then used to infect freshly grown *E. coli* TG1 cells.

Compared with elution of bound phage by directly adding *E. coli* TG1 cells to the wells, the results from the former methodology resulted in low colony counts (data not shown). Bound phage was therefore eluted by direct addition of *E. coli* TG1 cells to the wells in all subsequent experiments.

3.4.4.4 Panning against Fibronectin, apo-Transferrin and apo-Lactoferrin

Following successful optimization of the panning, elution, amplification and titration conditions, the phage display library of *C. jejuni* NCTC11168 was panned in separate experiments against Fn, apo-Tf, and apo-Lf [see Figure 3.8]. These yielded exponential increments in the number of bound phage after each successive panning, suggesting enrichment of specific clones. Furthermore, the amplification of eluted phage after each round also produced consistent titres. This ensured reproducibility of the panning experiments since it ensured that the number of phage particles applied to each well was the same at the beginning of each round for each panning experiment.

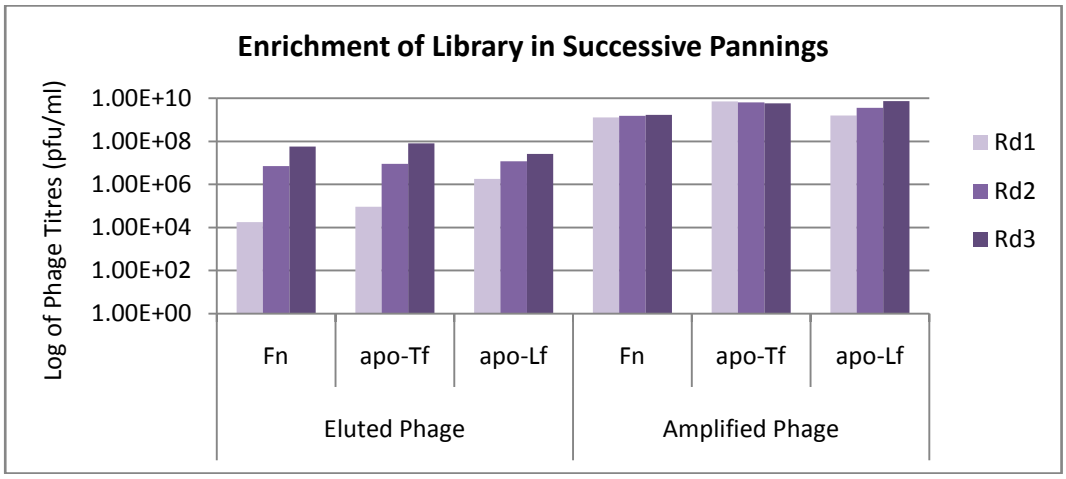

Figure 3. 8 | Phage titres showing exponential increases after each successive panning. After three successive rounds of panning *E. coli* TG1 cells were infected with the eluted phage solution as described. The *E. coli* cells were then plated on LB-Amp agar and incubated at 37°C overnight. The number of ampicillin-resistant colonies was then counted to determine the titres.

3.4.4.5 Repeat Panning against apo-Lactoferrin and holo-Lactoferrin

The panning of the phage display library of *C. jejuni* NCTC11168 against apo-Lf was repeated twice and then once against holo-Lf in separate experiments [see Figure 3.9]. Again, these pannings yielded exponential increments in the number of bound phage after each successive panning, indicating enrichment of specific clones. Furthermore, the amplification of eluted phage after each round also produced consistent titres, which were consistent with previous experiments.

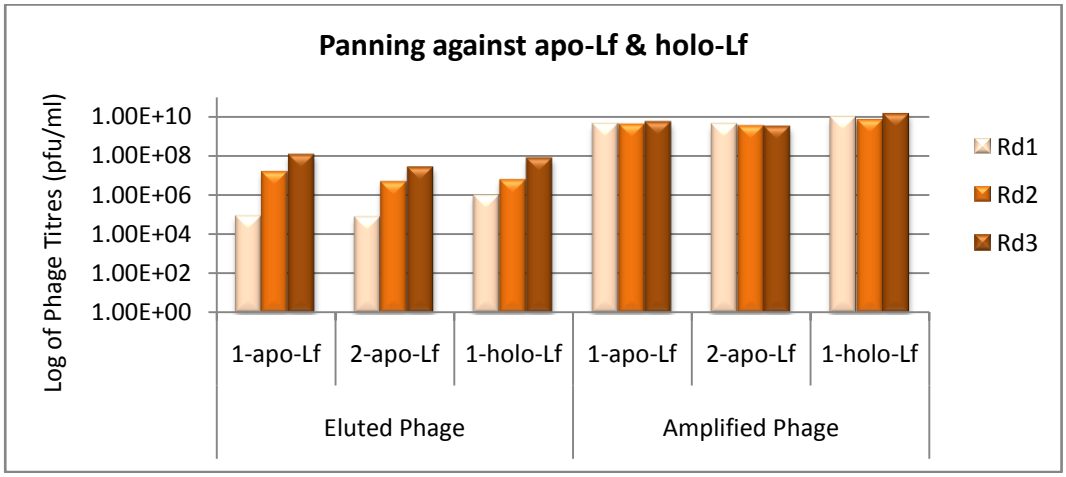

Figure 3. 9 | Phage titres showing exponential increases after each successive panning. The phage titres resulting from the pannings against apo-Lf and holo-Lf were consistent with previous experiments. Both the first and the second repeat panning against apo-Lf (1-apo-Lf and 2-apo-Lf respectively) compared very well with the panning against holo-Lf (1-holo-Lf).

3.4.5 Bio-informatic Analysis of Selected Clones

For each panning, 24 clones were randomly selected and the inserts sequenced. Usable sequences were analysed. A summary of the genes isolated from the pannings against the various ligands is provided in Table 3.1 below.

After analyses of the DNA inserts, clones were considered candidate genes if they were in the right orientation and in frame with gene 8 of the vector and had a unique predicted function of possible interest, with or without the presence of signal peptide and transmembrane domain (TMD).

Ligand	Isolated *C. .jejuni* Gene(s)	The Insert		Predicted Function (s)	SignalP (Y/N)	TMD (Y/N)
		Size (bp)	Fre-quency			
				Non-enriched Pannings		
BSA	i.Cj1564	827	2/20	Putative methyl-accepting chemotaxis protein	Y	Y
	ii. Cj1283	1159	2/20	Probable K+ uptake protein	Y	Y
	iii. Cj1075	1096	2/20	Conserved hypothetical protein	N	N
apo-Tf	i. None		0/20	Almost all of the isolated genes were in reverse orientation/combination of smaller inserts. The only inserts that were in the correct orientation encoded for ribosomal RNA.		
	ii. None		0/20			
	iii. None		0/20			
Fn	i.Cj1127c	992	2/40	Probable oligosaccharyl transferase	N	N
	ii.Cj0530	152	2/40	Probable periplasmic protein	N	Y
	iii.Cj1235	895	2/40	Probable periplasmic protein	N	N
				Enriched Pannings		
apo-Lf	i. Cj0609c	457	26/42	Probable periplasmic protein	Y	N
	ii.Cj1618c	586	4/42	Probable haemin uptake system/periplasmic haemin-binding protein	N	N
	iii.Cj0599	129	2/42	Putative OmpA family protein	Y	Y
holo-Lf	i. Cj0609c	457	14/24	Probable periplasmic protein	Y	N
	ii. Cj0525c	95	2/24	Putative penicillin-binding protein	Y	Y
	iii.Cj0697	65	2/24	Flagellar basal-body rod protein	N	N

Table 3. 1 | Summary of unique ligand-specific genes after three rounds of panning. All clones that produced readable sequences were analysed, and the inserts in the right orientation within the vector positively identified. Based on the probable function and location within *C. jejuni*, three clones were selected for each ligand and the presence of signal peptide or transmembrane domain determined. Y/N mean yes/no.

3.4.5.1 Enrichment versus non-Enrichment after Successive Pannings

Analyses of the DNA sequences of randomly selected clones from the non-enriched pannings (pannings that did not produce sequential increase in phage titres) revealed the isolation of unrelated *Campylobacter* genes (see Figures 3.5 – 3.7). Panning of library I led to the isolation of inserts whose sequences were concatamers of several *Bfu*CI-target sequences and hence did not match any genes in the *Campylobacter* genome database.

By contrast, analyses of the DNA sequences of selected clones from the enriched pannings (pannings that produced sequential increase in phage titres) revealed the isolation of identical *Campylobacter* genes in *ca.* >50% of clones (see Figures 3.8 – 3.9). Again, analyses of randomly selected clones from their second round pannings further revealed the isolation of genes that were related to those from the third round panning, with 30-40% of clones being identical.

3.4.5.2 Panning against Several Ligands Concurrently

Following panning of the *Cj*NCTC11168 phage display library concurrently against BSA, apo-Tf, Fn and apo-Lf, analyses of randomly selected clones from each panning revealed the isolation of identical clones. Obviously, these clones were not considered to be uniquely binding to any specific ligand. This confirmed Jacobsson's assertions that phage particles would easily cross-contaminate experiments and therefore warranted that panning against different ligands was done in separate experiments at different times.

3.5 Discussion

Phage display is a powerful technique with major applications in protein-protein interactions, and has been successfully used in the identification of novel interacting proteins of several pathogens with host proteins (Chen *et al.*, 2009; Jacobsson *et al.*, 2003; Segvich *et al.*, 2009; Wu *et al.*, 2008; Zhu *et al.*, 2008). By extension, this technique can be used to screen the genomes of pathogenic organisms for genes encoding proteins that interact with specific host proteins.

To date, phage display has only been used in the identification of potential adhesins in only a few Gram-positive pathogens (Bjerketorp *et al.*, 2004; Jacobsson *et al.*, 2003; Karlstrom *et al.*, 2004; Mullen *et al.*, 2006; Mullen *et al.*, 2008; Wall *et al.*, 2003; Zhang *et al.*, 1999). This therefore represents the first report of a phage display natural peptide library in, not only *Campylobacter*, but also in a Gram-negative organism.

To identify unique *Campylobacter* genes encoding specific ligand-binding proteins in such a library, several factors needed to be considered in the design and optimization of panning protocols. Firstly, in order to obtain consistent results from titration of the enriched libraries, the stage of bacterial growth at which optimal infectivity of the phage particles can be attained had to be identified. Following repeated experiments of infecting *E. coli* TG1 cells grown to different levels at optical density of 600 nm (OD_{600}) with same amount of phage particles, it was observed that maximal infectivity of phage was obtained at OD_{600} of 0.5-0.6. This was not surprising since this point of growth fell well within the log phase of growth of the *E. coli* TG1 cells. This obviously represents the growth phase at which the bacterial cells are actively dividing and are therefore likely to replicate effectively at the same rate following transformation with phage genes (Skarstad *et al.*, 1983). In fact, whilst obtaining OD_{600} of 0.4-0.6 as the optimal growth stage for maximal phage infectivity in most of their experiments, Jacobsson and co-workers recommended that this value was determined for each individual study (Jacobsson & Frykberg, 2001a; Jacobsson *et al.*, 2003).

The choice of Lf as the main ligand for panning in this study was informed by the recent study by Miller and co-workers that showed that supplementation of iron-deficient medium MEM with Lf-bound and Tf-bound iron was comparable to supplementation with FeSO4 in *C. jejuni* growth (Miller *et al.*, 2008). Huynh *et al.* have also recently contended that Lf, which has been considered a potential addendum to the treatment regime of *H. pylori*, is ineffective against the organism and can rather enhance growth and gastric inflammation (Huynh *et al.*, 2009).

Having selected Lf as the main ligand for panning in this library, it was important to ascertain the effect of Lf on *E. coli* TG1 cells and phage particles used in this library. This is due to the contradicting reports of the anti-bacterial and anti-viral, as well as growth promoting effects of lactoferrin (Huynh *et al.*, 2009; Kim *et al.*, 2004; Leon-Sicairos *et al.*, 2009). In fact, it was not surprising that Lf had no effect on the phage particles or the *E. coli* TG1 cells in this study.

An additional factor that needed to be clarified was the use of immuno-tubes and immuno-wells. Whereas both wells and tubes have been extensively used in various experiments, wells seemed more convenient than did tubes (Jacobsson & Frykberg, 2001a; Mullen *et al.*, 2007a). Although the initial panning experiments with Fn and BSA were all non-enriched pannings, they provided good indices that informed the decision to choose the well format in all subsequent experiments. The use of MaxiSorp Immunotubes was abandoned due simply to its cumbersome nature and all subsequent experiments were carried out in MaxiSorp Immunowells.

Generally, pannings are carried out in two or three successive experiments, and specific binding is manifested by exponential increases in the titres of the eluted-phage from the successive pannings (Jacobsson & Frykberg, 2001a; Jacobsson *et al.*, 2003). With a starting phage titre of 10^{11} in every single panning experiment, the elution of *ca.* 10^3 or 10^7 is an indication of enrichment of 10^4 or 10^8 in each round, which should in theory result in enrichment of binding sequences after only 2 or 3 rounds given that the library contains only 1,000,000 – 1,600,000 clones. Colonies can then be randomly selected from the second or the third round pannings and their inserts analysed, which should

result in the isolation of identical clones. Further rounds of panning will result only in selection of phage that have a growth advantage over the library phage (McCafferty, 1996). The absence of this exponential increase in the titres in the successive pannings (see Figures 3.5 and 3.7) was therefore an obvious indication of non-enrichment and hence warranted further optimisation of the panning protocol.

As indicated earlier, it was thought that bound phage can be eluted with an acidic solution and then neutralised before the solution was used to infect *E. coli* TG1 cells. However, this was abandoned and bound phage particles were eluted by directly adding *E. coli* TG1 cells to the wells. This methodology was considered superior since the phage particle binds ligands through the major coat protein, pVIII, whilst infecting through the minor coat protein, pIII (Azzazy & Highsmith, 2002). Thus, whilst elution with acidic solution can leave some phage particles un-dissociated from the ligand, elution with *E. coli* TG1 cells would result in the infection of the bacterial cells by all bound phage particles.

After elution of the bound phage in each panning cycle, the former was amplified to multiply the phage titres for use in subsequent experiments. The phage particles are obtained by super-infection of the *E. coli* TG1 cells with helper phage and further incubation overnight. The *E. coli* cells can be grown either in LB broth with the appropriate antibiotics or in 2.0% soft agar as previously described. Growth in soft agar yielded the highest level of phage titres comparable to published data (Jacobsson & Frykberg, 2001a; Jacobsson *et al.*, 2003), and also yielded consistent values. However, an explanation cannot be offered for this observation (Figure 3.6). The expected titres can still be achieved through amplification in LB broth providing small volumes of cells are cultured in larger containers, with aeration and proper shaking (Schofield *et al.*, 2007).

Following optimisation of the panning protocol, panning experiments were done against BSA, Fn, apo-Tf and apo-Lf concurrently, the results of which indicated not just non-enrichment, but also the isolation of genes identical for all four ligands. This can be attributed to the high sensitivity of the phage particles, and their ability to easily contaminate adjacent experiments. This observation can be

likened to Jacobsson's observation that working with several different libraries in the same laboratory led to a high enrichment after panning against a ligand but isolation of peptides that emanated from another library (Jacobsson *et al.*, 2003). Again, Jacobsson *et al.* reported that experiments with libraries made in different vectors resulted in isolation of inserts that were out of frame with the vector sequence.

An enrichment panning was manifested as exponential increases in the titres of the successive pannings, as depicted in Figure 3.8 by the panning against Fn, apo-Tf and apo-Lf. This exponential increase is the result of the fact that amplification of eluted phage results in the application of more of the same clones to the ligand in the subsequent panning resulting in an increase in the number of bound phage in that panning. These data were observed in the repeat experiment with apo-Lf as well as the panning against holo-Lf. Furthermore, sequence analyses of clones from these enriched pannings also revealed the isolation of identical *Campylobacter* genes in *ca.* >50% of the clones (see Table 3.1). Again, analyses of randomly selected clones from their second round pannings further revealed the isolation of genes that were identical to those from the third round panning, *ca.* 30-40% of clones. These sequences were considered as potentially encoding for peptides that can bind to Lf. The repeated isolation of Cj0609c in the enriched pannings against apo-Lf (*ca.* 62%) and holo-Lf (*ca.* 58%) is an interesting finding worth further investigating.

Whilst the isolation of Cj0609c in these panning can be reliable, the absence of the *cadF* gene in all of the pannings against Fn can be attributed to the fact that those pannings did not lead to enrichment. However, this may also be the result of the different conformations that Fn can assume in solution and after application to the wells. Again, the absence of the necessary glycosylation in the proteins expressed by the phage particles may also affect their ability to bind to the right target ligand. Further work is therefore needed to confirm or otherwise of the authenticity of the isolated genes.

Non-enriched pannings, on the contrary, manifested as the absence of exponential increases in the titres of the successive pannings, with sequence analyses of clones from these pannings also revealing the isolation of sequences

from non-identical *Campylobacter* genes (see Table 3.1). Thus, the pannings against BSA, apo-Tf, and Fn did not result in the isolation of any unique gene as depicted in Table 3.1.

Generally, if a gene appeared more than once (2-5×) in the sequenced clones, it was analysed in more detail. However, the most isolated gene for both apo- and holo-Lf remained Cj0609c.

Finally, it must be emphasised that the there is significant difficulty in preparing and panning phage display libraries as well as the utility of this approach but, conversely, this represents an approach that can be applied to the study of *C. jejuni* and other pathogens, and in particular, to the identification of previously unidentified protein-protein interactions that may be involved in host-pathogen interactions.

Chapter Four

Analysis of Enriched Phage Library

Identification of Host-pathogen Interacting Molecules of *Campylobacter jejuni* using Phage Display Technology and in silico Sequence Analysis

CHAPTER FOUR

4.0 Analysis of Enriched Phage Library

4.1 Introduction

Following enrichment of a phage library, it is important to confirm that the phage particles present in the enriched library after the third round panning do really bind to the selected ligand(s). This was achieved by comparing the binding of the first and second round enrichments with the third round enrichment.

Furthermore, after isolation of a number of clones encoding putative binding proteins specific for a specific ligand from the third round enrichment, it is important that the binding specificities of the encoded proteins are also confirmed.

This could be achieved through enzyme-linked immunosorbent assay or through comparative binding of the encoding phage to the initial target ligand and to other unrelated ligands (Jacobsson *et al.*, 2003). In the case of the latter, the resulting titres of the bound phage on the original target ligand are 1,000-10,000 folds higher than that obtained for the unrelated ligands (Jacobsson *et al.*, 2003).

Binding specificity could also be confirmed through a viral competitive inhibition assay, in which binding of phage stocks of single clones from the third round panning could be inhibited by antibodies to the ligand (Nilsson *et al.*, 2004b).

Phage stocks of single clones from the third round panning could also be used in phage ELISA experiments to ascertain which clone binds more specifically. This approach has been used in many recent phage display studies (McCafferty & Johnson, 1996; Schofield *et al.*, 2007).

This chapter therefore discusses the use of ELISA to analyse the enriched-pannings resulting from the panning of phage display library *Cj*NCTC11168 against apo-Lf and holo-Lf.

4.2 Materials and Methods

4.2.1 General Materials

4.2.1.1 Phage Library

[See Section 3.3.1.1 above]

4.2.1.2 Bacterial Strains and Culture conditions

[See Section 2.6.1 above]

4.2.1.3 R408 Helper Phage

[See Section 2.6.2 above]

4.2.1.4 Antibiotic Supplements

[See Section 2.6.3 above]

4.2.1.5 Culture Media

[See Section 2.6.4 above]

4.2.1.6 Miscellaneous Buffers and Solutions

[See Section 2.6.5 above]

4.2.1.7 Polymerase Chain Reaction

[See Section 2.6.8 above]

4.2.1.8 Ligands

[See Section 3.3.2 above]

4.2.1.9 ELISA Wells

[See Section 3.3.3 above]

4.2.2 Enriched Phage Library

Enriched phage libraries following three rounds of panning against each of apo-Lf (×2) and holo-Lf were used in the experiments described in this chapter.

4.2.3 HRP Anti-M13 Antibodies

Horseradish peroxidase (HRP) anti-M13 monoclonal conjugate (GE Healthcare) consists of horseradish peroxidase conjugated to mouse anti-M13

monoclonal antibody. The antibody purified from BALB/c mouse ascites fluid, reacts specifically with the bacteriophage M13 major coat protein product of gene 8.

4.2.4 Substrate Detection

ABTS® peroxidase substrate solution was obtained from KPL, Gaithersburg (USA). This two-component liquid substrate system develops a blue-green hue when reacted with peroxidase labelled conjugates in Microwell plates.

4.2.5 ELISA Confirmation of Binding Activity of Enriched Phage Library

100-µl aliquots (*ca.* 10-100 µg/ml) of the dissolved ligands, apo-Lf and holo-Lf, were immobilised in a row of 8 microtiter wells of ELISA-ready plates and incubated at 4°C overnight. The next day, the wells were washed once with PBS and then blocked with 200 µl of blocking solution [i.e. 1% BSA in PBS-0.05% Tween 20 (PBS-T)]. Plates were incubated at room temperature (RT) for 1 h.

Using rescued phage from the first, second and third round pannings, and 1 in 2 dilution of third round phage, alongside appropriate negative controls, 100 µl each of the above samples were applied to the wells as before [see Figure 4.1]. The wells were incubated at room temperature for 1 h and then at 4°C overnight. The next day, unbound phage particles were removed by washing the wells 3× with PBS–T, followed by two additional washes with PBS.

A 4-µl aliquot of HRP/anti-M13 monoclonal conjugate (GE Healthcare) was diluted in 21 ml of 1 × blocking solution to give a dilution of 1:5000 [adequate for 96-well plate], and 100 µl of this added to each well where appropriate. This was then incubated at RT for 3 h. The wells were washed twice with PBS–T, followed by two additional washes with PBS, and then 100 µl of ready-ABTS substrate solution added to each appropriate well. This was incubated at room temperature for 45 min before the absorbance was read in a microplate reader at 405 and 595 nm. The reading was repeated at 60 min. All samples were tested in triplicate, giving twelve readings in all for each sample.

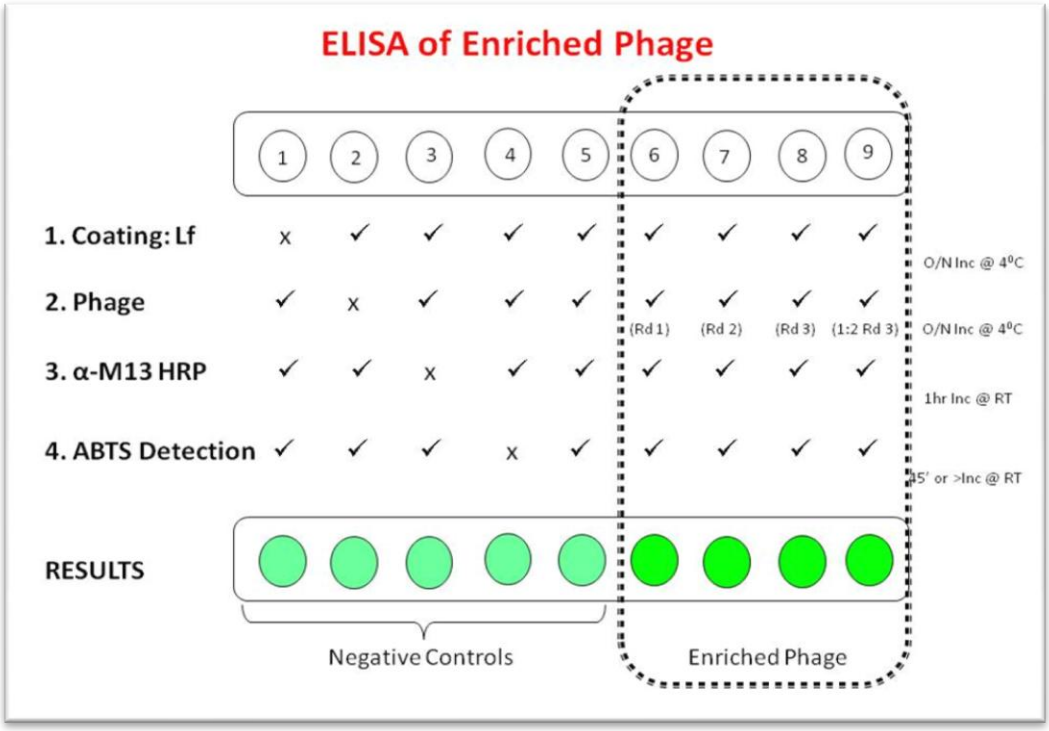

Figure 4. 1 | ELISA of enriched phage. Wells were coated with Lf and incubated at 4°C overnight (O/N). After washing and blocking for 1 h, phage was added and incubated again O/N. Wells were then washed to remove unbound phage and HRP/anti-M13 monoclonal conjugate added. This was incubated for 3 h at room temperature (RT), washed and then ready-ABTS substrate solution added to detect bound phage. After incubation at RT for 45 min, the absorbance was read in a microplate reader at 405 and 595 nm. The absorbance reading was repeated after 60 min incubation. To confirm binding of enriched panning, rescued phage from the first, second and third round pannings, and 1 in 2 dilution of third round phage; appropriate negative controls were used. Rd 3 phage was used in samples 1-4, whilst sample 5 used phage without insert (null phage). √, added; X, not added.

4.2.6 ELISA Screening of Candidate Phage Clones

Following the initial ELISA assays of the third round rescued phage, 24 colonies were selected from the LB plate of the third round elute and cultured in LB-Glu-Amp broth at 37°C overnight. A 10-µl aliquot of each culture was then grown in fresh LB-Amp broth for 1 h and R408 helper phage added at a multiplicity of infection of 10^{11} and incubated for a further 8 h. Phage from each colony was rescued by centrifugation at 9,000 × *g* for 45 minutes and the supernatant used in an ELISA as described above. The absorbance for each clone was measured at a wavelength of 405 nm and a reference wavelength of 595 nm after incubation for 45 min and then again at 60 min. All samples were tested in triplicate, giving twelve readings in all for each sample.

4.2.7 Validation of the ELISA

Since there is no commercial/ready-to-go kit available for phage ELISA, it was important that the ELISA results were validated in order to produce consistency in different phage ELISA experiments. A phage library was therefore constructed with a pG8SAET vector that had no insert, and then used in an ELISA alongside four other negative controls, each of which had one missing reagent. These data were then compared with three additional ELISAs that could potentially produce false positive results in determining the sensitivity of the assay (Figure 4.2).

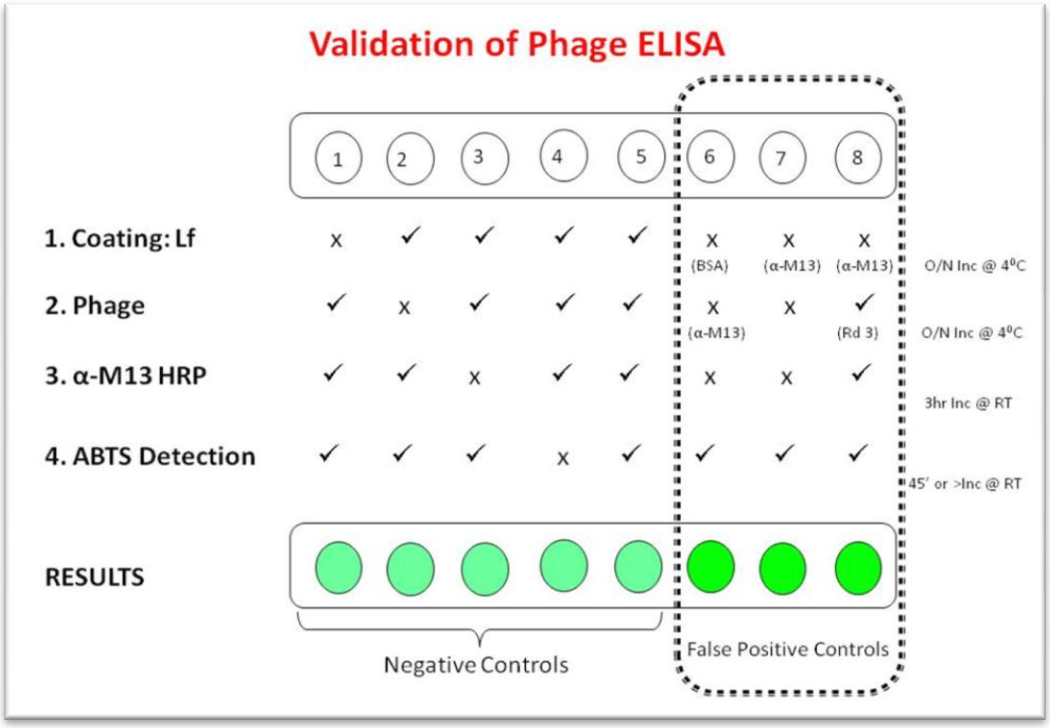

Figure 4. 2 | Validation of phage ELISA. Wells were coated with Lf and incubated at 4°C overnight (O/N). After washing and blocking for 1 h, appropriate substrate or phage was added and incubated again O/N. Wells were then washed to remove unbound phage/substrate and HRP/anti-M13 monoclonal conjugate added. This was incubated for 3 h at room temperature (RT), washed and then ready-ABTS substrate solution added for detection. After incubation at RT for 45 min, the absorbance was read in a microplate reader at 405 and 595 nm. The absorbance reading was repeated after 60 min incubation. Negative controls were obtained by omitting one of the substrates in the process. Scenarios that could lead to false positive results were also used as controls. Rd 3 phage was used in samples 1-4, whilst sample 5 used phage without insert (null phage). √, added; X, not added.

4.2.8 Statistics

Data were analyzed using the Minitab 15 software program (Minitab). The Chi squared test was performed to study the difference in positivity rate between individual clones and the mean of the negative controls. Differences were considered statistically significant at a p-value of less than 0.05.

4.2.9 Isolation of Plasmid from Positive Clones for PCR and Sequencing

Following confirmation of the binding affinity of the round three enriched phage and that of the individual clones, the twenty four colonies were prepared for PCR and sequencing. Aliquots of phage clones that yielded positive results were used to infect *E. coli* TG1 cells as described. Clones were cultured in 1.5 ml of LB broth containing appropriate antibiotics at 37°C overnight. Cells were harvested by centrifugation at 16,000 × *g* for 5 min and the pellet resuspended in the appropriate buffer for plasmid extraction using QIAprep Spin Miniprep kit (Qiagen). The chromosomal DNA inserts were amplified by PCR with the primers pG8SAET_F and pG8SAET_R_Long to confirm the presence or otherwise and the size of the insert for each clone [see Sections 2.6.8 and 2.6.18ii].

4.2.10 Nucleotide Sequencing of Chromosomal DNA Inserts

Sequencing of the chromosomal DNA inserts was performed with *Taq* polymerase-catalyzed cycle sequencing using fluorescent-labelled BigDye terminator kit, according to manufacturer's instructions, in an ABI 310 Genetic Analyser (ABI Perkin-Elmer) at the Biopolymer Synthesis and Analysis Unit, School of Biomedical Sciences, University of Nottingham. Primers pG8SAET_F and/or pG8SAET_R_Short were used to sequence the inserts in both the forward and reverse directions [see Section 2.6.8].

4.2.11 Bio-informatic Analysis of Chromosomal DNA Inserts

The Basic Local Alignment Search Tool (BLAST) of the Wellcome Trust Sanger Institute *C. jejuni* gene database

(http://www.genedb.org/genedb/cjejuni/blast.jsp) was used to analyse the sequences obtained for similarity. The results were also compared with similar BLAST searches at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nucleotide sequence data were analyzed using the DNAMAN software (Lynnon Corporation) and their orientation with respect to the *Campylobacter* gene itself and with respect to gene 8 or protein VIII of the pG8SAET vector determined.

Clones were considered candidates if their inserts were in the right orientation and in frame with gene 8 of the vector and had a unique predicted function of possible interest. Where an insert was not in frame with gene 8 but was in the right orientation in the *Campylobacter* genome and of a unique predicted function, it was also considered as candidate gene. This is because it is believed that ribosomal slippage can result in correction of frameshifts during translation (Chen *et al.*, 2009; Jacobsson *et al.*, 2003).

Selected genes were analysed for signal peptide-triggered and non-classical protein secretion using the signalP 3.0 and SecretomeP 2.0 servers for Gram-negative bacteria respectively (http://www.cbs.dtu.dk/services/).

Protein sequences of the selected genes were also used to perform BLASTP searches of the non-redundant protein database (nrdb95) and the Omniome pep database using the WU-BLAST2 servers at http://dove.embl-heidelberg.de/Blast2/ and http://tigrblast.tigr.org/cmr-blast/, respectively. Using the TMHMM 2.0 programme (http://www.cbs.dtu.dk/services/TMHMM-2.0/) an attempt was made to also identify transmembrane domains in the selected genes.

4.3 Results

4.3.1 Validation of Phage ELISA

Currently, there is no commercial ELISA kit available for evaluation of phage display libraries and the ELISA assays described by various researchers have been designed to suit their individual needs. Since the use of ELISA was necessary to confirm whether or not enriched phage or clones have the potential to bind to a specific ligand, it was important that reliable ELISA data were obtained. Several experiments were therefore carried out that made use of a phage display library made from pG8SAET vector without insert (Null phage) and a combination of the reagents added to the ELISA wells at different times (Figures 4.1 and 4.2). This approach provided information on the level of sensitivity and the specificity of target binding.

In contrast to the addition of whole proteins in conventional ELISA, phage ELISA involves the addition of phage particles. In the characterization of phage clones by ELISA, it is difficult to add more than 10^{12} virions per 100 µl well, which corresponds to a phage concentration of only 16 nM (Adey *et al.*, 1995). At this concentration, an unambiguously positive ELISA signal can only be observed if the binding affinity is in the micromolar range or better (McCafferty & Johnson, 1996). Signals obtained are therefore low, but significant.

The cut-off score for positivity was determined by calculating the mean absorbance of five sets of negative controls and three sets of false positive controls (0.048), plus three standard deviations (3×0.017). Any colour signal with an absorbance above this value (0.065) was regarded as positive (Figure 4.3). The three false positive controls were used as a way of increasing the sensitivity of the assay.

All samples in the ELISA assays were tested in triplicate, and read twice at different times, yielding 12 readings. The mean absorbance was then determined.

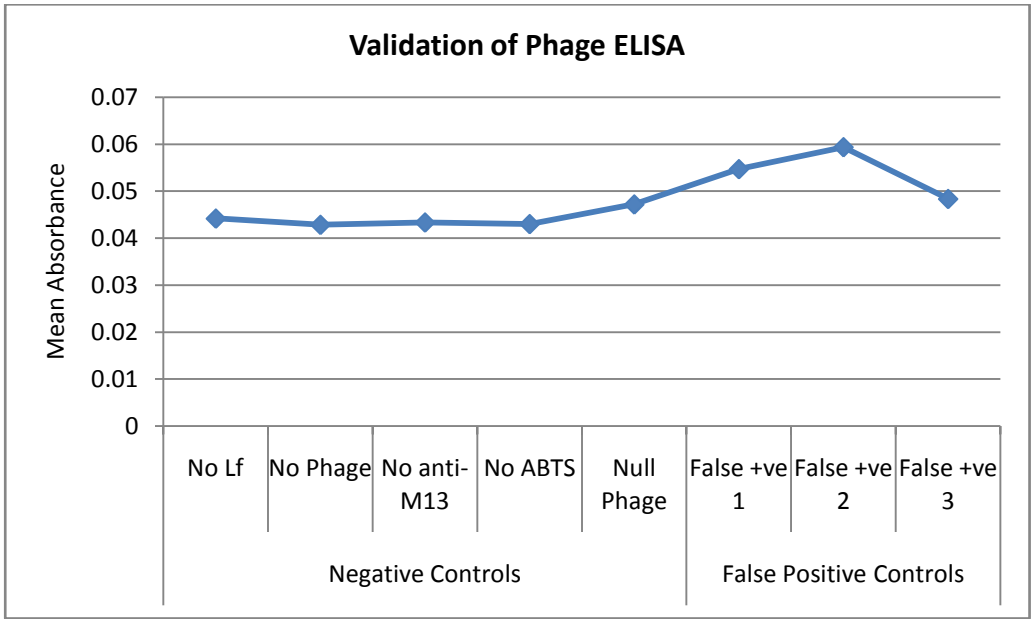

Figure 4. 3 | Validation of phage ELISA. The cut-off score of 0.065 for positivity was determined by calculating the mean absorbance of five sets of negative controls and three sets false positive controls, plus three standard deviations.

4.3.2 ELISA Confirmation of Binding Activity of Third Round Phage

Using enriched phage from the first, second and third round pannings, and 1 in 2 dilutions of third round enriched phage, alongside appropriate negative controls, ELISA was carried out to ascertain the binding of the final enriched phage to apo-Lf. All four samples yielded positive results. With the exception of the first round enriched phage which gave a mean absorbance of 0.068, the mean absorbance of the second and third round enriched phage, as well as the 1:2 dilution of the third round phage, were above 0.07 (Figure 4.4).

When the same populations were screened against holo-Lf, the first and second round enriched phage gave mean absorbance of 0.065 and 0.066 respectively, whereas the third round and its 1:2 dilution gave mean absorbance of 0.076 and 0.072 respectively (Figure 4.5).

There were no significant differences between the absorbance of the enriched libraries from the pannings against apo- or holo-Lf.

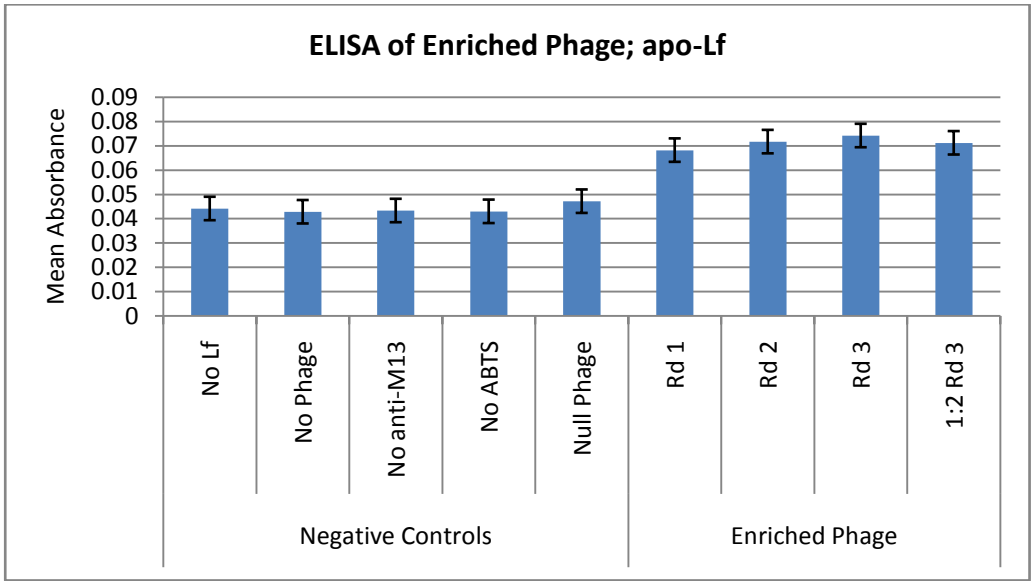

Figure 4. 4 | ELISA of enriched phage-1. ELISA was done using 5 sets of negative controls and aliquots from the first, second and third round enriched phage after three successive pannings against apo-Lf. A final sample of 1:2 dilutions of third round enriched phage was also used. All samples were positive in the ELISA although the first round phage gave a weakly positive result.

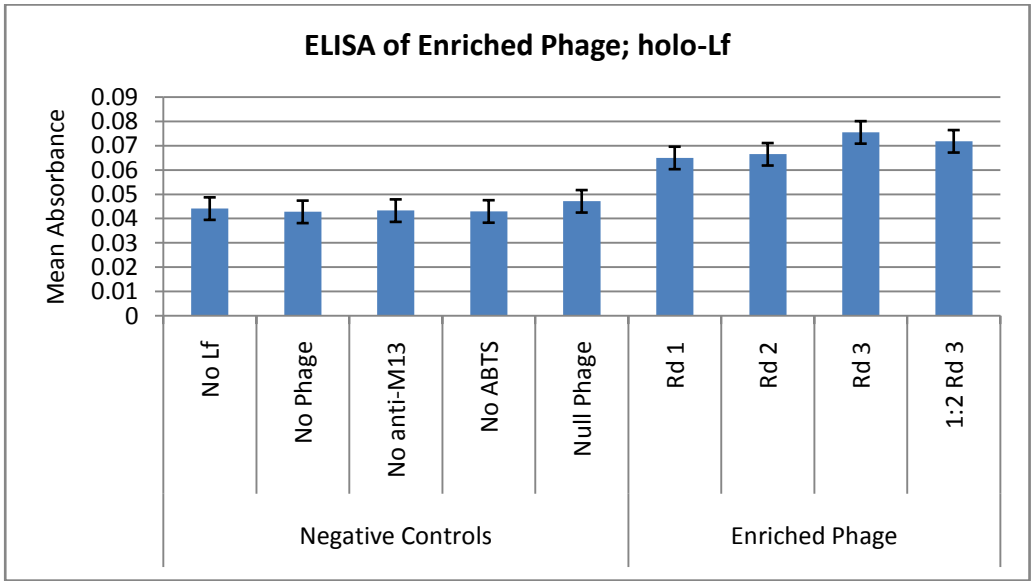

Figure 4. 5 | ELISA of enriched phage-2. ELISA was done using 5 sets of negative controls and aliquots from the first, second and third round enriched phage after three successive pannings against holo-Lf. A final sample of 1:2 dilutions of third round enriched phage was also used. All samples were positive in the ELISA although the first and second round phage gave weakly positive results.

4.3.3 ELISA Screening of Candidate Phage Clones

After confirmation of binding of the enriched library following two separate pannings against apo-Lf and one panning against holo-Lf, 24 colonies were randomly selected from each panning experiment and cultured further to generate individual phage clones, as described previously, and tested individually in ELISA assays. Of the twenty four clones selected from the enriched library from the first apo-Lf panning, twenty of them generated usable cultures. The lids of the other four cultures came off so they were presumed contaminated. All twenty clones (100%) gave positive ELISA results (Figure 4.6), with an average absorbance of 0.074, significantly greater than the cut-off point ($p<0.005$). All twenty four clones (100%) from the second apo-Lf panning also gave positive ELISA results (Figure 4.7), with an average absorbance of 0.084, significantly greater than the cut-off point ($p<0.005$). Twenty one of the twenty four clones (87.5%) from the holo-Lf panning gave positive ELISA results ($p<0.005$) (Figure 4.8), with an average absorbance of 0.085. the three clones, 14, 15 and 22, that yielded the negative results had mean absorbance each of 0.052, 0.063 and 0.054 respectively.

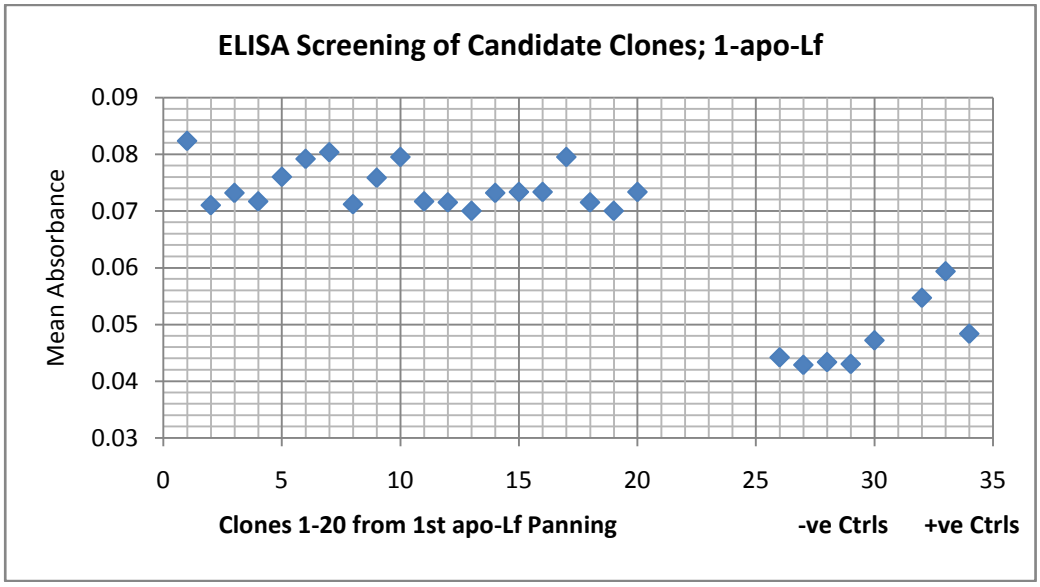

Figure 4. 6 | ELISA screening of candidate clones-1. 24 colonies were randomly selected from the enriched library following three successive pannings against apo-Lf and cultured to generate individual phage clones which were used in an ELISA assay. Of the twenty four clones, twenty yielded usable cultures, all yielding positive ELISA results. The negative controls (-ve Ctrls) and the false positive controls (+ve Ctrls) are given on the right hand side of the graph as samples 26-30, and 32-34 respectively.

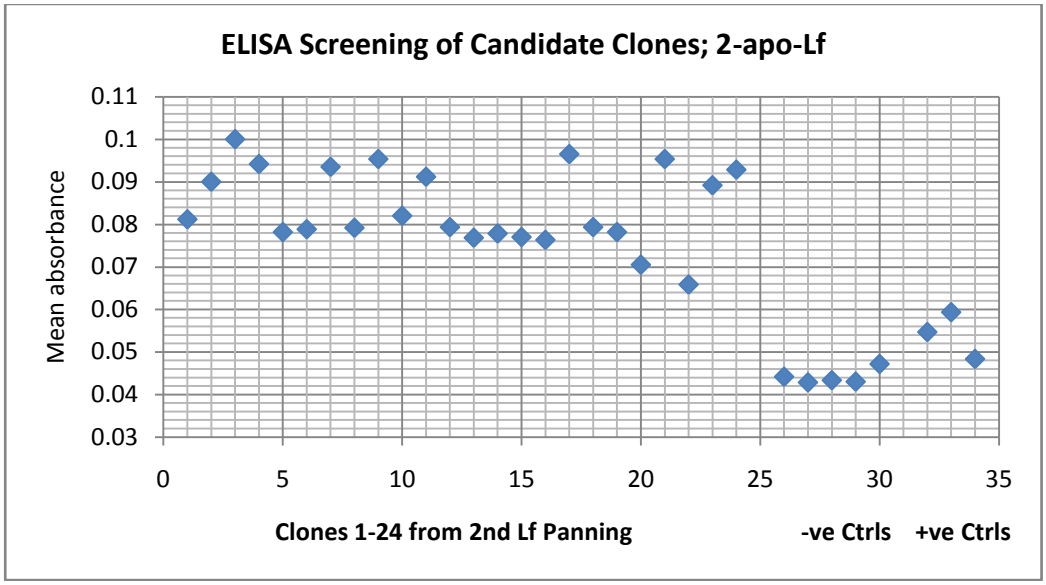

Figure 4. 7 | ELISA screening of candidate clones-2. 24 colonies were randomly selected from the enriched library following three successive pannings against apo-Lf and cultured to generate individual phage clones which were used in an ELISA assay. All twenty four clones yielded positive ELISA results, although clone 22 was weakly positive with a mean absorbance of 0.066. The negative controls (-ve Ctrls) and the false positive controls (+ve Ctrls) are given on the right hand side of the graph as samples 26-30, and 32-34 respectively.

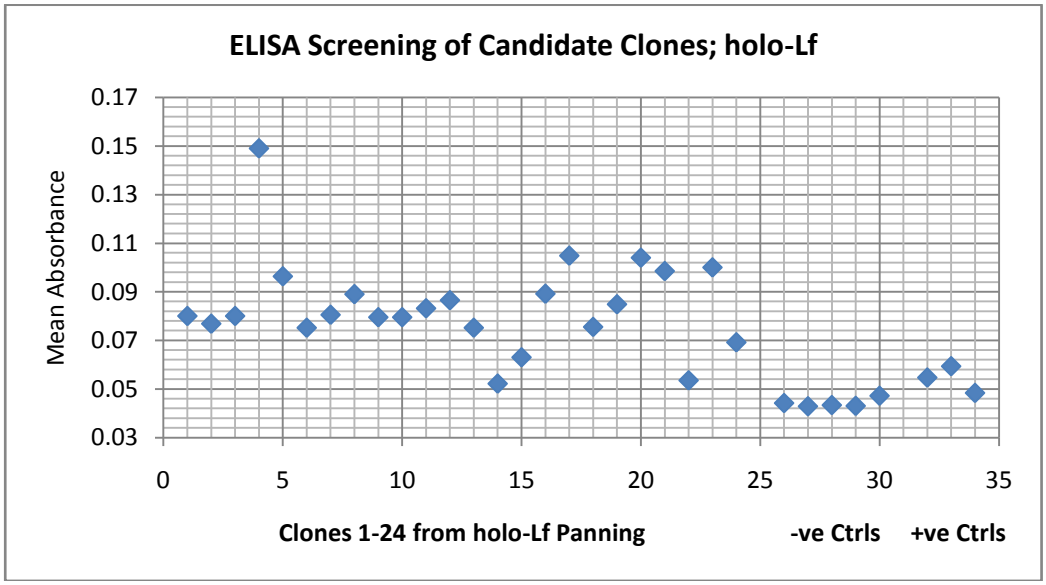

Figure 4. 8 | ELISA screening of candidate clones-3. 24 colonies were randomly selected from the enriched library following three successive pannings against apo-Lf and cultured to generate individual phage clones which were used in an ELISA assay. Twenty one clones yielded positive ELISA results, with clones 14, 15 and 22 yielding weakly positive results with a mean absorbance below 0.065. The negative controls (-ve Ctrls) and the false positive controls (+ve Ctrls) are given on the right hand side of the graph as samples 26-30, and 32-34 respectively.

4.3.4 Sequencing of Plasmid from Positive Clones

Following the ELISA assays, aliquots of the phage clones that yielded positive results were used to infect *E. coli* TG1 cells as described. These were cultured and plasmid DNA extracted for sequencing. Given that only three clones gave weakly positive results from the holo-Lf panning, the clones were also prepared for sequencing.

Usable sequence results were obtained for 42 of the 44 clones from the two apo-Lf pannings and all 24 clones from the holo-Lf panning.

4.3.5 Bio-informatic Analysis of Positive Clones

A summary of the genes isolated from the pannings against the various ligands is provided in Table 4.1 below. After analyses of the DNA inserts, clones were considered candidate genes if they were in the right orientation and in frame with gene 8 of the vector and had a unique predicted function of possible interest, with or without the presence of signal peptide and transmembrane domain (also see Table 3.1).

The gene, Cj0609c, was repeatedly isolated from the sequence results of both apo- and holo-Lf pannings. Whilst all clones that harboured sequence derived from Cj0609c yielded significantly positive ELISA results, the mean absorbance varied among the different clones. The sequence result of the insert of clone 4 from the holo-Lf panning, which had the highest mean absorbance of 0.15, revealed the isolation of Cj0609c.

The sequence results of clones 15 and 22 from the holo-Lf panning, which gave negative ELISA results, indicated that they had no inserts. However, clone 14, also from the holo-Lf panning, which also gave negative ELISA result, encoded for the gene Cj0697. This gene was also isolated in clone 8, which gave a positive ELISA result with mean absorbance of 0.089.

Ligand	Isolated *C. jejuni* Gene(s)	The Insert		Predicted Function (s)	SignalP (Y/N)	TMD (Y/N)
		Size (bp)	Fre-quency			
				Enriched Pannings		
apo-Lf	i. Cj0609c	457	26/42	Probable periplasmic protein	Y	N
	ii.Cj1618c	586	4/42	Probable haemin uptake system/periplasmic haemin-binding protein	N	N
	iii.Cj0599	129	2/42	Putative OmpA family protein	Y	Y
holo-Lf	i. Cj0609c	457	14/24	Probable periplasmic protein	Y	N
	ii. Cj0525c	95	2/24	Putative penicillin-binding protein	Y	Y
	iii.Cj0697	65	2/24	Flagellar basal-body rod protein	N	N

Table 4. 1 | Summary of unique ligand-specific genes after three rounds of panning. All clones that produced readable sequences were analysed, and the inserts in the right orientation within the vector positively identified. Based on the probable function and location within *C. jejuni*, three clones were selected for each ligand and the presence of signal peptide or transmembrane domain determined. All clones were ELISA-positive except one of Cj0697. Y/N mean yes/no.

4.3.6 The Gene, Cj0609c

The most frequently isolated gene in the panning against Lf, Cj0609c, encodes a *ca.* 45-kDa periplasmic protein predicted to be basic, with pI of 10.3. In silico analysis of Cj0609c was carried out using the SignalP 3.0, LipoP 1.0 and SecretomeP 2.0 servers (http://www.cbs.dtu.dk/services/). This revealed the presence of a signal peptidase I cleavage site at TSSYA↓^{22}QNLNT with a probability of 0.997 but a low signal anchor probability of 0.002. This is an indication that once this protein is exported out of the cytoplasm, it had no association with the inner membrane. This was confirmed with the TMHMM server which showed that the protein has no transmembrane helices. However, the SecretomeP 2.0 server predicted the possibility of Cj0609c being a non-classically secreted protein.

Whereas Cj0609c is identical to some conserved domain proteins in other *Campylobacter* strains such as *C. jejuni* subsp. *doylei* 269.97, *C. jejuni* RM1221, *C. jejuni* 81-176, *C. coli* and *C. upsaliensis*, it is also homologous to a number of uncharacterized periplasmic proteins in several Gram-negative bacteria notably *Helicobacter hepaticus*, *Helicobacter cinaedi*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Fusobacterium nucleatum*, and some Gram-positive bacteria such as *Bacillus cereus*, *Lactobacillus acidophilus*, *Clostridium botulinum*, and *Clostridium perfringens*. Cj0609c is also homologous to putative lipoproteins of *Proteus mirabilis*, *Pseudomonas*

fluorescens, and the virulence factor *mviN* of *Clostridium tetani*. However, Cj0609c had no homology with the Lf-binding proteins of *N. meningitidis* or *N. gonorrheae*.

Further analysis of conserved domains within this gene revealed that Cj0609c is a putative periplasmic member of the SGNH-family of hydrolases, a diverse family of lipases and esterases, which are composed of an active site, a catalytic triad and an oxyanion hole (Wei *et al.*, 1995); all these features are predicted in Cj0609c with high confidence [see Figure 4.9].

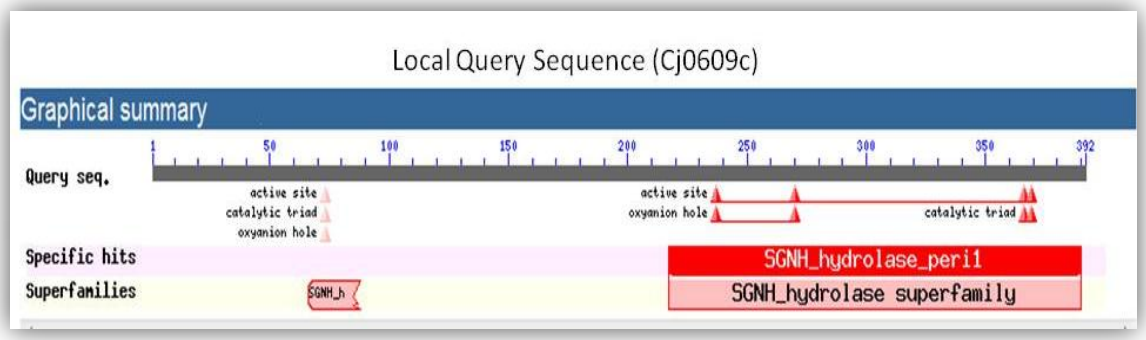

Figure 4. 9 | BLAST search of Cj0609c depicting SGNH hydrolase superfamily. All three features of the SGNH-family of hydrolases are present in Cj0609c, suggesting a role in hydrolysis of a protein(s).

Furthermore, Cj0609c is the first of four adjacent operon-like genes, Cj0609c-Cj0612c, with the first two encoding probable periplasmic proteins and the last two encoding a probable transmembrane transport protein and a probable ferritin respectively. The last of these four genes, Cj0612c, has been described in the past as *Campylobacter* ferritin (*cft*) gene but has not been extensively studied in conjunction with the other three, including Cj0609c (Wai *et al.*, 1996; Wai *et al.*, 1997).

4.4 Discussion

Since the use of ELISA was necessary to confirm whether or not enriched phage or clones in this study have the potential to bind to a specific ligand, it was important that reliable ELISA data were obtained, which necessitated initial validation to provide information on the level of sensitivity and the specificity of the response.

Usually, the cut-off score for positivity is determined by calculating the mean absorbance of a set of negative controls, plus three standard deviations (Robinson *et al.*, 1997). However, due to the low signals produced in phage ELISA it was important that signals that could present as positive results were eliminated and hence the use of the three false positive controls in addition to the conventional negative controls (Figure 4.2). In this regard, any absorbance above the determined value of 0.065 would have eliminated the background effect of the negative controls as well as that from the false positive controls, and could be regarded as true positive. This made the interpretation of ELISA results easier.

As stated earlier, it is difficult to add more than 10^{12} virions per 100 µl well during characterization of phage clones by ELISA (Adey *et al.*, 1995). At this concentration of *ca.* 16 nM, an unambiguously positive ELISA signal can only be observed if the binding affinity is in the micromolar range or better (McCafferty & Johnson, 1996). The iterative nature of phage selection permits identification of peptides with a broad range of affinities to a specific ligand, from sub-nanomolar to 1 millimolar, so lower affinity clones may even not show a positive ELISA signal (Adey *et al.*, 1995). However, it is worthwhile doing it since it could also provide invaluable information for further studies.

In the ELISA experiments described in this chapter, it was only possible to add approximately 10^{7} virions per 100 µl well based on the starting phage titres and the dilution factor of the blocking solution. This meant that if the binding affinity of any of the clones was low, then only minimal signal would be produced. Signals obtained in the optimization experiments were generally lower (data not shown) than the signals obtained in the substantive experiments.

This can be attributed to the fact that in the optimization experiments, incubation was carried out for only two hours following addition of the phage clones to Lf-coated wells, whilst in the substantive experiments incubation was done overnight as in the panning experiment.

Confirmation of the binding activity of the enriched libraries after three successive pannings was the starting point in clone isolation for further analysis. Thus, if a purportedly enriched library did not give a positive ELISA result, there would be no need to proceed to isolate single clones for further analysis. Obviously, all three pannings against apo-Lf and holo-Lf led to the enrichment of the phage display library *Cj*NCTC11168 for the respective ligands. Although the first and the second holo-Lf pannings yielded weak ELISA results, the third one produced a slightly higher absorbance of 0.076. However, there were no significant differences between the mean absorbance of the enriched libraries from the two pannings.

Majority of the selected individual clones (65/68 or 95.6%) gave positive ELISA results. Nevertheless, the mean absorbance for each individual clone varied among the clones, which could be a normal variation in binding affinity. Although clone 4 from the holo-Lf panning seemed like an outlier with a very high mean absorbance, it was interesting to know that its insert encoded for the most commonly isolated gene i.e. Cj0609c. Generally, if a gene appeared more than once (2-5×) in the sequenced clones, it was analysed in more detail.

C. jejuni contains both *N*- and *O*-linked glycosylation systems, which is an important determinant of the functions of its proteins (Szymanski *et al.*, 2003). The absence of the necessary glycosylation in the proteins expressed by the phage particles may therefore affect their ability to bind to the right target ligand. Further work is therefore needed to confirm or otherwise the authenticity of the isolated genes.

However, the repeated isolation of Cj0609c cannot be overlooked. The presence of a signal peptidase I cleavage site at TSSYA↓^{22}QNLNT in Cj0609c, without any association with the inner membrane indicates a *sec*-dependent transport (Kim & Kendall, 2000). Furthermore, the possibility of Cj0609c being a non-

classically secreted protein may also suggest a further transport beyond the periplasm.

Cj0609c is identical to some conserved domain proteins in other *Campylobacter* strains, and some Gram-positive bacteria. Although the functions of these proteins are unknown, it is likely that they play a vital role in the survival or pathogenesis of these organisms. It is therefore not surprising that Cj0609c is also homologous to putative lipoproteins of *Proteus mirabilis*, *Pseudomonas fluorescens*, and the virulence factor MviN of *Clostridium tetani* (Ling *et al.*, 2006; Rudnick *et al.*, 2001).

The presence of active sites, a catalytic triad and an oxyanion hole in Cj0609c in consonance with SGNH-family of hydrolases is an indication of a probable role as a hydrolase (Upton & Buckley, 1995; Wei *et al.*, 1995). Characterization of a SGNH hydrolase variant from the cyanobacterium, *Anabaena* species, has demonstrated its crucial role in substrate binding and catalytic activity (Bakshy *et al.*, 2009). Hydrolases are found in essentially all domains of life both as intracellular and extracellular enzymes and are largely involved in nutrient acquisition (Bakshy *et al.*, 2009). A recent characterization of a homologue of Cj0609c in a *Bacillus* species revealed the presence of a substrate binding region at the N-terminus of the protein (Navarro-Fernandez *et al.*, 2008).

The well-characterized *C. jejuni* adhesin, PEB1, is a periplasmic protein that has a signal peptidase II cleave site and has been demonstrated in culture supernatants (Young *et al.*, 2007). It is therefore possible that the protein encoded by Cj0609c, although predominant in the periplasm, is transported to the cell surface where it binds to Lf, hydrolyzing it in the process to aid iron transport into the bacterium. Further work is needed to confirm or otherwise that this gene encodes a Lf-binding protein in *C. jejuni* and possibly contributing to the utilization of Lf-bound iron in the organism.

Chapter Five

Identification and Expression of a *Campylobacter* Autotransporter

Identification of Host-pathogen Interacting Molecules of *Campylobacter jejuni* using Phage Display Technology and in silico Sequence Analysis

CHAPTER FIVE

5.0 Identification and Expression of a *Campylobacter* Autotransporter

5.1 Introduction

5.1.1 Protein Secretion Mechanisms in Gram-negative Bacteria

Bacterial proteins, manufactured within the cytoplasm, are usually subjected to compartmentalization, with as much as 50% transported to membrane compartments, the periplasm in Gram-negative bacteria or entirely out of the cell (Cao & Saier, 2003; Desvaux *et al.*, 2004b; Kim & Kendall, 2000). Transport across the inner membrane is aided by a ubiquitous mechanism, the *sec*-dependent pathway consisting of the Sec proteins, well-characterized in *E. coli* or through the *sec*-independent or twin-arginine translocation (Tat) and signal recognition particle (SRP) pathways (Binet *et al.*, 1997; Henderson *et al.*, 2004; Kim & Kendall, 2000). Bacterial pathogenesis is almost invariably defined by these proteinaceous factors that are secreted into the external milieu or to the cell surface (Finlay & Falkow, 1997; Henderson & Nataro, 2001a). These proteins are usually manufactured as precursors with N-terminal amino sequences, termed signal sequences that are important for efficient export (Omori & Idei, 2003). Whereas in Gram-positive bacteria the Sec pathway alone is capable of secreting proteins to the extracellular milieu, with cleavage of the signal peptide releasing the mature polypeptide, in Gram-negative bacteria another barrier in the form of the outer membrane has to be overcome (Binet *et al.*, 1997; Desvaux *et al.*, 2004b).

Gram-negative bacteria have, therefore, developed a number of specialised pathways or secretion systems by which proteins of diverse functions are transported across their double membrane system (Henderson *et al.*, 2000a; Henderson *et al.*, 2004). Recently, Desvaux, Hebraud, Talon and Henderson have proposed that these transport pathways are numbered from I to VIII for Gram-negative bacteria only, whilst the terms “Sec” (secretion), “Tat” (twin-arginine translocation), “FEA” (flagella export apparatus), “FPE” (fimbrilin-

protein exporter), "holin" (hole forming) and "Wss" (WXG100 secretion system) be applied to translocation systems across the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria (Desvaux *et al.*, 2009). For the purposes of this study, I shall briefly discuss types I to V.

The type I secretion system (T1SS) is energy-dependent but *sec*-independent bypassing the periplasm, and involves a multimeric ATP-binding cassette (ABC) protein and two accessory proteins that make up a channel spanning both the inner and outer membranes, and is exemplified by the pathway by which the *Escherichia coli* haemolysin A is secreted (Andersen, 2003; Fath & Kolter, 1993; Shimada *et al.*, 1994). This specific ATP-driven protein translocon of the ABC superfamily consists of two membrane-embedded hydrophobic and two conserved hydrophilic ATP-binding domains, which can either be parts of a single polypeptide or separate polypeptides (Binet *et al.*, 1997). The two accessory proteins are an inner membrane protein and an outer membrane protein, with the latter consisting of a short N-terminal hydrophobic domain anchoring it in the inner membrane, a large hydrophilic domain located in the periplasmic space and a C-terminal domain with a β-sheet structure interacting with the outer membrane (Binet *et al.*, 1997; Delepelaire, 2004). These proteins form a continuum represented in *E. coli* as HlyB, HlyD and TolC involved in the export of the HlyA toxin (Balakrishnan *et al.*, 2001; Holland *et al.*, 2005; Wandersman & Delepelaire, 1990; Wooldridge *et al.*, 2005).

Although the type II secretion system (T2SS) also spans both the inner and outer membranes, this requires as many as fourteen additional accessory proteins encoded on a single continuous operon as exemplified by the pullulanase (PulA) secretion system of *Klebsiella oxytoca* (Nunn, 1999; Possot & Pugsley, 1997; Pugsley *et al.*, 1997). Unlike the T1SS, the T2SS is dependent on the inner membrane *sec* translocase, which is made up of SecA and SecYEG, whilst SecB prevents folding of the substrates within the cytosol prior to secretion (Chapon *et al.*, 2001; Py *et al.*, 1999). The *sec* system is necessary for translocation into the periplasmic space, and once in the periplasmic space the resultant proteins are exported into the extracellular

space through the T2SS which is composed of many gene products, twelve *xcp* genes in the case of *P. aeruginosa* (Mori & Ito, 2006a; Mori & Ito, 2006b; Omori & Idei, 2003). The type II transport system is employed in the secretion of several exoproteins including elastase, exotoxin, protease, and phospholipase C (Crosby & Kachlany, 2007; Mansson *et al.*, 2007; Pradere *et al.*, 2007). Thus, both the Sec and the Tat pathways are used in the translocation of protein across the inner membrane, whilst T2SS exports the protein to the extracellular milieu (Desvaux *et al.*, 2004b).

First described in 1990 by Michiels and co-workers, the type III secretion system (T3SS) is a virulence mechanism by which some pathogenic bacteria inject proteins directly into the cytosol of eukaryotic cells modulating signalling responses during infection (Cornelis, 2002; Michiels *et al.*, 1990). It involves a complex apparatus of proteins which assemble into a tightly regulated oligomeric structure spanning both the inner and the outer membranes, and in some cases, the target host cell membrane (Henderson & Nataro, 2001a). The *sec* system is required for the translocation of some of the component proteins of the secretion apparatus across the inner membrane, but not in the secretion of the effector molecules (Galan & Collmer, 1999; Hueck, 1998; Veenendaal *et al.*, 2009). It is exemplified by the secretion or injection of the *Yersinia* outer proteins (Yops) into the cytosol of host cells that come into contact with the plague-causing agent, *Yersinia pestis* (Felek & Krukonis, 2009; Michiels *et al.*, 1990; Pan *et al.*, 2009). The shigellosis-causing Gram-negative bacterium, *Shigella flexneri*, also initiates infection by inducing a rapid burst of Ipa invasins secreted via this secretion system into colonic epithelial cells (Espina *et al.*, 2006; Picking *et al.*, 2005; Zurawski *et al.*, 2009). The T3SS and the Gram-negative flagellar export apparatus are thought to be homologous, leading researchers to classify the flagellar biosynthesis pathway as T3SS (Blocker *et al.*, 2003; Macnab, 1999).

The type IV secretion system (T4SS) comprises a diverse transporter family involved in competence and conjugative transfer of DNA and/or protein, enabling translocation of effector proteins from a Gram-negative bacterium to another or to a eukaryotic host cell (Burns, 2003; Craig & Li, 2008; Yen *et al.*,

2002). This system is therefore employed in maintaining genome plasticity and contributing to the evolution of pathogens through dissemination of antibiotic resistance and virulence genes, whilst contributing to pathogenesis (Juhas *et al.*, 2007; Juhas *et al.*, 2008). Although this secretion system also involves similar numbers of proteins as the type II and type III systems, these proteins are variously associated with the inner and outer membranes and are located within the periplasm and the cytoplasm, as exemplified by secretion of the pertussis toxin of *Bordetella pertussis* and *Bartonella* species (Burns, 1999; Dehio, 2008; Shrivastava & Miller, 2009).

The type V secretion system (T5SS) was previously thought to be the simplest of all Gram-negative transport mechanisms involving the translocation of proteins across the outer membrane via a membrane pore formed by a self-encoded β-barrel structure, and hence the name autotransporter (Desvaux *et al.*, 2004a; Yen *et al.*, 2002). However, recent developments have revealed minor variations in export of these proteins leading experts to suggest subdivisions [see Figure 5.1] such as the type Va (T5aSS) or autotransporter, the two-partner secretion (TPS) system or type Vb (T5bSS) and the Oca family or type Vc (T5cSS), although the generic name autotransporter still holds (Dautin & Bernstein, 2007; Desvaux *et al.*, 2004a; Desvaux *et al.*, 2009; Renn & Clark, 2008).

The T5aSS is the simplest described below, whilst the T5bSS or TPS involves the secretion of proteins such as adhesins and cytolysins whose translocator domains are transcribed and translated independently of the effector protein as TpsB and TpsA respectively (Jacob-Dubuisson *et al.*, 2001).

In 2004, Surana and co-workers described a novel subfamily of autotransporters in *Haemophilus influenzae*, characterised by a short trimeric C-terminal translocator domain and referred to these as trimeric autotransporters (Cotter *et al.*, 2005; Surana *et al.*, 2004). These trimeric autotransporters (T5cSS) have since been described in *E. coli*, *N. meningitidis*, *Bartonella* and *Haemophilus influenzae* (Leduc *et al.*, 2009; Pina *et al.*, 2009; Sjolinder *et al.*, 2008; Szczesny *et al.*, 2008; Szczesny & Lupas, 2008; Valle *et al.*, 2008).

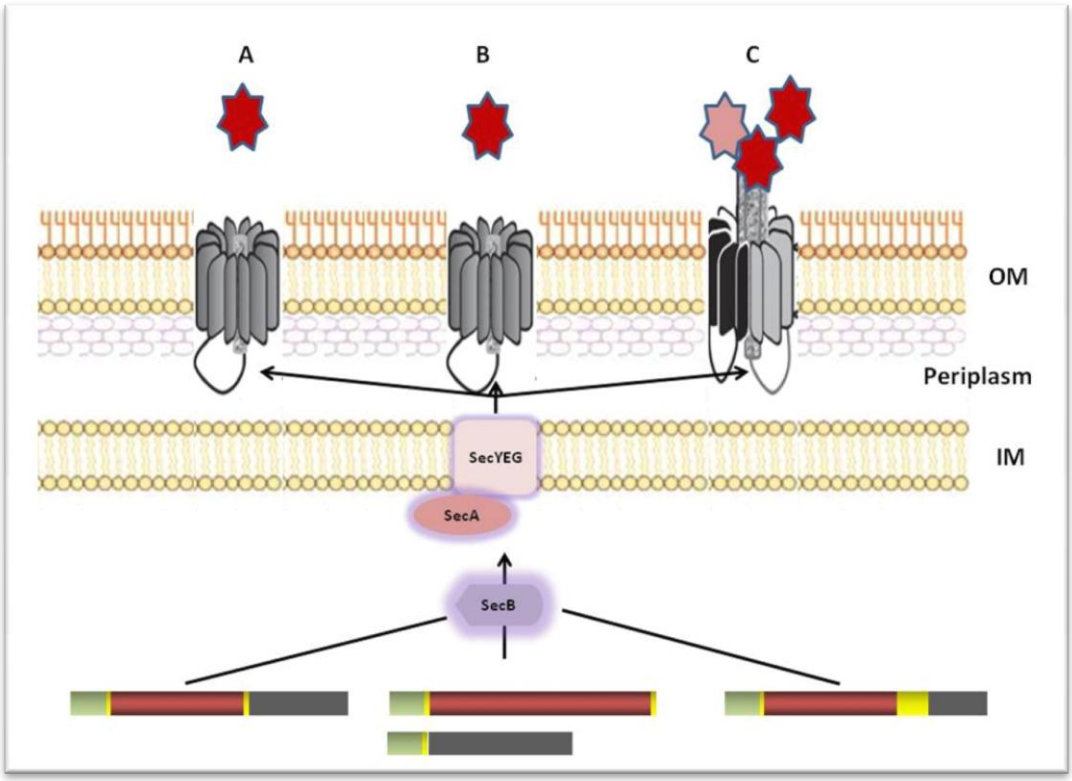

Figure 5. 1 | Schematic representation of the T5SSs. All three subdivisions of the system use the dedicated the inner membrane *sec* translocase, which is made up of SecA and SecYEG, with SecB serving as a chaperon to prevent cytosolic folding of substrate. Autotransporters (A) are produced as single polypeptide chains, which are then translocated via the β-barrel domain at their C-terminus. The TPS effector proteins (B) and their translocator domains are produced as separate polypeptides, which associate within the periplasm prior to secretion of the effector protein. Proteins secreted via the T5cSS (C) essentially behave as autotransporter proteins but have a shortened C-terminal domain encompassing one third of a translocation unit (predicted as four β-strands), which form a functional translocator as a trimer [Adopted with permission from Bacterial Secreted Proteins, edited by Karl Wooldridge, 2009].

The genome sequence of *C. jejuni* reveals the presence of both the *sec*-dependent and the *sec*-independent (Tat) protein export pathways for secretion of proteins across the inner membrane and the periplasmic space, as well as the signal recognition particle pathway (Fouts *et al.*, 2005; Parkhill *et al.*, 2000). Whilst there is no evidence of T1SS or T4SS in *Campylobacter*, the flagellar system of *C. jejuni* is known to function as both a flagellum and protein secretion system; this secretion system is known to be homologous to T3SS (Guerry, 2007). The transfer of genetic material is achieved through T2SS in *C. jejuni* (Konkel *et al.*, 2004a; Wang & Taylor, 1990; Wiesner *et al.*, 2003). The initial part of this study which involves a protein secreted by the T5SS (autotransporter) was recently also reported in *C. jejuni* (Ashgar *et al.*, 2007).

5.1.2 Autotransporter Proteins

This secretion pathway (T5aSS) was first described as being responsible for the secretion of IgA1 protease of *Neisseria gonorrheae*, the components of which consist of an N-terminal leader peptide required for *sec*-dependent secretion across the inner membrane, a functional secreted mature protein known as the passenger domain, and a C-terminal domain, which forms a β-barrel or pore in the outer membrane through which the passenger domain passes to the exterior [see Figures 5.1, 5.2 and 5.3] (Henderson *et al.*, 2000b; Henderson *et al.*, 2004; Meyer *et al.*, 1987; Pohlner *et al.*, 1987a).

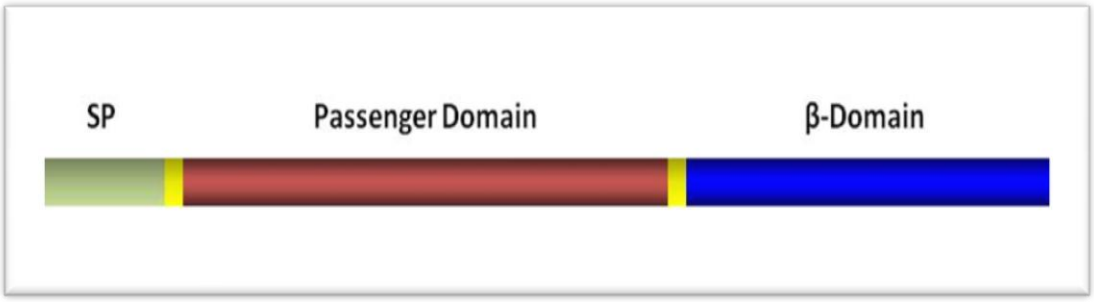

Figure 5. 2 | Schematic representation of the structure of autotransporter proteins. Proteins exported through the type V secretion system comprise of an N-terminal leader peptide, a functional secreted passenger domain, and a C-terminal β-barrel/domain through whose aperture the passenger domain passes to the exterior.

These auto-transported proteins or autotransporters have become a rapidly growing family of proteins among Gram-negative bacteria due to their diverse passenger domains and different functions (Henderson *et al.*, 2004; Jose & Meyer, 2007). Secretion via this pathway requires the *sec* system for transport across the inner membrane but further secretion beyond the outer membrane is thought to be determined by features that are contained within the secreted molecule (Henderson & Nataro, 2001a). It has been suggested that the size of β-barrel may define the structural properties of the secreted proteins (Henderson *et al.*, 1998; Henderson *et al.*, 2004; Oomen *et al.*, 2004). Again, it is believed that the physiological functions of the protein may determine or be determined by what happens once it is at the cell surface (Henderson *et al.*, 1998).

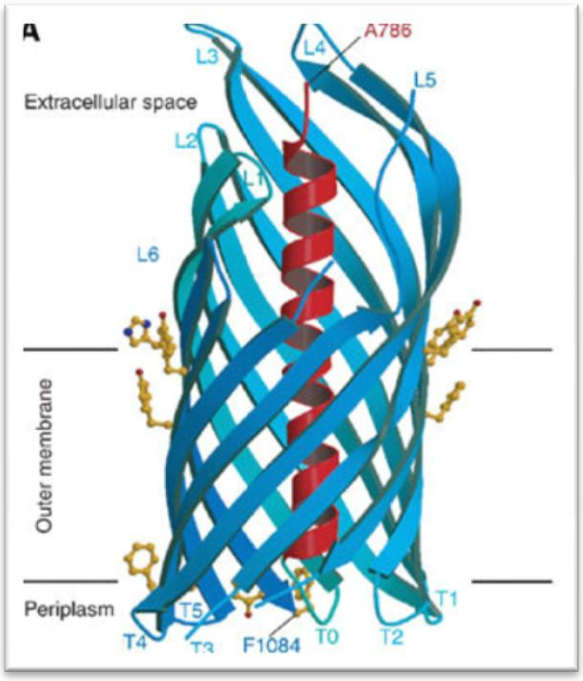

Figure 5. 3 | The Crystal structure of the autotransporter NalP of *N. meningitidis*. Side view of $NalP_{\beta}$ in space group $P6_122$ shows a 12-stranded β-barrel (blue ribbon representation). The hydrophobic membrane-embedded region is flanked by aromatic residues (yellow ball-and-stick). The periplasmic side is characterised by short turns (T0–T5) and the extracellular side by longer loops (L1–L6) connecting the alternating β-strands. An α-helical 'plug' (red ribbon representation) is connected to the barrel via T0 and positions the N-terminus of the translocator domain (Ala 786) at the extracellular side [Adopted from Oomen *et al.*, 2004].

The passenger domain may (i) remain attached to the β-domain and protrude from the bacterial surface as a large polyprotein; (ii) be cleaved from the β-domain, but remain loosely associated with it via non-covalent interactions; or (iii) be cleaved and totally detached from the β-domain to perform its functions in the external environment (Henderson *et al.*, 1998). A further level of variation exists – some proteins, such as the IgA proteases catalyse their own cleavage, while others, such as the IcsA of *Shigella flexneri*, require an accessory protease for cleavage (Shere *et al.*, 1997; Steinhauer *et al.*, 1999; Veiga *et al.*, 1999). A significant number of auto-transporter proteins have been characterized for several Gram-negative pathogens with most of these organisms secreting more than one protein and some proteins having multiple functions (Henderson *et al.*, 2004). In summary, characterized autotransporter proteins have been known to function as adhesins, proteases, mucinases, and toxins or to mediate serum resistance (Henderson & Nataro, 2001b; Lawrenz *et al.*, 2009; Reidl *et al.*, 2009; Swanson *et al.*, 2009; Van Gerven *et al.*, 2009; Wagner *et al.*, 2009).

5.1.3 Aim of the Study in This Chapter

In spite of the identification of several autotransporter proteins with virulence functions in several pathogenic organisms including *Neisseria* species, *Haemophilus* species, *Bordetella* species, *Shigella* species, *E. coli* and *H. pylori*, only one has so far been described for *Campylobacter* (Ashgar *et al.*, 2007).

Several of the autotransporter proteins which were discovered several years ago such as the MisL of *Salmonella enterica* serovar Typhimurium have only recently been characterized (Blanc-Potard *et al.*, 1999; Dorsey *et al.*, 2005; Tukel *et al.*, 2007), whilst others, such as the Ssa1 of *Mannheima (Pasteurella) haemolytica* A1, which were not considered members of this family, have recently been shown to possess autotransporter properties (Gioia *et al.*, 2006; Lo *et al.*, 1991).

The autotransporter mechanism of protein secretion has therefore become an area of intense research with ongoing studies leading to the identification and characterization of several complex arrays of proteins secreted in this way, and adding to our understanding of the fundamental features of these proteins and their roles in bacterial pathogenesis (Leduc *et al.*, 2009; Serruto *et al.*, 2009; van der Woude & Henderson, 2008).

Thus, given the varied pathogenic functions of autotransporters, an in silico analysis of the *C. jejuni* NCTC11168 genome was carried out to identify putative autotransporter proteins and further express one of them for characterization.

The main aim of this study was to express the full-length CapA protein to enable structural and further functional characterization of the protein.

A full-length CapA protein could be used in further studies including:

- identification of host cell ligands
- whole cell inhibition assay
- raising antibodies for western-blotting and immuno-precipitation
- cross-linking experiments

5.2 Materials & Methods

5.2.1 Bio-Informatic Searches and Sequence Analysis

In order to identify novel autotransporter proteins of *C. jejuni*, the amino acid sequences of previously described autotransporters IgA1 protease (Pohlner *et al.*, 1987b), AIDA-I (Benz & Schmidt, 1989), Hia (Barenkamp & St Geme, 1996), Hap (Hendrixson & St Geme, 1998), Pet (Eslava *et al.*, 1998), Pic (Henderson *et al.*, 1999), and AspA (Turner *et al.*, 2002) were employed to search the predicted coding sequences of *C. jejuni* NCTC11168 genome through the Basic Local Alignment Search Tool (BLAST) of the Wellcome Trust Sanger Institute *C. jejuni* gene database (http://www.genedb.org/genedb/cjejuni/blast.jsp).

The resulting protein sequences were examined for the typical characteristics of autotransporter proteins as given by Henderson (Henderson *et al.*, 2004). Protein sequences of the predicted passenger domains were used to perform BLASTP searches of the non-redundant protein database (nrdb95) and the Omniome pep database using the WU-BLAST2 servers at http://dove.embl-heidelberg.de/Blast2/ and http://tigrblast.tigr.org/cmr-blast/, respectively.

Using the Neural Network Promoter Prediction program (www.fruitfly.org/seq_tools/promoter.html) DNA segments were examined for potential promoter sequences. Ribosome-binding sites were also predicted using the Gene Locator and Interpolated Markov ModelER server (Glimmer 3.02) (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi). Identified proteins were analysed for signal peptide-triggered and non-classical protein secretion using the signalP 3.0 and SecretomeP 2.0 servers for Gram-negative bacteria respectively (http://www.cbs.dtu.dk/services/). Using the TMHMM 2.0 programme (http://www.cbs.dtu.dk/services/TMHMM-2.0/) an attempt was made to also identify transmembrane domains in the selected genes. Nucleotide sequence data were analyzed using the DNAMAN software (Lynnon Corporation).

Two genes were identified to encode autotransporter proteins in the available genome sequences of *C. jejuni*, which based on their initial functional

characterization, were later designated *Campylobacter* adhesion protein A, *capA* [Cj0628/Cj0629] and *Campylobacter* adhesion protein B, *capB* [Cj1677/Cj1678] (Section 5.3.1). The focus of this chapter was on the expression of *capA.*

5.2.2 General Materials

5.2.2.1 Antibiotic Supplements

[See Section 2.6.3 above]

5.2.2.2 Culture Media

[See Section 2.6.4 above]

5.2.2.3 Miscellaneous Buffers and Solutions

[See Section 2.6.5 above]

5.2.2.4 DNA Extraction, Purification and Quantification

[See Section 2.6.9, 2.6.10, and 2.6.11 above]

5.2.2.5 DNA Sequencing

[See Section 3.6.11 above]

5.2.2.6 Polymerase Chain Reaction

PCR was carried out using a Techne 96-well thermocycler. Primers used in this study (Table 5.1) were synthesized by Eurofins GmbH (Germany).

Primer	Sequence	Amplification of
capA_F1	5´ - ATTTCTTTTTTGGCAAGTTGTAC - 3´	Truncated *capA* ORF Cj0628, nt 114-1115
capA_R1	5´ - ATAGACATTGACCACCATTTAA - 3´	
capA_F2	5´ - GGATCCATGGGTGTAAATGTTCGTTC -3´	Truncated *capA* ORF Cj0629 , nt 751-3413
capA_R2	5´ - GTCGACTTACCAAAGATAATTAAACTGAGC - 3´	
capA_F3	5’ - ATGAATAAAACTGCATTAACTAAAACATAC - 3’	Full-length *capA* ORFs Cj0628/Cj0629, nt 1-3421
capA_R3	5’ - TACCCATACAGGATTTGCTCAGT - 3’	
capA_F4	5´ - TAACGGTGTTAGCATAGAAACC - 3´	Across poly-T/poly-G tract of Cj0628, nt 444-555
capA_R4	5´ - CAGTATTAGAGTGAATAGTTCCAC - 3´	

Table 5. 1 | PCR Primers used in the amplification of truncations of the *capA* gene. The *capA* gene comprises of two open reading frames (ORFs), Cj0628 and Cj0629 when out of frame, made up of nucleotides 1-541 and 721-3421 respectively. Deletion of a T or G between nucleotides 497 and 511 puts the two ORFs in frame, and can be translated as one protein. PCR was done to amplify fragments of the gene as well as the full-length *capA* after deletion of a G.

5.2.3 Bacterial Strains and Culture conditions

Wild type *Campylobacter jejuni* NCTC11168 was obtained from laboratory stock maintained in a 50:50 mixture of glycerol and Mueller-Hinton (MH) broth and stored at -80°C. *C. jejuni* NCTC11168 was grown at 42°C on charcoal-cefoperazone-desoxycholate agar (CCDA) or in MH broth (Oxoid) with shaking, in a MACS VA500 Microaerophilic workstation (Don Whitley Scientific) in atmospheric conditions of 6% O_2, 5% CO_2, 3% H_2 and 86% N_2.

Commercial *E. coli* strains, JM83 (Promega), Tuner (DE3) pLacI (Novagen) and BL21-CodonPlus™ (Stratagene) were used to express various forms of CapA protein. *E. coli* cells were cultured in LB broth or agar, containing the appropriate antibiotics, at 37°C overnight, with shaking where appropriate.

5.2.4 Cloning and Mutagenesis

The *capA* gene comprises of two ORFs, Cj0628 and Cj0629 when out of frame, made up of nucleotides (nt) 1-541 and 721-3421 respectively. Deletion of a G or T between nucleotide 497 and 511 puts the two ORFs in frame, and can be translated as one protein. PCR was done to amplify fragments of the gene as well as the full length *capA*.

5.2.4.1 Cloning of Full-length *capA*

Primers capA_F3 and capA_R3 were used to amplify either the *capA* gene from chromosomal DNA of wild type *C. jejuni* NCTC11168 or *capA* mutant harbouring a repaired-*capA* gene [see section 5.2.7], each approximately 3.5 kb in size. The sequences spanning the poly-T/poly-G tracts of *capA* in the two constructs were confirmed by PCR and sequencing using primers capA_F4 and capA_R4.

A 25-µl PCR reaction mixture contained 1 µl of DNA, 0.5 µM each of the respective forward and reverse primers, 10 mM dNTPs, 2.5 µl of 10 × PCR buffer, and 2 U of *Taq* Polymerase. Reactions were subjected to an initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, an annealing temperature of 50°C for 45 s, an extension at 68°C for 4 min, an annealing step at 50°C for 45 s and a final extension at

68°C for 3 min. PCR products were then analyzed by gel electrophoresis using ethidium bromide staining.

The resulting PCR products were labelled as original capA and repaired capA, *capAori*/*capArep* respectively. The PCR products were ligated into pETBlue-1 AccepTor vector (Novagen) according to manufacturer's instructions, generating two recombinant genes pET-*capAori* and pET-*capArep* respectively.

5.2.5 Generation of CapA Proteins

As stated earlier, the *capA* gene comprises of two ORFs, Cj0628 and Cj0629, when out of frame, encoded by nucleotides (nt) 1-541 and 721-3421 respectively. Deletion of a T or a G, or four nucleotides, between nucleotide 497 and 511 puts the two ORFs in frame to translate as one protein with a molecular mass of *ca*. 120 kDa.

5.2.5.1 Expression and Purification of Full-length CapA Protein

The resulting full-length *capA* gene products, pET-*capAori* and pET-*capArep*, encoded proteins pET-capAori [possibly one or two fragments] and pET-capArep respectively. The expected molecular mass of pET-capArep was calculated to be 116 kDa.

The two gene products were each used to transform two separate *E. coli* strains, Tuner (DE3) PLacI competent cells (Novagen) and BL21-CodonPlus competent cells (Stratagene) according to manufacturers' instructions. The transformed *E. coli* strains were cultured in LB broth to mid-log phase, and then induced using IPTG. Cells were grown for a further 4 h and then harvested by centrifugation at 4000 × *g*. These were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using Rabbit anti-serum raised against CapA (RαCapA) [see Section 5.2.6].

5.2.6 Repair of *capA*

To ensure that expression of a full-length CapA protein was fixed and no phase variation could occur, the *capA* gene was engineered using PCR so that the poly-T and adjacent poly-G tracts, which interrupt the full *capA* coding sequence, were replaced with a DNA sequence 4-bp shorter (to put *capA* in-frame) and encoding the same amino acids, but containing no homopolymeric sequences. A novel complementation strategy was developed to insert a single copy of *capA* into the genome of a *capA* mutant. This approach involved inserting *capA* and its upstream promoter sequence into the intergenic space between the Cj0652 and Cj0653c genes. Once this gene and a resistance marker had been introduced into the chromosome by double homologous recombination from a suicide vector, the genotype of the resulting strain was confirmed by PCR and sequencing. Immunoblotting experiments on equivalent amounts of whole cell protein were then carried out to confirm that CapA was being expressed in the complemented strain.

5.2.7 SDS-PAGE, Coomassie-staining, Western- & Immuno- Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using standard procedures. Western blotting was carried out using a TransBlot SD (BioRad) according to manufacturer's instructions. Gels were run in duplicate for Coomassie staining and immunoblotting.

Gels were stained using SimplyBlue^{TM} SafeStain for a minimum of 1 h, and de-stained with distilled water for a minimum of 2 h. Gels were photographed using the AlphaDigiDoc software via an Olympus digital camera. Duplicate gels were transferred in an appropriate buffer for Semi-dry Western transfer and immunoblotting analysis.

After western transfer, nitrocellulose membranes were blocked for 2 h with 20 ml of blocking solution [either in 5% non-fat milk solution or 2% BSA (Bovine Serum Albumin, Sigma) in phosphate buffered saline (PBS, Oxoid) and 0.05% Tween20]. Pre-adsorbed RαCapA, which had been diluted 1:500 in blocking solution, was added to each membrane covering it completely. This was incubated at room temperature overnight with shaking.

Blotting membranes were washed twice with PBS-Tween for 20 min in each case with shaking. A secondary antibody of Goat anti-rabbit HRP conjugate diluted 1:2000 in 20 ml of blocking solution was added to each membrane and incubated at room temperature for 2 h with shaking. Membranes were washed twice with PBS-Tween for 20 min with shaking, and radiographed.

5.2.8 Detection of Proteins on Blotting Membranes

Membranes were developed using Enhanced ChemiLuminescent (ECL) reagents (GE Healthcare) according to manufacturer's instructions.

ECL is known to be more sensitive. However, in some instances where membranes did not produce satisfactory images with ECL, they were washed with PBS-Tween and re-developed using the conventional 4-Chloro-1-naphthol (4-CN). Approximately 3 mg of 4-CN was added to 1 ml of ethanol in a separate tube. Another mixture containing 1 ml of 0.5M Tris-HCl (pH 6.8), 9 ml of dH_2O and 50 µl of H_2O_2 was prepared separately, and then the content of the two tubes added up, and placed on the membranes for about 10 min.

5.3 Results

5.3.1 In Silico Sequence Analysis of two *Campylobacter* Autotransporters

Using the amino acid sequences of known adhesion-associated and other well-characterized autotransporters to search the predicted coding sequences of *C. jejuni* NCTC11168, two genes, Cj0628 and Cj1677 [see Figure 5.4], were identified as coding for proteins that bear all of the typical characteristics of autotransporter proteins (Henderson *et al.*, 2004).

The C-terminal two-thirds of the protein sequences of the two genes (extending from ^{375}F in Cj0628 and ^{349}F in Cj1677) bear 100% identity (Figure 5.3), with portions (residues ^{862}S to ^{1144}W in Cj0628 and ^{839}S to ^{1121}W in Cj1677) encoding the predicted autotransporter β-barrel domain terminating in the "YLW" motif, which conforms to the typical autotransporter motif (Y/V/I/F/W)-X- (F/W) (Henderson *et al.*, 2004).

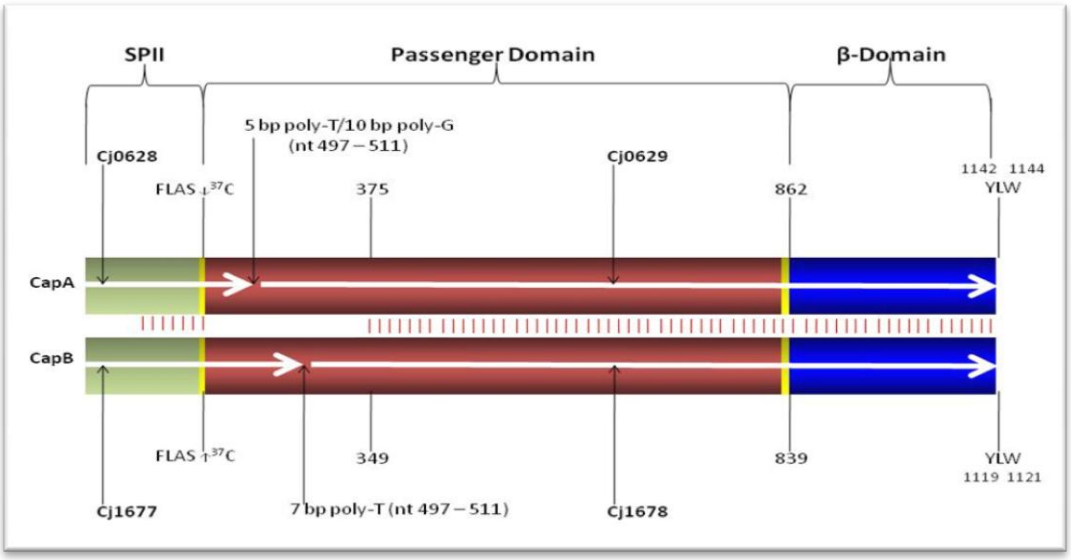

Figure 5. 4 | Schematic diagram of the predicted protein sequences of CapA (Cj0628/Cj0629) and CapB (Cj1677/Cj1678). Green shading, signal peptidase II-dependent signal peptides (SPII); Red shading, passenger domains; blue shading, β-domains. Predicted signal peptidase II cleavage sites are indicated. Both the Cj0629 and Cj1678 predicted protein sequences end with the motif "YLW," which conforms to the typical autotransporter motif (Y/V/I/F/W)-X-(F/W). Red vertical stripes indicate regions of identity between CapA and CapB. White solid arrows represent the ORFs encoding each protein; homopolymeric tracts resulting in frameshifts are also indicated. Unless otherwise indicated, numbers refer to amino acids [see Ashgar *et al.* 2007, *J Bacteriol* 189, 1856-1865].

They were later designated *Campylobacter* adhesion protein A, *capA* [Cj0628] and *Campylobacter* adhesion protein B, *capB* [Cj1677] respectively based on their adhesion properties (described later).

By contrast, the N-terminal regions are highly divergent except for 20 amino acids immediately preceding the likely signal peptidase II cleavage site. The only two cysteines found in the protein are contained in the predicted signal peptide, which also conforms to the low cysteine content of known autotransporter proteins (Henderson *et al.*, 2004).

The annotated sequence released by the Sanger Institute reveals, in the unspliced DNA of each gene, the presence of two separate ORFs, previously assigned Cj0628/Cj0629 and Cj1677/Cj1678 respectively, due to the presence of premature stop codons downstream of homopolymeric tracts. There is a 5-bp poly-T tract immediately followed by a 10-bp poly-G tract between nucleotides (nt) 497 and 511 in the case of *capA*, deletion of a single residue from either tract of which places the two adjacent coding sequences, Cj0628 and Cj0629, within a single ORF. In the case of *capB*, there is a 7-bp poly-T tract between nucleotides 578 and 584, deletion of a single residue thereof similarly places the adjacent coding sequences, Cj1677 and Cj1678, within a single putative gene.

The two pairs of ORFs were hypothesised to constitute a single gene each due to the presence of promoter sequences and ribosome-binding sites upstream of Cj0628 and Cj1677, but not Cj0629 or Cj1678. Again, using the SignalP algorithm, both Cj0628 and Cj1677 are predicted to encode proteins with signal peptidase II domains whose cleavage sites are predicted to be ^{33}**FLA**↓^{36}**SC**THA^{41}TL^{43}T and ^{33}**FLA**^{36}**SC**ANA↓^{41}KL^{43}N respectively. However, given that the sequences at both cleavage sites contain a putative lipid attachment motif and lipoprotein modification/processing site it would appear that the actual cleavage site may be at FLAS↓^{37}C in conformity with other lipoproteins (Hayashi & Wu, 1990; Juncker *et al.*, 2003).

It is also noteworthy that ^{37}C is the last of a stretch of 20 residues that are conserved between CapA and CapB. Cleavage at the predicted sites would result in the residues at +2 being cysteine and leucine respectively.

In *E. coli*, retention of lipoproteins within the inner membrane is dependent on the presence of aspartate at this position, in combination with aspartate, glutamate, glutamine, or asparagine at position 3 (Hara *et al.*, 2003). The absence of aspartate at position +2 would, therefore, suggest an outer membrane location for both CapA and CapB (Hara *et al.*, 2003).

Similarity searches of available gene databases with the passenger domains of *capA* and *capB* demonstrated the presence of both genes in a number of *C. jejuni* strains (Table 5.2).

Genome sequence	CapA	CapB
C. jejuni NCTC 11168	+	+
C. jejuni RM1221	-	-
C. jejuni 84-25	+	+
C. jejuni 81-176	-	-
C. jejuni CF93-6	+	+
C. jejuni 81116	-	-
C. jejuni CG8486	+	+
C. jejuni 260.94	-	-
C. jejuni 269.97	+	+
C. jejuni HB93-13	-	-
Total	**5/10**	**5/10**

Table 5. 2 | The presence of CapA and CapB in the available gene databases of other *Campylobacter* strains. In the case of *C. jejuni* subsp. *doylei* 269.97, the sequence is listed as autotransporter beta-domain-containing protein.

5.3.2 *Campylobacter* Adhesion Protein A, CapA

As stated earlier, the *capA* gene comprises of two ORFs, Cj0628 and Cj0629, when out of frame, encoded by nucleotides 1-541 and 721-3421 respectively. Deletion of a T or a G, between nucleotide 497 and 511 puts the two ORFs in frame to translate as one protein of 1,144 amino acids and a calculated molecular mass of *ca*. 120 kDa, becoming 116 kDa after cleavage of the predicted signal sequences. It is predicted to be weakly acidic, with pI of 6.8.

Protein	% ID with CapA	E-Value	Autotransporter β-Domain	SignalP	Organism
AIDA-I	43	9.5 e-07	Yes	Yes	*Escherichia coli*
TapA	43	2.5 e-14	Yes	Yes	*Acidithiobacillus ferrooxidans*
Mycobacterial PPE membrane proteins	41	2.5 e-14		Yes	*Mycobacterium tuberculosis*
Haemagglutinin-like autotransporter	44	6.8 e-18	Yes	Yes	*Ralstonia solanacearum*
Outer membrane protein/Haemolysin	43	4.5 e-09		Yes	*Fusobacterium nucleatum*
Putative Autotransporter	34	0.41	Yes	Yes	*Rhodopseudomonas palustris* TIE-1
Putative lipoprotein	45	3.0 e-20		Yes	*Helicobacter pullorum* MIT 98-548
rOMP B		9.1 e-07	Yes	Yes	*Rickettsia* Spp
Blr6013 protein	42	5.4 e-08	Yes	Yes	*Bradyrhizobium japonicum*
Outer membrane autotransporter	42	0.007	Yes	Yes	*Caulobacter sp. K31*
Outer membrane autotransporter	43	1.0 e-04	Yes	Yes	*Polynucleobacter necessarius*

Table 5. 3 | Similarity of CapA with other Gram-negative proteins. Whereas some of these closely identical proteins have been listed as autotransporters, others are listed simply as outer membrane proteins.

Further analysis revealed that the *capA* gene is flanked by an upstream gene encoding a probable hydrogenase isoenzyme (*hypA*) and a downstream gene encoding a 321-amino-acid conserved hypothetical protein. Whilst the *hypA* gene is in the same orientation as *capA* but separated from it by an 875-bp intergenic sequence containing a 14-bp inverted repeat, the downstream hypothetical gene is in the opposite orientation to *capA*, suggesting that *capA* is likely to be transcriptionally independent of its neighbouring genes.

Similarity searches of available gene databases with the passenger domain of CapA demonstrated similarity with a number of known autotransporters and/or surface proteins as well as some uncharacterised proteins (Table 5.3).

5.3.3 Complementary Experiments [By Ashgar & Oldfield, Ashgar *et al.* 2007]

The initial characterization of CapA and CapB involved experiments which are detailed in this section to clarify subsequent data and/or information. To avoid possible problems with slipped-strand mispairing at the homopolymeric tracts due to the presence of shared, as well as gene specific, regions within the *capA* and *capB*, separate fragments of *capA* were constructed: the gene-specific region of *capA*, which corresponds to aa 38 to 372 of the in silico-reconstructed CapA sequence and the entire downstream ORF of CapA (Cj0629), which corresponds to aa 243 to 1144 of the in silico-reconstructed CapA sequence and contains sequences common to *capA* and *capB*. Each fragment was cloned into the pMAL-c2X vector to produce plasmids pMAL-trCapA and pMAL-trCapAB respectively, in order to express maltose-binding protein (MBP) fusions of each truncated protein.

After induction of *E. coli* cells harbouring pMALtrCapAB, a recombinant protein (MBP-trCapAB) with a molecular mass of *ca.* 140 kDa was strongly expressed. This was consistent with the predicted molecular weight of MBP fused to the CapAB common region. The MBP-trCapAB fusion protein was affinity purified using amylase-resin columns and further purified by electroelution from sodium dodecyl sulfate-polyacrylamide gels (SDS-PAG) before being used to raise rabbit antibodies against both CapA and CapB

(RαCapAB). The antiserum recognized proteins with molecular masses of 140 kDa and 115 kDa in lysates of *E. coli* expressing the recombinant protein; these were assumed to be the full-length recombinant fusion protein and a breakdown product of this protein. Recombinant proteins containing the gene-specific region of CapA was expressed and purified in the same way from pMALtrCapA-containing *E. coli* cells, and used to generate the CapA-specific antiserum RαCapA, which strongly recognized proteins of the expected size in lysates of *E. coli* expressing CapA.

In order to obtain controls for expression studies and for phenotypic studies of CapA, single-knockout mutants of *capA* and *capB* (11168*capA* and 11168*capB*, respectively), as well as a double mutant (11168*capAcapB*) in which both genes were disrupted, were constructed. Each gene was disrupted by insertion of either a kanamycin or a chloramphenicol resistance cassette toward the beginning of the structural gene. Successful insertion of the respective antibiotic genes at the intended locations was confirmed by PCR and sequencing (Ashgar *et al.*, 2007).

Using RαCapAB to probe whole cell extracts of *C. jejuni* in immunoblotting experiments, Ashgar *et al.* observed that while CapA was consistently expressed in strain NCTC11168, there was no detectable expression of CapB under the culture conditions used (Ashgar *et al.*, 2007).

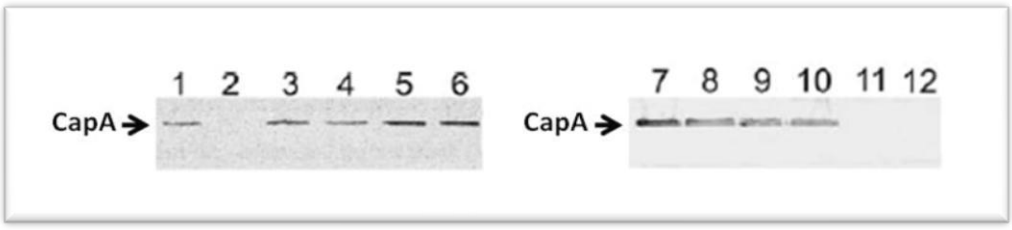

Figure 5. 5 | Expression of CapA in *C. jejuni*. RαCapAB recognizes a protein of *ca.* 116 kDa in whole-cell preparations of strain NCTC11168 (lane 1) and in four heterologous clinical isolates (lanes 3 to 6) but not in 11168capA (lane 2). A protein of the same size was also detected in extracts from isolates obtained from chickens experimentally inoculated with strain NCTC11168 at 2 (lane 7), 3 (lane 8), and 4 (lane 9) weeks post-inoculation and in post-mortem isolates (lane 10). No protein was detected in extracts of isolates from chickens inoculated with strain 11168capA at 1 week (lane 11) or 2 weeks post-inoculation (lane 12) [see Ashgar *et al.* 2007, *J Bacteriol* 189, 1856-1865].

In a further southern-blotting experiment and immunoblotting, Ashgar *et al.* observed that only 2/8 of multilocus sequence-typed (MLST) human isolates contained the *capA* gene, whilst 8/11 recent clinical isolates had the gene (Ashgar *et al.*, 2007). Of the 8 recent clinical isolates that had the gene, only 4 of them expressed detectable CapA protein, whilst both *capA*-containing MLST isolates expressed CapA [see Figure 5.5].

Using sub-cellular fractions of CapA-producing *C. jejuni* strains grown in the presence or absence of globomycin, Ashgar *et al.* confirmed that CapA is indeed a lipoprotein that is localised in the outer membrane (Ashgar *et al.*, 2007). They further showed that CapA plays a role in association and invasion of Caco2 cells, and that it is required for efficient colonization of chicken guts (Ashgar *et al.*, 2007).

5.3.4 Repair of *capA*

It was important that the CapA protein was expressed in its native form in order to further characterize the protein. Repair of *capA* was therefore carried out [**courtesy, Dr Neil Oldfield and Dr Karl Wooldridge**] to create a non-phase-variable variant of the *capA* gene to ensure the expression of a full-length CapA protein.

The poly-T and adjacent poly-G tracts were replaced with a DNA sequence 4-bp shorter (to put *capA* in-frame) and encoding the same amino acids, but containing no homopolymeric sequences (Figure 5.6).

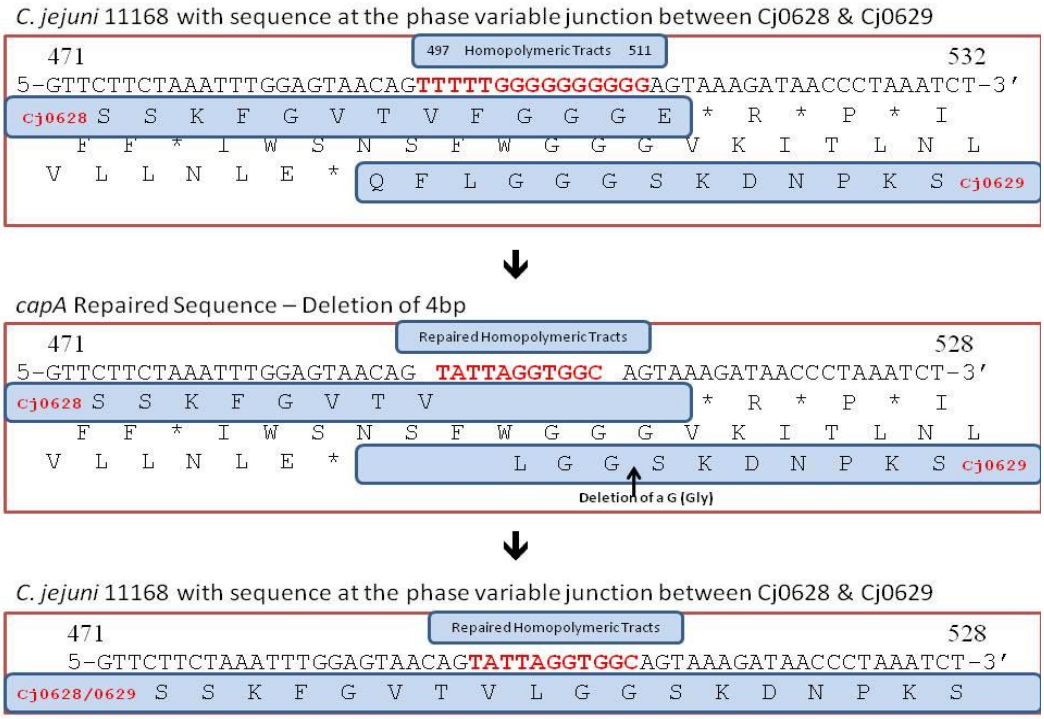

Figure 5. 6 | Repair of capA gene and complementation. The *capA* gene used in the complementation was engineered using PCR so that the poly-T and adjacent poly-G tracts, which interrupt the full *capA* coding sequence, were replaced with a DNA sequence 1-bp shorter (to put *capA* in-frame) and encoding the same amino acids, but containing no homopolymeric sequences. Thus, expression of a full-length CapA protein was fixed and no phase variation could occur.

The genotype of the resulting strain was confirmed by PCR and sequencing. By using immunoblotting experiments on equivalent amounts of whole cell protein were then carried out to confirm that CapA was being expressed in the complemented strain. These showed that CapA was being expressed, but at greatly reduced levels compared to the wild type [see Figure 5.7].

The adherence properties of the complemented strain were then compared to the wild type and *capA* mutant strains *in vitro*. These experiments showed that the complemented strain was indistinguishable from that of the mutant. This was interpreted as being a consequence of the low level of CapA expression in the complemented strain.

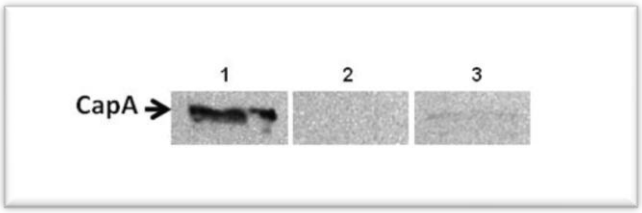

Figure 5. 7 | Expression of Full-length CapA. Rabbit polyclonal anti-CapA serum recognizes a protein of *c*. 116 kDa in a whole-cell preparation of *C. jejuni* NCTC11168 (lane 1) but not in the CapA-deficient derivative (lane 2). Complementation by inserting a single copy of *capA* into the genome of the *capA* mutant restores CapA expression, but not to wild type levels (lane 3).

5.3.5 Cloning of Full-length CapA

Using PCR the *capA* gene was amplified from chromosomal DNA of wild type *C. jejuni* NCTC11168 or *capA* mutant harbouring a repaired-*capA* gene [see section 5.2.7], each approximately 3.5 kb in size (Figure 5.8). The resulting PCR products, *capAori* and *capArep* respectively, were ligated into pETBlue-1 AccepTor vector (Novagen) according to manufacturer's instructions, generating two recombinant genes pET-*capAori* and pET-*capArep* respectively.

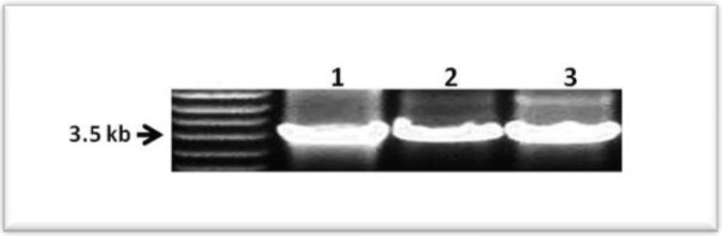

Figure 5. 8 | Agarose gel electrophoresis photo of PCR of *capA* gene. 1 and 2, repaired *capA* gene (*capArep*); lane 3, wild type *capA* (*capAori*) harbouring the homopolymeric tracts at nt 497-511. The repaired *capA* gene is only 4 nucleotides shorter than the original *capA* gene.

The presence of restriction sites within the recombinant plasmids (insert and vector) were used to ascertain the orientation of the insert before they were used in transformation of *E. coli* hosts [see Figures 5.9 and 5.10]. Insertion of *capA* in the same orientation as the T7 promoter of the vector creates an *Eco*RI site yielding three fragments of 420 bp, 3241bp and 3251 bp in a double digest with *Eco*RI and *Xba*I. In contrast, insertion of *capA* in a reverse orientation to the vector disrupts the *Eco*RI site yielding two fragments of 1236 bp and 5734 bp in a double digest with *Eco*RI and *Xba*I.

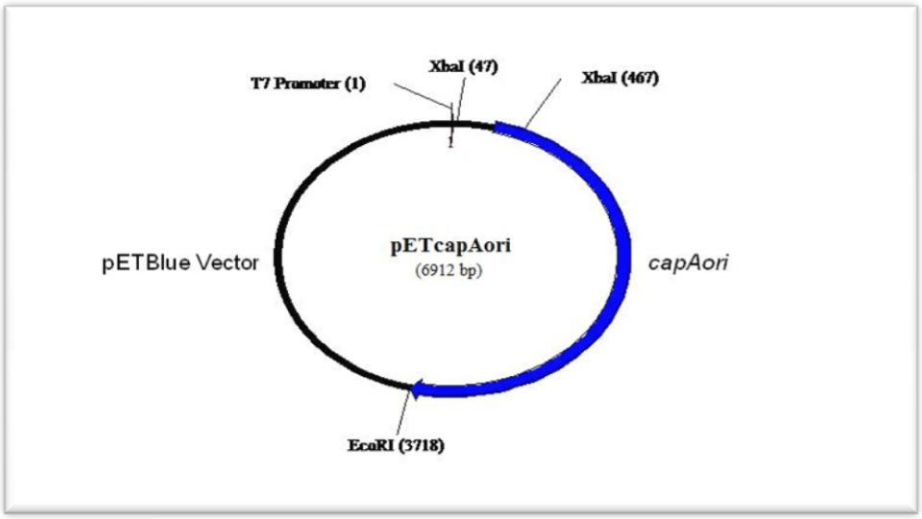

Figure 5. 9 | Schematic representation of the recombinant plasmid, pET-*capA*. The DNA sequence of the *capA* gene has an *Xba*I restriction site at position 99, but no *Eco*RI site, whilst pETBlue-1 vector has an *Eco*RI site within the multiple cloning region of the vector. Insertion of *capA* DNA sequence in the correct orientation to the T7 promoter results in the availability of the *Eco*RI site for digestion with the enzyme, whilst insertion of *capA* in the reverse orientation disrupts the *Eco*RI site. A double digest with *Xba*I and *Eco*RI, therefore, differentiates between a capA insert in the correct or reverse orientation.

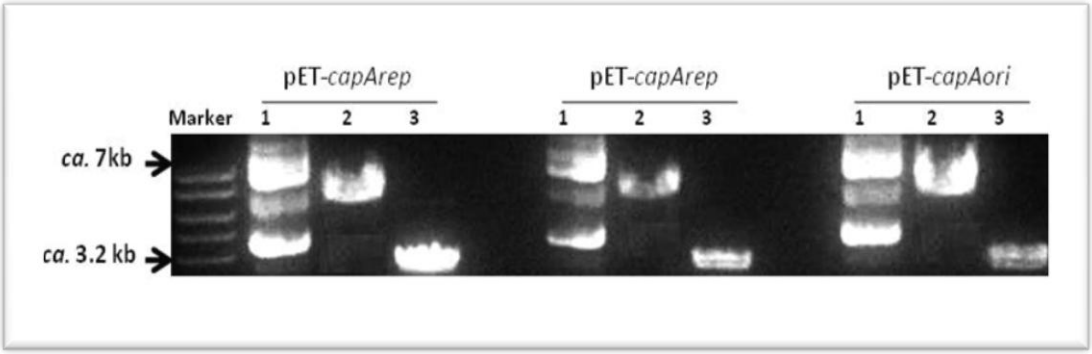

Figure 5. 10 | Agarose gel photo of *Xba*I/*Eco*RI double digestion reactions of pET-*capArep* and pET-*capAori*. Insertion of *capA* in the correct orientation to the T7 promoter of the vector creates an *Eco*RI site upstream, a double digest with *Eco*RI and *Xba*I of which yields two fragments of 3241bp and 3251 bp detectable as one fragment and a non detectable fragment of 420 bp, while a single digest with *Xba*I yields only one detectable fragment of 6.5 kb. In contrast, insertion of *capA* in a reverse orientation to the vector removes the *Eco*RI site yielding three detectable fragments of 1237 bp, 2434 bp and 3241 bp in a double digest with *Eco*RI and *Xba*I, while a single digest with *Xba*I yields two detectable fragments of 1237 bp and 5.6 kb. The above digest depict insertion of *capA* in the correct orientation to the T7 promoter. 1, undigested plasmid; 2, single digest with *Xba*I; and 3, double digest with *Xba*I and *Eco*RI.

5.3.6 Expression of Full-length CapA

Following initial cloning and verification of constructs in NovaBlue Singles™ competent cell host (Novagen) the recombinant plasmids were transformed into the Tuner (DE3) pLacI competent cells (Novagen) according to manufacturer's instructions for protein expression. As expected, no protein expression of CapAori was detected, whilst expression of CapArep was obtained. However, this was inconsistent in different experiments. Clones that expressed the protein in some experiments did not express it at other times.

After several unsuccessful attempts to express CapArep in Tuner cells, the pET-*capArep* plasmid was used to transform BL21-CodonPlus competent cells (Stratagene) according to manufacturer's instructions for protein expression. These cells contain some rare tRNAs that allow high level expression of many heterologous recombinant genes.

Using lysates from wild type *C. jejuni* strain NCTC11168 expressing CapA as a positive control and its mutant as a negative control, protein expression from clones containing the repaired *capA* construct (CapArep-1 and CapArep-2) produced a characteristic band at the expected size of CapA [see Figures 5.11 and 5.12]. However, this protein pattern was observed in clones that contained the pETBlue-1 vector without insert. Very strong protein bands with molecular weights of *ca.* 83 kDa and 175 kDa were also observed in the lysates.

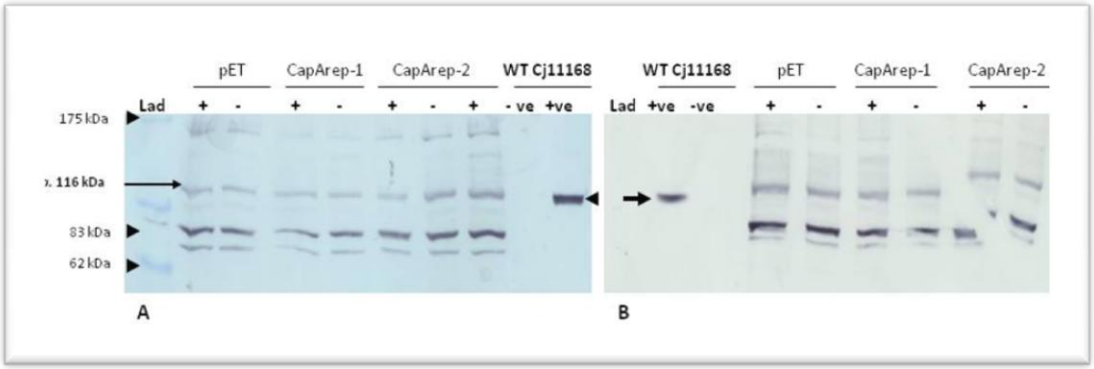

Figure 5. 11 | Immunoblotting analysis of CapA expression by two clones of the repaired *capA* gene, CapArep-1/2 in (A) BL21-CodonPlus cells and (B) Tuner Competent cells. WT *C. jejuni* strain NCTC11168 expressing CapA, its negative control in which the *capA* gene has been knocked out and pETBlue-1 vector without an insert (pET) were used as controls. Fresh overnight cultures of BL21-CodonPlus™ cells or Tuner (DE3) pLacI competent cells containing the *capA* clones and a pET-null were incubated in duplicate for two hours to mid-log phase growth. One of each duplicate culture was then induced with IPTG (+), the other un-induced (-) and incubated for further 3 h. Rabbit polyclonal anti-CapA serum recognizes a protein of *ca*. 116 kDa in a whole-cell preparation of the resultant cultures. Photo developed using 4-CN.

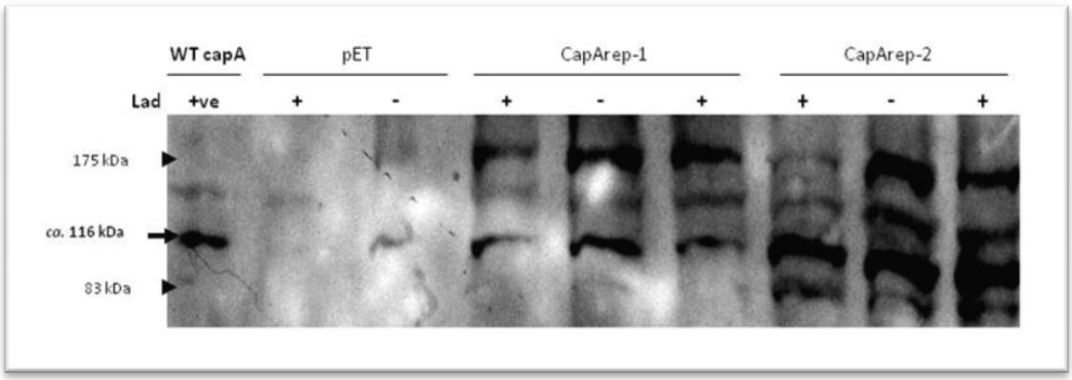

Figure 5. 12 | Immunoblotting analysis of CapA expression by two clones of the repaired *capA* gene, CapArep-1/2 in BL21-CodonPlus cells. WT *C. jejuni* strain NCTC11168 expressing CapA, and pETBlue-1 vector without an insert were used as controls. Fresh overnight cultures of BL21-CodonPlus™ cells the *capA* clones or a pET-null (pET) were incubated in triplicate for two hours to mid-log phase growth. Two each of the *capA* clones were then induced with IPTG (+), the other un-induced (-) and incubated for further 3 h. Rabbit polyclonal anti-CapA serum recognizes a protein of *ca*. 116 kDa in a whole-cell preparation of the resultant cultures. Photo obtained using ECL.

5.4 Discussion

The identification and initial characterization of *Campylobacter* adhesion protein A (CapA) has been reported in the Journal of Bacteriology, **189**(5): 1856-65 (Ashgar *et al.*, 2007). This constituted the first report of a *Campylobacter* autotransporter.

Although we have expressed truncated forms of the protein in *E. coli* for initial characterization, expression of the full-length CapA has only been consistently demonstrated in wild type *C. jejuni* strains.

The non-expression of the full-length of the original CapA in an *E. coli* host was not surprising because of the presence of the poly-T and poly-G tracts within its sequence and the fact that the gene was out of frame. Obviously, transcriptional slippage or stutter occurring during transcription resulting in frameshifts and phase variation could have led to the expression of the CapA protein, but this was not the case (van der Woude & Baumler, 2004).

Phase variation is a well-known phenomenon that has been observed in many aspects of *C. jejuni* biology (Bacon *et al.*, 2001; Coward *et al.*, 2006; Guerry *et al.*, 2002; Prendergast *et al.*, 2004). The sequenced genome of the *C. jejuni* NCTC11168 reveals the presence of short polymeric tracts of nucleotides within several genes making them highly phase variable (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000; Wassenaar *et al.*, 2002). Several *Campylobacter* genes such as the *wlaN* gene which encodes a galactosyltransferase, the *maf1*, *maf4* and *maf5* genes involved in flagellation are all known to contain homopolymeric tracts that render them phase variable (Karlyshev *et al.*, 2002; Linton *et al.*, 2000a). Thus, the presence of homopolymeric tracts at the 5' end of *capA*, as depicted by the gene sequence, suggests that its expression could also be phase-variable. Furthermore, whilst confirming that not all *C. jejuni* isolates had the *capA* gene, some clinical isolates that harboured the *capA* gene did not express detectable CapA protein (Ashgar *et al.*, 2007). Using micro-array comparative studies, Dorrell *et al.* identified genes in several NCTC 11168 loci that are either absent or highly divergent in these isolates (Dorrell *et al.*, 2001).

Based on the available data, it was difficult to interpret whether or not CapA was being expressed by the *capArep* constructs. The intensity of the protein detected in the null-vector-containing *E. coli* was identical to the intensity of the protein detected in the *capArep* constructs. The original *capA* gene was repaired using PCR so that the poly-T and adjacent poly-G tracts, which interrupt the full *capA* coding sequence, were replaced with a DNA sequence 4-bp and 1-amino acid shorter (to put *capA* in-frame) and encoding the same amino acids, but containing no homopolymeric sequences. Thus, expression of a full-length CapA protein was expected to be fixed and no phase variation could occur. This was, however, not the case, as the protein was expressed in some experiments but not in others. It is possible that CapA was being expressed but at a very low level. Although both *capArep*-1 and *capArep*-2 were identical, there were only a few instances where they gave identical protein expression profiles.

Low level expression was also observed following complementation of the *capA* mutant of *C. jejuni*, which also showed adherence properties identical to those of the original mutant. It is not clear whether the site of insertion of the repaired *capA* during complementation played a role in this. This location was selected because the Cj0652 and Cj0653c genes are orientated in a 'tail-to-tail' orientation, with a 123-bp gap in between, allowing for the insertion of DNA without polar effects.

It was therefore decided that the repaired *capA* be cloned into a vector and the protein expressed in an *E. coli* host. The pET*capA* plasmids were therefore transformed into Tuner competent cells since these cells contained the suppressor *lacI* gene. Since the pETBlue-1 vector does not contain a copy of the *lacI* gene, it requires an additional source of *lac* repressor to suppress basal expression of target genes in the absence of inducer. Thus, the presence of the pLacI plasmid in the Tuner cells provides this additional source of *lac* repressor after pETBlue recombinants have been transfected into the former.

Following unsuccessful attempts to consistently express CapA in Tuner competent cells, it was thought that a more specialized commercial *E. coli* would be needed for consistent expression. BL21-CodonPlus™ cells were therefore employed because of their ability to enable efficient expression of

heterologous proteins in *E. coli* due to the presence of extra copies of genes encoding tRNAs that are rare in *E. coli*. However, these also failed to consistently express the CapA protein. These observations could be attributed to the AT rich nature of the gene which might probably have resulted in promoter-like sequences and interfere with proper gene expression (Huerta *et al.*, 2006).

The expression of a full-length CapA may be dependent on other protein(s). Thus, in the complemented *C. jejuni* mutant strain or the *E. coli* hosts, where the other gene(s) may be showing phase variation or totally absent, the expression of CapA may also be hampered. Furthermore, the very low-level expression of the CapA protein in the complemented strain, which is likely to be the case in the *E. coli* hosts, could account for the absence of detectable levels of the protein in some experiments.

A comparison of the adherence properties of wild type *C. jejuni* NCTC11168 and its mutant deficient in *capA*, showed, in the initial characterization, that *capA* mutants lacked the ability to colonize chicken guts or adhere to Caco2 cells (Ashgar *et al.*, 2007). However, complementation of the *capA* mutant with repaired *capA* failed to confirm this, since the adherence properties of the two strains were indistinguishable. This could be attributed to the low level of CapA expression in the complemented strain.

That CapA is absent in some strains of *C. jejuni* despite being pathogenic could also be attributed to the roles of other known adhesins such as PEB-1, JlpA, CadF and MOMP described thus far. This is also likely to account for the residual adherence observed in the *capA* mutant strain. Thus, the very low-level expression of the CapA protein in the complemented strain was unlikely to detectably enhance the ability of this strain to adhere.

Through bio-informatic analysis, it has been demonstrated that CapA possesses all the features of autotransporter proteins (Henderson *et al.*, 2004) and shares homology with several Gram-negative autotransporters and adhesins as shown in Table 5.3. In spite of close homology of CapA with another *Campylobacter* protein, CapB, the transcription or expression of the latter has not been demonstrated, whilst that of CapA has.

In conclusion, Cj0628 encodes a *Campylobacter* outer membrane autotransporter protein, CapA, which plays some role in colonisation of chickens and adhesion to human epithelial cells *in vitro*. It would be necessary to produce a full-length CapA protein for structural and further functional characterization of this protein.

Chapter Six

General Discussion

Identification of Host-pathogen Interacting Molecules of *Campylobacter jejuni* using Phage Display Technology and in silico Sequence Analysis

CHAPTER SIX

6.0 Final Remarks

6.1 General Discussion

Campylobacter jejuni has established itself as the world's leading cause of food- and water-borne human gastroenteritis which is sometimes followed by unprecedented complications, ranging from localized peritonitis, pericarditis and encephalopathy to debilitating neuropathy and bacteraemia (Alzand *et al.*, 2009; Lang *et al.*, 2009; Roan *et al.*, 2009; Wesley *et al.*, 2009; Young *et al.*, 2009; Young *et al.*, 2007; Zilbauer *et al.*, 2008). Although much progress has been made in the past three decades in regards to its virulence determinants, our understanding of the molecular basis of *C. jejuni* pathogenesis, still lags behind that of other enteric pathogens (Poly & Guerry, 2008; Zilbauer *et al.*, 2008).

Bacterial pathogenesis is known to be dependent upon the panoply of proteinaceous virulence factors that are transported to the bacterial cell surface or released into the external environment to interact with host cellular molecules, including the extracellular matrix proteins (Casadevall & Pirofski, 2001; Finlay & Falkow, 1997; Patti *et al.*, 1994; Wilson *et al.*, 2002). Understanding the molecular basis of interactions between these proteins and host cells is therefore necessary in understanding and controlling infections.

The *C. jejuni* genome encodes as many as 1,643 proteins, of which 10-20% is predicted to be membrane, periplasmic or lipoproteins of unknown function (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000). Of these, 121 have been characterized and assigned as being involved in adhesion (10), motility (63), two-component systems (15), toxin production and drug resistance (19) (Fouts *et al.*, 2005; Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000). The sequencing of additional strains of *C. jejuni* has further revealed that the total number of uncharacterized membrane proteins ranges between 185 and 218 (Fouts *et al.*, 2005). Obviously, these individual proteins may well be involved in virulence processes, such as adhesion, colonization, iron acquisition, gut survivability and toxicity towards host cells.

To decipher all the genes encoding these proteins and their roles in pathogenesis calls for the use of different strategies in identifying these proteins and their respective functions. Several strategies have been described, including two-dimensional gel electrophoresis (2-DE), mass spectrometry, yeast two-hybrid screens, antibody screens, and comparative genomics (Cordwell *et al.*, 2008; LaGier & Threadgill, 2008; Parrish *et al.*, 2007; Shoaf-Sweeney *et al.*, 2008).

Two approaches have been employed in this study, namely, phage display technology and in silico sequence search/analysis of available gene databases. Whereas in silico search of gene databases and analysis of individual genes may lead to the identification of novel proteins, based on previously characterized homologues in other pathogens, the method could potentially miss other proteins that may play the same functions but have no homology to other characterized proteins. A detailed analysis of lactoferrin-binding proteins of *N. meningitidis* revealed homology in the sequences of some Gram-negative organisms, but also a lack of similarity to other known Lf-binding proteins of other organisms. For instance, two different Lf-binding proteins have been described in *N. meningitidis* (Pettersson *et al.*, 2006; Schryvers & Lee, 1989). This deficiency could be complemented by phage display technology which, not only screens libraries expressing many thousands of unique peptides and proteins by way of binding to specific ligands but also, has the capacity to maintain a molecular link between the displayed protein and the DNA sequence encoding it (Azzazy & Highsmith, 2002; Mullen *et al.*, 2006).

To my knowledge, this is the first description of a phage display library of *C. jejuni*, although other libraries have been described including a lambda gt11 library, genomic microarray library and a novel cloning of *C. jejuni* ORFs (Burucoa *et al.*, 1995; Parrish *et al.*, 2004; Poly *et al.*, 2004). This phage display library of *C. jejuni* NCTC11168 was constructed using the major coat protein of the M13 bacteriophage in a phagemid vector, pG8SAET (Jacobsson *et al.*, 2003). Based on the fact that minor coat proteins will display larger proteins more effectively than pVIII (Kehoe & Kay, 2005), two libraries were constructed; one that was made from size-selected DNA fragments for larger inserts and another that was not size-selected and hence contains smaller inserts.

Given that some short peptides cannot be displayed, whilst some proteins as large as penicillin G acylase, an 86-kDa heterodimer, have been displayed on both pIII and pVIII, this library should more than suffice for its intended purpose (Verhaert *et al.*, 1999).

The high insert rates in these phage display libraries of *C. jejuni* NCTC11168, together with the high complexities and high titres, are also indicative of good genomic libraries (Jacobsson *et al.*, 2003; Mullen *et al.*, 2007b). Since no such library has been described for *C. jejuni*, these will serve as rich resources for further host-pathogen protein interaction studies of *C. jejuni* for years to come.

Using these two approaches, therefore, we sought to identify host-pathogen interacting molecules of *C. jejuni*. On one hand, Lf has been implicated in a number of pathogenic processes including activation of macrophages via TLR4-dependent and -independent signalling pathways (Curran *et al.*, 2006), regulation of helper T cells, Th1 and Th2, responses (Fischer *et al.*, 2006), and above all the role of Lf in iron metabolism and the ability of some organisms to utilise Lf-bound iron (Furano & Campagnari, 2004; Perkins-Balding *et al.*, 2004). Thus, with the recent finding that *C. jejuni* could utilise Lf-bound iron (Miller *et al.*, 2008), which hitherto was not thought to be the case, Lf was considered a suitable ligand for the very first substantive panning of the phage display library.

As stated earlier, autotransporters, on another hand, have become a rapidly growing family of proteins among Gram-negative bacteria due to their diverse passenger domains and their unique roles in host-pathogen interactions including adhesion, toxigenicity and intracellular spread (Henderson *et al.*, 2004). Through in silico sequence analysis, Cj0628 was identified. Cj0628 encodes a protein which has been characterized and designated *Campylobacter* adhesion protein A (CapA) (Ashgar *et al.*, 2007).

Through phage display technology the gene, Cj0609c, was also identified and shown to encode a periplasmic protein. This study has provided the first evidence that this protein may be involved in Lf-binding and/or iron acquisition. However, it is yet to be fully characterized.

There are a number of iron sources to pathogens in the blood, tissue fluids and (particularly important for *Campylobacter*) in the mucosa. Lf is one such potential source in the latter habitat, although only a handful of pathogenic organisms including *H. pylori* and *N. meningitidis* have been reported to utilise iron from Lf and Tf sources (Husson *et al.*, 1993; Krewulak & Vogel, 2008; Pickett *et al.*, 1992).

Siderophores are low-molecular weight iron-chelating compound synthesised by Gram-negative and Gram-positive bacteria to scavenge iron from precipitates or host proteins in the microorganism's extracellular environment (Faraldo-Gomez & Sansom, 2003). Iron is typically obtained from these sources by Gram-negative bacteria through specialised uptake pathways made up of a TonB-dependent outer membrane receptor, a periplasmic binding protein (PBP), and an inner membrane ABC transporter as depicted in Figure 6.1 (Faraldo-Gomez & Sansom, 2003; Krewulak & Vogel, 2008). Whereas iron-siderophore complexes may enter the periplasm, iron-Lf/Tf and iron-haem complexes are prevented from doing so due to their sizes. An addition to the features of the TonB-dependent siderophore receptors include a β-barrel with an amino terminal segment that folds independently into a globular plug domain that opens or closes the β-barrel depending on the presence or absence of a siderophore (Faraldo-Gomez & Sansom, 2003; Krewulak & Vogel, 2008). Nevertheless, the processes involved in the removal of iron from Lf and Tf and its subsequent transport across the outer membrane are still not well-understood (Khan *et al.*, 2007) and, until recently *C. jejuni* was not known to utilise Lf as a source of iron.

Although a TonB dependent or ligand-gated outer membrane receptor for ferrienterochelin and colicins, Iha adhesin, has been predicted in *C. jejuni*, it is yet to be characterised (Gundogdu *et al.*, 2007). The protein is predicted to have channels created by a monomeric 22 strand anti-parallel β-barrel, ligand-binding large extracellular loops and a N-terminal 50-200 residues that form a plug from the periplasmic end of barrel (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000).

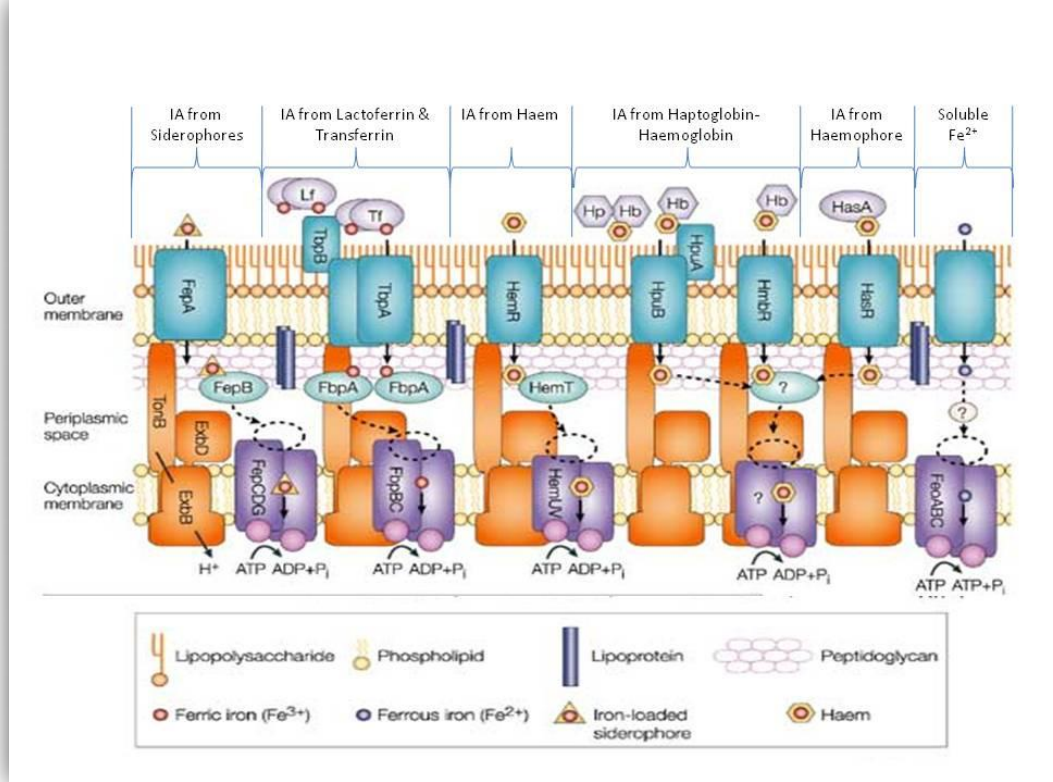

Figure 6. 1 | Schematic representation of iron-uptake systems in Gram-negative bacteria. Bacteria iron-acquisition (IA) starts with the binding of Fe^{3+}-containing proteins (light purple) such as iron-loaded siderophores or haem to high-affinity surface receptor proteins (Wooley & Blue). These are subsequently translocated into the periplasmic space with the aid of the Ton complex (TonB–ExbBD). Iron transported further into the cytoplasm through periplasmic binding proteins (light blue) and ATP-driven transporters (Purple *et al.*). In *E. coli*, the siderophore enterobactin is recruited through the outer membrane receptor, FepA and the FepBCDG system. IA in *Neisseria* species is achieved through receptor-mediated removal of iron from Lf/Tf, which is transported the Tf/Lf-binding protein (T/Lbp) AB and Ferric-binding protein (Fbp) ABC system. Haem is sequestered from haemoglobin (Hb) and haemoglobin–haptoglobin (Hb–Hp) by outer-membrane proteins such as HpuAB and HmbR or taken up directly by the HemRTUV system in *Yersinia enterocolitica*, or delivered by haemophores such as HasA in *Serratia marscensis* through HasR. Under anaerobic conditions, soluble Fe^{2+} can diffuse across outer-membrane porins, and is subsequently imported by energy-dependent systems such as the FeoABC [Adopted from Faraldo *et al*., 2003; *Nat Rev Mol Cell Biol* 4, 105-116].

The well-characterized *C. jejuni* adhesin, PEB1, is a periplasmic protein that has a signal peptidase II cleave site and has been demonstrated in culture supernatants (Young *et al.*, 2007). Furthermore, a putative periplasmic solute binding protein for ABC transport system, Cj0143c (ZntC), is predicted to be a metal-binding protein, that functions as an initial receptor in ABC transport of metal ions (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000). Another putative haemagglutination activity domain containing protein, Cj0737 (FhaB), which is

also a periplasmic protein is believed to possess exoproteins involved in haem-utilization or adhesion (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000).

Therefore, given the unique characteristics of Cj0609c as a periplasmic protein with a possibility of also being non-classically secreted, it is possible that the protein encoded by this gene could shuttle between the periplasmic space and the cell surface, enabling it to bind to Lf. Alternatively it may have a dual location and hence a dual role in binding Lf and delivering iron to the cells without 'shuttling' as such.

Furthermore, the *E. coli* thioesterase I (TAP), which belongs to the SGNH-hydrolase family, has been shown to be a multifunctional enzyme possessing activities of thioesterase, esterase, arylesterase, protease, and lysophospholipase (Lo *et al.*, 2003). The protein encoded by Cj0609c could therefore also possess a protease function, given that it belongs to the SGNH-hydrolase family, thus, capable of hydrolyzing Lf to release its Fe^{3+} in the process.

In the well-accepted model of TonB-dependent iron-acquisition from Lf/Tf, the role of the periplasmic binding protein in the process is overlooked, suggesting that transport across the outer membrane and subsequent capture of ligand by the periplasmic binding protein are independent processes. However, in a recent study by Khan and co-workers, it was observed that high-affinity binding of iron by the periplasmic binding protein is required to drive transport across the outer membrane (Khan *et al.*, 2007). In fact, the published affinity constants for Lf/Tf-iron complexes are near or below the threshold affinity observed by Khan in his periplasmic binding protein construct (Khan *et al.*, 2007). With the high pI of 10.3 predicted in Cj0609c, it is likely to have a higher affinity for Fe^{3+} than would Lf, and thus could competitively sequester Fe^{3+} from holo-Lf.

Furthermore, should the genes downstream of Cj0609c, which together are in reverse orientation to their flanking genes, be tightly regulated, then it could be hypothesized that Cj0609c-Cj0612c could form an operon for iron acquisition based on the accepted mechanism described above. This is because Cj0610c is periplasmic protein but has no signal peptide cleavage site, whilst Cj0611c is a probable transmembrane transport protein with acyltransferase functions, and Cj0612c encodes a ferritin. Being an enteropathogen, it is likely that

Campylobacter utilizes Lf-bound iron as part of its iron acquisition mechanisms for survival.

Lastly, with its role in iron homeostasis, it is believed that the internalization of both apo- and holo-Lf in mammalian cells is dependent on a clathrin-mediated endocytosis (Lopez *et al.*, 2008; McAbee *et al.*, 1993). Thus, some microorganisms have evolved Lf receptors which are not used in iron sequestration but employed to enable them to enter into host cells alongside Lf. For instance, it has been recently reported that *Streptococcus uberis*, an important causative agent of mastitis in dairy cattle throughout the world, utilises Lf as a molecular bridge for internalization into bovine mammary epithelial cells (Patel *et al.*, 2009). By extrapolation therefore, should Cj0609c not be involved in iron acquisition, it could be considered as a potential adhesin that binds to Lf within the human gut to aid internalization of the organism into the intestinal epithelial cell. In view of the above, we designate the product of Cj0609c as lactoferrin-interacting molecule of *Campylobacter jejuni* (LimC).

6.2 Conclusions

Based on the characterization of CapA and in silico analysis of Cj0609c, it is likely that these two genes, Cj0628 (*capA*) and Cj0609c (*limC*), which encode an autotransporter protein and a periplasmic protein in *Campylobacter jejuni* NCTC11168 respectively, may be involved in *Campylobacter* adhesion to host cells and *Campylobacter* utilization of lactoferrin-bound iron respectively, thus, contributing to the pathogenesis of the organism. Given that no mechanism for the utilization of Lf-bound iron has been reported in *Campylobacter*, and CapA is the first autotransporter with adhesion properties to be reported in the organism, these studies provide great insights that need further investigations.

6.3 Future Directions

While Cj0628 has been characterised genetically and shown to have a role in pathogenesis and colonisation, it has not been shown to date to interact directly with any host protein. Furthermore, the protein has not been functionally characterised since we could not consistently express the full-length protein.

Thus, further attempts at expression and purification of a full-length native protein should not be abandoned since it will constitute the first step towards these goals. In this regard, it is my wish that newer protein expression vectors and *E. coli* hosts be explored in order to consistently express CapA. This could then be used in adhesion and inhibition assays.

Here is the first evidence that the product of Cj0609c (*limC*) interacts with Lf. However, this needs to be confirmed using other methods to ascertain the role of Cj0609c in iron acquisition and hence the pathogenic processes of *C. jejuni*. Ideally, the first step would involve cloning the full-length Cj0609c into an appropriate vector to express the protein. This would then be used in ELISA assays, immuno-precipitation, far-Western analysis, surface plasmon resonance (SPR) spectroscopy etc. to confirm its binding to Lf.

Following confirmation of binding between Lf and Cj0609c, cellular fractionation would then be carried out to determine the final or other locations of the encoded protein. Mutagenesis of this gene would also help to determine whether this protein has a role in iron uptake from Lf. Several site-directed mutants could then be constructed to explore the functions of the encoded protein in iron-acquisition in *C. jejuni* or its involvement in internalization of *C. jejuni* into host cells.

In collaboration with researchers of protein structure and function, following successful initial characterization, the structure of the encoded protein could be elucidated to lend further understanding to the mechanisms employed by Gram-negative organisms in sequestration of iron from Lf and/or Tf.

We have a long way to go towards fully understanding the relationship between *C. jejuni* and its human and animal hosts. Phage display technology is one tool which may lead to new insights into this complex interaction.

Seven

Bibliography

Identification of Host-pathogen Interacting Molecules of *Campylobacter jejuni* using Phage Display Technology and in silico Sequence Analysis

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Eight

Appendix

Identification of Host-pathogen Interacting Molecules of *Campylobacter jejuni* using Phage Display Technology and in silico Sequence Analysis

Appendix

Appendix 1: Publications

1. **Abruquah, H. H., Oldfield, N. J., Worrall, K., Wooldridge, K. G., & Ala'Aldeen, D. A. (Drafted).** Identification of lactoferrin-interacting molecule of *Campylobacter jejuni* (LimC) using phage display technology and in silico sequence analysis. To be submitted to *J Bacteriol* or *Infect Immun.*

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

Appendix 2: Presentations

1. **Abruquah, H. H., Oldfield, N. J., Worrall, K., Wooldridge, K. G., & Ala'Aldeen, D. A. (2009).** Identification of Uncharacterized Host-pathogen Interacting Proteins of *Campylobacter jejuni* Using Phage Display; **Abstract accepted for poster presentation** at the 109TH General meeting of the American Society for Microbiology in Philadelphia, PA, **16th – 21st May 2009**.

2. **Abruquah, H. H., Oldfield, N. J., Worrall, K., Wooldridge, K. G. & Ala'Aldeen, D. A. (2008).** Investigating host-pathogen interaction of *Campylobacter jejuni* using phage display technology. **Abstract accepted for oral presentation** at the annual Postgraduate Research Day, School of Molecular Medical Sciences, Univ of Nottingham, Nottingham, **26th June 2008**.

3. **Abruquah, H. H., Oldfield, N. J., Worrall, K., Wooldridge, K. G., & Ala'Aldeen, D. A. (2007).** Construction of a phage display library of *Campylobacter jejuni* NCTC11168. **Abstract accepted for oral presentation** at the annual Postgraduate Research Day, School of Molecular Medical Sciences, Univ of Nottingham, Nottingham, **25th June 2007**.

4. **Abruquah, H. H., Worrall, K., Oldfield, N. J., Wooldridge, K. G. & Ala'Aldeen, D. A. (2007).** Identification and characterization of an autotransporter protein of *Campylobacter jejuni*, CapA. **Abstract accepted for poster presentation** at the annual Postgraduate Research Day, School of Molecular Medical Sciences, Univ of Nottingham, Nottingham, **25th June 2007**.

5. **Abruquah, H. H., Worrall, K., Oldfield, N. J., Wooldridge, K. G. & Ala'Aldeen, D. A. (2006).** Identification of an autotransporter protein of *Campylobacter jejuni*, CapA, using in silico sequence analysis. **Abstract accepted for poster presentation** at the annual Postgraduate Research Day, School of Molecular Medical Sciences, Univ of Nottingham, Nottingham, **29th June 2006**.

Appendix 3: Supplementary Information on Materials and Methods

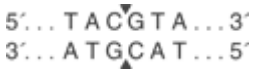
3.1 Enzymes

a. *Sna*BI

*Sna*BI is derived from an *E. coli* strain that carries the *Sna*BI gene from *Sphaerotilus natans*, has a recognition site of 5′...TAC▼GTA...3′ / 3′...ATG▲CAT...5′

b. *Sna*BI

*Sna*BI is derived from an *E. coli* strain that carries the *Sna*BI gene from *Sphaerotilus natans*, has a recognition site of



c. *Bgl*II

*Bgl*II is derived from an *E. coli* strain that carries the *Bgl*II gene from *Bacillus globigii*, has a recognition site of

d. *Bfu*CI

*Bfu*CI is derived from an *E. coli* strain that carries the *Bfu*CI gene from *Bacillus fusiformis*, has a recognition site of

e. *Bam*HI

*Bam*HI is derived from an *E. coli* strain that carries the *Bam*HI gene from *Bacillus amyloliquefaciens* H, has a recognition site of

f. *Sma*I

*Sma*I is derived from an *E. coli* strain that carries the *Sma*I gene from *Serratia marcescens*, has a recognition site of

g. *Eco*RV

*Eco*RV is derived from an *E. coli* strain that carries the *Eco*RV gene from the plasmid J62 pLG74, has a recognition site of

h. Alkaline Phosphatase Variants

a. Calf Intestinal Alkaline Phosphatase (CIAP)

This enzyme, obtained from calf intestine, catalyzes the removal of 5′ phosphate groups from DNA and RNA, thus making DNA and RNA deficient of the 5′ phosphoryl termini required by ligases for ligation (Sambrook & Rusell, 2001). This therefore prevents self-ligation, decreasing the vector background in the cloning experiments.

b. Shrimp Alkaline Phosphatase (SAP)

This enzyme, obtained from shrimp, catalyzes the removal of 5′ phosphate groups from DNA and RNA, thus making DNA and RNA deficient of the 5′ phosphoryl termini required by ligases for ligation (Sambrook & Rusell, 2001). This therefore prevents self-ligation, decreasing the vector background in the cloning experiments.

c. Antarctic Phosphatase (AP)

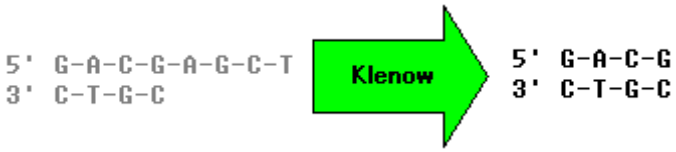
This enzyme, obtained from an *E. coli* strain that carries the TAB5 AP gene, originally cloned in pNI and re-cloned in plasmid pEGTAB7-4.1 (Rina *et al.*, 2000), catalyzes the removal of 5′ phosphate groups from DNA and RNA, thus making DNA and RNA deficient of the 5′ phosphoryl termini required by ligases for ligation (Sambrook & Rusell, 2001). This therefore prevents self-ligation, decreasing the vector background in the cloning experiments.

i. Klenow Fragment

Klenow Fragment (3′→ 5′ exo-), which is derived from an *E. coli* strain containing a plasmid with a fragment of the *E. coli* polA gene starting at codon 324, is an N-terminal truncation of DNA polymerase I, which retains polymerase activity, but has lost the 5′→ 3′ exonuclease activity and has a mutation which diminishes the 3′→ 5′ exonuclease activity (Derbyshire *et al.*, 1988).

A "fill-in" reaction, in the presence of Klenow and nucleotides, is used to create blunt ends on chromosomal DNA fragments with 5′overhangs following sonication such as this:

Although minimal, the 3′→ 5′ exonuclease activity of Klenow can digest away the protruding overhangs following sonication such as this.



In a Klenow reaction, removal of nucleotides from the 3′ ends will continue, but, in the presence of nucleotides, the polymerase activity will balance the exonuclease activity, yielding blunt ends. This reaction is more efficiently conducted with T4 DNA polymerase which has much more potent exonuclease activity.

j. T4 DNA Polymerase

T4 DNA polymerase, derived from a strain of *E. coli* that carries a T4 DNA polymerase overproducing plasmid, catalyzes the synthesis of DNA in the 5′→ 3′ direction in the presence of template and primer such as the sonicated-chromosomal DNA of *C. jejuni* NCTC11168. It also has a 3′→ 5′ exonuclease activity which is much more active than that found in the Klenow fragment or the complete DNA Polymerase I, whilst lacking a 5′→ 3′ exonuclease function (Sambrook & Rusell, 2001).

k. T4 DNA Ligase

T4 DNA ligase, derived from *E. coli* C600 pcl857 pPLc28 lig8, catalyzes the formation of a phosphodiester bond between juxtaposed 5′ phosphate and 3′ hydroxyl termini in duplex DNA or RNA and is employed in the ligation of blunt end and cohesive end termini as well as repair single stranded nicks in

duplex DNA, RNA or DNA/RNA hybrids (Rossi *et al.*, 1997; Sambrook & Rusell, 2001).

l. *Taq* DNA Polymerase

Taq DNA polymerase, a 95-kDa single polypeptide chain is used in Polymerase Chain Reaction (PCR). *Taq* DNA polymerase is a thermostable DNA polymerase, named after the thermophilic eubacterium *Thermus aquaticus BM* from which it was originally isolated and possesses a 5′→ 3′ polymerase activity and a double stranded specific 5′→ 3′ exonuclease activity but lacks a 3′→ 5′ exonuclease activity (Chien *et al.*, 1976; Lawyer *et al.*, 1993; Tindall & Kunkel, 1988). It was supplied with a 10× standard *Taq* reaction (PCR) buffer with magnesium chloride ($MgCl_2$).

m. Expand™ High Fidelity PCR System

The Expand™ High Fidelity (HiFi) PCR System, from Roche Diagnostics, is used in high fidelity PCR amplification. The Expand™ High Fidelity PCR System is composed of a mixture of *Taq* DNA polymerase and *Tgo* DNA polymerase, which has a 3′→ 5′ exonuclease activity. The combined effect of the proofreading activities of the *Taq* and the *Tgo* DNA polymerases is the generation of longer (3-27kb) PCR products of 3-fold yield, with high fidelity and high specificity from all types of DNA compared with a conventional *Taq* DNA polymerase alone (Barnes, 1992; Barnes, 1994).

3.2 Culture Media

vii. LB Media

Premade Luria-Bertani (LB), according to the Miller recipe, is prepared by dissolving 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in 1 litre of distilled water (Miller, 1972). The broth is then sterilized by autoclaving at 121°C, 15 lb. $inch^{-2}$ for 15 min.

LB agar is made in an identical fashion except for the addition of 1% (w/v) agar to LB broth prior to autoclave sterilization. Following cooling to approximately 40°C, any necessary antibiotics are added and the agar transferred to Petri dishes.

LB has been in use since time immemorial and continues to be one of the most commonly used media in molecular biology applications for the maintenance and propagation of recombinant strains of *Escherichia coli* (Anderson, 1946; Bertani, 2004). According to Giuseppe Bertani, the acronym LB was originally intended to stand for “lysogeny broth” (Bertani, 2004). However, it has been variously interpreted, perhaps flatteringly, but incorrectly, as Luria broth, Lennox broth, or Luria-Bertani media (Bertani, 1951; Lennox, 1955; Luria & Burrous, 1957). The low salt formulations, Lennox (5 g/l NaCl) and Luria (0.5 g/l NaCl), are ideal for cultures requiring salt-sensitive antibiotics media (Bertani, 1951; Lennox, 1955; Luria & Burrous, 1957).

3.3 Complementary Experiments [By Ashgar & Oldfield, Ashgar *et al.* 2007]

The following characterization experiments were carried out by Sammi Ashgar and Neil Oldfield. These experiments are therefore mentioned here to fill in the gaps in this chapter. Detailed descriptions of the procedures are given in Ashgar *et al.* 2007.

i. Cloning of Truncated-forms of *capA*

Two fragments of the *capA* gene, corresponding to the upstream and the downstream open reading frames (ORFs) Cj0628 and Cj0629, were amplified by PCR from nucleotide positions 114-1115 and 751-3413 using *C. jejuni* NCTC11168 genomic DNA and ORF-specific primers capA_F1/capA_R1 and capA_F2/capA_R2 respectively (Table 4.1). Using 1 µl of cDNA preparations for the PCR amplification reaction, a 50-µl reaction mixture contained 0.2 µM each of capA_F1/capA_R1 or capA_F2/capA_R2, 0.2 µM dNTPs, 5 µl of 10 × PCR buffer (Roche), 0.25 mM $MgCl_2$, and 4 U Biotaq (Roche). The reaction conditions were as follows: 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 60 s, and a terminal extension step at 68°C for 5 min. PCR products were then analyzed by gel electrophoresis using ethidium bromide staining.

The resulting PCR products were labelled as truncated capA and truncated capAB, tr*capA*/tr*capAB* respectively. The PCR products were ligated into pMAL-C2x vector (NEB) according to manufacturer's instructions, generating two recombinant genes pMAL-tr*capA* and pMAL-tr*capAB* respectively.

ii. Expression and Purification of Recombinant CapA Proteins

The resulting recombinant *capA* gene products, pMAL-tr*capA* and pMAL-tr*capAB*, encoded maltose-binding protein (MBP) fusions, MBP-trCapA and MBP-trCapAB, with calculated molecular masses of *ca.* 35 and 102 kDa respectively, compared to the full-length CapA protein of *ca.* 120 kDa. pMAL-trCapA and pMAL-trCapAB represented amino acid (aa) 38-372 and 243-1144 respectively on the *capA* gene, Cj0608/Cj0629.

E. coli JM83 cells harbouring either pMAL-trCapA or pMAL-trCapAB were cultured in LB broth to mid-log phase, and then induced using isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown for a further 4 h and then harvested by centrifugation at 4000 × *g*. Recombinant fusion proteins were affinity purified using amylase-resin columns (NEB), and further purified by electro-elution SDS-PAGE as described above.

iii. Mutagenesis of *capA* and *capB*

C. jejuni single-knockout mutants for *capA* and *capB* (11168*capA* and 11168*capB*, respectively), as well as a double mutant (11168*capAcapB*) in which both genes were disrupted, were constructed to serve as controls for expression studies and for phenotypic studies. Each gene was disrupted by insertion of either a kanamycin or a chloramphenicol resistance cassette toward the beginning of the structural gene. Successful insertion of the respective antibiotic genes at the intended locations was confirmed by PCR and sequencing (Ashgar *et al.*, 2007).

iv. Cloning and Expression of CapB

Two fragments of the *capB* gene, corresponding to the upstream and the downstream open reading frames (ORFs) Cj1677 and Cj1678 respectively, were amplified by PCR, cloned into the pMAL-C2x vector, expressed as MBP-trCapB, or MBP-trCapAB and used to raise rabbit anti-serum against the recombinant proteins. MBP-trCapAB obtained from *capA* and *capB* genes were identical due to the 100% homology between the C-terminal two-thirds of the two genes.

v. Generation of Rabbit Anti-serum against Recombinant CapA Proteins

New Zealand white female rabbits New Zealand white female rabbits were immunized four times at 2-week intervals with *ca.* 30 µg of purified MBP-trCapA, or MBP-trCapAB emulsified in Freund's complete (first immunization only) or incomplete adjuvant. The animals were test bled 7 days after the third dose and sacrificed 10 days after the final inoculation to generate rabbit antiserum against CapA or CapAB [RαCapAB or RαCapA]. Pre-immune sera were obtained prior to immunization for comparison.

Before use in immunoblotting experiments and other immuno-related experiments the sera were pre-adsorbed overnight at 4°C with a 20% (vol/vol) suspension of *C. jejuni* mutant 11168*capA* (for rabbit antiserum against CapAB [RαCapAB] or RαCapA) cells grown in MH broth before being cleared by centrifugation and filtration as previously described.

vi. Southern Blotting

Using GeneImages random prime labeling and CDP-star detection modules (Amersham) *Hin*dIII-digested genomic DNA of *C. jejuni* NCTC11168, southern blotting was carried out with *capA*-specific probes.

vii. Sub-cellular Fractionation

To determine the cellular localization of CapA, *C. jejuni* cells [wild type, CapA-carrying type and CapA null-mutant] were cultured in MH broth as described and then fractionated into the various sub-cellular fractions [secreted proteins, outer membrane, cytoplasmic membrane, periplasmic and cytosolic fractions]. These fractions were analyzed by SDS-PAGE and immunoblotting.

viii. Electron Microscopy and Immunogold Labelling

C. jejuni cells were cultured as described and prepared for immunogold staining and electron microscopy using JOEL JEM1010 electron microscope. Pre-adsorbed RαCapA was used as primary antibody, with preimmune serum from the same rabbit as negative control.

ix. *In vitro* Association and Invasion Assays

Association and invasion assays were carried out using Caco2 cells grown in Dulbecco's modified eagle medium (DMEM; Invitrogen) and *C. jejuni* cells cultured as described.

x. Chicken Colonization

Using day-of-hatch specific-pathogen-free Rhode Island Red chicks, colonization assays were carried out to determine the influence of CapA on chicken colonization (Ashgar *et al.*, 2007; Velayudhan *et al.*, 2004).